Carbamazepine Hypersensitivity: Linking Metabolism to the Immune Response

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor in Philosophy

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This thesis is the result of my own work and the material contained within the thesis has not been presented, nor is currently being presented, either wholly, or in part, for any other degree or qualification

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Abbreviations

2Me-CBZ	2-methylcarbamazepine
2OH-CBZ	2-hydroxycarbamazepine
3OH-CBZ	3-hydroxycarbamazepine
ADR	adverse drug reaction
AED	antiepileptic drug
AGEP	acute generalised exanthematous pustulosis
ANOVA	analysis of variance
APC	allophycocyanin
APCs	antigen presenting cells
C4C	consent for consent
CBZ	carbamazepine
CBZE	carbamazepine 10,11-epoxide
CD	cluster of differentiation
CDK	cyclin dependent kinases
CFSE	carboxyfluorescin siccinimidyl ester
CMV	cytomegalovirus
COX	cyclooxygenase
CPM	counts per minute
CV	coefficient of variation
CYP	cytochrome P450
DC	dendritic cells
DHR	drug hypersensitivity reaction
DIHS	drug-induced hypersensitivity syndrome
DILI	drug-induced liver injury
DiOH-CBZ	10,11-dihydro- 10,11-*trans*-dihydroxy-carbamazepine
DMSO	dimethyl sulfoxide
DPT	drug provovation testing
DRESS	drug reaction with eosinophilia and systemic symptoms
EDTA	ethylenediaminetetraacetic acid
ELISpot	enzyme-linked immunosorbent spot assay
ESI	electrospray ionisation

FDR	false discovery rate
FOCE	first-order conditional estimation
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSEA	gene set enrichment analysis
GSH	glutathione
GSTP	glutathione *S*-transferase pi
HBSS	Hank's balanced salt solution
HHV	human herpes virus
His-GSTP	hexahistidine-tagged human glutathione *S*-transferase pi
HIV	human immunodeficiency virus
HLM	human liver microsomes
HPLC	high performance liquid chromatography
HSA	human serum albumin
HSS	hypersensitivity syndrome
Ig	immunoglobulin
IL	interleukin
IFN-γ	interferon gamma
IPA	Ingenuity pathway analysis
IS	internal standard
LLOQ	lower limit of quantitation
LTT	lymphocyte transformation test
mEH	microsomal epoxide hydrolase
MHC	major histocompatibility complex
miRNA	micro ribonucleic acid
MPE	maculopapular exanthema
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
NAPQI	N-acetyl-p-benzoquinone imine
NES	normalised enrichment score
NONMEM	non-linear mixed effects modelling

NSAIDs	non-steroidal anti-inflammatory drugs
OFV	objective function value
PARP9	poly (ADP-ribose) polymerase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBPK	physiologically based pharmacokinetic
PCR	polymerase chain reaction
PE	phycoerythrin
PG	prostaglandin
P-gp	P-glycoprotein
PHA	phytohaemoagglutinin
PI	direct pharmacological interaction of drugs with immune receptors
PICME I	Pharmacokinetic Investigation into the formation of CBZ Metabolites and CBZ-protein conjugates in healthy volunteers
PICME II	Pharmacokinetic Investigation into the formation of CBZ Metabolites and CBZ-protein conjugates in Epilepsy patients
PK	pharmacokinetics
PPD	*p*-phenylenediamine
PXR	pregnane X receptor
QC	quality control
R_t	retention time
RIN	RNA integrity number
SD	standard deviation
SNP	single nucleotide polymorphism
SI	stimulation index
SJS	Stevens-Johnson syndrome
SMX-NO	nitroso-sulfamethoxazole
TCC	T cell clone
TCR	T cell receptor
TDM	therapeutic drug monitoring
TEN	toxic epidermal necrolysis
TFA	trifluoroacetic acid

TT	tetanus toxoid
UGT	uridine diphosphate glucuronosyltransferase
Vd	volume of distribution
VPCs	visual predictive checks
VZV	varicella zoster virus

Publications

1. **Yip VL**, Hawcutt DB, Pirmohamed M. Pharmacogenetic Markers of drug efficacy and drug toxicity. *Clin Pharmacol Ther.* 2015; 98(1):61-70
2. Plumpton CO, **Yip VL,** Alfirevic A, Marson AG, Pirmohamed M, Hughes DA. Cost-effectiveness of screening for *HLA-A*31:01* prior to initiation of carbmazepine in epilepsy. *Epilepsia*. 2015; 56(4):556-63
3. **Yip VL**, Alfirevic A, Pirmohamed M. Genetics of immune-mediated adverse drug reactions: a comprehensive and clinical review. *Clin Rev Allegy Immunol.* 2015; 48(2-3):165-75
4. Walker LE, Mirza N, **Yip VL**, Marson AG, Pirmohamed M. Personalized medicine approaches in epilepsy. *J Intern Med.* 2015; 277(2):218-34
5. **Yip VL**, Pirmohamed M. Expanding role of pharmacogenomics in the management of cardiovascular disorders. *Am J Cardiovasc Drugs.* 2013; 13(3): 151-62

Abstracts

1. **Yip VL**, Maggs J, Meng X, Marson AG, Park KB, Pirmohamed M. Covalent Adduction of Carbamazepine 10,11-Epoxide with Human Serum Albumin and Gluthathione S-Transferase pi: Implications for Carbamazepine Hypersensitivity. *Lancet*. 2014; 383: S114

   Academy of Medial Sciences spring meeting for clinician scientists in training, London, February 2014
2. **Yip VL**, Meng X, Maggs J, Marson AG, Park BK, Pirmohamed M. Carbamazepine hypersensitivity: linking metabolism to the immune response. British Pharmacological Society Winter meeting, London, December 2013

Abstract

Carbamazepine (CBZ) is an effective antiepileptic drug but has been associated with hypersensitivity reactions in up to 10% of patients. These reactions range from mild maculopapular exanthema to life-threatening conditions such as Stevens-Johnson syndrome and toxic epidermal necrolysis. The identification of CBZ-specific T cells and strong associations with specific human leukocyte antigen alleles provide evidence for immunological involvement. CBZ is extensively metabolised and forms several reactive metabolites. The aim of this thesis was to investigate the complex relationships between CBZ, its metabolism, the immune system, and genomics.

Direct and microsomal incubations demonstrated that carbamazepine 10,11-epoxide (CBZE), the major metabolite of CBZ, formed a protein conjugate with human serum albumin (HSA) at His146. The same CBZE-modified HSA was also detected in patients tolerant of CBZ therapy. A second His146 CBZ-modified HSA adduct was identified in microsomal incubations, formed as a product of arene oxide providing the first chemical evidence that reactive metabolites of CBZ can modify soluble proteins.

Healthy volunteers (n=8) and patients prescribed CBZ therapy (n=72) were recruited to investigate the influence of genetic variation on CBZ metabolism. Patient demographics and a mixture of rich and sparse pharmacokinetic (PK) samples were collected. Plasma levels of CBZ and four major metabolites were measured using a novel high performance liquid chromatography tandem mass spectrometric assay. There was significant variation in observed plasma concentrations of CBZ (14-fold) and its metabolites (approximately 30-fold).

A population PK model was developed with nonlinear mixed effects modelling using the PK and clinical data collected from patients. Completion of autoinduction, total daily dosage and concomitant therapy with phenytoin were significant covariates that influenced the CBZ PK. Analysis of variance demonstrated that two single nucleotide polymorphisms (SNPs) in the gene *ABCB1* and a single SNP in *EPHX1* were significantly associated with altered plasma concentrations of CBZE.

T cell clones (TCCs) were generated to CBZ and CBZE from two patients with a history of hypersensitivity to CBZ. All TCCs were CD4+ and secreted the cytokines IFN-γ, IL-13, granzyme B and perforin. TCC activation was MHC class II restricted and all TCCs were stimulated when CBZ was freshly added into the incubation mixture indicative of direct activation. A single TCC was activated when antigen presenting cells (APCs) were pulsed with CBZ indicative of a hapten mechanism.

Transcriptomic analysis of peripheral blood mononuclear cells (PBMCs) from patients with a known history of CBZ hypersensitivity identified 9 CBZ/CBZE-specific mRNA and 39 CBZ/CBZE- miRNA transcripts. Pathway analysis mRNA and miRNA changes showed that antiviral response, psoriasis and inflammation were the most significant functions associated with exposure of cells from cases to CBZ and CBZE.

In conclusion, these studies show that CBZ is transformed to stable and reactive metabolites, and these metabolites together with the parent drug, lead in susceptible individuals to an orchestrated response which involves transcriptional and immunological activation.

Chapter 1

General Introduction

1.1 Adverse Drug Reactions

1.1.1 Definition

Adverse drug reactions (ADR) are defined as “an appreciably harmful or unpleasant reaction resulting from an intervention related to the use of a medicinal product; adverse effects usually predict hazard from future administration and warrant prevention, or specific treatment, or alteration of the dosage regimen, or withdrawal of the product” (Aronson and Ferner 2005).

1.1.2 Epidemiology

ADRs are common and account for 6.5% of all hospital admissions and occur in 15% of inpatients increasing healthcare and societal costs. In the US, ADRs are between the fourth and sixth most common cause of mortality and responsible for > 100,000 deaths with similar estimates for the UK (Lazarou et al. 1998; Pirmohamed et al. 2004). In the UK, ADRs extend hospital admission by approximately eight days and account for 4% of the total capacity of hospital beds costing the NHS £466m each year (Pirmohamed et al. 2004). ADRs are also a major problem in drug development and a report by the FDA revealed that 28 drugs had been withdrawn from the US market between 1978 and 2005 because of safety issues and include high profile drugs such as cyclooxygenase (COX) II inhibitors (FDA Centre for Drug Evaluation and Research 2005). This has huge implications for the pharmaceutical industry.

1.1.3 Classification

ADRs were originally classified by Rawlins and Thompson into type A and type B (Rawlins 1981). Type A (augmented) reactions are predictable, typically dose dependent, and based on an exaggerated response to the known primary and/or secondary pharmacological action of the drug. This type of reaction is illustrated by hyperkalaemia with potassium sparing diuretics (Acker et al. 1998) or bleeding with warfarin therapy (Witt et al. 2016). In contrast type B (bizarre) reactions are unpredictable, totally aberrant effects that are unexpected from the known pharmacology of the drug when given at normal therapeutic doses to a patient whose body processes the drug in the normal way. An example of a type B reaction

would include Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN) in response to antiepileptic drugs (AEDs) (Ordonez et al. 2015). However, as knowledge of the mechanisms underlying ADRs increase it is hoped that type B reactions will become predictable in future. The original classification of ADRs was later expanded to include types C, D, E and F ADRs (Table 1.1) (Edwards and Aronson 2000).

Table 1.1 Classification of adverse drug reactions [adapted from Edwards and Aronson (2000)]

Type of Reaction	Mechanism	Example
Type A (augmented)	Predictable from known pharmacology of the drug; dose-dependent	Bleeding with warfarin therapy
Type B (bizarre or idiosyncratic)	Not predictable from pharmacological action of the drug; dose-independent	Stevens-Johnson syndrome with antiepileptic therapy
Type C (chemical or chronic)	Related to the chemical structure or a metabolite of the drug	Osteonecrosis of the jaw with bisphosphonates
Type D (delayed)	Develops years after drug therapy	Leucopaenia six weeks after a dose of lomustine
Type E (end of treatment)	Appear after drug withdrawal especially if stopped abruptly	Insomnia, anxiety and perceptual disturbances following withdrawal of benzodiazepines
Type F (failure)	Often caused by drug interactions	Failure of contraceptive during antibiotic therapy

More recently an alternative classification system for ADRs was developed based on dose relatedness, timing and patient susceptibility (DoTS) (Aronson and Ferner 2003). The new system incorporates not only the properties of the drug but includes characteristics of the reaction and individual patient susceptibility factors (Table 1.2). Neither classification system is perfect but both serve as important

guidance for pharmacovigilance in clinical practice and drug development whilst providing insights into the mechanisms of ADRs.

Table 1.2 DoTS classification criteria for adverse drug reactions

	Variable	Classification
Do	Dose relatedness	Supratherapeutic Standard therapeutic Subtherapeutic
T	Timing	Time independent Time dependent
S	Susceptibility	Age Gender Pathology Physiology Environmental

1.2 Drug Hypersensitivity Reactions

1.2.1 Epidemiology and Classification

Drug hypersensitivity reactions (DHRs) are idiosyncratic ADRs that result from inappropriate activation of the immune system by the offending drug or metabolite leading to heterogeneous clinical manifestations that affect multiple organ systems (Wheatley et al. 2015). DHRs are estimated to account for approximately 20% of all ADRs (Dodiuk-Gad et al. 2014). However, epidemiological studies in DHRs are complex because of the variable clinical presentation, absence of definitive diagnostic tests and lack of consensus on diagnostic criteria (Wheatley et al. 2015).

Classification of DHRs is challenging because for many drugs and clinical presentations, the underlying pathophysiological mechanisms are poorly understood. Clinically, DHRs are classified as immediate or delayed (Demoly et al. 2014). Immediate reactions are those that occur within 1 to 6 hours after the last drug administration and typically present with symptoms that can include uritcaria,

angioedema, bronchospasm and anaphylactic shock. Delayed DHRs commonly occur after multiple days of treatment and can present as maculopapular exanthema (MPE), hypersensitivity syndrome (HSS) and SJS amongst others (Bircher and Scherer Hofmeier 2012). Delayed DHRs are typically mediated by drug-specific T cells (Pichler 2003). Mechanistically, DHRs were originally classified into four main categories according to criteria originally devised by Gell and Coombs (Gell 1963) and subsequently expanded by Pichler (Pichler 2003) (Table 1.3). The most common DHRs are immunoglobulin (Ig) E- and T-cell mediated reactions (Demoly et al. 2014). In immediate DHRs, also known as type I reactions, IgE is produced by antigen specific B lymphocytes in response to drug-hapten complexes. IgE activates mast cells and basophils resulting in the release of preformed mediators (e.g. histamine and tryptase) and production of new mediators (e.g. leukotrienes and kinins). The preformed mediators lead to immediate symptoms whilst the inflammatory component occurs several hours later when the new mediators have been synthesised (Blanca et al. 2009). Some drugs such as beta-lactam antibiotics can cause both immediate and delayed hypersensitivity whilst AEDs typically cause mainly T-cell mediated reactions (Blanca et al. 2009; Ye et al. 2014).

Table 1.3 Mechanistic classification of drug hypersensitivity reactions [adapted from Demoly et al. (2014)]

Type	Immune Response	Effector Mechanism	Clinical Presentation	Reaction Time	Examples of Implicated Drugs
I	IgE	Mast cell and basophil degranulation	Anaphylaxis, angioedema, urticaria	Within 1 - 6 h	Penicillins, cephalosporins
II	IgG and complement	IgG and complement dependent cytotoxicity	Cytopaenia	5 - 15 days	Penicillin, quinidine
III	IgM or IgG and complement or FcR	Immune complex deposition	Serum sickness, urticarial vasculitis	7 - 21 days	Penicillin, tetracyclines
IVa	Th1: IFN-γ	Monocytic inflammation	Eczema	1 -21 days	*p*-phenylenediamine
IVb	Th2: IL-4 and IL-5	Eosinophilic inflammation	MPE, HSS	1 – 42 days	Anticonvulsants
IVc	Cytotoxic T cells (perforin, granzyme B, Fas ligand)	Keratinocyte death mediated by CD4 and CD8	MPE, SJS/TEN	1 – 28 days	Anticonvulsants
IVd	T cells (IL-8/CXCL8)	Neutrophilic inflammation	AGEP	Within 7 days	Antibiotics, carbamazepine

AGEP: acute generalised exanthematous pustulosis, FcR: Fc receptor, HSS: hypersensitivity syndrome, Ig: immunoglobulin, IL: interleukin, MPE: maculopapular exanthema, SJS: Stevens-Johnson syndrome, TEN: toxic epidermal necrolysis

1.2.2 Mechanisms of T Cell Activation by Drugs

The ability to recognise and respond to antigens is the fundamental basis of the immune system. An antigen is any molecule that can bind with high affinity to immunological receptors (Uetrecht and Naisbitt 2013). Antigens (bacterium, virus, drug-modified proteins) activate the innate immune system by stimulating pattern recognition receptors such as toll-like receptors and dendritic cells (DCs). The activated DCs act as antigen presenting cells (APCs) by taking up and processing the antigen into peptides. In lymphoid organs, such as lymph nodes, the DCs present these peptides on the cell surface in association with major histocompatibility complex (MHC) to T cell receptors (TCRs) on naive T cells. The DCs also secrete cytokines and express costimulatory receptors that contribute to polarisation of the immune response (e.g. Th1, Th2) (Uetrecht and Naisbitt 2013).

The interaction between the MHC, peptide and TCR determines the nature of the T cell response. In humans, the MHC encodes for human leukocyte antigens (HLA). Class I MHC consists of HLA-A, -B and -C whilst MHC class II is divided into HLA-DR, DQ and DP (The M. H. C. sequencing consortium 1999). MHC class I molecules are expressed by all nucleated cells and present peptides derived from intracellular compartments to CD8+ T cells (Neefjes et al. 2011). In contrast MHC class II expression is restricted to professional APCs but can also be induced in other cell types, including keratinocytes, by inflammatory processes such as psoriasis (Schonefuss et al. 2010). MHC class II molecules present peptides derived from extracellular compartments (Neefjes et al. 2011). Before entering the MHC class II processing pathway external proteins are internalised by phagocytosis and fused with lysosomes that contain proteases (Uetrecht and Naisbitt 2013). Type IV DHRs are characterised by activation of T cells by drugs or reactive metabolites (Pichler 2003). The hapten hypothesis, direct pharmacological interaction of drugs with immune receptors (PI) concept and the altered peptide repertoire model have been proposed as potential mechanisms for activation of T cells by drugs.

1.2.2.1 Hapten/Prohapten Hypothesis

The hapten hypothesis was originally outlined in the 1930s (Landsteiner and Jacobs 1935) and states that chemically reactive small molecules (including drugs and reactive metabolites) can act as a hapten and bind irreversibly to self proteins (Figure 1.1). The modified protein is recognised as an antigen and processed by APCs leading to generation of a pool of chemically modified peptides. These peptides are presented in association with MHC to TCRs which results in activation of the immune system (Park et al. 2001). Prohaptens are assumed to be chemically inactive and require bioactivation to chemically reactive compounds by metabolism (Knowles et al. 2000). Using mass spectrometry, immunogenic β-lactam-albumin and piperacillin-albumin conjugates (Whitaker et al. 2011) were characterised from patient plasma and found to activate drug-specific T cell responses from patients with known allergies (El-Ghaiesh et al. 2012; Jenkins et al. 2013).

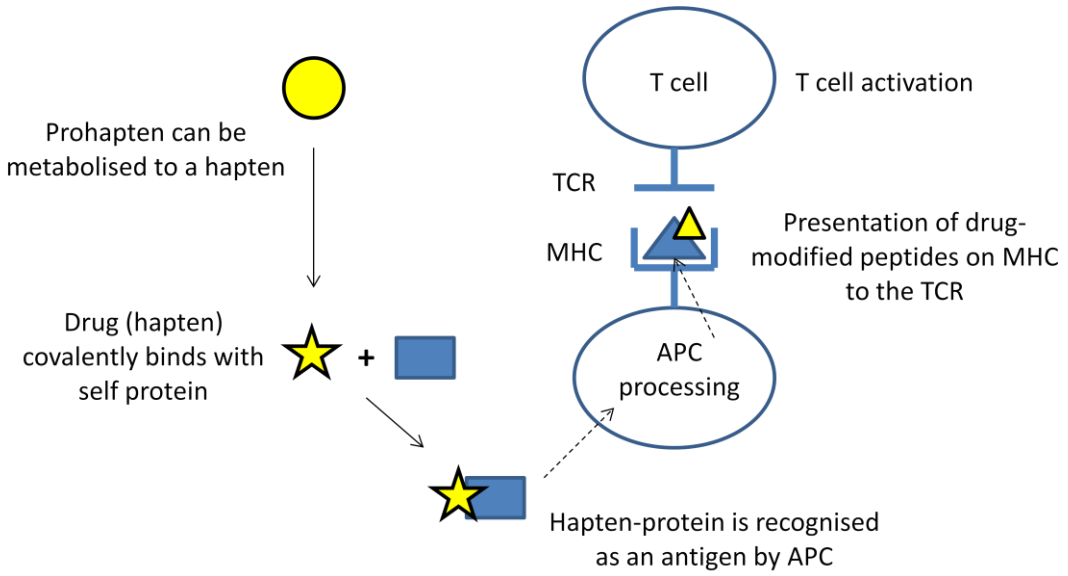


Figure 1.1 Prohapten/hapten model of T cell activation. The hapten hypothesis postulates that a drug or its metabolite (yellow star) is able to bind covalently with self-proteins (blue square). The hapten-protein complex is recognised as foreign by APCs which take up and process the hapten-protein complex producing a pool of drug modified peptides (yellow triangle and blue triangle). The drug modified peptide is presented in association with MHC and is recognised by the TCR as a neoantigen and activates the immune response.

1.2.2.2 Pharmacological Interaction of Drugs with Immune Receptors Concept

According to the PI model inert small molecules, such as drugs, are able to bind directly (non-covalently) to either the MHC or TCR (Figure 1.2). Provided that there is interaction between the TCR and MHC-peptide complex then the direct binding of drug to immune receptors is able to activate T cells (Pichler et al. 2006). Studies using lymphocytes from patients with CBZ and sulfamethoxazole hypersensitivity have revealed that these two drugs are able to directly activate lymphocytes without the need for processing and presentation by APC providing evidence for PI mechanism of DHR (Naisbitt et al. 2003; Castrejon et al. 2010).

1.2.2.3 Altered Peptide Repertoire Model

The altered peptide repertoire model states that low molecular weight drugs are able to bind noncovalently to regions within the antigen-binding cleft of HLA class I molecules. Binding of the drug changes the shape of the antigen-binding cleft resulting in alteration of the repertoire of peptides that are presented, which may now include self-peptides (Ostrov et al. 2012; Illing et al. 2012). A T cell response will be initiated if the patient is not tolerant to the new repertoire of peptides that are presented (Figure 1.2). Evidence for the altered peptide repertoire model has been derived from studies with abacavir (Norcross et al. 2012; Ostrov et al. 2012; Illing et al. 2012). Abacavir, an antiretroviral drug, is known to cause hypersensitivity reactions in patients positive for *HLA-B*57:01* (Hetherington et al. 2002). Abacavir binds to a specific pocket of the *HLA-B*57:01* molecule which alters its ability to bind large aromatic residues thereby leading to altered preference for peptides that bind to the HLA molecule. T cells from abacavir hypersensitive patients were able to recognise the altered peptide-HLA complexes (Norcross et al. 2012; Ostrov et al. 2012; Illing et al. 2012).

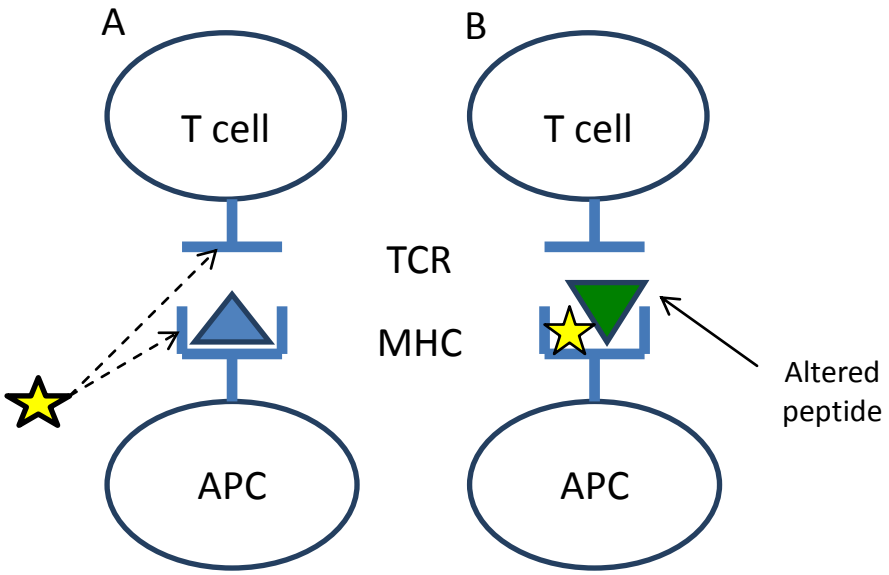


Figure 1.2 The PI and altered self-peptide repertoire models of T cell activation by small molecules. A. In the PI model the drug (yellow star) is able to bind noncovalently to either the TCR or MHC leading to activation of the immune system. B. In the altered self-peptide repertoire model the drug (yellow star) binds to the antigen binding cleft of the MHC. This results in presentation of novel peptides (green triangle) to the TCR and activation of the immune system.

1.2.3 Clinical Presentation of Type IV Drug Hypersensitivity Reactions

Type IV or delayed DHRs can affect many organ systems including the liver, kidneys, blood vessels and skin (Demoly et al. 2014). The focus of this thesis is cutaneous manifestations of delayed DHRs. Typically the skin is the most commonly affected organ because of its large surface area, extensive blood supply and fully competent immune system which usually helps to protect the body from harmful external pathogens (Bangert et al. 2011). The severity of cutaneous manifestations of type IV DHRs can range from mild self-limiting MPE to severe and life threatening conditions such as HSS, SJS, TEN and acute generalised exanthematous pustulosis (AGEP) (Pirmohamed et al. 2011).

1.2.3.1 Maculopapular Exanthema

Maculopapular rashes are the most common type of drug-induced skin rash and account for more than 90% of all cutaneous ADRs (Hunziker et al. 1997). The time to onset is typically 1-2 weeks after starting treatment and in the absence of other clinical symptoms, these drug rashes are not serious, and can resolve even if the drug is continued (Valeyrie-Allanore et al. 2007). However, it is important to remember that mild rashes such as MPE may progress to more severe conditions such as SJS or TEN and at present it is not possible to predict progression. Current clinical practice is to recommend withdrawal of drug that causes rash as this leads to better outcomes (Garcia-Doval et al. 2000).

1.2.3.2 Hypersensitivity Syndrome

HSS is a severe ADR with considerable morbidity and mortality that affects multiple organs including the kidney, liver, lung, gastrointestinal tract, central nervous and lymphoid systems. Because of its variable clinical presentation it has been hard to classify and phenotype patients. HSS has also been called drug-induced hypersensitivity syndrome (DIHS), drug reaction with eosinophilia and systemic symptoms (DRESS) and drug-induced delayed multiorgan hypersensitivity syndrome (Pirmohamed et al. 2011). Patients typically present within 3 months of starting treatment and skin rash is the most commonly reported feature along with fever, hypereosinophilia, liver involvement and lymphadenopathy (Cacoub et al. 2011). Anticonvulsants are the most common trigger for HSS accounting for about one third of cases followed by allopurinol and antibiotics (Kardaun et al. 2013). Several viral infections including Human Immunodeficiency Virus (HIV), Human Herpes Virus (HHV), Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) have been identified as risk factors for development of HSS (Dodiuk-Gad et al. 2014). The incidence of HSS secondary to phenytoin or CBZ has been estimated to be between 1.0-4.5 per 10,000 prescriptions (Tennis and Stern 1997). The overall incidence of long term sequelae was 11.5% in a Taiwanese cohort at least 1 year after resolution of symptoms. The most common complications were autoimmune disease (e.g. Graves's disease, type 1 diabetes) amongst younger patients and end organ failure (e.g. renal failure) in older patients (Chen et al. 2013). The mortality associated with

HSS has been estimated to range between 1.7% and 5.0%, although death rates of up to 10% have been reported when anticonvulsants are the culprit drugs (Dodiuk-Gad et al. 2014).

1.2.3.3 Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis

SJS and TEN are considered to be the same condition differentiated by the degree of skin detachment. Skin detachment of 1-10% is diagnosed as SJS whilst detachment greater than 30% is classified as TEN. Skin detachment between 10-30% is classified as overlap syndrome. Other clinical signs and symptoms include severe, often haemorrhagic, erosions of mucous membranes, severe pain and tenderness in the skin, target lesions to trunk and face and systemic manifestations (fever, deranged liver function tests, and lymphopaenia) (Bastuji-Garin et al. 1993). SJS and TEN are rare ADRs with an annual incidence of 1.2-6.0 and 0.4-1.2 per million individuals, respectively (Forman et al. 2002; Paquet and Pierard 2010). Drug exposure is the most common cause of SJS/TEN with antimicrobial sulphonamides, anticonvulsants, allopurinol and non-steroidal anti-inflammatory drugs (NSAIDs) most often implicated (Dodiuk-Gad et al. 2014). Most, but not all, cases of SJS/TEN present within 8 weeks of starting treatment with the culprit drug (Revuz and Roujeau 1996). The mortality rate of SJS/TEN is significant and has been reported to be 23% at 6 weeks and 34% at 1 year for TEN (Sekula et al. 2013; Lalosevic et al. 2015) and 5% for SJS (Hsu et al. 2016). Up to 80% of patients that survive acute TEN will suffer with long term complications which include scarring, visual loss, chronic kidney disease, intestinal strictures and bronchiectasis (Schwartz et al. 2013; Quirke et al. 2015).

1.2.3.4 Acute Generalised Exanthematous Pustulosis

The incidence of AGEP is estimated to be 1-5 cases/million/year (Sidoroff et al. 2001). Typical features include an acute widespread oedematous erythema followed by a sterile pustular eruption. The pustules occur initially in the neck, groin and axillae and become more widespread as the reaction progresses. Other features include fever and neutrophilia with mild eosinophilia (Pirmohamed et al. 2011). Clinically AGEP resembles pustular psoriasis and there have been reported

links between the two conditions (Roujeau et al. 1991). Greater than 90% of cases of AGEP are triggered by drugs and the most commonly associated drugs are antibiotics, NSAIDs and anticonvulsants (Sidoroff et al. 2001). Onset of AGEP after drug administration is faster than HSS and SJS/TEN; typically occurring within a few days (Pirmohamed et al. 2011). Outcome in these patients is good with resolution after 15 days and mortality less than 5% after drug withdrawal (Hotz et al. 2013).

1.2.4 Diagnosis of Delayed Drug Hypersensitivity Reactions

In cases of suspected DHR, the clinician needs to make an assessment of causality based upon the type and timing of onset of the reaction and its relationship with the suspected culprit drug. The aim is to identify the causative agent so that it can be avoided in future (Schrijvers et al. 2015). However, clinical history can be unreliable as patients usually take multiple medications simultaneously and there is significant heterogeneity in clinical presentation with many non-specific features (Demoly et al. 2014). This can lead to inappropriate exclusion of multiple drug classes and switch towards suboptimal therapies. Although several *in vivo* and *in vitro* diagnostic tests are available for the diagnosis of delayed DHRs, none are ideal in the clinical environment.

1.2.4.1 Skin Testing

Skin testing involves application of the suspected antigen onto the skin by a patch or intradermal injection and is particularly effective for detection of IgE-mediated hypersensitivity reactions (Demoly et al. 2014). Patch and intradermal testing have been used for the diagnosis of delayed hypersensitivity reactions but for most drugs, except beta-lactam antibiotics, standardised and validated test concentrations for use in testing protocols are not available and its use is limited (Brockow et al. 2013).

1.2.4.2 Drug Provocation Testing

Drug provocation testing (DPT) involves re-administration of the suspected drug antigen to the patient in a controlled environment at graded doses. It is usually recommended after full evaluation of hypersensitivity in order to demonstrate

tolerance to a particular drug rather than for confirmation of a DHR (Joint Task Force on Practice Parameters; American Academy of Allergy 2010). DPT is limited because it is contraindicated in patients who have experienced severe cutaneous or systemic reactions (e.g. HSS, SJS/TEN, AGEP and vasculitis) and requires expertly trained staff and resuscitation equipment (Demoly et al. 2014).

1.2.4.3 Lymphocyte Transformation Test

The lymphocyte transformation test (LTT) is an *in vitro* lymphocyte proliferation assay that is used for the detection of T cells that are sensitised to specific drugs or metabolites (Nyfeler and Pichler 1997). It is one of the most widely used tests for the diagnosis of T cell mediated drug hypersensitivity (Porebski et al. 2011). The test has also been used for diagnosis of immediate DHRs (Luque et al. 2001), drug-induced pancreatitis (Straumann et al. 1998) and hepatitis (Maria and Victorino 1997). In the LTT, peripheral blood mononuclear cells (PBMCs) are obtained from a drug sensitised patient and cultured in the presence of multiple concentrations of the suspected drug, stable metabolite or drug-protein conjugate (if available). Proliferation is quantified by measuring the uptake of tritium-labelled thymidine (^{3}H-thymidine) into the DNA of activated lymphocytes and calculation of the stimulation index (SI). The SI is the ratio of cell proliferation [counts per minute (cpm)] with antigen divided by the background proliferation (cpm) in absence of antigen. A SI ≥ 2 indicates a drug-specific T cell response (Naisbitt et al. 2014). In a retrospective analysis of 923 cases of patients with probable/definite drug hypersensitivity, the LTT demonstrated a sensitivity of 78% and specificity of 85% (Nyfeler and Pichler 1997). However, the LTT does have some limitations. Technically it is difficult to perform because it requires radioactivity and sterile culture for 6 days. The LTT is negative in many patients with SJS/TEN as these reactions are CD8+ T cell mediated and CD8+ T cells do not proliferate readily in the conditions of the LTT (Porebski et al. 2013). Most importantly, a negative LTT result cannot exclude drug hypersensitivity as it is impossible to include all possible antigens in the test (e.g. unstable reactive metabolites), and the assay is typically performed using the parent drug alone (Naisbitt et al. 2014).

1.2.4.4 Enzyme-Linked Immunosorbent Spot Assay

Enzyme-linked immunosorbent spot (ELISpot) is an antibody based assay used for the detection of cytokine secreting cells (e.g. TNF-α, IL-5 and IL-13) or release of cytotoxic proteases (e.g. granulysin, perforin and Fas ligand) from T cells that have been isolated from patients with drug hypersensitivity (Naisbitt et al. 2014). Briefly, PBMCs are isolated from patients and cultured in the presence of the suspected drug. Cells that secrete the cytokine of interest are displayed as spots with the number of spots corresponding to the number of cytokine secreting T cells. Data from cells incubated with drug are then compared with control T cells that have not been incubated with the drug (Earnshaw et al. 2014). Elispot assays have several advantages over the LTT including the lack of requirement for radioactive substances and shorter incubation period. The incubation period for ELISpot assays is shorter than the LTT, between 48-72 h, as production of cytokines by T cells occurs as soon as they are activated (Porebski et al. 2011). Measurement of cytokine activity provides basic insight into pathophysiological mechanisms of delayed DHRs and there is data to suggest that measuring the level of four cytokines (IL-2, IL-5, IL-13 and IFN-γ) may improve sensitivity and specificity for *in vitro* diagnosis of delayed DHRs (Lochmatter et al. 2009).

1.2.4.5 Flow Cytometry

After stimulation, T cells express a number of surface molecules including CD25, CD69 and CD71 (Wieland and Shipkova 2015). Flow cytometry has been utilised in DHRs to characterise T cell subsets by surface receptors and to measure specific cytokine production (e.g. IL-5, IL-10 and IFN-γ) (Martin et al. 2010). Furthermore, specific proliferation of drug specific T cells can be investigated using flow cytometry and fluorescent dyes such as carboxyfluorescin siccinimidyl ester (CFSE) to stain cell samples that contain drug specific T cells. The intensity of the CFSE is reduced by half following each cell division identifying the dividing population of antigen-specific T cells for further evaluation (Beeler et al. 2006).

1.2.4.6 T cell Cloning

T cell cloning experiments provide a valuable alternative for the detection of drug-specific T cells when other assays yield negative results. T cell clones (TCCs) are generated by serial dilution followed by repetitive mitogen stimulation. Several hundred single cell populations are generated and antigen-specificity is measured by culture with EBV-transformed B cells and antigen for 48 h. T cell proliferation is determined by incorporation of ^{3}H-thymidine. Clones that are antigen-specific (SI ≥ 2) are expanded and can be characterised with respect to phenotype, specificity and functionality by the techniques described earlier (Naisbitt et al. 2014). T cell cloning is a time-consuming process, taking weeks to months, so is unsuitable for acute diagnosis of delayed DHRs.

1.3 Carbamazepine

1.3.1 Indication

CBZ is a tricyclic anticonvulsant that is primarily used for the treatment of partial and secondary generalised seizures in epilepsy (Marson et al. 2007). It was originally licensed for epilepsy in the UK in 1965 and remains one of the most frequently prescribed anticonvulsants (Moran et al. 2004). The indications for CBZ have widened and it is now also prescribed for the treatment of neuropathic pain and psychiatric disorders (Bialer 2012).

1.3.2 Mechanism of Action

The mechanism of action of CBZ is incompletely understood but its main target is believed to be voltage-dependent sodium channels in the neuronal membrane (Ambrosio et al. 2002). CBZ is able to block high frequency repetitive spike firing, which occurs during the spread of seizure activity, leading to stabilisation of the neuronal membrane. The effect of CBZ on sodium channels is both voltage- and use- dependent so that only hyperactive neurons are affected. At hyperpolarized membrane potentials CBZ blocks sodium channels only weakly but when the neuron is depolarized, there is marked increase in inhibition. The inhibitory potency also accumulates with repetitive or prolonged activation. The effect on action potentials

translates into reduced neurotransmitter output at synapses, with glutamate release more strongly inhibited than that of other neurotransmitters such as GABA (Rogawski and Loscher 2004).

1.3.3 Pharmacokinetics

CBZ is typically used as an oral formulation and is absorbed slowly from the GI tract with a bioavailability of approximately 75% (Tolbert et al. 2015). Peak plasma concentrations of CBZ are typically reached between 4 and 8 h after oral administration and plateau for 10-30 h before declining (Rawlins et al. 1975). Plasma protein binding of CBZ reaches 70% to 80%. Carbamazepine 10,11-epoxide (CBZE) is the major metabolite of CBZ metabolism. It retains anticonvulsant activity and has plasma protein binding between 50% and 60%. CBZ and CBZE are both distributed rapidly and readily penetrate the blood brain barrier. Cerebrospinal fluid concentrations of CBZ have been reported to be between 17% to 31% of total plasma concentrations and CBZE levels are approximately 45% to 55% of corresponding plasma levels (Spina 2002).

CBZ is extensively metabolised in the body, with less than 2% of the oral dose eliminated unchanged in the urine (Faigle 1975). Metabolism occurs mainly in the liver and involves oxidation, hydroxylation and conjugation, catalysed by a number of enzymes including cytochrome P450 enzymes (CYP), microsomal epoxide hydrolase (mEH) and glucuronyltransferases. More than 30 different metabolites of CBZ have been reported (Lertratanangkoon and Horning 1982). Three major pathways account for 80% to 90% of total metabolism: the epoxide-diol pathway, aromatic hydroxylation and conjugation reactions (Figure 1.3) (Spina 2002). The epoxide-diol pathway is quantitatively the most important biotransformation pathway where CBZ is oxidised, primarily by CYP3A4 and CYP2B6, to the chemically stable CBZE (Kerr et al. 1994). CBZE is then extensively hydrolysed to the inactive metabolite *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (DiOH-CBZ) by mEH (Kitteringham et al. 1996).

The second CBZ metabolism pathway involves hydroxylation at different positions of the six-membered aromatic rings leading to the formation of several phenolic products: 1-, 2-, 3- and 4-hydroxycarbazmepine. These reactions are catalysed by various CYP enzymes including CYP3A4, CYP2E1, CYP2B6 and CYP1A2 (Pearce et al. 2002). There is evidence that reactive arene oxides are formed as intermediates during hydroxylation of the aromatic ring (Madden et al. 1996; Bu et al. 2007). These phenolic compounds are then conjugated with glucuronic acid by uridine diphosphate glucuronosyltransferase (UGT) 2B7 before excretion in urine (Staines et al. 2004). Direct conjugation of CBZ with glucuronic acid represents the third most important pathway in CBZ biotransformation. Glucuronidation can also occur as a secondary metabolic process in almost all CBZ metabolites that contain a free hydroxyl group to form their *O*-glucuronides (Spina 2002). Other reactive metabolites formed by CBZ metabolism have been reported and include CBZ-iminoquinone (Pearce et al. 2005) and 9-acridine carboxaldehyde (Furst et al. 1995) (Figure 1.3).

During long term therapy CBZ is able to induce its own metabolism (autoinduction) (Kudriakova et al. 1992) and concomitant therapy with other enzyme inducing anticonvulsants (e.g. phenytoin and phenobarbital) is able to further induce metabolism (heteroinduction) (Eichelbaum et al. 1979). Autoinduction occurs within 24 h of starting therapy or change in dose and the process is usually complete after one week (Kudriakova et al. 1992; Bernus et al. 1994). The autoinduction process primarily involves the epoxide-diol pathway with both the epoxidation and subsequent epoxide hydrolysis affected although the latter to a lesser extent (Bernus et al. 1996). The exact cellular mechanism of autoinduction is not well understood but may involve nuclear hormone receptors such as the pregnane X receptor (PXR) and hepatocyte nuclear factor 4α (Saruwatari et al. 2014). CBZ has been shown to be able to induce a broad spectrum of genes in the human liver that are involved with drug metabolism and drug transport (Oscarson et al. 2006). Reversal of autoinduction is rapid taking between 3-4 days if CBZ therapy is withdrawn meaning patients may need a reduced dose when restarting therapy after discontinuation (e.g. pre-operative EEG) (Schaffler et al. 1994).

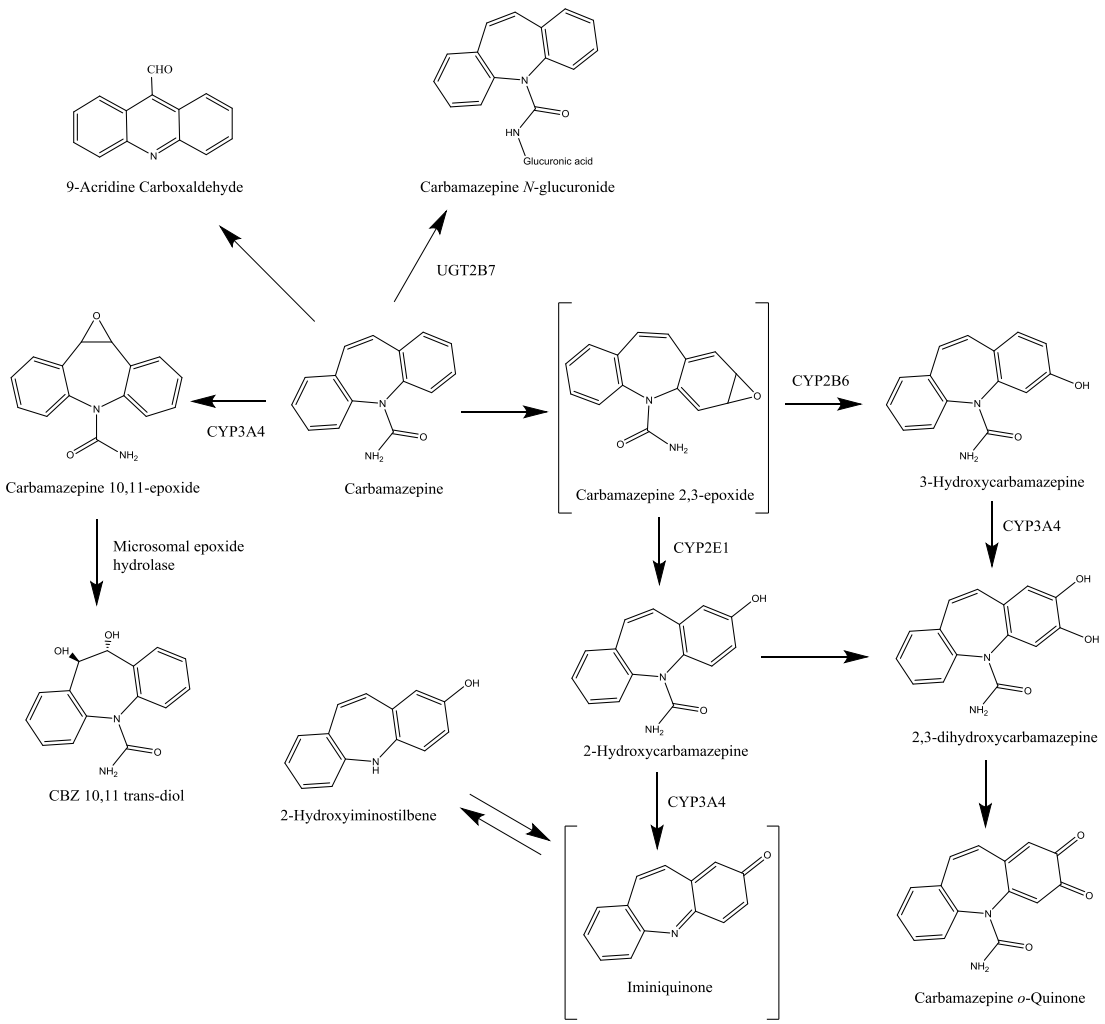


Figure 1.3 Proposed major pathways of bioactivation of CBZ in humans and the enzymes catalysing the major metabolic reactions. (CYP: cytochrome P450, UGT: uridine diphosphate glucuronosyltransferase)

Approximately 28% of an oral ^{14}C-labeled CBZ dose is excreted in the faeces (Faigle 1975) and 1% eliminated in the bile within 72 h (Terhaag et al. 1978). The major elimination pathway for CBZ is in the urine mainly as metabolites. 20% to 60% of the daily dose is excreted as DiOH-CBZ, 5% to 11% as 9-hydroxymethyl-10-carbamoyl acridan, 5% to 10% as the phenolic metabolites, 1 to 2% as CBZE and 0.5% as unchanged CBZ (Spina 2002).

1.3.4 Carbamazepine Hypersensitivity Reactions

1.3.4.1 Overview

CBZ is generally well tolerated but up to 10% of patients starting treatment experience a hypersensitivity reaction (Marson et al. 2007). The majority of these patients present with a skin rash which typically presents after a few days and can resolve spontaneously without further intervention. However, some patients present with more severe reactions that include HSS, SJS and TEN (Yip et al. 2012). The current clinical guidance is that patients should stop CBZ therapy if a rash appears as it is not possible to predict which patients will progress to more severe hypersensitivity and early discontinuation of the culprit drug leads to improved clinical outcomes (Garcia-Doval et al. 2000). The incidence of CBZ-induced HSS has an estimated frequency of 1.0-4.1 per 10,000 exposures (Tennis and Stern 1997). The estimated incidence of CBZ-induced SJS/TEN is greater in Asian populations (Chen et al. 2011) than European populations (McCormack et al. 2011), 25 cases per 10,000 compared with 1 to 6 cases per 10,000, respectively.

1.3.4.2 Pharmacogenetic Associations

Predisposition to CBZ hypersensitivity reactions is thought to have a genetic component because of cases reported in families and monozygotic twins (Edwards et al. 1999). Chemically reactive metabolites generated by metabolism of CBZ, such as epoxides and arene oxides, can cause direct cellular toxicity or lead to generation of neo-antigens that could activate the immune system leading to hypersensitivity reactions (Knowles et al. 2000). Metabolism of CBZ involves multiple CYPs and detoxification pathways (Figure 1.3). Genetic variation in these enzymes leads to altered metabolism of CBZ and could possibly increase the formation of chemically reactive metabolites (Jaramillo et al. 2014). Microsomal epoxide hydrolase is responsible for biotransformation of active CBZE to inactive DiOH-CBZ. A recent study has shown that a single nucleotide polymorphism (SNP) (c.337T>C) in *EPHX1*, the gene that encodes mEH, was significantly associated with development of CBZ-induced SJS/TEN in a Han Chinese population (He et al. 2014). However, a similar study in patients with heterogeneous clinical manifestations of CBZ hypersensitivity,

including SJS/TEN, hepatitis and pneumonitis, did not detect an association between genetic variation in *EPHX1* and susceptibility to CBZ hypersensitivity (Green et al. 1995). Polymorphisms in *ABCB1*, *CYP3A4*, *FAS, SCN1A, MICA* and *BAG6* were not associated with susceptibility to CBZ-induced SJS/TEN in a Han Chinese cohort from Taiwan (He et al. 2014). CBZ is also able to covalently modify CYP3A4 leading to a reduction in its catalytic activity (Kang et al. 2008). A summary of studies that have investigated the role of SNPs in metabolism enzymes and drug transporters involved in the metabolism of CBZ is outlined in Table 1.4.

A study in Han Chinese patients from Taiwan reported a very strong association (OR > 2,000) between patients with CBZ-induced SJS/TEN and the *HLA-B*15:02* allele (Chung et al. 2004). The association has subsequently been replicated in Han Chinese patients from other countries, including Hong Kong and China, as well as Thai, Malaysian and Indian patients (Yip et al. 2012). The association was not replicated in Caucasian, Japanese or Korean populations (Alfirevic et al. 2006; Kaniwa et al. 2008; Kim et al. 2011). The association between CBZ hypersensitivity and *HLA-B*15:02* is both ethnicity and phenotype specific. Ethnic specificity may be explained by the differing background frequency of *HLA-B*15:02* in different populations. The prevalence of *HLA-B*15:02* is highest amongst Asian populations (0.057-0.145 in Han Chinese, 0.085-0.275 in Thais and 0.12-0.157 in Malaysians) and lowest in Europeans (0.01-0.02), Japanese (0.002), and Koreans (0.004) (Lim et al. 2008). Subsequently, the drug label has been amended and physicians are warned and advised to undertake genotyping before initiation of CBZ treatment in patients of Asian descent (Ferrell and McLeod 2008). The utility of genotype testing has been confirmed in a prospective study in a Taiwanese population where pre-prescription genotyping reduced the incidence of CBZ-induced SJS/TEN from ten expected cases to zero (Chen et al. 2011). Despite these advances it is important that physicians receive education regarding pharmacogenetic tests. In Hong Kong introduction of *HLA-B*15:02* testing led to reduction in CBZ prescribing from 16.2% to 2.6% of all new AEDs and an increase in use of alternative AEDs such as phenytoin. The net effect was no reduction in overall incidence of AED-induced SJS as the alternative AEDs are also associated with risk of SJS/TEN (Chen et al. 2014).

More recently, a significant association between *HLA-A*31:01* and all phenotypes of CBZ hypersensitivity was reported in both Caucasian and Japanese patients (McCormack et al. 2011; Ozeki et al. 2011). Although the association was replicated in a Korean population (Kim et al. 2011), it was not significantly associated in Norwegian patients with non-bullous CBZ hypersensitivity (Shirzadi et al. 2015). Screening for *HLA-A*31:01* is not currently part of clinical practice although studies suggest that the number needed to test in order to avoid a hypersensitivity reaction ranges between 47 and 91 and an economic evaluation from the perspective of the NHS suggests that screening would be cost-effective (Plumpton et al. 2015). It is unclear at present why *HLA-B*15:02* predisposes only to SJS/TEN while *HLA-A*31:01* is a susceptibility factor for all forms of CBZ hypersensitivity and further functional studies are required to define the causal pathways.

Table 1.4 Studies that have investigated single nucleotide polymorphisms associated with pharmacokinetics of carbamazepine

Study	Population	No. of Subjects	SNP ID	Gene	Nucleotide change/allele	Effect of Polymorphism
(Ma et al. 2015)	Chinese	166	rs2242480	*CYP3A4*	*1G	NS
			rs776746	*CYP3A5*	*3	NS
			rs1051740	*EPHX1*	c.337T>C	Higher CBZ maintenance dose, lower lnCDR for CBZ and higher CBZE concentration
			rs7439366	UGT2B7	c.802T>C	Higher CBZ maintenance dose if also a carrier of *ABCC2* c.1249G>A
			rs3740066 rs2273697	*ABCC2*	c.3972C>T c.1249G>A	Higher CBZ maintenance dose and higher CBZE concentration Higher CBZ maintenance dose if also a carrier of *UGT2B7* c.802T>C
(Wang et al. 2015)	Chinese	88	rs4646440 rs2242480	*CYP3A4*	c.1023+608C>T *1G	NS NS
			rs15524 rs776746	*CYP3A5*	*1D *3	Higher dose adjusted CBZ and CBZE concentration in subjects on concomitant PHT/PB Higher dose adjusted CBZ and CBZE concentration

						in subjects on concomitant PHT/PB
			rs1045642 rs2032582 rs10234411 rs1128503	*ABCB1*	c.3435C>T c.2667G>T 2481+882A>T c.1236T>C	NS CBZ/CBZE ratio in subjects on concomitant PHT/PB CBZ/CBZE ratio in subjects on concomitant PHT/PB NS
(Caruso et al. 2014)	Italian	50	rs1051740 rs2234922	*EPHX1*	c.337T>C c.416A>G	NS NS
			rs35599367	*CYP3A4*	*22	NS
(Zhu et al. 2014)	Chinese	210	rs35599367 rs2242480	*CYP3A4*	*22 *1G	NS Lower adjusted levels of CBZ and CBZE
			rs776746	*CYP3A5*	*3	Higher dose adjusted levels of CBZ
			rs1057868	*POR*	*28	NS
			rs2234922 rs3738046	*EPHX1*	c.416A>G c.128G>C	Lower dose adjusted levels of DiOH-CBZ and DiOH-CBZ:CBZE ratio Higher DiOH-CBZ:CBZE ratio

			rs1051740		c.337T>C	NS
(Panomvana et al. 2013)	Thai	70	rs776746	*CYP3A5*	*3	Trend towards reduced clearance (not significant)
(Puranik et al. 2013)	African-American Caucasian	88	rs2740574	*CYP3A4*	*1B	Reduced clearance
			rs776746	*CYP3A5*	*3	Longer half life for CBZ (African-Americans)
			rs1051740	*EPHX1*	c.337T>C	Lower DiOH-CBZ:CBZE ratio (African-Americans)
			rs2234922		c.416A>G	NS
			rs7439366	*UGT2B7*	c.802T>C	NS
			rs11302069		c.1090+155delA	NS
			rs28365062		c.735A>G	NS
			rs28365063		c.372A>G	Increased clearance (African-American)
			rs4292394		c.1059C>G	NS
			rs1045642	*ABCB1*	c.3435C>T	NS
			rs1128503		c.1236T>C	Higher clearance (African-Americans)
			rs2032582		c.2677G>T	NS
			rs4148734		c.702+1789C>T	NS

			rs4148739 rs4148740		c.2482-236A>G c.2686-1911T>C	Higher DiOH-CBZ:CBZE ratio (African-American) Higher DiOH-CBZ:CBZE ratio (African-American)
			rs2273697 rs3740066 rs4148386 rs8187710	*ABCC2*	c.1249G>A c.3972C>A 208-3523G>A c.4544G>A	Higher clearance (Caucasians) Higher CBZE:CBZ ratio (Caucasian male) Reduced clearance and reduced CBZE:CBZ ratio (African-American) NS
			rs1523127 rs1523130 rs2461817 rs3814055 rs4688040 rs7643645	*NR1I2*	c.-131C>A c.-1663T>C c.-22-1425A>C c.-1135C>T c.-22-1985G>T c.-22-579A>G	NS NS Increased CBZE:CBZ ratio and reduced clearance (African-American) Reduced clearance Increased clearance (African-American) Increased CBZE:CBZ ratio
(Yun et al. 2013)	Chinese	83	rs1051740 rs2234922	*EPHX1*	c.337T>C c.416A>G	NS Higher adjusted plasma CBZ levels
			rs2242480	*CYP3A4*	*1G	NS

(Hung et al. 2012)	Taiwan	234	rs1051740	*EPHX1*	c.337T>C	Higher maintenance dosage
			rs2234922		c.416A>G	NS
			rs7668258	*UGT2B7*	c.-161T>C	NS
			rs7438135		c.-900G>A	NS
			rs28365062		c.735A>G	NS
			rs7439366		c.802T>C	NS
			rs1128503	*ABCB1*	c.1236T>C	NS
			rs2032582		c.2677G>T	NS
			rs1045642		c.3435C>T	NS
			rs717620	*ABCC2*	c.-24C>T	NS
			rs2273697		c.3972C>T	NS
(Sterjev et al. 2012)	Macedonian	162	rs1045642	*ABCB1*	c.3435C>T	Higher CBZ plasma levels
(Meng et al. 2011a)	Chinese	84	rs1045642	*ABCB1*	c.3435C>T	Lower plasma CBZ levels
			rs1128503		c.1236T>C	NS
			rs2032582		c.2677G>T	NS
(Park et al. 2009)	Korean	35	rs776746	*CYP3A5*	*3	Reduced clearance

(lnCDR- natural log dose adjusted concentration, NS- not significant, PB- phenobarbital, PHT- phenytoin

1.3.4.3 Functional Studies

The clinical features of CBZ hypersensitivity reactions suggest an immune-mediated aetiology, and the discovery of drug-specific T cells in hypersensitive individuals is consistent with this hypothesis (Naisbitt et al. 2003; Wu et al. 2006; Wu et al. 2007). Drug-specific T cells have been generated against CBZ and several CBZ metabolites including CBZE (Wu et al. 2006). Stimulation of these T cells occurred in the absence of metabolism and antigen processing suggesting that CBZ is able to activate T cells by direct interaction in accordance with the PI hypothesis (Wu et al. 2006; Wu et al. 2007). Because of the heterogeneity of clinical presentation of CBZ hypersensitivity reactions it is possible that several T cell activation pathways are involved. The metabolism of CBZ is postulated to generate chemically reactive metabolites including epoxides, arene oxides and iminoquinones (Figure 1.3) (Furst et al. 1995; Pearce et al. 2002; Pearce et al. 2005). These reactive metabolites could act as haptens and modify endogenous proteins leading to the generation of chemically modified peptides as outlined by the hapten hypothesis. Two glutathione (GSH) adducts were observed when GSH was incubated directly with CBZE (Bu et al. 2005). A third GSH adduct was identified in incubations containing CBZ and human liver microsomes (HLM) and postulated to be formed by an arene oxide intermediate of CBZ metabolism (Bu et al. 2007). CBZ is also able to form adducts with CYP3A4 which led to reduced catalytic activity of the enzyme (Kang et al. 2008). Anti-CYP3A antibodies have been detected in patients with aromatic anticonvulsant-induced hypersensitivity reactions, including CBZ, although the immunogenicity and their role in hypersensitivity reactions is not understood (Leeder et al. 1996). The minor metabolite, 9-acridine carboxaldehyde, has been shown to be able to covalently modify neutrophils and inhibit neutrophil function at high concentrations (Furst et al. 1995; Furst and Uetrecht 1995).

There has been evidence to suggest that CBZ-specific T cells may be activated by an altered peptide repertoire hypothesis. CBZ has been shown to be able to bind directly to the peptide binding groove of *HLA-B*15:02* and activate T cells but only in the presence of endogenous peptides (Wei et al. 2012). A separate study reported that CBZ binding to *HLA-B*15:02* leads to an alteration in the repertoire of

presented self peptides (Illing et al. 2012). A more recent structural model suggests that CBZ is located at the interface between the *HLA-B*15:02*/peptide and TCR, directly contacts the antigen peptide, and is bound within the TCR pocket (Zhou et al. 2015). Further evidence to support interaction between CBZ and TCR is the observation that CBZ-induced SJS/TEN patients have restricted TCR use (Ko et al. 2011). CBZ cytotoxicity could only be primed from PBMCs of individuals who were carriers of both *HLA-B*15:02* and TCR clonotype Vβ-11-ISGSY. This may explain why a significant number of patients who are *HLA-B*15:02* carriers do not experience SJS/TEN when prescribed CBZ as a specific TCR clonotype is also required to initiate hypersensitivity.

1.4 Liquid Chromatography Tandem Mass Spectrometry

1.4.1 Overview

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an analytical chemistry technique that combines the separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry enabling high specificity analysis of complex mixtures (Pitt 2009). Over the last 10-15 years the technology has grown in popularity in both scientific and clinical laboratories and has many applications including therapeutic drug monitoring (TDM), toxicology, metabolomics and proteomics (Seidler et al. 2010; Adaway et al. 2015).

1.4.2 High Performance Liquid Chromatography

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. Separation of components in a sample involves the movement of the sample through a HPLC column (stationary phase) in conjunction with mobile phase. Distinct molecules in the sample will interact differently with the stationary phase leading to separation of the components. Components that interact more strongly with the stationary phase will move more slowly through the column. Eventually each component of the analyte mixture elutes from the column and is detected as a peak on the data

display. High performance liquid chromatrography (HPLC) is a type of chromatography that is used to separate compounds that have been dissolved in solution (Kupiec 2004).

A typical system for HPLC will include a pump, injector, column, detection system and data acquisition system (Figure 1.4). The injector is responsible for injecting the sample of interest into the HPLC system with high reproducibility and under high pressure (up to 4000 psi). The sample is then pumped along with mobile phase through the HPLC column which is tightly packed. A steady pump pressure is needed to ensure reproducibility and accuracy (1000 - 2000 psi). Highest purity solvents and inorganic salts should be used to make the mobile phase to ensure reproducibility and accuracy. Wide selections of HPLC columns are available commercially with varying lengths, pore size and different stationary phases. Choice of column is dependent on the analyte of interest. A detector is used to sense the presence of a compound passing through the column. Typical detectors in HPLC include ultraviolet, refractive index and fluorescence (Kupiec 2004). In HPLC-MS/MS the chromatographically separated compounds are further characterised using MS.

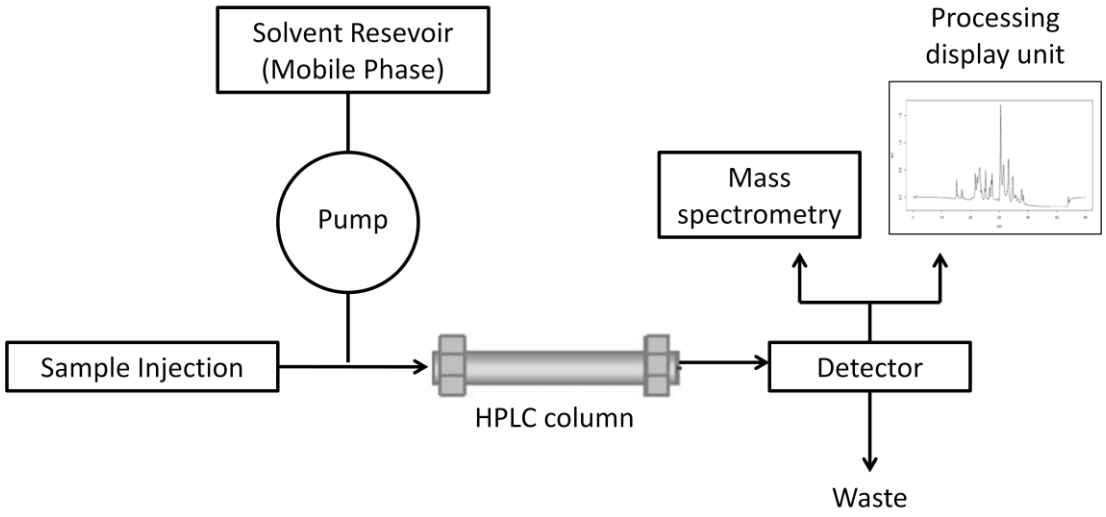


Figure 1.4 Schematic representation of high performance liquid chromatography. The sample is injected and pumped through the HPLC column at high pressure with mobile phase. Analytes are separated according to their interactions with the stationary phase in the HPLC column. As each analyte elutes from the column it is detected as a peak on the data display or ionised for analysis by mass spectrometry. (HPLC- high performance liquid chromatography).

1.4.3 Mass Spectrometry

Mass spectrometers function by applying a charge (ionisation) to analyte molecules, with subsequent analysis of the ions and any fragment ions generated, on the basis of their mass to charge ratio (*m/z*) (Pitt 2009). In HPLC-MS/MS, HPLC is used to separate the mixture before delivery to the MS instrument. The eluent from the HPLC is ionised, typically using electrospray ionization (ESI) (Whitehouse et al. 1985). The ionised analytes then pass into the mass analyser. Tandem MS involves two or more stages of independent mass analysis and can be achieved using a triple quadrupole mass analyser. Each quadrupole consists of four parallel metal rods that are able to modify their voltages so that only a narrow band of *m/z* values are able to travel along the axis of the rods at a particular time. The quadrupole can scan a range of *m/z* values or be programmed to monitor for specific *m/z* (Pitt 2009). In a triple quadrupole mass spectrometer the first quadrupole (Q1) is used to select a known parent ion based on *m/z*. The selected ion then passes into a collision cell (Q2), which contains a low pressure inert gas such as nitrogen or argon, where collision between the analyte and inert gas molecules result in fragmentation. The third quadrupole (Q3) can then also be programmed to isolate a specific product ion based on *m/z* and is termed a transition (Adaway et al. 2015).

For example, 25-hydroxy vitamin D3 produces a major $M+H^+$ ion of 401 *m/z* during ESI that loses water in the collision cell to produce a major 383 *m/z* product ion (Chen et al. 2008). Highly sensitive detection of 25-hydroxy vitamin D3 can be achieved by setting Q1 and Q3 to transmit ions of only 401 and 383 *m/z*, respectively. Monitoring of a single transition as described above ($401 \rightarrow 383$) is called selected reaction monitoring (Adaway et al. 2015). The first and third quadrupoles can also be set up to simultaneously monitor several *m/z* transitions in order to detect multiple targeted analyte transitions in a mixture and is termed multiple reaction monitoring (MRM) (Adaway et al. 2015). Product ion scanning involves fixing the *m/z* in the first quadrupole and application of scanning mode for product ions in the third quadrupole which generates structural information or unique “finger print” of the analyte that can be used to confirm its identity with great certainty (Pitt 2009).

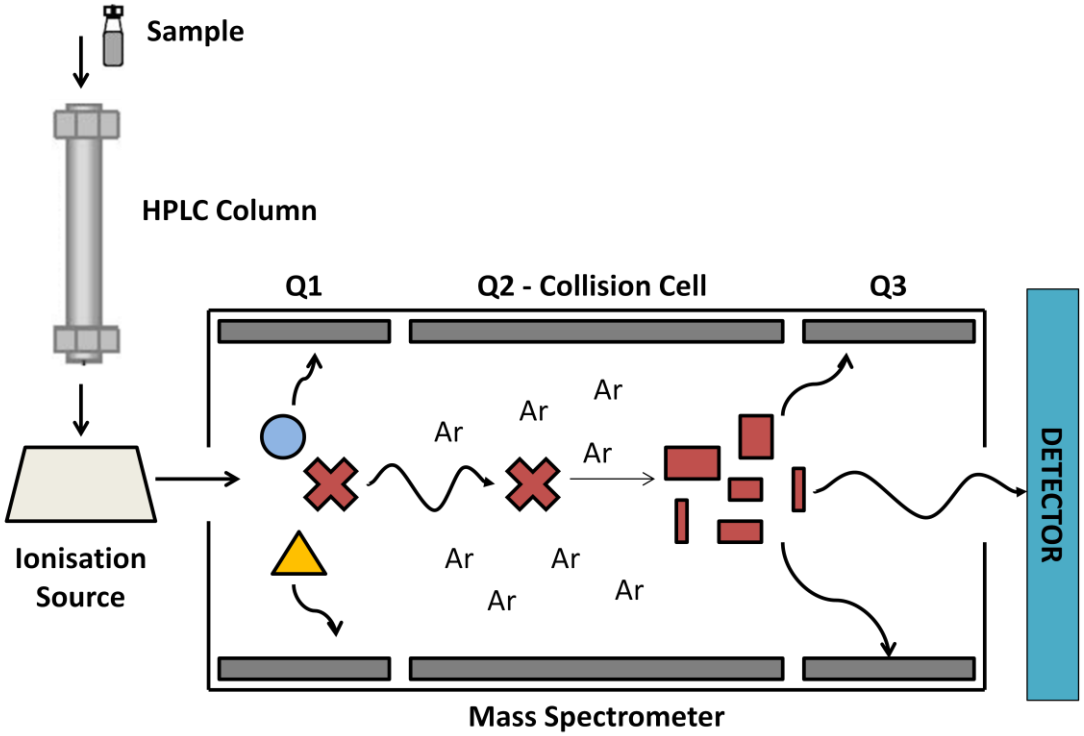


Figure 1.5 A simplified representation of high performance liquid chromatography tandem mass spectrometry. The sample is separated by HPLC before being charged in the ionisation source. At the first quadrupole (Q1) the analyte of interest is selected according to *m/z* before transition to the collision cell (Q2). In the collision cell collision between the selected analyte and Ar gas induces fragmentation. The fragments are subjected to selection by the third quadrupole (Q3) with only the selected *m/z* fragment reaching the detector. Product ion scanning can also be applied in Q3 to obtain structural information about the analyte. (Ar – argon).

1.4.4 Applications of High Performance Liquid Chromatography-Mass Spectrometry

Clinical applications of HPLC-MS/MS include TDM, toxicology, endocrinology, paediatrics and microbiology (Adaway et al. 2015). In TDM HPLC-MS/MS demonstrates several advantages over traditional immunoassays including reduced inter-laboratory variability and lower sample volume requirement (Adaway et al. 2015). A further advantage is the ability to multiplex several analytes into one run as illustrated by combined assays of multiple immunosuppressants (cyclosporin and tacrolimus, sirolumus and everolimus) (Ansermot et al. 2008) as well as parent drug and metabolite analyses (Breton et al. 2005).

HPLC-MS/MS is also increasingly used in proteomics for peptide sequencing, protein identification and detection of covalent modifications of proteins (Medzihradszky 2005). In HPLC-MS/MS peptides are fragmented by collision induced dissociation, occurring in Q2 of Figure 1.5, generating multiple fragment (product) ions. Correlation of the mass spectrometric data with sequence databases [e.g. TANDEM (Craig and Beavis 2004)] allows the rapid identification of proteins (Seidler et al. 2010). However, database recognition of peptides precludes the identification of peptides not present in the database or peptides with unknown modifications such as drug-modifications (Medzihradszky 2005). De novo peptide sequencing with HPLC-MS/MS can be used to identify these unknown or modified peptides (Seidler et al. 2010). Fragment ions provide information about the amino acid composition of the peptide as each of the 20 amino acids has a known and specific molecular mass (Table 1.5). Fragment ions are formed by peptide backbone cleavages and may occur between the alpha carbon and carbonyl group; at the peptide bond; between the amino group and the alpha carbon. When the charge is retained at the N-terminus **a**, **b** and **c** fragments are formed whereas **x**, **y** and **z** ions are produced when the charge is retained at the C-terminus (Figure 1.6) (Biemann 1990). The amino acid sequence of the peptide can then be determined by mass differences or additions. Drug-modification of peptides can be detected by combining peptide sequence information with knowledge of potential modification sites (e.g. cysteine), mass of drug or potential reactive metabolites and *m/z* of drug-modified peptides (Meng et al. 2014a).

Table 1.5 The 20 amino acids and their monoisotopic masses [adapted from (Medzihradszky and Chalkley 2015)]

Name	3 Letter Code	1 Letter Code	Elemental Composition	Monoistopic Residue Mass
Alanine	Ala	A	$C_3H_7NO_2$	71.03
Arginine	Arg	R	$C_6H_{14}N_4O_2$	156.10
Asparagine	Asn	N	$C_4H_8N_2O_3$	114.04
Aspartic Acid	Asp	D	$C_4H_8NO_4$	115.02
Cysteine	Cys	C	$C_3H_7NO_2S$	103.01
Glutamic Acid	Glu	E	$C_5H_9NO_4$	129.04
Glutamine	Gln	Q	$C_5H_{10}N_2O_3$	128.06
Glycine	Gly	G	$C_2H_5NO_2$	57.02
Histidine	His	H	$C_6H_9N_3O_2$	137.06
Isoleucine	Ile	I	$C_6H_{13}NO_2$	113.08
Leucine	Leu	L	$C_6H_{13}NO_2$	113.08
Lysine	Lys	K	$C_6H_{14}N_2O_2$	128.09
Methionine	Met	M	$C_5H_{11}NO_2$	131.04
Phenylalanine	Phe	F	$C_9H_{11}NO_2$	147.07
Proline	Pro	P	$C_5H_9NO_2$	97.05
Serine	Ser	S	$C_3H_7NO_3$	87.03
Threonine	Thr	T	$C_4H_9NO_3$	101.05
Tryptophan	Trp	W	$C_{11}H_{12}N_2O_2$	186.08
Tyrosine	Tyr	Y	$C_9H_{11}NO_3$	163.06
Valine	Val	V	$C_5H_{11}NO_2$	99.07

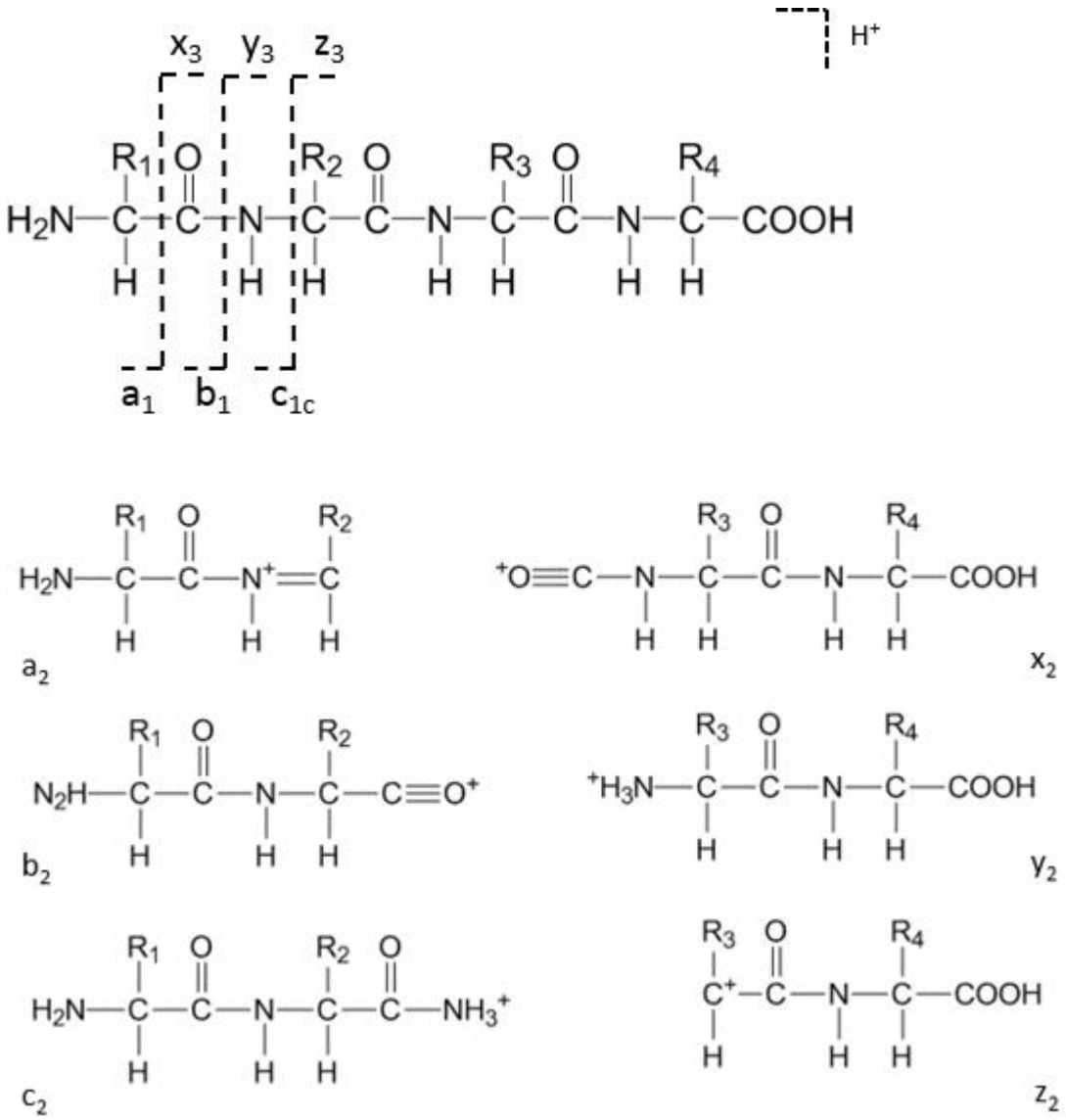


Figure 1.6 Nomenclature of sequence-specific peptide fragments; a-, b- and c-type ions contain the N terminus; x-, y-, and z-ions contain the C-terminus. R_n represent the side chains of the amino acid residues (Dass 2001).

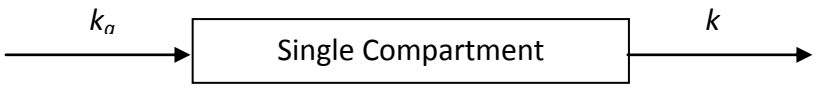
1.5 Pharmacokinetics

1.5.1 Overview

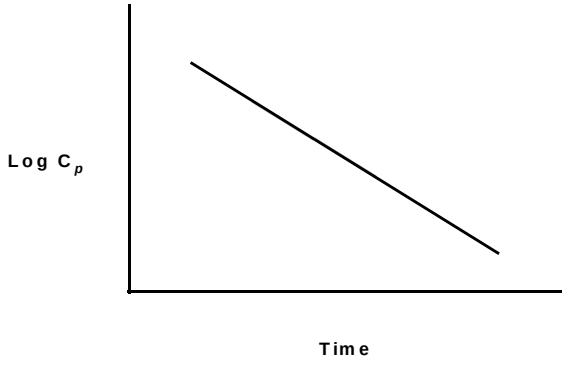
The relationship between drug dosage and the response to the drug is described by pharmacokinetics (PK) and pharmacodynamics. PK describes what the body does to the drug whilst pharmacodynamics explains what the drug does to the body. In order for a drug to produce its effect it has to be present at an appropriate concentration at the site of action (Rang et al. 2016). PK processes include absorption, distribution, metabolism and elimination. Quantification of these processes allows pharmacokineticists to understand, interpret and predict blood concentration-time profiles (Aarons 2005). However, intra- and inter-individual variability exists in these PK processes which contribute to the variability observed in the dose-concentration relationship.

1.5.2 Pharmacokinetic Models

PK models are mathematical schemes that attempt to describe the fate of a drug in a biological system after administration (Dhillon and Gill 2006). PK models can be classified by complexity. The simplest model is the one compartment model where the body is depicted as a kinetically homogenous unit. This model assumes that any drug administered is instantaneously distributed throughout the body and equilibration between tissues is also instant (Figure 1.7A). The relationship between log drug concentration and time will show a linear relationship (Figure 1.7B) (Dhillon and Gill 2006).



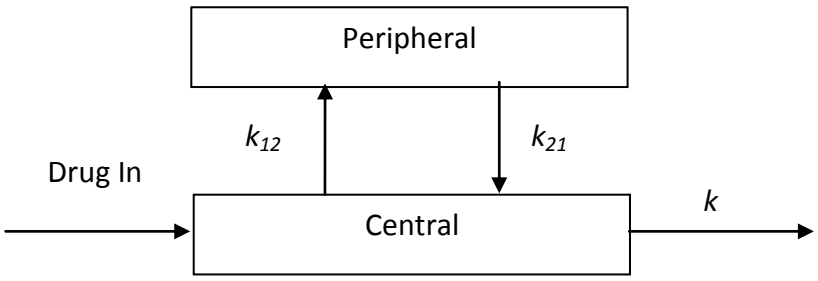
(A)



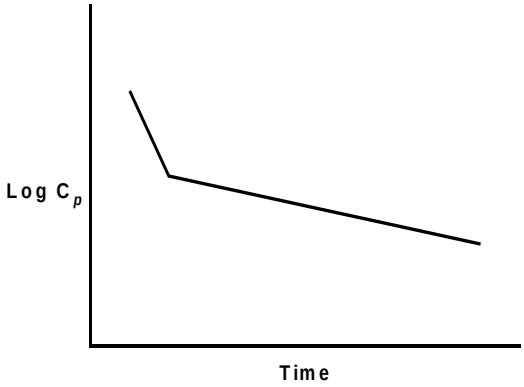
(B)

Figure 1.7 Theoretical Pharmacokinetic Models (A) One-compartment model k_a = absorption rate constant, k = elimination rate constant **(B) Time profile of a one-compartment model depicting log plasma concentration (C_p) against time**

A two compartment model depicts the body as a central and peripheral compartment (Figure 1.8A). These compartments have no physiological meaning or anatomical meaning but should be viewed as semi-mechanistic models as it is possible to relate parameters to physiological processes (e.g. effect of renal function on clearance) (Aarons 2005). The model assumes that after administration of the drug into a central compartment, the drug distributes between that compartment and the peripheral compartment but does not achieve instantaneous distribution (equilibration) between the compartments. The log drug concentration-time plot will show a biphasic response (Figure 1.8B) (Dhillon and Gill 2006).



(A)



(B)

Figure 1.8 Theoretical Pharmacokinetic Models (A) Two-compartment model. k_{12}, k_{21}, and k are first order rate constants; k_{12} = rate of transfer from central to peripheral compartment; k_{21} = rate of transfer from peripheral to central compartment; k = rate of elimination from central compartment **(B) Time profile of a two-compartment model depicting log plasma concentration (C_p) against time**

The most complex PK models are multi-compartmental and can be based on physiological considerations. Physiologically based pharmacokinetic (PBPK) models are compartmental models where each compartment represents an actual tissue or organ space (Figure 1.9) (Aarons 2005). While conventional PK models are defined by drug-related data, PBPK models integrate information from drug-related data, physiological, and biological parameters as they vary between subjects or with age and disease state (Espié et al. 2009). The major limitation of PBPK models is the lack of human data meaning they rely heavily on animal studies and intensive sampling of tissues which is not possible for all tissue types (Aarons 2005).

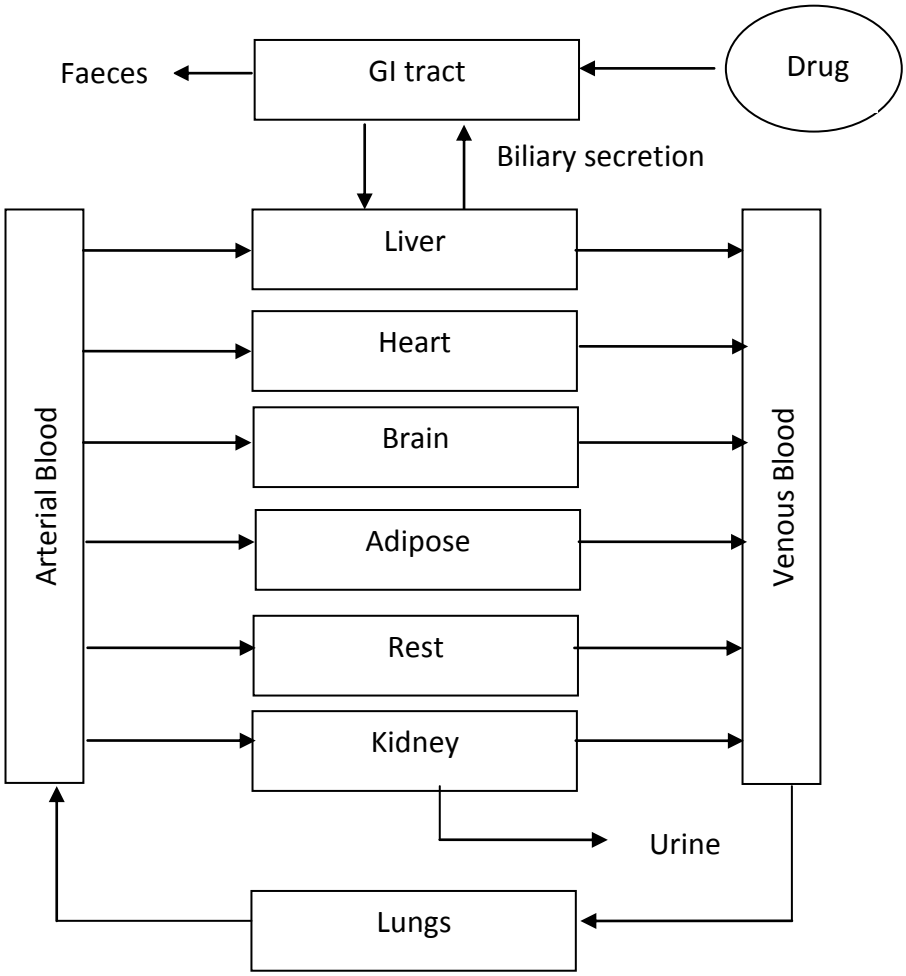


Figure 1.9 An example of a physiologically based pharmacokinetic model for an orally administered drug. The compartments represent tissues and organs; connecting arrows represent blood supplies.

In reality, because of the complexity of PBPK modelling and lack of sufficient data, partial PBPK or semi-mechanistic models often present more useful information (Aarons 2005). An elegant four-compartment semi-mechanistic model comprising the stomach, intestine, central and peripheral compartments was able to accurately describe that gastric emptying half-life was improved by a factor of 20 in patients intolerant to enteral nutrition after treatment with prokinetic agents (Figure 1.10) (Ogungbenro et al. 2011).

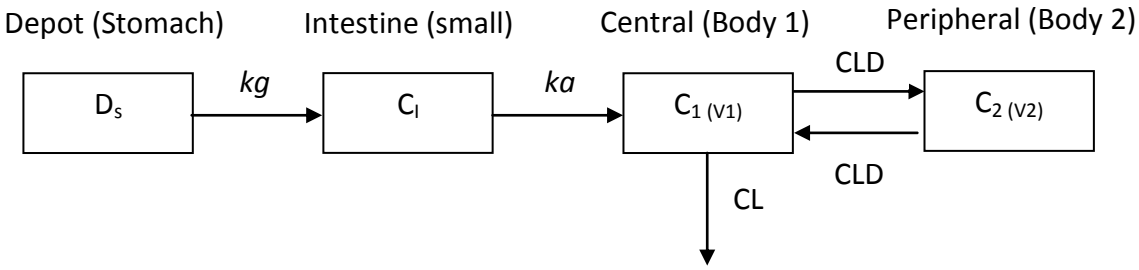


Figure 1.10 A four-compartment semi-mechanistic model with first order absorption. *kg* = gastric emptying rate constant, *ka* = absorption rate constant, CL = clearance, CLD = intercompartmental clearance, V1 = volume of central compartment, V2 = volume of peripheral compartment.

1.5.3 Population Pharmacokinetics

Population PK is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of the drug of interest (Aarons 1991). The aims of a population PK approach are (Ette and Williams 2004a):

- Identify and quantify sources of variability, such as inter-subject, intra-subject, and inter-occasion.
- Explain variability by identifying factors of demographic, pathophysiologic, environmental or drug-related origin that may influence the PK behaviour of the drug.
- Quantitatively estimate the magnitude of the unexplained part of the variability in the patient population.

Several methodological approaches for characterisation of population PK parameters have been proposed and include: naive pooled data analysis, two stage approaches (e.g. standard, Bayesian) and nonlinear mixed effects models (Ette and Williams 2004b). However the term population PK almost always means the use of nonlinear mixed effects modelling as it accounts for greater than 90% of published population PK methodology (Dartois et al. 2007).

"Nonlinear" refers to the nonlinear relationship between the dependent variable (e.g. concentration) and model parameters and independent variable(s). "Mixed

effects" refers to the parameters and are defined as "fixed effects" if they do not vary between individuals and "random effects" if the parameters demonstrate variation across individuals (Mould and Upton 2013). Random effects include inter-individual (e.g. inter-occasion) and residual variability (e.g. errors in dosing, sampling or recording time, assay errors, etc.) (Kiang et al. 2012). The population modelling approach considers the study population rather than each individual as the unit of analysis for estimating the distribution of PK parameters and their relationship with covariates within the population (Kiang et al. 2012). Covariates are factors such as weight, concomitant medications and genotype, that are predictive of PK variability in the target population (Mould and Upton 2012).

In traditional PK studies, the emphasis was to minimise sources of inter-individual variability by complex study designs and application of strict inclusion and exclusion criteria. A small number of subjects would be given the drug of interest and then intensively sampled over a fixed post-dose period generating "dense" PK data for each participant. However, these traditional studies are limited as they are performed in subjects that are not representative of the patient population (e.g. healthy volunteers), require multiple blood samples for each subject (greater than 6) and so are unsuitable for vulnerable patient groups (e.g. children) and unable to quantify effects of covariates (e.g. weight) on PK parameters (Ette and Williams 2004a). Population PK studies differ from traditional PK studies because participants are representative of the intended target population of the drug and although the numbers of subjects in these population studies tend to be greater each participant contributes relatively few PK samples generating "sparse" data (Kiang et al. 2012). Nonlinear mixed effects modelling is able to integrate dense and sparse PK data along with different dosages, multiple subject types and a heterogeneity of other factors (e.g. concomitant medications, pathophysiology) (Ette and Williams 2004a).

Population PK modelling requires skilled pharmacokineticists and pharmacometricians as well as specialised software (Ette and Williams 2004a). Several software programs are available for nonlinear mixed effects modelling and include: NONMEM, MONOLIX, Pmetrics, Phoenix NLME, Kinetica and S-ADAPT

(Kiang et al. 2012). NONMEM is the most widely used program in both academia and industry with approximately 70% of all population PK studies employing NONMEM for population modelling (Kiang et al. 2012). Population PK approaches have an important role in both drug development and routine drug therapy (Samara and Granneman 1997). In drug development population PK is useful for the integration of data from phase I, II and III of clinical studies which are likely to contain heterogeneous patients and both dense and sparse PK samples as well as longer term surveillance information (Food and Drug Administration 1999). In patient care population, PK can be used to individualise or optimise dosage regimens; for example, guidance for dose selection of metformin in patients with various stages of renal impairment where limited information was previously available as the drug was contraindicated in renal impairment (Duong et al. 2013).

1.5.4 Population Pharmacokinetics in Carbamazepine Metabolism

Because of the high intra- and inter-individual variability in relationship between dose and blood levels of CBZ, narrow therapeutic range, drug interaction profile and diverse treatment population (paediatric to elderly) several population PK models have attempted to describe the relationship between covariates and plasma levels of CBZ (Table 1.6). Patient factors that influence the PK of CBZ include gender, age and total body weight (Gray et al. 1998; Jiao et al. 2003; Milovanovic and Jankovic 2011). Concomitant therapy with valproic acid, phenytoin, felbamate and phenobarbital has also been associated with significant modification of CBZ metabolism (Graves et al. 1998; Jankovic et al. 2008). Phenytoin and phenobarbital are known to induce many CYP isoforms and glucuronyl transferases whilst valproic acid is an inhibitor of drug metabolism enzymes (Perucca 2006). Only one population PK model has investigated the role of genetic variation in metabolising enzymes on PK of CBZ and reported that homozygotes of *CYP1A2 -163A/A* achieved lower concentrations of CBZ compared with other genotype groups (Djordjevic et al. 2016). Nearly all of the reported population PK models consider only the PK of the parent drug CBZ with a single study also analysing the metabolism of the metabolite CBZE (Jiao et al. 2004).

Table 1.6 Population pharmacokinetic studies using NONMEM in patients receiving carbamazepine therapy

Study	Number of Subjects	Number of Observations	Timing of Samples	Ethnicity of Population	Mean Age	Analytes	Statistically Significant Covariates
(Djordjevic et al. 2016)	40	40	Trough	Serbian	11 (median)	CBZ	Gender Total daily dose *CYP1A2* -163C>A
(El Desoky et al. 2012)	302	302	Trough	Egyptian	Paediatric: 10.6 Adults: 29.4	CBZ	Nil
(Punyawudho et al. 2012)	121	555	Sparse	American Indian, Black, Hispanic, White	70.5	CBZ	Concomitant phenytoin
(Milovanovic and Jankovic 2011)	151	167	Sparse	Serbian	Paediatric: 8 Adults: 32	CBZ	Age Total daily dose
(Jankovic et al. 2008)	97	97	Trough 76% C_{max} 24%	Serbian	14.7	CBZ	Age Total body weight Concomitant valproic acid

(Vucicevic et al. 2007)	311	423	Sparse	Serbian	37	CBZ	Total body weight Total daily dose Concomitant phenobarbital Concomitant valproic acid
(Perumandla et al. 2005)	84	307	Sparse and rich	Indian	15.2	CBZ	Total body weight
(Jiao et al. 2004)	585	687	Trough	Chinese	23.3	CBZE	Total body weight Total daily dose Concomitant valproic acid
(Jiao et al. 2003)	585	687	Trough	Chinese	23.3	CBZ	Total body weight Total daily dose Age > 65 years Concomitant phenytoin Concomitant phenobarbital Concomitant valproic acid
(Chan et al. 2001)	193	302	Trough	Singaporean	12.5	CBZ	Age Total body weight Concomitant phenobarbital

(Deleu et al. 2001)	48	149	Sparse	Omani	27.8	CBZ	Nil
(Reith et al. 2001)	91	946	Sparse	Australian	18.1	CBZ	Surface area Total daily dose
(Gray et al. 1998)	72	118	Sparse	African and Indian	8.7	CBZ	Total body weight Concomitant valproate Concomitant phenytoin Concomitant phenobarbital
(Graves et al. 1998)	829	1834	Sparse	United States	35	CBZ	Age > 70 years Concomitant phenytoin Concomitant phenobarbital Concomitant felbamate
(Delgado Iribarnegaray et al. 1997)	201	387	Trough	Spain	9.5	CBZ	Total body weight Age Total daily dose Concomitant phenobarbital

1.6 Transcriptomics

1.6.1 Overview

The genes in DNA encode protein molecules which are responsible for carrying out all functions of an organism. Each gene encodes for a particular protein and the process of manufacturing each particular protein consists of two steps: transcription and translation. Transcription is transfer of the information in DNA to messenger RNA (mRNA). During the second stage, translation, the mRNA is "read" according to the genetic code, which relates the DNA sequence to the amino acid sequence in the protein (Clancy 2008). The transcriptome is the entire repertoire of transcripts in a species and represents the genes that are actively expressed at any given moment (Malone and Oliver 2011). There about 8.4^{23} RNA bases in the full human transcriptome (Velculescu et al. 1999). In addition to mRNA other RNA molecules exist. A microRNA (miRNA) is a small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression and so can also influence the transcriptome (Bartel 2004). Knowledge of the transcriptome can provide valuable insights into the pathophysiological processes involved in DHRs (Coulter et al. 2010; Bellon et al. 2010).

1.6.2 Gene Expression Microarrays

Gene expression microarrays have enabled rapid and high-throughput quantification of the transcriptome. A typical microarray experiment involves two components: the probe and target. Probes are cDNA or oligonucleotides that represent genomic DNA and are amplified and immobilised on a solid substrate (e.g. silicon wafer or glass slide). Target preparation involves extraction of transcripts from the experimental samples (e.g. cells or tissues) to be investigated. The target RNA is converted to cDNA by reverse transcription and labelled with fluorescent dyes. The labelled target cDNA is then hybridised to the array and scanned with a laser. Probes that correspond to the transcribed RNA hybridise to their complementary target and because transcripts are fluorescently-labelled light intensity can be used as a measure of gene expression (Figure 1.11) (Malone and Oliver 2011). Analysis of the large quantities of gene expression data generated by

microarrays is complex and typically consists of assessment of array quality, normalisation, summary of data, significance testing and biological interpretation (Reimers 2010). Commercial software (e.g. Genespring, GeneSifter) is available to help guide researchers through this process. Confirmation of gene expression changes on microarray should be validated using real-time polymerase chain reaction (PCR) (Reimers 2010). Construction of biological pathways from rich transcriptome data can help to explain how cellular decision making and responses are orchestrated in the whole cell/organism in response to specific stimuli (e.g. exposure to drug) leading to novel insights and hypotheses (Viswanathan et al. 2008). Ingenuity pathway analysis (IPA) is a web-based software application that enables researchers to analyse gene lists derived from expression microarrays. Using the IPA knowledge database biological models can be generated that incorporate upstream and downstream effects of the identified differentially expressed targets (Kramer et al. 2014).

1.6.3 Gene Expression Studies in Drug Hypersensitivity Reactions

Several studies have analysed gene expression in delayed DHRs (Table 1.7). However, most of these studies have investigated specific candidate genes (e.g. cytokines and cytotoxic proteins) rather than utilising a more global approach that does not rely on prior knowledge and is hypothesis generating. Two studies have used gene expression microarrays to investigate the transcriptome profile in delayed DHRs (Bellon et al. 2010; Coulter et al. 2010). Bellon et al. isolated PBMCs from patients during the acute phase of the hypersensitivity reaction and compared gene expression in 12,000 genes with PBMCs isolated from the same patients when the DHR had resolved. Elevated gene expression was reported in genes related to inflammation, cell cycle and lymphocytes and differences noted between bullous and non-bullous hypersensitivity reactions. A second study by Coulter et al. examined the gene expression profile of PBMCs isolated from patients with allergic contact dermatitis to *p*-phenylenediamine (PPD). PBMCs were then exposed to PPD *in vitro* and the expression profile of > 50,000 transcripts assessed. A network based analysis using IPA showed up-regulation of T helper 2 gene pathways in allergic individuals but a regulatory gene profile in tolerant individuals.

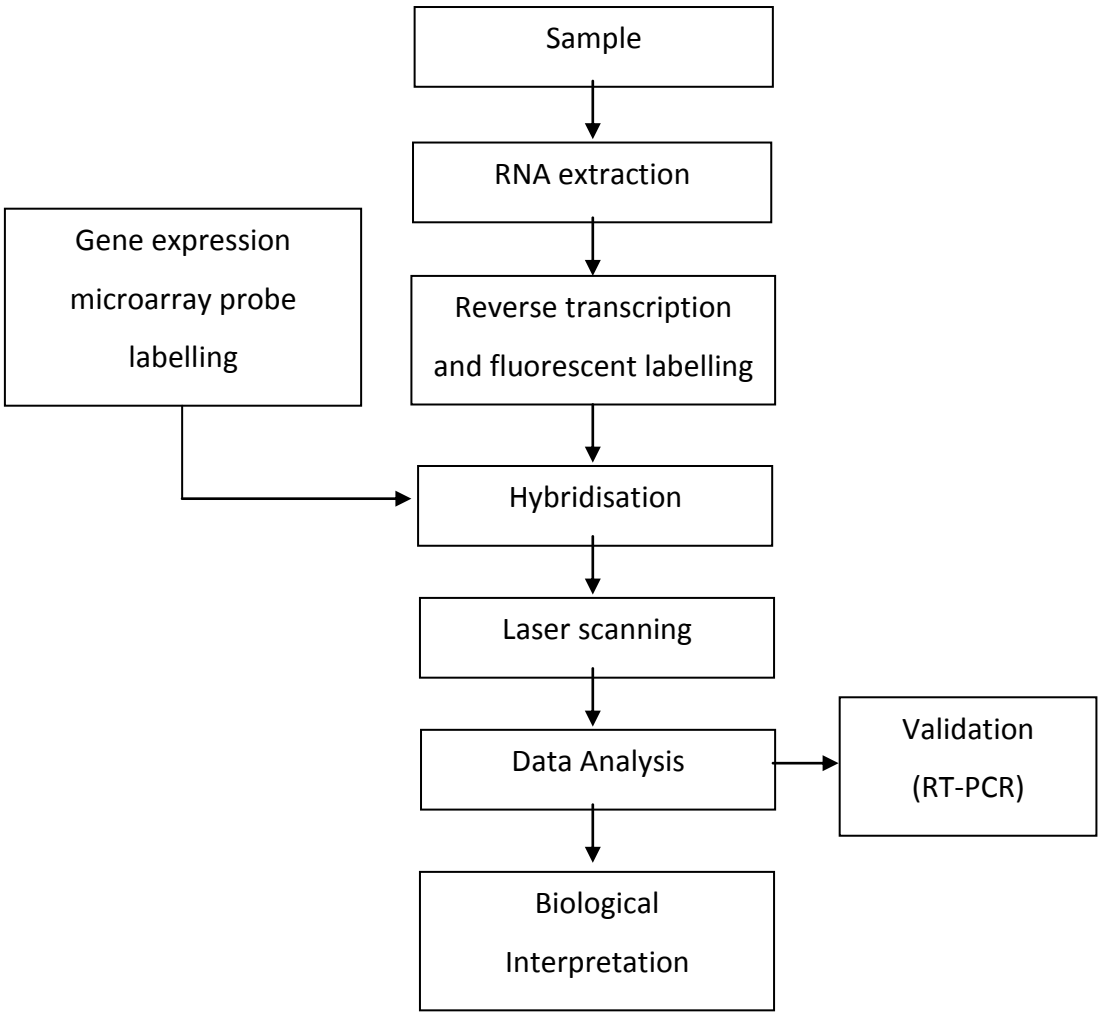


Figure 1.11 Overview of the process flow for gene expression microarray analysis (RT-PCR: real-time polymerase chain reaction)

Table 1.7 Summary of gene expression studies in drug hypersensitivity reactions

Study	No. of Patients	Reaction(s)	Suspected Drugs/Chemicals	Samples Analysed	Gene Expression Analysis Technique	Significant Genes
(Bhardwaj and Ishmael 2013)	1	DRESS	Cefepime, Vancomycin, Cefdinir, Cephalexin, Ampicillin	Isolated PBMCs from patient and stimulated *in vitro*	Real-time quantitative PCR	• Increases in GM-CSF, IL-5, IL-13, IL-2 and IFN-γ in hypersensitive subject
(Morel et al. 2011)	30	MPE, EM, SJS/TEN	Unknown	Isolated PBMCs from patients during acute phase and at least 1 month after resolution of DHR	Real-time quantitative PCR	• Overexpression of α-defensins 1-3 in patients with SJS/TEN
(Coulter et al. 2010)	5	Allergic contact dermatitis	*p*-Phenylenediamine	PBMCs from allergic and tolerant patients stimulated with PPD *in vitro*	Microarray	• Allergic patients' expressed genes related to positive regulation of cell proliferation, apoptosis, inflammatory response, humoral immune response and lymphocyte activation

(Bellon et al. 2010)	23	MPE, FDE, DRESS, EM, SJS, TEN	Diazepam, Erythromycin, Metamizole, Imatinib, Tamoxifen, Valproic acid, Clindamycin, Pseudoephedrine, Carbamazepine, Phenytoin, Ibuprofen, Amoxicillin, Spiramycin, Metronidazole, Allopurinol, Fluvastatin, Paracetamol, Furosemide	Isolated PBMCs from patients during acute phase and at least 1 month after resolution of DHR	Microarray	• During acute phase of DHR 92 genes differentially expressed • Genes expressed in lymphocytes are under-represented in PBMC of severe bullous reactions • Cell cycle genes are upregulated in severe reactions (SJS/TEN, EM and DRESS) • Expression of inflammation associated genes are strongly upregulated in bullous reactions
(Fernandez et al. 2008)	27	MPE	Amoxicillin, Cefuroxime, Co-amoxiclav, Metamizol, Paracetamol, Cefaclor, Ibuprofen, Spiramicin, Tetrazepam, Ebastine	Isolated PBMCs from patients during acute phase of reaction	Semi-quantitative real-time PCR	• Increased expression of IFN-γ, TNF-α, CXCR3, CCR6 and CCR10 in patients with MPE

(Cornejo-Garcia et al. 2007)	25	Urticaria, MPE, SJS/TEN	Amoxicillin, Cephalexin, Spiramicin, Metamizol, Phenobarbital, Carbamazepine Paracetamol, Diphenylidantoine, Allopurinol	Isolated PBMCs from patients during acute phase of reaction and 45 days after resolution	Semi-quantitative real-time PCR	• Increased expression of IFN-γ, TNF-α and T-bet in hypersensitivity reactions
(Posadas et al. 2002)	15	MPE, SJS/TEN	Ceftriaxone, Phenytoin, Amiodarone, Amoxicillin, Carbamazepine, Phenytoin, Ampicillin, Allopurinol, Sulphamethoxazole	Isolated PBMCs from patient during acute phase of reaction and 30 days later	Competitive PCR	• Increased expression of TNF-α, perforin, granzyme B in hypersensitivity reactions • Fas ligand expressed only in SJS/TEN reactions
(Leyva et al. 2000)	3	TEN	Phenytoin, Carbamazepine	Isolated PBMCs from patients during day 1, 3, 5, 15 and 24	Semi-quantitative reverse transcription-PCR	• Increased expression of IL-2, IL-4, TNF-α and IFN-γ

(Posadas et al. 2000)	9	MPE, SJS/TEN	Carbamazepine, Hidantoin, Amoxicillin, Ampicillin Amiodarone, Ceftriaxone,	Isolated PBMCs from patients during acute phase, 7, 15 and 30 days after reaction	Competitive PCR	• Increased expression of IL-2, IFN-γ and TNF-α in hypersensitivity reactions

CCR: chemokine receptor, CXCR: chemokine receptor, DRESS: drug reaction with eosinophilia and systemic symptoms, EM: erythema multiforme, GM-CSF: granulocyte macrophage colony stimulating factor, IFN-γ: interferon-gamma, IL: interleukin, MPE: maculopapular exanthema, PCR: polymerase chain reaction, SJS: Stevens-Johnson syndrome, T-bet: T helper type 1 cell transcription factor, TEN: toxic epidermal necrolysis, TNF-α: tumour necrosis factor alpha

1.7 Aims and Objectives of Thesis

The aim of this thesis was to investigate the chemical, metabolic, immunological and genetic factors that may cause or contribute to CBZ-induced hypersensitivity reactions. Healthy volunteers and patients prescribed CBZ therapy were recruited to several clinical studies to obtain blood samples for a series of immunochemical, mass spectrometric, PK and transcriptomic experiments in order to investigate the links between metabolism and the immune response in CBZ hypersensitivity reactions.

The specific aims of the thesis included:

- Investigation of the bioactivation and chemical reactivity of CBZ and its metabolites by assessment of covalent modification of proteins both *in vitro* and *in vivo*
- Development of a HPLC-MS/MS assay to accurately quantify CBZ and its major metabolites in human plasma
- Determine the demographic, clinical and genetic factors that influence the metabolism of CBZ using a population PK modelling approach
- Characterise T cell activation and response in patients with a history of CBZ hypersensitivity and to determine cross reactivity of these T cells to CBZ and CBZE
- Transcriptomic analysis of PBMCs (obtained from patients with a history of CBZ hypersensitivity) exposed to CBZ *in vitro* and construction of biological pathways to elucidate possible novel pathways in CBZ hypersensitivity reactions

Chapter 2

Assessment of the Reactivity of Carbamazepine Metabolites with Proteins *In vitro* and *In vivo*

2.1 Introduction

CBZ is an aromatic anticonvulsant that has been implicated in diverse hypersensitivity phenotypes that involve multiple organ systems including the skin and liver (Avancini et al. 2015; Chalasani et al. 2015). Chemically reactive metabolites of CBZ have been implicated as agents for hepatotoxicity in rat livers (Leone et al. 2014) and mortality from drug-induced liver injury (DILI) is significantly higher in individuals with concomitant skin reactions (Chalasani et al. 2015). Up to 10% of patients prescribed CBZ experience a cutaneous hypersensitivity reaction (Marson et al. 2007) including MPE, HSS, SJS and TEN (Roujeau et al. 1995). While the majority of affected patients experience only mild symptoms that resolve on cessation of the medication, others suffer significant morbidity or mortality. TEN is associated with an acute mortality rate of greater than 20% (Sekula et al. 2013; Lalosevic et al. 2015) and survivors can suffer long-term dermatologic (e.g. pigmentation) and ophthalmic (e.g. myopia, corneal scarring) sequelae (Schwartz et al. 2013; Quirke et al. 2015). At present the molecular mechanism(s) of these reactions is not fully understood. This chapter is focused primarily on cutaneous hypersensitivity reactions because of their frequency and strong associations with HLA alleles (Yip et al. 2012), although it should be noted that many manifestations of CBZ hypersensitivity involve multiple organ systems including liver, skin and kidneys (Mehta et al. 2014).

DHRs, such as those described above for CBZ, are a consequence of inappropriate activation of the immune system (Pavlos et al. 2015). These immune-mediated reactions are classified as type B ADRs as they are idiosyncratic and unpredictable with respect to the pharmacology of the drug (Rawlins 1981). According to the Gell-Coombs classification CBZ hypersensitivity is a Type IV reaction because of the delayed clinical presentation and presence of drug-reactive T lymphocytes (Gell 1963; Wu et al. 2007). Currently, there are three mechanistic hypotheses that attempt to explain how drugs activate T cells: the hapten hypothesis, the PI concept and the drug-dependent altered HLA peptide repertoire concept (Figure 2.1). The hapten hypothesis was originally outlined in the 1930's (Landsteiner and Jacobs 1935) and in its current form proposes that chemically reactive small molecules

(including drugs) or reactive metabolites irreversibly bind to an endogenous protein such as albumin. The small molecule is known as the hapten. The modified protein is then processed by APCs, generating a pool of chemically modified peptides. These peptides are presented on the MHC and act as antigens to trigger an immune response via TCR (Park et al. 2001). Under the PI model of drug hypersensitivity, the drug or metabolite binds non-covalently to either the TCR or MHC in a peptide independent manner and directly activates T cells (Pichler 2002). According to the altered peptide repertoire model, the offending drug attaches to the peptide-binding cleft of the MHC thereby altering the shape and chemistry of the binding cleft. As a result, altered self-peptide repertoires are presented by the MHC and recognised as "foreign" by the immune system with a subsequent T cell response (Illing et al. 2012).

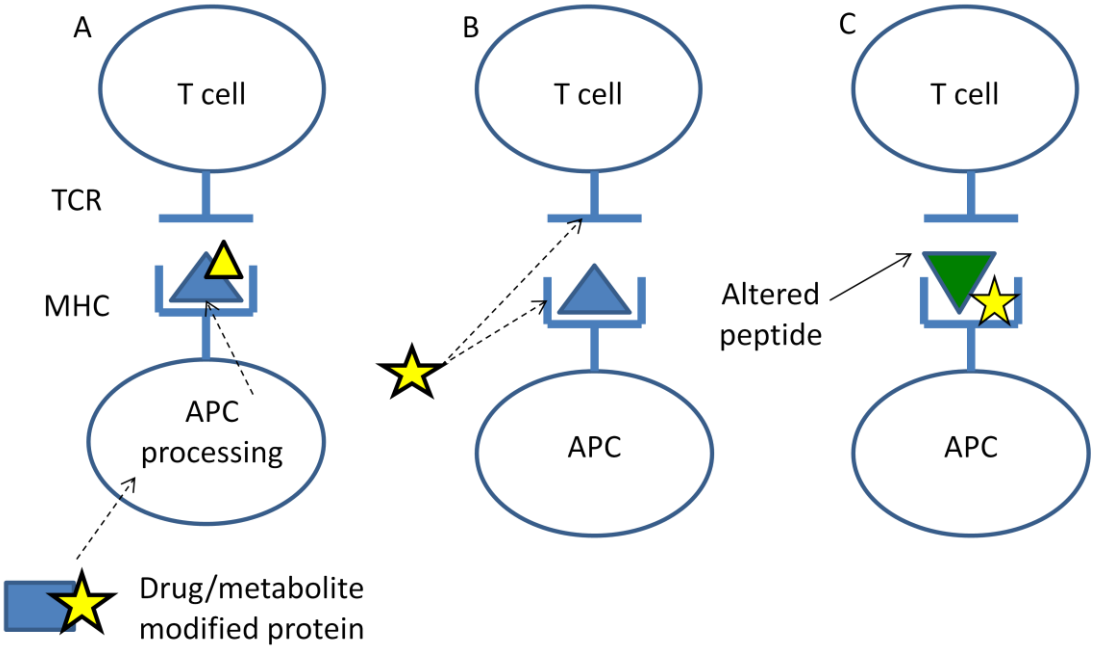


Figure 2.1 Models of T cell activation by small molecules. A. The hapten hypothesis postulates that the drug or its metabolite (yellow star) produces an antigen (yellow triangle) by binding covalently to protein (blue rectangle) which is then processed by APC, generating drug-modified peptides (blue triangle) that are presented on the MHC. The TCR recognises the haptenated peptides as neoantigens, activating the immune response. **B**. In the PI model the drug or metabolite binds noncovalently to either the TCR or MHC leading to activation of the immune system. **C**. The 'altered peptide repertoire' model proposes that the drug or metabolite (yellow star) binds noncovalently to the MHC binding cleft and alters the specificity of binding of natural peptides. This leads to the presentation of novel peptides (green triangle) that can elicit an immune response.

In vitro experiments have shown that CBZ and certain oxygenated derivatives, namely CBZE, a major metabolite of CBZ (Breton et al. 2005), and DiOH-CBZ, the major metabolite of oxcarbazepine (Flesch 2004), are able to activate T-cells obtained from patients with previous CBZ hypersensitivity reactions via a direct interaction with MHC consistent with the PI concept (Wu et al. 2007). The exact molecular interactions between CBZ, MHC and TCR are unclear but a recent structural modelling study proposes that CBZ can bind to the TCR, shifting TCR specificity and thereby altering the identification of self- and non-self peptides presented on the MHC; leading to aberrant activation of the immune system (Zhou et al. 2015).

The oxidative metabolism of CBZ in humans is complex, involving multiple P450 isoforms, leading to the generation of greater than 30 metabolites (Lertratanangkoon and Horning 1982) (Figure 2.2). Bioactivation of CBZ by P450 in HLM has been demonstrated by the formation of both cytotoxic and protein-reactive metabolites (Pirmohamed et al. 1992; Leone et al. 2007). Indeed chemically reactive metabolites of CBZ have been invoked as being responsible for hepatotoxicity (Leone et al. 2014; Iida et al. 2015). The reactive species were not identified but from their deactivation by conjugation with GSH were evidently soft electrophiles (Pirmohamed et al. 1992). Several reactive intermediates of CBZ have been proposed to be responsible for protein adduct formation, including an arene oxide (Pirmohamed et al. 1992; Madden et al. 1996; Elliott et al. 2012), 9-acridine carboxaldehyde (Furst et al. 1995), an iminostilbene (Ju and Uetrecht 1999; Pearce et al. 2005) and an *o*-quinone (Lillibridge et al. 1996) and CBZE (Bu et al. 2005) (Figure 2.2). Interestingly, CBZE, an abundant (Breton et al. 2005) and pharmacologically active (Potter and Donnelly 1998) plasma metabolite formerly believed to be chemically stable (Pirmohamed et al. 1992), has been demonstrated to be chemically reactive through the spontaneous formation of two isomeric CBZE-GSH adducts (CBZE-SG1 and CBZE-SG2) *in vitro* (Bu et al. 2005). The microsomal proteins CYP1A2 and CYP3A4 have also been demonstrated to form covalent adducts with reactive intermediates of CBZ (Wolkenstein et al. 1998; Kang et al. 2008). Molecular modelling has suggested that Cys239 of CYP3A4 could be a

haptenation site, creating a thioether antigen to which the immune response may be directed (Leeder et al. 1996; Kang et al. 2008). That deduction, the reaction of CBZE with GSH (Bu et al. 2005), and the trapping of CBZ's reactive metabolites by GSH in microsomal incubations predict modification of proteins at cysteine residues (Bu et al. 2007). The inferred structures of the multiple GSH adducts produced from CBZ by HLM included thioether derivatives of one or more arene oxides, namely products of nucleophilic conjugate addition, as well as the two adducts of CBZE (Bu et al. 2007). Nonetheless CBZE-SG1 and CBZE-SG2 were the most abundant adducts formed from CBZ. Consequently it has been suggested that CBZE is the principal protein-adducting, and thereby antigen-generating, metabolite of CBZ. This is supported by the significant association between polymorphisms in mEH (encoded by *EPHX1*), the enzyme responsible for deactivating CBZE (Pirmohamed et al. 1992), and severe cutaneous ADR in CBZ patients of Han Chinese ethnicity (He et al. 2014). However, this is an association rather than proof of causality as the polymorphisms are not invariably associated with higher plasma concentrations of CBZE (Caruso et al. 2014; He et al. 2014). The selective covalent modification of proteins at cysteine residues by alkane oxides is in fact a well-characterised reaction (Kim et al. 1997; Arafet et al. 2014; He et al. 2015).

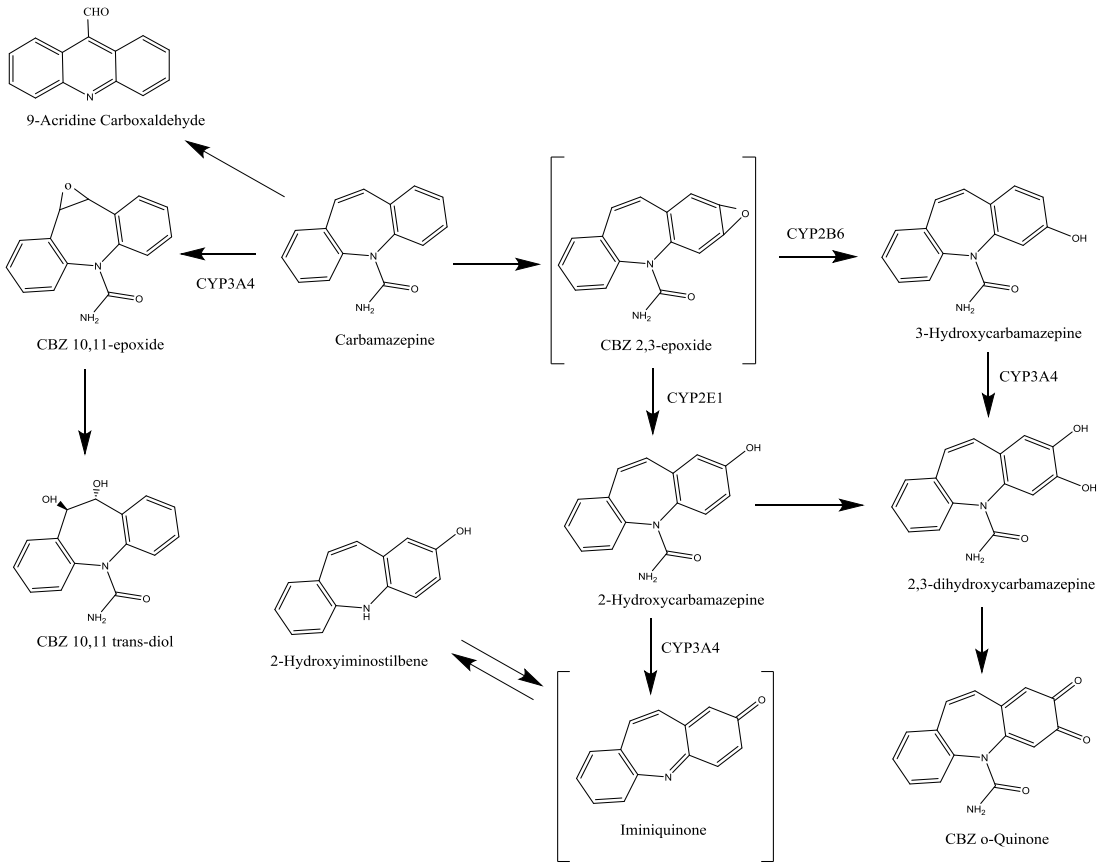


Figure 2.2 Proposed pathways of the oxidative bioactivation of CBZ in humans. The P450 isoforms shown are those reported to be the most active catalysts of the biotransformations (Pearce et al. 2008; Pearce et al. 2005; Pearce et al. 2002; Kerr et al. 1994). Based upon the relative expression of P450 isoforms in livers of Caucasians (Achour et al. 2014), CYP3A4 will usually be the predominant catalyst of both CBZ 3-hydroxylation and 10,11-epoxidation; while CYP2E1 will usually be the predominant catalyst of CBZ C-2 hydroxylation. However it was found in our study that CYP2B6 and CYP2E1, as well as CYP3A4 (Kerr et al. 1994), catalyze the 10,11-epoxidation of CBZ *in vitro*. The depiction of the 2,3-arene oxide as the sole product of aromatic epoxidation is purely representational; the number of arene oxides formed is unknown. The metabolic hydrolysis of CBZE, and therefore the epoxide's chemical (Pirmohamed et al. 1992; Bu et al. 2005) and pharmacological (Bourgeois and Wad 1984) deactivation *in vivo*, is catalysed by mEH (Kitteringham et al. 1996).

Although existing experimental evidence supports direct interaction between CBZ and MHC/TCR as the mechanism for hypersensitivity reactions (Wu et al. 2006; Wei et al. 2012; Zhou et al. 2015) it is possible that parallel or alternative processes such as the hapten mechanism, as a result of reactive metabolite formation, could also activate the immune system. None of the highly reactive CBZ metabolites described earlier has been tested *in vitro* because they are probably too unstable for chemical

synthesis, so their ability to activate T cells is unknown. Also, the phenotype of CBZ hypersensitivity is clinically diverse; raising the possibility that multiple pathophysiological mechanisms may be involved. The aims of this study were to characterize the structures of the protein conjugates, i.e. the potential antigens *in vivo*, formed by the reactive metabolites of CBZ and hence derive the metabolic pathways of their formation, using human serum albumin (HSA) and glutathione *S*-transferase pi (GSTP) as model proteins *in vitro*. GSTP is a proven biochemical reagent for trapping highly reactive small-molecule electrophiles (Jenkins et al. 2008; Callan et al. 2009; Boerma et al. 2011), its three nucleophilic cysteines exhibiting markedly disparate adduction capabilities (Boerma et al. 2011). HSA has a much more varied set of adductable nucleophilic side-chains (Meng et al. 2011b; Jenkins et al. 2013; Meng et al. 2013; Meng et al. 2014a). Previous experiments have isolated and identified conjugates of circulating HSA and intrinsically reactive drugs (Jenkins et al. 2009; Meng et al. 2011b; Meng et al. 2014b), circulating reactive drug metabolites (Meng et al. 2013; Hammond et al. 2014) and metabolic intermediates that might be confined to intracellular locations (Meng et al. 2013; Meng et al. 2014a). CBZE is a major plasma metabolite in patients (Breton et al. 2005), whereas the arene oxides are likely to be fleeting entities in blood (Lau et al. 1984). HSA isolated from patients prescribed CBZ therapy were also analysed to determine if conjugates are present *in vivo*. These conjugates and similar structures on other proteins have the potential to trigger the immunological signals responsible for hypersensitivity to CBZ.

2.2 Methods

2.2.1 Chemicals

HSA (97-99% pure) was purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom), sequencing-grade modified trypsin was from Promega (Southampton, United Kingdom), while liquid chromatography mass spectrometry (LC-MS) grade solvents were from Fisher Scientific UK Ltd. (Loughborough, United Kingdom). All other reagents were from Sigma-Aldrich (UK). CBZ and CBZE were both purchased from Sigma-Aldrich.

2.2.2 Microsomes and Supersomes

HLM preparations (UltraPool HLM 150; a mixed gender pool from 150 donors) were obtained from BD Gentest Corp (Oxford, United Kingdom). Microsomes from baculovirus-infected insect cells (Supersomes) expressing human P450 isoforms (CYP3A4, 2B6, or 2E1) were also purchased from BD Gentest Corp. All recombinant enzymes were co-expressed with human NADPH-CYP reductase and human cytochrome b_5 (Sigma Aldrich).

2.2.3 Bradford Assay

Calibration dilutions of a standard protein (0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/mL) were prepared in phosphate buffer (0.1 M, pH 7.4), using bovine serum albumin (Sigma-Aldrich Co). Each dilution (5 µL) was plated into 3 wells of a 96-well plate. The protein samples were plated in triplicate on the same 96-well plate. Bradford reagent (250 µL) was added to each well. The contents were mixed and any air bubbles removed using a sterile needle. Absorbance at 570 nm was measured using a plate reader. The means of the calibration values were calculated, and plotted on a standard calibration curve on Microsoft Excel. The protein concentrations of the samples were determined using the standard curve. The calculated concentration was the mean value of the three wells.

2.2.4 Conjugation of GSH with CBZE and Microsomally Generated Metabolites of CBZ

Incubations of synthetic CBZE with GSH were undertaken to confirm the spontaneous conjugation reaction of CBZE with thiols, using GSH as a biopeptide model as reported by Bu et al. (2005). Additionally, microsomal incubations were also undertaken with the parent drug, CBZ, and GSH to trap any distinctive dihydrohydroxydibenzoazepinyl thioether conjugates that may be formed by electrophilic [O]CBZ metabolites.

CBZE (80 μM; final methanol concentration, 1% v/v) was directly incubated with GSH (5 mM) in 0.1 M phosphate buffer (pH 7.4, final volume 0.25 mL) at 37^{o}C for 2 h. The reaction mixtures were analysed by LC-MS and LC-MS/MS without treatment. HLM and Supersome incubations were also set up by incubating CBZ (100 μM) with HLM (2 mg/mL) or Supersomes (20 nM), NADPH (2 mM), $MgCl_2$ (5 mM) and GSH (2 mM) for 2 h at 37^{o}C in 0.1 M phosphate buffer (pH 7.4; final volume, 0.2 mL). To terminate the enzymatic reaction, two volumes of ACN were added to the reaction mixtures. The samples were vortexed, and centrifuged at 12,000 *g* for 5 min at 4^{o}C. The supernatants were collected and evaporated to dryness under nitrogen. The dry residue was stored at -20^{o}C until analysed. On the day of analysis the samples were reconstituted in 0.1% aqueous formic acid (0.5 mL).

Aliquots (5-20 μL) were injected onto a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm; Agilent Technologies, Santa Clara, CA) fitted with a Zorbax C18 Reliance pre-column and connected to a PerkinElmer series 200 HPLC system (PerkinElmer, Norwalk, CT). Analytes were eluted at room temperature with a gradient program of acetonitrile (5% to 30% over 20 min; 30% to 60% over 0.5 min; 60% for 4.5 min; 60% to 5% over 0.1 min; 5% for 3.9 min) in 0.05% aqueous formic acid at 1.0 mL/min. This gradient resolved GSH adducts of [O]CBZ ([O]CBZ represents generically the reactive monoxygenated metabolites of CBZ, i.e. CBZE and any isomeric arene oxide), the CBZ phenols and CBZE. The split-flow to the mass spectrometer was approximately 150 μL/min. Analytes were detected in positive-

ion mode with a 4000 Qtrap instrument (Sciex, Warrington, United Kingdom) that was operated under the following conditions: source temperature, 450 ^{o}C; ionspray voltage, 4,500 V; desolvation potential, 100 V; entrance potential, 10 V; CAD gas setting, 5; collision energy, 25 eV; collision exit potential, 3 V; channel dwell time, 150 ms; curtain gas setting, 15; Gas-1 and Gas-2 settings, 50; scanning, m/z 100-1,000 over 5 s. The complete set of MRM monitoring transitions are listed in Table 2.1. Transitions for the GSH conjugates were taken from the data of Bu et al. (2005). Instrument management and data processing were accomplished through Analyst 1.5.1 software (Sciex).

Table 2.1. MRM transitions for CBZ, CBZ metabolites and GSH adducts

Analyte	Transition
DihydrohydroxyCBZ-*S*-glutathione conjugates	*m/z* 560 → 542 *m/z* 560 → 431 *m/z* 560 → 413
2-hydroxycarbamazepine	*m/z* 253 → 210
CBZ 10,11-epoxide	*m/z* 253 → 210; *m/z* 253 → 180
3-hydroxycarbamazepine	*m/z* 253 → 210
Carbamazepine	m/z 237 → 194

2.2.5 Covalent Modification of His-Tagged GSTP by Microsomally Generated Monoxygenated Metabolites of CBZ

Hexahistidine-tagged human glutathione *S*-transferase π (His-GSTP) was expressed in *E.coli* as described previously (Chang et al. 1999) and kindly provided for these experiments by Dr Holly Bryan, University of Liverpool. Reactive metabolites were generated from CBZ (1 mM; final methanol concentration, 2% v/v) in co-incubations of nickel bead-captured GSTP (40 μM), prepared as described by Jenkins et al. (*Proteomics*, 2008), with either HLM (protein concentration, 2 mg/mL) or Supersomes (P450 concentration, 40 mM), $MgCl_2$ (5 mM), and phosphate buffer (pH 7.4), for 6 h at 37^{o}C. The reactions were started by addition of NADPH (final

concentration, 1 mM). The final volume was 0.4 mL. Protein concentrations in this and all the other experiments were determined by the Bradford assay (section 2.2.3). After the 6 h incubation period, a 0.2 mL aliquot was removed from the reaction mixture, taking care not to remove any nickel beads, and stored at 4^{o}C for analysis of CBZ metabolite formation according to section 2.2.9. The nickel beads were recovered from the reaction mixture using magnetic separation and were washed 5 times with 1 mL phosphate buffer. Ammonium bicarbonate solution (50 µL of 50 mM, pH 8.0) was added to the beads and the protein subjected to on-bead tryptic digestion (1 µg of trypsin per 200 µg of protein) at 37^{o}C for 16 h. The digestion reaction was terminated by acidification of the incubation with trifluoroacetic acid (TFA 1%, final concentration 0.1%). Identification of covalently-modified GSTP was undertaken using LC-MS according to section 2.2.12.

2.2.6 Covalent Modification of HSA by Microsomally Generated Metabolites of CBZ

CBZ (50 mM) was freshly dissolved in methanol and then diluted 1:5 with phosphate buffer (0.1 M, pH 7.4). The CBZ (final concentration, 1 mM) was co-incubated with either HLM (2 mg/mL) or Supersomes (20 nM), NADPH (1 mM) and $MgCl_2$ (5 mM) and HSA (0.6 mM) in phosphate buffer, pH 7.4, for 2 h at 37^{o}C (final methanol concentration, 2% v/v). The final volume was 0.2 mL. Each microsome/Supersome incubation was completed in duplicate and the second mixture was used to confirm metabolism of CBZ to monoxygenated metabolites according to section 2.2.9. The incubations for analysis of adduct formation were ultra-centrifuged at 100,000 rpm for 30 min at 4^{o}C to remove microsomes and Supersomes from the mixture. HSA was isolated from the sample by using an anti-HSA affinity column (Agilent Technologies) attached to a PerSeptive Biosystems Vision Workstation (Applied Biosystems, Foster City, CA). The HSA was eluted with 12mM HCl and precipitated from the eluate with nine volumes of ice-cold methanol. The protein was re-suspended in 50 µL of phosphate buffer, then reduced with 10 mM dithiothreitol (15 min) followed by alkylation with 50 mM iodoacetamide (15 min) at room temperature. The protein was precipitated once more with methanol and the concentration of HSA was determined using the Bradford assay (section 2.2.3).

Trypsin (1 μg per 200 μg protein) was added to the isolated HSA and incubated overnight for 16 h at 37^{o}C before mass spectrometric characterisation of covalently-modified protein according to section 2.2.12.

2.2.7 Concentration-Dependent Modification of HSA by CBZE *in vitro*

CBZE (50 mM) was freshly dissolved in methanol and diluted in phosphate buffer (0.1M, pH 7.4) and incubated with HSA (1 mM, 40 μL) in phosphate buffer (0.1 M, pH 7.4) at 37^{o}C for 16 h. The molar ratios of CBZE to protein were 0.1:1, 1:1 and 5:1. The final concentration of methanol ranged between 0.1% (0.1:1 concentration) to 5% (5:1 concentration). Protein was precipitated twice with nine volumes of ice-cold methanol to remove non-covalently bound CBZ, re-suspended in 50 µL of phosphate buffer, and then reduced with 10 mM dithiothreitol (15 min) and alkylated with 50 mM iodoacetamide (15 min) at room temperature. The protein was precipitated once more with methanol and finally dissolved in 50 µL of 50 mM ammonium bicarbonate, pH 8.0. The protein concentration in each sample was determined using the Bradford assay (section 2.2.3). The protein (400 µg) was incubated with trypsin (2 µg) overnight for 16 h at 37^{o}C. Samples of the digest (50 µL) were processed for LC-MS/MS analysis according to section 2.2.12.

2.2.8 Time-Dependent Modification of HSA by CBZE *in vitro*

CBZE (50 mM) was freshly dissolved in methanol and diluted in phosphate buffer (0.1 M, pH 7.4) (1:5, v/v) before incubation with HSA (1 mM, 40 μL) in phosphate buffer (0.1 M, pH 7.4) at 37^{o}C (final CBZE concentration, 5 mM). The final concentration of methanol was 10% (v/v). Aliquots of 80 μL were removed after 0.2, 0.5, 1, 3, and 6 h and processed for LC-MS/MS analysis of tryptic digests as described in sections 2.2.7 and 2.2.12.

2.2.9 Analysis of CBZ Metabolites Produced by Microsomes and Recombinant P450 Isoforms

To confirm metabolism of CBZ, reaction mixtures obtained from incubations with microsomes and GSTP (section 2.2.5) or HSA (section 2.2.6) were processed by the addition of equal volumes of ACN and were then centrifuged at 400*g* for 10 min at 4^{o}C. The supernatants were filtered using 0.22 μM filter units (Millipore, Cork, Ireland) and evaporated to dryness using nitrogen. Dry residues were reconstituted in 200 μL methanol/water (1:1 v/v) and transferred to glass autosampler vials (VWR International, Lutterworth, Leicestershire, United Kingdom) for analysis of metabolites by LC-MS and LC-MS/MS. Aliquots (5-20 μL) were injected onto a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5μm; Agilent Technologies, Santa Clara, CA) fitted with a Zorbax C18 Reliance pre-column and connected to a PerkinElmer series 200 HPLC system (PerkinElmer, Norwalk, CT). Analytes were eluted at room temperature with a gradient programme of acetonitrile (15% to 33% over 12 min; 33% to 50% over 0.5 min; 50% for 5.5 min; 50% to 15% over 0.1 min; 15% for 4 min) in ammonium formate (2 mM, pH 3.0) at 1.0 mL/min. The split-flow to the mass spectrometer was approximately 150 μL/min. Metabolites were detected in positive-ion mode with a 4000 Qtrap instrument (Sciex, Warrington, United Kingdom) that was operated under the following conditions: source temperature, 450 ^{o}C; ionspray voltage, 4,500 V; desolvation potential, 100 V; entrance potential, 10 V; CAD gas setting, 5; collision energy, 41 eV; collision exit potential, 3 V; dwell time, 150 ms; curtain gas setting, 15; Gas-1 and Gas-2 settings, 50; scanning, m/z 100-1,000 over 5 s. The analyte transitions were *m/z* 253 → *m/z* 210 ([O]CBZ), 253 → *m/z* 180 (CBZE). Instrument management and data processing were accomplished through Analyst 1.5.1 software (Sciex). Analytes were identified by chromatographic and mass spectrometric comparisons with authentic standards.

2.2.10 Patients' Plasma Samples

Twenty plasma samples from patients receiving maintenance CBZ therapy for epilepsy were analysed for the presence of [O]CBZ-HSA adducts. None of the patients had experienced an adverse reaction to CBZ. The samples were obtained from the Walton Centre NHS Foundation Trust as part of the PICME II clinical trial (a PK investigation into the metabolism of CBZ in epilepsy patients). The study had received ethics committee approval from the National Research Ethics Service Committee North West – Haydock (ethics reference: 13/NW/0503). All the patients were prescribed a stable dose of CBZ for at least 4 weeks. Clinical history and patient demographics were recorded. Blood (5 mL) was collected into lithium heparin-containing tubes and centrifuged immediately at 1500 *g* for 10 min at 4^{o}C. The plasma was separated into 100 µL aliquots and frozen at -80^{o}C until required for analysis. Further details regarding the recruitment of the patients to the PICME II clinical study can be found in chapter 3.

2.2.11 Isolation of HSA from Patients' Plasma Samples by Depletive Resin Binding and Normalisation of the Albumin Concentration

Patient plasma samples (100 µL) were thawed at room temperature on the morning of analysis, and HSA was isolated from 50 µl of the aliquot using a Pierce albumin depletion kit (Thermo Fisher Scientific, Paisley, UK) according to manufacturer instructions. Briefly, HSA-binding resin (400 µL) was transferred to the spin column and centrifuged at 12,000 *g* for 1 min. The flow-through was discarded and 200 µL of binding buffer centrifuged through the spin column for 1 min at 12,000 *g*. The flow-through was discarded and 50 μL of plasma incubated with the resin for 2 min at room temperature. The plasma incubation was centrifuged at 12,000 *g* for 1 min. The flow-through was collected and centrifuged again through the resin. Unbound proteins were washed off the resin by adding wash buffer and centrifuging at 12,000 *g* for 1 min. The process was repeated three times. Albumin was eluted from the resin by centrifuging with 200 µL 1 M NaCl five times and retaining all of the flow-through. The albumin was reduced with 10 mM dithiothreitol for 15 min before alkylation with 50 mM iodoacetamide for 15 min, both steps at room temperature. An Amicon Ultra-0.5 mL centrifugal filter unit with Ultracel-50

membrane spin columns (Millipore, Watford, UK) was used to concentrate the albumin samples. The albumin samples were added to the spin column and centrifuged at 9200 *g* for 20 min. The spin columns were washed with 400 μL of LCMS-grade water at 9200 *g* for 20 min. LCMS-grade water (50 µL) was added to the albumin sample in the spin column and pipette into a new eppendorf tube. The albumin content was determined using the Bradford assay (section 2.2.3). All the albumin solutions were normalised to 100 pmol/µL and 2 µg of trypsin was added to each solution before overnight incubation. After trypsin digestion, incubations were diluted to 5 pmol/µL in 2% ACN/0.1% FA and K541 peptide (ATKEQLK) (internal standard) added such that its final concentration was 2.5 pmol/µL. Aliquots were analysed by mass spectrometry according to section 2.2.12.

2.2.12 Mass Spectrometric Characterisation of Covalently Modified Proteins

Chromatographic loading and mass spectrometric analysis of the tryptic digest samples was kindly undertaken by Dr Xiaoli Meng, University of Liverpool, according to the following standard method.

Tryptic digests of the recovered GSTP and the recovered or isolated HSA (50 µL) were desalted using C18 ZipTips (Millipore, Watford, United Kingdom). Briefly, the bed of the C18 ZipTip was humidified with acetonitrile and equilibrated with an aqueous solution of TFA (0.1%). The digests were then loaded onto the pipette tip, washed with TFA (0.1%) and finally eluted with a mixture of acetonitrile (50%) and TFA (0.1%). The eluate was evaporated to dry residue and reconstituted in 2% ACN/0.1% formic acid (v/v). Aliquots (2.4−5.0 pmol) of these solutions were delivered into a QTRAP 5500 hybrid triple quadrupole/linear ion trap mass spectrometer (Sciex, Framingham, MA, USA), fitted with a NanoSpray II source, by automated in-line liquid chromatography (U3000 HPLC System, 5 mm C18 nanoprecolumn and 75 µm × 15 cm C18 PepMap column [Dionex, California, USA]) via a 10 µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 60 min was applied at a flow rate of 300 nL/min. The ion spray potential was set to 2,200−3,500 V, the nebulizer gas to 19, and the interface heater to 150 °C. Spectra were acquired

automatically in positive-ion mode using information-dependent acquisition powered by Analyst 1.5 software, across mass ranges of 400−1000 amu in MS and 100−1000 amu in MS/MS. The 5 most intense ions were selected for MS/MS, using a threshold of 5000 counts per second, with dynamic exclusion for 30 s and rolling collision energy. Modified peptides were identified by filtering the product-ion signals for specific fragment ions in PeakView 1.2.0.3 (Sciex) and manual inspection of the spectra.

For MRM detection of modified tryptic peptides from both GSTP and HSA incubations, MRM transitions specific for [O]CBZ-modified peptides were selected as follows: the mass/charge ratio (*m/z*) values were calculated for all possible peptides containing a histidine, lysine or cysteine residue; to these was added the mass of the prospective hapten (for CBZE and an arene oxide that has not rearomatized after its reaction with the protein , 252 amu; for an arene oxide that has rearomatized after its reaction with the protein, 234 amu); and finally the precursor ion masses were paired with the mass of a fragment ion that was expected to be a generic fragment of the predicted adducts, namely the hydroxydibenzoazepinyl species of m/z 210 shown in Figure 2.6. Analysis of histidine, lysine and cysteine residues was undertaken as these residues have been found to be the most commonly modified when drug-protein adducts are characterised by LC-MS/MS analysis of tryptic peptides (Osaki et al. 2014). MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity. They were optimized for collision energy and collision cell exit potential, and the dwell time was 50 ms. MRM survey scans were used to trigger enhanced product ion scans of [O]CBZ-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC-MS and were used to normalize sample loading on the column. Relative quantification of modified peptides was performed by comparing the relative normalized intensity of MRM peaks for each of the modified residues against total ion counts across samples. Data were analysed using Analyst software (Sciex).

2.3 Results

2.3.1 Formation of GSH Conjugates by Microsomally Generated CBZ Metabolites

As reported (Bu et al. 2007), two isomeric dihydrohydroxy-*S*-glutathione conjugates of CBZE (CBZE-SG1 and CBZE-SG2) were produced by direct incubation of CBZE with GSH. The conjugates, which were detected using the MRM transition *m/z* 560→ 431 (the elimination of pyroglutamate from the glutamyl residue), which is effectively the generic fragmentation of *S*-glutathione conjugates in positive-ion analysis (Baillie and Davis 1993), eluted at 12.9 and 13.4 min, respectively (Figure 2.3). These two conjugates were also produced in the incubations of CBZ with HLM and all three CYP isoforms. The microsomal incubations also produced a singleton dihydrohydroxy-*S*-glutathione conjugate of CBZ at 11 min that was more polar, and consistently less abundant, than the isomeric metabolites derived from CBZE (Figure 2.4). By a process of elimination, the polar product was deduced to be a conjugate of an arene oxide. Bu et al. (2007) came to the same conclusion (see Figure 2B in that paper). By way of confirmation, the reaction mixture was also analysed for a rearomatized, i.e. a dehydrated, *S*-glutathione conjugate, which is generally considered to be the signature GSH conjugate of arene oxides (Maggs et al. 2000; Henderson et al. 2005); using by analogy the MRM transition *m/z* 542→ 413. Indeed there is tentative evidence that a dihydrohydroxyCBZ-*S*-glutathione conjugates of CBZ produced by isolated rat hepatocytes underwent this dehydration (Mahajan and Evans 2008). However, no MRM signal was detected in our experiments. Because certain dihydrohydroxyaryl thioether conjugates are in fact somewhat stable, and require acidic conditions to undergo appreciable rearomatization (Jerina et al. 1970; Jeffery and Jerina 1975), aliquots of the reaction mixture were incubated with 5% TFA at room temperature for 2-28 h. Although extensive rearrangements of the conjugates were evident, the rearomatization product was not seen. The proportions of the three GSH conjugates generated by CYP3A4 and CYP2E1 were similar but a relatively higher proportion of the more polar singleton conjugate was produced by CYP2B6 (Table 2.2). All three CYP isoforms generated CBZE, 2OH-CBZ and 3OH-CBZ metabolites (Figure 2.5).

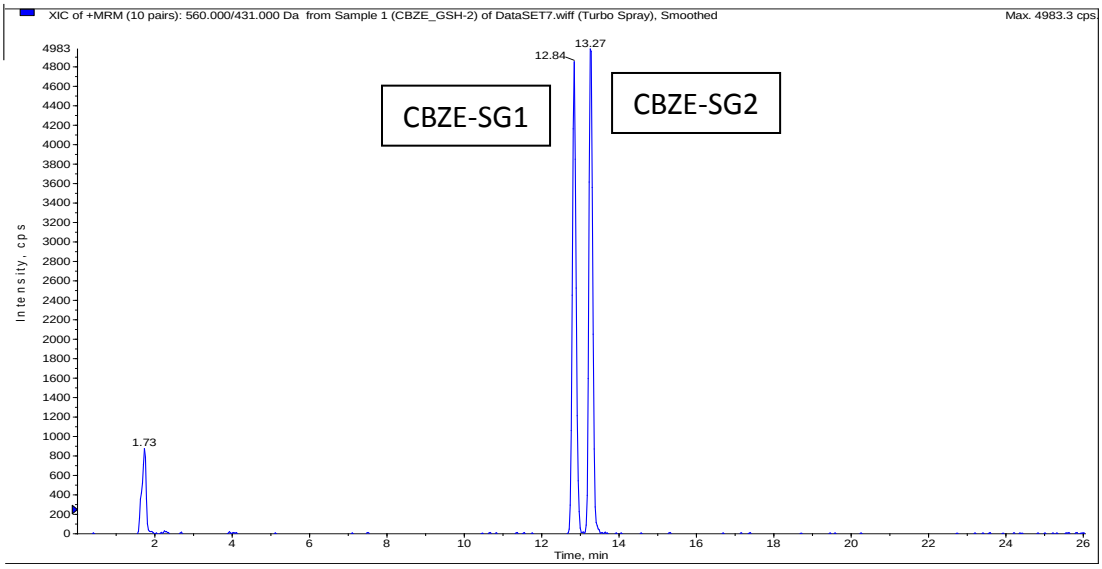


Figure 2.3 Isomeric GSH adducts of CBZE. Formed on the incubation of GSH (5 mM) with synthetic CBZE (80 μM) in phosphate buffer, 0.1M, pH 7.4, at 37 ^{o}C for 2 h. The adducts were resolved on a C8 column, and detected in positive-ion mode by MRM (*m/z* 560 $\rightarrow$ 431, the neutral loss of pyroglutamate from the protonated molecule).

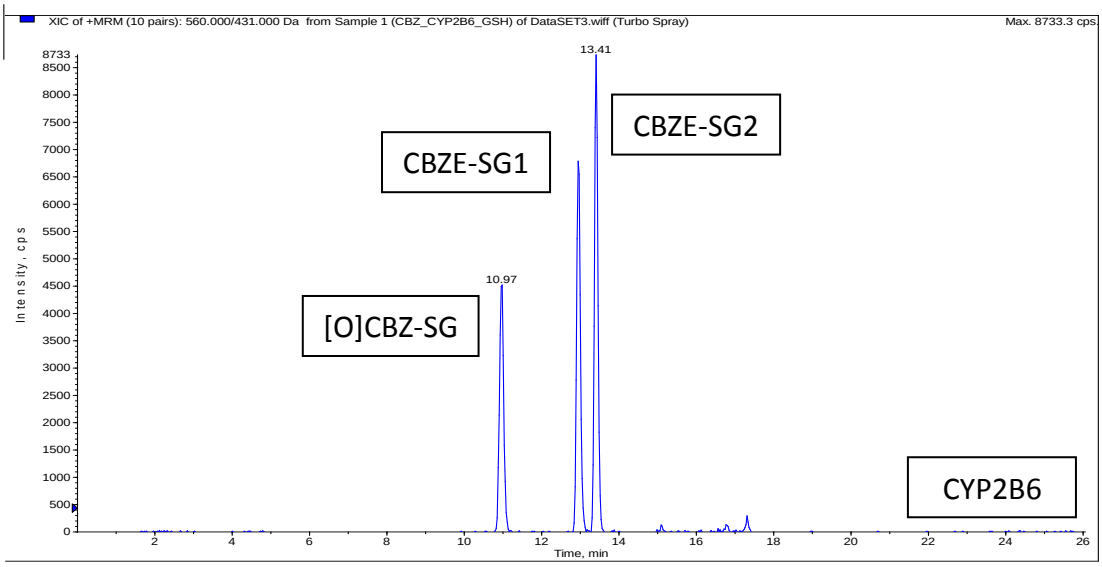


Figure 2.4 Isomeric GSH adducts of monoxygenated CBZ metabolites that were formed from CBZ incubated with HLM and Supersomes. The thioether metabolites produced by CYP2B6 Supersomes are shown. CYP2B6 is the most active CBZ C-2 hydroxylase (Pearce et al. 2002) and the most active CBZ hydroxylase overall (Table 2.2). CBZE-SG1 and CBZE-SG2 were assigned by comparison with standards prepared from synthetic CBZE. The assignment of the third product to an adduct of CBZ arene oxide was based on inference.

Table 2.2 Relative proportions of the three isomeric dihydrohydroxyCBZ-*S*-glutathione conjugates formed from CBZ by HLM and Supersomes

Enzymic Preparation	Ratio of GSH Adducts ([O]CBZ-SG: CBZE-SG1: CBZE-SG2)*	Aromatic Hydroxylase Activity (pmol/nmol P450/min)**
HLM	7.0 : 45.2 : 47.8	NA
CYP3A4	10.3 : 42.5 : 47.2	72.5
CYP2B6	25.5 : 34.5 : 40.0	186
CYP2E1	13.1 : 40.6 : 46.3	38

*Relative areas of peaks in *m/z* 560 → 431 MRM chromatograms acquired by LC-MS/MS (neutral loss of pyroglutamate from the protonated molecule)
**Combined C-2 and C-3 CBZ hydroxylase activities of human P450 isoforms taken from Pearce et al (2002)

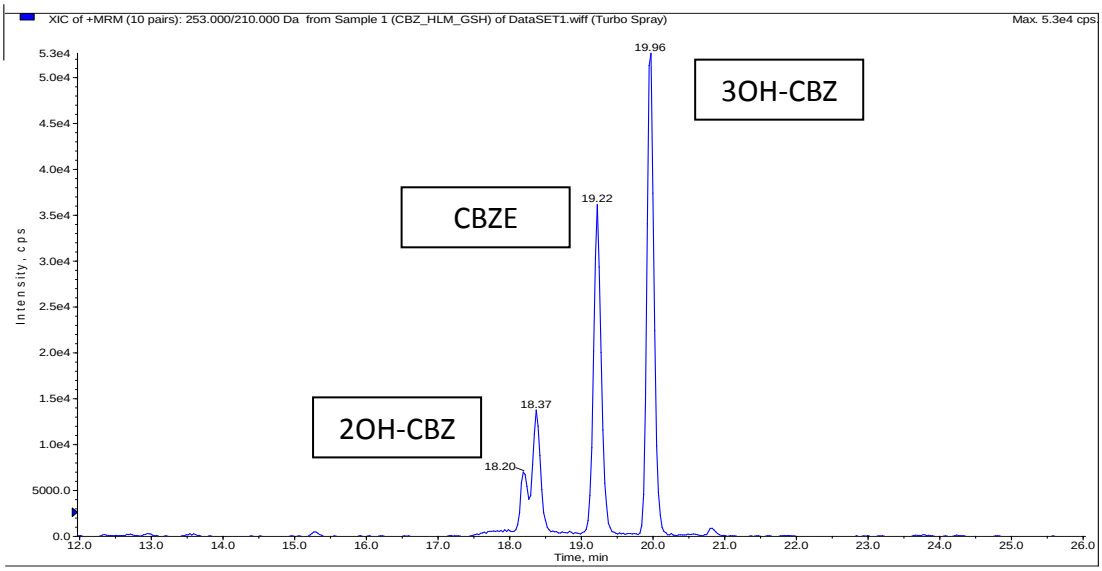


Figure 2.5 Representative MRM chromatogram of [O]CBZ metabolites formed from the incubation of CBZ (100 µM), HLM (2 mg/mL), NADPH (2 mM), MgCl2 (5 mM) and GSH (5 mM) in phosphate buffer (0.1 M, pH 7.4) for 2 h at 37 °C. The metabolites were resolved on a C8 column, and detected in positive-ion mode by MRM (m/z 253 → 210 CBZE and [O]CBZ).

2.3.2 Characterisation of GSTP Modified by Microsomally Generated Metabolites of CBZ

A [O]CBZ adduct was identified at Cys47 of GSTP that was isobarically consistent with the adduct identified previously when GSTP was reacted with synthetic CBZE (Yip et al. 2014). LC-MS/MS analysis of the tryptic digests demonstrated the unmodified Cys47-containing ^{45}ASCLYGQLPK^{54} peptide (+2 ion at *m/z* 540.3). Modification of Cys47 by one or more [O]CBZ species (CBZE and/or one or more arene oxides; the adduct's product ion spectrum does not enable a more precise chemical assignment) resulted in a doubly charged peptide ion at *m/z* 666.3 that eluted at 34 min, corresponding to a mass increase of 252 amu compared with the mass of the unmodified peptide. The sequence of the proposed [O]CBZ-modified peptide, ^{45}ASC*LYGQLPK^{54}, was confirmed by the peptide's product ion spectrum (Figure 2.7). An abundant ion at *m/z* 210.18 corresponded to a fragment ion derived from synthetic CBZE (the proposed structure is shown in Figure 2.6), providing the first-line evidence of the adduct's structure. The b3* ion (*m/z* 514.21), with an adduction of 252 amu, confirmed the addition of [O]CBZ species to Cys47. Although human GSTP contains four cysteine residues, three of which are nucleophilic (Antonini et al., 1997; Chang et al., 2001), only Cys47 was found to be modified by metabolites of CBZ. Metabolism of CBZ to CBZE and [O]CBZ metabolites was confirmed in all experiments (Figure 2.8).

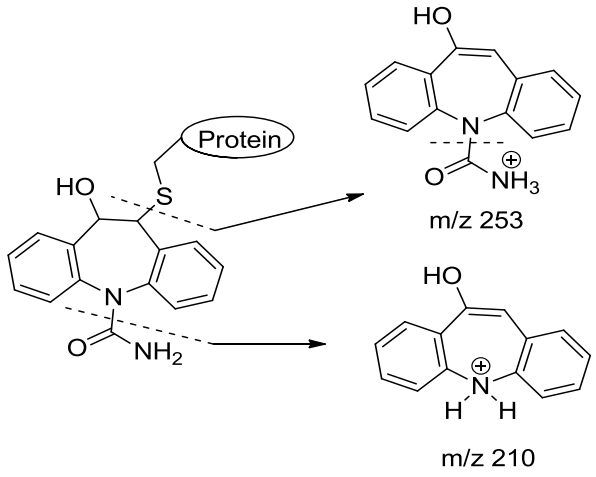


Figure 2.6 Proposed structure and mass spectrometric fragmentation of the conjugate formed by the reaction of enzymatically generated [O]CBZ with Cys47.

Modified GSTP tryptic peptide ^{45}ASC([O]CBZ)LYGQLPK^{54} (adducted at Cys47)

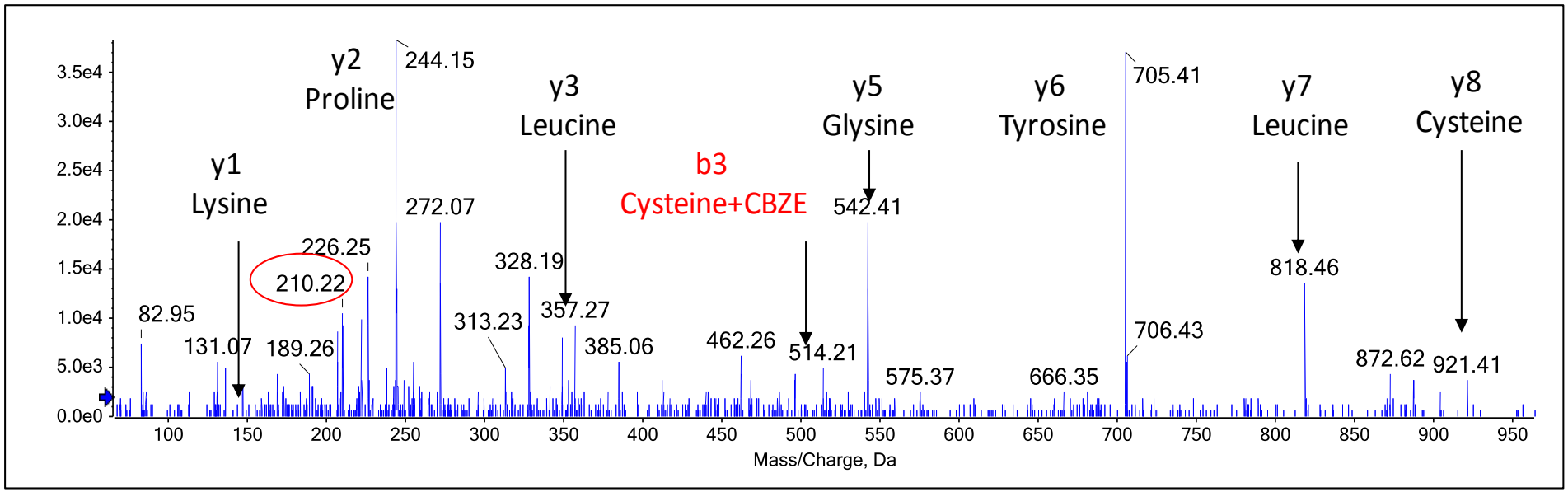


Figure 2.7 Adduct of microsomally generated [O]CBZ and human GSTP. CBZ was incubated with CYP2B6 Supersomes and recombinant human GSTP at pH 7.4 and 37^{o}C for 6 h. Representative product ion spectrum of the modified tryptic peptide adducted with one or more [O]CBZ species at Cys47. The red circle highlights the fragment ion *m/z* 210 consistent with fragmentation of [O]CBZ.

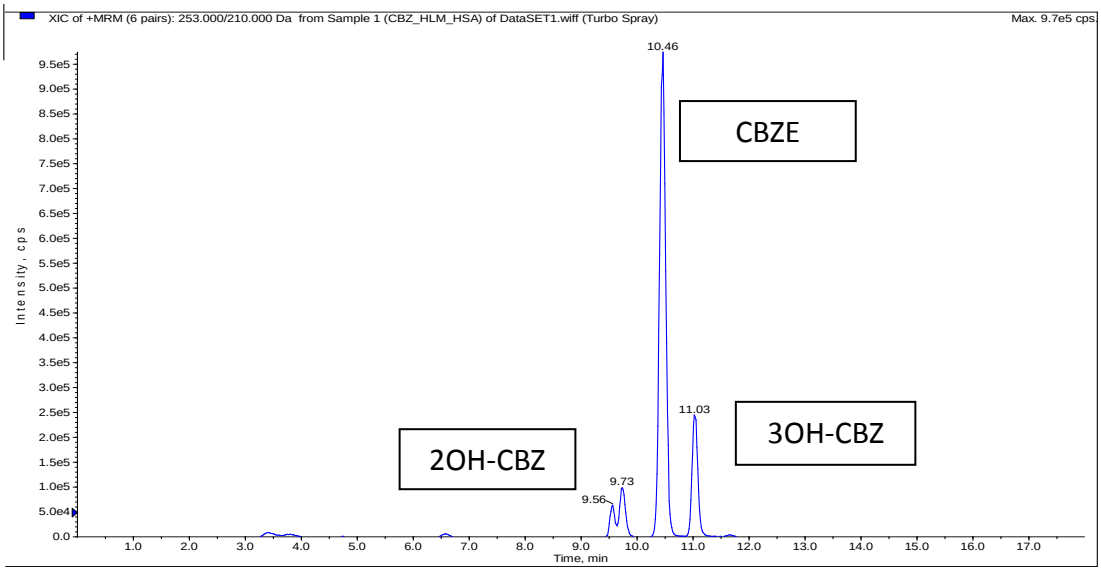


Figure 2.8 Representative MRM chromatogram of [O]CBZ metabolites formed from the incubation of CBZ (1 mM), Supersomes (CYP2B6 20nM), NADPH (2 mM) and HSA (0.6 mM) in phosphate buffer (0.1 M, pH 7.4) for 2 h at 37°C. The metabolites were resolved on a C8 column, and detected in positive-ion mode by MRM (*m/z* 253 → 210 CBZE and [O]CBZ).

2.3.3 Characterisation of Concentration-Dependent and Time-Course Modification of HSA by CBZE *in vitro*

Figure 2.9 outlines the proposed general structure and fragmentation of conjugate formed by the reaction of CBZE with His146 on HSA with abundant ions at *m/z* 180, 210, 236 and 253. A triply charged ion of *m/z* 717.8 was detected by LC-MS/MS at 70 min, corresponding to an adduct derived from the tryptic peptide ^{145}RHPYFYAPELLFFAK^{159} with an additional mass of 252 amu (Figure 2.10). The b2* ion (*m/z* 546) confirms the addition of CBZE to His146. The His146 adduct was detected at all concentrations and relative abundance was positively correlated with increasing CBZE concentration (Figure 2.11). The reaction between CBZE and HSA was first detected at 30 min and showed a positive trend towards greater relative abundance of adduct over time up to 6 h (Figure 2.12).

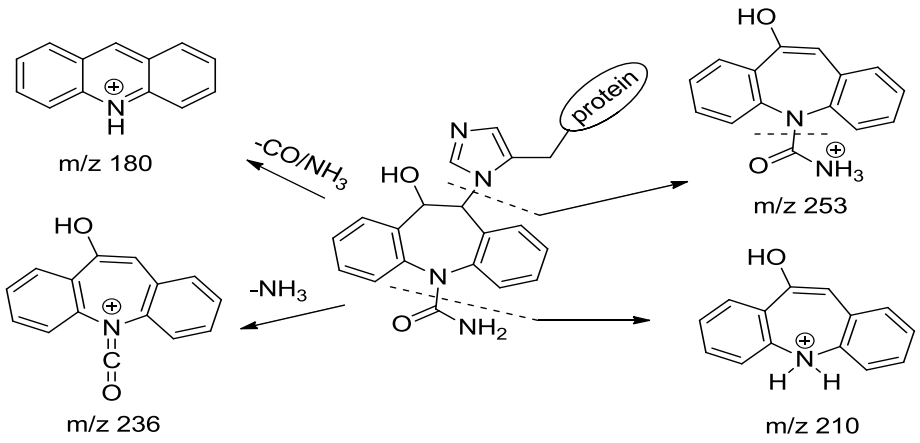


Figure 2.9 Proposed general structure and mass spectrometric fragmentation of conjugate formed by the reaction of CBZE with His 146 on HSA.

Modified HSA tryptic peptide ^{145}R**H**([O]CBZ)PYFYAPELLFFAK^{159} (adducted at His 146)

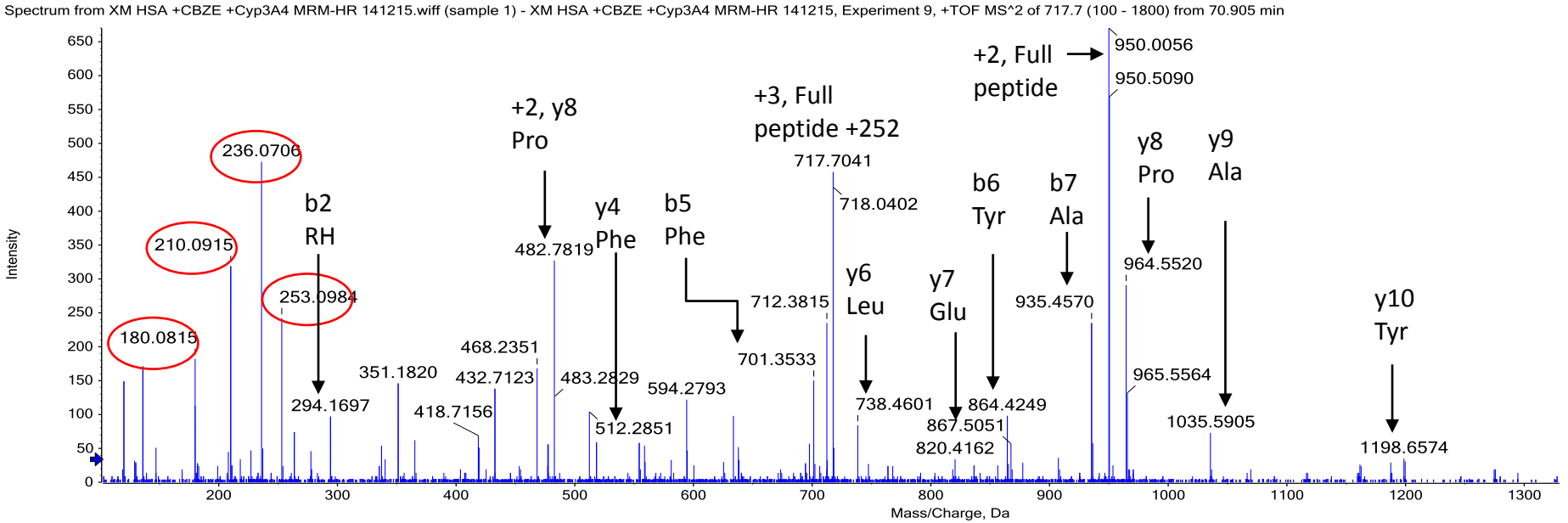


Figure 2.10 Representative product ion spectrum for adduct of CBZE and HSA (His 146). HSA (0.6 mM) was incubated with CBZ (1 mM), CYP3A4 Supersomes (20 nM), NADPH (1 mM) and $MgCl_2$ (5 mM) at 37^{o}C for 2 h. The red circles highlight the fragments of the CBZE-derived moiety of the adduct.

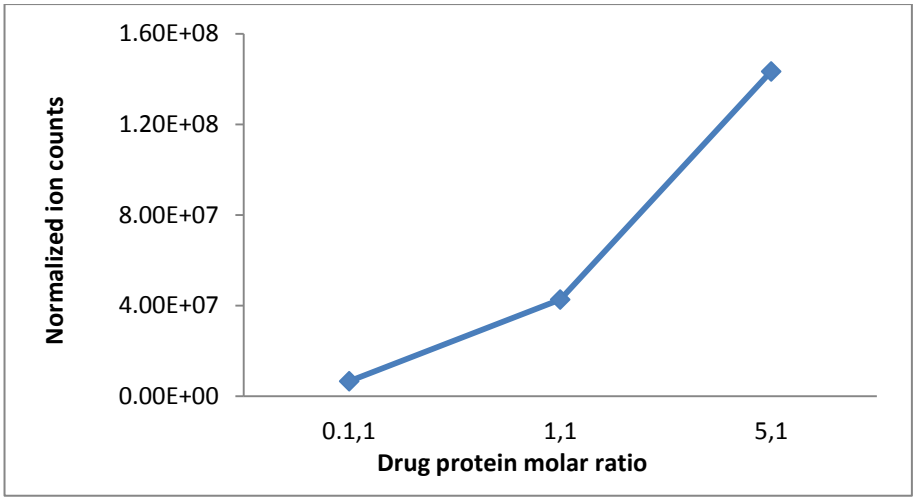


Figure 2.11 Abundance of the CBZE-HSA His146 adduct relative to CBZE:HSA molar ratios. CBZE was incubated with HSA at pH 7.4 and 37^{o}C for 16 h.

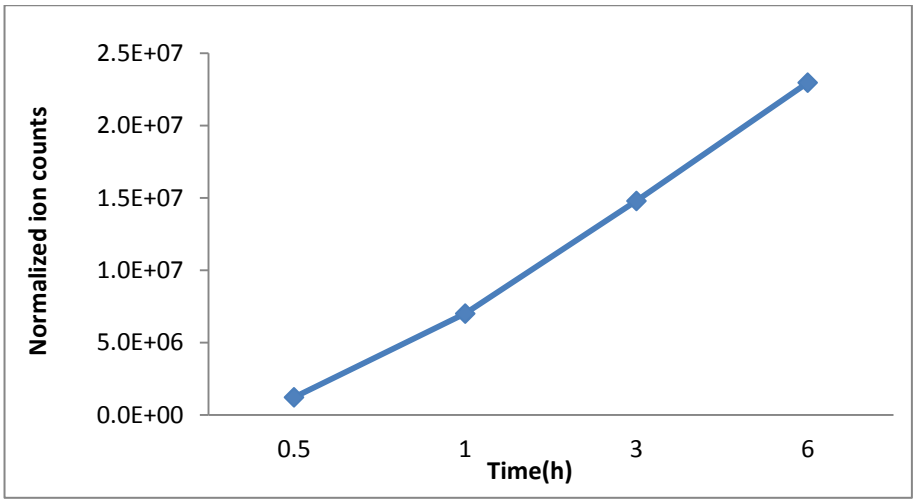


Figure 2.12 Abundance of CBZE-HSA His146 adduct relative to incubation time. CBZE was incubated with HSA at pH 7.4 and 37^{o}C, at a molar ratio of 10:1 (CBZE:HSA). Adduct was first detected at 0.5 h; no adduct was detectable at 0.2 h.

2.3.4 Characterisation of HSA Modified by Microsomally Generated Metabolites of CBZ

LC-MS/MS analysis of tryptic digests of HSA recovered from incubations with CBZ and HLM revealed the same [O]CBZ adduct at His146 as described above (Figure 2.10). There was also tentative evidence for formation of a CBZ adduct at His146, that is, a stable adduct produced by dehydration of a primary, dihydrohydroxyaryl, structure derived from an arene oxide. Figure 2.13 outlines the proposed mechanism for formation of the arene oxide adduct. A triply charged ion of *m/z* 711.7 detected at 63 min corresponded to an adduct of ^{145}RHPYFYAPELLFFAK^{159} with an additional mass of 234 amu (Figure 2.14) and the highlighted b and y fragments confirm the peptide sequence.

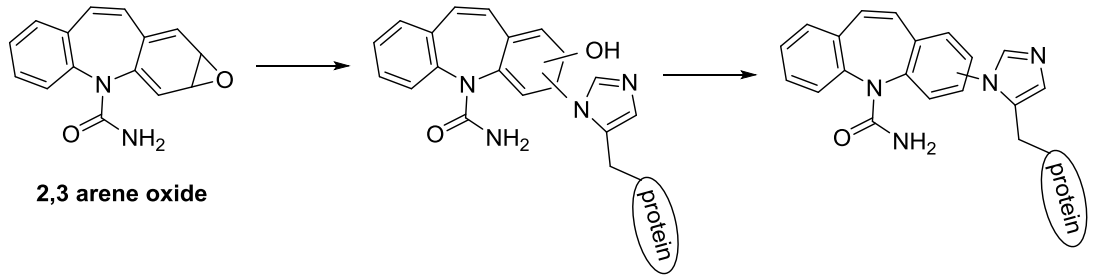


Figure 2.13 Proposed reaction of arene oxide metabolite with his-146 residue of HSA

Modified HSA tryptic peptide ^{145}RH([O]CBZ)PYFYAPELLFFAK^{159} (adducted at His146)

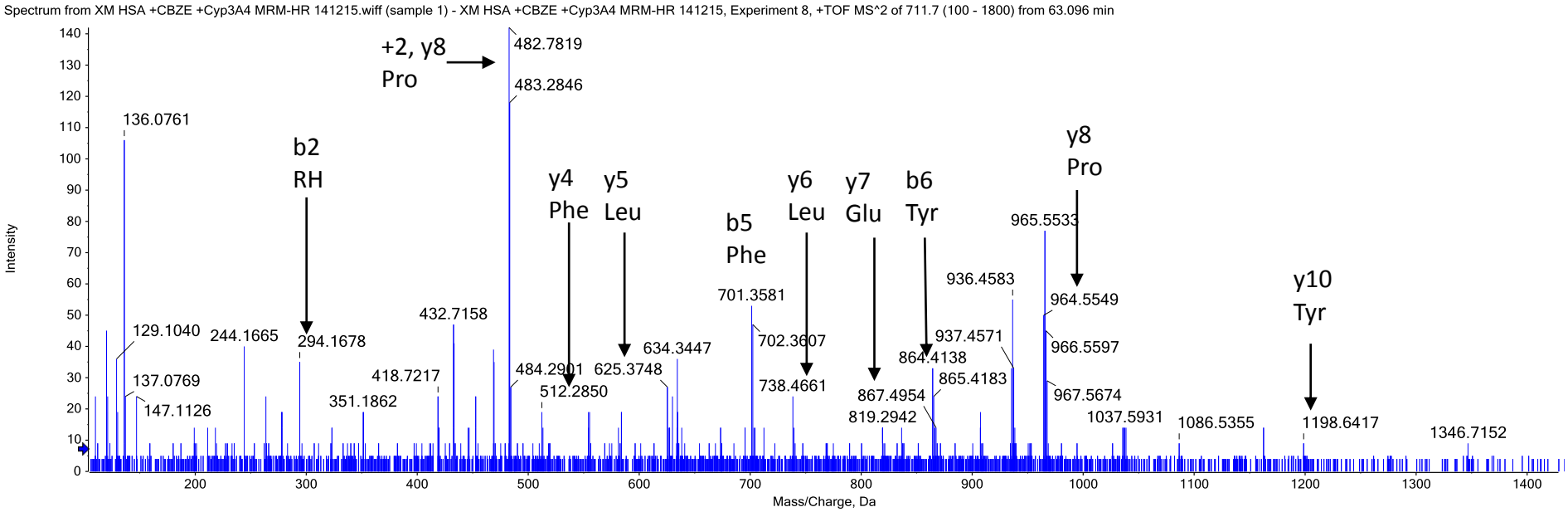


Figure 2.14 Representative product ion spectrum for possible adduct of monoxygenated CBZ (arene oxide) and HSA formed in microsomal incubations. HSA (0.6 mM) was incubated with CBZ (1 mM), CYP3A4 Supersomes (20 nM), NADPH (1 mM) and $MgCl_2$ (5 mM) at 37^{o}C for 2 h.

2.3.5 Detection of CBZ-Derived Covalent Modification of HSA in Epilepsy Patients

The clinical details for the patients who kindly donated the plasma samples are outlined in Table 2.3. The same [O]CBZ-modified His146 peptide, ^{145}RH*PYFYAPELLFFAK^{159}, was identified in all twenty plasma samples (Figure 2.10). There was a 16-fold variability between the lowest level of [O]CBZ-HSA adduct (subject C009) and the highest (subject C036). Some subjects donated two plasma samples from separate visits and these are denoted as (b) in Figure 2.15 and Table 2.3. The greatest intra-subject variability for amount of adduct observed was 3.6-fold for subject C037 despite remaining on the same 1000mg total daily dose of CBZ during the 91 day interval between blood sampling. There was a moderate positive correlation between total daily CBZ dosage and the relative amount of the adduct (r^2 = 0.3932, p = 0.0031) (Figure 2.16A). Plasma levels of CBZ, CBZE, 2OH-CBZ and 3OH-CBZ were measured using HPLC-MS/MS for these subjects (Table 2.3) and further details regarding this method can be found in chapter 3. The strongest positive correlation was observed between CBZ plasma levels and relative amount of adduct observed (r^2 = 0.4387, p = 0.0015) (Figure 2.16B). Moderate positive correlations were also reported between the relative amount of adduct observed and plasma CBZE levels (r^2 = 0.3545, p = 0.0056, Figure 2.16C) and total plasma [O]CBZ levels (r^2 = 0.3568, p = 0.0054, Figure 2.16E). The weakest positive correlation was observed between plasma levels of 2OH- and 3OH-CBZ with relative amount of adduct (r^2 = 0.2619, p = 0.0211, Figure 2.16D).

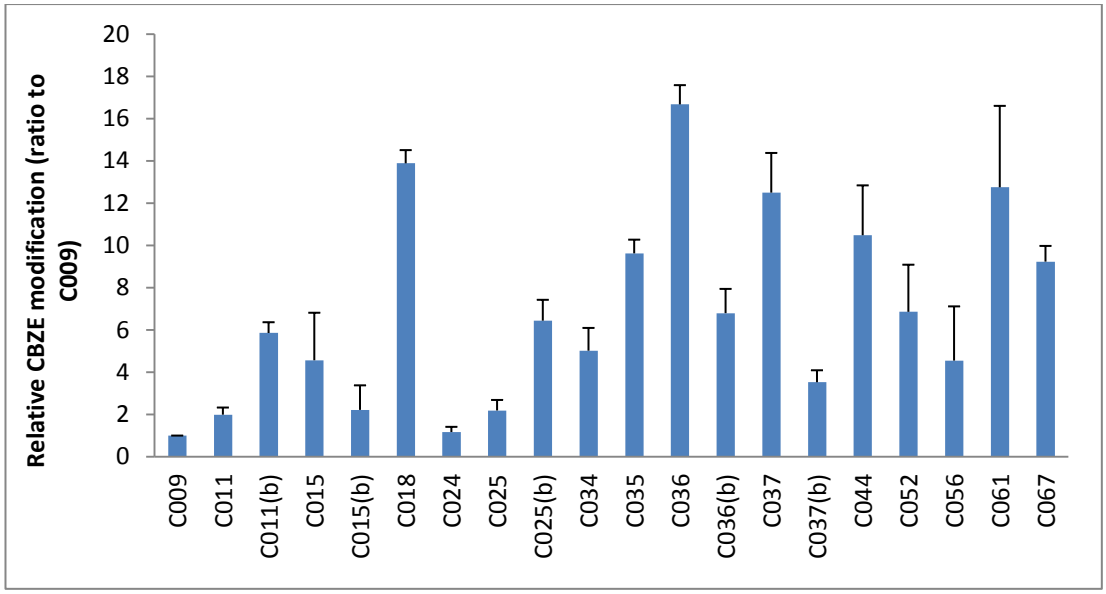


Figure 2.15 Relative quantitation of [O]CBZ-HSA adduct in patients' plasma samples [(b) denotes second plasma sample from same patient at separate visit; data represents mean (n=3) and standard deviation.]

Table 2.3 Patients' clinical details and the relative levels of [O]CBZ-HSA adduct in their plasma [(b) represents second plasma sample from same patient at separate visit].

Patient ID	Gender	Age (years)	Days between visits	Total daily CBZ dosage (mg)	Relative quantity of [O]CBZ-HSA adduct (mean, n=3)*	Standard Deviation	CBZ level (µg/mL)	CBZE level (µg/mL)	2OH-CBZ level (ng/mL)	3OH-CBZ level (ng/mL)
C009	M	34	X	200	1.00	0.00	1.56	0.16	1.76	2.96
C011	M	53	X	800	1.99	0.34	1.31	0.231	1.04	1.82
C011 (b)	M	53	245	800	5.85	0.51	3.8	1.46	6.41	23.70
C015	M	46	X	600	4.56	2.25	6.97	1.2	5.85	15.9
C015 (b)	M	46	202	600	2.22	1.16	6.76	0.918	6.77	15.2
C018	F	46	X	1200	13.89	0.63	8.06	2.57	6.51	16.1
C024	M	50	X	200	1.17	0.25	2.04	0.181	2.56	5.51
C025	M	75	X	200	2.19	0.50	4.46	0.851	4.06	10.5
C025 (b)	M	75	210	200	6.44	0.99	5.05	1.18	10.5	21.6
C034	M	60	X	800	5.02	1.08	8.86	1.32	10.3	17.1
C035	F	62	X	1000	9.62	0.65	11.0	2.93	14.9	31.6
C036	M	51	X	800	16.67	0.91	8.56	1.5	15.3	33.3
C036 (b)	M	51	53	800	6.79	1.15	11.7	1.68	16.4	38.5
C037	F	27	X	1000	12.50	1.88	11.7	2.13	9.84	23.9
C037 (b)	F	27	91	1000	3.52	0.57	11.6	2.88	14.1	31.2

Patient ID	Gender	Age (years)	Days between visits	Total daily CBZ dosage (mg)	Relative quantity of [O]CBZ-HSA adduct (mean, n=3)*	Standard Deviation	CBZ level (µg/mL)	CBZE level (µg/mL)	2OH-CBZ level (ng/mL)	3OH-CBZ level (ng/mL)
C044	F	48	X	1200	10.48	2.36	9.22	5.2	10.7	22.6
C052	F	53	X	600	6.86	2.23	7.86	1.85	11.4	23.9
C056	M	46	X	300	4.55	2.57	5.56	0.57	10	17.1
C061	F	61	X	1000	12.75	3.85	8.09	2.68	11.5	26.9
C067	F	58	X	1600	9.23	0.74	14.3	3.77	33.3	59.2

* [O]CBZ-HSA adduct: an adduct derived from one or more reactive [O]CBZ species, namely CBZE and/or one or more arene oxides.

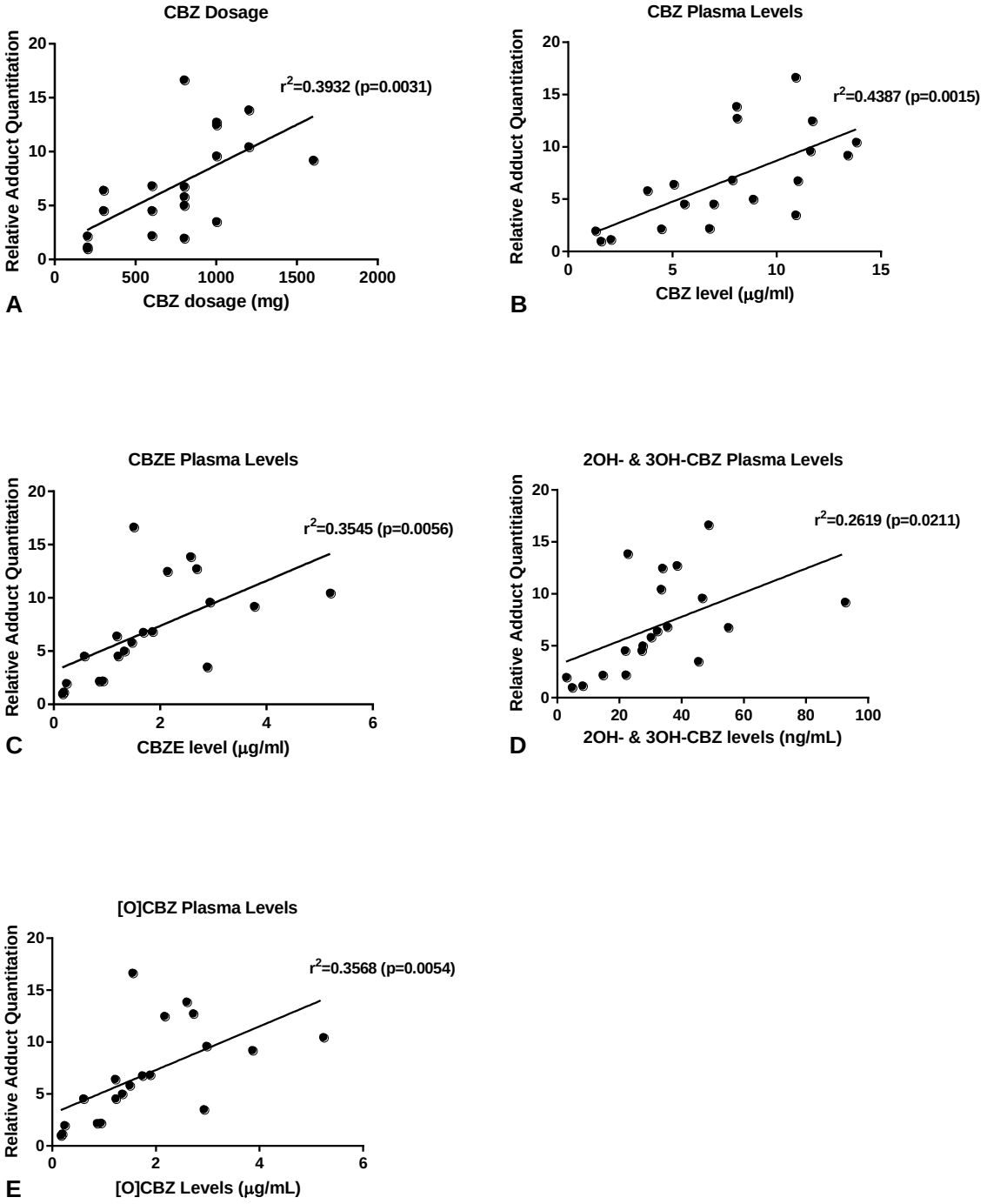


Figure 2.16 Correlation between relative amounts of [O]CBZ-HSA adduct in patients' plasma samples when compared with (A) total daily dosage, (B) CBZ plasma levels, (C) CBZE plasma levels, (D) 2OH- and 3OH-CBZ plasma levels and (E) total [O]CBZ plasma levels (CBZE + 2OH-CBZ + 3OH-CBZ).

2.4 Discussion

This study demonstrates that microsomally generated electrophilic monoxygenated metabolites of CBZ are able to form covalent adducts with co-incubated human GSTP and HSA as well as with GSH (Bu et al., 2005) (Figure 2.17). Reactive metabolite trapping by exogenous protein incubated with hepatic microsomes has been employed previously to identify the metabolites and characterize the protein adducts. Yukinaga et al. (2012) captured reactive intermediates of paracetamol and raloxifene on Cys47 of human GSTP. Damsten et al. (2007) produced an adduct of N-acetyl-p-benzoquinone imine (NAPQI), the reactive metabolite of paracetamol, on Cys34 of HSA by incubating whole plasma with the drug and rat liver microsomes. The [O]CBZE-GSTP adduct at Cys47 was indistinguishable by LC-MS/MS from that formed in earlier incubations of synthetic CBZE and GSTP (Yip et al. 2014). That is, the adducts were isobaric and yielded the same diagnostic fragment ions. The Cys47 residue of human GSTP is a proven biochemical reagent for trapping highly reactive small molecule electrophiles; for example, the residue has been shown to trap the reactive quinoneimine metabolite of paracetamol *in vitro* (Jenkins et al. 2008). Direct incubation between CBZE and GSH yielded two isomeric dihydrohydroxyCBZ-*S*-glutathione conjugates of CBZE (CBZE-SG1 and CBZE-SG2). These two conjugates had been identified previously (Bu et al. 2005). Incubation of GSH with CBZ and HLM or Supersomes yielded an additional singleton dihydrohydroxyCBZ-*S*-glutathione conjugate ([O]CBZ-SG) (Figure 2.4). The singleton adduct was consistently less abundant than the other two conjugates and hypothesised to be formed via an arene oxide (Bu et al. 2007). Comparison of the relative aromatic hydroxylase activity of the three CYP isoforms demonstrated positive correlation with the relative abundance of [O]CBZ-SG (Table 2.2). It has been proposed that a reactive arene oxide intermediate is formed during hydroxylation of CBZ to 2OH-CBZ and 3OH-CBZ (Pirmohamed et al. 1992; Bu et al. 2007). The greater formation of [O]CBZ-SG by CYP2B6, the isoform with greatest aromatic hydroxylase activity, as presented in the results, is therefore consistent with this hypothesis.

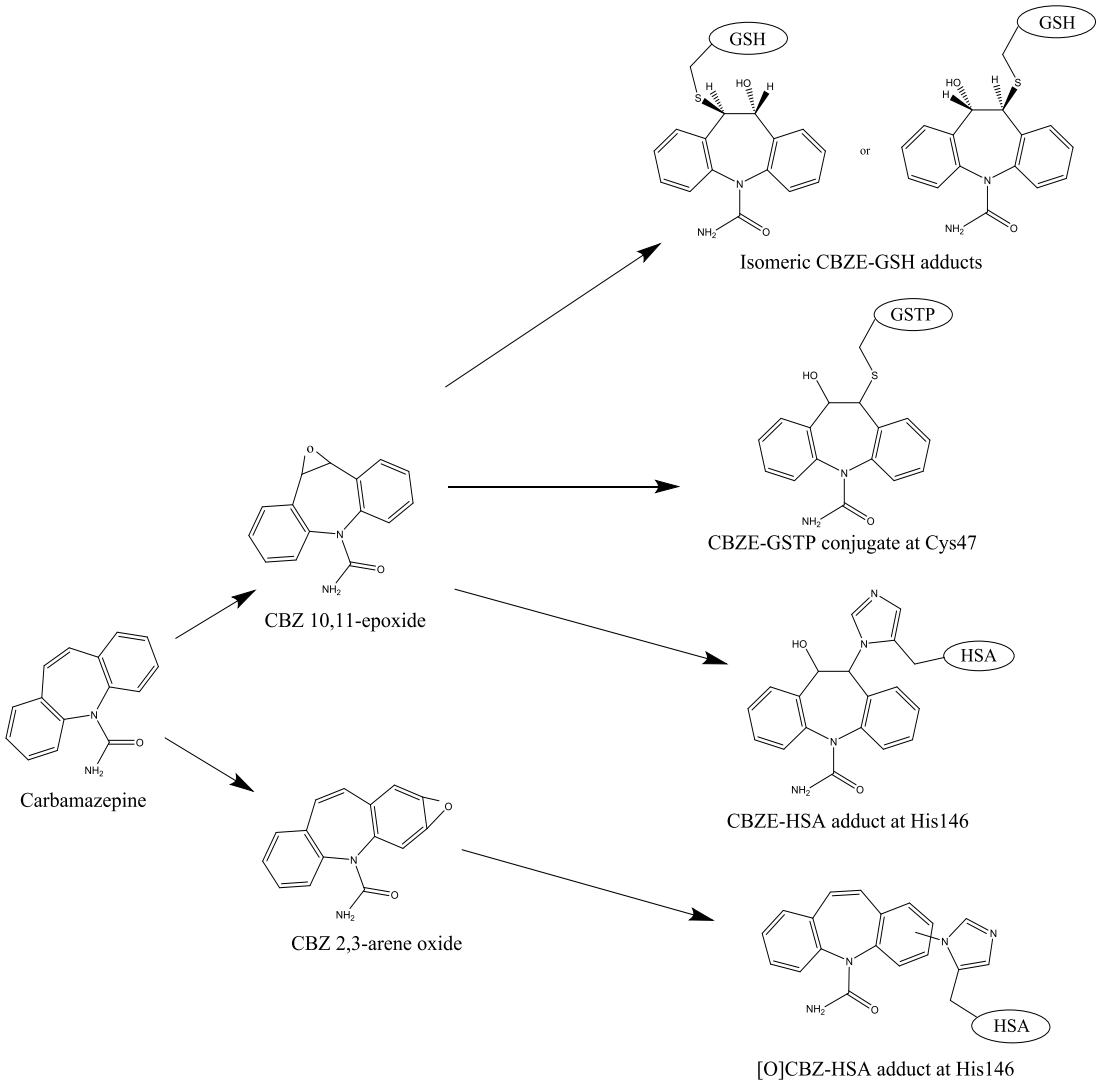


Figure 2.17 Proposed conjugation of CBZE and CBZ 2,3-arene oxide with cysteine and histidine residues. Synthetic CBZE forms two isomeric thioether adducts when it reacts spontaneously with GSH at pH 7.4. Under identical conditions it reacted with human GSTP at Cys47, but with HSA at His146 and His338, by nucleophilic conjugate addition. HSA recovered from an incubation with human liver microsomes, CBZ and NADPH was modified at His146 by addition of monoxygenated CBZ ([O]CBZ; CBZE and/or one or more arene oxides, which are anticipated to yield dihydrohydroxyCBZ adducts indistinguishable by LC-MS/MS). HSA isolated from patients receiving CBZ therapy was modified only at His146 by [O]CBZ. The depiction of the 2,3-arene oxide as the sole product of aromatic epoxidation is purely representational; the number of arene oxides formed is unknown. The HSA incubated with CBZ and microsomes was also adducted at His146 by a residue of mass 234 amu, which was assigned tentatively to the dehydration product of the conjugate of an arene oxide (Figure 2.14).

Intracellular GSTP can be modified by chemically reactive drug metabolites *in vivo*: it is one of the adduction targets of NAPQI in mouse liver (Qiu et al. 1998). However, although the GSTP1 subunit is expressed in many human tissues it is not normally expressed in human liver (Sherratt et al. 1997). The protein is normally present in human plasma (Steinkellner et al. 2005) but at concentrations (approx. 20-50 ng/mL) that are presently far too low for it to be used as a molecular dosimeter. Whereas HSA (66.5 kDa, t½ = 19 days), and specifically its Cys34 thiol (the anticipated reaction site of CBZE), has been recognised as a possible high-abundance target for reactive drug metabolites (Damsten et al. 2007) and electrophilic species generally (Rappaport et al. 2012). Firstly because it is synthesized extensively in the liver (Ballmer et al. 1990), and thereby in close proximity to a major site of drug bioactivation, and secondly because is the most abundant plasma protein, normally constituting about 60% of the total protein in plasma (Quinlan et al. 2005; Goto et al. 2013) (Figure 2.18). Consequently, HSA might be an intracellular target of short-range reactive metabolites and an extracellular target of persistent circulating electrophiles such as CBZE. It has been widely used as a model protein to detect drug-mediated protein modifications (Meng et al. 2011b; Whitaker et al. 2011; Jinno et al. 2011; Ariza et al. 2012; Hammond et al. 2014). A CBZE-HSA adduct, at His146, was identified on direct incubation of CBZE and HSA, and was detectable at low concentrations (0.1:1, CBZE:HSA) after an incubation period of 30 min. An analogous (isobaric) His146 [O]CBZ-HSA adduct was formed in microsomal incubations that also generated CBZE, 2OH-CBZ and 3OH-CBZ in the presence of HSA (Figure 2.10). A second adduct at His146, having the form of CBZ-HSA, was also found in the microsomal incubations of CBZ with HSA (Figure 2.14), and was assigned preliminarily to a rearomatized derivative of an arene oxide adduct. If confirmed, this would represent the first chemical evidence of a CBZ arene oxide that has the ability to escape the microsomal microenvironment and modify soluble proteins. The His146 residue of HSA has been shown to react with diverse electrophilic species including nevirapine metabolites (Meng et al. 2013), abacavir (Meng et al. 2014a) and delta 12-prostaglandin J2 which functions as a regulator of inflammation (Yamaguchi et al. 2010). Although the Cys34 residue is conventionally regarded as the

predominant site of HSA's reactions with organic electrophiles (Rappaport et al. 2012), no adducts were detected with CBZE or [O]CBZ metabolites. However, a number of other thiophilic compounds have also failed to react with Cys34 on HSA (Yamaguchi et al. 2010; Yang et al. 2016). Docking studies have shown that the cavity lined by Cys34 is narrow which can therefore restrict access by thiophilic compounds (Aldini et al. 2008).

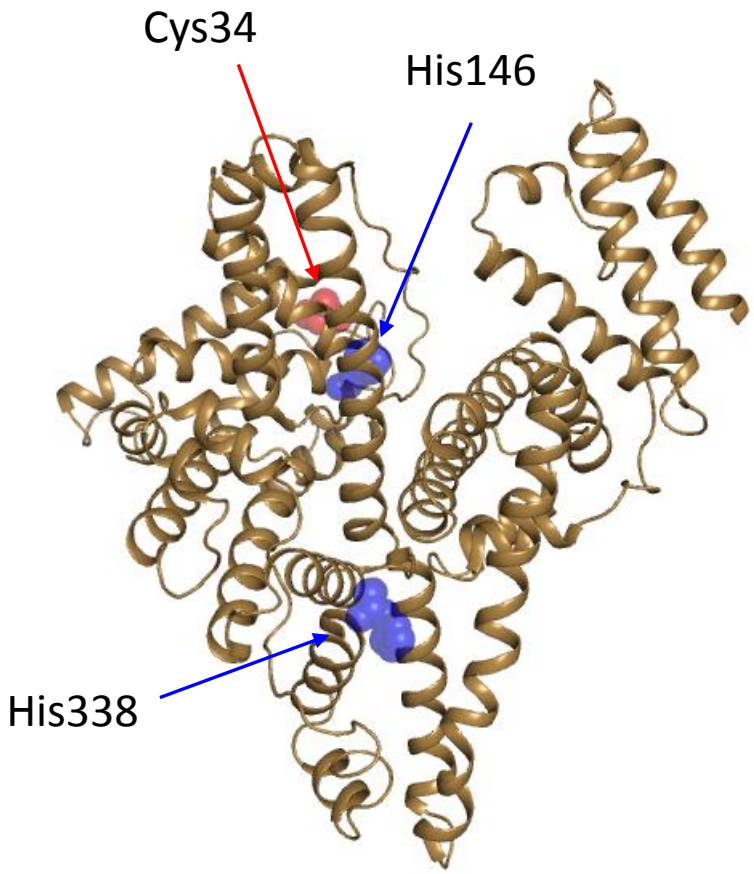


Figure 2.18 Ribbon diagram of HSA showing the locations of the two residues adducted by synthetic CBZE *in vitro*: His146 and His338 (blue residues). Only His146 was modified detectably, by monoxygenated CBZ (CBZE and/or CBZ arene oxide), in hepatic microsomal incubations and patients. The single free cysteine residue, Cys34, is shown as a red residue. It was not modified detectably, either *in vitro* or *in vivo*. The diagram was drawn with PyMol software (Schrödinger, Munich, Germany).

If the adverse skin reactions associated with CBZ (Roujeau et al. 1995; Avancini et al. 2015) are initiated by local protein haptenation, the haptenating species might be CBZE formed in the liver and delivered via the blood (Breton et al. 2005). However, protein-reactive metabolites of CBZ might also be produced locally.

Sulfamethoxazole and dapsone are two effective antimicrobials for the treatment of *Pneumocystis carinii* pneumonia but their use is limited by cutaneous ADRs which includes MPE, HSS and SJS/TEN (Svensson et al. 2001). Bioactivation of sulfamethoxazole and dapsone to reactive metabolites has been proposed to be a critical step for initiation of cutaneous ADR (Svensson 2003). Intracellular drug/reactive metabolite protein adducts were detected from incubations of sulfamethoxazole or dapsone with either normal human epidermal keratinocytes or HaCaT (an immortalised human keratinocyte cell line) (Roychowdhury et al. 2005; Vyas et al. 2006a; Vyas et al. 2006b). These studies provide evidence that keratinocytes are able to bioactivate drugs and form drug-protein adducts which may represent the antigenic determinants for T cells in hypersensitivity reactions. Interestingly, bioactivation in keratinocyte cell lines was mediated by flavin-containing monoxygenases and peroxidases with limited involvement of CYP450 or COXs (Vyas et al. 2006a; Vyas et al. 2006b). Human skin has been shown to express multiple CYP450 isoforms including CYP3A4 and CYP2B6 (Yengi et al. 2003). Levels of CYP2B6 mRNA demonstrated the largest inter-individual variation with CYP2B6 mRNA undetectable in some skin samples. Taken together, these findings suggest that metabolism of CBZ to reactive [O]CBZ metabolites could occur in keratinocytes and there would be variation between patients in the levels of [O]CBZ generated. Patients susceptible to hypersensitivity to CBZ may generate higher levels of [O]CBZ, overwhelming detoxification pathways and leading to keratinocyte death and manifestation of hypersensitivity.

For the first time [O]CBZ-modified HSA was also detected *in vivo*, in patients prescribed standard CBZ therapy for epilepsy and without any indications of drug toxicity; including any indications of the neurotoxicity that has been associated with unusually high plasma concentrations of CBZE (Russell et al. 2015). The His146 [O]CBZ-HSA protein adduct was identified in plasma samples from all of the 20 patients. There was 16-fold inter-patient variation and 3.5-fold intra-patient variation in relative abundance of the CBZE-HSA adduct (Table 2.3). The presence of significant intra-patient variation in relative adduct abundance implies that even within a single patient the levels of [O]CBZ -HSA adduct are likely to be variable and

dependent on factors other than the size and time of dose. It is possible that all patients who receive CBZ therapy have [O]CBZ-HSA adducts in their circulation, irrespective of the imminence or presence of hypersensitivity, implying that other factors need also to be present to trigger a hypersensitivity reaction. Similar analyses conducted in patients receiving flucloxacillin therapy also identified parent drug- and metabolite-modified HSA in all (n=8) tolerant patients (Jenkins et al. 2009). These data combined suggest that circulating drug-modified HSA alone is insufficient to trigger hypersensitivity.

CBZE-HSA adducts appeared detectably *in vitro* from 30 min and increased over time (Figure 2.12). The half-life of human albumin is 19 days, so it is expected that as patients commence CBZ therapy and titrate their dosages the levels of CBZE-HSA adduct will also increase. It is possible that hypersensitivity reactions to CBZ may develop only when a critical threshold of adducts is exceeded. The amount of time taken to reach this threshold could partially explain the two to six week delay between starting CBZ therapy and manifestation of hypersensitivity (Knowles et al. 1999). Cystic fibrosis patients receive multiple intravenous courses of antibiotics because of recurrent infections and are known to have a high incidence of piperacillin hypersensitivity (30-50%) (Parmar and Nasser 2005). Studies in these patients demonstrated that as the plasma concentration of piperacillin is increased then the number of modified residues on albumin increases, generating a larger number of epitopes that could be recognised by the immune system (Whitaker et al. 2011). Similarly, the current study has identified a moderate but significant positive correlation between the dosage of CBZ and relative abundance of [O]CBZ-HSA adduct (r^2 = 0.3932, p= 0.0031). The strongest positive correlation was observed between plasma CBZ levels and relative abundance of [O]CBZ-HSA adduct (r^2= 0.4387, p = 0.0015). However, comparison of relative amount of [O]CBZ-HSA adduct with plasma CBZE concentrations (r^2 = 0.3545, p = 0.0056) and all plasma [O]CBZ metabolites (CBZE, 2OH-CBZ and 3OH-CBZ) concentrations (r^2 = 3568, p = 0.0054) resulted in weaker positive correlations. The assay for measuring the amount of plasma CBZE-HSA adduct is only semi-quantitative, because it was not possible to synthesis CBZE-modified HSA peptides during the study, leading to

inaccurate measurement of adducts present. CBZE has also been previously shown to form covalent adducts with other (unidentified) plasma proteins and HLM, which were not measured as part of this study and may have led to underestimation of total amount of CBZE adducts formed (Bu et al. 2005).

High concentrations of CBZ (1mM) were chosen for the *in vitro* experiments in comparison with the typical therapeutic plasma levels of CBZ observed in patients (0.05mM) in order to maximise generation of minor reactive metabolites thereby increasing the potential for detection of metabolite-modified protein adducts by HPLC-MS/MS. Incubation of synthetic CBZE and HSA generated a second CBZE-HSA adduct at His338 (Yip et al. 2014). However, no adduct other than the [O]CBZ-HSA at His146 was observed in microsomal or patient samples because microsomally generated and plasma levels of CBZE are unlikely to reach the molar ratios tested *in vitro*. The greatest concentration of CBZE observed in patients was 5.2 µg/mL (0.02 mM) and when compared with a typical plasma albumin concentration of 0.6 mM results in a molar ratio of 0.03:1 (CBZE:HSA) in comparison with molar ratios of 0.1:1 to 5:1 (CBZE:HSA) tested *in vitro*. Although unlikely it is possible that a small fraction of patients form a second hapten with CBZ therapy. Russell et al. found a plasma CBZE concentration of 54 µg/mL (214 µM) or 450% of the parent drug after multiple medication overdose, including 17.8 g of CBZ, which would translate to molar ratios in the range of the *in vitro* incubations (Russell et al. 2015). The relationship between plasma levels of drug/metabolite-protein adduct and susceptibility to hypersensitivity is poorly understood and warrants further investigation.

It has been suggested that CBZ hypersensitivity is mediated through a direct PI mechanism (Wei et al. 2012). However, most of this evidence is derived from *in vitro* experiments which cannot replicate the complexity of *in vivo* situations where drug concentrations can vary in different tissues and genetic variation in drug metabolism enzymes might lead to differences between the levels of reactive metabolites (Yip et al. 2015b). *HLA-B*15:02* and *HLA-A*31:01* have both been associated with specific phenotypes of CBZ hypersensitivity reaction but both

associations have very low positive predictive values (Yip et al. 2012), implying that factors other than HLA genotype are involved in pathogenesis of hypersensitivity. The PI mechanism alone is unable to account for these findings. It is possible that CBZ may modify the MHC or TCR directly but CBZ or metabolite-modified peptide fragments are also required to be presented with MHC to activate T-cells. If the threshold of drug/metabolite-peptide is not reached or reactive metabolites detoxified then the peptide fragments may not be presented and activation of T cells avoided. There is significant heterogeneity of immune response in CBZ hypersensitivity which could imply that multiple complementary molecular mechanisms are involved in different patients or organ systems. There is also evidence from CBZ hypersensitivity consistent with the altered peptide repertoire model, where 15% of eluted peptides from CBZ-treated APCs expressing *HLA-B*15:02* were distinct from those bound to *HLA-B*15:02* in the absence of CBZ exposure (Illing et al. 2012); highlighting the possibility of multiple aetiological mechanisms. Similar heterogeneity in mechanism of hypersensitivity has also been postulated for abacavir. Abacavir, a nucleoside reverse transcriptase inhibitor used for treatment of HIV, is associated with hypersensitivity reactions in patients positive for *HLA-B*57:01*, and crystal structure and modelling studies have provided evidence for the altered peptide repertoire model of immune system activation (Illing et al. 2012; Ostrov et al. 2012). Despite these findings analysis of HSA from patients prescribed abacavir revealed that the metabolic intermediate abacavir aldehyde was also able to modify multiple amino acid residues on HSA, including His146, leading to the possible generation of multiple antigens (Meng et al. 2014a). Current *in vitro* assays for diagnosis and investigation of DHRs (e.g. LTT) are limited because only stable compounds, such as parent drug and stable metabolites, are routinely investigated (Schrijvers et al. 2015). Future studies need to focus on assay systems that enable the generation of reactive metabolites and incorporate drug-modified peptides to determine their ability to elicit T-cell responses (Whitaker et al. 2011).

In conclusion, this study has provided the first chemical evidence for the microsomal production of [O]CBZ species that are able to escape the microsomal

domain to react covalently with exogenous soluble proteins. If those species are equally able to escape from the endoplasmic reticulum of living cells they might haptenate numerous intracellular proteins (in fact the ER membrane is significantly more permeable to small organic molecules than other cellular membranes (Le Gall et al. 2004). Most importantly, this study has demonstrated the presence of circulating [O]CBZ-modified HSA in patients without ADR who were receiving standard CBZ therapy and did not have exceptionally high concentrations of CBZE in their plasma.

Chapter 3

The development of a high-performance liquid chromatography-tandem mass spectrometry technique for measurement of carbamazepine and four metabolites in human plasma

3.1 Introduction

The metabolism of CBZ in humans is complex, with multiple pathways leading to the generation of over 30 metabolites (Lertratanangkoon and Horning 1982). CBZ is almost completely metabolised in the liver, with only approximately 5% excreted unchanged in the urine (Thorn et al. 2011). CBZ is oxidised by several CYP450 isoforms and is known to induce its own metabolism through transcriptional up-regulation of multiple CYPs in the liver (Oscarson et al. 2006). Autoinduction is usually complete within the first two weeks of starting therapy but contributes to drug-drug interactions when CBZ is prescribed as part of polytherapy (Kudriakova et al. 1992; Dresser et al. 2000).

It has been persistently hypothesised that reactive drug metabolites could be responsible for hypersensitivity to some drugs in certain patients (Park et al. 2001; Faulkner et al. 2014). The exact identities of those reactive metabolites remain unknown but arene oxide, o-quinone, iminoquinone and acridine species have been hypothesised in the case of CBZ (Madden et al. 1996; Pearce et al. 2002; Pearce et al. 2005; Pearce et al. 2008). The major oxidative route of metabolism for CBZ is conversion to the pharmacologically active CBZE, which is primarily catalysed by CYP3A4 and accounts for approximately 70% of total metabolism (Thorn et al. 2011). CBZE is further metabolised in humans by mEH to DiOH-CBZ (Kroetz et al. 1993; Kerr et al. 1994). The second most important pathway in metabolism of CBZ is the formation of 2OH-CBZ and 3OH-CBZ, which are postulated to proceed through the highly reactive arene oxide intermediate, CBZ 2,3-epoxide (Madden et al. 1996; Bu et al. 2007) (Figure 3.1).

CYP2E1 is the most active catalyst for the formation of 2OH-CBZ in HLMs, whilst CYP2B6 is the most active catalyst for 3-hydroxylation (Pearce et al. 2002). However, CYP3A4, which is generally the most abundant P450 isoform in adult human liver (Achour et al. 2014), also possesses substantial C-3 hydroxylase activity (Pearce et al. 2002), and additionally has minor C-2 hydroxylase activity (Section 2.3). From a combination of specific activities and the isoform abundance in livers of adult Caucasians, it is probable that CYP2E1 and CYP3A4 are usually the

predominant hepatic catalysts of CBZ C-2 hydroxylation and C-3 hydroxylation, respectively. Breton et al. (2005) found that 3OH-CBZ was the principal phenolic metabolite of CBZ in plasma from a set of chronically treated patients: 2OH-CBZ, 0.023–0.057 μg/mL (9/30 patients); 3OH-CBZ, 0.023–0.72 μg/mL (25/30 patients). This finding conformed with the documented enzymology of CBZ's hydroxylation: the specific C-3 hydroxylase activity of CYP3A4 is approximately five-fold greater than the specific C-2 hydroxylase activity of CYP2E1 (Pearce et al. 2002).

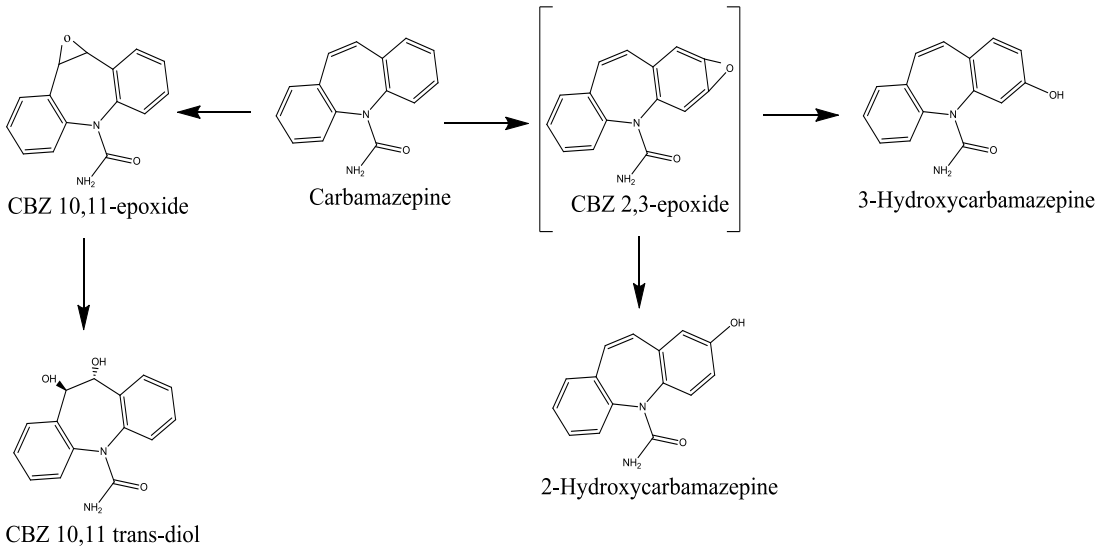


Figure 3.1 Simplified representation of the oxidative and hydrolytic pathways of the metabolism of CBZ in humans. Showing all of the plasma metabolites assayed in the present investigations (CBZ 2,3-epoxide is shown in brackets as this intermediate species is a hypothetical structure, based on the known chemical structures of the stable phenolic metabolites). The complete oxidative pathways of the metabolism of CBZ in humans, as currently known, are shown in Figure 1.3.

CBZE is a reactive metabolite that forms covalent adducts with HSA *in vitro*, and probably contributes to the production of circulating CBZ[O]-HSA adducts in patients established on CBZ therapy (Figure 2.10). The formation of 2OH- and 3OH-CBZ implies the bioactivation of CBZ to a reactive arene oxide intermediate (Pearce et al. 2002). Indeed, arene oxide metabolites have been shown to contribute to the covalent binding of CBZ to hepatic microsomal protein (Pirmohamed et al. 1992), and GSH adducts of epoxides have been identified in the bile of rats given CBZ (Madden et al. 1996). The liver contains multiple detoxification pathways as it is

exposed to potentially harmful exogenous chemical substances each day from the environment (Grant 1991).

Chemical deactivation (bioinactivation) of CBZ, and of pre-reactive and reactive CBZ metabolites, is proposed to be through glucuronidation by UGT2B7 (Staines et al. 2004), reduction by DT-diaphorase, methylation by catechol-O-methyltransferase (Lillibridge et al. 1996), hydrolysis by mEH (Kitteringham et al. 1996; Nakajima et al. 2005) and conjugation with GSH (Madden et al. 1996; Bu et al. 2005; Bu et al. 2007). These pathways could play an important role in limiting the hypersensitivity reactions to CBZ. Intrinsic or acquired insufficiency of those detoxification pathways or generation of excessive reactive metabolites could lead to the initiation of cellular damage and CBZ-induced hypersensitivity (Green et al. 1995; Zhu et al. 2014).

To investigate the roles of bioactivation and bioinactivation pathways in the development of CBZ hypersensitivity reactions, it is necessary to measure human plasma levels of CBZ and its metabolites. In clinical practice TDM of CBZ can help to guide dosing especially as CBZ interacts with many drugs, including other antiepileptics (Neels et al. 2004). Correlation between CBZ dosage and plasma levels is poor so TDM is important in detecting elevated CBZ levels in patients with signs of toxicity or low levels in those with no therapeutic effect (Bondareva et al. 2006). Therapeutic levels of CBZ are between 4-12 mg/L and fatalities have been reported at levels >20 mg/L (Schulz et al. 2012). Because CBZE contributes to the clinical response - the therapeutic range is considered to be 0.4-4 mg/L - its plasma concentration should not be greater than 9 mg/L to prevent toxicity (Burianova and Borecka 2015). Other metabolites of CBZ are not routinely measured in clinical practice. It was therefore necessary to develop an assay capable of measuring plasma levels of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ in patients. These analytes were selected because of their hypothesised role as reactive metabolites *in vivo* (CBZE) or as downstream products of reactive metabolites (DiOH-CBZ, and 2OH- and 3OH-CBZ; Figure 3.1)

Several HPLC-MS/MS techniques to assay CBZ and one or more of its metabolites in human plasma or serum have been published (Eto et al. 1995; Wad et al. 1997; Van Rooyen et al. 2002; Breton et al. 2005; Lionetto et al. 2012; Shibata et al. 2012). Two of the methods quantify only CBZ and CBZE (Van Rooyen et al. 2002; Lionetto et al. 2012) whilst two other methods quantify only individual minor metabolites (Eto et al. 1995; Wad et al. 1997). The most comprehensive HPLC-MS/MS assay quantified CBZ and nine metabolites with 2-methyl-carbamazepine (2Me-CBZ) as internal standard (IS) (Breton et al. 2005). This method included all of the analytes of current interest: CBZ, CBZE, DiOH-CBZ, 2-hydroxycarbamazepine (2OH-CBZ) and 3-hydroxycarbamazepine (3OH-CBZ), and acted as a basis for the development of the HPLC-MS/MS assays described below. Assay development was undertaken according to the guidelines for bioanalytical method validation outlined by the FDA (Food and Drug Administration 2001).

3.2 Methods

3.2.1 Chemicals and Reagents

CBZ (Sigma-Aldrich Co, Poole, UK), CBZE (Sigma-Aldrich Co), DiOH-CBZ (NewChem Technologies, Durham, UK), 2OH-CBZ (NewChem Technologies) and 3OH-CBZ (NewChem Technologies) were measured using the HPLC-MS/MS assay. The purities for each compound were reported by the manufacturers and are as follows: CBZ ≥98%, CBZE ≥98%, DiOH-CBZ 98.5%, 2OH-CBZ 99.4% and 3OH-CBZ 97.6%. 2Me-CBZ (NewChem Technologies) was used as the internal standard for all the assays of plasma analytes and its purity was reported as 99.7%. The ammonium formate buffer solution (pH 3) consisted of the formic acid salt (126 mg/L) dissolved in purified water (HPLC eluent A). Purified water was generated by a PURELAB Ultra water system (Veolia Water Technologies UK, High Wycombe, Buckinghamshire). Acetonitrile (Fisher Scientific, Loughbrough, UK) used in eluent B was LCMS grade. All reagents were LCMS grade unless otherwise specified.

For validation of the method, blood samples from healthy volunteers were collected in lithium heparin tubes and centrifuged at 1500*g* for 10 min at 4^{o}C to obtain plasma. 1 mL aliquots of plasma were separated, and frozen at -80^{o}C. Plasma aliquots were thawed at room temperature when required during the study for the preparation of calibration and quality control (QC) standards. The study protocol was approved by the University of Liverpool research ethics committee.

Stock solutions of the analytes and internal standard were prepared by dissolving each compound in acetonitrile (Table 3.1). Stock solutions were stored at 4^{o}C and protected from light during the analyses. Working solutions were prepared fresh daily by diluting stock solutions with acetonitrile-water (50:50, v/v) (Breton et al. 2005).

Table 3.1 Concentrations of stock solutions (all analytes were dissolved in acetonitrile)

Analyte	Concentration (mM)
Carbamazepine	45
Carbamazepine-10,11-epoxide	12.5
10,11-dihydro- 10,11-*trans*- dihydroxy-carbamazepine	4
2-hydroxycarbamazepine	2.5
3-hydroxycarbamazepine	10
2-methylcarbamazepine	4

3.2.2 Preparation of Calibration and Quality Control (QC) Standards

The calibration set consisted of eight concentrations and they were prepared by adding 3 µL of each analyte (CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ) and 3 µL of internal standard (2Me-CBZ, 0.2mM) into 482 µL of drug-free human plasma. After addition of the analytes and internal standard the samples were vortexed for

30 s to ensure thorough mixing. The calibration concentrations for each analyte are recorded in Table 3.2. The plasma was pooled from six volunteer donors to minimise volunteer-specific matrix effects. QC standards used for assay validation, and thereby the acceptance of the assays of each batch of analytical samples, were prepared in the same way as the calibration standards. Three QC standards, corresponding to low (Q1), middle (Q2) and high (Q3) concentration points of the calibration curve, were prepared by mixing volunteer plasma and 3 µL of working solution for each analyte and 3 µL of internal standard (0.2 mM) (Table 3.3). To ensure that the physico-chemical integrity of the matrix was maintained, the total volume of working solution added to the matrix (human pooled plasma) was always less than 4% of the final volume of the spiked plasma. Acetone (500 µL) was added to each calibration and QC standard and then vortexed for 30 s. The mixtures were centrifuged at 1400*g* for 10 min at room temperature. The supernatant was collected carefully, leaving behind the precipitate. The supernatants were evaporated to dry residue using a SpeedVac vacuum concentrator (Eppendorf, Germany). The dried residues were then reconstituted in 500 µL of acetone and water (50:50 v/v). The samples were vortexed to ensure complete homogenisation. An aliquot of the vortexed solution (100 µL) for analysis of CBZ, CBZE and DiOH-CBZ was transferred into a glass HPLC autosampler vial (VWR, Leicestershire, UK) ready for immediate analysis.

For analysis of the plasma QC and calibration standards for 2OH- and 3OH-CBZ, 100 µL of each standard was aliquoted from the deproteinized and reconstituted supernatants, and centrifugally filtered at 450 *g* for 15 min at 4^{o}C through a 0.45 µm hydrophilic PTFE filter plate (Merck Millipore, Watford, UK). The filter plates were prepared by adding 100 µL of water and centrifuging at 450 *g* for 15 min at 4^{o}C prior to sample processing. The filtrates were subsequently transferred into glass HPLC autosampler vials (VWR) ready for immediate analysis. The PTFE filter plates were used to further remove any proteins that may have still been present.

Table 3.2 Calibration ranges for each analyte

Calibration Points	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
1	0.33	0.10	0.10	0.92	4.73
2	0.66	0.20	0.20	1.85	9.46
3	1.33	0.39	0.41	3.69	18.91
4	2.66	0.79	0.81	7.39	37.83
5	5.32	1.58	1.62	14.77	75.66
6	7.97	3.15	3.24	29.54	151.32
7	10.63	4.73	4.86	59.09	302.64
8	15.95	6.30	6.49	118.18	605.28

Table 3.3 Quality control sample concentrations

Quality Control	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
QC1 (low)	0.475	0.150	0.150	2.000	10.000
QC2 (middle)	4.750	1.500	1.500	10.000	50.000
QC3 (high)	9.500	6.000	6.000	100.000	500.000

3.2.3 Liquid Chromatography-Tandem Mass Spectrometric Analysis of Calibration and QC Standards

Aliquots (20 µL) of the calibration and QC standards for CBZ, CBZE and DiOH-CBZ were injected onto an Agilent 5-µM Zorbax Eclipse XDB-C8 column (150 mm x 4.6 mm; Agilent Technologies, Santa Clara, CA, USA) also attached to a 5 µM Eclipse XDB-C18 guard column (Agilent Technologies) without further treatment for determination of CBZ, CBZE and DiOH-CBZ levels. The samples were separated by

gradient elution at room temperature with acetonitrile in ammonium formate buffer (2 mM, pH 3). The eluent was delivered by a PerkinElmer series 200 HPLC system (pump and autosampler; PerkinElmer, Norwalk, CT, USA). The MS analysis was performed using a 4000 QTrap hybrid quadrupole mass spectrometer (Sciex, Warrington, UK) with a Turbo V electrospray source that was connected to the column using a Valco flow-splitting T-piece. The split flow of the eluate was approximately 150 µL/min. CBZ, CBZE and DiOH-CBZ were eluted with the following optimized gradient of acetonitrile: 15% to 33% over 12 min; 33% to 50% over 0.5 min; 50% for 2 min; 50% to 15% over 0.5 min; 15% for 4 min. The eluent flow rate was 1.0 mL/min. The operating parameters of the mass spectrometer (source and Q1) were as follows: source temperature, 450^{o}C; ionspray (electrospray capillary) voltage, 4,500 V; entrance potential, 10 V; curtain gas setting, 15; spray gas (Gas-1) setting, 50; heater gas (Gas-2) setting, 50. Table 3.4 outlines the analyte specific mass spectrometric parameters for CBZ, CBZE, DiOH-CBZ and 2Me-CBZ. The instrument was set up for MRM acquisitions. Instrument management and data processing were accomplished through Analyst 1.5.2 software (Sciex, Framingham, MA, USA).

Table 3.4 Mass spectrometer parameters for CBZ, CBZE,DiOH-CBZ and 2Me-CBZ

MS Parameter	CBZ	CBZE	DiOH-CBZ	2Me-CBZ
Dwell time (msec)	150	150	150	150
Desolvation potential (V)	121	76	46	121
Exit potential (V)	10	10	10	10
Collision energy (V)	27	33	41	27
Collision exit potential (V)	12	48	10	12

To measure levels of 2OH-CBZ and 3OH-CBZ, 5 µL of the calibration and QC standards were injected onto a Waters 2.5 µM Xbridge BEH C18 column (2.1 mm x 150 mm; Waters Limited, Herts, UK) which was also attached to a 2.5 µM Xbridge BEH C18 VanGuard pre-column (Waters Limited, UK). The samples were separated by optimized gradient elution at 40^{o}C with acetonitrile containing 0.1% formic acid in ammonium formate buffer (2 mM, pH 3). The eluent was delivered by an UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA). The MS analysis was performed using a 4000 QTrap hybrid quadrupole mass spectrometer (Sciex, UK) with a Turbo V electrospray source that was connected directly to the column. 2OH-CBZ and 3OH-CBZ were eluted with the following gradient of acetonitrile: 15% to 20% over 2 min; 20% to 25% over 2 min; 25% to 30% over 2 min; 30% to 60% over 2 min; 60% for 4 min; 60% to 15% over 0.1 min; and 15% for 6.9 min. The eluent flow rate was 200 µL/min. The operating parameters of the mass spectrometer (source and Q1) were as follows: source temperature, 450^{o}C; ion spray (electrospray capillary) voltage, 5,500 V; entrance potential, 10 V; curtain gas setting, 30; spray gas (Gas-1) setting, 40; heater gas (Gas-2) setting, 40. Table 3.5 outlines the analyte specific mass spectrometer parameters for 2OH-CBZ, 3OH-CBZ and 2Me-CBZ. The instrument was set up for MRM acquisitions. Management of the HPLC pump was achieved using the Chromeleon 6.80 software (Dionex Corporation, CA, USA) linked using Dionex chromatography MS link version 2.7.0.2551 (Dionex Corp, USA) to Analyst 1.5.1 software (Sciex, UK), which was used to the control the MS. Data processing was accomplished through Analyst 1.5.1 software (Sciex, UK).

Table 3.5 Mass spectrometer parameters for 2OH-CBZ, 3OH-CBZ and 2Me-CBZ

MS Parameter	2OH-CBZ	3OH-CBZ	2Me-CBZ
Dwell time (msec)	100	100	100
Desolvation potential (V)	56	61	121
Exit potential (V)	10	10	10
Collision energy (V)	27	27	27
Collision exit potential (V)	14	16	12

For each analyte and the internal standard, two ions were selected: the protonated molecule ($M + H^+$), as the parent ion, and a daughter ion (Table 3.6). The most abundant ions following full scanning acquisitions were chosen as the daughter ions. Secondary, less abundant, confirmation-of-identity transitions were also selected for each analyte from the full scanning acquisition data (Table 3.6).

Table 3.6 Selected parent and daughter ions for each compound

Drugs	Parent Ions ($M + H^+$), (*m/z*)	Daughter Ions (*m/z*)	Confirmation-of-Identity Ions (*m/z*)
CBZ	237	192	194
CBZE	253	180	208
DiOH-CBZ	271	180	210
2OH-CBZ	253	210	167
3OH-CBZ	253	210	180
2Me-CBZ	251	208	206

3.2.4 Data Analysis

Mass spectrometric data analysis was undertaken using the quantitation facility of the relevant Analyst 1.5-generation software (Sciex, UK). From the automatically computed and visually inspected peak areas, the ratio of each analyte to the internal standard was calculated and plotted against analyte concentrations to confirm the linearity of each standard curve. The analyte concentrations were calculated from the equation $y = mx + b$, as determined by the weighted (1/x) linear regression of the standard line. The regression was not forced through zero. The correlation coefficient (r^2) was determined for each calibration curve and only accepted if >0.99.

3.2.5 Accuracy and Precision

Intra- and inter-day accuracy and precision (collectively, repeatability) for each analyte in the assays were determined. Intra-day repeatability was assessed by analysing low, middle and high QC standards for each analyte in pooled plasma five times in a single day against a set of freshly prepared calibration standards. Inter-day repeatability was assessed by repeating the above procedure on five separate days using freshly prepared low, middle and high QC standards against freshly prepared sets of calibration standards. The accuracy of the method was expressed by [(mean observed concentration)/ (spiked concentration)] x 100. Precision was determined by calculating the coefficient of variation (CV) (%).

3.2.6 Analyte Specificity Studies

To investigate the specificity of the assays, drug-free plasma was obtained from five healthy volunteers and processed separately as described in 3.2.2 to determine if signal-conflicting endogenous constituents coeluted with the different analytes. Elution times and relative intensities of endogenous MRM signals were then compared with those of the analytes of interest.

3.2.7 Determination of the Lower Limit of Quantitation

The lower limit of quantitation (LLOQ) for each analyte was defined as the lowest concentration which could be determined with an accuracy of 100 +/- 20% and a precision of ≤20%, in line with FDA recommendations.

3.2.8 Recovery and Matrix Effects

Recovery and matrix effects for the assays were measured according to a strategy outlined by Matuszewski et al. (2003). Matrix effect is the effect on an analytical method caused by all other components of the sample except the analyte to be quantified and may result in ion suppression or enhancement of the target analyte in the presence of co-eluting compounds (Matuszewski et al. 2003). The presence of matrix effects needs to be investigated to ensure that the assay is valid and accurate for assessment of patient plasma samples (Food and Drug Administration 2001). Three sets of calibration samples were prepared using pooled plasma from six healthy volunteers. Set 1 consisted of 3 µL of each analyte added to 485 µL acetone:water (50:50, v/v) without further processing prior to injection onto the HPLC-MS/MS. Set 2 was prepared by evaporating to dry residue 500 µL of volunteer plasma in a SpeedVac (Eppendorf, Germany) before reconstitution in 485 µL acetone:water (50:50, v/v) and addition of 3 µL of each analyte. The final set consisted of 485 µL volunteer plasma spiked with 3 µL of each analyte that was evaporated to dry residue in a SpeedVac vacuum concentrator (Eppendorf) and finally reconstituted in 500 µL acetone:water (50:50, v/v). An aliquot (20 µL) of each sample was injected onto the HPLC-MS/MS in accordance with section 3.2.3.

Recovery (%) was calculated as the ratio of the mean peak area (n=3) of an analyte spiked before extraction (set 3) to the mean of the peak area of an analyte spiked post extraction (set 2) multiplied by 100. Matrix effect was expressed as the ratio of the mean peak area of an analyte spiked post extraction (set 2) to the mean peak area of the same analyte standards in solvent (set 1) multiplied by 100.

3.2.9 Storage of Plasma Samples and Stability of the Analytes

QC standards containing all five analytes and internal standard were prepared according to section 3.2.2 and analysed in triplicate on the day of preparation according to section 3.2.3; thereby establishing the starting concentrations for the storage stability assessments (day 1). Aliquots of the QC standards were frozen at -80^{o}C for storage stability analyses. Storage stability for 2OH-CBZ, 3OH-CBZ and DiOH-CBZ were analysed on day 90 and 246.

New QC standards containing CBZ, CBZE and internal standard had to be prepared approximately 96 days into the original storage stability analyses because CBZ and CBZE had precipitated out of solution in the original working solutions. Precipitation had occurred secondary to evaporation of acetonitrile because of the small volumes that were originally used to dissolve the analytes (CBZ: 1167µL and CBZE: 824.5µL). New stock solutions for CBZ and CBZE were made according to the concentrations in Table 3.1 using significantly larger volumes of acetonitrile. New QC standards containing CBZ, CBZE and internal standard were prepared and analysed in triplicate on the day of preparation according to section 3.2.3, thereby establishing the starting concentration for storage stability assessment (day 1). Aliquots of the new QC standards for CBZ and CBZE were frozen at -80^{o}C and analysed on day 151. The new CBZ and CBZE working solutions were used in calibration and QC standards for all patient analyses.

All of the QC aliquots for storage stability analyses were thawed and re-analysed in triplicate on the same day using a freshly prepared set of calibration standards. The average concentration values for the storage samples were compared against the average concentration values from the day 1 analyses, and expressed as a percentage to represent analyte stability in plasma.

3.2.10 Linear Dilution of Samples

It was possible that plasma samples obtained during the study might have demonstrated analyte concentrations above the upper limit of the standard curve. In those instances the samples would have been diluted 1:1 using pooled plasma from six healthy volunteers prior to extraction and processing of samples. An assessment to confirm linear dilution of CBZ was undertaken by 1:1 serial dilution of the top calibration point using pooled human plasma until a dilution factor of 1:32 was obtained and checked for linearity.

3.2.11 Recruitment Sites for Patients

Participants were recruited from two study sites: The Royal Liverpool and Broadgreen University Hospitals NHS Trust and the Walton Centre NHS Foundation Trust. The Clinical Research Unit at the Royal Liverpool Hospital is a phase I accredited unit with facilities for conducting clinical trials and access to the Consent 4 Consent (C4C) database. The C4C database contains contact details for prospective trial participants who have already provided consent to be contacted when suitable clinical trials arise. The PICME I (a PK Investigation into the formation of CBZ Metabolites and CBZ-protein conjugates in healthy volunteers) clinical trial was conducted at the Royal Liverpool Hospital.

The Walton Centre is a tertiary referral centre for neurology and neurosurgery and the Neuroscience Research Centre is attached to the outpatients department with clinic rooms and equipment for sample collection and processing as part of the study. Daily outpatient epilepsy clinics are undertaken by neurologists and epilepsy nurse specialists. The PICME II (a PK Investigation into the formation of CBZ Metabolites and CBZ-protein conjugates in Epilepsy patients) clinical trial was conducted at the Walton Centre.

3.2.12 Study Populations

PICME I Clinical Trial:

Healthy adult male volunteers were recruited using the C4C database.

The inclusion criteria were:

- Subject willing and able to give written and informed consent
- Healthy male subjects between 18 and 55 years of age inclusive
- Subject's body weight between 50 and 100 kg
- Subject's body mass index between 18 and 32 kg/m^2

The exclusion criteria were:

- Subject not willing to take part or unable to give written informed consent
- Subject with clinically significant abnormal medical history or physical exam
- Subject with history of febrile illness within 4 weeks prior to admission
- Subject with a clinically significant abnormal laboratory test at screening including HBV/HCV/HIV
- Subject taking any interacting prescription or non-prescription drug, or dietary supplements within 2 weeks prior to study admission. Herbal supplements had to be discontinued at least 4 weeks prior to admission to the clinical research facility
- Subject positive for either the *HLA-B*15:02* or *HLA-A*31:01* genotype
- Subject with a clinically significant ECG abnormality – prolonged corrected QT>450ms, 2^{nd} or 3^{rd} degree atrioventricular conduction block
- Subject with known hypersensitivity to CBZ or structurally related drugs (e.g. tricyclic antidepressants) or any other component of the formulation
- Subject with history of bone marrow depression
- Subject with history of hepatic porphyrias (e.g. intermittent porphyria, variegate porphyria, porphyria cutanea tarda)
- Subject who had taken part in another research study within 90 days of commencement
- Subject with any condition which in the opinion of the investigator will interfere with the study

PICME II Clinical Trial:

Two groups of patients were recruited for the PICME II clinical trial: an autoinduction group and a maintenance group. The autoinduction group comprised patients with epilepsy for whom a neurologist had decided to newly initiate CBZ modified release therapy. The maintenance group consisted of patients with epilepsy who had been established on a stable dose of CBZ modified release for at least 4 weeks. Patients were recruited according to the following inclusion and exclusion criteria.

<u>Autoinduction group:</u>

Inclusion criteria:

- Subject willing and able to give written informed consent
- Subject aged 18 or over
- Subject newly prescribed CBZ by their attending physician

Exclusion criteria:

- Subject not willing to take part or unable to give written informed consent
- Subject who in the past 4 weeks received another medication that was a CYP3A4 inducer or inhibitor
- Subject with any condition which in the opinion of the investigator will interfere with the study

<u>Maintenance Group:</u>

Inclusion criteria:

- Subject willing and able to give written informed consent
- Subject aged 18 or over
- Subject had received CBZ therapy at the same dosage for at least 4 weeks

Exclusion criteria:

- Subject not willing to take part or unable to give written informed consent
- Subject with any condition which in the opinion of the investigator was likely to interfere with the study

3.2.13 Population Sample Size

An accurate calculation of the population sample size required for these investigations could not be performed because the variables that affect the metabolism of CBZ and the extent of any effects remained under investigation. For the PICME I trial, 8 healthy male volunteers were recruited. In the PICME II trial the aim was to recruit 8 patients for the autoinduction group and to collect 150 blood samples from the maintenance group. Due to difficulty with recruitment to the PICME II trial, only 3 patients were recruited to the autoinduction group and 77 blood samples were collected from the maintenance group. Despite the reduction in number of subjects recruited the number of samples would still be sufficient to provide rich and sparse PK data and is consistent with other studies of a similar nature (Yun et al. 2013; Panomvana et al. 2013).

3.2.14 Ethical Approval

Ethical approval for the PICME I study was granted by the North West Research Ethics Committee – Greater Manchester (12/NW/0780). Approval covered collection of blood for measurement of routine clinical parameters (full blood count, urea and electrolytes, and liver function tests), viral safety (hepatitis B/C, HIV), storage and analysis of DNA, and the identification and analysis of CBZ metabolites and CBZ-HSA adducts.

Ethical approval for the PICME II study was granted by the North West Research Ethics Committee – Haydock (13/NW/0503). Approval covered collection of blood for measurement of routine clinical parameters (full blood count, urea and electrolytes, and liver function tests), storage and analysis of DNA, and the identification and analysis of CBZ metabolites and CBZ-HSA adducts.

3.2.15 Recruitment

PICME I

Prospective healthy volunteer participants were identified from the consent 4 consent database held by the Clinical Research Unit at the Royal Liverpool University Hospital. They were contacted by e-mail and those expressing an interest in participation were e-mailed the participant information leaflet (PIL) and consent form and invited for a screening visit. During the screening visit, the research physician [Vincent Yip (VY)] confirmed that the PIL and consent form had been read and understood. If the person consented and satisfied the inclusion and exclusion criteria, they were recruited to the study.

PICME II

Prospective participating patients were identified from outpatient epilepsy clinics at the Walton Centre. In the autoinduction group, neurologists referred patients who were newly prescribed CBZ. If the patient was interested in participating in a trial, a copy of the PIL and consent form were given to them and a consultation with the research physician (VY) was organised. If the patient satisfied the inclusion and exclusion criteria, consent was obtained. The patient was advised to refrain from taking CBZ until their first study visit, which could be up to 72 h after their outpatient appointment.

For the maintenance group, patients attending epilepsy outpatient clinics who were prescribed CBZ therapy were identified by the neurologist or nurse. On arrival for the clinic appointment, the PIL and consent form was given to the patient. If the patient was interested in taking part they were invited for a separate consultation with the research physician (VY). During the study consultation, the research physician confirmed the patient understood the PIL and consent form, and the patient was recruited to the study if all inclusion and exclusion criteria were satisfied. All the required personal information and the blood samples were collected immediately.

3.2.16 Data Collection

The following data were collected from the medical case notes and in consultation with the patient:

- Demographic details including age, sex, ethnicity, smoking status, alcohol intake
- Physical characteristics including height, weight, BMI, blood pressure, temperature
- Past medical history including allergies
- Concomitant medications (type, dose and formulation)
- Co-morbid illnesses
- Physical examination (cardiovascular, respiratory, abdominal and neurological) (PICME I only)
- Electrocardiogram (PICME I only)

3.2.17 Venepuncture and Sample Processing Procedures

Venepuncture was undertaken by qualified health professionals at both recruitment sites. In the PICME I trial, samples for full blood count, urea and electrolytes, and liver function tests were undertaken by the Blood Sciences Laboratory at the Royal Liverpool University Hospital. Samples for hepatitis B and hepatitis C testing, HIV screening and HLA genotyping were undertaken in the Immunology Department at the Royal Liverpool University Hospital. The above blood samples were taken at baseline as part of the screening visit. Blood samples for storage and analysis of DNA were frozen immediately at -80^{o}C. Blood samples for analysis of CBZ and its metabolites were centrifuged at 1500*g* for 10 min at 4^{o}C. Aliquots of plasma were pipetted into cryovials and stored at -80^{o}C.

In the PICME II trial, blood samples for full blood count, urea and electrolytes, and liver function tests were undertaken by the Blood Sciences Laboratory at Aintree University Hospital NHS Foundation Trust. Blood samples for storage and analysis of DNA were frozen immediately at -80^{o}C. Blood samples for analysis of CBZ and its

metabolites were centrifuged at 1500*g* for 10 min at 4^{o}C. Aliquots of plasma were pipetted into cryovials and stored at -80^{o}C until further analysis.

3.2.18 PK Sample Collection Time Points

In the PICME I clinical trial, PK blood samples were collected immediately prior to dosing and at the following time points after ingestion of a 400-mg CBZ tablet: 15 min and 30 min, and 1, 2, 4, 6, 8, 12, 24, 48 and 72 h. The choice of CBZ dosage was based on the recommended starting dose according to the summary of product characteristics (Electronic Medicines Compendium 2015).

At visit 1 of the autoinduction group of PICME II, a pre-dose PK sample was collected immediately before administration of 200 mg CBZ. Subsequent PK samples were collected at 1, 2, 4, 6, 8 and 24 h post-dose. Participants were then discharged, and they self-administered CBZ (200 mg) once daily. On day 14, participants returned to the clinical trials unit for visit 2; when PK samples were collected pre-dose and then 1, 2, 4, 6 and 8 h post dose. After this visit the dosage of CBZ was increased to 200 mg twice daily. The participant was asked to return to the clinical trials unit on day 28 and undergo PK sampling at the times listed above. At the end of this visit the participant was asked to increase the dosage of CBZ to 200 mg in the morning and 400 mg at night. The participant returned on day 42 and underwent PK sampling at the time points described above. The dose titration regimen is standard practice for initiation of CBZ therapy at the Walton Centre NHS Foundation Trust.

Participants in the maintenance group of the PICME II clinical trial underwent PK sampling either before or after their scheduled clinical appointment with the neurologist. Timing of the PK sample was calculated by asking the subject the timing and dosage of the previous dose of self-administered CBZ.

3.2.19 Patient Sample Processing for HPLC-MS/MS

Aliquots of patients' plasma (300 µL) were thawed at room temperature on the day of analysis and 10 µL of IS (0.2 mM) was added to each plasma sample and mixed thoroughly. Acetone (300 µL) was added to the plasma aliquot and vortexed for 30 s. The mixtures were then centrifuged at 1400*g* for 10 min at room temperature. Supernatant was collected carefully, discarding the precipitate. The supernatant was evaporated to dryness using a SpeedVac (Eppendorf, Germany). The dried residues were reconstituted in 300 µL of acetone and water (50:50 v/v), and vortexed briefly to ensure complete homogenisation. An aliquot of 100 µL of the vortexed solution was transferred to an autosampler vial (VWR, UK) ready for injection onto the column for analysis of CBZ, CBZE and DiOH-CBZ as described in section 3.2.3. For analysis of 2OH-CBZ and 3OH-CBZ, 100 µL of the vortexed sample was centrifugally filtered at 450 *g* for 15 min at 4^{o}C through a 0.45-µm hydrophilic PTFE filter plate (Merck Millipore, Watford, UK) to further remove any protein. The filter plates were prepared by adding 100 µL of water and centrifuging at 1500 rpm for 15 min at 4^{o}C prior to sample processing. The filtrates were transferred into glass autosampler vials (VWR, UK) ready for immediate analysis. Analysis was undertaken according to section 3.2.3. The PK variables, C_{max}, T_{max}, t½ and AUC, were computed for CBZ using the PKSolver program and compared with existing data for CBZ. PKSolver is a freely available program for solving basic problems in PK and pharmacodynamic data analysis (Zhang et al. 2010).

3.2.20 Assessment for the Possibility of Analytical Conflicts with Concomitant Medications

The theoretical possibility of analytical conflicts, i.e. LC-MS/MS signal conflicts, between the concomitant medications and the analytes was assessed by comparing the molecular mass of drug and its known metabolites to determine whether or not the protonated molecules had *m/z* of any of the analytes.

3.3 Results

3.3.1 Chromatographic Resolution and Analyte Specificity

Representative LC-MS/MS chromatograms obtained with the Zorbax Eclipse XDB-C8 column are shown in Figure 3.2 for (A) drug-free volunteer plasma and (B) pooled volunteer plasma spiked with the analytes and the internal standard. The retention times (R_t) during assay validation are outlined in Table 3.7. Figure 3.2a contains a broad peak in the MRM channel for CBZ extending from 13.5 to 14.5 min, which coincides with the R_t for CBZ and 2Me-CBZ. The peak was present amongst all five samples of drug-free plasma from healthy volunteers. The source of this peak was unclear as it persisted despite changing to a new HPLC column and alternative HPLC pump. However, the intensity of the interfering peak was low relative to the anticipated and actual intensities of CBZ and 2Me-CBZ peaks from calibration and QC standards, and ultimately did not affect the accuracy and precision of the assay.

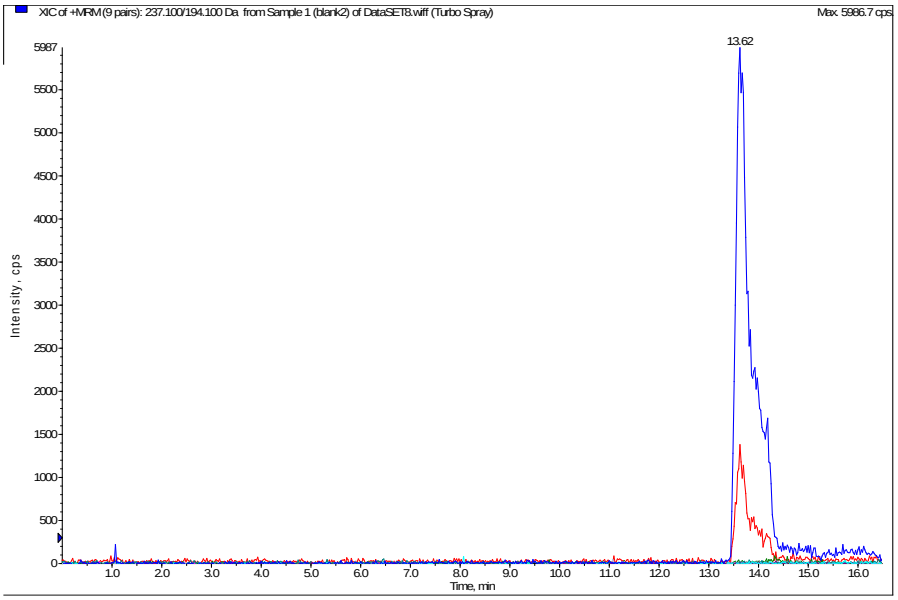
Table 3.7. Retention times for each analyte using the Zorbax Eclipse XDB-C8 column

Analyte	Retention Time (min)
DiOH-CBZ	6.8 (± 0.0)
2OH-CBZ	9.0 (± 0.1)
CBZE	9.7 (± 0.2)
3OH-CBZ	10.4 (± 0.1)
CBZ	13.6 (± 0.1)
2Me-CBZ	14.2 (± 0.0)

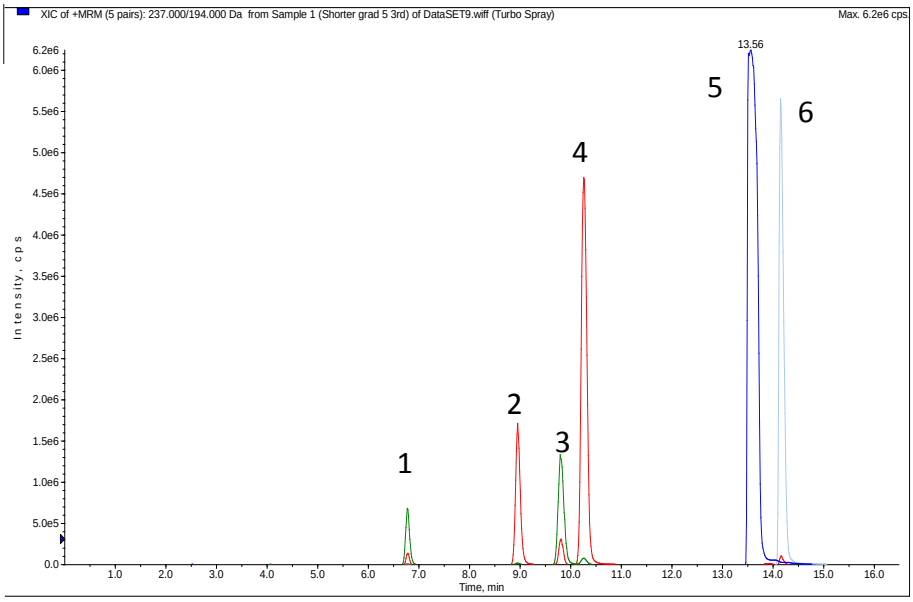
Typical chromatograms obtained using the Xbridge BEH C18 column, representing (A) drug-free plasma and (B) volunteer plasma spiked with analytes and the internal standard, are shown in Figure 3.3. R_t for each analyte are outlined in Table 3.8. Figure 3.3a is representative of all five healthy volunteer samples and does not demonstrate any chromatographic interference or coincidence in time with any analytes in the MRM channel for 2OH- and 3OH-CBZ.

Table 3.8 Retention times for each analyte using the Xbridge BEH C18 column

Analyte	Retention Time (min)
DiOH-CBZ	8.2 (± 0.1)
2OH-CBZ	9.9 (± 0.2)
CBZE	11.0 (± 0.2)
3OH-CBZ	11.3 (± 0.2)
CBZ	13.2 (± 0.2)
2Me-CBZ	13.9 (± 0.1)

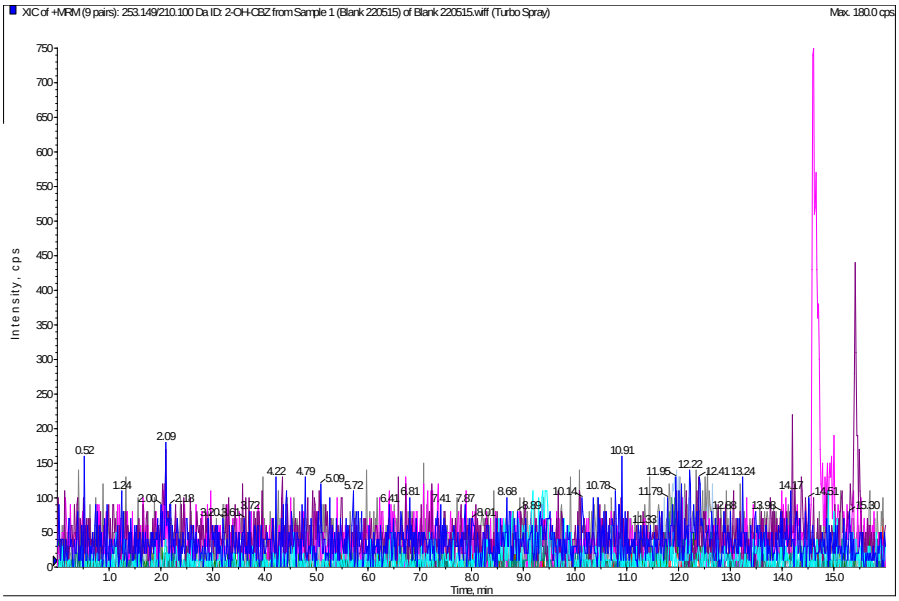


(A)

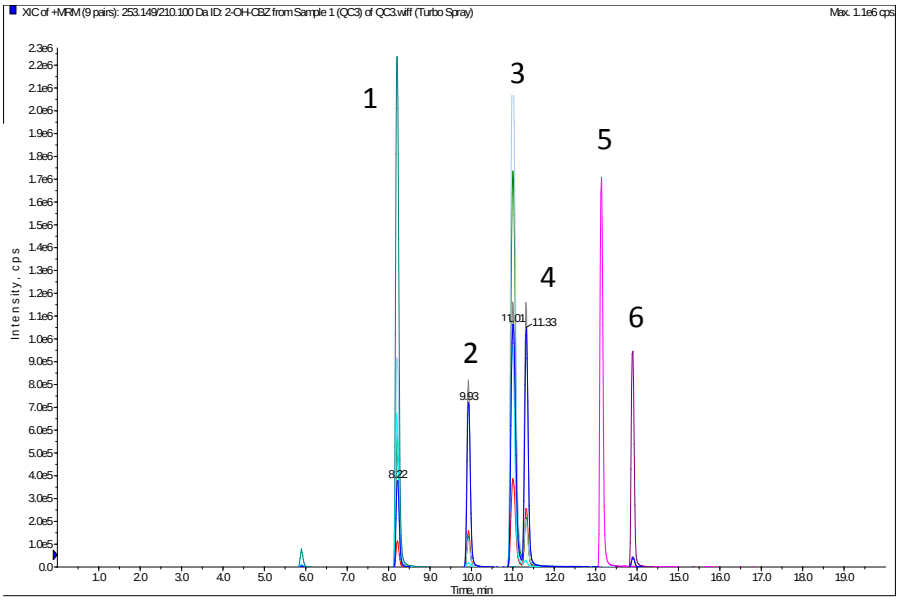


(B)

Figure 3.2 Typical LC-MS/MS (MRM) chromatograms of (a) drug-free human plasma, and (b) drug-free human plasma spiked with CBZ, CBZE, DiOH-CBZ, 2OH-CBZ, 3OH-CBZ and 2Me-CBZ, on a Zorbax Eclipse XDB-C8 column. Secondary, less abundant peaks represent the confirmation of identity transitions. The samples were chromatographed as described in 3.2.3. 1: DiOH-CBZ, 2: 2OH-CBZ, 3: CBZE, 4: 3OH-CBX, 5: CBZ, 6: 2Me-CBZ.



(A)

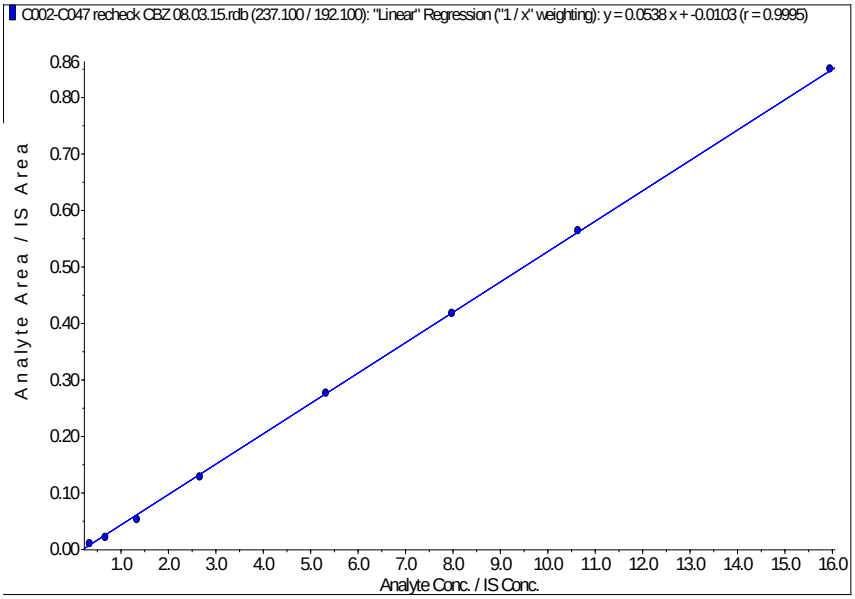


(B)

Figure 3.3. Typical LC-MS/MS (MRM) chromatograms of (A) drug-free human plasma, and (B) drug-free human plasma spiked with CBZ, CBZE, DiOH-CBZ, 2OH-CBZ, 3OH-CBZ and 2Me-CBZ, on a XBridge BEH C18 column. Secondary, less abundant peaks represent the confirmation of identity transitions. The samples were chromatographed as described in 3.2.3. 1: DiOH-CBZ, 2: 2OH-CBZ, 3: CBZE, 4: 3OH-CBX, 5: CBZ, 6: 2Me-CBZ.

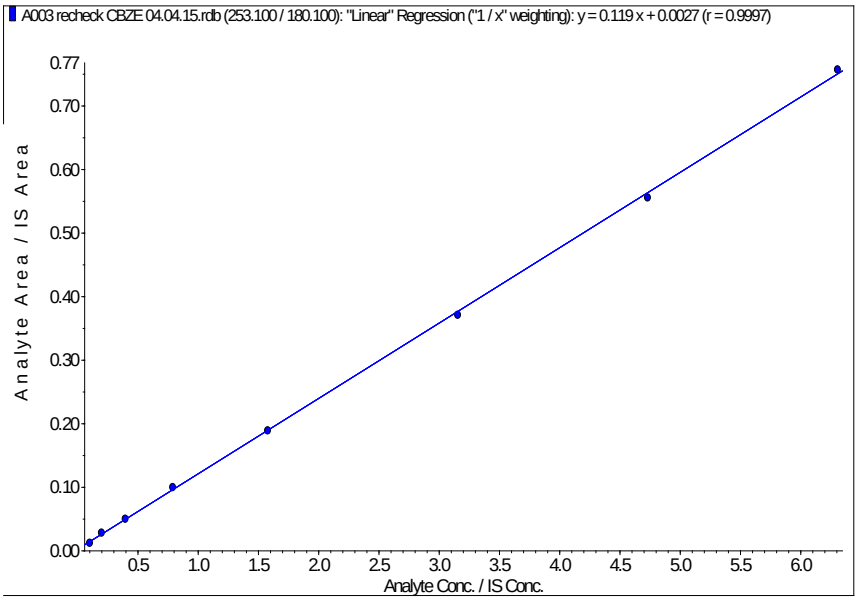
3.3.2 Calibration Curves

Linearity of calibration was observed amongst all five analytes throughout the investigated calibration ranges and the coefficient (r^2) of all the calibration curves was greater than 0.99. Figure 3.4 demonstrates representative calibration curves for each analyte.



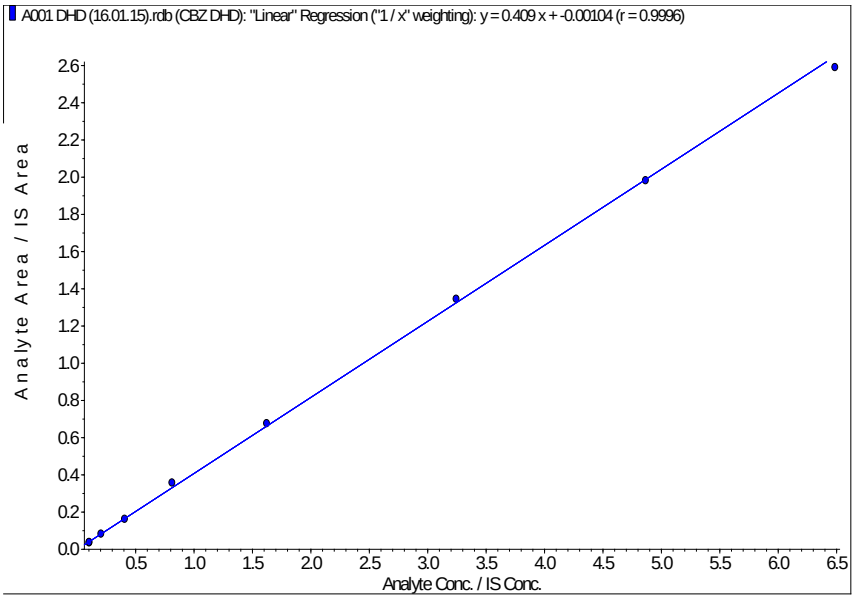
CBZ Calibration range (µg/mL): 0.33 – 15.95

(A)



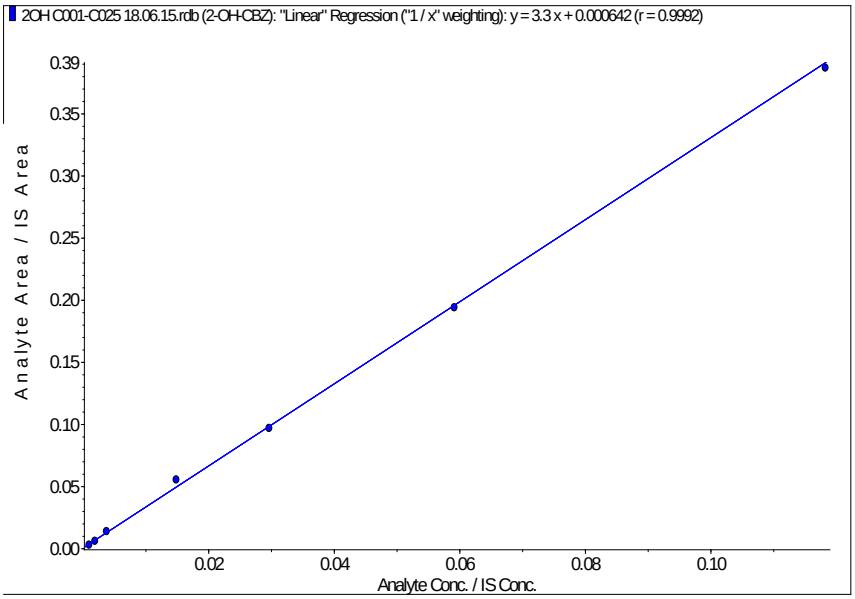
CBZE Calibration range (µg/mL): 0.10 – 6.30

(B)



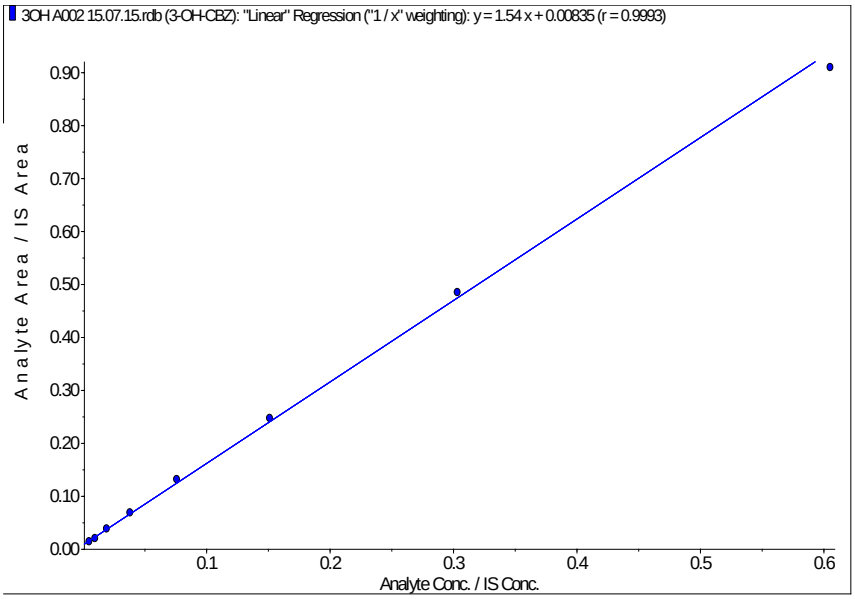
DiOH-CBZ Calibration range (µg/mL): 0.10 – 6.49

(C)



2OH-CBZ Calibration range (ng/mL): 0.92 – 118.18

(D)



3OH-CBZ Calibration range (ng/mL): 4.73 – 605.28

(E)

Figure 3.4 Representative calibration curves for all the analytes: (A) CBZ, (B) CBZE, (C) DiOH-CBZ (D) 2OH-CBZ and (E) 3OH-CBZ.

3.3.3 Accuracy and Precision

All of the assays achieved the required accuracy and precision parameters (CV of ±15%) as outlined by the FDA for both intra- and inter-day analysis (n=5) (Table 3.9 and Table 3.10).

Table 3.9 Intra-day accuracy and precision

Analyte	Nominal Conc.	Set 1	Set 2	Set 3	Set 4	Set 5	Mean	Accuracy (%)	Precision (% CV)
CBZ (µg/mL)	0.33	0.36	0.38	0.39	0.38	0.38	0.38	114.2	2.7
	2.66	2.35	2.34	2.41	2.54	2.45	2.42	91.0	3.4
	10.6	11.6	10.2	11.2	10.8	11.4	11.0	103.9	5.0
CBZE (µg/mL)	0.10	0.10	0.09	0.09	0.09	0.10	0.09	92.9	4.9
	1.57	1.69	1.71	1.62	1.73	1.65	1.68	106.6	2.7
	6.31	5.87	6.03	5.85	5.85	5.92	5.90	93.6	1.3
DiOH-CBZ (µg/mL)	0.10	0.10	0.09	0.07	0.08	0.09	0.09	85.0	11.8
	1.62	1.69	1.71	1.64	1.69	1.63	1.67	103.2	2.1
	6.49	6.26	6.30	6.01	6.22	6.19	6.20	95.5	1.8
2OH-CBZ (ng/ml)	2.00	2.16	2.03	2.33	2.29	2.13	2.2	109.4	5.6
	10.0	11.1	11.2	11.4	11.5	11.7	11.4	113.8	2.1
	100	94.9	92.6	109	100	101	99.5	99.5	6.4
3OH-CBZ (ng/ml)	10.0	10.8	11.1	11.1	11.3	10.5	11.0	109.6	2.9
	50.0	57.6	55.1	57.5	57.4	57.1	56.9	113.9	1.8
	500	480	464	538	490	495	493	98.8	5.6

Table 3.10 Inter-day accuracy and precision

Analyte	Nominal Conc.	Set 1	Set 2	Set 3	Set 4	Set 5	Mean	Accuracy (%)	Precision (% CV)
CBZ (µg/mL)	0.33	0.31	0.35	0.41	0.39	0.39	0.37	111.4	11.8
	2.66	2.41	2.64	2.50	2.43	2.46	2.49	93.6	3.7
	10.6	10.8	11.1	11.3	10.3	11.4	11.0	103.3	4.0
CBZE (µg/mL)	0.15	0.14	0.14	0.17	0.15	0.14	0.15	96.8	8.2
	1.50	1.65	1.43	1.62	1.65	1.57	1.58	105.6	5.8
	6.00	6.15	5.40	6.08	6.20	6.08	5.98	99.7	5.5
DiOH-CBZ (µg/mL)	0.10	0.10	0.09	0.09	0.11	0.10	0.10	94.3	7.1
	1.62	1.69	1.81	1.59	1.66	1.55	1.66	102.4	6.1
	6.49	6.26	6.47	5.84	6.29	5.92	6.16	94.9	4.3
2OH-CBZ (ng/mL)	2.00	2.16	2.03	2.13	1.84	2.01	2.03	101.7	6.2
	10.0	11.1	11.2	10.6	8.58	11.1	10.5	105.2	5.6
	100	94.9	92.6	94.6	91.7	103	95.3	95.4	4.7
3OH-CBZ (ng/mL)	10.0	10.8	11.1	9.83	10.9	9.95	10.5	105.2	5.6
	50.0	57.6	55.1	55.4	55.2	57.2	56.1	112.2	2.1
	500	480	464	433	435	457	454	90.8	4.4

3.3.4 Recovery and Matrix Effects

Recovery and matrix effects were assessed for all analytes throughout the calibration range (Table 3.11). A negative matrix effect was most pronounced with CBZ, with peak areas reduced by an average of 35.8% across the calibration range. CBZE, 2OH-CBZ, 3OH-CBZ and DiOH-CBZ had mean matrix effects of 100.1%, 97.4%, 96.1% and 94.0%, respectively. The CV of matrix effects for all the analytes were between 3.5% and 9.2%. Mean recovery of all the analytes ranged between 76.9% for 3OH-CBZ to 80.7% for CBZE, and CV values for recovery were between 4.4% and 7.5% (Table 3.11).

Table 3.11 Table of recovery (RE) and matrix effects (ME) (set 1: solvent; set 2: plasma spiked after processing; set 3: plasma spiked before processing)

		Mean Peak Area [cps ($x10^6$)]						
Analyte	**Conc point**	**Set 1**	**Set 2**	**Set 3**	**ME (%)**	**CV ME (%)**	**RE (%)**	**CV RE (%)**
CBZ (µg/ml)	0.33	0.68	0.40	0.34	58.7		84.6	
	0.66	1.41	0.87	0.71	61.6		81.2	
	1.33	2.92	1.87	1.38	64.1		73.8	
	2.66	5.47	3.82	2.81	69.8		73.6	
	5.32	11.7	7.78	6.03	66.7		77.5	
	10.6	21.3	14.7	11.4	68.9		77.6	
	21.3	36.1	21.6	17.9	59.9		82.7	
				Mean ME (%)	64.2	6.8	78.7	5.4
CBZE (µg/ml)	0.10	0.51	0.48	0.38	94.5		79.2	
	0.20	0.98	0.94	0.78	95.9		82.9	
	0.39	1.93	1.94	1.47	100.3		75.8	
	0.79	3.43	3.51	2.77	102.4		79.0	
	1.58	6.33	6.41	5.13	101.3		80.1	
	3.15	10.9	11.6	9.42	107.1		81.0	
	6.31	18.2	18.0	15.7	98.9		87.1	
				Mean ME (%)	100.1	4.2	80.7	4.4

		Mean Peak Area [cps ($x10^6$)]						
Analyte	**Conc point**	**Set 1**	**Set 2**	**Set 3**	**ME (%)**	**CV ME (%)**	**Analyte**	**Conc point**
DiOH-CBZ (µg/ml)	0.10	0.84	0.73	0.58	86.9		79.3	
	0.20	1.65	1.50	1.14	90.7		76.2	
	0.41	3.53	3.23	2.33	91.6		72.2	
	0.81	6.39	6.26	4.74	97.9		75.7	
	1.62	12.8	12.4	9.41	96.6		75.9	
	3.24	24.4	24.0	18.2	98.1		76.0	
	6.49	39.6	38.1	33.4	96.1		87.7	
				Mean ME (%)	94.0	4.6	77.6	6.3
2OH-CBZ (ng/ml)	7.40	0.06	0.05	0.04	87.9		78.1	
	14.8	0.12	0.10	0.09	88.5		84.6	
	29.6	0.24	0.24	0.18	102.2		74.9	
	59.1	0.45	0.48	0.36	107.2		75.5	
	118	0.93	0.93	0.72	101.0		76.9	
	236	1.80	1.90	1.40	105.4		73.7	
	473	3.41	3.22	2.78	94.3		86.3	
				Mean ME (%)	97.4	9.2	78.6	6.3
3OH-CBZ (ng/ml)	18.9	0.08	0.08	0.06	98.8		70.0	
	37.8	0.15	0.16	0.13	102.2		79.8	
	75.7	0.33	0.34	0.25	103.7		72.1	
	151	0.60	0.65	0.51	108.1		78.4	
	302	1.25	1.34	1.01	106.9		75.7	
	605	2.52	2.67	2.00	105.9		74.9	
	1210	4.38	4.36	3.82	99.5		87.6	
				Mean ME (%)	103.6	3.5	76.9	7.5

3.3.5 Analyte Storage Stability in Plasma

All of the QC standards for the assessments of the analytes' storage stability were prepared on day 1 and frozen at -80^{O}C until further analysis. Samples containing 2OH-CBZ, 3OH-CBZ and DiOH-CBZ were analysed on days 1, 90 and 246 against freshly prepared calibration standards. Due to precipitation of the CBZ and CBZE working solutions, new QC standards for CBZ and CBZE had to be prepared for storage stability analysis on days 1 and 151. All of the QC standards for each analyte were within ±15% for accuracy and precision, confirming that the analytes were stable in human plasma at -80^{o}C (Table 3.12).

3.3.6 Linear Dilution

CBZ demonstrated a linear dilution pattern (r^2 = 0.9951) throughout the serial dilution range (Figure 3.5).

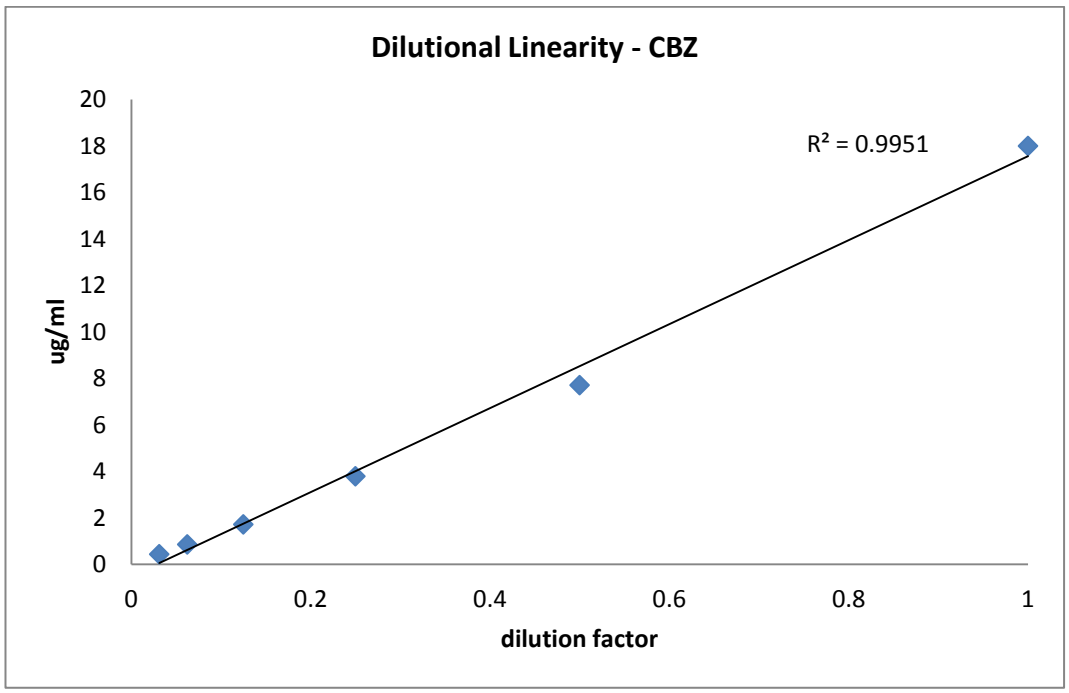


Figure 3.5 Serial dilution of CBZ in pooled volunteer plasma up to 1:32

Table 3.12 Storage stability analyses (*day 90 applicable to 2OH-CBZ, 3OH-CBZ and DiOH-CBZ, day 151 applicable to CBZ and CBZE)

Analyte	Theoretical Conc.	Day 1 (mean)	Day 90 or 151* (mean)	Day 246 (mean)	Overall Mean	Accuracy (%)	Precision (CV) (%)
CBZ (µg/mL)	0.475	0.516	0.508	NA	0.512	107.8	1.2
	4.75	4.85	4.96	NA	4.91	103.3	1.7
	9.50	10.80	10.70	NA	10.8	113.3	0.9
CBZE (µg/mL)	0.15	0.14	0.14	NA	0.14	93.1	0.7
	1.50	1.68	1.62	NA	1.64	109.2	1.6
	6.00	6.00	6.41	NA	6.20	103.4	4.7
DiOH-CBZ (µg/mL)	0.15	0.17	0.138	0.14	0.15	99.6	12.0
	1.50	1.61	1.52	1.48	1.54	102.5	4.4
	6.00	5.89	5.96	6.03	5.96	99.3	1.1
2OH-CBZ (ng/mL)	10.0	11.3	9.90	11.3	10.8	108.2	7.6
	100	110	95.1	110	105	105.0	8.2
	400	434	389	434	419	104.7	6.2
3OH-CBZ (ng/mL)	33.0	37.5	35.0	34.4	35.7	108.0	4.6
	330	366	329	351	349	105.6	5.4
	1000	1053	1030	1053	1045	104.5	1.3

3.3.7 Failure of Sensitivity and Specificity in 2OH-CBZ and 3OH-CBZ Analysis Using Agilent 5-µM Zorbax Eclipse XDB-C8 column

It was the initial intention for all five analytes to be quantified using the Agilent 5-µM Zorbax Eclipse XDB-C8 column. However, data from patient samples demonstrated significant interference in the MRM channel for 2OH- and 3OH-CBZ, which meant that sensitivity and specificity for quantitation of both 2OH-CBZ and 3OH-CBZ were insufficient (Figure 3.6). The interference was present in all patient samples. The lack of sensitivity and specificity was isolated to patient samples only and not present in either the calibration or QC standards from pooled plasma (Figure 3.7). Therefore, quantitation of 2OH-CBZ and 3OH-CBZ was undertaken using a Waters 2.5-µM Xbridge BEH C18 column as outlined above (section 3.2.3).

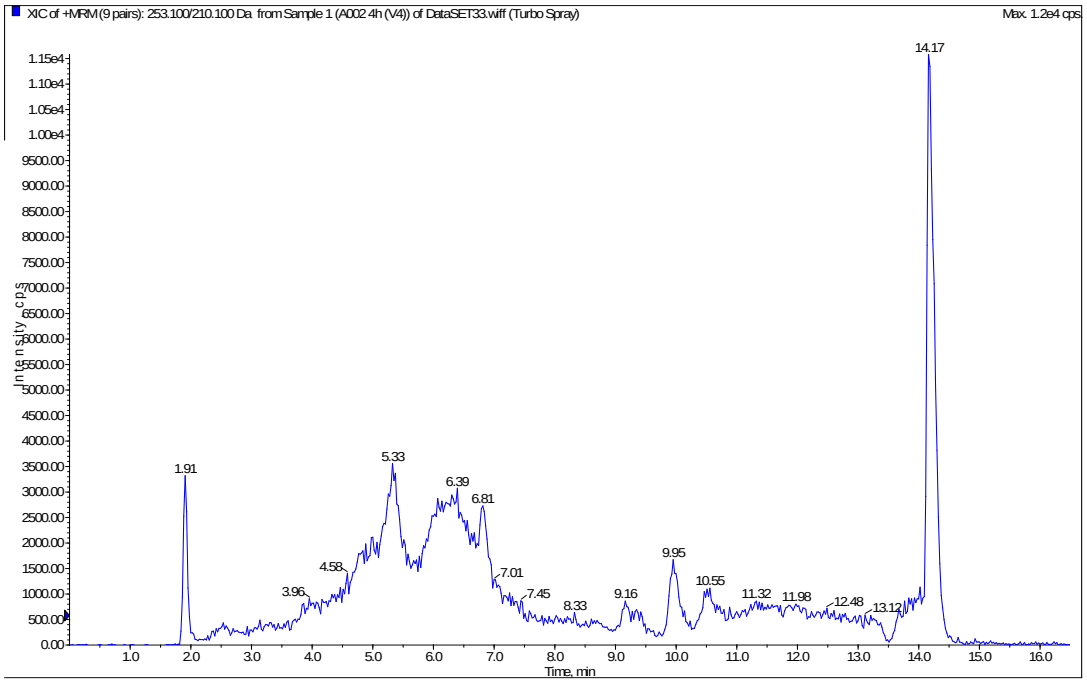


Figure 3.6 Representative LC-MS/MS (MRM) chromatogram demonstrating insufficient specificity for 2OH-CBZ and 3OH-CBZ peaks (Table 3.4) in patient samples when analysed on a Zorbax Eclipse XDB-C8 column.

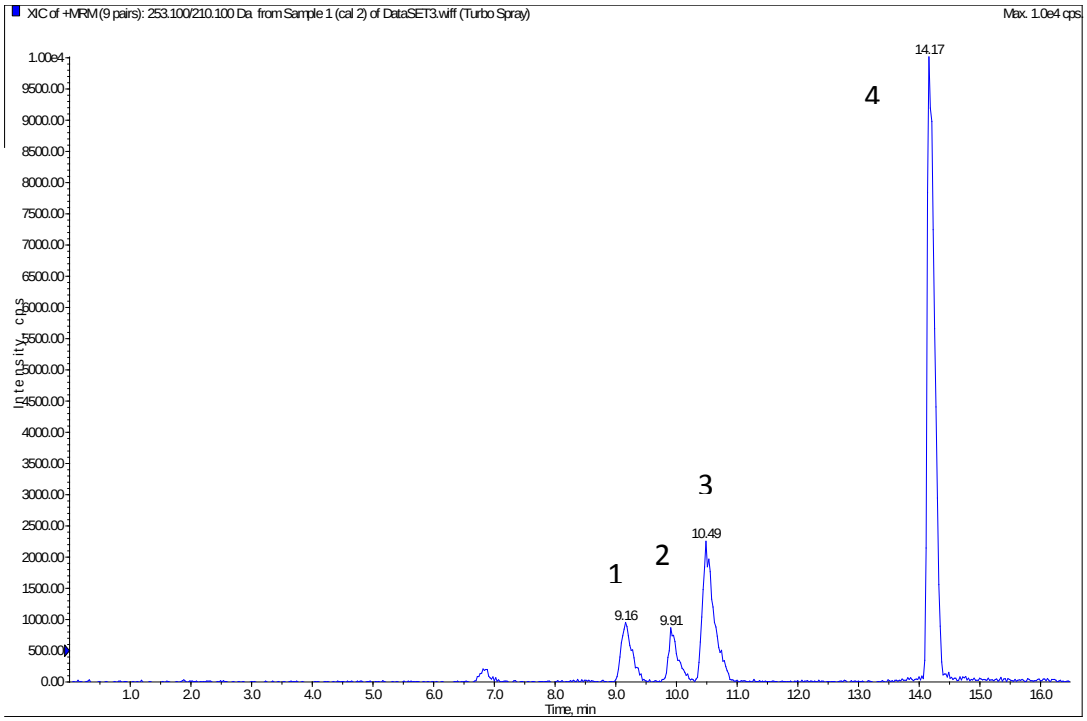


Figure 3.7 Representative LC-MS/MS (MRM) chromatogram of calibration standards for CBZE, 2OH-CBZ and 3OH-CBZ when analysed on a Zorbax Eclipse XDB-C8 column. (1: 2OH-CBZ, 2: CBZE, 3: 3OH-CBZ, 4: 2Me-CBZ)

3.3.8 Patient Demographics and Plasma Assays

In total, 8 healthy male volunteers were recruited to the PICME I clinical trial. Their demographics are outlined in Table 3.13. Three participants, two female and one male, were recruited to the autoinduction group of the PICME II clinical trial and their clinical details are represented in Table 3.14. In the maintenance group of PICME II, 77 plasma samples were obtained from 69 participants (Table 3.15). Eight of those subjects were sampled on two separate visits. Thirty seven of the participants in the maintenance group were male and 32 female. Median age in the group was 48 with a range between 25 and 75. CBZ dosages ranged from 100 mg per day to 1600 mg per day (Table 3.15). All of the patients in the PICME II clinical trial had been diagnosed with epilepsy.

Table 3.13 Demographics of subjects recruited to the PICME I clinical trial

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking status	Medical History	Medication*	Allergies
P001	M	29	1.76	90.0	128/77	70	36.3	European	Ex-smoker	Depression	Nil	Nil
P002	M	21	1.78	99.4	115/64	65	36.5	European	Non smoker	Nil	Nil	Penicillin
P003	M	26	1.81	69.8	130/80	55	36.8	European	Non smoker	Eczema	Eumovate	Nil
P004	M	26	1.78	87.0	122/73	73	37.1	European	Non smoker	Osteoarthritis	Ibuprofen	Nil
P005	M	24	1.72	70.6	114/58	70	37.5	European	Non smoker	Nil	Nil	Nil
P006	M	32	1.72	84.8	137/80	80	36.7	European	Ex-smoker	Nil	Nil	Penicillin
P007	M	29	1.81	97.1	132/74	59	36.8	European	Non smoker	Nil	Nil	Nil
P008	M	28	1.84	77.8	116/69	75	36.7	Egyptian and UK	Non-smoker	Eczema	Nil	Shellfish

* No analytical conflicts were detected between concomitant medications and the analytes.

Table 3.14 Demographics of subjects recruited to the auto-induction group of PICME II clinical trial

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
A001	M	42	1.78	88.0	150/98	80	37.0	European	Ex-smoker	Epilepsy	Mirtazapine, olanzapine, methadone
A002	F	43	1.63	124.5	149/86	88	35.9	European	Non Smoker	Epilepsy, type 2 diabetes, congenital small kidney	Atorvastatin
A003	F	44	1.67	73.1	144/95	82	35.7	European	Ex-smoker	Epilepsy, hypertension, Raynaud's, previous intraventricular haemorrhage	Venlafaxine, co-codamol

*No analytical conflicts were detected between concomitant medications and analytes.

Table 3.15 Demographics of subjects recruited to the maintenance group of PICME II clinical trial

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C001	M	32	1.64	112.5	145/85	77	36.5	European	Non-smoker	Fatty liver, anxiety	Citalopram
C002	F	48	1.62	86.6	107/71	68	37.3	European	Current smoker	-	-
C003	F	27	1.70	50.0	132/76	85	37.6	European	Non-smoker	Asthma	Venlafaxine
C004	F	32	1.54	59.5	123/80	68	37.8	European	Non-smoker	Asthma, irritable bowel syndrome	Clobazam
C005	F	71	1.55	39.0	224/102	78	35.6	European	Current smoker	Osteopaenia	Multivitamins,Vitamin D
C006	F	67	1.63	62.5	142/77	77	36.7	European	Current smoker	Chronic obstructive pulmonary disease	Lamotrigine, symbicort, salbutamol, spiriva, pyroxidine,

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C007	M	53	1.72	93.5	170/100	66	36.3	European	Non-smoker	Knee replacement	Levetiracetam, cetirizine, lansoprazole, pravastatin
C008	M	46	1.73	140.5	149/73	57	36.8	European	Ex-smoker	Asthma, chronic obstructive pulmonary disease, atrial fibrillation, osteoarthritis, sleep apnoea	Bisoprolol, calichew D3 forte, fostair, clobazam, phenytoin, salbutamol
C009	M	34	1.75	83.0	139/81	48	36.2	European	Ex-smoker	-	-
C010	F	37	1.60	60.0	124/74	82	37.0	European	Non-smoker	Panic attacks	Senna

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C011	M	53	1.84	107.5	140/87	68	36.2	European	Non-smoker	Inflammatory bowel disease, urolithiasis	Levetiracetam, Sodium valproate, clobazam, Asacol, phenytoin, Vitamin D
C012	M	60	1.88	91.0	185/104	52	37.0	European	Non-smoker	-	Folic acid, zonisamide, clobazam,
C013	F	34	1.61	75.5	110/84	80	36.8	European	Current smoker	-	Quetiapine, levetiracetam, folic acid
C014	F	28	1.63	90.5	128/84	70	36.9	European	Non-smoker	Irritable bowel syndrome, migraines	Paracetamol, Ibuprofen
C015	M	46	1.80	75.0	154/94	65	36.7	European	Current smoker	-	Levetiracetam
C016	M	47	1.82	86.5	136/90	71	36.8	European	Ex-smoker	Seborrheic dermatitis	Topiramate

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C017	F	58	1.59	50.0	111/61	73	37.5	European	Non-smoker	Irritable bowel syndrome, osteoporosis	Adcal D3, alendronic acid, levetiracetam, movicol, lactulose
C018	F	46	1.55	84.5	143/77	74	37.4	European	Current smoker	-	Levetiracetam
C019	M	43	1.70	67.0	133/94	81	36.6	European	Current smoker	-	Levetiracetam, citalopram, ranitidine
C020	F	57	1.63	57.5	142/87	89	36.9	European	Current smoker	Hypertension, indigestion, recurrent urinary tract infection	Lansoprazole, citalopram, ramipril, trimethoprim, nitrofurantoin
C021	F	60	1.80	124.5	126/65	85	36.6	European	Non-smoker	Osteoarthritis, hayfever	Adcal D3, clonidine, folic acid, levocetirizine, zonisamide

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C022	F	48	1.64	55.0	150/91	74	37.7	European	Current smoker	Asthma, chronic obstructive pulmonary disease	Salbutamol
C023	M	61	1.77	93.5	149/88	82	36.0	European	Ex-smoker	Hip replacement, brain tumour	Levetiracetam
C024	M	50	1.76	86.5	156/90	45	35.8	European	Non-smoker	-	Sodium valproate
C025	M	75	1.81	99.0	148/97	67	37.1	European	Non-smoker	-	Levetiracetam, clobazam, duloxetine
C026	M	36	1.73	76.5	150/92	71	38.8	European	Non-smoker	-	Lamotrigine, levetiracetam
C027	F	30	1.75	71.0	111/79	76	37.3	European	Non-smoker	-	Levetiracetam, clobazam, citalopram
C028	M	50	1.78	68.0	135/86	55	36.6	European	Ex-smoker	Overactive bladder	-

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C029	F	59	1.60	98.0	207/109	70	37.0	European	Non-smoker	Angina, hypertension, prev stroke, aortic and mitral valve regurgitation, diverticular disease, cervical spondylosis	clopidogrel, bisoprolol, clonidine, ramipril, telmisartan, bendroflumethiazide, atorvastatin, isosorbide mononitrate
C030	M	25	1.81	87.0	105/63	71	36.6	European	Ex-smoker	-	-
C031	M	45	1.90	106	153/101	77	35.3	European	Non-smoker	Encephalitis, hermiparesis	Calcichew, folic acid
C032	M	33	1.71	69.5	141/95	76	36.7	European	Non-smoker	-	-
C033	M	69	1.77	97.5	156/81	69	37.1	European	Non-smoker	Hypertension, stroke constipation, bladder cancer	Aspirin, atorvastatin, amlodipine, ramipril, senna, docusate

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C034	M	60	1.77	84.5	123/76	67	36.5	European	Current smoker	Spinal stenosis, sciatica	Zonisamide, dihydrocodeine, pregabalin
C035	F	62	1.60	77.7	155/85	72	37.1	European	Non-smoker	Pacemaker, stroke	Amolodipine, atorvastatin, clobazam, clopidogrel, lansoprazole, levetiracetam, paroxetine, ramipril
C036	M	51	1.62	69.0	185/93	66	36.7	European	Non-smoker	Hypertension, diabetes	Amlodipine, aspirin, atenolol, atorvastatin, irbesartan, perampanel levetiracetam, novomix 30

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C037	F	27	1.63	78.5	140/83	87	36.0	European	Non-smoker	Constipation, cerebral palsy	Levetiracetam, lacosamide, clobazam, midazolam
C038	F	38	1.68	69.0	157/91	67	36.1	European	Current smoker	Irritable bowel syndrome, hydrocephalus, temporal mandibular joint dysfunction	Levetriacetam, alverine citrate, premarin, tramadol, morphine sulphate continus, vitamin D, domperidone, ibuprofen, paracetamol
C039	F	43	1.69	77.5	141/89	98	37.3	European	Non-smoker	-	-
C040	M	37	1.70	68.0	127/60	82	36.1	European	Current smoker	-	-

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C041	F	47	1.55	83.5	140/84	70	35.9	European	Ex-smoker	hypertension	Perindopril, folic acid
C042	M	49	1.77	68.5	118/71	57	36.4	European	Non-smoker	Brain tumour, visual impairment	Clobazam
C043	F	43	1.68	66.5	139/100	85	36.9	European	Current smoker	Constipation	Levtiracetam, clobazam, mirtazepine, omeprazole, codeine, Oxycontin (morphine sulphate), paracetamol, HRT patch
C044	F	48	1.66	68.0	122/65	82	36.9	European	Ex-smoker	Urinary incontinence, osteoarthritis, thyroidectomy	Levetiracetam, clobazam, levothyroxine, calcichew

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C045	F	40	1.60	61.0	115/86	84	37.5	European	Ex-smoker	Behcet's disease	Clobazam, topiramate, mirtazapine, pregabalin
C046	M	28	1.75	87.5	127/63	84	36.8	European	Non-smoker	Psychosis, depression	Citalopram, fexofenadine, levetiracetam
C047	M	54	1.70	62.5	151/95	53	37.0	European	Non-smoker	Osteoporosis, fibroma	Phenobarbital
C048	F	58	1.56	52.0	109/66	75	37.1	European	Current smoker	Chronic obstructive pulmonary disease, constipation, osteoporosis, psoriasis	Zonisamide, clobazam, venlafaxine, salbutamol, symbicort, alendronic acid, beconase nasal spray, duloxetine, fexofenadine

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C049	M	40	1.73	88.0	143/95	67	36.3	European	Current smoker	-	Levetiracetam, clobazam
C050	F	33	1.71	104.0	142/72	66	37.0	European	Current smoker	Asthma	Phenytoin, symbicort, Salbutamol, clobazam, midazolam
C051	M	44	1.67	66.0	116/81	69	36.6	European	Non-smoker	-	Clobazam, atorvastatin, levetiracetam, topiramate
C052	F	53	1.59	75.0	165/99	68	36.1	European	Ex-smoker	Hypertension, high cholesterol	Levetiracetam, ramipril, lercanidipine, atorvastatin, adcal D3, salbutamol

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C053	M	45	1.75	98.0	135/98	115	37.1	European	Non-smoker	Hypertension	Felodipine, lisinopril, pregabalin, omeprazole
C054	F	60	1.58	86.5	154/77	65	36.4	European	Non-smoker	Irritable bowel syndrome, osteoarthritis, osteoporosis, calcium deficiency, hypothyroid, schizophrenia, dementia	Procyclidine, simvastatin, aripiprazole, calcichew D3 forte, calcitriol, donepezil, haloperidol, lamotrigine, levothyroxine, mebeverine, naproxen, omeprazole, paroxetine

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C055	M	59	1.70	76.5	174/94	64	36.2	European	Non-smoker	Asthma, eczema	Salbutamol, Qvar, phenytoin, calcichew D3 forte, midazolam
C056	M	46	1.88	95.0	159/100	93	36.8	European	Non-smoker	-	sodium valproate
C057	M	59	1.77	78.6	125/74	55	35.6	European and South African	Non-smoker	Hypertension	Lamotrigine, omeprazole, indapamide atorvstatin, atenolol, amlodipine, ramipril
C058	M	45	1.75	106.6	132/90	91	36.7	European	Non-smoker	Indigestion	Diazepam, levetiracetam, omeprazole, setraline, sulpiride, thiamine, vitamin B co strong, zopiclone

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C059	F	68	1.51	48.5	162/82	75	36.3	European	Non-smoker	Osteoporosis	Pregabalin, zonisamide
C060	M	50	1.65	69.0	143/81	72	36.8	European	Current smoker	-	Levetiracetam, atorvastatin
C061	F	61	1.64	50.5	128/74	67	36.2	European	Current smoker	Osteoporosis, osteoarthritis	Adcal D3, alendronic acid, clobazam, co-codamol, levetiracetam, lansoprazole, senna
C062	M	40	1.81	69.5	143/85	68	37.0	European	Ex-smoker	-	lacosamide, levetiracetam
C063	M	43	1.86	80.0	134/78	59	36.2	European	Non-smoker	-	Topiramate, levetiracetam, lacosamide

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C064	M	54	1.74	95.0	153/102	65	37.0	European	Non-smoker	Angina, osteoarthritis	Bisoprolol, isosorbide mononitrate xl, lansoprazole, co-dydramol, atorvastatin, aspirin, thiamine
C065	M	55	1.72	103.0	167/101	79	36.8	European	Non-smoker	-	-
C066	M	59	1.58	69.0	135/82	55	36.9	European	Current smoker	Osteoarthritis, migraine	Levetiracetam
C067	F	58	1.60	78.5	189/113	62	36.7	European	Current smoker	Hypertension, headaches	Calcichew D3, bisoprolol, lisinopril
C068	F	44	1.75	89.0	112/75	72	36.8	European	Non-smoker	Asthma	salbutamol, Qvar

*No analytical conflicts were detected between concomitant medications and analytes.

ID	Gender	Age (y)	Height (m)	Weight (kg)	Blood Pressure (mmHg)	Pulse (bpm)	Temp (^{o}C)	Ethnicity	Smoking Status	Medical History	Concomitant Medications*
C069	F	58	1.58	95.5	169/93	78	37.5	European	Ex-smoker	Chronic back pain, multiple strokes	Gabapentin, omeprazole, senna, atorvastatin, amitriptyline, clobazam, paracetamol, lactulose, citalopram, naproxen

* No analytical conflicts were detected between concomitant medications and analytes.

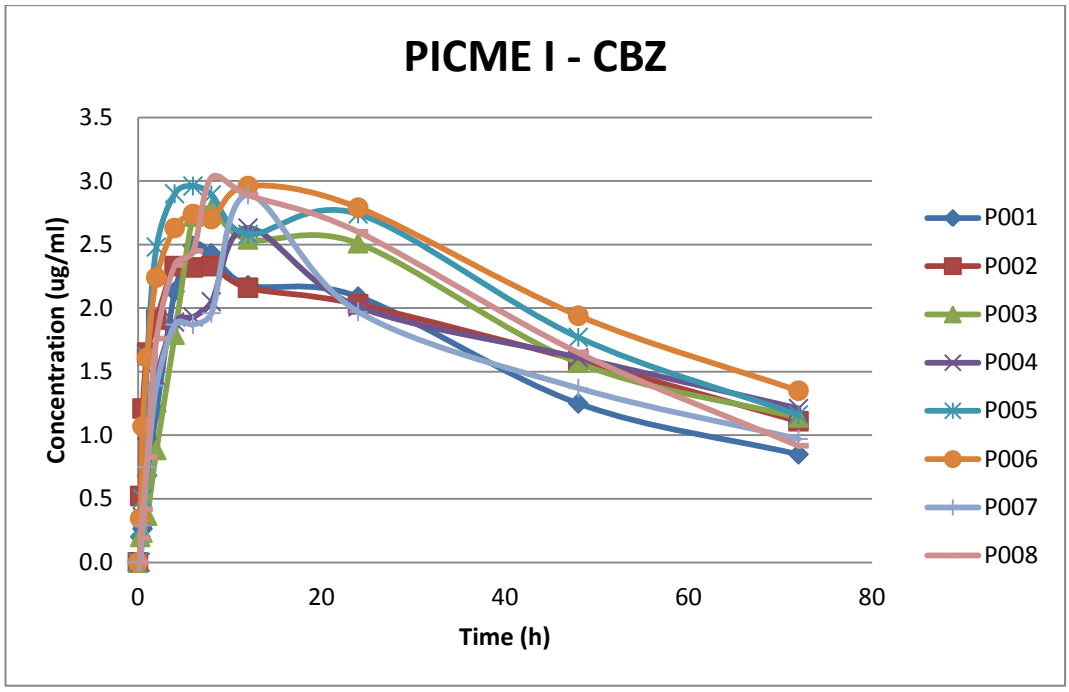
Plasma PK profiles of CBZ and its metabolites for the single-dose healthy volunteers are presented in Table 3.16 and Figure 3.8. C_{max} for CBZ ranged between 2.33 µg/mL and 3.03 µg/mL with corresponding T_{max} between 6 and 12 h. The mean half-life (t½) for CBZ was 46.5 h (±12.2 h) amongst the 8 participants (Table 3.17). T_{max} for CBZE was reached at 24 h except in one volunteer when it was 48 h. C_{max} ranged from 0.08 µg/mL to 0.22 µg/mL. C_{max} for DiOH-CBZ ranged between 0.20 µg/mL and 0.30 µg/mL with T_{max} at 48 h. C_{max} for 2OH-CBZ ranged between 1.84 ng/mL and 5.69 ng/mL, and time taken to reach T_{max} was typically between 12 and 24 h. Finally, C_{max} for 3OH-CBZ ranged between 1.82 ng/mL and 6.83 ng/mL with T_{max} typically between 24 and 48 h. It should be noted that due to the low levels of 3OH-CBZ only two out of eight patients had C_{max} values above the LLOQ (P004 and P005).

Table 3.16 Plasma levels of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ in the PICME I clinical trial

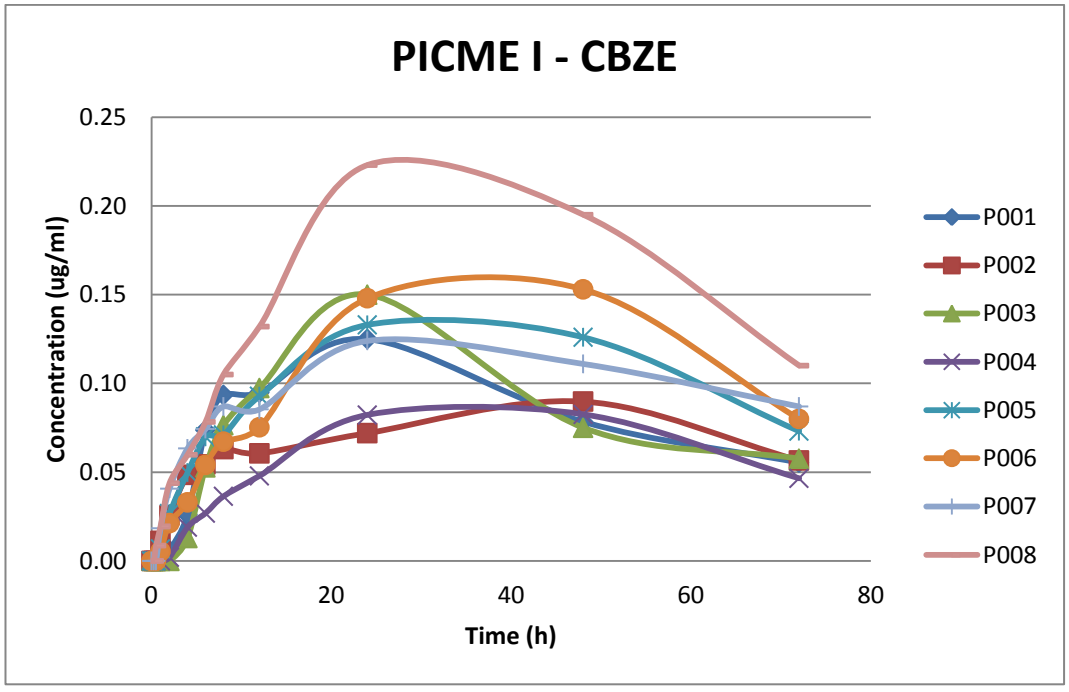
Analyte	Participant	Time post dose (h)											
		0	**0.25**	**0.5**	**1**	**2**	**4**	**6**	**8**	**12**	**24**	**48**	**72**
CBZ (µg/ml)	**P001**	0.00	0.20	0.26	0.68	1.19	2.13	2.49	2.43	2.18	2.09	1.25	0.850
	P002	0.00	0.52	1.21	1.65	1.91	2.33	2.32	2.33	2.16	2.03	1.59	1.11
	P003	0.00	0.20	0.23	0.37	0.88	1.79	2.72	2.78	2.54	2.51	1.57	1.14
	P004	0.00	0.00	0.36	1.04	1.46	1.89	1.93	2.05	2.63	2.01	1.61	1.21
	P005	0.00	0.27	0.50	1.62	2.48	2.90	2.96	2.89	2.58	2.74	1.77	1.16
	P006	0.00	0.35	1.07	1.61	2.24	2.63	2.74	2.70	2.96	2.79	1.94	1.35
	P007	0.00	0.00	0.29	0.749	1.39	1.87	1.87	1.96	2.89	1.97	1.37	0.97
	P008	0.00	0.19	0.42	0.83	1.76	2.34	2.45	3.03	2.89	2.60	1.65	0.92

Analyte	Participant	0h	0.25h	0.5h	1h	2h	4h	6h	8h	12h	24h	48h	72h
CBZE (µg/ml)	P001	0.00	0.00	0.00	0.00	0.01	0.03	0.07	0.09	0.10	0.13	0.08	0.06
	P002	0.00	0.00	0.00	0.01	0.03	0.05	0.05	0.06	0.06	0.07	0.09	0.06
	P003	0.00	0.00	0.00	0.00	0.00	0.01	0.05	0.08	0.10	0.15	0.08	0.06
	P004	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.04	0.05	0.08	0.08	0.05
	P005	0.00	0.00	0.00	0.01	0.03	0.05	0.07	0.07	0.09	0.13	0.13	0.07
	P006	0.00	0.00	0.00	0.01	0.02	0.03	0.05	0.07	0.08	0.15	0.15	0.08
	P007	0.00	0.00	0.00	0.02	0.04	0.06	0.07	0.09	0.09	0.12	0.11	0.09
	P008	0.00	0.00	0.01	0.02	0.04	0.06	0.08	0.11	0.13	0.22	0.20	0.11
DiOH-CBZ (µg/ml)	P001	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.08	0.17	0.20	0.15
	P002	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.05	0.07	0.12	0.20	0.15
	P003	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.04	0.08	0.18	0.29	0.24
	P004	0.00	0.00	0.00	0.00	0.01	0.02	0.03	0.04	0.07	0.14	0.22	0.18
	P005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.14	0.27	0.22
	P006	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.16	0.27	0.22
	P007	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.05	0.13	0.20	0.18
	P008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.24	0.30	0.23

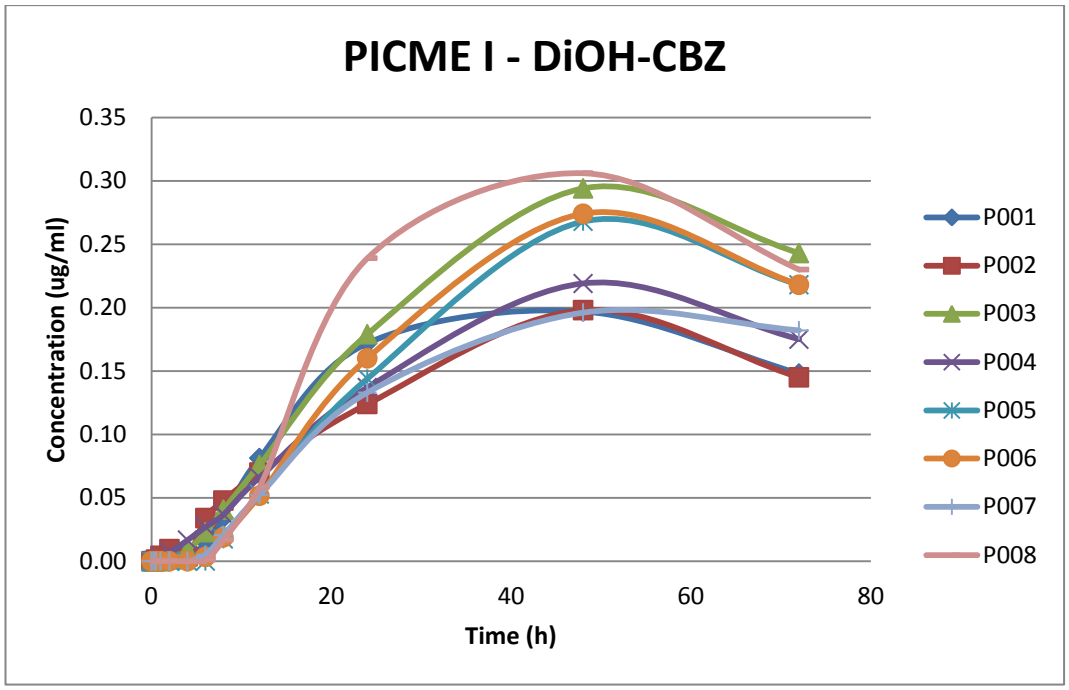
Analyte	Participant	0h	0.25h	0.5h	1h	2h	4h	6h	8h	12h	24h	48h	72h
2OH-CBZ (ng/ml)	P001	0.00	0.00	0.00	0.57	0.67	1.85	2.16	2.69	2.70	2.63	1.88	1.65
	P002	0.00	0.29	1.00	1.49	1.93	2.23	2.36	2.32	2.31	2.36	1.91	1.65
	P003	0.00	0.00	0.00	0.00	0.17	0.52	0.79	0.87	0.87	1.02	1.84	1.63
	P004	0.00	0.00	0.38	2.13	2.40	3.08	3.88	4.36	5.56	5.69	4.64	2.66
	P005	0.00	0.16	0.72	2.62	3.15	3.68	4.05	4.67	4.42	4.91	4.19	3.02
	P006	0.00	0.20	0.87	1.55	1.61	1.93	2.07	2.55	2.84	2.96	2.87	1.73
	P007	0.00	0.04	0.23	0.70	1.16	1.60	1.66	1.76	1.80	2.17	1.81	1.41
	P008	0.00	0.04	0.45	0.83	1.37	1.99	2.33	2.41	2.72	3.45	2.82	1.64
3OH-CBZ (ng/ml)	P001	0.00	0.00	0.00	0.06	1.75	1.52	1.79	2.59	2.93	2.54	1.75	1.46
	P002	0.00	0.00	0.48	1.49	2.19	2.74	3.18	3.11	3.00	3.12	2.56	1.83
	P003	0.00	0.00	0.00	0.00	0.00	0.55	1.36	1.44	1.56	2.06	2.55	1.97
	P004	0.00	0.00	0.00	0.00	0.73	1.92	3.62	4.42	5.67	6.83	4.88	1.49
	P005	0.00	0.00	0.00	0.11	1.71	3.36	4.60	5.62	5.04	6.35	4.69	2.44
	P006	0.00	0.00	0.00	0.00	0.00	0.10	0.41	1.08	1.38	1.73	1.82	0.00
	P007	0.00	0.00	0.00	0.00	1.04	1.92	2.09	2.44	2.39	2.84	2.68	1.61
	P008	0.00	0.00	0.00	0.00	0.60	1.26	1.58	1.94	2.31	3.25	2.98	1.00


PICME I - CBZ
Concentration (ug/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Time (h)
P001
P002
P003
P004
P005
P006
P007
P008

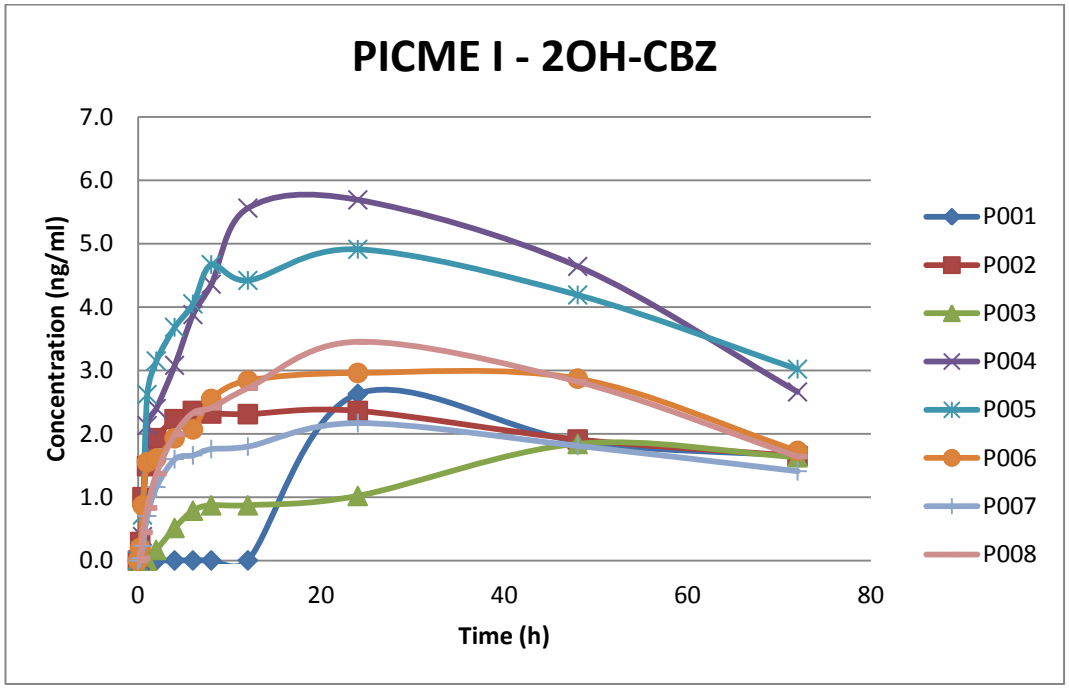
(A)


PICME I - CBZE
Concentration (ug/ml)
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
Time (h)
P001
P002
P003
P004
P005
P006
P007
P008

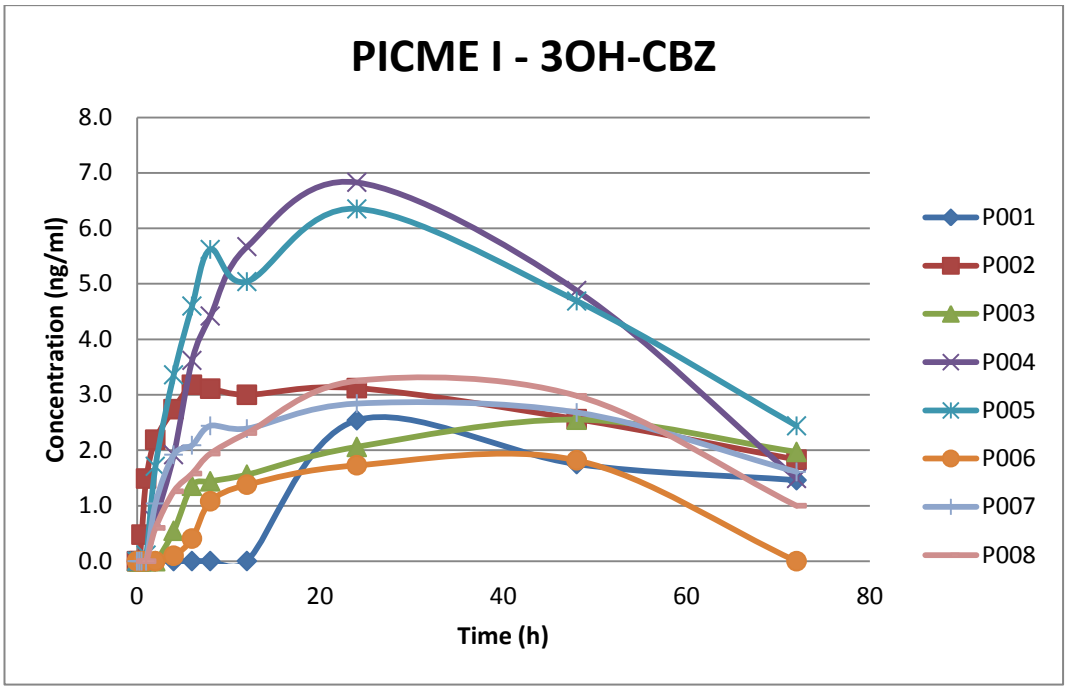
(B)


PICME I - DiOH-CBZ
Concentration (ug/ml)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
Time (h)
P001
P002
P003
P004
P005
P006
P007
P008

(C)


PICME I - 2OH-CBZ
Concentration (ng/ml)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
Time (h)
P001
P002
P003
P004
P005
P006
P007
P008

(D)



(E)

Figure 3.8 Plasma PK profiles for healthy volunteers following a single 400 mg dose of CBZ: (A) CBZ, (B) CBZE, (C) DiOH-CBZ (D) 2OH-CBZ and (E) 3OH-CBZ.

Table 3.17 PK parameters for CBZ following a single 400 mg dose

	C_{max} (µg/mL)	T_{max} (h)	t½ (h)	AUC (µg/mL*h)
P001	2.49	6	37.0	114.2
P002	2.33	4	63.7	126.2
P003	2.78	8	42.2	136.0
P004	2.63	12	65.6	127.3
P005	2.96	6	38.7	151.9
P006	2.96	12	45.8	160.6
P007	2.89	12	47.0	119.3
P008	3.03	8	31.9	142.7

Plasma PK profiles for CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ in the autoinduction patients in the PICME II clinical trial are presented in Table 3.18. For all three subjects, as the dosage of CBZ increased during the study, plasma levels of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ also increased. There was variability in levels of all the analytes despite subjects being prescribed the same dosages of CBZ. Subject A003 demonstrated the highest plasma levels of CBZ, CBZE, DiOH-CBZ and 3OH-CBZ; while subject A002 consistently had the lowest plasma levels of the same analytes. Plasma levels of 2OH-CBZ were highest throughout the course of therapy in subject A001 and lowest in A003. Figure 3.9 represents the mean concentrations of plasma CBZ at each visit as dosage was increased.

Table 3.18 Plasma concentrations of CBZ and its metabolites in the autoinduction group of the PICME II clinical trial

		Time Post Dose (h)						
CBZ (µg/mL)	**Participant**	**0**	**1**	**2**	**4**	**6**	**8**	**24**
Visit 1	**A001**	0.00	1.48	1.77	2.06	1.57	1.60	1.54
	A002	0.00	0.20	0.29	0.77	0.72	0.90	0.89
	A003	0.00	0.18	0.37	1.00	1.42	1.22	1.34
Visit 2	**A001**	2.18	2.93	3.27	3.46	3.58	3.65	X
	A002	2.21	2.30	2.59	3.10	2.65	2.26	X
	A003	3.77	4.38	4.05	4.21	4.39	4.96	X
Visit 3	**A001**	3.79	4.35	4.97	4.98	4.54	4.68	X
	A002	2.79	2.97	3.06	2.69	2.94	2.69	X
	A003	6.39	6.14	6.52	7.27	6.57	6.55	X
Visit 4	**A001**	4.72	5.03	5.52	6.01	5.03	4.93	X
	A002	3.69	3.36	3.63	3.82	3.62	3.42	X
	A003	7.33	7.67	7.02	6.79	7.42	6.58	X

		Time Post Dose (h)						
CBZE (μg/mL)	**Participant**	**0**	**1**	**2**	**4**	**6**	**8**	**24**
Visit 1	**A001**	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	A002	0.00	0.05	0.01	0.03	0.00	0.07	0.04
	A003	0.00	0.00	0.00	0.00	0.02	0.03	0.08
Visit 2	**A001**	0.30	0.30	0.30	0.23	0.24	0.26	X
	A002	0.23	0.15	0.17	0.25	0.17	0.13	X
	A003	0.53	0.40	0.45	0.48	0.48	0.46	X
Visit 3	**A001**	0.87	0.72	0.66	0.62	0.67	0.87	X
	A002	0.33	0.27	0.33	0.29	0.40	0.29	X
	A003	1.34	1.18	1.21	1.23	1.26	1.26	X
Visit 4	**A001**	1.46	0.89	1.18	1.22	1.10	0.86	X
	A002	0.48	0.34	0.44	0.47	0.44	0.43	X
	A003	1.34	1.27	1.34	1.21	1.42	1.22	X

DiOH-CBZ (µg/mL)		Time Post Dose (h)						
	Participant	**0**	**1**	**2**	**4**	**6**	**8**	**24**
Visit 1	**A001**	0.00	0.01	0.01	0.01	0.02	0.02	0.08
	A002	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	A003	0.00	0.00	0.00	0.00	0.01	0.02	0.09
Visit 2	**A001**	0.62	0.60	0.60	0.52	0.58	0.54	X
	A002	0.40	0.37	0.36	0.39	0.38	0.40	X
	A003	1.06	0.80	0.85	0.85	0.90	0.89	X
Visit 3	**A001**	1.47	1.34	1.33	1.28	1.32	1.69	X
	A002	0.74	0.70	0.70	0.68	0.75	0.74	X
	A003	2.48	2.71	2.68	2.50	2.47	2.44	X
Visit 4	**A001**	3.23	2.22	1.98	2.49	2.46	2.02	X
	A002	1.30	1.26	1.27	1.30	1.40	1.35	X
	A003	4.23	4.34	4.24	4.08	4.27	3.98	X

2OH-CBZ (ng/mL)		Time Post Dose (h)						
	Participant	**0**	**1**	**2**	**4**	**6**	**8**	**24**
Visit 1	**A001**	0.00	0.42	0.60	0.89	0.62	0.74	0.75
	A002	0.00	0.00	0.00	0.52	0.72	0.73	0.97
	A003	0.00	0.00	0.00	0.13	0.28	0.36	0.58
Visit 2	**A001**	1.90	2.91	1.86	3.16	3.15	2.95	X
	A002	4.61	4.60	4.04	4.96	4.69	4.19	X
	A003	1.97	1.94	2.59	2.69	3.93	2.76	X
Visit 3	**A001**	6.67	7.49	6.28	6.12	5.59	6.96	X
	A002	5.89	6.29	6.50	7.76	7.0	6.28	X
	A003	3.32	3.90	4.13	3.48	3.93	4.03	X
Visit 4	**A001**	10.8	8.26	9.59	8.80	7.99	7.00	X
	A002	6.40	6.11	7.47	7.59	8.42	8.20	X
	A003	6.62	6.18	7.01	7.02	6.76	6.71	X

3OH-CBZ (ng/mL)		Time Post Dose (h)						
	Participant	**0**	**1**	**2**	**4**	**6**	**8**	**24**
Visit 1	**A001**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	A002	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	A003	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Visit 2	**A001**	1.49	1.52	1.53	1.77	1.93	1.56	X
	A002	2.00	2.27	1.90	2.71	2.57	1.84	X
	A003	5.64	4.55	5.82	5.62	6.56	6.16	X
Visit 3	**A001**	7.68	8.74	6.85	6.66	6.04	7.11	X
	A002	6.18	6.65	6.73	7.45	7.12	6.02	X
	A003	9.89	12.10	12.40	10.40	11.30	11.40	X
Visit 4	**A001**	12.6	9.82	12.50	11.10	10.00	7.79	X
	A002	6.28	5.98	8.48	8.73	9.96	6.01	X
	A003	19.50	16.40	17.80	20.70	19.00	17.50	X

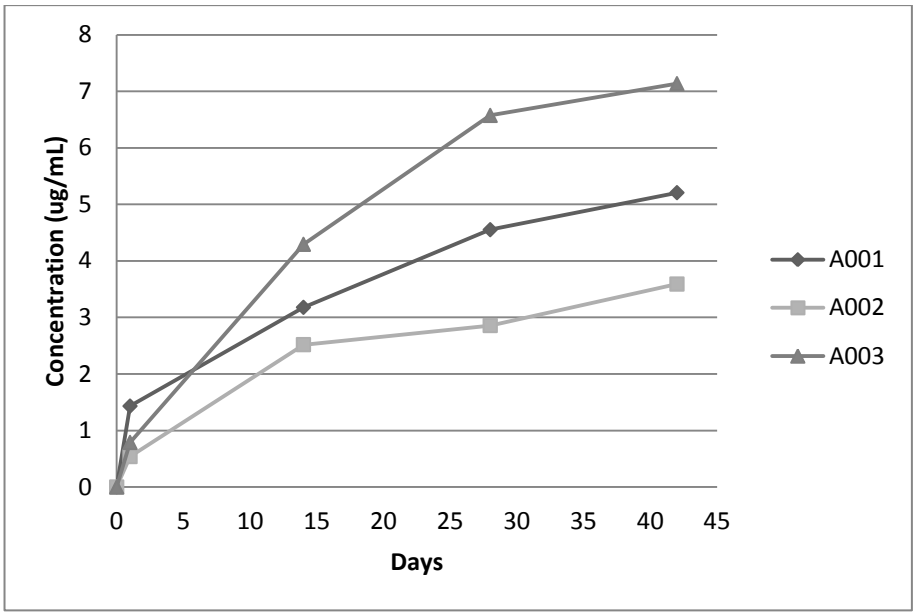


Figure 3.9 Mean CBZ plasma levels on PK sampling days as dosage increased in the autoinduction group of the PICME II clinical trial (day 1: 200 mg once daily, day 14: 200 mg twice daily, day 28: 200 mg in the morning and 400 mg at night, day 42: 200 mg in the morning and 400 mg at night)

Plasma levels of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ for the maintenance group subjects in the PICME II clinical trial are presented in Table 3.19. There was no correlation between levels of CBZ and PK sampling time post dose (r^2= 0.0443, p=0.0662) (Figure 3.10). There was a weak correlation between total daily CBZ dosage and plasma levels of CBZ (r^2= 0.1903, p<0.0001) (Figure 3.11). One subject (C020) in the maintenance dosage group did not have any detectable peaks for CBZ or any of the metabolites. It is most likely that this subject had not taken the prescribed CBZ. CBZ, CBZE, DiOH-CBZ and 2OH-CBZ were detectable in all other plasma samples at concentrations greater than the LLOQ of the assay. Plasma concentrations of CBZ ranged from 0 – 18.9 µg/mL; CBZE, 0-5.20 µg/mL; DiOH-CBZ, 0-6.9 µg/mL; and 2OH-CBZ, 0-33.3 ng/mL. 3OH-CBZ was detected in all the other patients but was below the LLOQ in three patients (C009, C011 and C046). 3OH-CBZ plasma concentrations ranged from 0 – 60.8 ng/mL.

Table 3.19 Plasma concentrations of CBZ, CBZE, 2OH-CBZ, 3OH-CBZ and DiOH-CBZ at sparse pharmacokinetic time points (BD: twice daily, TDS: three times a day, OM: morning, PM: afternoon, ON: night time)

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C001	M	32	200	BD	220	7.96	0.53	1.23	3.22	6.95
C002	F	48	400	ON	800	8.93	1.47	2.67	17.4	30.80
C003	F	27	700	BD	500	6.81	1.79	4.25	14.6	43.90
C004	F	32	600	BD	385	7.55	1.54	5.08	9.92	25.30
C005	F	71	200	BD	560	5.11	0.659	2.79	7.62	14.60
C006	F	67	400	BD	405	8.52	1.73	3.78	11.1	17.40
C007	M	53	200	BD	222	5.29	0.455	0.62	4.78	14.80
C008	M	46	600	BD	145	9.37	1.83	4.91	5.39	18.80
C009	M	34	200	ON	580	1.56	0.16	0.20	1.76	2.96
C010	F	37	400/600	OM/ON	410	7.83	1.75	3.09	11	35.50
C010 (2)	"	"	400/600	OM/ON	140	3.42	0.598	1.33	7.99	14.30
C011	M	53	400	BD	325	1.31	0.231	0.75	1.04	1.82
C011 (2)	"	"	400	BD	245	3.8	1.46	2.07	6.41	23.70
C012	M	60	400/600	OM/ON	1030	5.4	1.07	3.40	6.54	16.10

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C013	F	34	600	BD	390	8.44	2.86	4.48	6.3	29.80
C014	F	28	200	BD	900	4.82	0.468	1.00	3.76	13.70
C015	M	46	200	TDS	220	6.97	1.2	1.36	5.85	15.9
C015 (2)	"	"	200	TDS	225	6.76	0.918	1.00	6.77	15.2
C016	M	47	400/600	OM/ON	90	8.23	1.34	3.16	5.83	12.9
C017	F	58	400/600	OM/ON	580	5.46	2.35	4.99	11.8	38.0
C018	F	46	400/200/600	OM/PM/ON	155	8.06	2.57	4.60	6.51	16.1
C019	M	43	800	BD	120	13.6	1.02	0.99	10.5	19.3
C020	F	57	800	BD	240	0.00	0.00	0.00	0.00	0.00
C021	F	60	400/200/800	OM/PM/ON	430	8.51	1.81	4.82	4.66	12.7
C022	F	48	200/400	OM/ON	230	5.89	1.16	2.78	12.9	45.6
C023	M	61	400/500	OM/ON	425	6.98	1.32	3.34	6.75	20.4
C024	M	50	200	OM	130	2.04	0.181	0.39	2.56	5.51
C025	M	75	100	BD	240	4.46	0.851	0.78	4.06	10.5
C025 (2)	"	"	200/100	OM/ON	195	5.05	1.18	1.34	10.5	21.6

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C026	M	36	400/800	OM/ON	200	9.09	2.01	3.54	7.78	26.4
C027	F	30	200/400	OM/ON	380	6.95	1.44	2.20	12.9	22.6
C027(2)	"	"	200/400	OM/ON	155	5.06	0.941	1.98	9.62	18.9
C028	M	50	400	BD	265	8.79	1.21	3.00	14	21.7
C029	F	59	200/400	OM/ON	265	10.4	1.13	2.75	7.88	24.4
C030	M	25	800	ON	785	10.2	1.72	3.95	15.2	42.1
C031	M	45	600	BD	280	10.7	1.92	3.98	11.9	36.9
C032	M	33	400/300	OM/ON	525	6.46	0.854	2.07	7.95	11.3
C033	M	69	200/400	OM/ON	435	6.01	1.21	3.00	6.87	24.0
C034	M	60	400	BD	415	8.86	1.32	3.18	10.3	17.1
C034 (2)	"	"	400	BD	123	7.22	1.11	2.28	7.58	13.1
C035	F	62	400/600	OM/ON	135	11.0	2.93	5.66	14.9	31.6
C036	M	51	400	BD	100	8.56	1.5	3.22	15.3	33.3
C036 (2)	"	"	400	BD	387	11.7	1.68	2.90	16.4	38.5
C037	F	27	400/600	OM/ON	70	11.7	2.13	3.88	9.84	23.9

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C037 (2)	"	"	400/600	OM/ON	221	11.6	2.88	4.73	14.1	31.2
C038	F	38	400/800	OM/ON	225	9.86	1.88	3.20	8.32	20.1
C039	F	43	200	BD	110	6.19	1.12	1.97	6.95	11.8
C040	M	37	500/600	OM/ON	360	9.54	1.48	4.16	8.33	21.3
C041	F	47	400/600	OM/ON	140	5.99	1.45	4.36	9.32	23.2
C042	M	49	600	BD	70	9.90	2.51	5.74	8.8	26.1
C043	F	43	600	ON	805	4.45	0.83	1.74	7.41	18.8
C044	F	48	600	BD	395	9.22	5.2	6.90	10.7	22.6
C045	F	40	400	BD	362	5.32	0.80	1.58	5.82	16.9
C046	M	28	200	ON	1028	2.6	0.26	0.35	2.29	3.37
C047	M	54	200	TDS	253	5.42	1.49	3.59	7.12	16.1
C048	F	58	300	BD	263	7.88	1.81	3.81	6.48	15.7
C049	M	40	600/400	OM/ON	995	6.51	1.79	2.52	11	26.1
C050	F	33	600/800	OM/ON	170	3.88	1.15	2.90	6.09	21.2
C051	M	44	400/200/400	OM/PM/ON	343	8.57	2.51	3.89	9.38	20.8

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C052	F	53	200/400	OM/ON	314	7.86	1.85	2.77	11.4	23.9
C053	M	45	200	BD	115	6.28	0.87	1.13	5.08	15.5
C054	F	60	200/400	OM/ON	185	5.7	1.77	4.90	10.4	27.1
C055	M	59	200	BD	410	3.01	0.44	0.74	3.89	8.5
C056	M	46	100/200	OM/ON	185	5.56	0.57	0.73	10	17.1
C057	M	59	400	BD	275	10	1.46	3.93	19.4	44.0
C058	M	45	400	BD	889	8.09	1.23	1.85	6.02	15.8
C059	F	68	300/400	OM/ON	330	10.4	2.21	5.52	13.4	38.3
C060	M	50	200/400	OM/ON	220	7.14	1.18	2.03	11.5	23.4
C061	F	61	400/600	OM/ON	225	8.09	2.68	5.63	11.5	26.9
C062	M	40	400/600	OM/ON	275	8.89	2.75	4.38	11.8	33.2
C063	M	43	400	BD	440	4.67	1.43	2.55	8.05	12.8
C064	M	54	600/800	OM/ON	245	18.9	2.59	4.29	25.7	60.8
C065	M	55	200	BD	195	7.47	0.98	1.34	7.70	8.8
C066	M	59	200/400	OM/ON	165	9.90	2.78	3.75	11.7	36.4

ID	Gender	Age (years)	CBZ Dose (mg)	Frequency	PK time post-dose (mins)	CBZ (µg/mL)	CBZE (µg/mL)	DiOH-CBZ (µg/mL)	2OH-CBZ (ng/mL)	3OH-CBZ (ng/mL)
C067	F	58	600/400/600	OM/PM/ON	210	14.3	3.77	6.26	33.3	59.2
C068	F	44	200	BD	200	9.34	1.41	3.40	15.1	31.5
C069	F	58	200/400	OM/ON	310	8.95	1.76	4.21	8.88	14.1

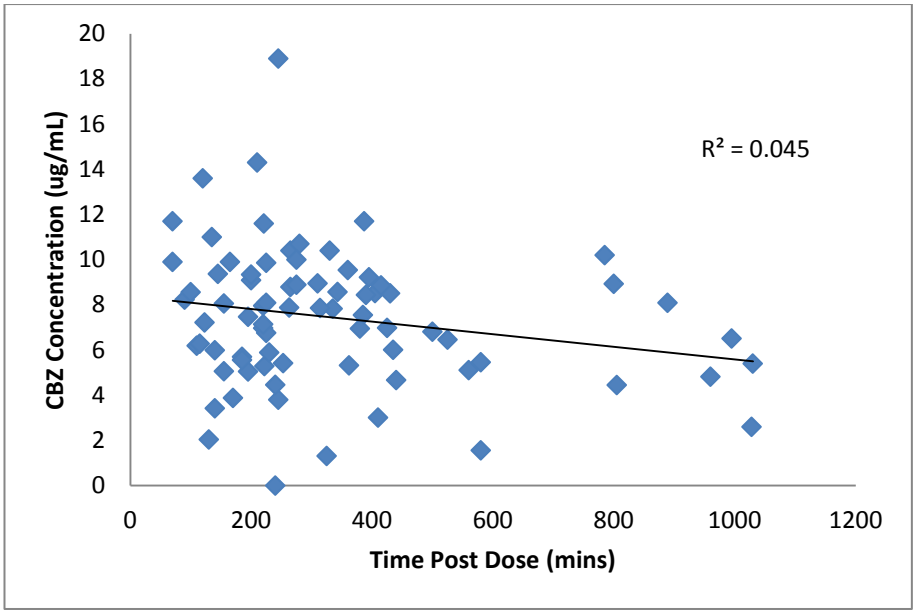


Figure 3.10 Correlation between PK sampling time post dose and plasma levels of CBZ

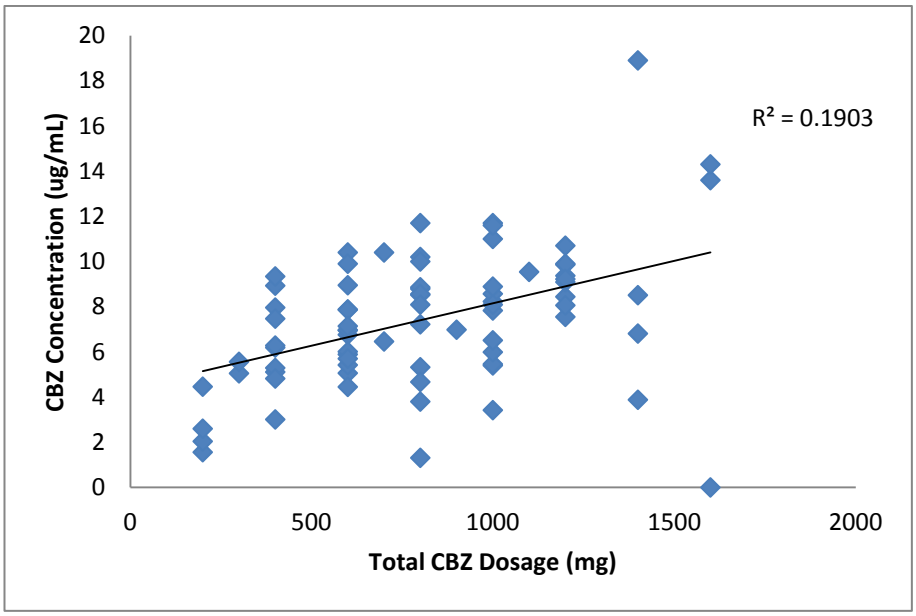


Figure 3.11 Correlation between total daily CBZ dosage and plasma levels of CBZ

3.4 Discussion

Two HPLC-MS/MS assays for quantification of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ in human plasma have been developed and validated according to FDA guidance (Food and Drug Administration 2001). It was initially the intention to utilise a single assay to quantify all five analytes. Unfortunately due to insufficient specificity for 2OH-CBZ and 3OH-CBZ in patient samples (Figure 3.6) a second assay had to be utilised. The interference on the MRM chromatogram preventing quantitation of 2OH-CBZ and 3OH-CBZ was absent from the pooled healthy volunteer plasma calibration and QC samples and not present in pre-dose patient samples. The samples were stored and treated identically. Therefore it is possible that the interference was caused by drug excipients, or their metabolites, although it was not possible to ascertain their identities. A Waters XBridge C18 BEH column was used for assaying the phenols and the reconstituted deproteinized plasma samples were filtered through a hydrophilic PTFE filter plate for further removal of proteins prior to injection onto the HPLC column. These adjustments for the new assay delivered the required specificity for 2OH-CBZ and 3OH-CBZ, and the assay was validated for use in patient plasma samples.

Matrix effect is a physico-chemical phenomenon that is essentially intrinsic to the ESI process used in LC-MS/MS assays. It refers to the matrix-induced suppression and/or enhancement of analyte ionisation when other ions or substances co-elute with the analyte, and can lead to sample-to-sample inconsistencies in analyte response versus concentration (Matuszewski et al. 2003). Although the matrix (plasma) was qualitatively consistent throughout the study, inter-individual variations of plasma composition, and especially of phospholipids (Ismaiel et al. 2013), might lead to differential effects. To assess ME, the peak areas of analytes dissolved in acetonitrile were compared with those of the analytes spiked into deproteinized and reconstituted pooled plasma (from 6 volunteers). Minor ME were observed in respect of four of the analytes: CBZE, 2OH-CBZ, 3OH-CBZ and DiOH-CBZ (Table 3.11). However, a ME on CBZ of 64.2% was observed. Although a substantial ME on CBZ was present it was similar across the whole calibration range and demonstrated a low CV of 5.4%, minimising any adverse effects on quantitation of

CBZ. The recovery of all 5 analytes ranged between 76.9% and 80.7% and was consistent throughout the concentration range, with low CVs ranging from 4.4% to 7.5% thereby minimising adverse effect on quantitation of analytes.

Acetone was chosen as the plasma protein precipitant because it has previously been demonstrated to provide the highest analyte recovery efficiencies compared with methanol or acetonitrile (Breton et al. 2005), and the same method was utilised for the current assays. 2Me-CBZ was utilised as the internal standard in both assays to account for the loss of any sample during the extraction process and for any variation in ionisation at the mass spectrometer source (Annesley 2003). Although stable isotopically labelled analogues are considered the best type of internal standard because of its nearly identical chemical and physical properties to target analytes, it was not possible to use them for the current assay as they were not easily commercially available (Hewavitharana et al. 2014). In addition, structurally similar compounds can perform as well as stable isotope IS and both new assays reported here satisfied bioanalytical assay validation criteria using 2Me-CBZ as the IS (Wieling 2002). Assessment of the freeze-thaw cycle stability of analytes was not undertaken during assay validation because as intended all the plasma samples, having been separated into multiple aliquots, were frozen at -80^{o}C and thawed only for analysis and not returned to the freezer.

Although the assays were based upon existing HPLC-MS/MS methods, these new assays improved sensitivity and specificity whilst reducing the time required to analyse each sample. The only method in the literature to quantify all of the analytes of interest had a total cycle time of 50 min (Breton et al. 2005) whereas the total cycle time for each of the new assays is 20 min. Although other assay cycle times are shorter, ranging from four to ten minutes, they do not quantify as many analytes (Van Rooyen et al. 2002; Lionetto et al. 2012; Shibata et al. 2012). The new assays improve sensitivity for all the analytes compared with the original assay devised by Breton et al. (2005). Two other assays demonstrated lower LLOQ for CBZ and CBZE but they were complicated by difficult extraction procedures or the

requirement for much larger volumes of serum (Van Rooyen et al. 2002; Lionetto et al. 2012).

In total 248 individual plasma samples were analysed using the newly developed HPLC-MS/MS assays of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ. The PK variables for CBZ reported in the PICME I clinical trial, namely a T_{maz} of 6-12 h and an average t½ of 46.5 h (Table 3.17), were consistent with previously reported data for single doses in healthy volunteers (Bertilsson 1978). In the PICME II autoinduction group subjects A001 and A003 had reached therapeutic levels of CBZ (4-12 μg/mL) (Schulz et al. 2012), 4.93 μg/mL and 6.58 μg/mL, respectively, after the dose titration regimen. CBZ levels in subject A002 remained below the therapeutic window, with a concentration of 3.59 μg/mL (Figure 3.9). On review of the clinical history, subject A002 was noted to have a very large BMI (46.8) compared with the other two subjects and the increased weight is most likely to account for the reduced plasma levels of CBZ. In addition subject A002 had been commenced on atorvastatin therapy by her general practitioner during the dose titration process. Data from pre-clinical mouse models have demonstrated that chronic treatment with atorvastatin reduces the anticonvulsant potency of CBZ (Stepien et al. 2012). The exact mechanism by which this occurs is unclear but atorvastatin, like CBZ, is metabolised by CYP3A4 and it is possible that reduction of anticonvulsant activity is mediated by an unidentified PK effect (Shitara et al. 2006).

The plasma level range of CBZ in the maintenance group of the PICME II clinical trial (0-18.9 μg/mL) was found to be similar to that reported in the literature (Breton et al. 2005; Lionetto et al. 2012). Plasma levels of CBZ were below the therapeutic range in nine participants and above the therapeutic range in three cases. In all three cases of elevated levels, the total daily dosage of CBZ was greater than 1400 mg. The recommended daily dosage for CBZ according to the summary of product characteristics is between 800-1200mg daily (Electronic Medicines Compendium 2015). Plasma concentrations of CBZE (0-5.2 μg/mL) and DiOH-CBZ (0-6.9 μg/mL) measured in the current study were comparable to the results of previous studies (Breton et al. 2005; Lionetto et al. 2012). It is interesting to note that 2OH-CBZ was

above the LLOQ in all 77 plasma samples in this study whereas a previous study only identified the metabolite in 9 out of 30 subjects (Breton et al. 2005). However, the previous assay was not as sensitive as the updated assay. The LLOQ in the older assay was 23 ng/mL, compared with the new assay's LLOQ of 0.9 ng/mL. If the calibration range from the older assay had been applied to the plasma samples in the current clinical trial, only two samples would have been above the LLOQ. Additionally, the previous study obtained PK samples only at the trough of the dosing interval whereas the current study sampled across a much wider post-dose time frame (70 to 1030 min). Also, 3OH-CBZ was assayable in only 25 out of 30 subjects in the previous study, with concentrations between 23.0 to 720 ng/mL. Whereas 3OH-CBZ was above the LLOQ in 73 out of 77 plasma samples assayed during the present study, with concentrations ranging from 0 to 60.8 ng/mL. The increased detection rate is attributable to the lower LLOQ in the new assay: 4.73 ng/mL against 23.0 ng/mL (Breton et al. 2005). There was only a weak correlation between CBZ dosage and plasma CBZ levels (r^2=0.1903, p<0.0001) (Figure 3.11). This highlights the significant variability associated with CBZ metabolism. Polymorphisms of drug metabolising enzymes and transporters as well as external factors such as concomitant medications are likely to play an important role in explaining variability in plasma levels (Yun et al. 2013; Caruso et al. 2014; Wang et al. 2015). Detailed population PK modelling of CBZ and its metabolites has been undertaken, incorporating multiple covariates, gene polymorphisms and patient variables. It is described and analysed in the next chapter.

In conclusion, two HPLC-MS/MS assays were developed and validated according to FDA guidance, and utilised for the measurement of the concentrations of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ, and 3OH-CBZ in 248 patient and volunteer plasma samples.

Chapter 4

A Population PK Model to Investigate the Role of Patient Demographics and Genetic Variation on the Metabolism of Carbamazepine

4.1 Introduction

CBZ is a tricyclic anticonvulsant that is used for the treatment of epilepsy (Marson et al. 2007). CBZ is almost completely metabolised in the liver and the major route of metabolism is conversion to CBZE (Pearce et al. 2008). CBZE is a pharmacologically reactive metabolite that can also form protein conjugates *in vivo* (Figure 2.10). CBZE is converted to DiOH-CBZ by mEH (Tomson et al. 1983). Other metabolites include 2OH-CBZ and 3OH-CBZ, presumed to be formed via a reactive arene oxide intermediate (Pearce et al. 2002). Multiple CYP isoforms are involved in formation of these metabolites including CYP3A4, CYP3A5, CYP2B6 and CYP2C9 (Thorn et al. 2011). UGT2B7 is responsible for glucuronidation of CBZ (Staines et al. 2004) and metabolism of 2OH-CBZ and 3OH-CBZ by myeloperoxidase (MPO) may lead to formation of free radicals (Lu and Uetrecht 2008). Formation of multiple reactive metabolites may be implicated in hypersensitivity reactions observed with CBZ therapy (Knowles et al. 2000).

CBZ undergoes autoinduction through upregulation of multiple CYP isoforms and drug transporters meaning patients typically need to undergo dose titration regimens at the beginning of therapy (Oscarson et al. 2006). The role of drug transporters, such as P-glycoprotein (P-gp), in CBZ response is unclear at present but there is some evidence to suggest that variable transport across the blood brain barrier could influence clinical outcomes with CBZ treatment (Sisodiya and Goldstein 2007). Clinically, CBZ has a narrow therapeutic index and therapeutic levels are estimated to range between 4 and 12 μg/mL (Schulz et al. 2012). There is large inter-individual variability in plasma levels of CBZ with little apparent relationship to dose as demonstrated in Figure 3.12. This has led researchers to postulate that factors other than dose are responsible for variation in CBZ plasma levels. These factors include: concomitant medications (Spina et al. 1996) and genetic polymorphisms in metabolising enzymes and transporter proteins (Puranik et al. 2013).

In subjects receiving CBZ treatment concomitantly with phenytoin or phenobarbital, dose-adjusted plasma concentrations of CBZ and CBZ plus CBZE concentrations

were significantly associated with SNPs in *CYP3A5* (rs776746 and rs15524) and the haplotype (rs15524-rs776746 GT, AC) (Wang et al. 2015). A second study in Korean patients reported that oral clearance of CBZ was significantly higher in *CYP3A5* non-expressors (rs776746) compared with wild type and heterozygotes (Park et al. 2009). However, two studies did not find a significant association between CBZ levels and *CYP3A5* polymorphisms (Panomvana et al. 2013; Zhu et al. 2014). The *CYP3A4*1B* SNP was significantly associated with reduced clearance for CBZ (Puranik et al. 2013). Several other studies have investigated the association between polymorphisms in *CYP3A4* and CBZ metabolism but no significant effects have been reported (Yun et al. 2013; Caruso et al. 2014; Zhu et al. 2014; Wang et al. 2015).

P-gp is a drug efflux transporter that is encoded for by ATP-binding cassette sub-family B member 1 (*ABCB1)* and plays a significant role in drug absorption and disposition (Lin and Yamazaki 2003). SNPs in *ABCB1* (rs2032582 and rs10234411) were associated with altered CBZE/CBZ ratio in subjects receiving CBZ therapy with phenytoin or phenobarbital (Wang et al. 2015). Patients who were wild-type for *ABCB1* c.3435C>T were found to have significantly higher dose adjusted concentrations of CBZ, CBZE and DiOH-CBZ (Meng et al. 2011a; Zhu et al. 2014). A third study reported that subjects with the CC genotype had a higher likelihood of response to CBZ compared with patients with CT or TT genotypes (Sterjev et al. 2012).

CBZE is metabolised to DiOH-CBZ by mEH, which is encoded by *EPHX1* gene. The *EPHX1* SNPs, c.416A>G and c.128G>C, were significantly associated with higher and lower DiOH-CBZ:CBZE ratios, respectively (Zhu et al. 2014). Higher adjusted CBZ plasma concentrations were reported in Chinese epilepsy patients with the variant *EPHX1* c.416A>G compared to those with the wild-type genotype. Another study in Japanese patients reported that specific haplotype structures of *EPHX1* were significantly associated with increased or decreased ratios of DiOH-CBZ/CBZE ratios (Nakajima et al. 2005). Carriers of *EPHX1* c.337T>C required higher doses of CBZ (Hung et al. 2012; Ma et al. 2015) and potentially showed increased susceptibility to

CBZ-induced SJS/TEN (He et al. 2014). However, one study did not find any significant association between carriers of *EPHX1* c.337T>C and c.416A>G with plasma CBZE levels (Caruso et al. 2014). These data suggest that combinations of SNPs in multiple genes involved in the metabolism of CBZ are likely to contribute to the inter-individual variability in plasma levels of CBZ.

Population PK modelling aims to evaluate sources and correlates of PK variability in target populations receiving a specific pharmacological agent (Kiang et al. 2012). Traditional PK studies employed "intensive" PK sampling from relatively small numbers of subjects that were not representative of the target population meaning physicians did not have enough information to use the drug properly in the patient group (Aarons 1991). Population PK studies employ a "sparse" sampling approach whereby only a small number of PK samples are collected from each patient but the patients are representative of the target population (Ette and Williams 2004a). The "sparse" sampling approach for characterising population PK enables better estimation of inter-subject variability. PK variability is affected by several factors including demographics (e.g. age, weight, gender, etc), environmental factors (e.g. smoking, alcohol), genetic variation in metabolising enzymes (e.g. CYP), drug-drug interactions and disease states (e.g. hepatic or renal impairment) (Ette and Williams 2004a). Population PK modelling is often carried out with nonlinear mixed effects approach, where fixed effects and random effects parameters are estimated to describe a dataset and its variability. Fixed effects are generally the population average PK parameters while the random effects are used to account for variability in the data from the trends described by the fixed effects; such as inter-individual, inter-occasion and residual variability (the latter including errors in dosing, sampling or recording time or assay noise) (Kiang et al. 2012). In drug development, population PK modelling can be used to guide the first dose in humans from preclinical data (Kiang et al. 2012). In direct patient care, population PK modelling can allow for dosage individualisation once variables such as weight, concomitant medications and medical history for the intended patient is obtained through history and physical examination (Ette and Williams 2004a). NONMEM (non-linear mixed effects modelling) is the most widely used software program for population

PK modelling and has gained wide acceptance in both industry and academia (Dartois et al. 2007).

Therapy with CBZ is complicated because of its complex PK profile. The drug has high inter-individual variability in response, is metabolised to an active metabolite (CBZE), it has the ability to autoinduce its own metabolism and is subject to a high number of drug interactions due to metabolism by several CYP isoforms (Thorn et al. 2011). Due to the unpredictable response to CBZ therapy, several population PK models have been developed to investigate factors responsible for this variation and to enable personalised dosing (Milovanovic and Jankovic 2013). Previous population PK models have investigated clinical covariates such as gender, dose, age, weight and concomitant medications with only a single study using population PK modelling to investigate the role of genetic variants in metabolising enzymes on variability in the metabolism of CBZ (Djordjevic et al. 2016). However, this recent study only examined the effect of two SNPs in a single gene (*CYP1A2*) against plasma levels of CBZ. The aim of this chapter was to generate a comprehensive population PK model with NONMEM using the plasma concentrations of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ obtained from patients described in chapter 3. Twenty SNPs in multiple CBZ metabolism enzymes as well as traditional covariates (e.g. age, gender, weight, etc.) have been incorporated to improve our understanding of the metabolism of CBZ in patients.

4.2 Methods

4.2.1 Study Population

Blood samples were obtained for the study from the PICME I and PICME II clinical trials. Further details regarding these two studies can be found in chapter 3. There were three groups of patients:

- Healthy volunteers receiving a single 400mg dose of CBZ with rich PK sampling over 72 h (n= 8).
- Subjects with a diagnosis of epilepsy that were newly started on CBZ with rich PK samples at two weekly intervals during dose titration for the first six weeks of therapy (n= 3).
- Sparse PK samples from epilepsy patients who had been on the same dosage of CBZ for at least four weeks (n= 69).

4.2.2 Measurement of Plasma CBZ and Metabolite Levels in Patient Plasma

Blood samples for measurement of CBZ and its metabolites were collected from study participants in tubes containing lithium heparin and centrifuged at 1500 *g* for 10 min at 4^{o}C. Aliquots of plasma were pipette into cryovials and frozen at -80^{o}C until analysis. Plasma was thawed at room temperature on day of analysis. Plasma levels of CBZ, CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ were determined using HPLC-MS/MS according to Section 3.2.19.

4.2.3 DNA Extraction Procedure

Whole blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and frozen immediately at -80^{o}C until further analysis. Blood samples were thawed at room temperature on the same day as DNA extraction. DNA was extracted from 4-5 mls of whole blood using a Chemagic magnetic separation module (Chemagen Biopolymer-Technologie AG, Baeweiler, Germany) according to the manufacturer's protocol. The concentration of DNA was measured using NanoDrop (Thermo Scientific, Wilmington, USA). The extracted DNA was stored in aliquots at 4^{o}C until ready for further analysis.

4.2.4 Selection of Genetic Polymorphisms

The role of genetic variation on the metabolism of CBZ was investigated by analysis of SNPs. The selection of these SNPs was based upon known pathways of CBZ metabolism, expected minor allele frequencies (MAF) in the study population and review of previous polymorphism association studies (Table 1.4). A total of 20 SNPs were chosen in 11 genes (Table 4.1).

4.2.5 Analysis of Single Nucleotide Polymorphisms

SNPs were typed using either the Sequenom MassArray iPLEX platform (Sequenom Inc, Sand Diego CA) or TaqMan real-time PCR SNP genotyping assays (Life Technologies, Paisley, UK) according to the manufacturer's protocols (Table 4.1). Genotype frequencies for each SNP were examined for deviation from Hardy-Weinberg equilibrium (HWE) using the chi-squared test.

Table 4.1 Selection of genes and single nucleotide polymorphisms for genotyping studies

SNP ID	Gene	Nucelotide Change/Allele	Potential Change in Function	References	SNP Typing Platform
rs1045642	*ABCB1*	3435C>T	Reduced expression	(Hodges et al. 2011)	Taqman
rs1128503	*ABCB1*	1236T>C	Inconclusive	(Hodges et al. 2011)	Sequenom
rs2032582	*ABCB1*	2677G>T	Inconclusive	(Hodges et al. 2011)	Sequenom
rs2273697	*ABCC2*	1249G>A	Increased activity	(Bruhn and Cascorbi 2014)	Sequenom
rs3740066	*ABCC2*	3972C>T	Inconclusive	(Bruhn and Cascorbi 2014)	Sequenom
rs717620	*ABCC2*	-24C>T	Reduced activity	(Bruhn and Cascorbi 2014)	Taqman
rs28365062	*UGT2B7*	735A>G	Increased activity	(Kwara et al. 2009)	Sequenom
rs28365063	*UGT2B7*	372A>G	Increased activity	(Puranik et al. 2013)	Sequenom
rs7438135	*UGT2B7*	-900G>A	Increase activity	(Matic et al. 2014)	Sequenom
rs1051740	*EPHX1*	337T>C	Increased activity	(Nakajima et al. 2005)	Sequenom
rs2234922	*EPHX1*	416A>G	Reduced activity	(Nakajima et al. 2005)	Sequenom
rs11572080	*CYP2C8*	*3	Inconclusive	(Aquilante et al. 2013)	Sequenom
rs1058930	*CYP2C8*	*4	Reduced activity	(Aquilante et al. 2013)	Sequenom
rs4244285	*CYP2C19*	*2	Loss of function	(Scott et al. 2012)	Sequenom
rs192154563	*CYP2C19*	*17	Gain of function	(Scott et al. 2012)	Sequenom
rs3745274	*CYP2B6*	*6	Reduced activity	(Thorn et al. 2010)	Taqman

SNP ID	Gene	Nucelotide Change/Allele	Potential Change in Function	References	SNP Typing Platform
rs2242480	*CYP3A4*	*1G	Increased activity	(Zhu et al. 2014)	Sequenom
rs776746	*CYP3A5*	*3	Loss of function	(Lamba et al. 2012)	Sequenom
rs2333227	*MPO*	-463G>A	Reduced activity	(Hansson et al. 2006)	Taqman
rs1057868	*POR*	*28	Increased activity	(Lunde et al. 2014)	Taqman

4.2.6 Development of Population PK Model

Development of the population PK model was undertaken under the supervision of Dr. Henry Pertinez (Department of Molecular and Clinical Pharmacology, University of Liverpool). NONMEM (Sheiner et al. 1972) (version 7.2) was used with the first-order conditional estimation (FOCE) method for modelling the plasma dataset. Model selection was based on goodness of fit and the likelihood ratio test, using the difference in the NONMEM minimum objective function value (OFV) to discriminate between nested models with increasing degrees of freedom provided by extra model parameters. Covariate model building was carried out using the method of forward addition: with the NONMEM OFV (-2 x log likelihood) being approximately χ^2 distributed, a significant improvement in OFV from one model to the next was judged to have been achieved at the $p < 0.05$ significance level if the OFV was reduced by 3.84 by the addition of 1 additional structural model parameter (i.e. one degree of freedom) to the model, 3.84 being the critical χ^2 value for a p value of 0.05 for 1 degree of freedom. Goodness of modelled fits was assessed with the standard errors and correlation matrix of parameter estimates and graphically with diagnostic plots (observed vs. population predicted data, observed vs. individual predicted data, residual plots) and visual predictive checks (VPCs). Prediction corrected VPCs (Bergstrand et al. 2011) were produced in R (version 0.98.1091), with 1000 simulations (of the fitted dataset) carried out using the PK model parameters and residual error estimates given by the final NONMEM model fitting.

4.2.7 Correlation of Plasma CBZ Metabolite Concentrations with Single Nucelotide Polymorphisms in CBZ Metabolising Enzymes

Analysis of variance (ANOVA) was undertaken using GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Dose-adjusted plasma concentrations of CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ were compared against SNPs involved in the pharmacokinetics of CBZ to determine if there were any significant associations between genotype and plasma levels.

4.3 Results

4.3.1 Subject Demographics and Genetic Polymorphisms

In total, 80 subjects were recruited into the study: 8 healthy volunteers, 3 patients with diagnosis of epilepsy and newly started on CBZ treatment and 69 subjects on a stable dose of CBZ for at least 4 weeks. A summary of patient demographics and medication data is outlined in Table 4.2. Rich PK samples were obtained from the 8 healthy volunteers and 3 patients newly commenced on CBZ therapy. Sparse PK samples were collected from the 69 patients identified in clinic who had been on a stable dose of CBZ for at least 4 weeks. More detailed clinical information for each patient was previously reported in chapter 3 (Tables 3.13, 3.14, and 3.15).

The distribution of the 20 SNPs amongst subjects is outlined in Table 4.3. For a single SNP, CYP2C19*17 (rs192154563), all subjects were homozygous for the wild-type allele. Each genotype frequency was consistent with HWE and minor allele frequencies ranged from 4% to 55%.

Table 4.2 Demographic and Medication Data for Subjects included in Population Analysis. Data are expressed as mean values ± standard deviation (ranges)

Parameter		Index		
Number of subjects		80		
Number of observations		248		
Gender (%):	Male	46 (57.5%)		
	Female	34 (42.5%)		
Age (years)		45.8 ± 12.7 (21.0 – 75.0)		
Total body weight (kg)		80.8 ± 18.4 (39.0 – 140.5)		
Smokers (%)	Yes	20 (25%)		
	No	60 (75%)		
Sparse PK sampling time points* (mins)		337.5 ± 230.9 (70 – 1030)		
CBZ dose (mg/day)		765 ± 359 (200 – 1600)		
CBZ therapy without concomitant AEDs or CYP3A4 inducer/inhibitor (%)		29 (36.2%)		
Concomitant with 1 AED (%)		26 (32.5%)		
Concomitant with 2 AEDs (%)		16 (20.0%)		
Concomitant with 3 AEDs (%)		5 (6.3%)		
Concomitant with 1 CYP3A4 inducer/inhibitor (%)		13 (16.3%)		
Concomitant with 2 CYP3A4 inducer/inhibitors (%)		3 (3.8%)		
Number of subjects for each AED:	Valproate	3	Zonisamide	5
	Phenytoin	4	Topiramate	4
	Phenobarbital	1	Lacosamide	3
	Clobazam	18	Pregabalin	4
	Lamotrigine	5	Perampanel	1
	Levetiracetam	26		
Number of subjects for each CYP3A4 inducer/inhibitor:	Omeprazole	6		
	Statin	13		

*time points applicable only to sparse PK sampling group; AED: antiepileptic drug; CBZ: carbamazepine, PK: pharmacokinetic

Table 4.3 The Distribution of SNPs Amongst Study Subjects (n= 80)

SNP ID	Gene	Allele	Genotype Frequency (%)			Minor Allele Frequency	Hardy Weinberg Equilibrium
			Wild type	Heterozygote	Homozygote		
rs1045642	*ABCB1*	3435C>T	14 (17.5%)	44 (55%)	22 (27.5%)	0.55	0.32
rs1128503	*ABCB1*	1236T>C	14 (17.5%)	42 (52.5%)	24 (30%)	0.56	0.55
rs2032582	*ABCB1*	2677G>T	24 (30.0%)	39 (48.8%)	17** (21.2%)	0.46	0.88
rs2273697	*ABCC2*	1249G>A	49 (61.3%)	29 (36.3%)	2 (2.4%)	0.21	0.34
rs3740066	*ABCC2*	3972C>T	25 (31.3%)	42 (52.5%)	13 (16.2%)	0.43	0.51
rs717620	*ABCC2*	-24C>T	50 (62.5%)	26 (32.5%)	4 (5.0%)	0.21	0.80
rs28365062	*UGT2B7*	735A>G	62 (77.5%)	18 (22.5%)	0 (0.0%)	0.11	0.26
rs28365063	*UGT2B7*	372A>G	56 (70.0%)	24 (30.0%)	0 (0.0%)	0.15	0.11
rs7438135	*UGT2B7*	-900G>A	27 (33.8%)	39 (48.7%)	14 (7.5%)	0.42	0.99
rs1051740	*EPHX1*	337T>C	36 (45.0%)	37 (46.3%)	7 (8.7%)	0.32	0.56
rs2234922	*EPHX1*	416A>G	53 (66.3%)	24 (30.0%)	3 (3.7%)	0.19	0.89
rs11572080	*CYP2C8*	*3	62 (77.5%)	16 (20.0%)	2 (2.5%)	0.13	0.44
rs1058930	*CYP2C8*	*4	70 (87.5%)	10 (12.5%)	0 (0.0%)	0.06	0.55
rs4244285	*CYP2C19*	*2	57 (71.3%)	19 (23.7%)	4 (5.0%)	0.17	0.17
rs192154563	*CYP2C19*	*17	80 (100%)	0 (0.0%)	0 (0.0%)	0.00	NA
rs3745274	*CYP2B6*	*6	52 (65.0%)	26 (32.5%)	2 (2.5%)	0.19	0.55

SNP ID	Gene	Allele	Genotype Frequency (%)			Minor Allele Frequency	Hardy Weinberg Equilibrium
			Wild type	Heterozygote	Homozygote		
rs2242480	*CYP3A4*	*1G	71 (88.8%)	9 (11.2%)	0 (0.0%)	0.06	0.59
rs776746	*CYP3A5*	*3	73 (91.3%)	7 (8.7%)	0 (0.0%)	0.04	0.68
rs2333227	*MPO*	-643G>A	12 (15%)	41 (51.3%)	27 (33.7%)	0.59	0.58
rs1057868	*POR*	*28	40 (50.0%)	27 (33.8%)	13 (16.2%)	0.33	0.53

**included 3 TA subjects, NA: not applicable

4.3.2 Base Model Fitting

The plasma exposure data for CBZ only was adequately described with a one-compartment, first order absorption model. Residual error was modelled with a proportional error model. Parameter estimates from the base model fitting (without incorporating any covariates into the model) are given in Table 4.4. Log normal distributions were assumed for the description of inter-individual variability in PK parameters, as shown in the following equation:

$$\theta_{xi} = \theta_x * \exp(\eta_{xi}) \quad (1)$$

where θ_{xi} is the PK parameter '*x*' of the i^{th} individual; θ_x is the fixed effect population parameter estimate; and η_{xi} is the log inter-individual variability for parameter '*x*' in individual '*i*' drawn from a normal distribution with a mean of zero and a variance of ω^2.

Table 4.4 Parameter estimates and standard errors for the base model fitting

	CL/F (L h^{-1})	**V/F (L)**	**k_a (h^{-1})**	**IIV CL/F (%)**	**IIV V/F (%)**	**IIV k_a (%)**	**Proportional residual error variance**	**OFV**
Estimates	2.47	132	0.579	52.1%	10.4%	82.0	0.0354	255.685
%RSE	7%	5%	22%	18%	73%	29%	23%	

CL/F: apparent clearance, IIV: interindividual variability, k_a: absorption constant, OFV: objective function value, RSE: residual standard error, V/F: apparent volume of distribution.

4.3.3 Covariate Model Fitting

Patient demographic variables, concomitant medications and 19 SNPs were explored as covariates on the CL/F structural parameter of the model and effect on OFV are outlined in Table 4.5. The SNP rs192154563 (*CYP2C19*17*) was not explored as a covariate as all subjects in the study were wild-type.

Dichotomous and trichotomous covariates were introduced as a power model and continuous variables were modelled using a power model with the covariate value normalised to the dataset population mean value, i.e.:

$$\theta_x = \theta_x \times \theta_{COV}^{\ zi} \times \theta_{COV}^{\ z2i} \quad (2)$$

$$\theta_{xi} = [\theta_x \times (COV_i/COV_{mean})^{\theta COV}] * \exp(\eta_{xi}) \quad (3)$$

where θ_{xi} is PK parameter 'x' in the i^{th} individual and θ_x is the population parameter estimate as previously; in equation 2 (dichotomous and/or trichotomous covariates) θ_{COV} is the ratio value for the typical value of parameter '*x*' in individuals according to their dichotomous classification Z_i or Z_{2i} which can both be equal to 0 or 1. In equation 3 (for continuous covariates), COV_i is the value of the covariate for the i^{th} individual, COV_{mean} is the mean value in the population dataset, and θ_{COV} is the exponent describing the covariate effect. For weight as a covariate, an allometric model (Anderson and Holford 2008) was applied to standardise the CL and V PK parameters using a standard weight (WT_{std}) of 70 kg in equation 3 above instead of the mean for the dataset, and fixing the exponent to 0.75 for CL and 1 for V.

Age, weight and gender were patient variables that were explored as covariates on the CL/F structural parameter of the model. Plots of the post hoc individual subject estimates of CL (from the base model fitting) vs weight and CL (from the base model model fitting) vs subject age are given in Figure 4.1. A box and whisker plot of the individual subject estimates of CL categorised according to gender is given in Figure 4.2. The lack of an obvious trend in the plot of individual CL estimates against

weight, age or gender was confirmed by non-significant drops in OFV when these covariates were incorporated into the population PK model (Table 4.5).

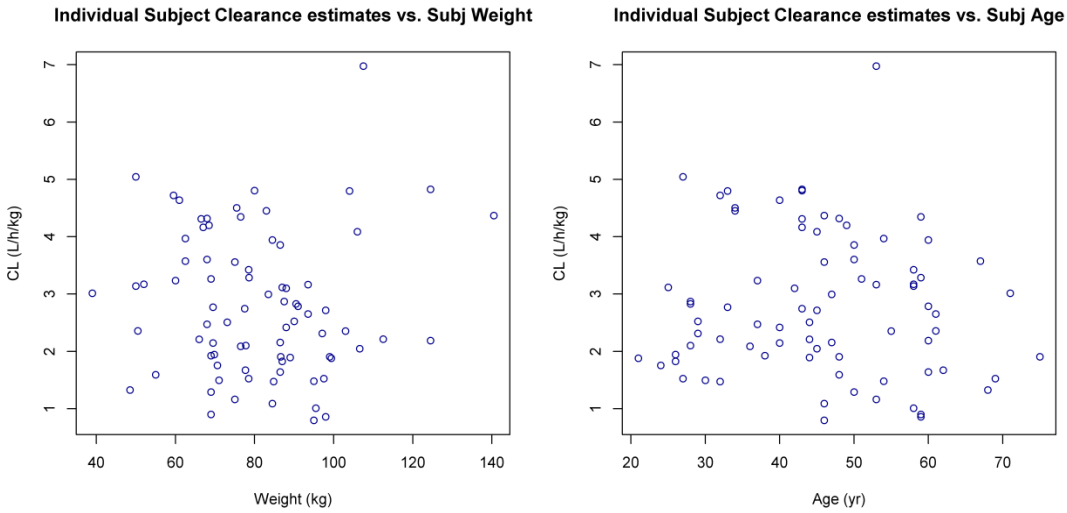


Figure 4.1 Individual subject estimates of CL vs. individual patient weights and individual subject estimates of CL vs.individual patient ages

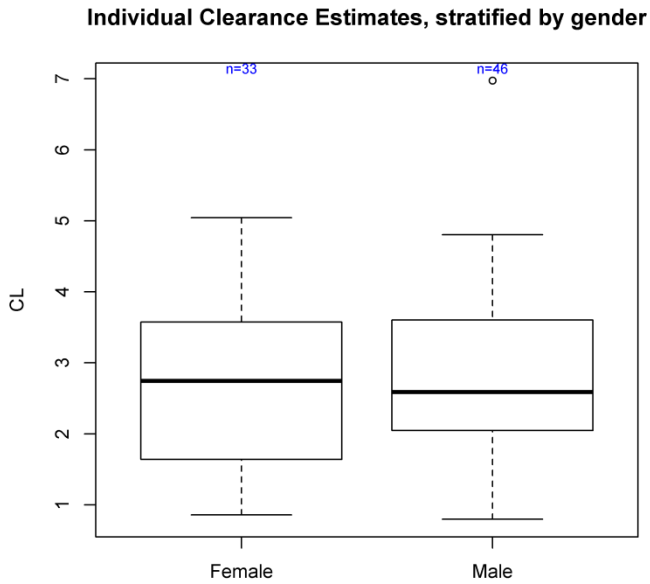


Figure 4.2 Box and whisker plot of individual subject estimates of CL from the base model fitting, categorised by gender

Table 4.5 Effect of covariates on objective function value in univariate analysis

Covariate			ΔOFV
Total CBZ daily dose (mg)			-53.974
Study group (PICME I, PICME II: autoinduction, or PICME II: maintenance)			-6.953
Occasion			-84.933
Age			0.01
Gender			26.901
Weight (kg)			-6.42
Smoker			-1.281
Concomitant therapy with:			
Sodium valproate			-0.143
Phenytoin			-10.399
Phenobarbital			-1.671
Clobazam			-3.126
Lamotrigine			-0.022
Levetiracetam			-2.967
Zonisamide			-0.231
Topiramate			-1.99
Lacosamide			-0.105
Pregabalin			-0.945
Perampanel			-0.219
Omeprazole			-0.08
Statin			-1.323
Single Nucleotide Polymorphisms			
SNP ID	**Gene**	**Allele**	
rs1045642	*ABCB1*	3435C>T	-1.301
rs1128503	*ABCB1*	1236T>C	-1.65
rs2032582	*ABCB1*	2677G>T	-1.856
rs2273697	*ABCC2*	1249G>A	-0.499
rs3740066	*ABCC2*	3972C>T	-2.906
rs717620	*ABCC2*	-24C>T	-2.011
rs28365062	*UGT2B7*	735A>G	-0.085
SNP ID	**Gene**	**Allele**	**ΔOFV**

rs28365063	*UGT2B7*	372A>G	-0.843
rs7438135	*UGT2B7*	-900G>A	-5.086
rs1051740	*EPHX1*	337T>C	-2.949
rs2234922	*EPHX1*	416A>G	-3.272
rs11572080	*CYP2C8*	*3	-6.756
rs1058930	*CYP2C8*	*4	-4.842
rs4244285	*CYP2C19*	*2	-1.977
rs3745274	*CYP2B6*	*6	-0.729
rs2242480	*CYP3A4*	*1G	-0.108
rs776746	*CYP3A5*	*3	-1.962
rs2333227	*MPO*	-643G>A	-0.486
rs1057868	*POR*	*28	-3.512

Concomitant medications, both AEDs and other medications, were also explored as covariates in the model. Representative box and whisker plots of individual subject estimates of CL categorised according to statin therapy and phenytoin are presented in Figure 4.3. Apart from phenytoin, no other concomitant medications were significant covariates. Multiple SNPs in CBZ metabolism enzymes were also explored in the PK model. None of the SNPs were found to significantly reduce the OFV of the population PK model. Figure 4.4 is a depiction of representative box and whisker plots comparing individual subject estimates of CL to SNPs in 4 genes (wild type, heterozygotes and homozygotes): *ABCC2* (3972C>T), *UGT2B7* (-900G>A), *EPHX1* (337T>C) and *CYP2C8*3*. The effect of mutants (combining heterozygotes and homozygotes) and wild-types on individual subject estimates of CL was also investigated for the SNPs, but none were found to significantly reduce OFV (Figure 4.5).

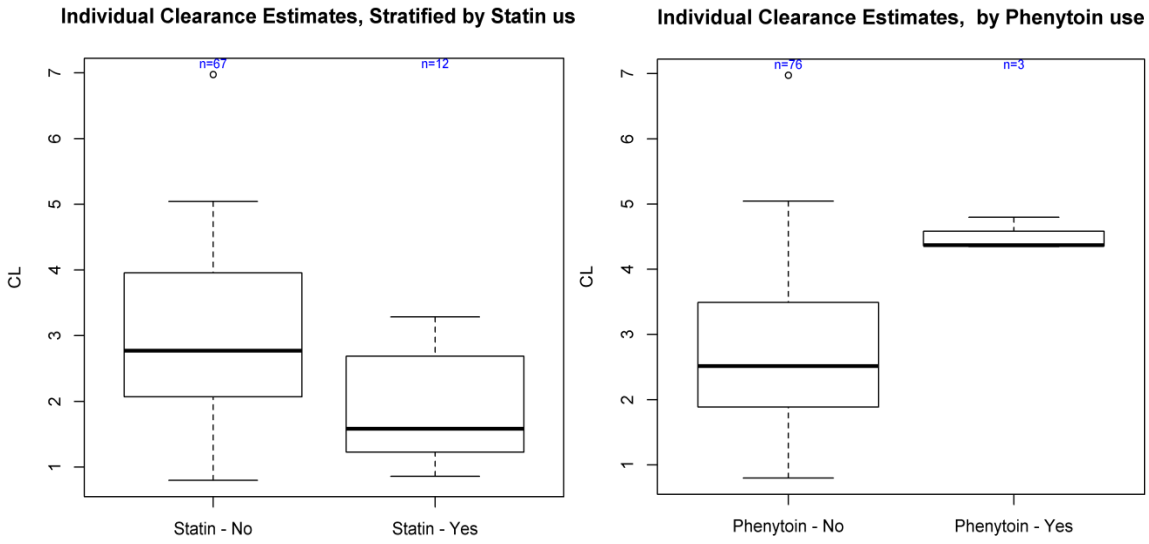


Figure 4.3 Box and whisker plots of individual subject estimates of CL from base model fitting, categorised by statin or phenytoin therapy

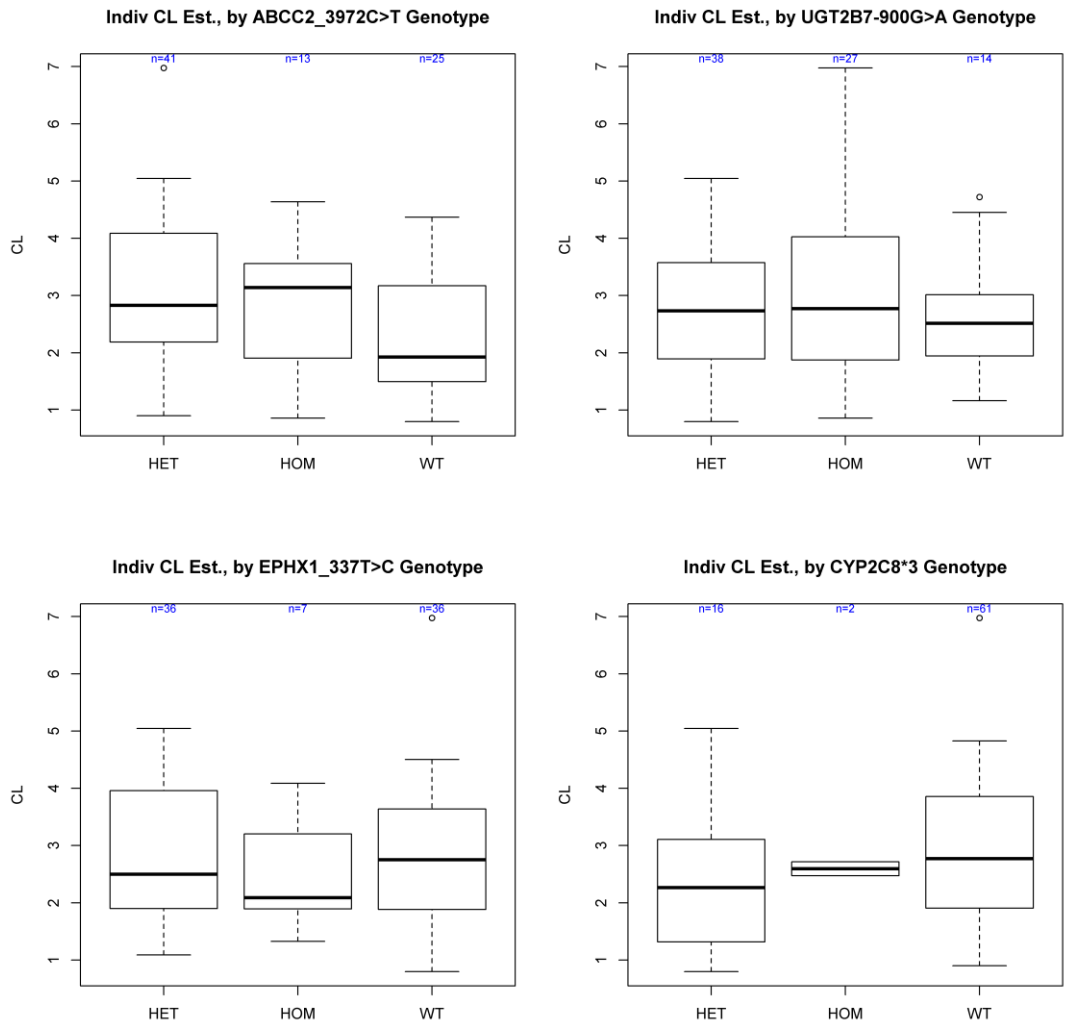


Figure 4.4 Box and whisker plots of individual subject estimates of CL from the base model fitting, characterised by genotype for *ABCC2* (3972C>T), *UGT2B7* (-900G>A), *EPHX1* (337T>C) and *CYP2C8*3.* (Het: heterozygote, HOM: homozygote, WT: wild type)

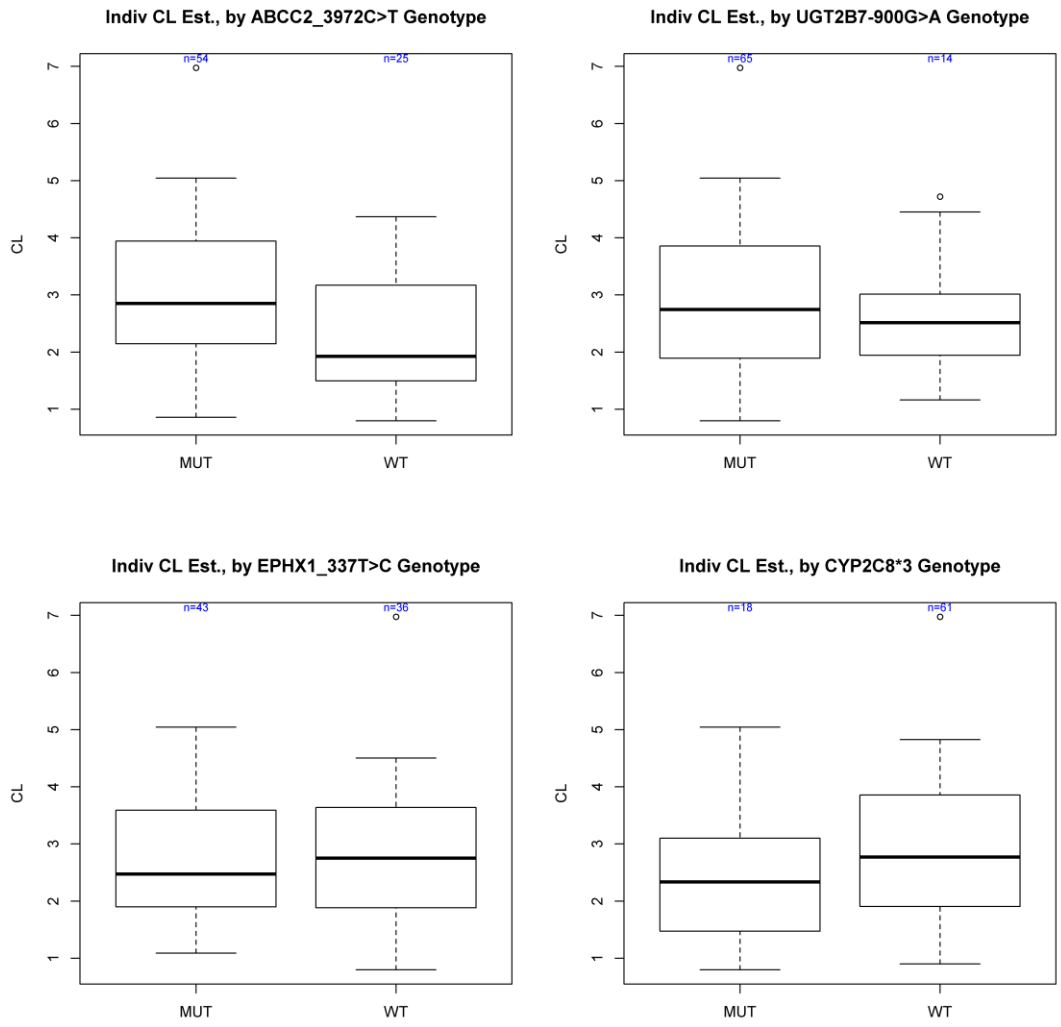


Figure 4.5 Box and whisker plots of individual subject estimates of CL from the base model fitting, characterised by genotype for *ABCC2* (3972C>T), *UGT2B7* (-900G>A), *EPHX1* (337T>C) and *CYP2C8*3.* [MUT: mutant (homozygote and heterozygote), WT: wild type]

The largest significant drop in OFV was seen with the occasion covariate (-84.9). Occasion was used to describe completion of autoinduction in the final visit for the three subjects in the PICME II: autoinduction group. Visual inspection of the observed vs. predicted concentration demonstrated that the base model over-predicted the final visit. At the final visit for these subjects, autoinduction was completed as the dose of CBZ had been stable for two weeks. Addition of the occasion variable significantly improved the prediction of the model and this was confirmed by significant reduction in OFV. A box and whisker plot comparing the individual subject estimates of CL to occasion is given in Figure 4.6. Concomitant therapy with phenytoin and total daily dose of CBZ were the other covariates that resulted in a significant drop in OFV (Table 4.5). A box and whisker plot comparing the individual subject estimates of clearance to the total daily dose of CBZ is given in Figure 4.7.

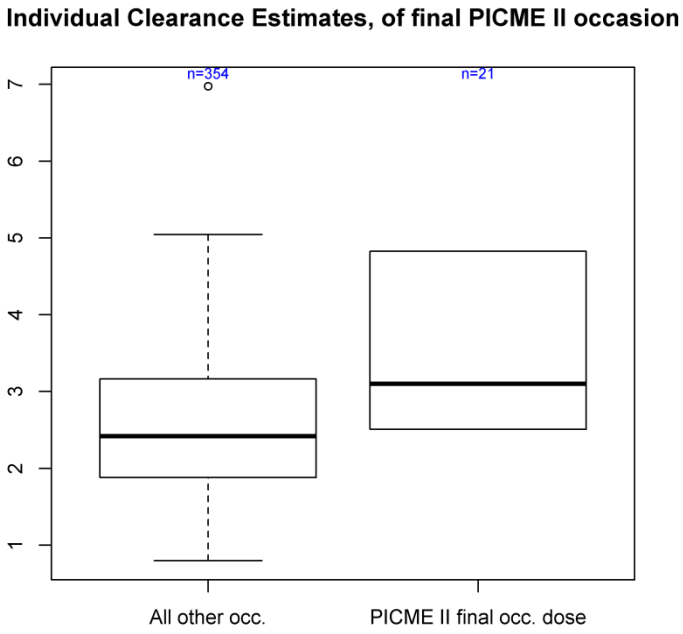


Figure 4.6 Individual subject estimates of CL vs. final dose occasion in subjects newly started on CBZ therapy (indicative of completion of autoinduction)

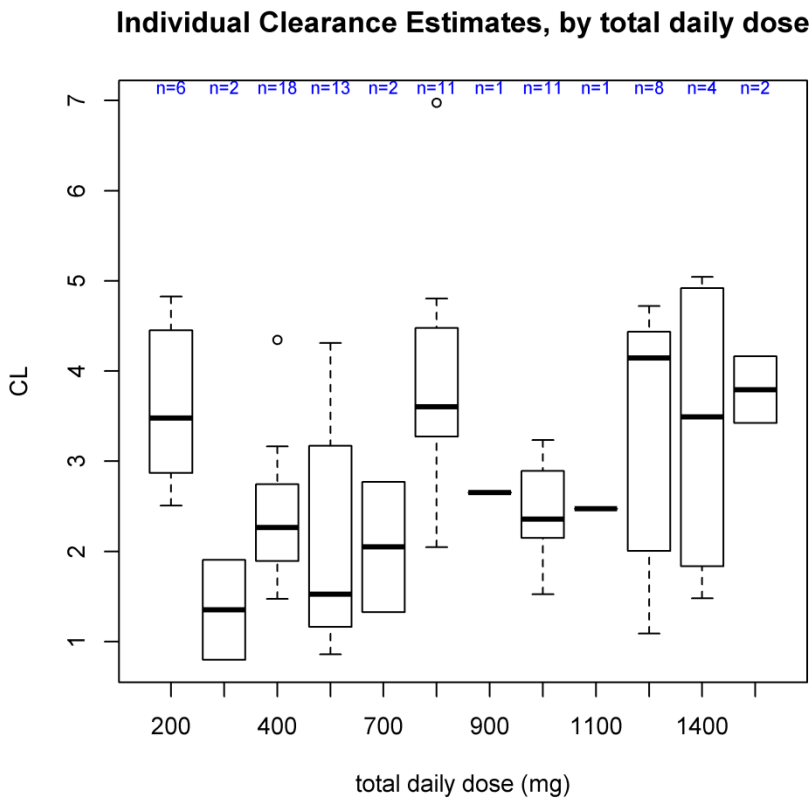


Figure 4.7 Box and whisker plots for individual subject estimates of CL vs. total daily dose of CBZ

A summary of the final model selection is illustrated in Table 4.6. Parameter estimates from the final model fitting with the covariate effect are given in Table 4.7. Prediction corrected VPCs demonstrating the goodness of fit for each of the study groups are given in Figure 4.8, Figure 4.9 and Figure 4.10. Figure 4.11 represents the VPC for the final PK model fitting for all patient groups combined. Plot of observed vs. population predicted concentrations in Figure 4.12 and with observed vs. individual predicted concentrations in Figure 4.13. The population PK model provides a satisfactory description of the PK profile data and its variability.

The final model equation:

$$CL_i = (\theta_{CL} \times \theta_{occ} \times \theta_{PHT} \times Dose^{\theta TDD}) \times \exp(\eta_{xi}) \quad (4)$$

where θ_{CL} is the fixed effect coefficient for population mean clearance, θ_{occ} is the fractional effect of full autoinduction at the end of dose titration for subjects newly started on CBZ therapy, θ_{PHT} is the fractional effect if the subject is taking phenytoin and θ_{TDD} is the exponent effect of the total daily dose.

Table 4.6 Summary of final model selection

Model	ΔOFV	CL/F ($L\ h^{-1}$)	V/F (L)	k_a (h^{-1})	ε
Base model	0	2.47	132	0.579	0.0354
Occasion (completion of autoinduction)	-84.933	2.60	127	1.75	0.0195
Concomitant phenytoin	-93.068	2.52	127	1.76	0.0194
Total CBZ daily dose	-99.695	0.969	126	0.899	0.0162

ΔOFV: change in objective function value, ε = proportional residual error variance, CL/F: apparent clearance, IIV: interindividual variability, k_a: absorption constant, OFV: objective function value, RSE: residual standard error, V/F: apparent volume of distribution.

Table 4.7 Parameter estimates and standard errors for the final model fitting incorporating occasion, concomitant phenytoin therapy and total CBZ daily dose covariates

	θ_{CL}	V/F (L)	k_a (h^{-1})	θ_{OCC}	θ_{PHT}	θ_{TDD}	IIV CL/F (%)	IIV V/F (%)	IIV k_a (%)	ε	OFV
Est	0.969	126	0.899	1.57	2	0.15	50.5	11.5	123	18	155.99
%RSE	86%	5%	29%	18%	23%	91%	17%	54%	56%	23%	

ε = proportional residual error variance, CL: clearance, Est: estimates, IIV: interindividual variability, k_a**:** absorption constant**,** OFV: objective function value, RSE: residual standard error, V/F: apparent volume of distribution.

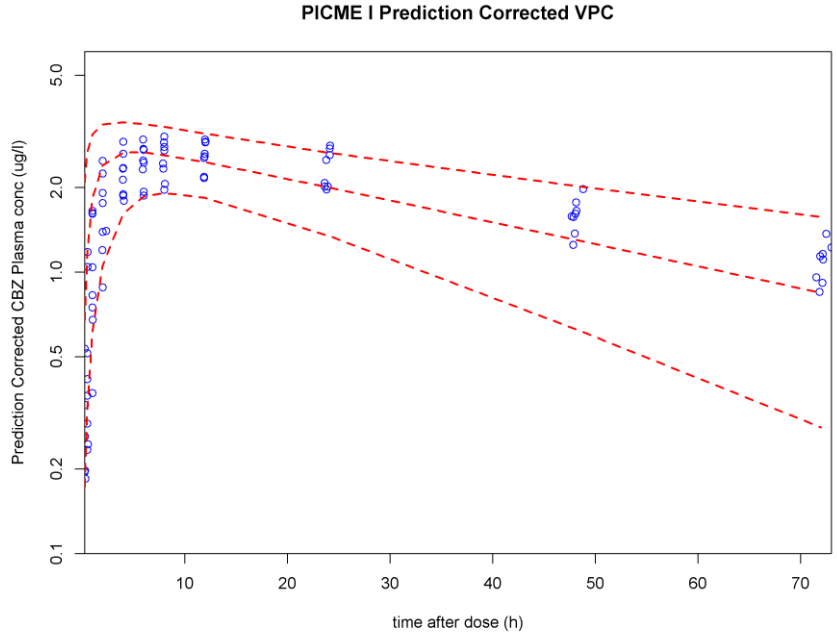


Figure 4.8 Visual predictive check (VPC) for the final pharmacokinetic model fitting in the healthy volunteer subjects (PICME I). The red lines represent a 90% prediction interval (5th, 50th and 95th percentiles) for the fitted dataset based on 1000 simulations of the dataset using the final model parameter estimates and the dataset covariates.

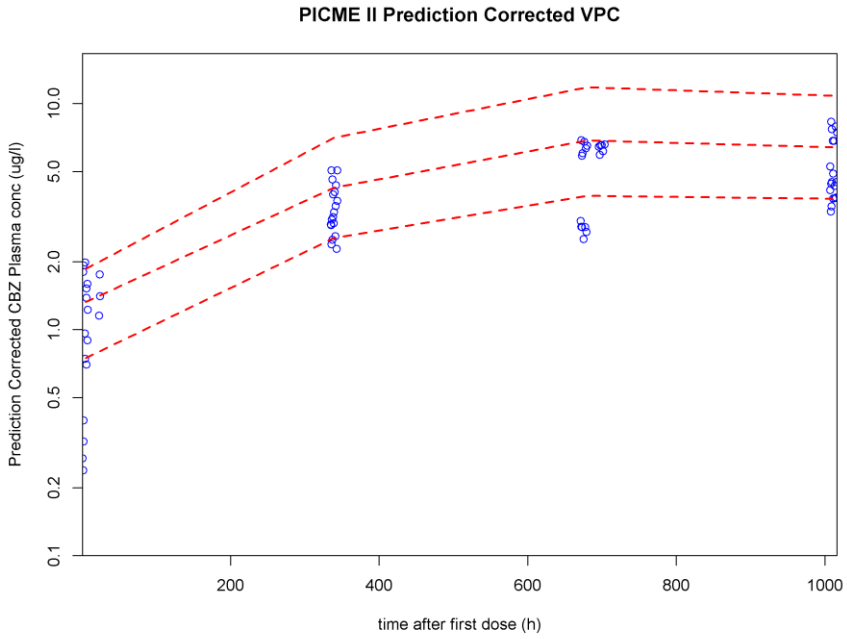


Figure 4.9 Visual predictive check (VPC) for the final pharmacokinetic model fitting in the newly diagnosed epilepsy patients (PICME II). The red lines represent a 90% prediction interval (5^{th}, 50^{th} and 95^{th} percentiles) for the fitted dataset based on 1000 simulations of the dataset using the final model parameter estimates and the dataset covariates.

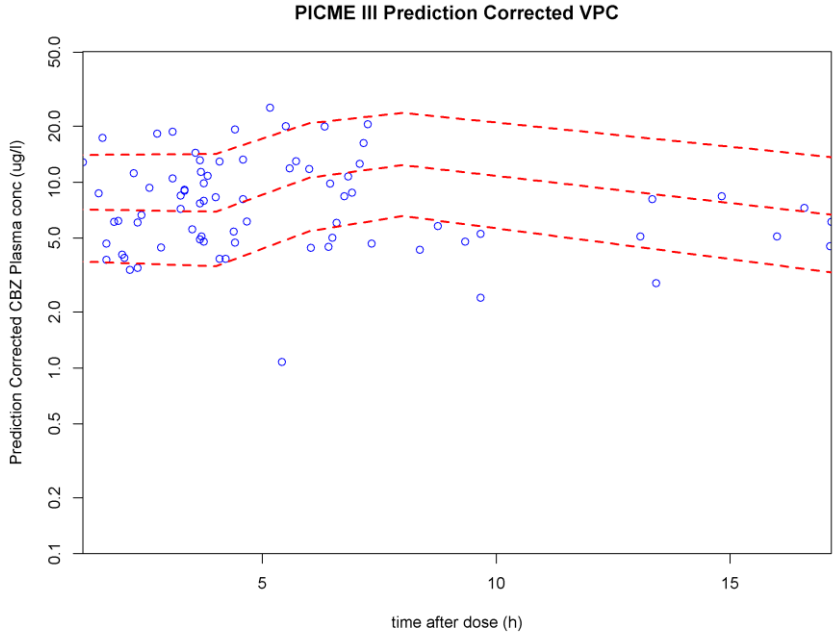


Figure 4.10 Visual predictive check (VPC) for the final pharmacokinetic model fitting in epilepsy patients prescribed a stable dose of CBZ (PICME III). The red lines represent a 90% prediction interval (5th, 50th and 95th percentiles) for the fitted dataset based on 1000 simulations of the dataset using the final model parameter estimates and the dataset covariates.

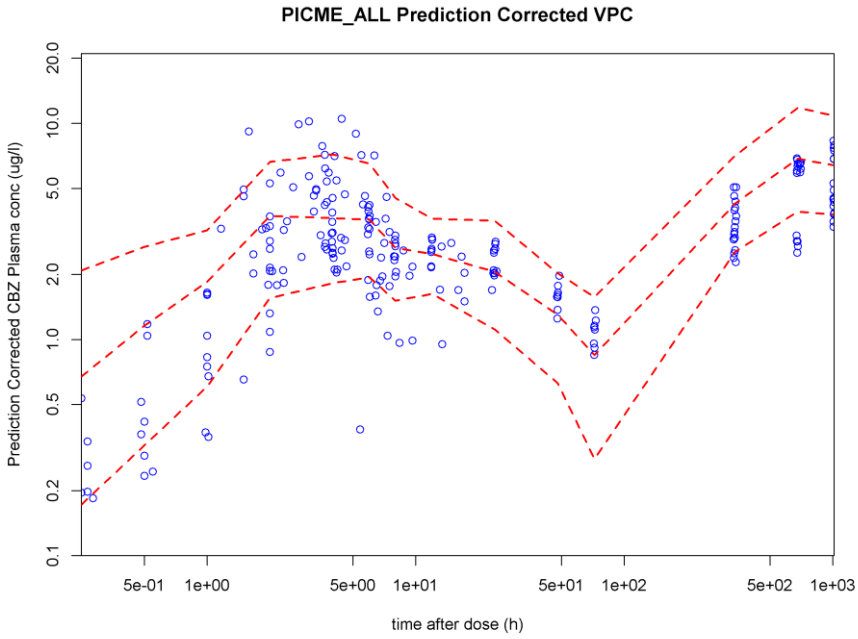


Figure 4.11 Visual predictive check (VPC) for the final pharmacokinetic model fitting for all patient groups. The red lines represent a 90% prediction interval (5th, 50th and 95th percentiles) for the fitted dataset based on 1000 simulations of the dataset using the final model parameter estimates and the dataset covariates. Note both the time and plasma concentration are in logarithmic scale.

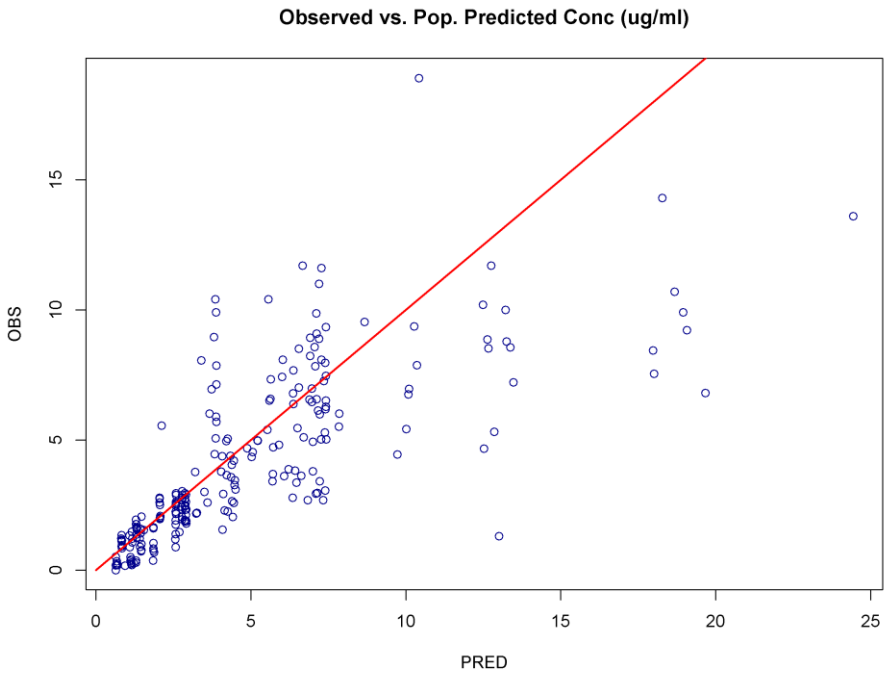


Figure 4.12 Observed vs population predicted CBZ plasma concentrations for final covariate model fitting to plasma exposure data.

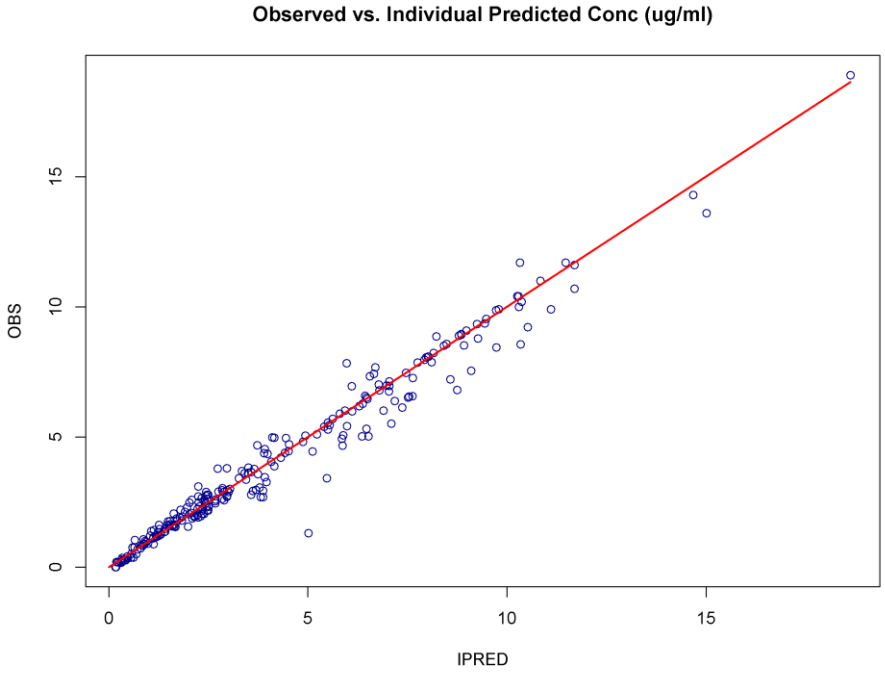


Figure 4.13 Observed vs individual predicted CBZ plasma concentrations for final covariate model fitting to plasma exposure data.

4.3.4 Incorporation of Multiple CBZ Metabolites into Population PK Model

It was originally intended for the population PK model to include the major metabolites of CBZ: CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ (Figure 4.14). This model was identifiable, parameterised successfully and able to generate initial estimates. Details of the model differential equations are included in the appendix (Figure 9.1), but briefly, to constrain the model to make the parameters identifiable in a fitting exercise, the model was parameterised with a total clearance for CBZ with the rates of formation of the 3 primary metabolites being fractions of this total value (FM1, FM2, FM3 for CBZE, CBZ-2OH and CBZ-3OH respectively). In addition, only 3 volumes of distribution (Vd) were estimated: one for parent CBZ, one for CBZ-DiOH and a third which was used for each of CBZE, CBZ-2OH and CBZ-3OH - i.e. the Vd of these 3 metabolites was fixed to the same value; this was required to make the parameterisation identifiable and was also plausible given the physicochemical properties of each of the analytes. However, when the metabolites were incorporated with parent in a single analytical run, the description for oral absorption of CBZ was lost.

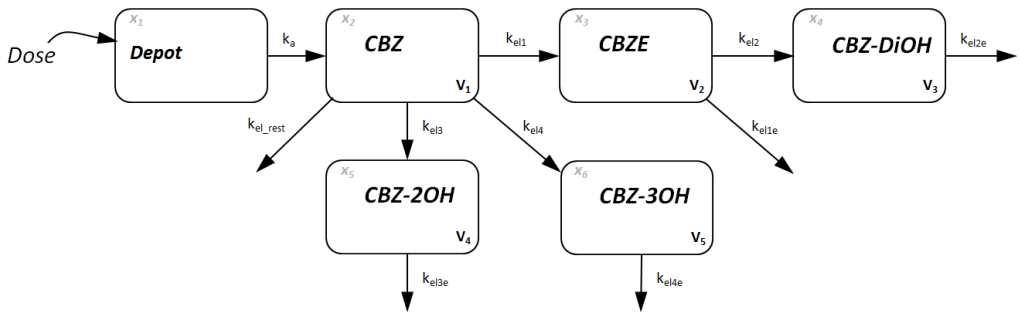


Figure 4.14 Proposed semi-mechanistic CBZ metabolite model

Various efforts were made to improve the model and address the issue of description of the absorption phase of parent CBZ. Principally these included implementations of various structural model alternatives to a first order absorption model for the description of oral absorption including:

- a zero order absorption model (where the oral absorption is modelled as if it were an infusion but where the duration of infusion is estimated during the fitting process).
- a transit compartment model (Savic et al. 2007) where the number of transit compartments was both estimated and fixed to n = 2, 3, and 4.
- a simultaneous zero order and first order absorption model, which had been used for CBZ PK modelling in the past (Riad et al. 1986).
- a sequential zero order and first order absorption model (where the lag-time for the 1st order absorption (i.e. when the 1st order phase begins) was set to equal the duration of the zero order phase (i.e. when the infusion ends), the value of which was estimated as a parameter in the fitting.

Attempts were also made where the model was simplified by one analyte at a time to potentially narrow down if the loss of description of the absorption of CBZ was due to the data of any one metabolite component in particular. Unfortunately, all versions of the model including ones with only one metabolite being directly produced were still unable to describe the profile of parent CBZ as successfully as a 1-compartment 1st order model was able to describe CBZ modelled alone.

Also attempted was a full re-parameterisation of the model with the CBZ-DiOH metabolite removed according to the method set out by Bazzoli et al where the ratios of elimination rate constant parameters to volumes of distribution are estimated instead of the parameters directly themselves (Bazzoli et al. 2011).

4.3.5 Dose-adjusted CBZE plasma concentrations are associated with SNPs in ABCB1 and EPHX

Dose-adjusted plasma levels of CBZE were found to be significantly associated with two SNPs in the gene *ABCB1* (c.1236T>C and c.2677G>T) and a single SNP in the gene *EPHX1* (c.416A>G) (p = 0.0019). Plasma concentrations of CBZE were lower in subjects that were heterozygotes or homozygotes for *ABCB1* c.1236T>C (p = 0.0055). Homozygous mutants for *ABCB1* c.2677G>T were found to have higher plasma concentrations of dose-adjusted CBZE. Finally, homozygous mutants for *EPHX1* c.416A>G were found to have significantly higher dose-adjusted plasma concentrations of CBZE compared with wild-type and homozygotes (p = 0.003) (Figure 4.15). No other SNPs or dose-adjusted metabolites for CBZ demonstrated significant associations.

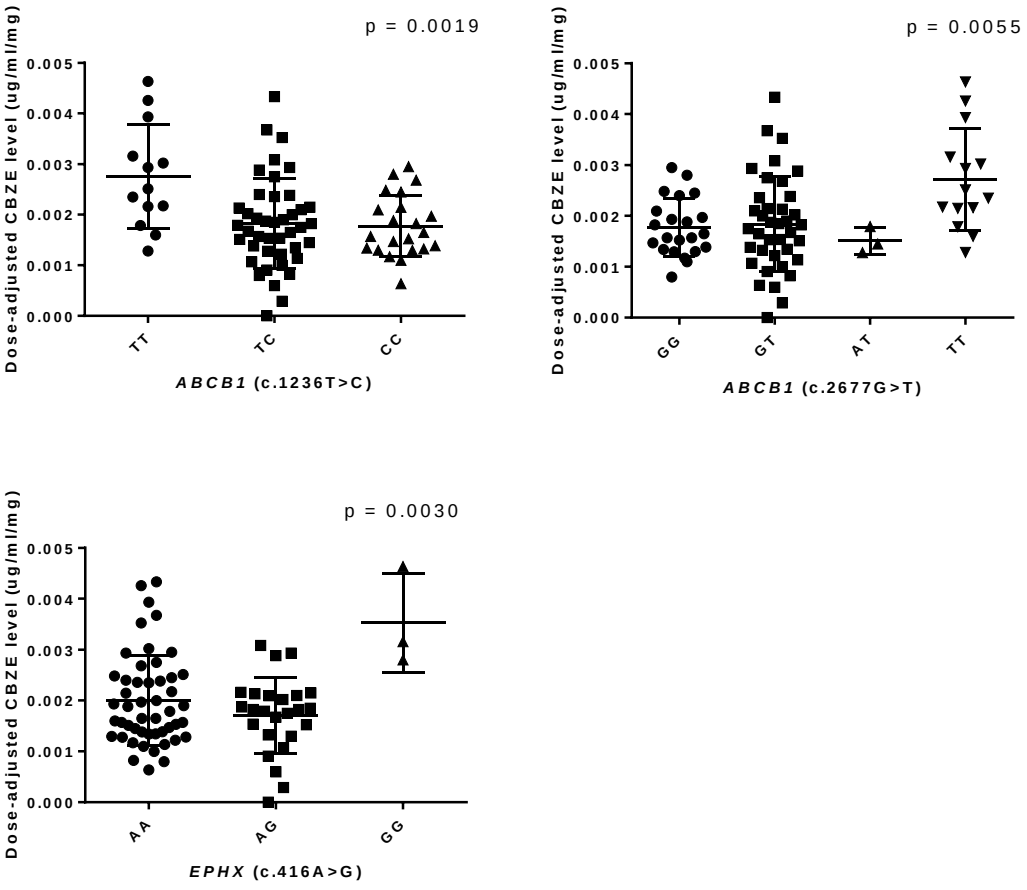


Figure 4.15 Significant associations between SNPs in *ABCB1* and *EPHX1* with dose-adjusted plasma levels of CBZE by ANOVA (data plotted are mean and standard deviation)

4.4 Discussion

A population PK model was developed in order to investigate the role SNPs in genes involved in the metabolism of CBZ have on the plasma exposure and clearance of CBZ in healthy volunteers and epilepsy patients. In addition, the influence of different demographic and clinical characteristics of the patients on CBZ clearance was also investigated. The model revealed that completion of autoinduction, concomitant therapy with phenytoin and the total daily dose of CBZ were significant factors that affect the clearance of CBZ. Despite investigating 19 SNPs from genes suspected to be involved in metabolism of CBZ, there was no demonstrable effect of these on CBZ PK.

CBZ is known to induce its own metabolism (autoinduction) through activation of nuclear hormone receptors (Saruwatari et al. 2014) and induction of a broad spectrum of genes involved with drug metabolism and drug transport (Oscarson et al. 2006). Autoinduction starts within 24 h after starting therapy or increasing the dose and the process is typically complete in one week (Kudriakova et al. 1992). The CBZ population PK model that has been developed is consistent with these findings. The clearance of CBZ in all three subjects that had been newly started on treatment stabilised once the total daily dose of CBZ was fixed at 600mg for 2 weeks.

Concomitant medication with phenytoin significantly increased clearance of CBZ in the population PK model. The influence of phenytoin therapy on CBZ metabolism has been recognised in several other population PK models previously (Graves et al. 1998; Gray et al. 1998; Jiao et al. 2003; Punyawudho et al. 2012). Phenytoin increases metabolism of many drugs including immunosuppressants (D'Souza et al. 1988), chemotherapeutic agents (Pursche et al. 2008) and antiretroviral drugs (Lim et al. 2004) through induction of multiple CYP isoforms and upregulation of P-gp (Brodie et al. 2013). Patients who are co-prescribed CBZ with phenytoin are likely to require a larger dose of CBZ to maintain plasma levels and caution should be taken if discontinuing phenytoin therapy as this may lead to toxicity with CBZ as the inductive effect of phenytoin is removed.

Total daily dose of CBZ was the final covariate that was found to be significant in the CBZ population PK model. All subjects in this study were prescribed controlled release formulations of CBZ. There was a positive correlation between total daily dose of CBZ and clearance of CBZ. The effect of dose on clearance of CBZ may be explained by a reduction in the bioavailability of CBZ and increase of its clearance through greater autoinduction at higher doses (Kudriakova et al. 1992). Several other population PK models of CBZ have reported that the total daily dose is a significant covariate in the PK of CBZ (Delgado Iribarnegaray et al. 1997; Reith et al. 2001; Vucicevic et al. 2007; Milovanovic and Jankovic 2011; Djordjevic et al. 2016).

Concomitant therapy with sodium valproate (Gray et al. 1998; Jiao et al. 2003; Vucicevic et al. 2007; Jankovic et al. 2008), phenobarbital (Delgado Iribarnegaray et al. 1997; Graves et al. 1998; Gray et al. 1998; Chan et al. 2001; Jiao et al. 2003; Vucicevic et al. 2007) and felbamate (Graves et al. 1998) had previously been reported to be significant covariates in population PK models of CBZ. None of these AEDs were significant in the current model and this is most likely secondary to the small numbers of subjects in the study receiving these particular AEDs. Other AEDs that were investigated using the current population PK model included clobazam, lamotrigine, levetiracetam, zonisamide, topiramate, lacosamide, pregabalin and perampanel but none were found to significantly affect the PK of CBZ. Apart from levetiracetam (n=26) and clobazam (n=18) the other AEDs were prescribed to fewer than 5 patients in the study population making any significant effects on CBZ PK very difficult to detect.

Two other concomitant medications that were not AEDs were also investigated as covariates in the population PK model: omeprazole (n=6) and statin therapy (n=13). Omeprazole is a proton pump inhibitor that is subject to drug-drug interactions secondary to inhibition of CYP2C19 and CYP3A4 (Shirasaka et al. 2013). Statins are plasma lipid lowering drugs that act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Brown and Goldstein 1986). They have been reported to inhibit CYP enzymes (Transon et al. 1996) and act as substrates of the organic anion transporters OATP1B1 and OATP1B3 and P-gp (Lennernas and Fager

1997). These characteristics mean that omeprazole and statins could interact with the metabolism of CBZ but this was not identified in the current population PK model.

Age and total body weight have previously been reported to be significant covariates (Delgado Iribarnegaray et al. 1997; Graves et al. 1998; Chan et al. 2001; Jiao et al. 2003; Perumandla et al. 2005; Jankovic et al. 2008; Milovanovic and Jankovic 2011; Djordjevic et al. 2016) but neither of these patient factors was identified as significant in the current model. Clearance of CBZ can be affected by extremes of age, with increased clearance in the paediatric population (Delgado Iribarnegaray et al. 1997) and reduced clearance in the elderly (Jiao et al. 2003) when compared with adults. The effect of total body weight on clearance of CBZ is significant in studies where a significant proportion of the study subjects include paediatric patients and it has been postulated that in children, total body weight more accurately represents physiologic conditions and metabolic capacity compared with age (Jiao et al. 2003). The current study population did not include any paediatric subjects and only 6 patients were over the age of 65 meaning it is unlikely to have the power to detect any significant effects on the clearance of CBZ exerted by age and total body weight.

Two SNPs in the drug transporter gene *ABCB1*, c.1236T>C and c.2677G>T, were significantly associated with dose-adjusted plasma concentrations of CBZE by ANOVA. Up to one third of patients with epilepsy do not respond to AED therapy and the transporter hypothesis proposes that over-expression of efflux transporters such as ABCB1, in the blood-brain barrier limits the access of AEDs to the epileptic focus (Aronica et al. 2012). In this study, subjects with the genotype 1236TT (wild-type) and 2677TT (homozygous mutants) were found to have higher dose-adjusted plasma concentrations of CBZE. Elevated concentrations of CBZE may lead to greater formation of the CBZE-modified HSA adduct identified in chapter 2, which could lead to increased susceptibility to CBZ hypersensitivity reactions. There remains significant controversy regarding the influence of genetic variation on metabolism of CBZ with conflicting results from many studies (see Table 1.4). For

example, CYP3A4 is the major CYP isoform responsible for metabolism of CBZ and the allele *CYP3A4*1G* has been associated with lower serum levels of CBZ and CBZE in Chinese patients with epilepsy implying that the SNP was associated with increased function (Zhu et al. 2014). However, three other studies were not able to replicate this association (Yun et al. 2013; Ma et al. 2015; Wang et al. 2015). Similarly, the SNP c.337T>C in the gene *EPHX1*, which encodes for mEH responsible for metabolism of CBZE to DiOH-CBZ (Thorn et al. 2011), has been associated with increased levels of CBZE in one study (Ma et al. 2015) but no differences were detected in the current study or studies by others (Yun et al. 2013; Caruso et al. 2014; Zhu et al. 2014). Subjects that were homozygous for the SNP c.416A>G in *EPHX1* were found to have higher dose-adjusted CBZE levels. This result is consistent with the observation that *EPHX1* c.416A>G has reduced metabolic activity (Nakajima et al. 2005). Elevated levels of plasma CBZE may predispose subjects to greater formation of the CBZE-modified HSA adduct identified previously.

Limitations of the current study include the relatively small number of subjects, absence of paediatric patients and limited number of elderly patients compared with other population PK studies meaning that it may not have had sufficient power to detect significant covariates with small effects or covariates affecting extremes of age only. *CYP1A2* (c.-163C>A) genotype has been shown to affect CBZ PK in children (Djordjevic et al. 2016) but was not investigated as part of the current study as CYP1A2 had not previously been associated with metabolism of CBZ. In future, it would be worthwhile using stored DNA samples to genotype subjects in the study for polymorphisms in CYP1A2. It was originally intended for the population PK model to explore the metabolism of CBZ including its metabolites CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ. The metabolite model was not able to describe the entire dataset accurately (performance was adequate for the metabolites themselves, but the description of the parent CBZ was poor) and due to its complexity (a 6-state system of ordinary differential equations whose numerical solution for fitting is not a parallelisable computer task) led to impractical extended runtimes (>48h) during parameter estimation, particularly when the full dataset was used and random

effects parameters to account for inter-individual variability were being included. For these reasons, work on the metabolite data remains incomplete for the purposes of this thesis; however it is the intention of the authors to continue exploring this model in the future.

In conclusion, a population PK model has been developed for CBZ with the completion of autoinduction, concomitant therapy with phenytoin and total daily dose of CBZ as significant covariates which influence PK of CBZ in an adult epilepsy population from the UK.

Chapter 5

Characterisation and Activation of T Cell Clones from Patients with Carbamazepine Hypersensitivity

5.1 Introduction

DHRs are defined as adverse events that resemble clinical allergy with evidence of either drug-specific antibodies or T cells to an otherwise safe and effective therapeutic agent. They account for approximately 15% of all ADRs and can affect up to 7% of the general population (Demoly et al. 2014). Accurate diagnosis of DHR is important to ensure optimum treatment and prevention of future episodes but is very difficult in the clinical setting. Currently, assessment of causality in DHRs is based upon clinical history and examination of the relationship between drug intake, type of drug, time of onset of reaction and clinical presentation of DHR (Schrijvers et al. 2015). This approach usually leads to exclusion of multiple drug classes and switch towards suboptimal therapies. After recovery from the initial reaction definitive workup to identify the culprit drug, potential cross-reactive compounds and safe alternatives requires the use of *in vivo* tests such as skin testing and drug provocation tests (Demoly et al. 2014). However, *in vivo* tests are contraindicated in very severe reactions because of the risk for re-eliciting symptoms and it is not possible to test all chemical entities (e.g. drug metabolites) using these methods (Aberer et al. 2003). Therefore, there is a clinical need for an *in vitro* validated assay for diagnosis of DHRs.

The only common aspect for all delayed type DHRs is the activation of drug-specific T lymphocytes, which cannot be detected in drug-tolerant subjects (Naisbitt et al. 2014). This observation has led to the development of test systems for detection of these drug-specific T cells. The LTT is an *in vitro* assay that measures lymphocyte proliferation and is used for the detection of sensitised T cells to a specific drug, metabolite and/or drug-protein conjugate (if available) in order to diagnose T cell mediated DHRs (Earnshaw et al. 2014). The assay is based on incubation of fresh PBMCs from hypersensitive patients with titrated concentrations of the suspected culprit drug(s) and vehicle control for 6 days (Nyfeler and Pichler 1997). Comparison of proliferation between drug and control, assessed by incorporation of [^{3}H] thymidine, is determined by the stimulation index (SI) with SI ≥ 2 indicative of T cell sensitisation. The LTT has a reported specificity of at least 85% (Porebski et al. 2011) and sensitivity of 60-70% (Pichler and Tilch 2004). Limitations of the LTT include the

need for technical expertise, the prolonged duration of the assay, use of radioactive [^{3}H] thymidine and the inability of a negative LTT to conclusively exclude DHR. Despite these shortcomings the LTT has been successfully used to confirm CBZ-induced hypersensitivity reactions in patients (Naisbitt et al. 2003; Wu et al. 2006; Wu et al. 2007).

To determine the profile of drug-specific T cells, alternative techniques such as T cell cloning (Naisbitt 2004), cell surface marker detection using flow cytometry (Beeler et al. 2006) and measurement of cytokines using enzyme-linked immunosorbent spot (ELISpot) assay (Naisbitt et al. 2003) have been utilised. Generation of TCCs provides a platform in which the pathophysiology of the hypersensitivity reaction can be investigated with respect to the phenotype, specificity and functionality of T cells that have been isolated from patients with hypersensitivity (Pichler et al. 2002). Cytokine release from T cells act as effectors for the immune system and quantification of their secretion using ELISpot assay can provide important insights into the mechanisms of DHRs; there is also the added benefit of shorter incubation time (48-72 h) and the ability to detect much lower numbers of drug-specific T cells (1:8000 to 1:30000) (Rozieres et al. 2009) in comparison with the LTT (1:250 to 1:10000) (Beeler et al. 2006). Measurement of panels of cytokines, including interferon gamma (IFN-γ), interleukin (IL) -4, IL-5, IL-13, IL-17, IL-22, FasL, TNF-α, granzyme B and perforin have the potential to increase sensitivity of diagnostic assays and are currently being investigated (Naisbitt et al. 2014). Previous work with TCCs isolated from patients with CBZ hypersensitivity has identified multiple cytokine secretion patterns from T cells including IFN-γ, IL-4, IL-5 and IL-10 (Naisbitt et al. 2003).

CBZ undergoes extensive metabolism and multiple reactive metabolites have been postulated as the species responsible for triggering hypersensitivity reactions (Pearce et al. 2002; Pearce et al. 2005; Pearce et al. 2008). Due to the unstable nature of these reactive metabolites none have been tested using *in vitro* assays so their ability to activate T cells is not known. T cell responses have been detected against some stable CBZ metabolites including CBZE and 10-hydroxycarbamazepine

but not others (2OH-CBZ, 3OH-CBZ, DiOH-CBZ) (Wu et al. 2006). Activation of these T cells was through direct interaction with the MHC via a pathway consistent with PI mechanism of hypersensitivity. However, earlier in chapter 2 of this thesis we demonstrated that CBZE, the most abundant stable metabolite, is able to form protein conjugates with albumin (Figure 2.10). CBZE-modified HSA was also detected *in vivo* from the circulation of patients prescribed long-term CBZ therapy for epilepsy raising the possibility that CBZE could act as a hapten by modification of endogenous proteins that could then be detected by the immune system (Faulkner et al. 2014). Based on the multiple phenotypes of CBZ hypersensitivity including MPE, HSS, SJS and TEN it is possible that a combination of pathophysiological mechanisms are responsible for hypersensitivity.

In this chapter using the techniques described above, freshly isolated PBMCs were obtained from 12 patients with a known history of hypersensitivity to CBZ and incubated with different concentrations of CBZ and/or CBZE in the LTT to assess lymphocyte proliferation. TCCs were generated from two patients using CBZ and CBZE and cell surface markers characterised using flow cytometry. Cross-reactivity of TCCs to CBZ and CBZE, the role of antigen processing and MHC class in activation of TCCs and cytokine secretion profile were assessed using the ELISpot assay.

5.2 Methods

5.2.1 Chemicals and Reagents

CBZ (Sigma-Aldrich Co, UK) and CBZE (Sigma-Aldrich Co) were prepared as stock solutions (10 mg/mL) in T cell medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich Co). The stock solutions were made up fresh and diluted to the appropriate concentrations directly before use. Tetanus toxoid (TT) was obtained from the Statens Serum Institut in Denmark. Nitroso-sulphamethoxazole (SMX-NO) was purchased from Dalton chemical laboratories Inc. (Toronto, Canada). Tritiated [^{3}H]-methyl thymidine was purchased from Moravek (California, USA). ELISpot kits including antibodies (IFN-γ, IL-13, granzyme B and perforin) and substrate solution were purchased from Mabtech (Stockholm, Sweden). ELISpot plates were bought from Millipore Corporation (Millipore, Watford, UK).

5.2.2 Cell Culture Medium

T cell culture medium consisted of RPMI 1640 medium supplemented with 10% human pooled AB serum (Innovative Research, USA), 25 mM HEPES buffer, 2 mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 25 µg/mL transferrin. To maintain T cells in long term culture, the medium was supplemented with 200 U/mL IL-2 (Preprotech, UK). B-cell medium comprised of RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS; Invitrogen, UK), 25 mM HEPES buffer, 100 µg/mL streptomycin and 100 U/mL penicllin.

5.2.3 Isolation of Peripheral Blood Mononuclear Cells

PBMCs were isolated from the whole blood of patients with CBZ hypersensitivity by density gradient centrifugation. The blood was layered on top of an equal volume of Lymphoprep (Axis Shield, UK) and centrifuged at 400*g* for 25 min at room temperature. The layer of PBMCs was extracted carefully with a Pasteur pipette and washed twice in Hank's balanced salt solution (HBSS) to remove any excess Lymphoprep. Isolated PBMCs were re-suspended in T cell culture medium.

5.2.4 Lymphocyte Transformation Test

The LTT was performed on PBMCs using an established protocol (Nyfeler and Pichler 1997). Briefly, PBMCs (1.5 x 10^5; 100 µL) were cultured with either CBZ (12.5, 25 and 50 µg/mL; 100 µL) or CBZE (12.5, 25, 50 µg/mL; 100 µL) in a 96-well U bottom plate in triplicate for six days. TT (1 µg/mL) was used as a positive control and cell culture medium acted as a negative control. [^{3}H]-thymidine (Moravek, USA) was added for the final 16 h of incubation. Cells were harvested and proliferation determined by [^{3}H]-thymidine incorporation as cpm using a MicroBeta TriLux β-counter (Perkin Elmer, USA). Proliferative responses were calculated as SI = cpm in drug treated cultures/cpm in medium control. An SI ≥ 2 was considered a positive response.

5.2.5 Generation of EBV-Transformed B-Cells

Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines were used as antigen-presenting cells (APC). B-lymphoblastoid cell lines were generated by transforming PBMCs using supernatant from the EBV-producing cell line B9.58 (Neitzel 1986). PBMCs (5 x10^6) were resuspended with 5 mL of filtered (0.45 µM syringe filter) supernatant from the B95.8 cell line. Cyclosporin A (1 µg/mL) was added to inhibit T cell proliferation before the PBMCs were incubated overnight at 37^{o}C under an atmosphere of 95% air/5% CO_2. After incubation the cells were centrifuged at 400*g* for 5 min and resuspended in 2 ml B-cell medium that had been supplemented with cyclosporin A (1 µg/mL). The cells were cultured in a 24-well plate and the cyclosporin A supplemented B-cell medium was changed twice per week. Cyclosporin A treatment was stopped after 2 weeks in order to enhance the proliferation of B-cells. Cells were transferred to a tissue culture flask when confluent and maintained with fresh B-cell medium twice a week.

5.2.6 Generation of Drug-Specific T Cell Clones

PBMC (1 x 10^6, 1 mL) from CBZ hypersensitive patients were cultured in T cell medium containing either CBZ (25 µg/mL) or CBZE (25 µg/mL) for 14 days in a 48-well plate at 37^{o}C. Culture medium was supplemented with IL-2 (200 IU/mL) on days 5 and 9 to expand the number of antigen specific T cells. On day 14, T cell bulks

were separated into CD4+ and CD8+ T cells using magnetic sorting beads (Miltenyl Biotech, UK). T cells were incubated with CD8 beads and separated over a magnetic column in a syringe. The flow through contained CD4+ T cells and CD8+ T cells were collected by flushing the syringe using the plunger. CD4+ and CD8+ TCCs were cloned by serial dilution using an established method (Naisbitt et al. 2003). Briefly, cells were transferred into a 96-well U bottomed plates at concentrations of 0.3, 1.0 and 3.0 cells per well. Allogeneic irradiated PBMCs (5 x 10^5/well), phytohaemoagglutinin (PHA, 5 μg/mL) and IL-2 (200 U/mL) were added to stimulate T cell growth. T cell growth was assessed every other day and indicators of good growth included change in colour of the culture medium and increasing size of the cell pellet. Well growing clones were expanded and split into new plates when necessary.

Antigen specificity was assessed by culturing irradiated EBV-transformed B-cells (1 x 10^4/ well) and CBZ (12.5 μg/mL) or CBZE (12.5 μg/mL) with clones (5 x 10^4/well; 200 μL) for 48 h. Proliferation was measured by the addition of [^{3}H]-thymidine followed by scintillation counting. Clones with a stimulation index greater than 2 were further expanded by repetitive stimulation with irradiated allogeneic PBMCs (5 x 10^5/well), PHA (5 μg/mL) in IL-2 (5 μg/mL) containing medium.

5.2.7 T Cell Phenotyping Using Flow Cytometry

CD4 and CD8 cell surface expression of clones were determined by flow cytometry. Clones (50 μL) were stained with phycoerythrin (PE) labelled anti-CD4 antibodies (3 μL) and allophycocyanin (APC) labelled anti-CD8 antibodies (3 μL) for 20 min at 4^{o}C in the dark. After incubation the clones were washed and resuspended in HBSS containing 1% FBS and 0.02% sodium azide. Fluorescence was measured using a BD FACS Canto II flow cytometer (BD Biosciences) and data analysed using Cyflogic software (CyFlo Ltd, Finland).

5.2.8 Enzyme-Linked Immunospot Assay (ELISpot) for Measurement of Cytokine Secretion

Cytokine secretion (IFNγ, IL-13, granzyme B and perforin) was determined by ELISpot assay according to the manufacturer's protocol. Multiscreen HTS filter plates (Millipore, UK) were coated with the cytokine-specific capture antibody and incubated overnight at 4^{o}C. Following incubation the wells were washed five times using sterile phosphate buffer saline (PBS) and then blocked with T cell culture medium (200 µL) for 30 min at room temperature. Drug-specific TCCs (5 x 10^4; 50 µL), autologous EBV-transformed B-cells (1 x 10^4; 50 µL) and multiple concentrations of CBZ (5, 10, 20 µg/mL; 100 µL) or CBZE (5, 10, 20 µg/mL; 100 µL) were added to the wells and incubated for 48 h at 37^{o}C. The cells were discarded and the wells were washed five times with 200 µL PBS. Biotin-labelled detection antibody (1 µg/mL) was added to the wells and incubated at room temperature for 2 h. Wells were then washed again using PBS. Streptavidin-alkaline phosphatase (100 µL), diluted in PBS containing 0.5% FBS (1:1000), was added to each well and incubated for 1 h at room temperature. Wells were washed five further times using PBS (200 µL). Spots were developed by addition of BCIP/NBT substrate solution for approximately 10-15 min until distinct spots were visible. The development reaction was terminated by running the plate under tap water. Plates were left to air dry and the spots visualised and counted using an AID ELISpot reader (Cadama Medical, Stourbridge, UK).

5.2.9 Confirmation of Antigen Specificity and Concentration Response of TCCs

Antigen specificity and concentration response of TCCs were assessed by measuring IFN-γ secretion using the ELISpot assay. TCCs (5 x 10^4 cells/well; 50 µL) were cultured with irradiated EBV-transformed B-cells (1 x 10^4 cells; 50 µL) and one of CBZ (0, 5, 10, 20 µg/mL; 100 µL), CBZE (0, 5, 10, 20 µg/mL; µL) or SMX-NO (25 µM; 100 µL; SMX-NO) according to section 5.2.8. PHA (10 µg/mL) acted as positive controls for each experiment.

5.2.10 Antigen Pulsing Assays

The role of hapten formation and APC processing in the activation of TCCs was investigated by pulsing EBV-transformed B-cells (1 x 10^6 cells; 1mL) with CBZ (20 μg/mL) or CBZE (20 μg/mL) for 16 h at 37^0C before extensive washing with HBSS to exclude free drug. The pulsed EBV-transformed B-cells (1 x 10^4 cells/well; 50 μL) were then incubated with drug-specific TCCs (5 x10^4 cells/well; 50 μL) and T cell culture medium (100 μL). Activation of TCCs was measured by IFN-γ secretion using the ELISpot assay (section 5.2.8). Comparison of IFN-γ secretion was made with TCC incubations with EBV-transformed B-cells and freshly added CBZ or CBZE. Negative control incubations consisted of TCCs, EBV-transformed B-cells and T cell culture medium. Each condition was repeated in duplicate and mean IFN-γ secretion reported.

5.2.11 MHC Restriction Assay

To determine if presentation of CBZ or CBZE to drug-specific TCCs is MHC class I or class II restricted anti-human HLA-A, -B, -C (MHC I) and anti-human HLA-DP, -DQ, -DR (MHC II) antibodies (5 μg/mL) were pre-incubated with autologous EBV-transformed B-cell lines (1 x 10^4, 50 μL) for 30 mins at 37^oC. The APCs were then co-cultured with either CBZ- or CBZE-specific TCCs with or without CBZ/CBZE for 48 h. IFN-γ secretion determined by ELISpot assay was used as a measure of TCC activation according to section 5.2.8. Isotype antibody (5 μg/mL) incubations were used as controls in this experiment. Each experiment was repeated in duplicate and mean IFN-γ secretion reported.

5.2.12 Statistical Analysis

Mean values and standard deviations or standard error of the mean were calculated and statistical analysis was performed using paired T-test on the GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

5.3 Results

5.3.1 Patient Characteristics and Results of Lymphocyte Transformation Test

Twelve patients with a history of CBZ hypersensitivity were recruited and their PBMCs tested in the LTT. Their clinical characteristics and responses to the LTT are outlined in Table 5.1. The interval between onset of hypersensitivity reaction and *in vitro* testing using LTT ranged from one year to greater than 25 years. The time to reaction following initiation of CBZ ranged from one day to seven months. The clinical presentation of hypersensitivity was variable and ranged from MPE to descriptions that could be compatible with SJS and TEN. Unfortunately, due to the extensive time that had passed since the hypersensitivity reactions, some subjects were unable to recall their symptoms and the clinical records were poorly documented. Six patients (50%) demonstrated positive stimulation index (SI ≥ 2) on LTT to CBZ. The SI ranged from 3.0 to 16.4. Three subjects (50%) were positive to CBZE on LTT with SI of 2.6, 3.7 and 5.9. Two subjects were positive to both CBZ and CBZE and a single patient was positive to CBZE only. The concentration response relationships for subjects with positive LTT to CBZ and CBZE are illustrated in Figure 5.1 and Figure 5.2.

Table 5.1 Clinical Characteristics of Patients with CBZ Hypersensitivity (mth: months, NA: not applicable SI: stimulation index, unk: unknown)

Subject	Clinical Presentation	Time to Reaction	CBZ LTT (SI)	CBZE LTT (SI)
RLH001	1989 – unknown	21 d	+ve (3.6)	NA
RLH002	1991 – generalised rash, flu symptoms, mucosal inflamed	8 d	-ve	NA
RLH005	2013 – red blisters to palms, soles and mouth. Rash on arms and legs	4 d	+ve (3.0)	NA
RLH007	1989 – generalised erythematous rash	6 d	+ve (16.4)	+ve (3.7)
RLH010	2002 – ulceration of oral cavity and lips	Unk	+ve (3.2)	-ve
WAL003	2006 – generalised, itchy erythematous macules	1 mth	+ve (6.0)	+ve (2.6)
WAL004	2004 - generalised , itchy, scaly erythematous rash	7 mth	-ve	+ve (5.9)
WAL005	2012 – Generalised, itchy, flaking skin, lethargy, hair loss	6 d	-ve	-ve
WAL006	2008 – generalised papular erythema and fever	1 d	-ve	-ve
WAL007	1994 – generalised maculopapular exanthema	Unk	+ve (3.1)	NA
WAL008	2013 – generalised itchy maculopapular exanthema	35 d	-ve	NA
WAL009	2002 – Itchy erythema over arms and legs	7 d	-ve	NA

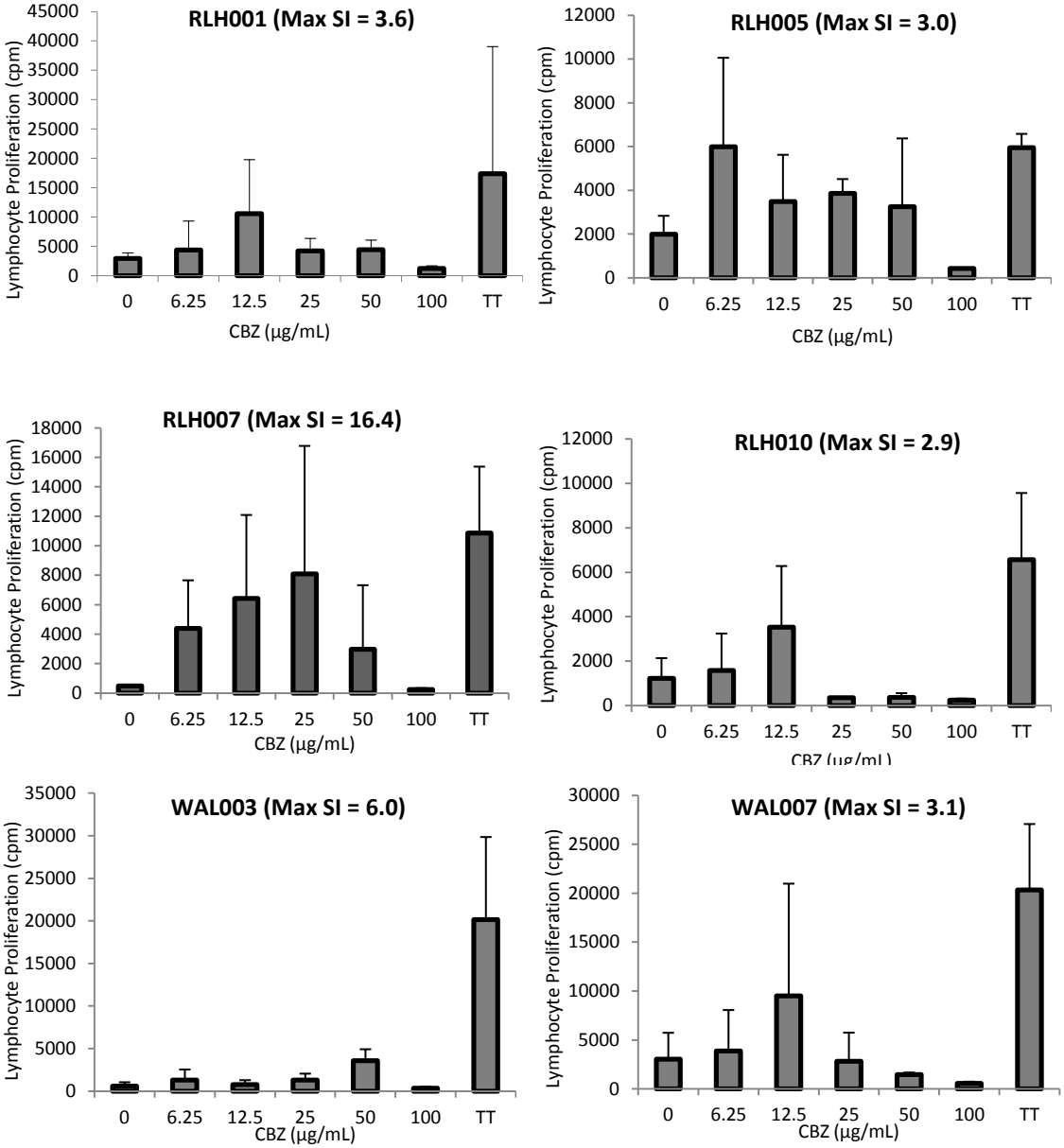


Figure 5.1 Positive LTT to CBZ in six patients. PBMCs (1.5 x 10^4 cells, 100µL) were incubated with graded concentrations of CBZ (6.25 - 100 µg/mL) in 96-well U bottomed plates. Plates were incubated at 37^{o}C for 5 days before addition of [^{3}H]-thymidine (0.5 µCi) and a further 16 h incubation. T cell proliferation was measured using scintillation counting. (Data represents mean proliferation and standard deviation of triplicate cultures).

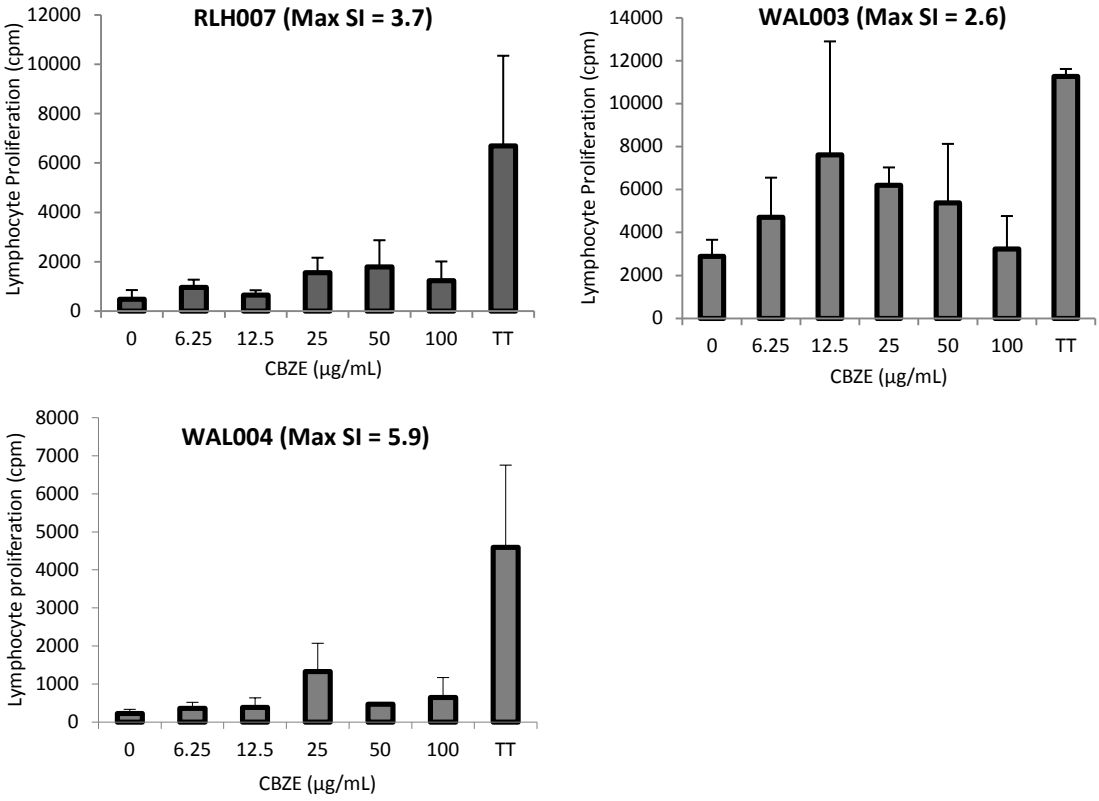


Figure 5.2 Positive LTT to CBZE in 3 patients. PBMCs (1.5 x 10^4 cells, 100µL) were incubated with graded concentrations of CBZE (6.25 - 100 µg/mL) in 96-well U bottomed plates. Plates were incubated at 37^{o}C for 5 days before addition of [^{3}H]-thymidine (0.5 µCi) and a further 16 h incubation. T cell proliferation was measured using scintillation counting. (Data represents mean proliferation and standard deviation of triplicate cultures).

5.3.2 Characterisation of Drug-Specific T Cell Clones

Based upon the results of the LTT, TCCs to CBZ and CBZE were generated from subjects RLH001 and RLH007 using serial dilution as described above (section 5.2.6). The reactivity of clones was tested using a 48 h proliferation assay. Seventeen CBZ-specific TCCs were generated after testing 638 clones (Table 5.2). Eleven CBZE-specific TCCs were generated after testing 579 clones (Table 5.3). The phenotype of all 28 drug-specific TCCs were CD4+ despite attempts to isolate CD8+ T cells using magnetic MultiSort beads (section 5.2.6) (Figure 5.3). An attempt was made to generate TCCs from WAL004 because this subject expressed positive lymphocyte proliferation to CBZE exclusively. Unfortunately the TCC generating procedure was not successful.

Table 5.2. Characteristics of CBZ-specific TCCs

Subject	Clones tested (n)	Specific clones (n)	Proliferation (cpm)		CD phenotype (%)		
			Control	CBZ (25 µg/mL)	CD4+	CD8+	CD4+ CD8+
TOTAL	**638**	**17**	**1406.4 (±395.1)**	**13434.6 (±10472.8)**	**100**	**0**	**0**
RLH001	350	8	1456.2 (±530.8)	14910.9 (±11048.1)	100	0	0
RLH007	288	9	1362.1 (±223.5)	12122.3 (±10066.4)	100	0	0

Table 5.3. Characteristics of CBZE-specific TCCs

Subject	Clones tested (n)	Specific clones (n)	Proliferation (cpm)		CD phenotype (%)		
			Control	CBZE (25 µg/mL)	CD4+	CD8+	CD4+ CD8+
TOTAL	**579**	**11**	**2180.7 (±2007.7)**	**17137.6 (±12178.5)**	**100**	**0**	**0**
RLH001	363	4	3610.9 (±2894.4)	23551.6 (±16620.1)	100	0	0
RLH007	216	7	1363.5 (±150.5)	13472.4 (±7146.2)	100	0	0

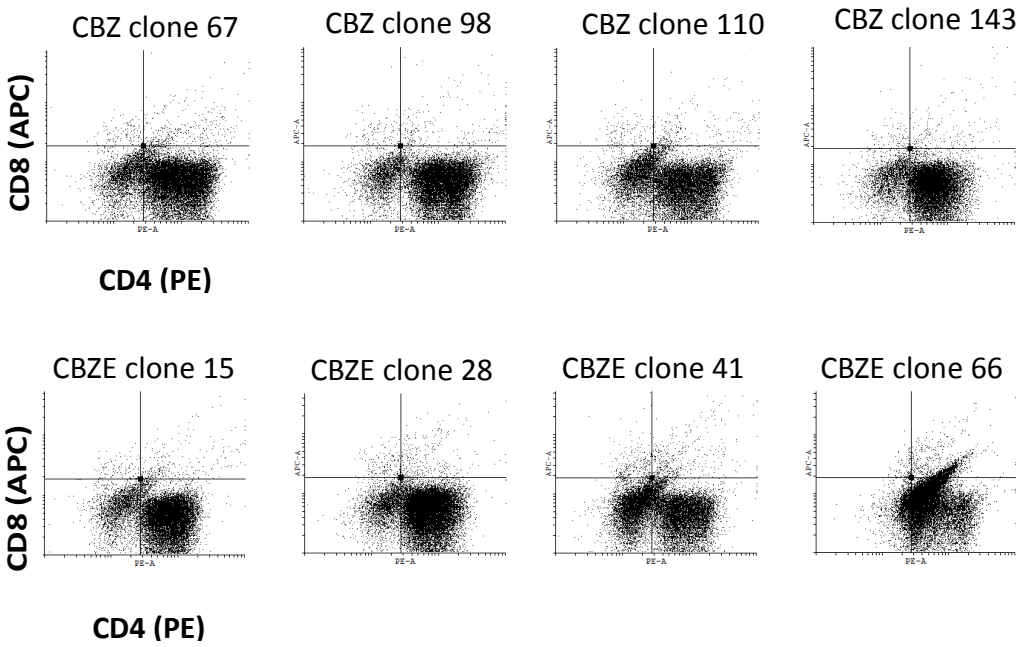


Figure 5.3 Representative CD4 and CD8 phenotyping by FACs for a selection of CBZ- and CBZE-specific clones. TCC suspensions (50 µL) were stained with phycoerythrin (PE) labelled anti-CD4 antibodies (3 µL) and allophycocyanin (APC) labelled anti-CD8 antibodies (3 µL) for 20 min. Cells were washed and signals acquired by flow cytometry and analysed using cyflogic.

5.3.3 Concentration Response and Cross Reactivity of Drug-specific T Cell Clones

Concentration response and cross reactivity of drug-specific TCCs was determined by measurement of IFN-γ secretion using ELISpot. All drug-specific TCCs demonstrated concentration-dependent secretion of IFN-γ (Figure 5.4). For CBZ-specific TCCs, 11/17 (64.7%) were cross reactive to CBZE (Figure 5.5A). For CBZE-specific TCCs, 9/11 (81.8%) were cross reactive to CBZ (Figure 5.5B). Interestingly, a single TCC generated initially by stimulation of lymphocytes to CBZ secreted IFN-γ only in the presence of CBZE and not CBZ.

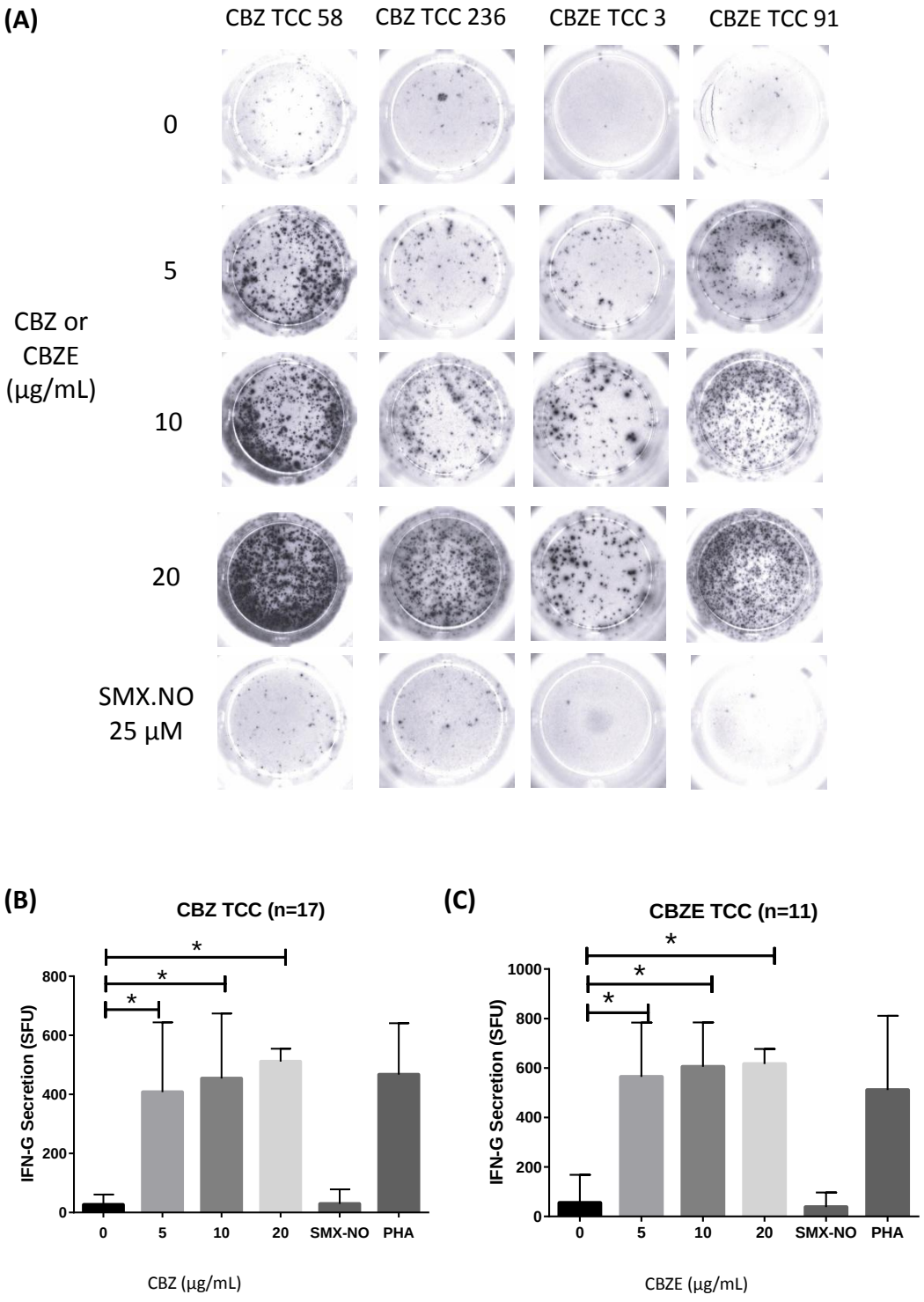


Figure 5.4 (A) Representative drug specificity and concentration response secretion of IFN-γ by drug-specific TCCs using ELISpot. (B) CBZ-dependent secretion of IFN-γ by CBZ-specific TCCs. (C) CBZE-dependent secretion of IFN-γ by CBZE-specific TCCs. TCCs were incubated with autologous EBV-transformed B-cells and CBZ or CBZE at indicated concentrations for 48 h. Data represent mean spot forming units (SFU) ± SEM of n clones. Statistical analysis was performed using paired T-test. (SMX-NO: nitroso-sulphamethoxazole, PHA: phytohaemagglutinin). *$p < 0.001$.

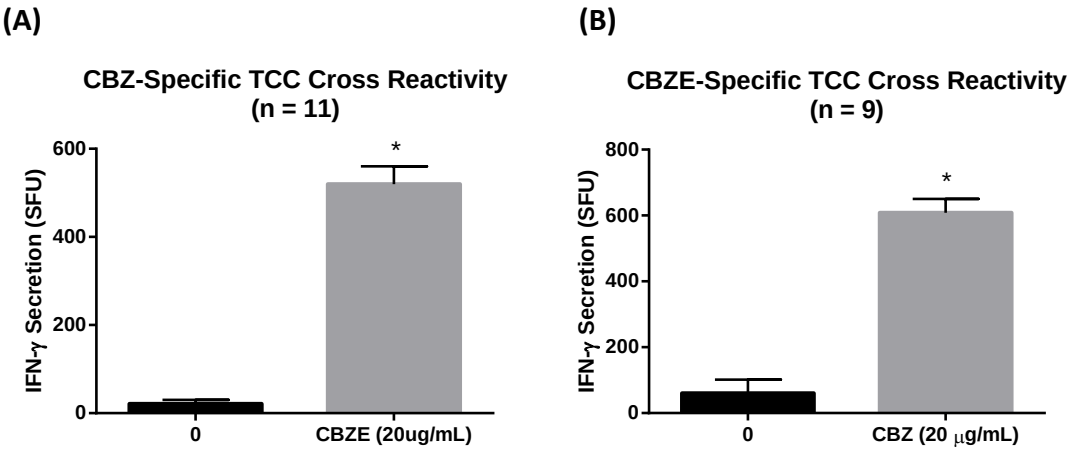


Figure 5.5 (A) Cross reactivity of 11/17 (64.7%) CBZ-specific TCC to CBZE measured by IFN-γ secretion using ELISpot. (B) Cross reactivity of 9/11 (81.8%) CBZE-specific TCC to CBZ measured by IFN-γ secretion using ELISpot. TCCs were incubated with autologous EBV-transformed B-cells and CBZ or CBZE at indicated concentrations for 48 h. Data represent mean spot forming units (SFU) ± SEM of n clones. Statistical analysis was performed using paired T-test. *$p < 0.0001$.

5.3.4 Cytokine Secretion Profile of Drug-Specific T Cell Clones

The secretion of IFN-γ, IL-13, granzyme B and perforin by drug-specific TCCs was measured using ELISpot. Concentration-dependent secretion of IFN-γ by CBZ- and CBZE-specific TCCs was previously demonstrated in Figure 5.4. All 28 drug-specific TCCs secreted IL-13 in response to CBZ or CBZE. Maximal secretion of IL-13 was typically reached with the lowest concentration of CBZ or CBZE tested (5 μg/mL) with only a limited concentration response (Figure 5.6A and B). Granzyme B secretion was measured by ELISpot in 7 drug-specific clones (3 CBZ-specific TCCs and 4 CBZE-specific TCCs). All 7 drug-specific TCCs secreted granzyme B in response to incubation with CBZ or CBZE (Figure 5.6C and D). Perforin secretion was measured in the remaining 21 drug-specific TCCs but due to technical failure, the experiment worked in only five CBZE-specific TCCs. However, perforin secretion was detected in all five clones at multiple concentrations of CBZE (Figure 5.6E).

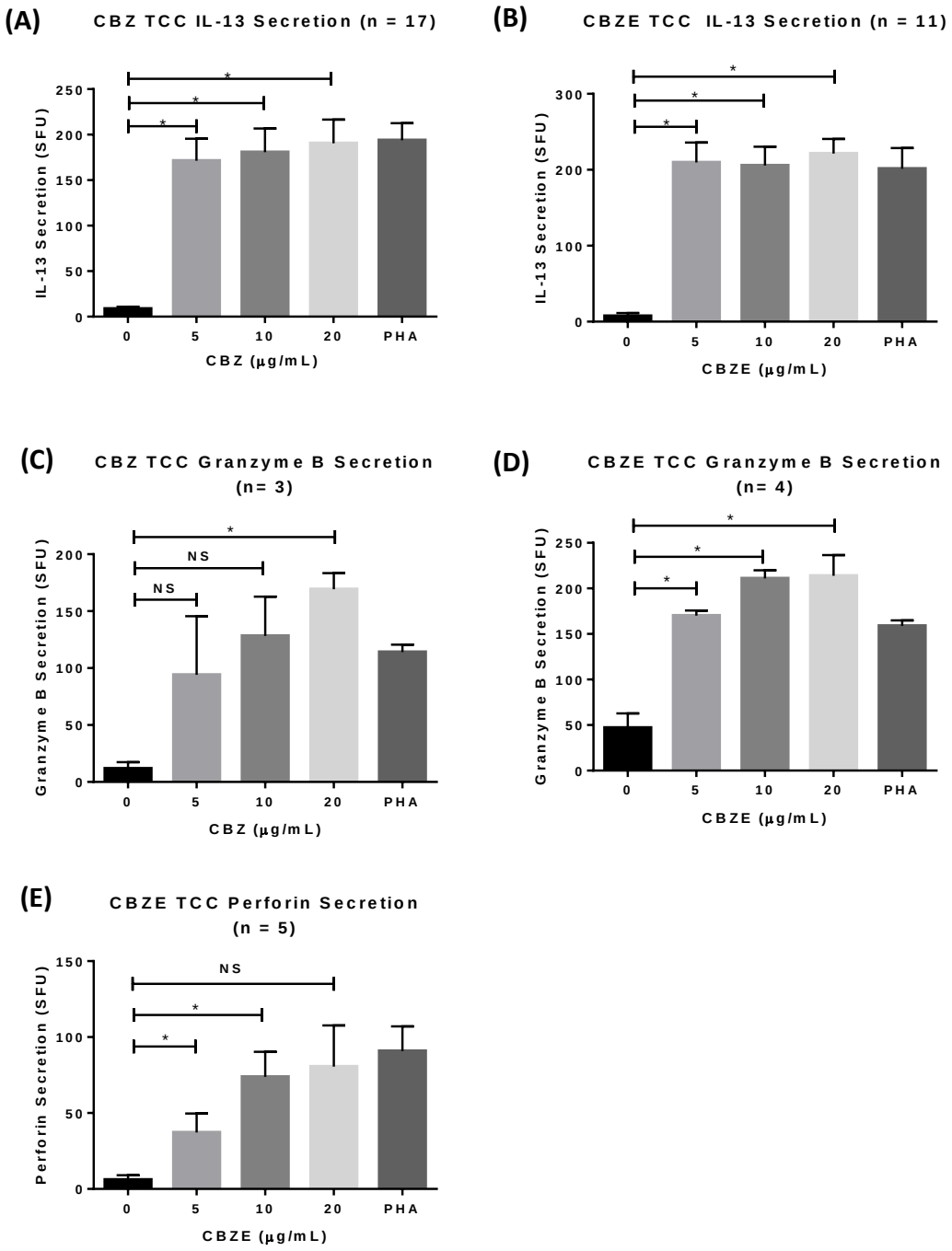


Figure 5.6 (A) IL-13 secretion by CBZ-specific TCCs in response to ascending concentrations of CBZ (B) IL-13 secretion by CBZE-specific TCCs in response to ascending concentrations of CBZE (C) Granzyme B secretion by CBZ-specific TCCs in response to ascending concentrations of CBZ (D) Granzyme B secretion by CBZE-specific TCCs in response to ascending concentrations of CBZE (E) Perforin secretion by CBZE-specific TCCs in response to ascending concentrations of CBZ. TCCs were incubated with autologous EBV-transformed B-cells and CBZ or CBZE at indicated concentrations for 48h. Data represent mean spot forming units (SFU) ± SEM of n clones. Phytohaemoagglutinin (PHA) acted as a positive control. Statistical analysis was performed using the paired T-test. *$p < 0.05$.

5.3.5 The Role of Antigen Pulsing in Activation of T cell Clones

The role of antigen processing was investigated by comparing IFN-γ secretion in response to TCC incubations with EBV-transformed B-cells that had been pulsed with CBZ or CBZE against TCC incubations with EBV-transformed B-cells and freshly added CBZ or CBZE. Pulsed EBV-transformed B-cells were thoroughly washed prior to incubation with TCCs to ensure that any excess drug had been removed. All 17 CBZ-specific TCCs and all 11 CBZE-specific TCCs responded to incubations with freshly added CBZ or CBZE supportive of a P-I mechanism of hypersensitivity (Figure 5.7B). Interestingly, one CBZ-specific clone (CBZ TCC 109) was also activated by the pulsed EBV-transformed B-cells (Figure 5.7A). None of the 11 CBZE-specific TCCs were responsive to the pulsed EBV-transformed B-cells (Figure 5.7A).

5.3.6 The Role of MHC Class I and Class II in Activation of T Cell Clones

The role of MHC class I and class II in the activation of TCC was investigated by incubation of MHC class I or MHC class II blocking antibodies with autologous EBV-transformed B-cells before addition of TCCs and drug or T cell culture medium. Activation of TCCs was measured as IFN-γ secretion by ELISpot. Activation was blocked by anti-MHC II antibodies in 5/17 (29.4%) CBZ-specific TCCs (Figure 5.8). For CBZE-specific TCCs, anti-MHC II antibodies blocked activation in 6/11 (54.5%) clones (Figure 5.8). MHC I blocking antibodies did not prevent activation of any drug-specific TCCs.

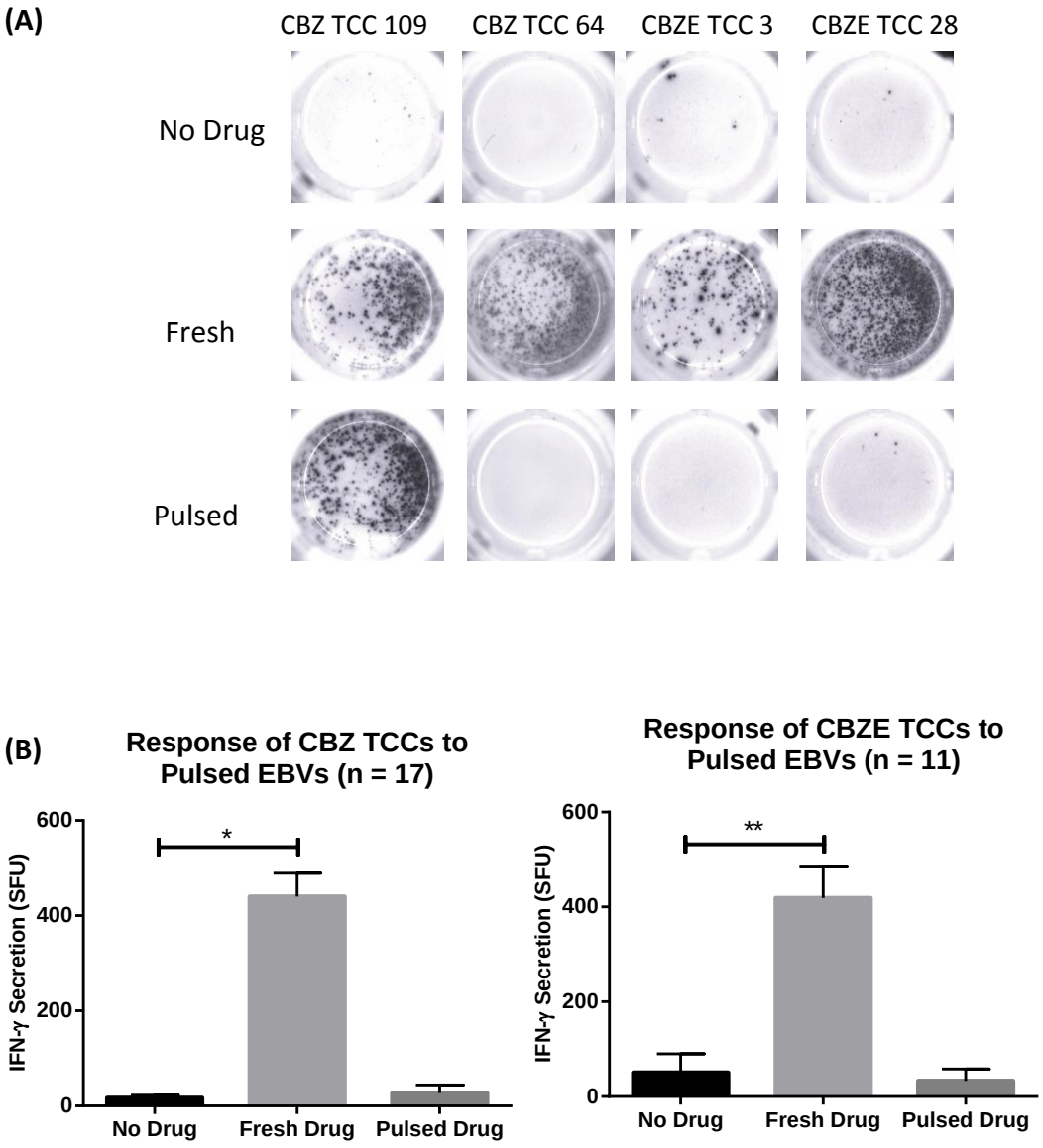


Figure 5.7 (A) Representative IFN-γ secretion measured by ELISpot for drug-specific TCCs in response to negative control, freshly added drug and EBV-transformed B-cells pulsed with drug for 16 h. (B) Effect of fresh drug and pulsed drug on activation of TCCs in response to CBZ or CBZE as measured by IFN-γ secretion using ELISpot. EBV-transformed B-cells were pulsed with drug for 16 h. Excess drug was washed off after incubation period before incubation with TCCs for 48 h. Comparison of IFN-γ secretion was made with TCC incubations containing EBV-transformed B-cells with fresh drug or T cell culture medium only. Data represent mean spot forming units (SFU) ± SEM of n clones. Statistical analysis was performed using paired T-test. (*$p < 0.0001$, ** $p = 0.0015$)

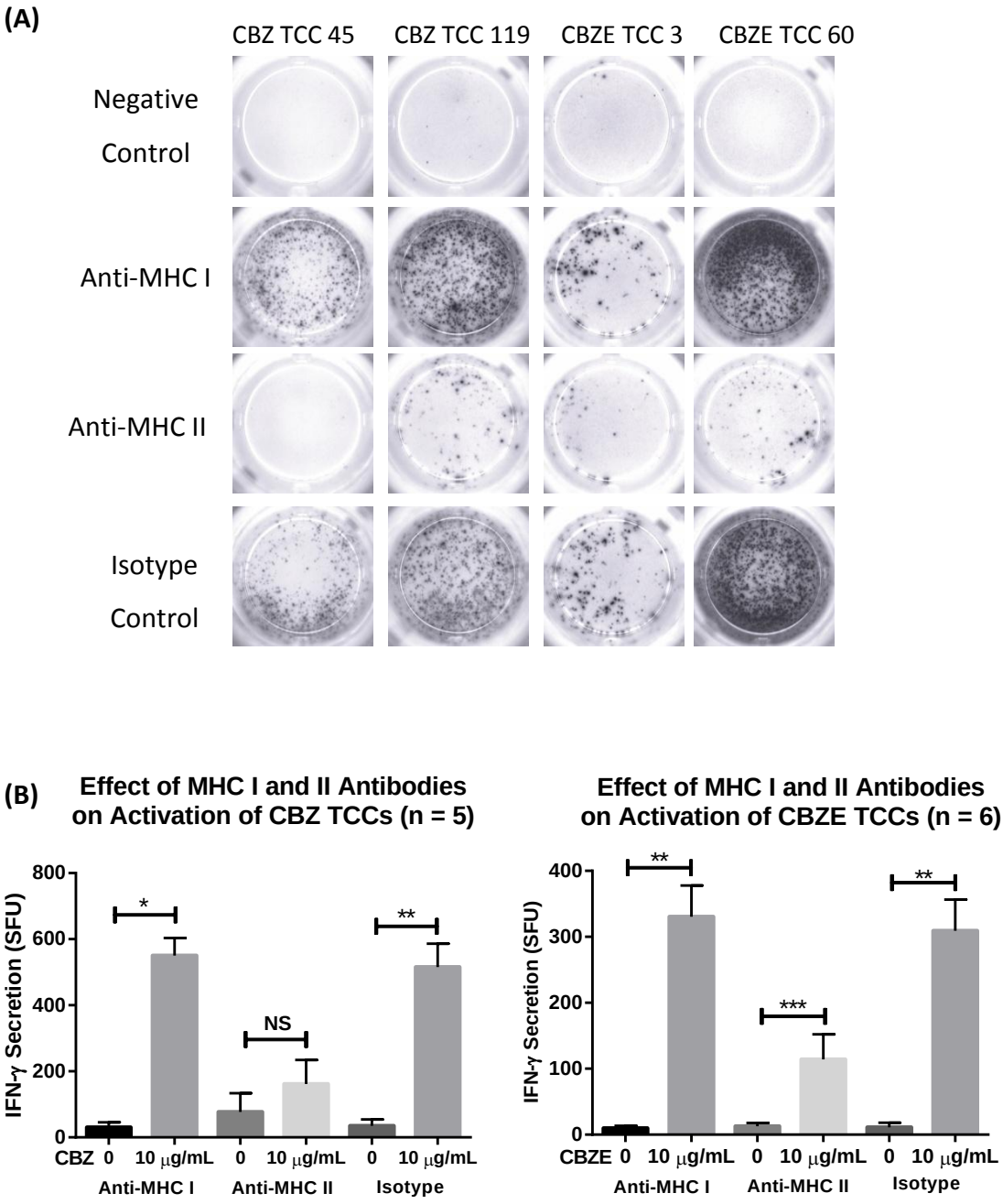


Figure 5.8 (A) Representative secretion of IFN-γ by drug-specific TCCs using ELISpot in response to MHC I, MHC II and isotype blocking antibodies. (B) Effect of MHC I and II blocking antibodies on activation of TCCs in response to CBZ or CBZE as measured by IFN-γ secretion using ELISpot. EBV-transformed B-cells were incubated with anti-MHC I, anti-MHC II or isotype control antibody for 30 min. TCCs were then added with either T cell culture medium or CBZ/CBZE at indicated concentrations before culture for 48 h. Data represent mean spot forming units (SFU) ± SEM of n clones. Statistical analysis was performed using paired T-test. (*p = 0.003, **p = 0.001, ***p= 0.03, NS = not significant)

5.4 Discussion

During the study, 12 subjects with a clinical history of CBZ hypersensitivity were identified. The clinical presentations of CBZ hypersensitivity were highly variable with case descriptions possibly consistent with MPE, HSS and SJS/TEN which correlates with the varied clinical manifestations of hypersensitivity reported previously (Yip et al. 2012). Unfortunately, clinical and patient descriptions of the lesions were poor and documentation incomplete, which is a limitation. There were no photographs making definitive diagnosis more difficult. The interval between onset of reaction and commencement of therapy was between 1 day and 4 weeks for most patients although one subject reported hypersensitivity after 28 weeks. This is broadly consistent with previously reported time frames for CBZ-induced hypersensitivity reactions of 2-8 weeks after starting treatment (Knowles et al. 1999). Only half of the patients demonstrated a positive response on the LTT which is lower than the reported specificity for detection of CBZ-induced hypersensitivity of greater than 80% in other studies (Naisbitt et al. 2003; Wu et al. 2006; Wu et al. 2007). The reasons for this could be inability of the LTT to detect the T cell population responsible for a specific phenotype of hypersensitivity or misdiagnosis of CBZ hypersensitivity reaction. The LTT is often negative in patients who present with drug-induced SJS/TEN (Tang et al. 2012; Porebski et al. 2013). CD8+ T cells are the primary effector cells in SJS/TEN (Nassif et al. 2002) and it has been postulated that they do not proliferate readily in the LTT (Naisbitt et al. 2014). The inability to generate CBZ- or CBZE-specific CD8+ TCCs in this chapter would corroborate this hypothesis. Secondly, clinical diagnosis of drug-induced hypersensitivity reactions is difficult as patients are likely to be taking multiple concomitant medications and the signs and symptoms of hypersensitivity can be non-specific leading to incorrect diagnosis. New guidelines for the investigation and evaluation of suspected drug-induced skin reactions should improve the situation in the future (Pirmohamed et al. 2011).

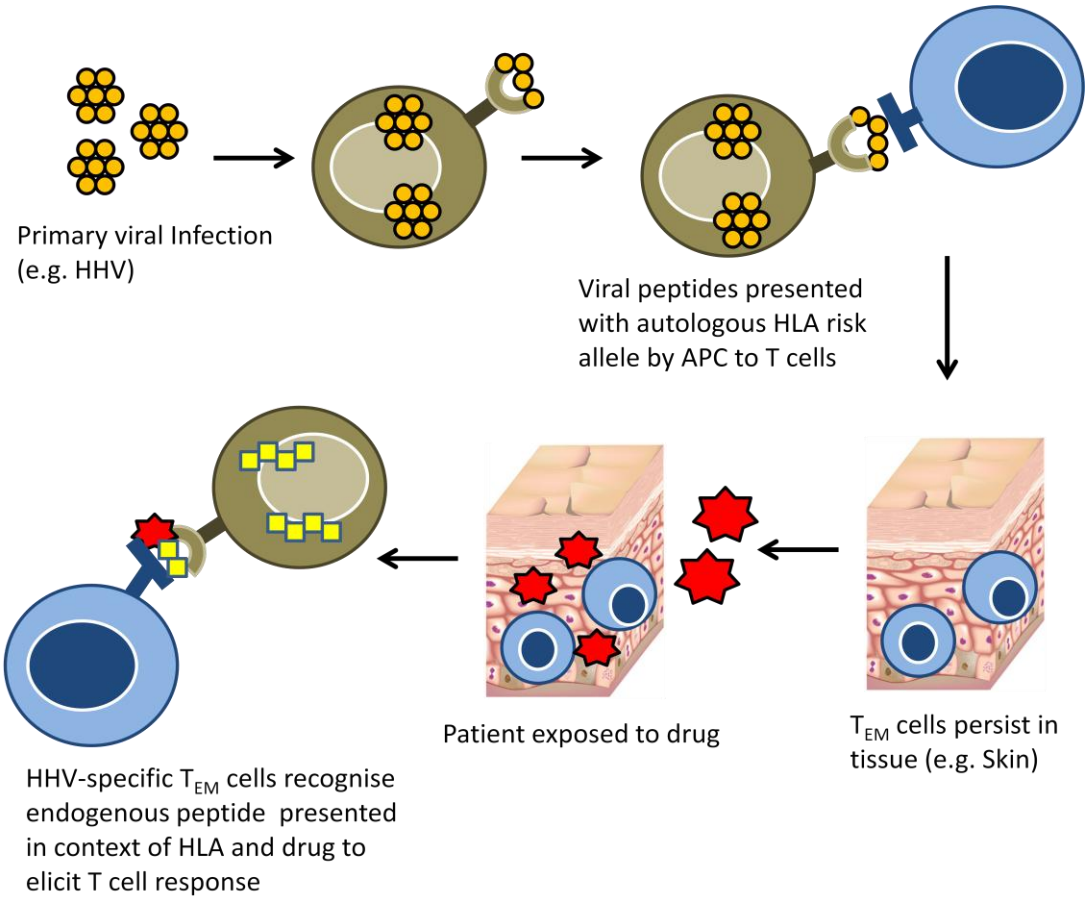
The LTT was undertaken using CBZE in six of the participants. Two subjects tested negative to both CBZ and CBZE, two subjects demonstrated positive lymphocyte proliferation to both CBZ and CBZE, one participant demonstrated a positive

lymphocyte response to CBZ only and the final patient demonstrated lymphocyte proliferation to CBZE only. These results suggest that some drug-specific T-cells from patients with CBZ hypersensitivity may respond only to parent drug or CBZE whilst others demonstrate a pattern of cross-reactivity. Previous work by Wu et al investigated the proliferation of lymphocytes to CBZ and several stable metabolites in five patients with a history of CBZ hypersensitivity using the LTT (Wu et al. 2006). Lymphocyte proliferation was observed with CBZ, CBZE, 10-hydroxycarbamazepine and oxcarbazepine. No proliferation was seen with 2OH-CBZ, 3OH-CBZ or DiOH-CBZ. All lymphocytes that proliferated in the presence of the parent drug also proliferated with CBZE, 10-hydroxycarbamazepine and oxcarbazepine. There were no LTT responses only to the parent compound or a specific metabolite.

Two subjects had experienced hypersensitivity reactions greater than 25 years ago yet still retained drug-specific T cells which proliferated when incubated with CBZ in the LTT in the case of subject RLH001 and both CBZ and CBZE for subject RLH007. This suggests that drug-specific T cells to CBZ could persist lifelong within a memory pool in the peripheral circulation despite avoidance of the culprit drug. Previously, drug-specific T cells had been observed for up to 12 years following a hypersensitivity reaction to sulfamethoxazole (Beeler et al. 2006). At present the antigens that maintain these long lived T cell responses are unknown but viral antigens have been proposed by the heterologous immunity model (White et al. 2015) (Figure 5.9). According to this model, the subject acquires primary infection by a viral pathogen (e.g. HHV or varicella zoster virus [VZV]). Viral peptides are then presented in the context of a HLA risk allele (e.g. *HLA-B*15:02*) leading to the generation of a polyclonal $CD8^+$ T cell response that contains the virus. The virus becomes latent and the memory T cells persist at the site of antigen interaction (e.g. skin). The T cells are then intermittently stimulated by viral antigens during viral activation but effector memory T cells ensure the virus does not cause clinical symptoms leading to persistence of T cells over many years. The patient is then exposed to the drug that causes hypersensitivity (e.g. CBZ). The offending drug or metabolite then interacts with the HLA risk allele through mechanisms such as the hapten hypothesis, PI concept or altered peptide repertoire concept. The offending

drug peptide-MHC complex is subsequently recognised by the TCR that was originally primed against the viral pathogen leading to activation of the memory T cells and clinical ADR. This possible mechanism needs further investigation to either confirm or refute the hypothesis.

Generation of TCCs provides a platform to further investigate the pathophysiology of the hypersensitivity reaction with respect to phenotype, specificity and functionality of the T cells isolated from the hypersensitive patients (Naisbitt 2004). In total, 28 drug-specific TCCs were generated from two subjects. Seventeen clones were specific to CBZ and eleven clones specific to CBZE. All of the clones were CD4+ phenotype despite attempts to generate CD8+ clones using CD8 magnetic beads. Although one CD4+CD8+ TCC and two CD8+ TCCs were initially identified as CBZE-specific in the cloning procedure, all became nonresponsive to CBZE in functional analyses and were therefore classed as non-specific. Antigen specific immune tolerance or anergy is a recognised feature of repeatedly stimulating cloned CD8+ T cells (Wu et al. 2007). The failure to generate drug-specific CD8+ TCCs emphasises the reduced proliferation capacity of CD8+ T cells compared to CD4 T cells (Naisbitt et al. 2014) although it should be noted that CBZ-specific CD8+ TCCs have been successfully generated previously (Wu et al. 2006; Wu et al. 2007).



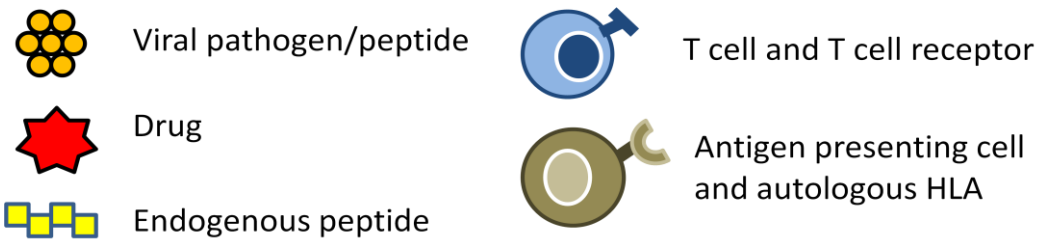


Figure 5.9. Proposed mechanism for generation of heterologous immune responses in pathogenesis of T cell-mediated ADRs. Patient is known carrier of a risk HLA allele (e.g. *HLA-B*15:02*) and acquires viral infection (e.g. HHV). Viral peptides are presented with the autologous risk HLA allele by APCs leading to activation of T cell responses that contain viral infection. T_{EM} persist in the circulation and other tissues (e.g. skin). Intermittent reactivation of viruses lead to persistence of T_{EM} cells. Patient is then exposed to offending drug (e.g. CBZ). The offending drug interacts with the risk autologous HLA, possibly by hapten, PI or altered repetoire mechanisms. The drug-peptide-MHC complex is recognised by the T cell and TCR that was originally primed by the viral pathogen leading to activation of T cell response and hypersensitivity. (HHV – human herpes virus, HLA- human leukocyte antigen T_{EM} – memory effector T cells).

Cytokines are an important component of the effector phase of the immune response and IFN-γ is considered as a dominant cytokine in multiple drug-induced exanthemas including with CBZ, lamotrigine and penicillins (Naisbitt et al. 2003; Halevy et al. 2005; Khalil et al. 2008). Consistent with previous findings, all 28 drug-specific TCCs secreted IFN-γ, as measured by the ELISpot assay, in a concentration-responsive manner when incubated with CBZ and/or CBZE. IL-13 secretion was detected in all 28 drug-specific TCCs when co-incubated with CBZ or CBZE. IL-13 is typically secreted by CD4+ T helper type 2 (Th2) cells in conjunction with IL-4 leading to activation of type 2 inflammation commonly manifesting clinically as asthma or allergy (Bao and Reinhardt 2015). Both IFN-γ and IL-13 have been detected in cutaneous lesions of erythema multiforme and SJS/TEN (Caproni et al. 2006). It has been proposed that measurement of a combination of cytokines including IFN-γ and IL-13 (as well as IL-2 and IL-5) from patients with suspected DHR could improve *in vivo* detection of T cell sensitisation to drugs (Lochmatter et al. 2009). Perforin and granzyme B have also been detected from blood and blister fluid of subjects with MPE, SJS and TEN with levels of both cytokines demonstrating strong positive correlation with disease severity (Posadas et al. 2002). Activated T cells and natural killer cells secrete perforin, which leads to the formation of channels in the target cell membrane (e.g. keratinocyte), allowing granzyme B to enter the cell to activate the caspase cascade with resultant apoptosis (Lowin et al. 1995). In line with the above findings, secretion of granzyme B and perforin was detected in all of the clones tested (7 for granzyme B and 5 for perforin).

Cross-reactivity to CBZ or CBZE, measured by IFN-γ secretion using ELISpot, was observed in 20 out of 28 drug-specific TCCs. Six CBZ-specific TCCs were responsive to CBZ only whilst eleven were responsive to both CBZ and CBZE. Two CBZE-specific TCCs were responsive to CBZE exclusively whilst nine were responsive to both CBZE and CBZ. These results suggest that patients with CBZ hypersensitivity possess a range of T cells that are responsive to both CBZ and CBZE *in vivo*. These findings are in line with previous studies that have identified CBZ-specific T cells from hypersensitive patients which are responsive to CBZE, other stable metabolites such as 10-hydroxycarbamazepine and oxcarbazepine, an alternative anticonvulsant with

structural similarities to CBZ (Naisbitt et al. 2003; Wu et al. 2006). It is possible that CBZ- and CBZE-specific TCCs generated in this study may also respond to other CBZ metabolites. In chapter 2 of this thesis a CBZE-modified albumin conjugate was detected in patients receiving CBZ therapy for clinical indications. Similar albumin modifications have also been detected with piperacillin. Antigenicity of the piperacillin-modified albumin was confirmed by stimulation of T cell responses using characterised synthetic conjugate and TCCs from patients with known piperacillin hypersensitivity (Whitaker et al. 2011). In future, similar experiments using synthetic CBZE-protein conjugates and TCCs from CBZ hypersensitive patients could be undertaken to investigate the ability of CBZ- and CBZE-specific TCCS to respond to metabolite-modified proteins.

The antigen pulsing assay provides indirect evidence for the hapten mechanism of drug hypersensitivity. In the current study, APCs, in this case EBV-transformed B cells, were incubated with CBZ or CBZE for 16 h before thorough washing to ensure removal of any drug that had not been taken up into the APC. These pulsed APCs were then incubated with the drug-specific TCCs and activation of TCCs measured by IFN-γ secretion. Comparisons of activation were made against incubations with TCCs, APCs and freshly added CBZ/CBZE or drug negative controls. All 28 drug-specific TCCs secreted IFN-γ when fresh drug was added indicative of a direct PI mechanism for hypersensitivity. These findings are consistent with previous findings that CBZ is able to directly interact with T cells or HLA to initiate hypersensitivity (Wei et al. 2012; Zhou et al. 2015). However, one CBZ specific clone also responded to pulsed EBVs suggestive of antigen-dependent presentation and hapten mechanism for hypersensitivity. Previously, two [O]CBZ-HSA conjugates were identified *in vitro* (Figure 2.10 and Figure 2.14). It is possible that these protein conjugates were formed during the incubation of CBZ with APC as the T-cell culture medium contains HSA and it is not possible to exclude metabolism of CBZ to [O]CBZ metabolites during the 16 h overnight incubation. The [O]CBZ-HSA conjugate could then have been processed and presented by the APC to the TCC. In future, to assess for the presence of protein conjugates and drug metabolism an aliquot of the supernatant could be removed from the incubation of CBZ and APC, prior to

addition of TCC, for analysis by HPLC-MS/MS as described in chapters 2 and 3. It is possible that multiple T cell activation pathways are triggered during a CBZ hypersensitivity reaction.

Predisposition to multiple DHRs have been associated with multiple HLA alleles (Yip et al. 2015a). *HLA-B*15:02* has been strongly associated with CBZ-induced SJS/TEN in Asian patients (Chung et al. 2004) whilst *HLA-A*31:01* is strongly associated with all phenotypes of CBZ hypersensitivity in multiple ethnicities (McCormack et al. 2011). Antibodies to MHC class I and MHC class II were used in the study to investigate the role HLA in activation of CBZ- and CBZE-specific TCCs. As expected, activation of drug-specific TCCs was not MHC class I restricted as all TCCs were CD4+ phenotype. Activation of drug-specific TCCs was MHC class II restricted in 11 out of 28 drug-specific TCCs (5 for CBZ and 6 for CBZE). The remaining 17 drug-specific TCCs were responsive in the presence of both MHC class I and II antibodies. These TCCs may have been directly activated by CBZ without presentation on MHC or the experimental conditions for antibody blocking of MHC were not optimised. In future studies it will be important to test a range of concentrations for both CBZ/CBZE and MHC blocking antibodies to investigate the sensitivity of response of individual TCCs to antigen and MHC blocking antibodies. Recently, it has been shown that specific HLA class I and class II alleles are associated with particular phenotypes of nevirapine hypersensitivity; *HLA-B*35:01* and *HLA-Cw*04* were associated with cutaneous hypersensitivity, *HLA-Cw*08* was associated with eosinophilia and *HLA-DRB*01:01* was associated with hepatitis. Cellular studies in these patients identified specific combinations of class I restricted CD8+ T cells and class II restricted CD4+ T cells that contributed to pathogenesis of specific phenotypes of nevirapine hypersensitivity (Keane et al. 2014). The exact relationship between HLA class I and class II alleles in CBZ hypersensitivity requires further investigation. The HLA class II allele, *HLA-DRB1*04:04*, has been reported to be functionally significant for the activation of T cells generated from a *HLA-A*31:01* positive patient (Lichtenfels et al. 2014). The authors reported strong linkage disequlibrium between the two alleles and suggest that a common haplotype may contribute to the multiclonal response seen in Caucasian patients with CBZ hypersensitivity.

Carriage of the risk HLA alleles alone is insufficient to generate a hypersensitivity response when exposed to CBZ (Yip et al. 2012). CBZ responsive CD8+ T cells isolated from *HLA-B*15:02* positive patients with a history of CBZ-induced SJS were all found to use a common restricted TCR repertoire and over express specific TCR Vβ profiles (Ko et al. 2011). These results suggest that specific TCRs could also act as a risk factor in development of specific phenotypes of CBZ hypersensitivity. In future studies it would be informative to investigate the range of TCR profiles expressed in CBZ- and CBZE-specific TCCs generated from subjects with multiple phenotypes of CBZ hypersensitivity.

In conclusion, positive lymphocyte proliferation responses can be generated from patients with a history of CBZ hypersensitivity even if presentation of the original symptoms were longer than 25 years ago. It is possible that these drug-specific memory T cells persist in the circulation lifelong and are possibly maintained by the heterologous immunity model. In this study, twenty eight CD4+ drug-specific TCCs were generated to CBZ or CBZE. Specificity of TCCs was variable with some clones responsive only to original stimulating antigen whilst others were also responsive to both CBZ and CBZE. Activation of clones was through a direct PI mechanism although a single CBZ TCC did respond to pulsed APCs consistent with the hapten hypothesis. MHC class II is responsible for presentation of antigen to CD4+ TCCs. Secretion of IFN-γ, IL-13, granzyme B and perforin were detected in the TCCs providing evidence for the pathophysiological mechanism of hypersensitivity. These data demonstrate that CBZ hypersensitivity is characterised by plurality of T cell responses reacting with multiple antigens using different mechanistic pathways.

Chapter 6

Gene Expression Analysis of Peripheral Blood Mononuclear Cells from Patients with Carbamazepine Hypersensitivity Exposed to Carbamazepine and Carbamazepine 10,11-Epoxide

6.1 Introduction

The clinical presentation of CBZ hypersensitivity reactions is diverse, ranging from mild eruptions such as MPE to more severe systemic manifestations, which include HSS, SJS and TEN (Yip et al. 2012). Up to 10% of patients experience hypersensitivity when prescribed CBZ (Marson et al. 2007) and the presence of rash warrants withdrawal of the drug as it is currently not possible to predict who will progress to the more severe systemic conditions (Mehta et al. 2014). Early withdrawal of CBZ improves prognosis and survival, and if possible, other aromatic anticonvulsants should be avoided in the future (Garcia-Doval et al. 2000; Błaszczyk et al. 2015).

Despite the importance of early recognition, diagnosis of hypersensitivity reactions remains difficult in everyday clinical practice. Diagnostic tests are not routinely available and acute diagnosis is typically based on limited clinical history and examination (Polak et al. 2013). Even after recovery, testing is required to determine the culprit drug/chemical in order for the patient to avoid it in the future. *In vivo* investigations, such as skin prick and patch testing, have been utilised for the diagnosis of CBZ hypersensitivity but there has been a high variability in response rates (Alanko 1993; Seitz et al. 2006). In addition, *in vivo* tests are only recommended in cases of mild exanthems because of the risk of inducing life-threatening reactions, and are not suitable for acute diagnosis (Mirakian et al. 2009). Therefore, there is a clinical need for accurate *in vitro* diagnostic tests.

The LTT, also known as the lymphocyte proliferation assay, is the most widely reported *in vitro* diagnostic test for drug hypersensitivity (Pichler and Tilch 2004). It detects the presence of drug-specific T-cells and measures the proliferation of these T-cells when re-exposed to titrated concentrations of the suspected drug (Nyfeler and Pichler 1997). The LTT has been used for the diagnosis of CBZ hypersensitivity but the sensitivity and specificity for the test are influenced by the clinical manifestation of hypersensitivity and the timing of the test since the reaction (Elzagallaai et al. 2009). The LTT does have other limitations: it is technically demanding, requires thymidine (a radioactive isotope) and the typical duration for the assay is 5-7 days (Nyfeler and Pichler 1997). In chapter 5, twelve patients had

been clinically diagnosed with CBZ hypersensitivity but only six tested positive (50%) on LTT using CBZ. The reasons for this variation remain unknown and require further investigation.

The identification of CBZ-specific T-cells (Wu et al. 2007) and strong associations with specific HLA-alleles (Chung et al. 2004; McCormack et al. 2011) provide evidence that CBZ hypersensitivity reactions are immune-mediated. The utility of screening for *HLA-B*15:02* for prevention of CBZ-induced SJS/TEN in Asian populations has been demonstrated prospectively (Chen et al. 2011) and the drug label for CBZ has been changed by many drug regulatory agencies, including the European Medicines Agency and US Food and Drug Administration, to mandate screening. *HLA-A*31:01* is associated with all phenotypes of CBZ hypersensitivity in European (McCormack et al. 2011) and Japanese populations (Ozeki et al. 2011) but screening is not mandatory at present. However, not all patients that carry the risk HLA alleles will experience hypersensitivity if exposed to CBZ, resulting in the denial of therapy to many patients who would have tolerated treatment (Yip et al. 2012). These data suggest that HLA are necessary for a CBZ hypersensitivity reaction but insufficient to trigger hypersensitivity on their own. There remains a need to understand the other factors that lead to a CBZ hypersensitivity reaction in order that better treatments and diagnostic tests can be developed.

Transcriptomics is the study of the transcriptome - the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell - using high throughput technologies such as RNA sequencing and microarray analysis (Wang et al. 2009). Comparison of transcriptomes allows identification of genes that are differentially expressed in distinct cell populations (e.g. diseased and non-diseased) (Costello et al. 2005), recognition of subtypes of known pathology (Bradley et al. 2015), and detection of novel biomarkers for prognosis of a disease (Moreno and Sanz-Pamplona 2015). MicroRNAs are small noncoding RNAs that are approximately 22 nucleotides long and regulate the expression of mRNA (Bartel 2004). More than 1000 miRNAs have been identified in

humans and they have been implicated in the immune response, inflammation and autoimmune diseases (Friedman et al. 2009; Furer et al. 2010).

The aims of this chapter were to obtain and compare transcriptome profiles from PBMCs of patients with known clinical CBZ hypersensitivity in order to unravel the underlying mechanisms related to these ADRs. PBMCs were isolated from patients with heterogeneous responses to the LTT and incubated with CBZ, CBZE, TT or cell culture medium alone to determine if differentially expressed genes could explain variability in response to LTT and offer novel insight for development of new treatments and diagnostic biomarkers.

6.2 Methods

6.2.1 Patient Characteristics

Five subjects with a clinical history of hypersensitivity to CBZ were recruited retrospectively from the Royal Liverpool University Hospital and the Walton Centre for Neurology and Neurosurgery. The subjects were chosen based on their known responses to the LTT (Figure 6.1). Clinical details for the patients are summarised in Table 6.1. The study was approved by the local ethics committee and informed consent was obtained from each patient.

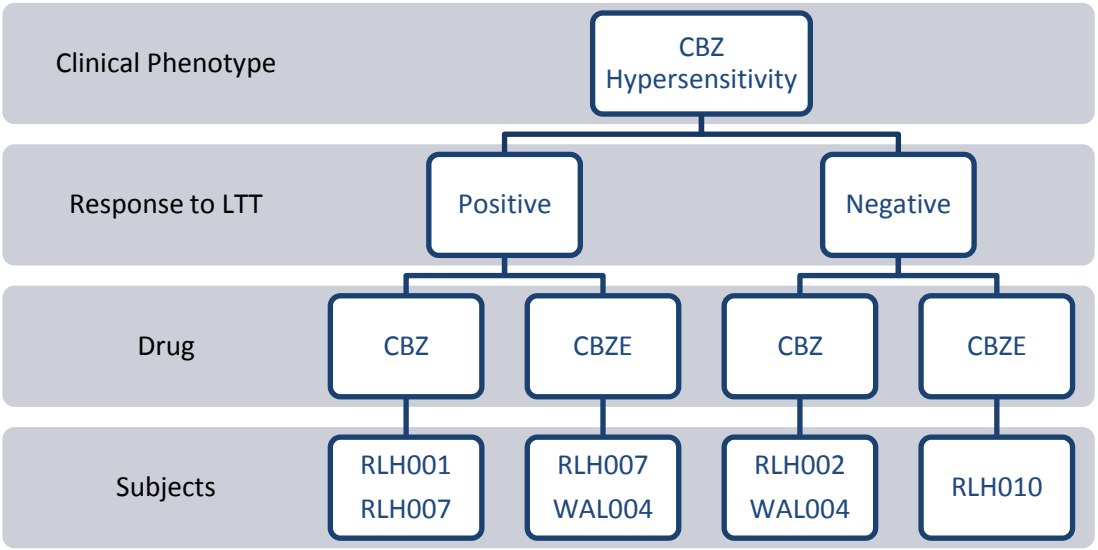


Figure 6.1 Selection of subjects for gene expression analysis based on response to lymphocyte transformation test (LTT)

Table 6.1 Clinical Characteristics of Patients Selected for Transcriptome Analysis (SI: stimulation index, d: days, mth: months, NA: not applicable)

Subject	Clinical Presentation	Time to reaction	CBZ LTT (SI)	CBZE LTT (SI)
RLH001	1989 – unknown	21 d	3.6	NA
RLH002	1991 – generalised rash, mucosal involvement and flu symptoms	8 d	negative	NA
RLH007	1989 – generalised erythematous rash	6 d	16.4	3.7
RLH010	2002 – ulceration of oral cavity and lips	Unknown	3.2	Negative
WAL004	2004 – generalised, itchy and scaly erythematous rash	7 mth	negative	5.9

6.2.2 Chemicals and Reagents

CBZ (Sigma-Aldrich Co, UK) and CBZE (Sigma-Aldrich Co) were prepared as stock solutions (10 mg/mL) in T-cell medium containing 10% DMSO (Sigma-Aldrich Co). The stock solutions were made up fresh and diluted to the appropriate concentrations directly before use. TT was obtained from the Statens Serum Institut in Denmark. RNA extraction was undertaken using the miRNeasy extraction kit (Qiagen, Manchester, UK). All other reagents were from Sigma-Aldrich Co unless otherwise stated.

6.2.3 Cell Culture Medium

T-cell culture medium consisted of RPMI 1640 medium supplemented with 10% human pooled AB serum (Innovative Research, USA), 25 mM HEPES buffer, 2 mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 25 µg/mL transferrin.

6.2.4 Isolation of Peripheral Blood Mononuclear Cells

PBMCs were isolated from whole blood of patients by density gradient centrifugation on the same day that blood samples were obtained. The blood was layered on top of an equal volume of Lymphoprep (Axis Shield, UK) and centrifuged at 400*g* for 25 min at room temperature. The layer of PBMCs was extracted carefully with a Pasteur pipette and washed twice in HBSS to remove any excess Lymphoprep. Isolated PBMCs were re-suspended in T-cell culture medium.

6.2.5 Culture of Isolated PBMC with CBZ and CBZE

Isolated PBMCs (3.0×10^6; 2 mL) were cultured with CBZ (25 µg/mL) or CBZE (25 µg/mL) in triplicate in a 24-well plate and incubated at 37^oC under an atmosphere of 95% Air/ 5% CO_2 for 24 h. TT (1 µg/mL) and culture medium were used as positive and negative controls, respectively. After the incubation period, the cells were harvested into sterile collection tubes and centrifuged at 400 *g* for 10 min. Cell

culture medium was carefully removed and the PBMCs were re-suspended in RNA later (Qiagen, UK) and stored at -80^{o}C until RNA extraction.

6.2.6 RNA Extraction and Quantification

RNA extraction was undertaken according to manufacturer instructions using the miRNeasy kit (QIAgen, UK). Briefly, PBMCs suspended in RNA later were thawed at room temperature before centrifugation at 2000*g* for 10 min. The supernatant was removed carefully from the pelleted cells and 700 µL of QIAzol lysis reagent was added to each sample. The cells were homogenized by vortexing for 1 min and then placed on the benchtop at room temperature for 5 min. Chloroform (140 µL) was added to each sample and thoroughly mixed for 15 sec, and then left at room temperature for 3 min. The homogenates were then centrifuged for 15 min at 12000*g* at 4^{o}C. The upper aqueous phase was transferred into a new collection tube and 1.5 volumes of 100% ethanol added to each sample before mixing. The sample was then centrifuged in an RNeasy mini spin column at 8000*g* for 15 s at room temperature. Buffer RWT (700 µL) was added to wash the spin column and centrifuged for 15 s at 8000*g*. The spin columns were washed two further times by addition of 500 µL RPE buffer and centrifugation at 8000*g* for 15 s and 2 min, respectively. To eliminate any carryover of buffer or ethanol, the spin column was centrifuged at 12000*g* *for* 1 min. All flow-through from the above steps was discarded. The RNeasy mini spin columns were transferred to a new collection tube and RNA was eluted by addition of 50 µL RNAse-free water directly onto the spin column membrane and centrifuged for 1 min at 8000*g.* A second elution step with a further 50 µL RNAse-free water was undertaken to obtain a greater concentration of RNA. The concentration of RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). RNA samples were frozen at -80^{o}C until ready for microarray analysis.

6.2.7 Microarray Data Expression Analysis

Expression profiles of mRNA and miRNA were undertaken by Dr Lucille Rainbow at the Centre for Genomics Research (University of Liverpool, UK) using the Affymetrix GeneChip Human Transcriptome Array 2.0 and the Affymetrix GeneChip 4.0 miRNA array (Santa Clara, CA, USA) according to manufacturer's instructions. The Centre for Genomics Research is a core facility and all RNA underwent QC procedures. Quality and concentration of RNA was checked using nanodrop and Qubit (ThermoFisher Scientific, Wilmington, USA). The RNA integrity number (RIN) (Schroeder et al. 2006) for all samples was determined using an Agilent bioanalyzer (Santa Clara, CA, USA). The RIN values for the RNA samples ranged from 7.6 to 9.2. Data was normalised against internal controls using the Affymetrix expression console (build 1.4.1.46).

Expression data from cells treated with CBZ, CBZE or TT were compared with untreated control samples in cell culture medium. Differential gene expression analysis was performed by Dr Kim Clarke (Institute for Integrative Biology, University of Liverpool, UK) using the LIMMA package (Smyth 2005) in the R statistical software (https://www.r-project.org/). Genes known to be specifically associated with gender were removed prior to analysis to minimise the effects of genetic heterogeneity between patient groups. The genes were ranked in a list according to their differential expression. The associated RNA expression levels were normalised against the control expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Similarly, the associated miRNA expression levels were normalised against the control expression of HSA-miR-92a-3p. Plots were then generated using the R statistical software package (version 3.2.1). Within the plots, the y-axis value is the normalised mRNA or miRNA expression level and the x-axis denotes the test group of either CBZ/CBZE, negative control or TT. The points from CBZ are coloured black and the points from CBZE are coloured red. The plots were kindly generated by Dr Ben Francis (Department of Biostatistics, University of Liverpool, UK).

Pathway analyses were performed using IPA software (Qiagen, Redwood City, Ca, USA). Dr. Eunice Zhang (Institute of Translational Medicine, University of Liverpool, UK) and Dr. Olga Vasieva (Institute of Integrative Biology, University of Liverpool, UK) provided supervision and guidance when using IPA. Significant genes derived from the LIMMA analysis were uploaded for core analysis within IPA. Significance was defined as $p < 0.05$ and Log_2-fold change < -1 or > 1. Comparison analyses were undertaken using IPA in order to determine pathways and networks that were specific to CBZ or CBZE when compared with TT.

Gene set enrichment analysis (GSEA) (Subramanian et al. 2005) was undertaken by Dr Kim Clarke (Institute of Integrative Biology, University of Liverpool, UK). GSEA aims to overcome problems associated with conventional single gene methods by evaluating microarray data at the level of gene sets. Gene sets are defined based on prior biological knowledge of particular processes (e.g. apoptosis). The goal of GSEA is to determine whether members of a gene set tend to occur toward the top (or bottom) of the ranked gene list, in which case the gene set is correlated with the phenotypic class distinction (e.g. responsive to drug) (Subramanian et al. 2005). The entire gene list was used for GSEA analysis irrespective of fold change and p-value.

6.3 Results

6.3.1 Gene Expression Profiles in Subjects with CBZ Hypersensitivity Reactions

We performed whole genome mRNA and miRNA expression profiling using PBMCs isolated from a cohort of 5 patients with a clinical history of CBZ hypersensitivity. A mixture of patients who were positive and negative to CBZ/CBZE in the LTT (Figure 6.1) were included to determine if specific mRNA or miRNA transcripts could be used to identify T cell activation to drug irrespective of response to LTT. For each incubation with CBZ or CBZE the PBMCs were also incubated with cell culture medium (negative control) and TT (positive control). Subject RLH007 and WAL004 donated PBMCs twice for incubation with both CBZ and CBZE. In total 21 RNA samples were analysed by microarray for mRNA and miRNA. Differential gene

expression was determined by comparing PBMCs incubated with CBZ, CBZE or TT against PBMCs from the same subjects that had been incubated with cell culture medium alone. A significant change in expression for both mRNA and miRNA was classified as a Log_2-fold change > 1 or < -1 in conjunction with an unadjusted p-value < 0.05. PBMCs incubated with TT demonstrated the greatest number of significant differentially expressed mRNA and miRNA, 159 and 104, respectively (Table 6.2 and Table 6.3). The expression values, precise p-values and identity of all the mRNA and miRNA differentially expressed are listed in the appendix (Tables 9.1 to 9.8 and Tables 9.18 to 9.25).

Table 6.2 Number of significant differentially expressed genes according to incubation conditions

Incubation Condition (all compared with cell culture medium)	LTT Result (number of genes differentially expressed)	Number of Significant Differentially Expressed Genes (mRNA) (duplicates removed)
CBZ	Positive (n=24)	25
	Negative (n=1)	
CBZE	Positive (n=14)	32
	Negative (n=18)	
TT	NA	159

Table 6.3 Number of significant differentially expressed miRNA according to incubation conditions

Incubation Condition (all compared with cell culture medium)	LTT Result (number of miRNA differentially expressed)	Number of Significant Differentially Expressed miRNA (duplicates removed)
CBZ	Positive (n=24)	35
	Negative (n=14)	
CBZE	Positive (n=13)	41
	Negative (n=30)	
TT	NA	104

No mRNA transcripts were differentially expressed across all three conditions or in incubations between CBZ and CBZE. Two mRNA were differentially expressed in both CBZ and TT incubations. Ten mRNA were differentially expressed between CBZE and TT (Figure 6.2). A single miRNA (miR-181a-5p) was differentially expressed across all three incubation conditions and one miRNA (MIR6124) was differentially expressed in both CBZ and CBZE incubations. Sixteen miRNA were differentially expressed in both CBZ and TT and thirteen miRNA were differentially expressed in both CBZE and TT incubations (Figure 6.2).

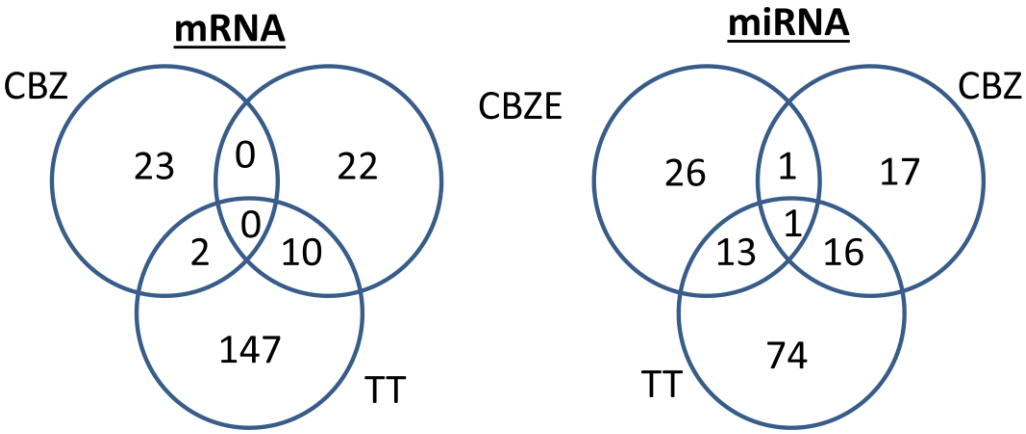
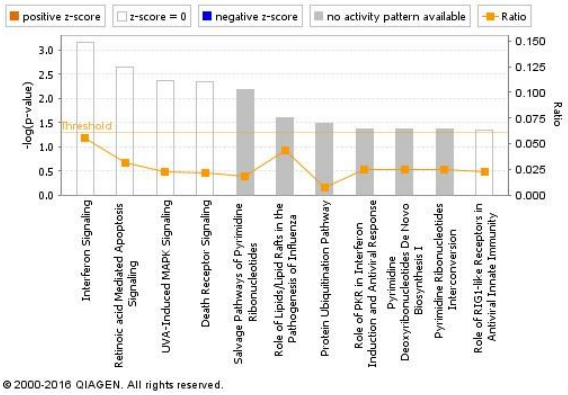


Figure 6.2 Differential RNA expression in PBMC incubated with CBZ, CBZE or TT compared with negative controls (significance was defined as Log2-fold change <-1 or >1 and p < 0.05).

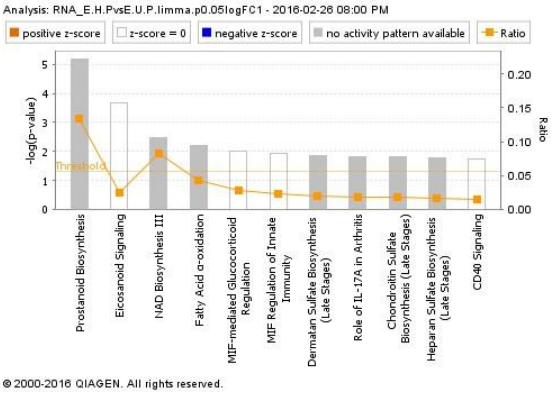
6.3.2 Ingenuity Pathway Analyses for mRNA

To define the molecular mechanisms involved in the development of CBZ hypersensitivity reactions, we initially used the "canonical pathways" function in IPA on the gene expression lists generated in section 6.3.1. In subjects where the LTT was positive to CBZ, multiple pathways involving the immune system were enriched in comparison with PBMCs incubated with cell culture medium alone (Figure 6.3A). These included the following pathways: interferon signalling, death receptor signalling and antiviral responses. In PBMCs that were positive on LTT to CBZE, different canonical pathways were significant when compared with those that were positive to CBZ. The most significant pathways with CBZE were prostanoid biosynthesis and eicosanoid signalling (Figure 6.3B). Pathways involving the immune

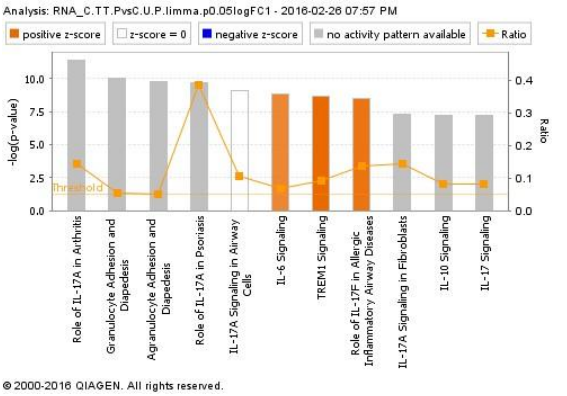
system were also significant. Interestingly, pathways involving biosynthesis of dermatan sulfate, chondroitin sulfate and heparan sulphate were also significant. No significant canonical pathways were reported in PBMCs where the LTT was negative to CBZ when compared with negative control PBMCs. In PBMC incubations with TT, multiple pathways involving regulation of the immune system, cytokine signalling and antiviral response were significant (Figure 6.3C, D and E). No significant pathways were reported in PBMCs that were negative on LTT to CBZE.



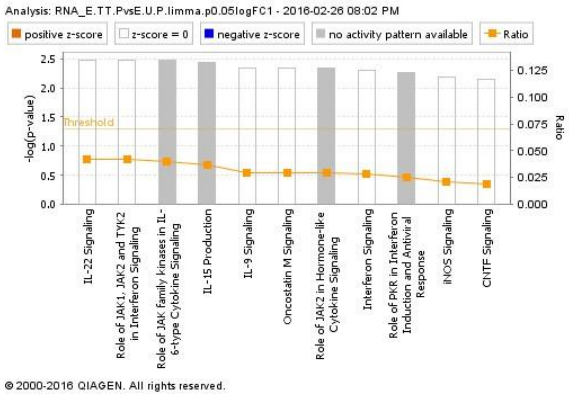
(A)



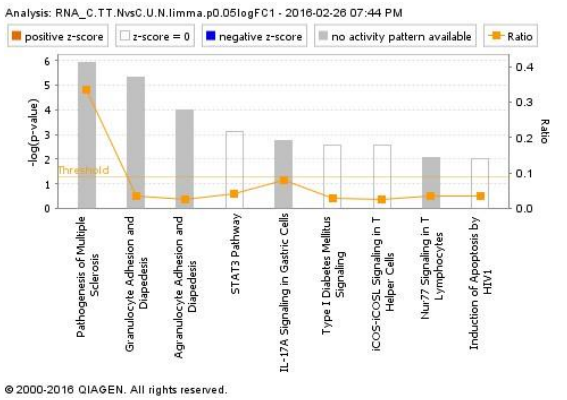
(B)



(C)



(D)



(E)

Figure 6.3 Top canonical pathways identified in IPA when compared with negative controls. (A) CBZ LTT positive (B) CBZE LTT positive (C) TT in CBZ LTT positive PBMC (D) TT in CBZE LTT positive PBMC (E) TT in CBZ negative LTT PBMC

Comparison analyses were undertaken in IPA to identify canonical pathways that were only differentially regulated in drug treated PBMC when compared with TT treated cells (Table 6.4). Twelve genes were identified to be either significantly upregulated or downregulated in PBMCs that were positive on LTT to CBZ or CBZE. The list of CBZ/CBZE-specific genes was reduced to nine when duplicates were removed by comparing with the TT gene list generated by LIMMA analysis (Section 6.3.1). No significant canonical pathways were identified when comparison analyses were undertaken between PBMCs that were negative on LTT to both CBZ and CBZE in comparison with TT. Using the 9 CBZ/CBZE-specific genes a network of interactions was generated in IPA (Figure 6.4). The top 5 diseases and functions involving at least 3 drug-specific molecules from the network were viral infection, psoriasis, antiviral response, infection cells and infection by RNA virus (Table 6.5).

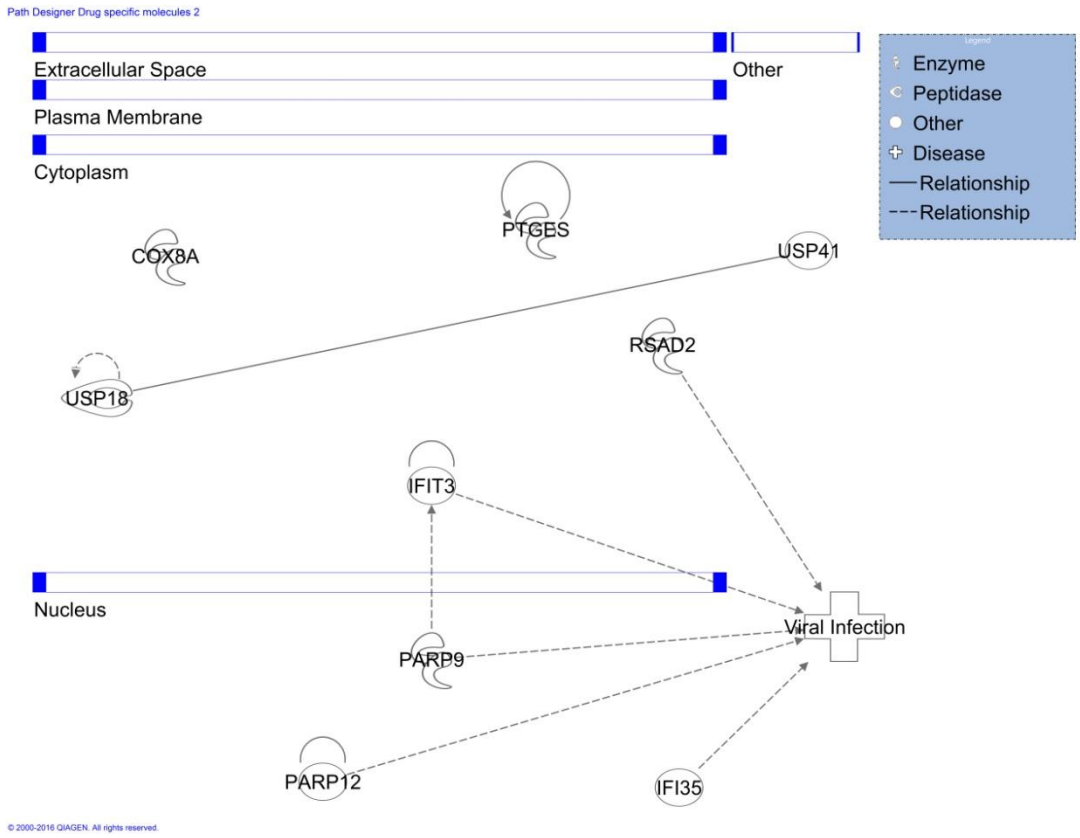


Figure 6.4 Network representation of interactions between the 9 CBZ/CBZE-specific genes identified from comparison analyses of PBMCs treated with CBZ/CBZE against TT treated PBMCs. Viral infection has been added to illustrate the 5 molecules involved with the specific function.

Table 6.4 CBZ and CBZE-specific canonical pathways derived from comparison analyses with TT positive controls. The differential expression of genes responsible for each significant canonical pathway are expressed as exp log ratio with associated p value.

Comparison Analyses	Canonical Pathways	Molecules	Exp Log Ratio	P-Value
CBZ LTT positive VS TT positive control	Interferon signalling	IFI35	↑1.142	0.046
		IFIT3	↑2.419	0.045
	Retinoic acid mediated apoptosis signalling	PARP9	↑1.424	0.035
	UVA-induced MAPK signalling	PARP12	↑1.091	0.041
	Role of lipids/lipid rafts in the pathogenesis of influenza	RSAD2	↑2.825	0.030
	Protein ubiquitination pathway	USP18	↑1.882	0.033
		USP41	↑1.259	0.050
CBZE LTT positive VS TT positive control	Prostanoid biosynthesis	PTGES	↓-1.147	0.029
	Eicosanoid signalling	PTGS2	↓-6.122	0.045
	NAD biosynthesis III	NAMPT	↓-2.367	0.047
	Dermatan sulphate biosynthesis Chondroitin sulphate biosynthesis Heparan sulphate biosynthesis	CHST15	↓1.176	0.035
	Oxidative phosphorylation	COX8A	↑1.133	0.016
	Fatty acid α-oxidation	PTGS2	↓-6.122	0.045

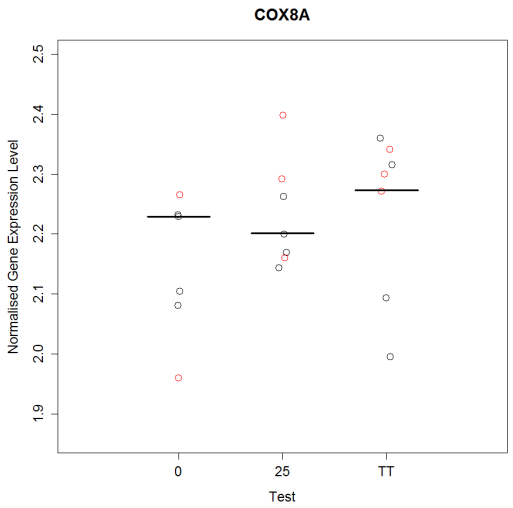
	MIF-mediated glucocorticoid regulation MIF regulation of innate immunity Role of IL-17A in arthritis IL-17 signalling CD40 signalling Role of MAPK signalling in the pathogenesis of influenza PPAR signalling HGF signalling LXR/RXR activation PI3K/AKT signalling Endothelin-1 signalling ILK signalling			

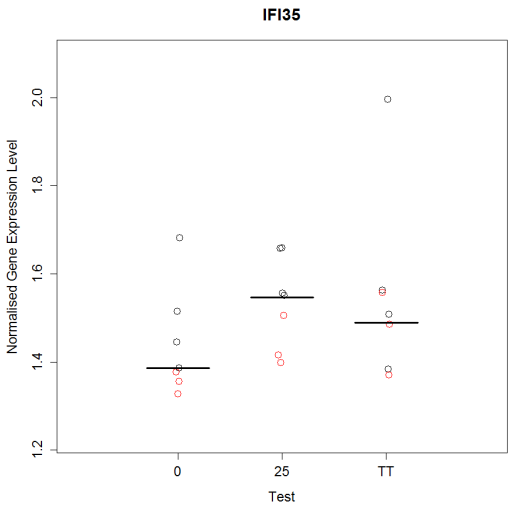
Table 6.5 Top 5 diseases and functions associated with the CBZ/CBZE-specific gene network

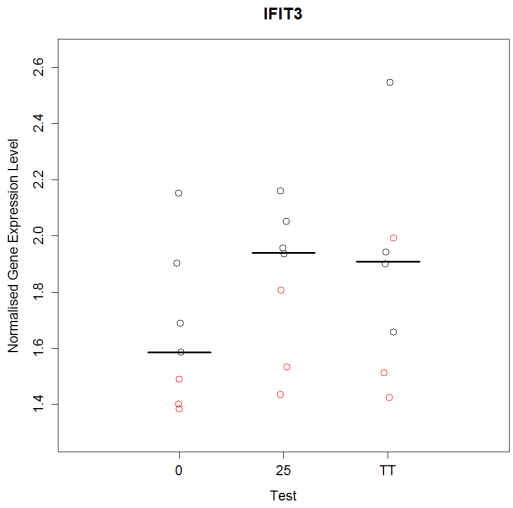
Diseases and Functions	P-value	Molecules
Viral infection	1.02×10^{-3}	IFI35, IFIT3, PARP12, PARP9, RSAD2
Psoriasis	7.86×10^{-5}	IFI35, IFIT3, PARP9, RSAD2
Antiviral response	1.09×10^{-4}	COX8A, IFIT3, RSAD2
Infection of cells	4.51×10^{-3}	IFI35, PARP9, RSAD2
Infection by RNA virus	8.39×10^{-3}	IFI35, PARP9, RSAD2

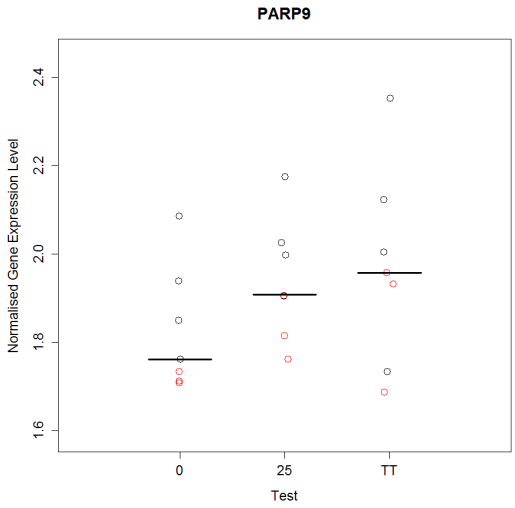
6.3.3 CBZ/CBZE-Specific mRNA Expression

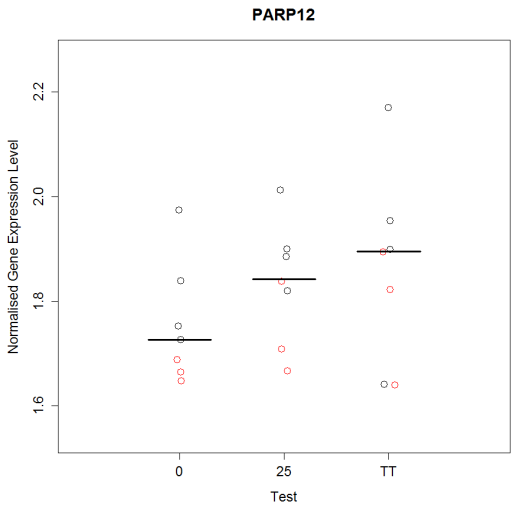
Expression values for the 9 CBZ/CBZE-specific genes were normalised according to GAPDH (Appendix, Table 9.9) and expression of each gene according to exposure are outlined in Figure 6.5. Analysis of mean expression levels of CBZ/CBZE-specific genes reveals that expression tends to be increased in PBMCs incubated with either drug or TT. However, it is not possible to identify genes that were upregulated specifically in CBZ/CBZE-incubated samples only.

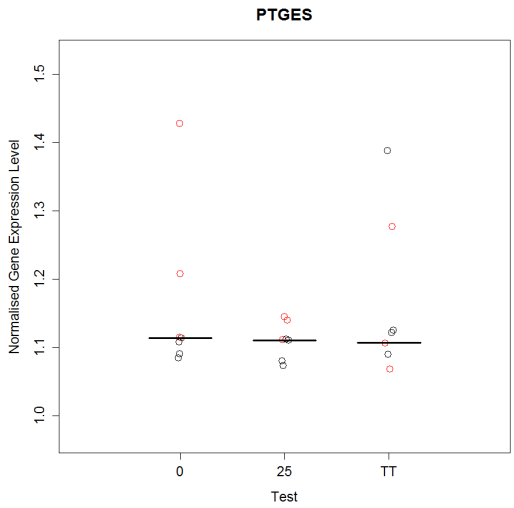

COX8A
Normalised Gene Expression Level
1.9
2.0
2.1
2.2
2.3
2.4
2.5
0
25
TT
Test


IFI35
Normalised Gene Expression Level
1.2
1.4
1.6
1.8
2.0
0
25
TT
Test


IFIT3
Normalised Gene Expression Level
1.4
1.6
1.8
2.0
2.2
2.4
2.6
0
25
TT
Test


PARP9
Normalised Gene Expression Level
1.6
1.8
2.0
2.2
2.4
0
25
TT
Test


PARP12
Normalised Gene Expression Level
1.6
1.8
2.0
2.2
0
25
TT
Test


PTGES
Normalised Gene Expression Level
1.0
1.1
1.2
1.3
1.4
1.5
0
25
TT
Test

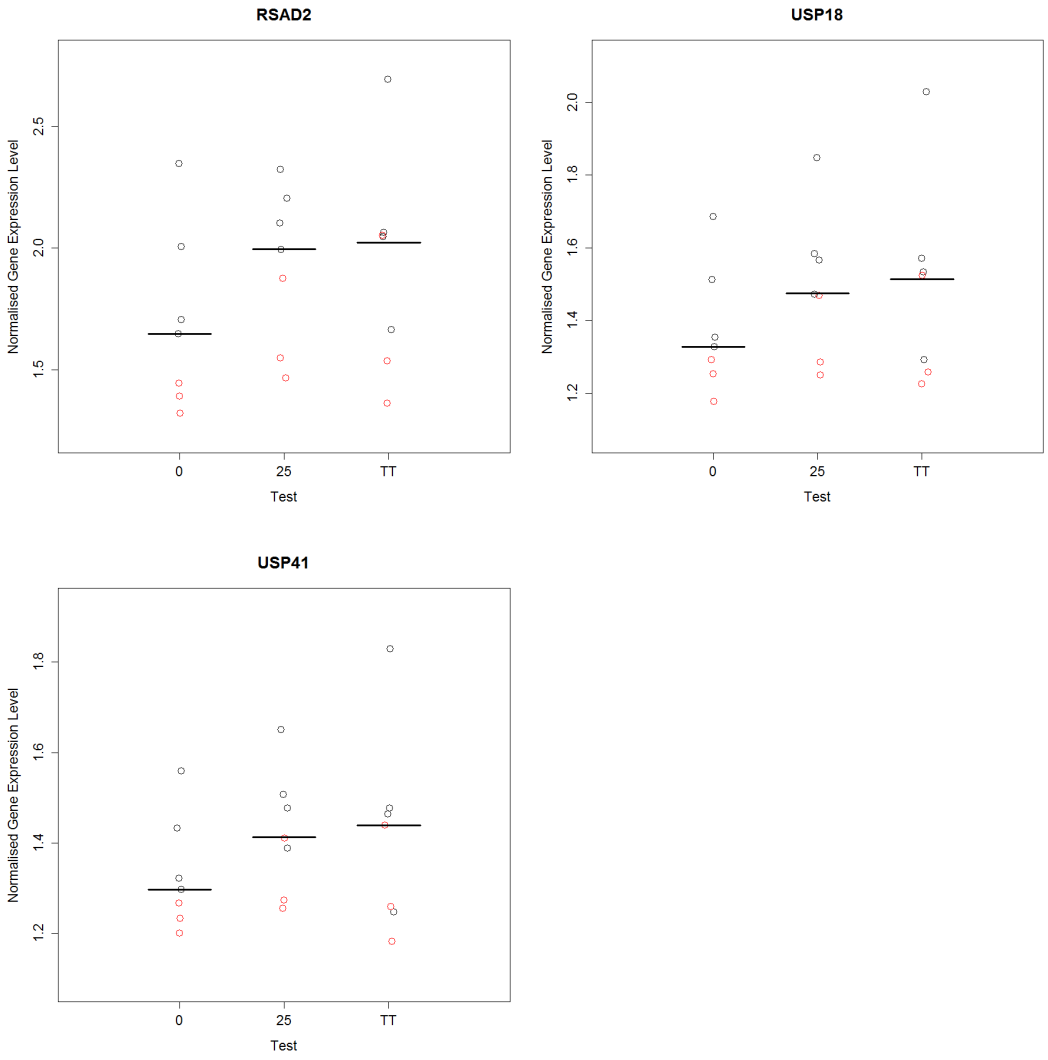


Figure 6.5 Relative expression levels of CBZ/CBZE-specific mRNA generated from comparison analyses in IPA. (mRNA expression normalised to GAPDH, black circles represent CBZ incubations, red circles represent CBZE incubations, horizontal line represents mean expression).

6.3.4 Gene Set Enrichment Analysis

GSEA was undertaken using the ranked gene lists generated from LIMMA analysis (section 6.3.1). GSEA directly scores pre-defined gene sets for differential expression and is able to identify gene sets with subtle but coordinated expression changes that cannot be detected with traditional candidate gene analysis that are based upon arbitrarily chosen threshold levels (e.g. fold changes and p-values) (Nam and Kim 2008). Gene sets with a false discovery rate (FDR) < 0.05 were considered significant. Only the top 10 enriched gene sets are included in the results section but full lists for each incubation condition are included in the appendix (Tables 9.10 to 9.16).

Gene sets involving the immune system (chemokines, cytokines and viral response) were the most significant (lowest FDR) and demonstrated the greatest enrichment when PBMCs positive to CBZ LTT were compared with PBMCs incubated in cell culture medium alone (Table 6.6). Interestingly, different gene sets were enriched in PBMCs that were positive to CBZE in LTT when compared with enriched gene sets of LTT CBZ positive PBMCs (Table 6.7). Gene sets involved in protein synthesis, RNA processing and mitochondria dominated. Only a single gene set, structural constituent of ribosome, was significantly enriched in PBMCs that were negative in the LTT to CBZ. No significantly enriched gene sets were identified from PBMCs that were negative to CBZE in the LTT. The top 10 most significantly enriched gene sets when PBMCs were incubated with TT are outlined in Table 6.8 and Table 6.9. There is significant overlap between the top 10 enriched gene sets in PBMCs incubated with TT and those incubated with CBZ or CBZE.

In total there were 51 gene sets that were exclusively enriched in PBMCs that were incubated with CBZ or CBZE and not with TT. Table 6.10 outlines the top 10 most significantly enriched gene sets specific to CBZ and CBZE incubations. Drug specific gene sets included those that regulate T lymphocytes, bind collagen and are involved in control of the cell cycle. The full list of gene sets is available in the appendix (Table 9.17).

Table 6.6 Top 10 enriched gene sets when PBMCs positive to CBZ in LTT are compared with PBMCs incubated with cell culture medium (NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR
Chemokine receptor binding	41	2.89	0
Chemokine activity	40	2.87	0
G-protein coupled receptor binding	51	2.86	0
Cytokine activity	108	2.76	0
Structural constituent of ribosome	77	2.74	0
Locomotory behavior	89	2.66	0
Response to virus	42	2.56	0
Behaviour	139	2.54	0
Response to external stimulus	296	2.35	0
Inflammatory response	124	2.34	0

Table 6.7 Top 10 enriched gene sets when PBMCs positive to CBZE in LTT are compared with PBMCs incubated with cell culture medium (NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR
Structural constituent of ribosome	77	3.05	0
RNA binding	240	2.95	0
Ribonucleoprotein complex	135	2.77	0
Ribonucleoprotein complex biogenesis and assembly	80	2.63	0
Nucleolus	115	2.63	0
RNA processing	164	2.62	0
Mitochondrion	314	2.56	0
Organelle membrane	280	2.56	0
Mitochondrial inner membrane	58	2.54	0
Intracellular organelle part	1110	2.51	0

Table 6.8 Top 10 enriched gene sets when PBMCs positive to CBZ in LTT are incubated with TT and compared with cell culture medium (NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR
Cytokine activity	108	2.93	0
Extracellular space	230	2.84	0
Extracellular region part	320	2.82	0
G-protein coupled receptor binding	51	2.75	0
Chemokine receptor binding	41	2.75	0
Chemokine activity	40	2.69	0
Response to external stimulus	296	2.68	0
Inflammatory response	124	2.62	0
Locomotory behavior	89	2.61	0
Negative regulation of cell proliferation	148	2.60	0

Table 6.9 Top 10 enriched gene sets when PBMCs positive to CBZE in LTT are incubated with TT and compared with cell culture medium (NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR
Structural constituent of ribosome	77	3.00	0
RNA binding	240	2.87	0
Ribonucleoprotein complex	135	2.78	0
Organelle membrane	280	2.67	0
RNA processing	164	2.66	0
Organelle part	1115	2.61	0
Intracellular organelle part	1110	2.60	0
Ribonucleoprotein complex biogenesis and assembly	80	2.59	0
Endoplasmic reticulum part	91	2.56	0
Nucleolus	115	2.49	0

Table 6.10 Top 10 CBZ/CBZE-specific significantly enriched gene sets (NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR	Drug
Nuclease activity	53	1.96	0.005	CBZE
Negative regulation of DNA metabolic process	17	1.93	0.007	CBZE
Collagen binding	14	1.89	0.036	CBZ
Mitochondrial small ribosomal subunit	10	1.89	0.010	CBZE
Centrosome	54	1.87	0.012	CBZE
Cellular protein complex disassembly	12	1.87	0.011	CBZE
Caspase activation	26	1.87	0.041	CBZ
Regulation of cyclin dependent protein kinase activity	42	1.83	0.049	CBZ
Positive regulation of T cell activation	20	1.82	0.017	CBZE
Positive regulation of lymphocyte activation	23	1.80	0.019	CBZE

6.3.5 Ingenuity Pathway Analysis for miRNA

Significant miRNA (Log_2-fold change < -1 or > 1, unadjusted $p < 0.05$) identified from LIMMA analysis were uploaded into IPA and comparison analyses were undertaken with CBZ and CBZE incubated PBMCs against TT incubated PBMCs to identify the miRNA transcripts differentially expressed only in drug treated cells. There were 39 miRNA molecules identified as being differentially expressed in CBZ or CBZE treated samples (Appendix, Table 9.26). The miRNA target function in IPA was used to identify mRNA targets for these 39 CBZ/CBZE-specific miRNA. In total 5794 mRNA targets were identified for 30 of the miRNA molecules. Five of the drug-specific miRNA molecules were found to target 4 CBZ/CBZE-specific mRNA (Table 6.11). The network of interactions is illustrated in Figure 6.6. The top 5 diseases and functions involving at least 3 drug specific molecules from the network were viral infection, psoriasis, inflammation of body region, inflammation of organ and antiviral response (Table 6.12).

Table 6.11 CBZ/CBZE specific miRNA with their CBZ/CBZE mRNA targets

miRNA	Exp Log Ratio	P-value	mRNA target	Exp Log Ratio	P-value
miR-433-3p	-1.087	0.020	COX8A	1.133	0.016
miR-3194-5p	-1.106	0.009	IFIT3	2.419	0.045
miR-455-3p	1.779	0.046	PARP9	1.424	0.035
miR-4723-5p	-1.786	0.047			
miR-345-5p	-1.579	0.020	RSAD2	2.825	0.030

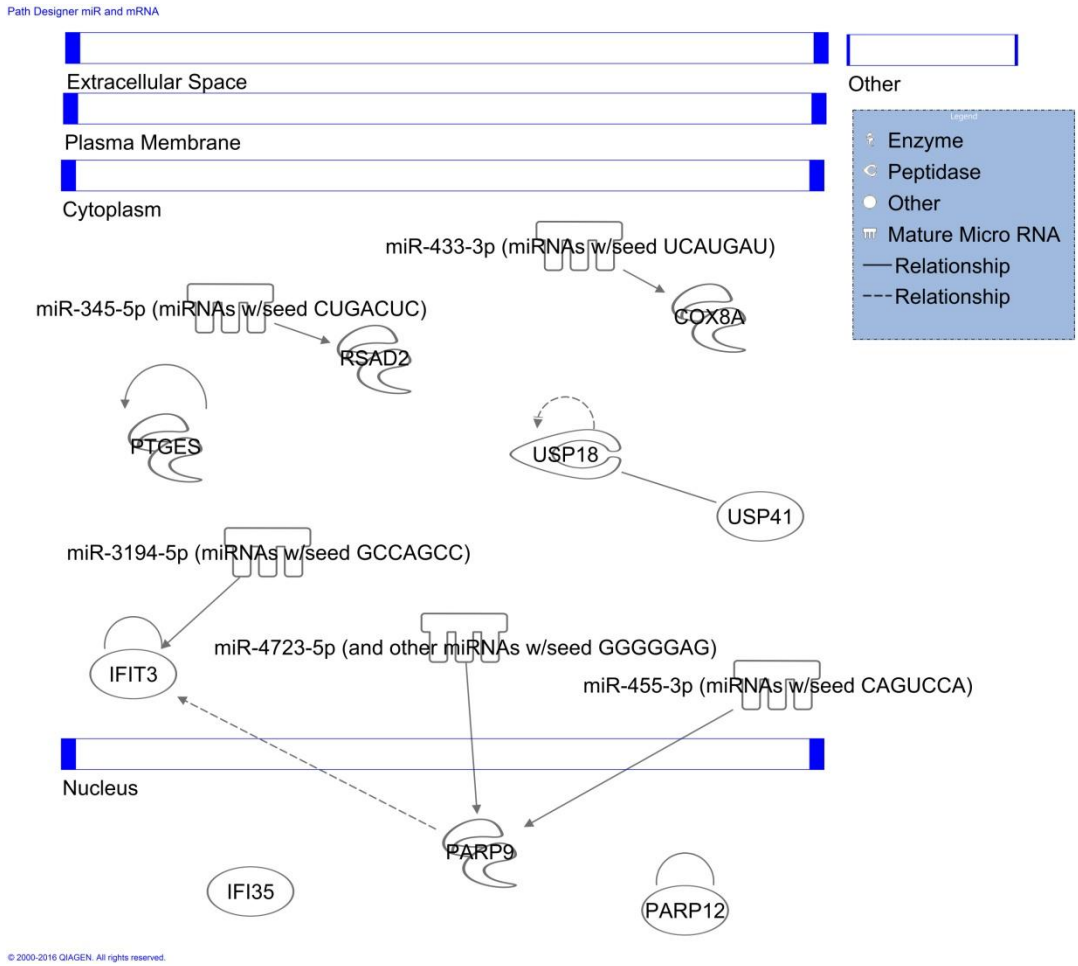


Figure 6.6 Network representation of interactions between the 9 CBZ/CBZE-specific genes and 5 overlapping CBZ/CBZE-specific miRNA identified from comparison analyses of PBMCs treated with CBZ/CBZE against TT treated PBMCs

Table 6.12 Top 5 diseases and functions associated with the CBZ/CBZE-specific mRNA and miRNA network

Diseases and Functions	P-value	Molecules
Viral infection	4.91×10^{-3}	IFI35, IFIT3, PARP12, PARP9, RSAD2
Psoriasis	2.88×10^{-4}	IFI35, IFIT3, PARP9, RSAD2
Inflammation of body region	9.40×10^{-3}	miR-345-5p, miR433-3p, PTGES, USP18
Inflammation of organ	1.71×10^{-2}	miR-345-5p, miR433-3p, PTGES, USP18
Antiviral response	2.78×10^{-4}	IFIT3, RSAD2, COX8A

6.3.6 CBZ/CBZE-Specific miRNA Expression

Expression values for the CBZ/CBZE-specific miRNA involved in the CBZ/CBZE-specific network analysis were normalised to HSA-miR-92a-3p and expression plotted in Figure 6.7 (Appendix, Table 9.27). The top 5 most upregulated CBZ/CBZE-specific miRNA identified by IPA are outlined in Table 6.13 and their expression under different incubation conditions in Figure 6.8.

Table 6.13 Top 5 up-regulated CBZ/CBZE-specific miRNA identified in IPA comparison analyses

miRNA	Exp Log Ratio	Exp P-Val	Drug
hsa-miR-548f-3p	3.052	2.44E-04	CBZE
hsa-miR-711	2.184	2.76E-02	CBZ
hsa-miR-197-5p	2.174	1.08E-02	CBZE
hsa-miR-532-3p	1.921	1.55E-02	CBZE
hsa-miR-455-3p	1.779	4.58E-02	CBZ

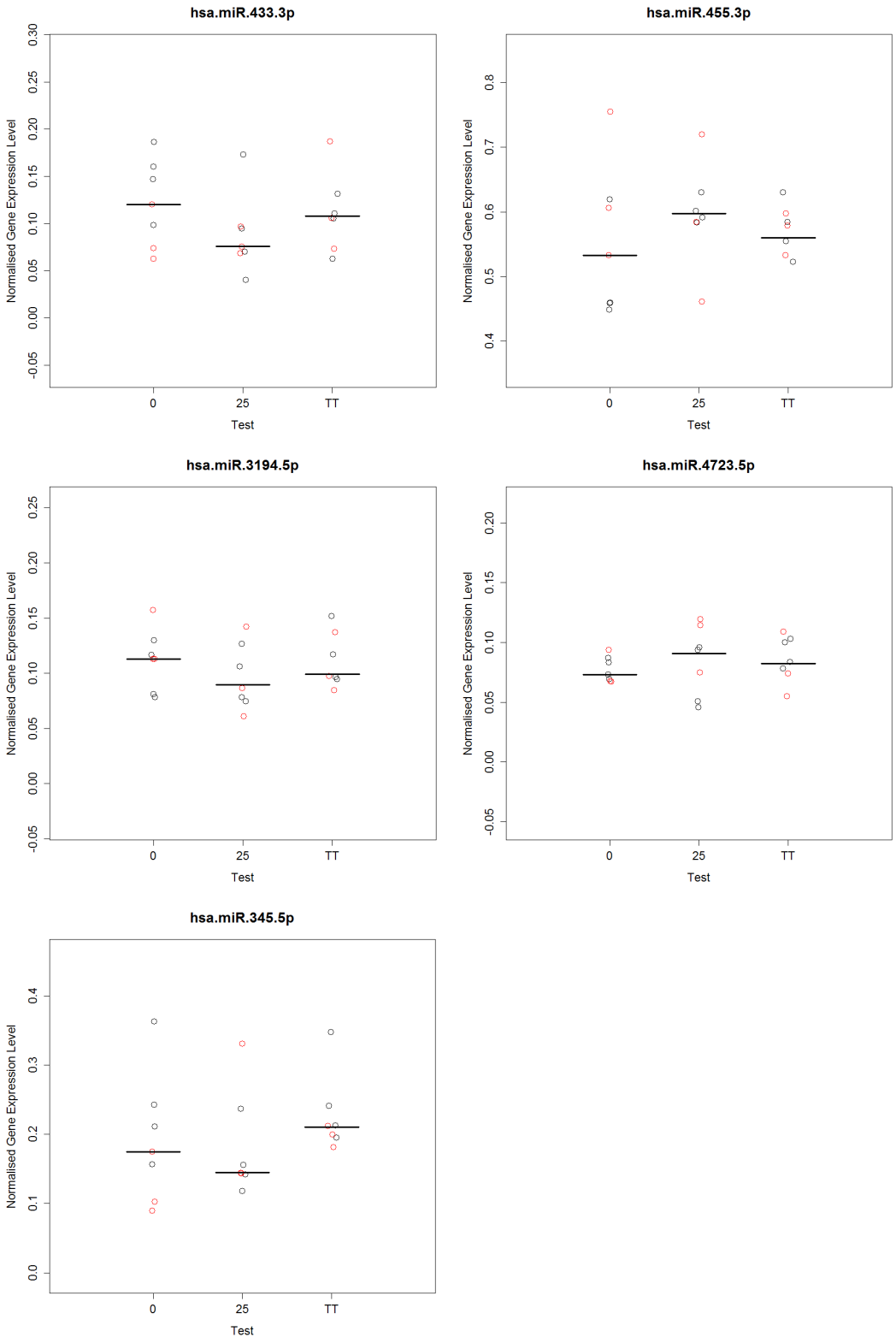


Figure 6.7 Relative expression levels of CBZ/CBZE-specific miRNA identified from the CBZ/CBZE-specific network analysis. (Expression normalised to HSA-miR-92a-3p, black circles represent CBZ incubations, red circles represent CBZE incubations, horizontal line represents mean expression).

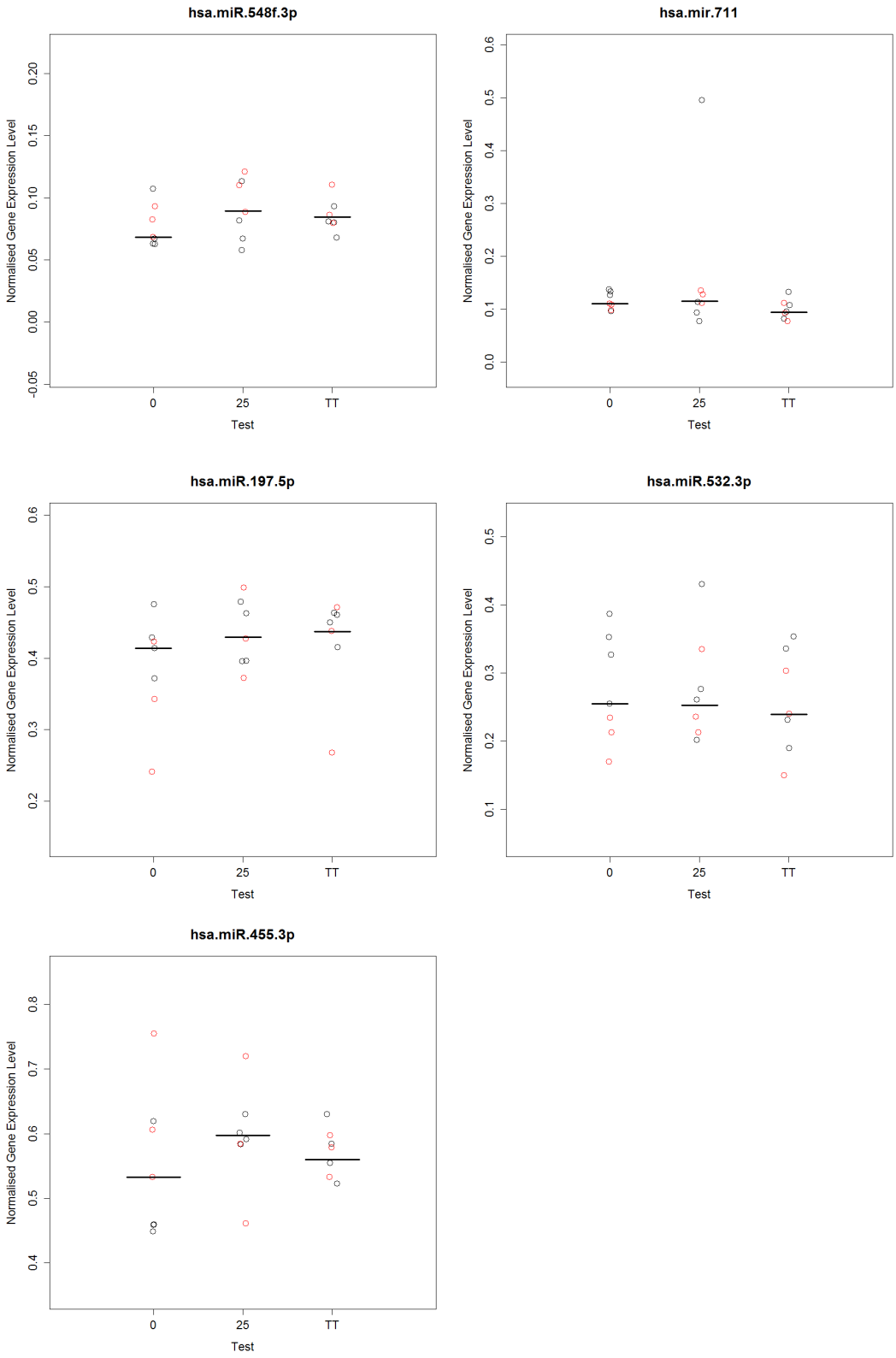


Figure 6.8 Relative expression levels of top 5 CBZ/CBZE-specific miRNA generated from comparison analyses in IPA. (Expression normalised to HSA-miR-92a-3p, black circles represent CBZ incubations, red circles represent CBZE incubations, horizontal line represents mean expression).

6.4 Discussion

Gene expression profiles were generated from PBMCs of 7 patients with a known history of CBZ hypersensitivity. The PBMCs were incubated with CBZ, CBZE, TT (positive control) or cell culture medium (negative control) *in vitro*. Differential expression of mRNA and miRNA between different incubation conditions was used to further investigate the molecular mechanisms of these reactions with IPA network analyses and GSEA.

When comparing differential gene expression using unadjusted p-value < 0.05 and Log_2-fold change <-1 or >1 there is very limited overlap in mRNA and miRNA between CBZ and CBZE incubations (Figure 6.2). This finding suggests that CBZ and CBZE activate different molecular pathways when incubated with PBMCs from CBZ hypersensitive patients *in vitro*. This was also borne out in IPA pathway analyses where the top canonical pathways for CBZ and CBZE incubations were different (Figure 6.3). Canonical pathways involving antiviral responses, apoptosis/death receptor signalling and pyrimidine processing were the most significant pathways when PBMCs were incubated with CBZ. In contrast, the most significant canonical pathways in PBMCs incubated with CBZE were involved with eicosanoid synthesis and signalling, synthesis of multiple glycosaminoglycans (GAGs) and pathways involved in regulation of the immune system.

The role of immune responses to viruses has been implicated in both HLA-restricted and non-HLA associated hypersensitivity reactions (Picard et al. 2010; Lucas et al. 2015). A prospective assessment of a cohort of 40 patients diagnosed with DRESS to CBZ, allopurinol and sulphamethoxazole identified reactivation of EBV, HHV-6, or HHV-7 reactivation in 76% of the patients. The authors propose that the culprit drug(s) induce viral reactivation and antigenic presentation of quiescent forms of EBV or other herpes viruses which trigger a multi-organ immune response (Picard et al. 2010). Abacavir-reactive memory T cells have been identified in drug-naive individuals who are *HLA-B*57:01* positive. T cell responses to persistent viral infections (e.g. HHV) have been shown to mediate allogenic organ rejection via TCR cross reactivity and a similar heterologous immunity model may explain the

existence of drug-reactive T cells in absence of prior exposure to the culprit drug (Lucas et al. 2015). In SJS/TEN, cytotoxic CD8+ T cells and the cytotoxic molecules FasL (Fas ligand) and granulysin lead to disseminated keratinocyte apoptosis (Viard et al. 1998; Chung et al. 2008). Activation of canonical pathways involved in apoptosis and death receptor signalling in PBMCs activated by CBZ is therefore consistent with the pathogenesis of SJS/TEN. Interestingly, retinoic acid mediated apoptosis signalling is the second most significant canonical pathway activated by CBZ in PBMCs. It has been hypothesised that medications implicated in SJS/TEN synergise with endogenous retinoids leading to accumulation of retinoids in the liver and cholestatic dysfunction (Mawson et al. 2015). This leads to toxic retinoid compounds that are normally stored in the liver and excreted harmlessly into the intestinal tract to be spilled into the circulation inducing cytotoxicity and apoptosis via granulysin with manifestation of clinical symptoms similar to SJS/TEN (Mawson et al. 2015).

Eicosanoids, which include prostaglandins (PG), are locally acting bioactive signalling lipids derived from arachidonic acid which regulate a diverse set of homeostatic and inflammatory processes (Funk 2001). In PBMCs treated with CBZE there is a reduction in the expression of the gene PTGES. PTGES encodes for the enzyme PGE synthase which catalyses the formation of PGE2 (Jegerschold et al. 2008). PGE2 has been shown to compromise immunity to influenza A virus through inhibition of macrophage antigen presentation and T cell immunity (Coulombe et al. 2014). Reduced expression of PTGES in PBMCs exposed to CBZE may be in response to activation of antiviral pathways in order to enhance antiviral immunity. Dermatan sulphate, chondroitin sulphate and heparan sulphate are GAGs (Esko et al. 2009). GAGs have been shown to play important roles in the immune system which includes modulation of cytokine function, detection of tissue injury, activation of inflammation and as potential targets for bacterial and viral virulence factors that contribute to immune system evasion (Trowbridge and Gallo 2002; Davis and Parish 2013). CBZE leads to reduced expression of the gene *CHST15* in PBMCs which encodes for a sulfotransferase protein important in synthesis of dermatan sulphate, chondroitin sulphate and heparan sulphate (Wu et al. 2011).

CBZE therefore reduces GAG synthesis in PBMCs but the effect on hypersensitivity reactions is unclear as GAGs have both pro-inflammatory and anti-inflammatory effects (Davis and Parish 2013). Patients prescribed CBZ will be exposed to both the parent drug and CBZE *in vivo* as CBZE is the major metabolite of CBZ metabolism (Kerr et al. 1994). These data suggest that exposure to both CBZ and CBZE could contribute to hypersensitivity via multiple molecular pathways and may explain the diverse clinical phenotypes observed in CBZ hypersensitivity reactions.

Comparison analyses were undertaken between PBMCs incubated with CBZ/CBZE and TT using IPA to identify canonical pathways and genes significant in CBZ/CBZE treated samples only. Nine drug-specific genes were identified and a network involving all nine genes was created. Viral infection was the most significant disease/function associated with the CBZ/CBZE-specific pathway providing further evidence to support the role of heterologous immunity in pathogenesis of CBZ hypersensitivity reactions. Heterologous immunity proposes that effector memory T cells are generated when viral peptides (e.g. HHV) are presented with risk HLA alleles (e.g. *HLA-A*31:01*). When a patient is subsequently exposed to the culprit drug/metabolite (e.g. CBZ or CBZE) it is presented with the risk HLA and the effector memory T cells originally primed against viral pathogens are cross-reactive to the drug/metabolite leading to hypersensitivity (White et al. 2015). Psoriasis was the second most significant disease associated with the CBZ/CBZE-specific network. Psoriasis is a chronic inflammatory autoimmune disease characterised by aberrant hyperproliferation of keratinocytes. The pathogenesis of psoriasis shares similarities with hypersensitivity reactions and involves a complex interplay of immune cell functions, cytokines and T cell responses (Deng et al. 2016).

Gene set analysis aims to overcome the problems associated with individual gene analysis methods where selection of significant genes is typically based upon arbitrarily chosen threshold values (Nam and Kim 2008). Alteration of the chosen thresholds can result in alternative biological conclusions and the omission of genes with moderate but meaningful expression changes if the thresholds selected are too strict reducing statistical power (Pan et al. 2005). GSEA evaluates the entire

microarray dataset at the level of gene sets (Subramanian et al. 2005). The gene sets are defined based on existing biological knowledge and enable the identification of sets of genes with subtle but coordinated expression changes that would not be detectable using individual gene analysis (Nam and Kim 2008). In contrast to individual gene and canonical pathway analysis above where there was little overlap between different incubation conditions, GSEA demonstrated considerable overlap in enriched gene sets of PBMCs from the same subjects that were incubated with CBZ or CBZE when compared with TT. Lack of overlap in statistically significant genes when different groups study the same biological systems is a problem frequently encountered with individual gene analysis (Fortunel et al. 2003).

Fifty one gene sets were significantly enriched in CBZ or CBZE treated PBMCs only. Gene sets involved in collagen binding, positive regulation of T cell activation and caspase activation were significantly enriched in drug treated cells and are consistent with the known pathogenesis of hypersensitivity reactions to CBZ, with identification of drug-specific T cells and keratinocyte apoptosis in SJS/TEN (Naisbitt et al. 2003; Chung et al. 2008). Other gene sets that were significantly enriched involved regulation of cell cycle and processes pertaining to synthesis of DNA and protein complexes (Table 6.10). Cyclins and cyclin-dependent kinases (CDKs) are important regulators in the control of the cell cycle (Malumbres 2014). Owing to their role in cell proliferation and other diverse processes (e.g. apoptosis and regulation of transcription), development of drugs that target CDK have been developed for cancer and other therapeutic areas (Roskoski 2016). Current management options for drug-induced hypersensitivity reactions are limited and include withdrawal of the suspected culprit drug and systemic corticosteroids (Shiohara et al. 2012). CDK inhibitor drugs have been demonstrated experimentally to have anti-inflammatory and pro-resolution properties through promotion of neutrophil and lymphocyte apoptosis (Leitch et al. 2009). These findings raise the possibility that CDK inhibitors could be used to treat acute hypersensitivity reactions in the future.

Five CBZ/CBZE-specific miRNAs were found by network analysis in IPA to interact with the CBZ/CBZE-specific gene network. The top 5 functions of the CBZ/CBZE-specific miRNA and mRNA network was similar to the network involving only the mRNA with viral infection and psoriasis incorporating the most number of mRNA and miRNA transcripts. However, addition of miRNA has also highlighted inflammation as being an important function regulated by the network. This is unsurprising as the clinical manifestation of CBZ hypersensitivity reactions involves inflammation of multiple organs (Yip et al. 2015a). Inspection of the CBZ/CBZE-specific network reveals that *PARP9* is the gene that is most highly regulated. PARP9 [poly (ADP-ribose) polymerase] has recently been shown to enhance cellular responses to interferon which markedly improved antiviral function in transgenic mice and transduced human cells (Zhang et al. 2015). In these studies PARP9 formed a complex with DTX3L to promote interferon-stimulated gene expression by interaction with STAT1 and degradation of viral proteases through ubiquitin ligase functionality. These functions share similarities with the CBZ/CBZE-specific canonical pathways that were identified in IPA analysis where interferon signalling and protein ubiquitination were upregulated in CBZ treated PBMCs. Small molecule interferon signal-enhancer compounds have been identified using high throughput screening as possible candidates for new antiviral therapy (Patel et al. 2014). It may be possible that these new antiviral agents could also be used to treat CBZ hypersensitivity reactions.

There have been a very limited number of studies that have studied transcriptomic profiles in drug hypersensitivity (Bellon et al. 2010; Ichihara et al. 2014). Bellon et al. (2010) isolated PBMCs from 23 patients during an acute hypersensitivity reaction with a repeat sample upon resolution of the symptoms. Eighty-five genes were differentially expressed during the acute phase of hypersensitivity reactions with the majority involved in apoptosis, the cell cycle and the immune response. Although the functions of the genes that were differentially expressed are similar to the results observed in this chapter there is very little overlap in individual mRNA transcripts. These discrepancies could be explained by different experimental design. Bellon et al. (2010) recruited patients with hypersensitivity to multiple drugs

(including 2 cases of CBZ), PBMCs were exposed to the suspected culprit drug/metabolite *in vivo* rather than *in vitro* and analysis of gene expression was undertaken only at the individual gene level with arbitrarily defined significance levels (1.5-fold change and unadjusted $p < 0.005$). Upregulation of miR-18a-5p was detected in skin and serum samples from patients with acute TEN (Ichihara et al. 2014). The miR-18a-5p was found to mediate keratinocyte apoptosis through downregulation of the expression of B-cell lymphoma/leukaemia-2-like protein 10, an anti-intrinsic apoptotic molecule. The authors concluded that serum miR-18a-5p levels could be a useful disease marker for drug eruptions. Significant upregulation of miR-18a-5p was also observed in our experiments when PBMCs were incubated with CBZE implying that it may also have a role in CBZ hypersensitivity. However, it was also detected in TT incubations suggesting that it may not be drug-specific *in vitro*.

Diagnosis of drug hypersensitivity is complex and dependent on patient history and clinical judgement. *In vitro* tests (e.g. LTT) are time-consuming, lack sufficient sensitivity and specificity; whilst *in vivo* tests (e.g. patch testing or drug provocation) are not applicable in all cases of hypersensitivity (Schrijvers et al. 2015). In this chapter, 9 CBZ/CBZE-specific mRNA and 39 CBZ/CBZE-specific miRNA have been identified. Analysis of expression profiles of these transcripts was unable to identify any that were specific to CBZ or CBZE *in vitro* as expression was also elevated in TT incubations (Figure 6.5, Figure 6.7 and Figure 6.8). However, they may demonstrate utility *in vivo*, during the acute hypersensitivity reaction, as outlined above with miR-18a-5p (Ichihara et al. 2014).

The limitations of this study include the small number of patients analysed and the lack of validation of gene expression with RT-PCR due to time constraints. Also, PBMCs were incubated with CBZ or CBZE and it is unknown if the parent drug or major metabolite are responsible for hypersensitivity *in vivo*.

In conclusion, gene expression profiles were generated using PBMCs obtained from 5 patients with a history of CBZ hypersensitivity. A list of CBZ/CBZE-specific mRNA

and miRNA were generated that may serve as biomarkers for diagnosis of CBZ hypersensitivity in future. The differential gene expression profiles have provided further evidence for involvement of the immune system in CBZ hypersensitivity reactions and identified specific therapies, such as CDK inhibitors or interferon signal enhancers, which could be treatment options for CBZ hypersensitivity reactions in the future.

Chapter 7

Final Discussion

The overall aim of this thesis was to identify and connect the metabolic and immunological risk factors that are associated with the development of CBZ hypersensitivity reactions. Despite extensive research into CBZ hypersensitivity, the exact pathophysiological mechanism(s) of these reactions is still not fully understood but is likely to involve a combination of genetic, immunological and metabolic factors. Predisposition to CBZ hypersensitivity is thought to be genetic because of cases reported in families and monozygotic twins (Edwards et al. 1999). The clinical features of CBZ hypersensitivity and discovery of CBZ-specific T cells provide evidence that the reactions are mediated by the immune system (Naisbitt et al. 2003; Wu et al. 2006; Wu et al. 2007). Specific HLAs, *HLA-B*15:02* and *HLA-A*31:01*, have been strongly associated with susceptibility to CBZ hypersensitivity (Chung et al. 2004; McCormack et al. 2011). However, the HLA associations are both phenotype- and ethnicity-specific and carriage of the risk HLA allele alone is insufficient to trigger a hypersensitivity reaction when exposed to CBZ (Yip et al. 2012). These findings imply the involvement of other as yet unidentified factors.

The metabolism of CBZ is complex and leads to the formation of greater than 30 metabolites (Lertratanangkoon and Horning 1982). Several reactive metabolites have been proposed to be responsible for CBZ hypersensitivity and include an arene oxide (Pirmohamed et al. 1992), 9-acridine carboxaldehyde (Furst et al. 1995), an iminostilbene (Ju and Uetrecht 1999), an *o*-quinone (Lillibridge et al. 1996) and CBZE (Bu et al. 2005). Taken together, these studies provide some evidence that CBZ hypersensitivity reactions involve both metabolic and immunological risk factors.

The exact chemical identity of the antigen that triggers hypersensitivity to CBZ is unknown – of course, it may be that multiple antigens are involved, and the antigen profile may vary from patient to patient. CBZ is metabolised by multiple CYP450 isoforms but the major route of metabolism is conversion to CBZE (Pearce et al. 2008). CBZE has been demonstrated to be chemically reactive through the spontaneous formation of two isomeric adducts with GSH *in vitro* (Bu et al. 2005). In chapter 2 of this thesis, CBZE was demonstrated to form protein-adducts not only

with GSH but also with HSA. HSA is the most abundant plasma protein constituting around 60% of total body proteins and is able to circulate freely throughout the body. It has thus been extensively used *in vitro* as a model protein to study the formation of active drug metabolites because it contains many nucleophilic amino acid residues (Osaki et al. 2014). CBZE was found to modify HSA at his146 (^{145}R**H**([O]CBZ)PYFYAPELLFFAK^{159}) in a concentration- and time-dependent manner *in vitro*. The same CBZE-modified HSA adduct was also identified *in vivo* from 20 patients with epilepsy who had been taking CBZ at a stable dose for at least 4 weeks. However, it is important to note that these patients were tolerant of CBZ and did not have any symptoms of hypersensitivity. Despite this, it remains possible that the CBZE-modified HSA adduct is one of the antigens responsible for hypersensitivity. Other, as yet unidentified risk factors, such as immune system variation (e.g. HLA, TCR subtype) or polymorphisms in drug metabolising enzymes (e.g. reduced clearance of CBZE) may influence susceptibility. Indeed, there was 16-fold variation in the relative quantity of CBZE-modified adduct identified in patients and only moderate correlation with measured plasma levels of CBZ (r^2 = 0.4387, p = 0.0015) and CBZE (r^2 =0.3545, p = 0.0056) suggesting that variability in exposure will occur between patients and consequently may be a factor in determining susceptibility. Adducts between beta-lactam antibiotics (flucloxacillin, piperacillin and amoxicillin) and HSA have been isolated from patients and demonstrated to activate T cells isolated from hypersensitive individuals *in vitro* (Jenkins et al. 2013; Yaseen et al. 2015). A strategy to determine whether the CBZE-modified HSA adduct is able to stimulate T cell responses would involve chemical synthesis of the CBZE-modified peptide and incubation with T cells isolated from patients with CBZ hypersensitivity in experiments similar to those described in chapter 5.

The second quantitatively most important metabolic pathway for CBZ involves the formation of several phenolic products (e.g. 2OH-CBZ and 3OH-CBZ) by hydroxylation at different positions of the six-membered aromatic rings (Spina 2002). These reactions are catalysed by various CYP450 isoforms including CYP3A4, CYP2E1 and CYP2B6 (Pearce et al. 2002). There is indirect evidence that formation of these phenolic compounds proceeds through a highly reactive arene oxide

intermediate (Madden et al. 1996; Bu et al. 2007). In chapter 2, relative formation of the presumed arene oxide conjugate with GSH was positively correlated with the aromatic hydroxylase activity of specific CYP isoforms. Mass spectrometric evidence for the existence of the arene oxide intermediate was generated by incubation of CBZ with microsomes/supersomes and HSA. The arene oxide modified HSA at his146 which is consistent with the amino acid residue modified by CBZE. These results provide the first chemical evidence for the existence of an arene oxide intermediate and its ability to escape the immediate microsomal environment and modify proteins. The clinical manifestations of CBZ hypersensitivity are highly variable affecting multiple organ systems including the skin and liver (Yip et al. 2012). It is possible that localised metabolism of CBZ, for example in the skin, to a highly reactive arene oxide intermediate may overcome the limited detoxification pathways leading to keratinocyte death. Incubation of CBZ with HaCaT cells (an immortalised keratinocyte line) led to the generation of low levels of CBZE, 2OH-CBZ and 3OH-CBZ as detected by mass spectrometry (Yip, unpublished data). This provides evidence for the generation of reactive metabolites in keratinocytes *in vitro* but detection of drug metabolites in skin biopsy from patients with an acute hypersensitivity reaction *in vivo* using techniques such as mass spectrometric imaging (Cobice et al. 2015) or Raman spectroscopy (Franzen and Windbergs 2015) would provide direct evidence for such a mechanism.

Based on the identification of the CBZE and arene oxide intermediates, it was hypothesised that bioactivation of CBZ to greater amounts of the two reactive metabolites may increase susceptibility to CBZ hypersensitivity reactions through formation of protein adducts. A HPLC-MS/MS assay was set up and validated in chapter 3 to measure plasma levels of CBZ, CBZE, 2OH-CBZ, 3OH-CBZ and DiOH-CBZ in patient plasma. CBZE is metabolised to DiOH-CBZ (Thorn et al. 2011) and 2OH-CBZ and 3OH-CBZ act as surrogates for arene oxide formation (Madden et al. 1996). Variation in plasma levels of CBZ and metabolites of CBZ may be secondary to genetic variation in drug metabolising enzymes and transporters known to be involved in the disposition of CBZ (Table 1.4). However, there are numerous conflicting reports regarding the effect of genetic variation on the metabolism of

CBZ and this is likely to be secondary to inadequate sample sizes and analysis of the parent compound alone in the majority of studies. Healthy volunteers (n=8) and patients with epilepsy who were prescribed CBZ (n=72) were recruited into two clinical studies. A mixture of rich and sparse PK samples, blood for genotyping and medical history for each patient were collected. All patients were genotyped for 20 SNPs in drug metabolising enzymes and transporters known to be involved in CBZ disposition. The data showed that there was 14-fold variation in levels of CBZ and approximately 30-fold variation for each of the metabolites.

Population PK is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of the drug of interest (Aarons 1991). It was the original intention to set up a population PK model using NONMEM to describe the PK of CBZ and its metabolites and to examine the effects of the clinical demographics and genotypes as covariates. Unfortunately, the final population PK model did not include metabolites as it was too complex, with simulations that were very time-consuming and which ultimately did not describe the data accurately. Due to time constraints, a population PK model examining CBZ alone was developed in chapter 4 although work on the multiple metabolite PK model is ongoing. The population PK model for CBZ reported that completion of autoinduction, total daily dosage and concomitant medication with phenytoin significantly influenced the clearance of CBZ which is consistent with covariates reported in other studies (Table 1.6). None of the SNPs in metabolising enzymes were reported as significant in the current population PK model. This is possibly secondary to small population size meaning the study was underpowered to detect significant differences or the genotypes exert their influence on the metabolism of specific metabolites that were not part of the current population PK model. Alternatively, in order to influence the PK of CBZ it may require a combination of SNPs in metabolising enzymes and the presence of specific clinical factors. For example, one study found that SNPs in the drug transporter ABCB1 influenced the CBZ/CBZE ratio only in patients receiving concomitant phenytoin or phenobarbital therapy and no effect was seen with the SNPs alone (Wang et al. 2015). Another study reported that patients required

higher dosages of CBZ only if they possessed a combination of SNPs in both *UGT2B7* and *ABCC2* (Ma et al. 2015).

ADRs represent a significant clinical problem accounting for 6.5% of UK hospital admissions and cost the NHS £466 million each year (Pirmohamed et al. 2004). ADRs also lead to withdrawal of medications after licensing at great cost to the drug industry (FDA Centre for Drug Evaluation and Research 2005). Hypersensitivity to CBZ affects up to 10% of patients that are prescribed the drug and clinical presentation includes MPE, HSS, SJS and TEN. CBZ hypersensitivity is a type B ADR that is unpredictable from the known pharmacology of the drug and is (probably) dose-independent (Edwards and Aronson 2000). These reactions are T cell mediated, and represent type IV hypersensitivity reactions (Demoly et al. 2014), as CBZ-specific T cells have been isolated from patients with a history of hypersensitivity to CBZ (Naisbitt et al. 2003; Wu et al. 2006; Wu et al. 2007). HLA also play an important role in hypersensitivity to CBZ with carriage of *HLA-B*15:02* or *HLA-A*31:01* being associated with significantly increased susceptibility to CBZ hypersensitivity reactions (Chung et al. 2004; McCormack et al. 2011). However, a significant number of patients that possess the risk HLA alleles do not develop hypersensitivity when prescribed CBZ indicating other factors that affect susceptibility (Yip et al. 2012). Three mechanisms have been proposed to explain how drugs or reactive metabolites activate T cells: the hapten hypothesis (Landsteiner and Jacobs 1935), the direct PI concept (Pichler et al. 2006) and the altered peptide repertoire model (Ostrov et al. 2012; Illing et al. 2012; Norcross et al. 2012). There is evidence to suggest that CBZ can activate T cells through multiple mechanisms including direct interaction (Naisbitt et al. 2003; Zhou et al. 2015), altered peptide repertoire (Illing et al. 2012) and formation of haptens (Chapter 2).

In chapter 5, TCCs were generated from patients with a known history of hypersensitivity to CBZ to further investigate the pathophysiological mechanisms in CBZ hypersensitivity. TCCs were successfully generated to CBZ and CBZE from two subjects who reported hypersensitivity reactions to CBZ more than 25 years ago. Seventeen clones were generated to CBZ and 11 clones to CBZE all of which were

CD4+ phenotype. The TCCs secreted the cytokines IFN-γ, IL-13, granzyme B and perforin. These cytokines have been detected in skin biopsy, blood samples and biopsy fluid from subjects with MPE, SJS and TEN (Posadas et al. 2002; Caproni et al. 2006). A significant number of TCCs generated were cross reactive to CBZ or CBZE. Previous experiments have shown *in vitro* lymphocyte proliferation to CBZ, CBZE, 10-hydroxycarbamazepine and oxcarbazepine, but lack of proliferation to the metabolites 2OH-CBZ, 3OH-CBZ and DiOH-CBZ (Wu et al. 2006). The antigen pulsing assay provides indirect evidence for the hapten mechanism of hypersensitivity. The drug is incubated with APCs for 16h before washing to remove excess drug followed by incubation with drug specific TCCs. All TCCs responded when incubated with fresh drug supporting the PI concept for T cell activation. Interestingly, a single TCC also responded in the incubation containing only washed APCs implying a hapten mechanism of activation. The identity of the proposed hapten could not be determined, and it is not possible to exclude metabolism of CBZ in the T cell culture medium which also contained abundant HSA.

Two HLA class I alleles, *HLA-B*15:02* (Chung et al. 2004) and *HLA-A*31:01* (McCormack et al. 2011), have been associated with susceptibility to CBZ hypersensitivity. However, all of the TCCs generated in this study were CD4+ and as expected TCC activation was blocked by MHC class II antibody but not by an antibody to MHC class I. These results suggest that CD4+ lymphocytes also play a role in the pathogenesis of CBZ hypersensitivity. This is further supported by the generation of CBZ-specific CD4+ TCCs from a patient positive for *HLA-A*31:01*; the CD4+ TCCs were activated in a *HLA-DBRB1*04:04* restricted manner (Lichtenfels et al. 2014). The pathogenesis and phenotype of nevirapine hypersensitivity reactions has been associated with specific combinations of class I and class II HLA alleles (Keane et al. 2014) and a similar pathophysiological mechanism may be applicable to CBZ hypersensitivity reactions. T cells that are responsive to CBZ and or its metabolites persist in the circulation of patients for many years (25 years in this study). It has been proposed that these long lasting T cells are maintained by heterologous immune responses to other antigens such as viruses (e.g. HHV and EBV) (White et al. 2015). There is significant overlap in the clinical presentation of

drug hypersensitivity and viral illness (e.g. exanthema and liver inflammation) and reactivation of HHV6 in CBZ induced HSS is associated with slower recovery (Aihara et al. 2003). Taken together these data demonstrate that CBZ hypersensitivity is characterised by plurality of T cell responses reacting with multiple antigens using different mechanistic pathways.

The diagnosis of hypersensitivity to CBZ and other drugs is very difficult as there is significant heterogeneity in clinical presentation and clinical history can be limited with many patients taking multiple medications. Incorrect diagnosis leads to the inappropriate exclusion of effective treatments and switching to suboptimal therapies (Demoly et al. 2014). There are a variety of *in vivo* (skin testing and drug provocation testing) and *in vitro* tests (LTT, ELISpot and flow cytometry) available but none are routinely used in clinical practice (Schrijvers et al. 2015). *In vivo* testing involves re-administration of the suspected drug to the patient but is associated with risk of serious ADR and cannot be used in patients who have experienced severe forms of hypersensitivity (e.g. SJS/TEN) (Demoly et al. 2014). *In vitro* tests are more acceptable to patients but are time-consuming, involve radiation in the case of LTT, require specialised cell culture laboratories and cannot be used during the acute hypersensitivity reaction (Naisbitt et al. 2014). If the identity of the culprit antigen is unknown or it is a reactive metabolite, then it is not possible to test hypersensitivity *in vitro* at present. Pre-treatment HLA genotyping for *HLA-B*57:01* has demonstrated clinical utility and cost effectiveness in abacavir therapy (Hughes et al. 2004). Currently, pre-treatment screening for *HLA-A*31:01* is not mandated for CBZ but *HLA-B*15:02* screening is recommended in patients of South East Asian origin (Ferrell and McLeod 2008). There is an urgent clinical need for diagnostic tests for drug hypersensitivity. It is likely that a combination of tests will be necessary with rapid *in vivo* biomarkers able to confirm drug hypersensitivity in the acutely unwell patient whilst longer term *in vitro* tests enable the mechanisms of hypersensitivity to be unravelled and to allow structurally similar compounds to the culprit drug to be tested and avoided in the patient if they test positive.

In chapter 6, gene expression profiles and transcriptomic analyses were undertaken using PBMCs from patients with known CBZ hypersensitivity in order to investigate the pathophysiological mechanisms of hypersensitivity to CBZ and to explore the possibility of using mRNA or miRNA as biomarkers for diagnosis. Using IPA, 9 mRNA and 39 miRNA molecules were identified to be differentially expressed in CBZ/CBZE incubated cells when compared with negative controls and TT incubations. A network analysis incorporating the CBZ/CBZE specific mRNA and miRNA was generated in IPA and the top 5 functions associated with the network were viral infection, psoriasis, inflammation of body region/organ and antiviral response. These functions are consistent with the clinical presentation of CBZ hypersensitivity (Yip et al. 2012) and provide further evidence for the association between CBZ hypersensitivity and viral reactivation (White et al. 2015). Interestingly, psoriasis was also a function reported to be associated with the CBZ/CBZE specific mRNA and miRNA network. There is evidence to suggest overlap of disease mechanisms between autoimmune diseases, such as psoriasis, and drug mediated hypersensitivity reactions. Both autoimmune disease and drug hypersensitivity are associated with dysregulation of the immune system and increased susceptibility with carriage of specific HLA alleles (Michels and Ostrov 2015). In psoriasis, T cells are directed against the dermis (Liu et al. 2008) and dysregulation of T cells may impair the immune system's ability to correctly self-regulate (Soler et al. 2016). Susceptibility to psoriasis has also been associated with carriage of specific HLA alleles (Indhumathi et al. 2016) and response to treatment may also be predicted by carriage of the same *HLA-C*06* allele (Talamonti et al. 2016).

MicroRNAs are small noncoding RNAs that are 22 nucleotides on average and regulate mRNA expression in a large variety of cellular processes including immune system regulation, cell development and apoptosis (Bartel 2004). MicroRNAs are attractive biomarkers for diagnosis because they are likely to play an important role in onset of disease and progression and are easily measureable in biofluids and biological samples (Aziz 2016). MicroRNAs have been explored for the diagnosis of acute coronary syndrome (Ahlin et al. 2016), drug-induced kidney injury (Pavkovic and Vaidya 2016) and cancer (Shi 2016). Elevated levels of the miR-18a-5p were

reported in the serum and skin biopsy samples of patients with hypersensitivity to multiple medications (including 2 cases of CBZ) (Ichihara et al. 2014). The association was strongest with TEN and serum concentrations demonstrated a positive correlation with severity of hypersensitivity. The miR-18a-5p was also detected in chapter 6 but expression was elevated in both CBZE and TT incubated PBMCs suggesting that it may not be drug specific *in vitro*. Further investigation of miR-18a-5p levels in patients with viral or bacterial infections is necessary to determine if it is an acceptable diagnostic biomarker *in vivo*. The list of 9 mRNA and 39 miRNA moelcules identified in chapter 6 represent a collection of potential diagnostic biomarkers but requires validation in a larger cohort of patients with hypersensitivity to CBZ. It would also be worth investigating if the mRNA and miRNA are applicable biomarkers in hypersensitivity to other drugs. In the future, it will be important to obtain skin biopsy and blood samples from patients with acute hypersensitivity to determine if the mRNA and miRNA transcripts are applicable for acute *in vivo* diagnosis.

The current management for hypersensitivity reactions to CBZ and other medications is withdrawal of the suspected culprit drug, supportive therapy and corticosteroid therapy (Shiohara et al. 2012). There is limited evidence for the benefits of corticosteroids and no specific treatments have yet been identified (Lee et al. 2012). GSEA was undertaken using the entire gene list generated from the *in vitro* gene expression experiments irrespective of fold change or p value (Subramanian et al. 2005). Fifty one gene sets were identified as significantly enriched in CBZ or CBZE treated PBMCs only. Multiple gene sets related to the cell cycle including regulation of cyclins and CDKs were significantly enriched. Identification of the involvement of CDKs in hypersensitivity to CBZ raises the possibility that CDK inhibitors, which are currently in development for cancer (Roskoski 2016), may be useful to treat acute hypersensitivity reactions, but of course, this will require well-designed prospective trials. CDK inhibitors have been reported to have anti-inflammatory and pro-resolution properties through promotion of neutrophil and lymphocyte apoptosis (Leitch et al. 2009). The *PARP9* gene was identified as being the most regulated gene in the CBZ/CBZE specific

mRNA and miRNA network. PARP9 functions to promote interferon-stimulated gene expression to enhance antiviral function (Zhang et al. 2015). Interferon signal enhancing small molecules are currently in development as novel antiviral agents (Patel et al. 2014) and identification of the importance of the interferon signalling in CBZ hypersensitivity raises the possibility that these compounds could also be used for the treatment of acute hypersensitivity reactions.

There are a number of outstanding questions that arise from this research. There is significant *in vitro* evidence to suggest that CBZ is able to directly activate T cells through a PI mechanism (Naisbitt et al. 2003; Zhou et al. 2015). However, the identification of two [O]CBZ-HSA protein conjugates suggest that alternative T cell activation pathways including those consistent with the hapten hypothesis need to be considered. CBZE modified HSA conjugates were also detected in patients tolerant of CBZ. Similarly, abacavir-HSA conjugates have also been detected in patients tolerant to abacavir (Meng et al. 2014a). Piperacillin modified HSA was found to stimulate T cells *in vitro* (Whitaker et al. 2011) but whether all drug modified HSA conjugates are immunogenic is unknown. Synthesis of the [O]CBZ modified peptides for testing *in vitro* would provide direct evidence as to whether [O]CBZ-HSA conjugates are immunogenic. If the [O]CBZ-HSA conjugates are immunogenic, then further work should focus on identification of other susceptibility factors as the CBZE modified HSA adduct was detected in CBZ tolerant patients. These could include carriage of the risk HLA allele, specific TCR clonotype or formation of excessive amounts of reactive metabolites that overcome detoxification processes. Animal models of CBZ induced liver injury have demonstrated that CYP3A- mediated metabolism and GSH depletion in rats contribute to CBZ induced liver injury (Iida et al. 2015) in conjunction with activation and stimulation of immune responses (Higuchi et al. 2012). However, these results have to be treated with caution, because it is unclear how well these animal models reflect the situation in man.

It has been hypothesised that reactive metabolites of CBZ are responsible for triggering hypersensitivity reactions (Furst and Uetrecht 1995; Pearce et al. 2002)

and that genetic polymorphisms in CBZ metabolism enzymes contribute to risk (He et al. 2014). None of the 19 SNPs implicated in CBZ metabolism or transport significantly affected the PK of CBZ as described by population PK modelling using NONMEM. This may be secondary to insufficient sample size. Although the levels of other metabolites including CBZE, DiOH-CBZ, 2OH-CBZ and 3OH-CBZ were also measured in these patients it was not possible to develop a PK model that included all of the metabolites due to its complex nature and time constraints on the project.

However, two SNPS in *ABCB1* (c.1236T>C and c.2677G>T) and a single SNP in *EPHX1* (c.416A>G) were found to be significantly associated with dose-adjusted plasma CBZE concentrations by ANOVA. Patients who were heterozygous or homozygous mutants for *ABCB1* c.1236T>C demonstrated lower plasma levels of dose-adjusted CBZE compared with wild-type patients. Patients who were homozygous mutants for *ABCB1* c.2677G>T were found to have higher dose-adjusted plasma concentrations of CBZE compared with heterozygous and wild-type patients. Homozygous mutants for *EPHX1* c.416A>G demonstrated higher levels of dose-adjusted plasma CBZE concentrations compared with heterozygous and wild-type patients, which is consistent with reduced activity of the mutant mEH as demonstrated in earlier studies (Nakajima et al. 2005). These results demonstrate that genetic variation in CBZ metabolising enzymes can affect the plasma concentrations of CBZE. Elevated concentrations of plasma CBZE could trigger hypersensitivity through increased formation of the CBZE-modified HSA identified earlier in chapter 2. However, this hypothesis would require further study and proof that CBZE-modified HSA can act as an antigen.

Transcriptome analysis of PBMCs incubated with CBZ or CBZE identified 9 mRNA and 39 miRNA molecules that are specific for CBZ and CBZE. Validation of these markers is required in a larger number of patients and their applicability to other drugs that can cause hypersensitivity also needs to be investigated. To determine if any mRNA or miRNA are applicable *in vivo*, then skin biopsies and blood samples should be taken from patients with acute hypersensitivity reactions. The ideal

biomarker for diagnosis should be applicable in both the acute hypersensitivity reaction but also as an outcome measure in post hypersensitivity *in vitro* testing.

Because hypersensitivity reactions are relatively rare, a recurring problem in studies of drug hypersensitivity is inadequate sample size. In addition, patients present with clinically heterogeneous signs and symptoms that mimic many other conditions and there is currently no diagnostic test meaning diagnosis is complex. Patients may also be prescribed multiple medications making it harder for the clinician to be sure of the culprit medication. Correct diagnosis is very important as some pharmacogenetic associations (e.g. *HLA-B*15:02* and CBZ induced SJS/TEN) are applicable only to one phenotype of hypersensitivity (Chung et al. 2004). Inadequate diagnosis and phenotyping of patients with hypersensitivity will reduce the power of any study to detect a true association. The phenotype standardisation project for drug induced skin injury outlines clear diagnostic criteria and investigations to be undertaken for suspected drug hypersensitivity which will hopefully standardise the clinical information collected for these patients in future (Pirmohamed et al. 2011). To ensure sufficient numbers of patients are recruited for clinical studies repositories such as the RegiSCAR (Kardaun et al. 2013) and the international serious adverse event consortium (http://www.saeconsortium.org/) have been established where clinical samples are collected and stored.

In conclusion, this thesis has investigated the links between metabolism and immunological mechanisms in CBZ hypersensitivity. Two novel [O]CBZ-HSA adducts were identified both *in vitro* and *in vivo* although their ability to stimulate the immune system requires further investigation. A novel HPLC-MS/MS analysis was developed to measure CBZ and four of its metabolites in healthy volunteers and patients prescribed CBZ for epilepsy. A population PK model was developed to investigate the role of genetic polymorphisms in metabolising enzymes on the PK of CBZ although none were found to be significant. *In vitro* experiments demonstrated that CBZ-specific T cells are activated by multiple mechanisms and several antigens. Transcriptomic analysis provided further evidence to support involvement of viral infection processes and revealed novel pathways that may be targets for treatment

such as CDK inhibitors and interferon signalling enhancers. A selection of CBZ/CBZE specific mRNA and miRNA has the potential to act as diagnostic biomarkers, but requires validation in larger cohorts of patients. The results in this thesis provide further evidence for the role of immunological and metabolic factors in the susceptibility and pathogenesis of CBZ hypersensitivity reactions. Future research into drug induced hypersensitivity reactions requires the establishment of infrastructure and guidelines for the diagnosis and investigation of suspected patients with joint working between clinicians, scientists, government, industry and patient advocates.

Chapter 8

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Chapter 9

Appendix

CLINICAL PROTOCOL

PICME Study

A pharmacokinetic investigation into the formation of carbamazepine metabolites and carbamazepine-protein conjugates in healthy volunteers

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REC: 12/NW/0780

UoL Reference: UoL000900

RLBUHT NHS R&D: 4444

EUDRACT: 2012-004700-35

Personnel Involved

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Dr Amitava Ganguli, Clinical Research Facility Co-Director, Royal Liverpool University Hospital

Dr Richard FitzGerald, Clinical Research Facility Co-Director, Royal Liverpool University Hospital

Dr Xiaoli Meng, Postdoctoral Fellow, University of Liverpool

Dr Vincent Yip, MRC Clinical PhD Fellow, University of Liverpool

CONTENTS

1. INVESTIGATIONAL SITE

The study will be conducted at the Clinical Research Facility in the Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP, UK

Dr Amitava Ganguli will be the principal investigator responsible for the conduct of the study.

Prof Munir Pirmohamed will act as the chief investigator for the study.

2. LABORATORIES

The Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, L69 3BX, UK will conduct the relevant analyses required for the study

3. ETHICS

The trial will be performed in compliance with the guidelines of the Declaration of Helsinki on biomedical research involving human volunteers (Hong Kong revision, 1989 and the 48th General Assembly, Somerset West, Republic of South Africa, October 1996, updated in October 2000), ICH-GCP guidelines, relevant regulatory guidelines, and the study protocol. The protocol and relevant substantive data will be submitted for consideration by the Local Research Ethics Committee and written approval from the Chair of the Ethics Committee is required before the study is initiated and clinical activities of the study can commence.

Any major changes to the protocol will be made by means of a formal written protocol amendment and submitted for approval by the Research Ethics Committee. The committee will also be kept informed of the study progress and will receive a copy of the final study report.

On completion of the study, subjects will be paid £250. This compensation is based on an assessment of the loss of time and inconvenience as a result of participation in this study. Subjects who are withdrawn from the study by the Investigator for medical reasons (either due to study medication or procedures) will receive

payment for the visits completed. Subjects who do not complete the study for other *bona fide* reasons (e.g. intercurrent illness, major unexpected change in personal circumstances) will be compensated *pro rata*. Withdrawal of consent without any reason or violation of the protocol may, however, result in partial or complete forfeiture of compensation. Protocol violation is defined as the disobeying of instructions communicated verbally or in writing.

4. BACKGROUND

Carbamazepine (CBZ) is an important drug that is used in the treatment of epilepsy, trigeminal neuralgia and bipolar disorder (1, 2). Although generally well tolerated, it can cause cutaneous adverse drug reactions (ADR) in up to 10% of patients (1). These reactions can range from mild maculopapular exanthema (MPE) to severe life threatening conditions such as the hypersensitivity syndrome (HSS), Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (3). MPE consists of diffuse cutaneous erythema with areas of skin elevation that may evolve to vesicles or papules but usually resolve once the offending agent is stopped (4). HSS is a severe adverse reaction that typically occurs within three months of the start of drug treatment and is characterised by fever, eosinophilia, lymphadenopathy, cutaneous exanthematous eruption and involvement of extracutaneous organs (5). The most severe phenotypes, SJS and TEN, are associated with significant mortality (up to 30%) and long-term morbidity. SJS and TEN are characterised by a blistering rash affecting a variable percentage of the body surface area, erosion of mucous membranes, fever, malaise and systemic involvement. SJS and TEN represent a spectrum of the same disease with skin detachment of 1-10% in SJS, 10-30% in the overlap syndrome and >30% in TEN (5, 6).

The pathophysiology of these CBZ hypersensitivity reactions is not well understood but predisposition is thought to be genetic because of cases reported in families and monozygotic twins (7). One of the presently accepted mechanisms of drug hypersensitivity is based on the hapten hypothesis. This hypothesis suggests that drugs and/or their metabolites can act as haptens binding and irreversibly

modifying carrier proteins in patients. These drug-protein conjugate are then able to stimulate the immune system via T-cells in susceptible hypersensitive patients (8). The clinical features of CBZ hypersensitivity suggest an immune-based aetiology and the discovery of CBZ specific T-cells in hypersensitive individuals is consistent with this hypothesis (9). Human leukocyte antigens (HLA) play a central role in the immune response to antigens and it has been hypothesised that specific HLA molecules may present a drug or its metabolites to specific T-cells which subsequently triggers an immune response. This has been borne out by studies which have demonstrated strong associations between CBZ hypersensitivity and two HLA alleles in particular HLA-B*1502, which was first reported in Han Chinese patients and HLA-A*3101 which has been reported in Japanese and Caucasian patients (10-12).

Whitaker *et al* have used mass spectrometry to identify and characterise the identity of two haptens formed by piperacillin and its metabolites with human serum albumin; these haptens were also detected in plasma isolated from patients receiving piperacillin therapy. The antigenicity of these haptens was confirmed by stimulation of piperacillin-specific T-cells in vitro from patients with known penicillin allergy using synthetic conjugates (13). Similar studies and drug haptens have been identified with benzylpenicillin and flucloxacillin (14, 15). The identity of the CBZ metabolites and CBZ-protein conjugates responsible for CBZ hypersensitivity reactions are currently not known.

Hapten formation and susceptibility to drug hypersensitivity reactions is also dependent upon drug metabolism. CBZ is metabolised primarily in the liver by CYP3A4 to form the major metabolite CBZ-10,11-epoxide (16). Other metabolites include 2-OH-CBZ and 3-OH-CBZ which are formed by ring hydroxylation via chemically reactive arene oxide intermediates which have the potential to cause cellular damage if not detoxified (17, 18). Secondary oxidation of 2-OH-CBZ and 3-OH-CBZ by CYP3A4 leads to the formation of thiol-reactive metabolites via iminoquinone intermediates that are capable of inactivating CYP3A4 and formation of protein adducts (19, 20). Genetic variation in CBZ metabolising and detoxification

enzymes has been hypothesised to contribute to susceptibility of CBZ hypersensitivity reactions. Most studies have focused on polymorphisms in the microsomal epoxide hydrolase enzyme which is responsible for the detoxification of chemically reactive arene oxide metabolites but researchers did not find a significant association (21, 22). The role of genetic polymorphisms in CYP450 enzymes, transporter proteins and other detoxification pathways have not been fully characterised in relation to CBZ metabolism or susceptibility to CBZ hypersensitivity reactions.

Our current understanding of how CBZ triggers hypersensitivity reactions in genetically susceptible individuals is lacking. Further research is needed to link CBZ metabolism, CBZ hapten formation and activation of the immune system to enable better diagnostic tests and safer drug design in future.

5. STUDY RATIONALE, OBJECTIVES AND DESIGN

5.1 Rationale

An improved mechanistic understanding of CBZ hypersensitivity is crucial because CBZ is an effective and widely prescribed medication. However, a significant number of patients (up to 10%) develop hypersensitivity reactions that if severe are associated with significant mortality and long-term morbidity. Current methods for the diagnosis of CBZ hypersensitivity are time consuming, have low sensitivity and low specificity and would not be applicable in clinical practice. Although pharmacogenetic tests for HLA exist that can identify patients at risk of hypersensitivity they are not employed routinely in clinical practice. The mechanistic studies described herein will link CBZ metabolism which leads to antigen formation to the immune response and individual susceptibility. This will improve our understanding of the relationship between antigenicity and immunogenicity which will help in future drug design; and how individuals with specific genetic biomarkers respond to CBZ in-vivo and in-vitro which will improve the basis for developing predictive tests. The lessons from this research will also

provide valuable information for other drugs that are known to cause hypersensitivity reactions.

5.2 Aims and Objectives

- To obtain rich pharmacokinetic (PK) data from plasma and urine of healthy volunteers following a single dose of CBZ to improve our understanding of CBZ metabolism and CBZ-protein adduct formation.
- To study the effect of genetic polymorphisms in drug metabolising enzymes and transporter proteins on metabolism and adduct formation following CBZ therapy.
- Data from this study will subsequently be incorporated into a larger population PK model for CBZ.

5.3 *Study Design*

It is intended that 8 healthy male volunteers will complete the study. Subjects will attend an initial screening visit within 14 days prior to Day 1. Subjects will then be admitted to the Clinical Research Facility at the Royal Liverpool University Hospital on day 1. Subjects will be requested to fast from midnight prior to admission. Subjects will have an intravenous cannula inserted into the forearm and blood samples will be collected from the cannula and venepuncture at regular intervals over the next 72 hours

5.4 *Statistical Considerations*

The number of subjects planned for this study is consistent with other studies of a similar nature (23-25). As this is a novel exploratory study to characterise metabolite and hapten formation it is difficult to provide an accurate sample size in advance.

6. SUBJECTS AND SCREENING

6.1 *Source of Subjects*

The study population will consist of healthy male volunteers between the ages of 18-55 years inclusive and will be recruited from a variety of sources. These will include the Royal Liverpool University Hospital clinical research facility volunteer panel, the University of Liverpool volunteer research database and new recruitment through advertising in the Royal Liverpool Hospital and the University of Liverpool. Prospective volunteers will be contacted in writing and telephone. Subjects will be assigned the numbers CBZ1, CBZ2, etc, in the order of their inclusion in the study which will be defined as the time they consent to participation.

6.2 *Inclusion Criteria*

- Subject is willing and able to give written informed consent
- Healthy male subjects between 18 and 55 years of age inclusive
- Subject's body weight is between 50 and 100 kg
- Subject's body mass index is between 18 and 32 kg/m^2

6.3 *Exclusion Criteria*

- Subject is not willing to take part or unable to give written informed consent
- Subject has clinically significant abnormal medical history or physical exam
- Subject has history of febrile illness within 4 weeks prior to admission
- Subject has clinically significant abnormal laboratory test at screening including HBV/HCV/HIV
- Subject has taken any interacting prescription or non-prescription drug, or dietary supplements within 2 weeks prior to study admission. Herbal supplements must be discontinued at least 4 weeks prior to admission to the clinical research facility
- Subject possesses either the HLA-B*1502 or HLA-A*3101 genotype
- Subject has a clinically significant ECG abnormality – prolonged corrected QT>450ms, 2^{nd} or 3^{rd} degree atrioventricular conduction block

- Subject has known hypersensitivity to carbamazepine or structurally related drugs (e.g. tricyclic antidepressants) or any other component of the formulation
- Subject with history of bone marrow depression
- Subject with history of hepatic porphyrias (e.g. intermittent porphyria, variegate porphyria, porphyria cutanea tarda)
- Subject has taken part on another research study within 90 days of commencement
- Subject has any condition which in the opinion of the investigator will interfere with the study

6.4 *Screening Procedures*

The first clinic visit will be the pre-study (screening) visit. Subjects will be invited to attend this visit up to 14 days before the start of the study. This visit will take approximately 60 minutes in total and further details can be found in section 7.1.1.

6.5 *Subject Informed Consent Form*

Voluntary written informed consent will be obtained from each subject prior to performing any trial-related procedures. Each subject will be given both verbal and written information describing the nature and duration of the clinical trial. The informed consent process will take place under conditions where the subject has adequate time to consider the risks associated with his participation in the clinical trial. Subjects will not be screened until they have signed an approved informed consent form (ICF) written in a language that is understandable to the subject.

The investigator's local research and ethics committee (REC)-approved ICF will be personally signed and dated by the subject and the investigator or designee who conducted the informed consent discussion. Each subject will receive a copy of the signed and dated written ICF along with any other written information provided to the subject.

The investigator is responsible for ensuring that informed consent is obtained from each subject in accordance with all applicable regulations and guidelines. The original signed ICF will be retained within the subject's source documents.

7. **STUDY CONDUCT**

7.1 *Study Schedule*

Blood sampling of patients will take place at the clinical research facility in the Royal Liverpool Hospital as part of a planned 12 hour admission with subsequent blood sampling at 24, 48 and 72 hours as ambulatory visits.

7.1.1 *Screening Visit*

The first clinic visit will be the pre-study (screening) visit. Subjects will be invited to attend this visit up to 14 days before the start of the study. The visit will take approximately 60 minutes and proceed accordingly:

1. Consent	Written and verbal consent for inclusion into study
2. Interview	(a) Past medical history
	(b) Concomitant medications
	(c) Social history – smoking, alcohol, caffeine, diet, physical activity
	(d) Contact details – GP
3. Physical Examination	(a) Height and Weight
	(b) Blood Pressure
	(c) Temperature
	(d) Cardiovascular, respiratory and abdominal examination
	(e) ECG
4. Blood Sampling	(a) 2 x 9ml EDTA monovette tube (FBC, HLA genotyping)
	(b) 1 x 9ml Serum gel monovette tube (U&E, LFTs)
	(c) 1 x 9ml Serum Z gel monovette tube (Hep B&C and HIV screen)
5. Administration	(a) Screen subject against over-volunteering using the TOPS (The Over-volunteering Protection System) database
	(b) GP questionnaire to confirm subject's past medical

	history

7.1.2 *Visit 1*

Subjects will be admitted in at 0700 on day 1 and undergo routine observations (temperature, blood pressure and pulse rate). Blood sampling will commence following insertion of a cannula into the arm at approximately 0800. Samples will be obtained by an appropriately qualified person from the clinical research facility. If the cannula becomes blocked a new one will be sited or samples taken by standard venepuncture. Blood will be collected for analysis in EDTA and Li-heparin monovette tubes and 24 hour urine collected. Subjects will receive a single dose of oral CBZ 400mg after cannula insertion and first blood sample. Subsequent blood sampling will be according to the timeline below. A standard operating procedure for blood sampling including aseptic technique will be utilised by all practitioners involved in the study. The following timeline will be followed for all subjects:

1. Consent	Ensure patient still happy to continue with study	
2. Case Note Review	(a) Review laboratory results	FBC, U&E, LFTs, Hep B&C, HIV
	(b) Review HLA genotype	
	(c) Review ECG	
	(d) Review GP questionnaire	
3. Interview	(a) Ensure that patient well (b) New medications since screening visit	
4. Physical Examination	(a) Temperature	
	(b) Blood Pressure	
	(c) Pulse	
5. Inclusion/Exclusion Criteria	Review and update inclusion/exclusion criteria	
If all inclusion and exclusion criteria are satisfied then continue		
6. Cannulate and Blood Sampling (Time=0)	(a) 1x 9ml EDTA monovette (genetic polymorphisms) (b) 1x 5ml Li Heparin monovette	

7. Carbamazepine	Single oral dose 400mg Carbamazepine administered at 0800
8. Blood Sampling	Blood sampling from cannula into 1x9ml Li Heparin monovette at the following times: • 0815 (15 minute sample) • 0830 (30 minute sample) • 0900 (1 hour blood sample) • 1000 (2 hour blood sample) • 1200 (4 hour blood sample) • 1400 (6 hour blood sample) • 1600 (8 hour blood sample) • 2000 (12 hour blood sample)
9. Urine Collection	Commence 1st 24 hour urine collection
10. Discharge	Removal of cannula and discharge from clinical research facility following 12 hour blood sample at 2000

Subjects will be discharged following the 12 hour blood sample at 2000 hours. Blood and urine samples will be analysed for CBZ metabolites and CBZ protein adducts. Blood taken pre-carbamazepine at time 0 will also be analysed for genetic polymorphisms in drug metabolising enzymes and transporter proteins.

7.1.3 *Visit 2*

Visit 2 will take place at 0800 on day 2 and subjects will undergo the following consultation:

1. Interview	(a) Ensure patient happy to continue with study	
	(b) Monitor for adverse events	Complete AE and SAE forms as necessary
2. Physical Examination	(a) Temperature	
	(b) Pulse	
	(c) Blood pressure	
3. Blood Sampling	1x 5ml Li Heparin monovette at • 0800 (24 hour blood sample)	
4. Urine Collection	Completion of 1st 24 hour urine collection Commence 2nd 24 hour urine collection	

7.1.4 *Visit 3*

Visit 3 will take place at 0800 on day 3 and subjects will undergo the following consultation:

1. Interview	(a) Ensure patient happy to continue with study	
	(b) Monitor for adverse events	Complete AE and SAE forms as necessary
2. Physical Examination	(a) Temperature	
	(b) Pulse	
	(c) Blood pressure	
3. Blood Sampling	1x 5ml Li Heparin monovette at • 2000 (48 hour blood sample)	
4. Urine Collection	Completion of 2nd 24 hour urine collection Commence 3rd 24 hour urine collection	

7.1.5 *Visit 4*

Visit 4 will take place at 0800 on day 4 and is the final study visit.

1. Interview	(a) Ensure patient happy to continue with study	
	(b) Monitor for adverse events	Complete AE and SAE forms as necessary
2. Physical Examination	(d) Temperature	
	(e) Pulse	
	(f) Blood pressure	
3. Blood Sampling	1x 5ml Li Heparin monovette at • 0800 (72 hour blood sample)	
4. Urine Collection	Completion of 3rd 24 hour urine sample	

In total approximately 100mls of blood will be drawn over the whole study period. A summary of study visits and interventions are provided in appendix A.

7.2 *Study Restrictions*

7.2.1 *Concurrent Medication*

Subjects must refrain from taking any interacting prescription or non-prescription drug, or dietary supplements within 2 weeks of admission to the clinical unit. Herbal

supplements must be discontinued at least 4 weeks prior to admission to the clinical research facility. This is because other medications and herbal supplements may induce or inhibit cytochrome P450 enzyme function.

7.2.2 *Diet*

Seville oranges, grapefruits and grapefruit juice are known inhibitors of CYP3A4, the principal CYP450 enzyme responsible for CBZ metabolism, subjects will be asked to avoid consuming pomelo oranges, grapefruits or grapefruit juice for at least 48 hours prior to day 1 of the clinical trial.

7.2.3 *Alcohol*

Subjects must refrain from alcohol consumption from 48 hours prior to admission to the clinical research facility until all blood and urine sampling is completed.

7.2.4 *Caffeine*

Subjects must refrain from caffeine and caffeine-containing products from 48 hours prior to admission to the clinical research facility and until blood and urine sampling is completed.

7.2.5 *Smoking*

Subjects must have refrained from smoking for at least 6 months prior to the screening visit and should not smoke until the end of the study.

7.2.6 *Physical Activity*

Subjects should not perform excess strenuous exercise beyond his/her normal exercise routine.

7.2.7 *Sexual Intercourse*

Subjects should utilise single barrier contraception (such as condoms) if they engage in sexual intercourse until the end of the study.

7.2.8 *Blood Donation*

Subjects must refrain from blood donation for one month prior to screening visit, during the study and 2 months after completion of the study.

7.3 *Methods for Clinical Procedures*

7.3.1 *Blood Sampling*

This study will be conducted with close attention to patient safety. Venepuncture poses minimal risk of bruising or bleeding. This procedure will be carried out by trained, competent health professionals.

Blood samples for determination of CBZ metabolites, CBZ-protein conjugates and genetic polymorphisms will be collected via a cannula or direct venepuncture. Prior to each sample being taken from the cannula 0.5mls of blood will be drawn and discarded. The cannula will be flushed with 2mls of 0.9% saline each time a sample is taken. At each designated time point 5mls of blood will be taken into lithium heparin tubes. Blood sampling at time 0 will include a further 9ml sample taken into an ethylene-diamine-tetra-acetic-acid (EDTA) monovette tube for metabolising enzyme and transporter protein genetic polymorphism analysis. Blood samples will be centrifuged within 15 minutes of collection or stored overnight at 4^{o}C for centrifuge the following day. 1mL and 100µL aliquots will then be transferred to appropriate tubes and stored at approximately -80^{o}C prior to bioanalysis.

No more than 20mls of blood will be extracted at time 0 and no more than 10mls for each subsequent sampling. Over a 24 hour period no more than 60mls of blood will be sampled. Total volume of blood sampled for the study will be approximately 100mls.

7.3.2 *Urine Sampling*

Three 24 hour urine bottles for each subject will be collected. Total volume of each bottle will be measured and a 50mls sample stored at -20^{o}C for metabolite and carbamazepine-protein conjugate analysis.

7.4 *Laboratory Evaluations*

7.4.1 *Haematology*

Blood samples will be taken into an EDTA monovette for full blood count.

7.4.2 *Biochemistry*

Blood samples will be taken into a serum gel monovette for urea and electrolytes and liver function tests.

7.4.3 *Virology*

Blood samples will be taken into a serum Z monovette for hepatitis B and C and HIV serology.

7.4.4 *Analysis of Genetic Polymorphisms*

Blood samples will be taken into EDTA monovette tubes for HLA-A*3101 and HLA-B*1502 genotyping and for identification of polymorphisms in drug metabolising enzymes and drug transporter proteins.

7.5 *CBZ Metabolite and CBZ-Protein Conjugate Analysis*

High sensitivity mass spectrometry techniques already in use at the MRC Centre for Drug Safety Science at the University of Liverpool will be utilised to characterise and quantify CBZ metabolites and CBZ-protein conjugates.

7.6 *Sample storage*

All samples will be transferred/delivered in appropriate packaging in dry ice (solid carbon dioxide) to the University of Liverpool, Department of Pharmacology Laboratories. Samples will be stored securely as described above and the University will act as custodian.

7.7 *End of Study*

The end of study is defined as the last scheduled protocol activity of the last subject in the study. The conclusion of the study will be notified by the University of

Liverpool to the Ethics Committee within the required timelines. All research data will be archived at the University of Liverpool for at least 15 years.

7.8 *Criteria for Termination of the Study*

There are no criteria for termination of the study. Details regarding the withdrawal of subjects are given in section 10.

8. CASE REPORT FORMS

Paper case report forms (CRFs) will be used for recording all data collected during the study. All CRFs are to be completely filled out by personnel administering the study procedures and reviewed and signed by the chief investigator or nominated designee. All CRFs are to be completed in a clear and legible manner. Black ink must be used to ensure accurate interpretation of data. Any changes or corrections must be made by drawing a line through the data to be changed, entering corrected information, and signing (or initialling) and dating the change. Erasing, overwriting, or the use of "liquid paper" is not permitted on CRFs. Every effort should be made to have the CRFs completed as soon as possible following a subject's study visit. All data from the recruited patients will be transcribed onto an electronic spreadsheet. All study documentation as defined above will be made available to the Ethics Committee and to regulatory authorities for inspection on request.

9. ADVERSE EVENTS AND SAFETY REPORTING

An adverse event (AE) is defined as any undesirable event occurring to a subject during a clinical study. All adverse events will be coded and reported as follows:

- Mild: Awareness of sign or symptom but easily tolerated
- Moderate: Discomfort enough to cause interference with usual activity
- Severe: Incapacitating with inability to work or do usual activity

Laboratory results that are outside the normal range will be recorded as an adverse event. If patients are found to be HIV or hepatitis positive then the investigator will invite the patient to the CRF to explain the results and refer them to the infectious

diseases team at the Royal Liverpool University Hospital. The severity of each event will be assessed by the investigator. When recording the outcome of the adverse event its maximum severity will also be recorded. Adverse events, if any, not resolved by the end of the study, will be followed up by the investigator.

Serious adverse events (SAE) or suspected unexpected serious adverse reactions (SUSAR) are defined as any adverse event that results in death, is life-threatening, requires hospitalisation, results in persistent or significant disability or incapacity and consists of a congenital anomaly or birth defect. All SAEs have to be reported, whether or not considered causally related to the investigational product, or to the study procedure(s). If any SAE occurs in the course of the study, the investigator must inform the Sponsor within 24 hours of when he or she becomes aware of it. The investigator must inform the Sponsor of any follow-up information on a previously reported SAE immediately, or no later than 24 hours of when he or she becomes aware of it. SUSARs that are fatal or life-threatening will be reported to the MHRA as soon as possible but no later than 7 days. SUSARs which are not fatal or life-threatening will be reported to the MHRA as soon as possible but no later than 15 days. Causality for SAEs and SUSARs will be checked against the SmPc. All SAE and SUSAR will be managed according to the Royal Liverpool University Hospital research and development standard operating procedures.

10. WITHDRAWAL OF SUBJECTS

Subjects will be informed that they are free to withdraw from the study at any time. The investigator may remove a subject if, in his opinion, it is in the best interest of the subject. Although a subject is not obliged to give his reason for premature withdrawal, the investigator will make a reasonable effort to obtain the reason while fully respecting the subject's rights. If there is a medical reason for withdrawal of the subject then this will be reported as an adverse event and followed up by the investigator. If in the opinion of the investigator it is considered necessary, the subject's GP will be informed of the medical reason for the withdrawal. The subject's consent will be obtained prior to informing the GP. Subjects that withdraw consent or are withdrawn at the discretion of the

investigator prior to completion of study procedures may be replaced to ensure that there are 8 evaluable subjects.

11. DATA ANALYSIS

As this is an exploratory/pilot study it is most likely that only descriptive statistics will be required.

12. RECORDS AND CONFIDENTIALITY

12.1 *Records*

Adequate records as required by ICH GCP will be maintained for the study. This includes medical records, case report forms, laboratory reports, signed consent forms, and worksheets. All records from the study will be kept for 15 years.

12.2 *Confidentiality*

To maintain subject privacy, all case report forms, study reports and communications will identify the subject by initials where permitted and/or by the assigned subject number. The subject's confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations.

12.3 *Report and Publication*

The results of the study will be incorporated into scientific papers for publication. All identifiable patient information will be removed. Appropriate precautions will be taken to maintain confidentiality of medical records and personal information.

13. APPENDIX A

13.1 Summary of Study Visits

		Day 1	Day 2	Day 3	Day 4
	Screening	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
Informed Consent	X				
Medical History	X				
Clinical Examination	X				
Height and Weight	X				
Vital Signs	X	X	X	X	X
FBC,U&E, LFT	X				
Hepatitis B & C test	X				
HIV test	X				
HLA Genotyping	X				
Genetic polymorphisms in drug metabolising enzymes		X			
Carbamazepine blood tests over 12 hours		X			
Single Carbamazepine blood tests			X	X	X
AE Monitoring		X	X	X	X

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A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN HEALTHY VOLUNTEERS

Participation Information Sheet

Invitation

You are being invited to participate in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read this sheet carefully and discuss it with relatives, friends and the study team. You may also wish to discuss this with your GP. Ask us about anything that is not clear and take time to decide whether or not you wish to take part.

What is the purpose of the study?

This study is looking at the formation of breakdown products produced by the body following carbamazepine ingestion and how genetic variation affects this process. Carbamazepine is a medication that is used to treat epilepsy, trigeminal neuralgia and some psychiatric conditions. We know that some patients develop adverse reactions, which are termed hypersensitivity reactions, to carbamazepine. These hypersensitivity reactions include skin rash, fever and lethargy, and usually occur with multiple doses. At the moment we do not know what causes these hypersensitivity reactions but we believe that they are caused by the way the body handles carbamazepine and forms breakdown products. These breakdown products (also called metabolites) then cause hypersensitivity reactions in genetically susceptible patients. The main purpose of this study is to find out how genetic variation can affect the formation of carbamazepine metabolites.

Do I have to take part?

It is up to you to decide whether or not to take part in the study. Participation is entirely voluntary. If you do decide to participate you will be given this information sheet to keep and be asked to sign a consent form. You will receive a copy of this signed consent form to keep. If you decide to take part you are still free to

withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

You will be given sufficient time to consider whether or not you wanted to take part. If you agree to take part in the study you will be participating for a maximum of 5 days. Over this period of time you will be asked to visit the clinical research facility 5 times. On one of these visits you will have to stay for 12 hours.

During the study you will be asked to take a single 400mg tablet of carbamazepine.Your partner should not become pregnant whilst taking part in the study. You will need to use one of the following methods of contraception until you have finished the study.

- Complete abstinence from intercourse from 24 hours prior to taking study medication, throughout the study and for at least 1 week after stopping study medication
- Single barrier method (male condom, diaphragm)
- Any intrauterine device (IUD) with published data showing the expected failure rate is <1% per year
- Any other method with published data showing that the expected failure rate is <1% per year
- Hormonal contraception plus a barrier method. Hormonal contraception alone cannot be used

During the study you will not be able to eat grapefruit or pommello oranges or any product that contains grapefruit or pommello oranges.

If you need to take any other medication you should contact the study team before taking it. You are not allowed to take any interacting prescription or non-prescription drug, recreational drugs (such as St John's wort, cannabis, cocaine), or dietary supplements. You are allowed to take paracetamol and multi-vitamins.

During the study we will ask you to refrain from alcohol consumption and caffeine products from 48 hours prior to admission to the clinical research facility until the end of the study.

We will ask you not to perform any extra physical exercise during the study. It is ok to carry on with your normal exercise routine.

Screening Visit

You will need to bring a form of photographic identification and your National Insurance number with you. This visit will be used to see if you are suitable to take part. Details about yourself will be taken such as your age, ethnic group, past medical problems and details of any medication you are taking. A clinical examination will be undertaken. We will also take some blood samples to see if you can take part in the study. This blood will also be tested for HIV and hepatitis. If you want further information or to discuss these tests please ask the study doctor or nurse who can give you more information. If we find out that you have HIV or hepatitis we will make sure you receive the right care. If you are suitable to take part in the study we will arrange for you to attend the study days.

Visit 1

You will be asked to attend the clinical research facility at 0730 and be required to stay until 2000. You will be required to fast from midnight before this visit. On this visit we check your temperature, pulse and blood pressure. We will ask you about any medication you may be taking and about your general health. We will insert a small tube (cannula) into a blood vessel and we will take two blood samples at this time (time=0). Following this you will be given a single 400mg tablet of carbamazepine. We will use the cannula to take blood samples at 15mins, 30mins, 1hr, 2hrs, 4hrs, 6hrs, 8hrs and 12hrs after you took the tablet. The total amount of blood taken will be approximately 54 mls. We will also request that you collect your urine for the next 72 hours using three 24 hour urine collection bottles. You will be allowed home following the 12 hour blood sample and cannula will be removed.

Visit 2

You will be asked to return to the clinical research facility at 0730 the day following visit 1. At this visit we will check your temperature, pulse and blood pressure. We will ask if you had any problems with the medication. We will require a blood test and return of the first 24 hour urine sample bottle.

Visit 3

On day 3, 48 hours since you took the carbamazepine tablet, we will ask you to return to the clinical research facility at 0730. We will check your temperature, pulse and blood pressure. We will ask if you had any problems with the medication. We will require a blood test and return of the second 24 hour urine sample bottle.

Visit 4

This is the final study visit and will take place at 0730 at the clinical research facility one day after visit 3. We will check your temperature, pulse and blood pressure. We will ask if you had any problems with the medication. We will require a blood test and return of the third 24 hour urine sample bottle.

Withdrawal Visit

If you decide to stop the study before the end you will need to attend for a final visit. We will check your blood pressure, pulse, temperature and urine.

What are the possible side effects/risks of taking part?

You may have side effects whilst taking part in this study. However, we will monitor everyone in the study for side effects. If you are worried about your symptoms then you can contact the study team at any time. You will be given contact numbers of the study team.

Carbamazepine side effects

Dizziness, ataxia (loss of co-ordination), double vision, drowsiness, and nausea and vomiting may occur with single doses in about 10% of people. However, these are usually short lasting. Other side effects which have been reported with

carbamazepine occur with multiple doses, which you will not be receiving. These include low platelets, fluid retention, dry mouth, elevated liver enzymes and skin rashes.

Abnormal blood test results

If any blood tests taken during your participation in the study are abnormal then we will invite you back to the clinical research facility to explain the results to you. We will also ensure that you are referred to the appropriate clinician for further management.

Other possible risks

When you give blood you may feel faint or experience bruising or tenderness at the site of the needle puncture. You will be required to remain in hospital for most of the day on day 1 of the clinical trial which may cause inconvenience.

What are the benefits of taking part?

There are unlikely to be any direct benefits to you by taking part in the study. However, in the long-term, the information we get from this study may help improve the treatment of people with carbamazepine**.**

What happens if something goes wrong?

If you are unhappy with any aspect of the study then complaints should be made to the chief investigator in the first instance. There are no special compensation arrangements. If you are harmed because of negligence then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you.

Will my taking part in this study be kept confidential?

All the information collected about you during the course of the study will be kept strictly confidential. Any information about you which leaves the clinic will have your name and address removed so that you cannot be recognised from it. All samples will be coded and anonymised. All identifying details (e.g. name, address, date of birth) will be removed, but the sample will remain linked to data collected from this study (e.g. weight, age). Your medical records may be inspected by research staff for the purposes of checking that the information we have is accurate and complete. We will seek your consent to notify your GP about your participation in this study.

What will happen to my samples?

Your blood and urine samples will be analysed for levels of carbamazepine and its breakdown products. We will look at genes in your DNA samples to see how this determines the way your body handles carbamazepine.

What happens when the research study stops?

You will be asked if we can store the samples from this study for use in other projects. The samples will be kept in a locked freezer in the University of Liverpool. ALL THESE SAMPLES WILL BE CODED (but will be linked to details that we will collect about your drug levels). We will keep your sample until all of it is used up. The samples will be considered a gift to the University of Liverpool, which will act as a custodian of all the samples obtained as part of this project. Rarely, a small amount of your sample may be provided to other researchers in the UK or other parts of the world. However, it is important to remember that this will only be identified by a unique code, and your name will not be given out.

We are asking for your permission to store your samples and linked details for these future studies. Your samples will not be sold for profit. (It is not the purpose of this study to produce commercial gain, and in any case, any commercial value in the future will only come from findings in groups of people rather than from samples from a single person).

Once the studies are completed your anonymised data will be kept for a minimum of 15 years.

What will happen to the results of the study?

We will combine all the results from the subjects taking part in the study, and publish any important results in medical journals. Results may also be presented at scientific meetings. No individuals will be specifically identified in any publication.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the area that is being studied. We will share this information with you right away if it is important to your health. If you have any concerns about it you should discuss this with the research team.

Prevention of over volunteering (TOPs database)

You must not take part in too many studies because it is not good for you. We and other units like ours in the UK keep a database of healthy volunteers and when they take part in studies. We will enter into the database:

- Your National Insurance number (if you're a UK citizen); or
- Your passport number and country of origin (if you're not a UK citizen); and
- The date of your last dose of study medicine

If you withdraw from the study before you receive any study medicine, the database will show that you never received a dose. Only staff at the Royal Liverpool NHS Hospital and other medicines research units can use the database. We may call other units, or they may call us, to check your details. We will keep your details for at least 2 years. If we need to contact you about the study after you have finished it, but we can't because you have moved or lost contact with your GP, we might be able to trace you through the information in the database.

Who is funding the research?

This study is funded by the Medical Research Council.

Who has reviewed the study?

This study has been reviewed by the National Research Ethics Service Committee North West – Greater Manchester Central

Reimbursement

You will be reimbursed a total of £250 for participating in this study. This payment includes travel expenses. If you withdraw before completion of the study you will be reimbursed for the time you participated. Screening visit £40; visit 1- 12 hour blood sampling £90; visit 2- blood sampling £40; visit 3- blood sampling £40; final visit- blood sampling £40. Withdrawal of consent without any reason or violation of the protocol may, however, result in partial or complete forfeiture of compensation. Protocol violation is defined as the disobeying of instructions communicated verbally or in writing. You will be asked to provide details of your bank account so this payment can be made. You will be responsible for paying tax on any payment received.

Involvement of your General Practitioner

With your permission, your general practitioner will be contacted to confirm your past medical history. Your signature on the consent form will authorise the study team to do this.

For further information on this study please contact:

Dr Vincent Yip, MRC Clinical Fellow in Clinical Pharmacology and Therapeutics, Department of Molecular and Clinical Pharmacology, The Wolfson Centre for Personalised Medicine, The University of Liverpool, L69 3GL

Email: vyip@liv.ac.uk

Tel: 0151 795 5407

Study Investigator _____

Tel Number _____

E-mail _____

Summary of Study Visits

		Day 1	Day 2	Day 3	Day 4
	Screening	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
Informed Consent	X				
Medical History	X				
Clinical Examination	X				
Height and Weight	X				
Vital Signs	X	X	X	X	X
FBC,U&E, LFT	X				
Hepatitis B & C test	X				
HIV test	X				
HLA Genotyping	X				
Genetic polymorphisms in drug metabolising enzymes		X			
Carbamazepine blood tests over 12 hours		X			
Single Carbamazepine blood tests			X	X	X
AE Monitoring		X	X	X	X

Thank you for reading this. This sheet (and a copy of the consent form) should be retained by you if you decide to participate.

A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN HEALTHY VOLUNTEERS

Participant Consent Form

The volunteer should complete the whole of this sheet himself/herself

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Participant identification number: _____

Name of Researcher: _____

Contact Details for Research Team: _____

PART 1 Please initial boxes

1. I confirm that I have read and understand the information sheet dated 26/11/12 (version 3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I agree to give blood samples for research in this project. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw at any time without giving any reason, without medical care or legal rights being affected. ☐

3. I understand that once the study has ended the samples will be fully anonymised and once gifted to the University of Liverpool cannot be withdrawn. ☐

4. I understand that relevant sections of my study records and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I understand that I will not benefit financially if this research leads to the development of a new medical test. ☐

6. I agree to my GP being informed of my participation in the study and I understand that the GP will be required to complete a questionnaire about my past medical history before I can participate in the study. ☐

7. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

8. I agree to take part in the above study ☐

9. I understand that I will receive up to £250 for participating in this study. ☐

10. I agree to my details being entered into the TOPS database. ☐

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

Please see overleaf for part 2 of the consent form.

PART 2

11. **Consent for storage and use in possible future research projects**

I agree that the samples I have given and the information gathered about me can be stored by the University of Liverpool for possible use in future projects as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than the Liverpool Pharmacology who ran the first project. ☐

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

Thank you for agreeing to participate in this research.

Prof M Pirmohamed, Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool L69 3GL

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes

NHS
Health Research Authority
National Research Ethics Service
NRES Committee North West - Greater Manchester Central
HRA NRES Centre - Manchester
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7825
Facsimile: 0161 625 7299

18 December 2012

Professor M Pirmohamed
Professor of Clinical Pharmacology and Honorary Consultant Physician
University of Liverpool
The Wolfson Centre for Personalised Medicine
Department of Pharmacology
Brownlow Street
Waterhouse Building
Liverpool
L69 3GL

Dear Professor Pirmohamed

Study title:	**A Pharmacokinetic Investigation Into the Formation of Carbamazepine Metabolites and Carbamazepine-Protein Conjugates in Healthy Volunteers**
REC reference:	**12/NW/0780**
Protocol number:	**UoL000900**
EudraCT number:	**2012-004700-35**
IRAS project ID:	**112522**

Thank you for your letter of 26 November 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Kath Osborne, nrescommittee.northwest-gmcentral@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Advertisement	2	12 October 2012
Investigator CV	2 (MP)	24 September 2012
Investigator CV	Dr V L M Yip	18 October 2012
Letter from Sponsor	Royal Liverpool Intention to Sponsor	24 September 2012
Letter of invitation to participant	2	12 October 2012
Participant Consent Form	2	26 November 2012
Participant Information Sheet	3	26 November 2012
Protocol	4	26 November 2012
Questionnaire: GP	2	26 November 2012
REC application	112522/373929/1/650	16 October 2012
Referees or other scientific critique report	MRC Review	10 September 2012
Response to Request for Further Information		26 November 2012

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NW/0780	Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Signed on behalf of
Professor S J Mitchell
Chair

Email:nrescommittee.northwest-gmcentral@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Dr V Yip, University of Liverpool

Ms H Rogers, Royal Liverpool and Broadgreen University Hospital Trust

CLINICAL PROTOCOL

PICME II Study

A pharmacokinetic investigation into the formation of carbamazepine metabolites and carbamazepine-protein conjugates in epilepsy patients

Department of Molecular and Clinical Pharmacology

The Wolfson Centre for Personalised Medicine

The University of Liverpool

Block A: Waterhouse Buildings

1-5 Brownlow Street

Liverpool, L69 3GL

REC: 13/NW/0503

UoL Reference: UoL000969

EUDRACT: 2013-002743-28

Personnel Involved

Prof Munir Pirmohamed, NHS Chair of Pharmacogenetics, University of Liverpool

Prof Anthony Marson, Professor of Neurology, University of Liverpool

Prof Kevin Park, Director of MRC Centre for Drug Safety Science, University of Liverpool

Dr Xiaoli Meng, Postdoctoral Fellow, University of Liverpool

Dr Vincent Yip, MRC Clinical PhD Fellow, University of Liverpool

Chief Investigator Signature:_____ **Date:**_____

Chief Investigator Name :_____

CONTENTS

1. INVESTIGATIONAL SITE

The study will be conducted at the Walton Centre NHS Foundation Trust, Lower Lane, Fazakerley, Liverpool, L9 7LJ, UK.

Dr Vincent Yip will be the principal investigator responsible for the conduct of the study.

Prof A Marson will act as the chief investigator for the study.

2. LABORATORIES

The department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, L69 3BX, UK, will conduct the relevant analyses required for the study.

3. ETHICS

The trial will be performed in compliance with the guidelines of the Declaration of Helsinki on biomedical research involving human volunteers (Hong Kong revision, 1989 and the 48th General Assembly, Somerset West, Republic of South Africa, October 1996, updated in October 2000), ICH-GCP guidelines, relevant regulatory guidelines, and the study protocol. The protocol and relevant substantive data will be submitted for consideration by the Local Research Ethics Committee and written approval from the Chair of the Ethics Committee is required before the study is initiated and clinical activities of the study can commence.

Any major changes to the protocol will be made by means of a formal written protocol amendment and submitted for approval by the Research Ethics Committee. The committee will also be kept informed of the study progress and will receive a copy of the final study report.

On completion of the study, only subjects in the **autoinduction group** will be paid £200 (£40 per visit). This compensation is based on an assessment of the loss of time and inconvenience as a result of participation in this study. All participants will have their travel costs reimbursed. Subjects who are withdrawn from the study by the Investigator for medical reasons (either due to study medication or procedures)

will receive payment for the visits completed. Subjects who do not complete the study for other *bona fide* reasons (e.g. intercurrent illness, major unexpected change in personal circumstances) will be compensated *pro rata*. Withdrawal of consent without any reason or violation of the protocol may, however, result in partial or complete forfeiture of compensation. Protocol violation is defined as disobeying of instructions communicated verbally or in writing.

4. BACKGROUND

Carbamazepine (CBZ) is an important drug that is used in the treatment of epilepsy, trigeminal neuralgia and bipolar disorder.[1,2] Although generally well tolerated, it can cause cutaneous adverse drug reactions (ADR) in up to 10% of patients.[1] These reactions can range from mild maculopapular exanthema (MPE) to severe life threatening conditions such as the hypersensitivity syndrome (HSS), Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).[3] MPE consists of diffuse vesicles or papules but usually resolve once the offending agent is stopped.[4] HSS is a severe adverse reaction that typically occurs within three months of the start of drug treatment and is characterised by fever, eosinophilia, lymphadenopathy, cutaneous exanthematous eruption and involvement of extracutaneous organs.[5] The most severe phenotypes, SJS and TEN, are associated with significant mortality (up to 30%) and long term morbidity. SJS and TEN are characterised by a blistering rash affecting a variable percentage of the body surface area, erosion of mucous membranes, fever, malaise and systemic involvement. SJS and TEN represent a spectrum of the same disease with skin detachment of 1-10% in SJS, 10-30% in the overlap syndrome and >30% in TEN.[5,6]
The pathophysiology of these CBZ hypersensitivity reactions is not well understood but predisposition is thought to be genetic because of cases reported in families and monozygotic twins.[7] One of the presently accepted mechanisms of drug hypersensitivity is based on the hapten hypothesis. This hypothesis suggests that drugs and/or their metabolites can act as haptens binding and irreversibly modifying carrier proteins in patients. These drug-protein conjugates are then able to stimulate the immune system via T-cells in susceptible hypersensitive patients.[8]

The clinical features of CBZ hypersensitivity suggest an immune-based aetiology and the discovery of CBZ specific T-cells in hypersensitive individuals is consistent with this hypothesis.[9] Human leukocyte antigens (HLA) play a central role in the immune response to antigens and it has been hypothesised that specific HLA molecules may present a drug or its metabolites to specific T-cells which subsequently triggers an immune response. This has been borne out by studies which have demonstrated strong associations between CBZ hypersensitivity and two HLA alleles in particular HLA-B*1502, which was first reported in Han Chinese patients and HLA-A*3101 which has been reported in Japanese and Caucasian patients.[10-12]

Whitaker *et al* have used mass spectrometry to identify and characterise the identity of two haptens formed by piperacillin and its metabolites with human serum albumin; these haptens were also detected in plasma isolated from patients receiving piperacillin therapy. The antigenicity of these haptens was confirmed by stimulation of piperacillin-specific T-cells in vitro from patients with known penicillin allergy using synthetic conjugates.[13] Similar studies and drug haptens have been identified with benzylpenicillin and flucloxacillin.[14,15] The identity of the CBZ metabolites and CBZ-protein conjugates responsible for CBZ hypersensitivity reactions are currently not known.

Hapten formation and susceptibility to drug hypersensitivity reactions is also dependent upon drug metabolism. CBZ is metabolised primarily in the liver by CYP3A4 to form the major metabolite CBZ-10,11-epoxide.[16] Other metabolites include 2-OH-CBZ and 3-OH-CBZ which are formed by ring hydroxylation via chemically reactive arene oxide intermediates which have the potential to cause cellular damage if not detoxified.[17,18] Secondary oxidation of 2-OH-CBZ and 3-OH-CBZ by CYP3A4 leads to the formation of thiol-reactive metabolites via iminoquinone intermediates that are capable of inactivating CYP3A4 and formation of protein adducts.[19,20] The metabolism of CBZ is unique because it undergoes autoinduction which takes between one week and one month.[21-23] Genetic variation in CBZ metabolising and detoxification enzymes has been hypothesised to

contribute to susceptibility of CBZ hypersensitivity reactions. Most studies have focused on polymorphisms in the microsomal epoxide hydrolase enzyme which is responsible for the detoxification of chemically reactive arene oxide metabolites but researchers did not find a significant association.[24,25] The role of genetic polymorphisms and autoinduction in CYP450 enzymes, transporter proteins and other detoxification pathways have not been fully characterised in relation to CBZ metabolism or susceptibility to CBZ hypersensitivity reactions.

Our current understanding of how CBZ triggers hypersensitivity reactions in genetically susceptible individuals is lacking. Further research is needed to link CBZ metabolism, CBZ hapten formation and activation of the immune system to enable better diagnostic tests and safer drug design in future.

5. STUDY RATIONALE, OBJECTIVES AND DESIGN

5.1. Rationale

An improved mechanistic understanding of CBZ hypersensitivity is crucial because CBZ is an effective and widely prescribed medication. However, a significant number of patients (up to 10%) develop hypersensitivity reactions, which if severe, are associated with significant mortality and long-term morbidity. Current methods for diagnosis of CBZ hypersensitivity are time consuming, have low sensitivity and low specificity and would not be applicable in clinical practice. Although pharmacogenetic tests for HLA exist that can identify patients at risk of hypersensitivity they are not employed routinely in clinical practice. The mechanistic studies described herein will link CBZ metabolism which leads to antigen formation to the immune response and individual susceptibility. This will improve our understanding of the relationship between antigenicity and immunogenicity which will help in future drug design; and how individuals with specific genetic biomarkers respond to CBZ in-vivo and in-vitro which will improve the basis for developing predictive tests. The lessons from this research will also provide valuable information for other drugs that are known to cause hypersensitivity reactions.

5.2. Aims and Objectives

- To obtain pharmacokinetic (PK) data from plasma and urine of patients who have been newly prescribed CBZ to improve our understanding of the role of auto-induction in CBZ metabolism and CBZ protein adduct formation.
- To obtain sparse pharmacokinetic (PK) data from plasma and urine of patients who have been on maintenance CBZ treatment to improve our understanding of CBZ metabolism and CBZ-protein adduct formation.
- To study the effects of genetic polymorphisms in drug metabolising enzymes and transporter proteins on metabolism and adduct formation in patients receiving CBZ therapy.
- Data from this study will subsequently be incorporated into a larger population PK model for CBZ.

5.3. *Study Design*

The study will consist of two groups:

Autoinduction group:
It is intended that 8 patients with epilepsy who are newly commenced on CBZ therapy will complete the study. Subjects will be identified from the Walton Centre NHS Foundation Trust. Subjects will be required to attend the clinical research facility at the Walton Centre for blood and urine sampling.

Maintenance group:
It is intended that 150 plasma samples will be obtained from patients who have been receiving maintenance CBZ therapy at the same dosage for at least 4 weeks. Subjects will be identified from outpatient clinics at the Walton Centre NHS Foundation Trust. If a subject consents to participation then a blood sample will be taken by venepuncture and a spot urine sample collected.

5.4. Statistical Considerations

The number of patients planned for this study is consistent with other studies of a similar nature.[22,23] As this is a novel exploratory study to characterise metabolite and drug-protein conjugate formation it is difficult to provide an accurate sample size in advance.

6. SUBJECTS AND SCREENING

6.1. Source of Subjects

The study population will consist of male or female patients age 18 or over and will be recruited from the Walton Centre NHS Foundation Trust. Subjects will be assigned trial numbers, in the order of their inclusion in the study which will be defined as the time they consent to participation.

6.2. Inclusion Criteria

Autoinduction group:

- Subject is willing and able to give written informed consent
- Subject is aged 18 or over
- Subject is newly prescribed CBZ by their attending physician

Maintenance group:

- Subject is willing and able to give written informed consent
- Subject is aged 18 or over
- Subject has received CBZ therapy at the same dosage for at least 4 weeks

6.3. Exclusion Criteria

Autoinduction group:

- Subject is not willing to take part or unable to give written informed consent
- Subject has in the past 4 weeks received other medication that is a CYP3A4 inducer or inhibitor (see study restrictions- section 7.2)

- Subject has any condition which in the opinion of the investigator will interfere with the study

Maintenance group:

- Subject is not willing to take part or unable to give written informed consent
- Subject has any condition which in the opinion of the investigator will interfere with the study

6.4. Screening Procedures

Subjects will be identified from neurology clinics at the Walton Centre NHS Foundation Trust. Subjects will be asked if they are willing to take part following their clinic appointments.

6.5. Subject Informed Consent Form

Voluntary written informed consent will be obtained from each subject prior to performing any trial-related procedures. Each subject will be given both verbal and written information describing the nature and duration of the clinical trial. The informed consent process will take place under conditions where the subject has adequate time to consider the risks associated with his/her participation in the clinical trial. No trial procedures will be undertaken until subjects have signed an approved informed consent form (ICF) written in a language that is understandable to the subject.

The Investigator's local research and ethics committee (REC) - approved ICF will be personally signed and dated by the subject and the investigator or designee who conducted the informed consent discussion. Each subject will receive a copy of the signed and dated written ICF along with any other written information provided to the subject.

The investigator is responsible for ensuring that informed consent is obtained from each subject in accordance with all applicable regulations and guidelines. The original signed ICF will be retained within the subject's source documents.

7. **STUDY CONDUCT**

7.1. *Study Schedule*

Blood sampling of patients will take place at the clinical research facility at the Walton Centre NHS Foundation Trust.

7.1.1. *Patient Recruitment and Consent*

Autoinduction Group:

Patients that are issued with a new prescription for carbamazepine will be identified and invited for a short consultation where the study will be explained and they will be provided with the patient information leaflet and consent form. If the patient is happy to consent at this time then this will be allowed. **Otherwise, patients will be given at least 24 hours to decide and a member of the study team will contact the patient to determine if the patient is willing to participate. In the meantime the patient should be advised not to start CBZ therapy. Delaying CBZ therapy by up to 5 days will have no negative effects for patient care.**

Maintenance Group:

Patients who have received carbamazepine treatment at the same dosage for at least 4 weeks will be identified and invited for a short consultation where the study will be explained and they will be provided with the patient information leaflet and consent form. If the patient is happy to consent at this time then this will be allowed because the study does not interfere with patient care and consists of venepuncture only.

7.1.2. *Autoinduction Group Visit Schedule*

7.1.2.1. *Visit 1*

All potential participants will be asked to the clinical research facility at the Walton Centre NHS Foundation Trust at approximately 0800 on the first day that they are due to start CBZ treatment. During this visit subjects will be consented (if not already completed). They will also undertake a brief interview with physical

examination and blood and urine sampling according to the timeline below. Blood samples will be sent for full blood count (FBC), urea & electrolytes (U&E), liver function tests (LFT) and analysis of genetic polymorphisms in drug metabolism and transporter proteins. Patients will be allowed to leave the unit after the 8 hour blood sample and return the following morning for the 24 hour blood sample. Patients will also be asked to provide a 24 hour urine sample. After this visit subjects will be advised to continue carbamazepine as prescribed by their physician and will be given the date to return for their next blood sampling appointment.

1. Consent	Written and verbal consent for inclusion into study
2. Interview	(a) Past medical history (b) Concomitant medications (c) Carbamazepine – dosage, frequency, formulation, time of last dosage (d) Social history – smoking, alcohol and ethnicity (including parents) (e) Contact details - GP
3. Physical Examination	(a) Height and Weight (b) Body mass index
4. Cannulate and blood sampling at time=0	(a) 2 x 9ml EDTA tube (FBC, Genetic polymorphisms) (b) 1 x 9ml serum gel tube (U&E, LFT) (c) 1 x 5ml Li Heparin tube (CBZ, metabolite and adducts)
5. CBZ administration	(a) Administer CBZ
4. Blood Sampling	Blood sampling from cannula into 1 x 5ml Li heparin tube at the following times: • 1 hour post dose • 2 hours post dose • 4 hours post dose • 6 hours post dose • 8 hours post dose
5. Urine Sampling	(a) 1 x 24 hour urine sample

7.1.2.2. *Visit 2*

Subjects will be asked to return to the Walton Centre on day 2 following commencement of CBZ when they will undergo the following consultation:

1. Interview	(a) Ensure patient happy to continue with study (b) Concomitant medications (c) Carbamazepine – dosage, frequency and formulation
2. Blood Sampling	Blood sampling by venepuncture into 1 x 5ml Li heparin tube prior to administration of CBZ (pre dose)
3. Urine Sampling	(a) 1 x 24 hour urine sample

7.1.2.3. *Visit 3*

Subjects will be asked to return to the Walton Centre on day 14 after starting CBZ when the following consultation will take place:

1. Interview	(a) Ensure patient happy to continue with study (b) Concomitant medications (c) Carbamazepine – dosage, frequency and formulation
2. Cannulation and Blood Sampling	Blood sampling from cannula into 1x 5ml Li heparin tube at the following times • 0 hours (pre dose) then administer CBZ • 1 hour post dose • 2 hours post dose • 4 hours post dose • 6 hours post dose • 8 hours post dose
3. Urine Sampling	(a) 1 x 24 hour urine sample

7.1.2.4. *Visit 4*

Subjects will be asked to return to the Walton Centre on day 28 after starting CBZ when the following consultation will take place.

1. Interview	(a) Ensure patient happy to continue with study (b) Concomitant medications

	(c) Carbamazepine – dosage, frequency and formulation
2. Cannulation and Blood Sampling	Blood sampling from cannula into 1x 5ml Li heparin tube at the following times • 0 hours (pre dose) then administer CBZ • 1 hour post dose • 2 hours post dose • 4 hours post dose • 6 hours post dose • 8 hours post dose
3. Urine Sampling	(a) 1 x 24 hour urine sample

7.1.2.5. *Visit 5*

Subjects will be asked to return to the Walton Centre on day 42 since starting CBZ for the following consultation.

1. Interview	(a) Ensure patient happy to continue with study (b) Concomitant medications (c) Carbamazepine – dosage, frequency and formulation
2. Cannulation and Blood Sampling	Blood sampling from cannula into 1 x 5ml Li heparin tube at the following times: • 0 hours (pre dose) then administer CBZ • 1 hour post dose • 2 hours post dose • 4 hours post dose • 6 hours post dose • 8 hours post dose
3. Urine Sampling	(a) 1 x 24 hour urine sample

7.1.2.6. *Visit Schedule Variations*

If subjects are not able to attend on the exact dates outlined above then they will be allowed to reschedule appointments up to 48 hours before and after the specified date.

7.1.3 *Maintenance Group Visit Schedule*

7.1.3.1 *Visit 1*

Once a patient has consented for the study two blood samples will be taken by venepuncture and a spot urine sample collected. A short interview will be conducted to ascertain patient demographics, past medical history, concurrent medications and social history. A brief physical examination will also be undertaken to determine the subject's height and weight.

1. Consent	Written and verbal consent for inclusion into study
2. Interview	(a) Past medical history (b) Concomitant medications (c) Carbamazepine – dosage, frequency, formulation, time of last dosage (d) Social history – smoking, alcohol and ethnicity (including parents)
3. Physical Examination	(a) Height and Weight (b) Body mass index
4. Venepuncture	(a) 1 x 9ml EDTA tube (Genetic polymorphisms) (b) 1 x 5ml Li Heparin tube (CBZ, metabolite and adducts) (c) 1 x 5ml serum gel tube (U&E, LFTs)
5. Urine Sampling	(b) 1 x spot urine sample

7.1.3.2 *Subsequent Visits*

If the same subject presents to the Walton Centre for further clinic visits then they will be allowed to donate further blood and urine samples as long as they are happy to do so. The original consent will be valid for one year. If a patient presents more than one year after their initial consent then they will be re-consented for the study.

7.2 *Study Restrictions (<u>applicable only to autoinduction group</u>)*

7.2.1 *Concurrent Medication*

Participants will be expected to complete all 5 visits for blood and urine sampling. CBZ is metabolised by CYP3A4 and other drugs may influence its metabolism

through induction or inhibition of CYP3A4[26]. Therefore, patients will be excluded from the study if they have taken any of the following list of medications in the past 4 weeks:

Inducers	**Inhibitors**		
Rifampicin	Amiodarone	Fluvoxamine	Saquinavir
Rifampin	Amprenavir	Indinavir	S-Verapamil
Isoniazid	Atazanavir	Lopinavir	Verapamil
Phenytoin	Azithromycin	Mifepristone	
Phenobarbital	Grapefruit juice	Nelfinavir	
Omeprazole	Clarithromycin	Norverapamil	
Clotrimazole	Cyclosporine A	Ritonavir	
HMG-CoA reductase inhibitors	Delavirdine	*N*-desmethylerythromycin	
Cyclophosphamide	Erythromycin	Roxithromycin	
Spironolactone	Fluoxetine	R-Verapamil	

7.2.2 *Diet*

Seville oranges, grapefruits and grapefruit juice are known inhibitors of CYP3A4. Subjects will be asked to avoid consuming these substances for the study period.

7.2.3 *Alcohol*

Subjects will be advised to adhere to the government recommendations for alcohol intake for the duration of the study. For men that is 3-4 units per day and women 2-3 units per day.

7.3 *Methods for Clinical Procedures*

7.3.1 *Blood Sampling*

This study will be conducted with close attention to patient safety. Venepuncture and cannulation poses minimal risk of bruising or bleeding. This procedure will be carried out by trained, competent health professionals.

Blood samples for determination of CBZ, CBZ metabolites and CBZ-protein conjugates and genetic polymorphisms will be collected via a cannula or direct

venepuncture. Prior to each sample being taken from the cannula 0.5mls of blood will be drawn and discarded. The cannula will be flushed with 2mls of 0.9% saline each time a sample is taken. Blood samples will be centrifuged within 15 minutes of collection or stored overnight at 4^{o}C for centrifuge the following day. 1ml and 350µL aliquots will then be transferred to appropriate tubes and stored at approximately -80^{o}C prior to bioanalysis.

In the autoinduction group no more than 35mls of blood will be extracted at time 0 on day one and no more than 5mls for each subsequent sampling on day one. Over a 24 hour period no more than 65mls of blood will be sampled. Total volume of blood sampled for the autoinduction group will be approximately 155mls.

In the maintenance group no more than 20mls of blood will be extracted at each visit.

7.3.2 *Urine Sampling*

Subjects in the autoinduction group will be asked to provide a 24 hour urine sample at each visit. The samples will be stored at -20^{o}C for metabolite and carbamazepine-protein conjugate analysis.

Subjects in the maintenance group will be asked to provide a spot urine sample at each visit. The samples will be stored at -20^{o}C for metabolite and carbamazepine-protein conjugate analysis.

7.4 *Laboratory Evaluations*

7.4.1 *Haematology*

Blood samples will be taken into EDTA tube for full blood count.

7.4.2 *Biochemistry*

Blood samples will be taken into serum gel tube for urea and electrolytes and liver function tests.

7.4.3 *Analysis of Genetic Polymorphisms*

Blood samples will be taken into EDTA tube for identification of polymorphisms in drug metabolising enzymes and drug transporter proteins.

7.5 *CBZ Metabolite and CBZ-Protein Conjugate Analysis*

High sensitivity mass spectrometry techniques already in use at the MRC Centre for Drug Safety Science at the University of Liverpool will be utilised to characterise and quantify CBZ metabolites and CBZ-protein conjugates.

7.6 *Sample Storage*

All samples will be transferred/delivered in appropriate packaging in dry ice (solid carbon dioxide) to the University of Liverpool, Department of Pharmacology Laboratories. Samples will be stored securely as described above and the University will act as custodian.

7.7 *End of Study*

The end of study is defined as the last scheduled protocol activity of the last subject in the study. The conclusion of the study will be notified by the University of Liverpool to the Ethics Committee within the required timelines. All research data will be archived at the University of Liverpool for at least 15 years.

7.8 *Criteria for Termination of the Study*

There are no criteria for termination of the study. Details regarding the withdrawal of subjects are given in section 10.

8. CASE REPORT FORMS

Paper case report forms (CRFs) will be used for recording all data collected during the study. All CRFs are to be completely filled out by personnel administering the study procedures and reviewed and signed by the chief investigator or nominated designee. All CRFs are to be completed in a clear and legible manner. Black ink must be used to ensure accurate interpretation of data. Any changes or corrections must be made by drawing a line through the data to be changed, entering corrected information, and signing (or initialling) and dating the change. Erasing, overwriting, or the use of “liquid paper” is not permitted on CRFs. Every effort should be made to have the CRFs completed as soon as possible following a subject’s study visit. All

data from the recruited patients will be transcribed onto an electronic spreadsheet. All study documentation as defined above will be made available to the Ethics Committee and to regulatory authorities for inspection on request.

9. SAFETY ASSESSMENT AND ADVERSE EVENT REPORTING

An adverse event (AE) is defined as any undesirable event occurring to a subject during a clinical study. All adverse events will be coded and reported as follows:

- Mild: Awareness of sign or symptom but easily tolerated
- Moderate: Discomfort enough to cause interference with usual activity
- Severe: Incapacitating with inability to work or do usual activity

Laboratory results that are outside the normal range will be recorded as an adverse event. The severity of each event will be assessed by the investigator. When recording the outcome of the adverse event its maximum severity will also be recorded. Adverse events, if any, not resolved by the end of the study, will be followed up by the investigator.

Serious adverse events (SAE) or suspected unexpected serious adverse reactions (SUSAR) are defined as any adverse event that results in death, is life-threatening, requires hospitalisation, results in persistent or significant disability or incapacity and consists of a congenital anomaly or birth defect. . All SAEs possibly or probably definitely related to CBZ or to the study procedure(s) will be reported by the investigator to the Sponsor within 24 hours of when he or she becomes aware of it. The investigator must inform the Sponsor of any follow-up information on a previously reported SAE immediately, or no later than 24 hours of when he or she becomes aware of it. SUSARs that are fatal or life-threatening will be reported to the MHRA as soon as possible but no later than 7 days. SUSARs which are not fatal or life-threatening will be reported to the MHRA as soon as possible but no later than 15 days. Causality for SAEs and SUSARs will be checked against the SmPc. All SAE and SUSAR will be managed according to the Royal Liverpool University Hospital research and development standard operating procedures.

10. WITHDRAWAL OF SUBJECTS

Subjects will be informed that they are free to withdraw from the study at any time. The investigator may remove a subject if, in his opinion, it is in the best interest of the subject. Although a subject is not obliged to give his reason for premature withdrawal, the investigator will make a reasonable effort to obtain the reason while fully respecting the subject's rights. If there is a medical reason for withdrawal of the subject then this will be reported as an adverse event and followed up by the investigator. If in the opinion of the investigator it is considered necessary, the subject's GP will be informed of the medical reason for the withdrawal. The subject's consent will be obtained prior to informing the GP. Subjects that withdraw consent or are withdrawn at the discretion of the investigator prior to completion of study procedures may be replaced to ensure that there are 8 evaluable subjects.

11. DATA ANALYSIS

Initially descriptive statistics will be used to analyse the data. Subsequently a population pharmacokinetic model will be generated using the software NONMEM (non-linear mixed effects modelling).

12. RECORDS AND CONFIDENTIALITY

12.1 *Records*

Adequate records as required as required by ICH GCP will be maintained for the study. This includes medical records, case report forms, laboratory reports, signed consent forms, and worksheets. All records from the study will be kept for 15 years.

12.2 *Confidentiality*

To maintain subject privacy, all case report forms, study reports and communications will identify the subject by initials where permitted and/or by the assigned subject number. The subject's confidentiality will be maintained and will not be made publicly available to the extent permitted by the applicable laws and regulations.

12.3 *Publication*

The results of the study will be incorporated into scientific papers for publication. All identifiable patient information will be removed. Appropriate precautions will be taken to maintain confidentiality of medical records and personal information.

APPENDIX A

13.1 *Summary of Study Visits for Autoinduction Group*

	Day 1	**Day 2**	**Day 14**	**Day 28**	**Day 42**
	Visit 1	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**
Informed consent	X				
Interview	X	X	X	X	X
Medication review	X	X	X	X	X
Physical examination	X				
Cannulation	X		X	X	X
Safety bloods	X				
Bloods for genetic variation	X				
Carbamazepine blood test	X	X	X	X	X
24 hour urine sample		X	X	X	X
Adverse event monitoring	X	X	X	X	X

13.2 *Summary of Study Visits for Maintenance Group*

	Visit 1	**Subsequent Visits**
Informed consent	X	Not necessary if within 1 year
Interview	X	X
Medication review	X	X
Physical examination	X	X
Blood test for genetic variation	X	
Carbamazepine blood test	X	X
Spot urine sample	X	X

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A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN EPILEPSY PATIENTS

Participation Information Sheet (Auto-Induction Group)

Invitation

You are being invited to participate in a research study **because you have been newly prescribed carbamazepine by your doctor**. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read this sheet carefully and discuss it with relatives, friends and the study team. You may also wish to discuss this with your GP. Ask us about anything that is not clear and take time to decide whether or not you wish to take part.

What is the purpose of the study?

This study is looking at the formation of breakdown products produced by the body following carbamazepine ingestion and how genetic variation affects this process. Carbamazepine is a medication that is used to treat epilepsy, trigeminal neuralgia and some psychiatric conditions. We know that some patients develop adverse reactions, which are termed hypersensitivity reactions, to carbamazepine. These hypersensitivity reactions include skin rash, fever and lethargy, and usually occur with multiple doses. At the moment we do not know what causes these hypersensitivity reactions but we believe that they are caused by the way the body handles carbamazepine and forms breakdown products. These breakdown products (also called metabolites) then cause hypersensitivity reactions in genetically susceptible patients. The main purpose of this study is to find out how genetic variation can affect the formation of carbamazepine metabolites.

Do I have to take part?

It is up to you to decide whether or not to take part in the study. Participation is entirely voluntary. If you do decide to participate you will be given this information sheet to keep and be asked to sign a consent form. You will receive a copy of this signed consent form to keep. If you decide to take part you are still free to

withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

You will be given sufficient time to consider whether or not you wanted to take part. If you agree to take part in the study you will be required to make **5 visits** to the Walton Centre. For each visit you will be asked to donate blood and urine samples. Each visit will take at least 8 hours. During the study you will be asked to take your carbamazepine as prescribed by your doctor. The first visit will take place on the same day as you take your first tablet of carbamazepine. Subsequent visits will take place on days 2, 14, 28 and 42 following commencement of treatment.

During the study you will not be able to eat grapefruit or pommello oranges or any product that contains grapefruit or pommello oranges.

If you need to take any other medication you should contact the study team before taking it. You are not allowed to take any interacting prescription or non-prescription drug, recreational drugs (cannabis, cocaine), or dietary supplements. You are allowed to take paracetamol and multi-vitamins.

During the study we will ask you to adhere to the governments recommendations with regards to alcohol consumption. For men that is 3-4 units per day and women 2-3 units per day.

During the study visits you will be studying in the clinical research unit at the Walton Centre. You will have your own room and bed. There is also a common room where a TV is available. Wi-Fi is available throughout the unit and you are free to bring laptops or tablets. Breakfast and lunch are provided with unlimited tea and coffee throughout the day.

Visit 1

Please do not take your first dose of carbamazepine before your first visit. **Your details will be checked against your clinical notes and we will record** your age, ethnic group, past medical problems and details of any medication you are taking. A

clinical examination will be undertaken. If you are suitable to take part in the study we will insert a small tube (cannula) into a blood vessel and take blood samples for full blood count, kidney and liver function and genetic variation in the proteins responsible for metabolism of carbamazepine. We will then ask you to take your first carbamazepine tablet. Blood will be taken from the cannula at regular intervals following the carbamazepine dose at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours. We will also ask you to keep a 24 hour urine collection. The total amount of blood taken will be approximately 65mls. You will be allowed home following the 8 hour blood sample and the cannula will be removed.

Visit 2

You will be asked to return to the clinical research facility the following morning, 24 hours after taking your first carbamazepine tablet. Please do not take the 2nd dose of carbamazepine before this visit. We will undertake a brief interview, take a single blood sample and ask you to return your first 24 hour urine sample bottle. You will be given a new 24 hour urine bottle to collect urine on the day before (day 6) you are due for the next visit. Visit 2 will last approximately 30 minutes.

Visit 3

Visit **3** will take place on day 14 after starting carbamazepine therapy. You will be asked to omit your carbamazepine tablet that morning and to return to the clinical research facility with your completed 24 hour urine collection bottle. A brief interview will be undertaken and a cannula will be inserted and a blood sample taken from it. Following this you will be given your carbamazepine tablet and blood will be taken at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours from the cannula. The total amount of blood taken will be approximately 30mls. We will provide you with a 24 hour urine sample bottle to complete the day before (day 20) you are due to return for your next visit.

Visit 4

Visit **4** will take place on day 28 after starting carbamazepine therapy. You will be asked to omit your carbamazepine tablet that morning and to return to the clinical

research facility with your completed 24 hour urine collection bottle. A brief interview will be undertaken and a cannula will be inserted and a blood sample taken from it. Following this you will be given your carbamazepine tablet and blood will be taken at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours from the cannula. The total amount of blood taken will be approximately 30mls. We will provide you with a 24 hour urine sample bottle to complete the day before (day 27) you are due to return for your next visit.

Visit 5

This is the final study visit and will take place on day 42 after starting carbamazepine therapy. You will be asked to omit your carbamazepine tablet that morning and to return to the clinical research facility with your completed 24 hour urine collection bottle. A brief interview will be undertaken and a cannula will be inserted and a blood sample taken from it. Following this you will be given your carbamazepine tablet and blood will be taken at 1 hour, 2 hours, 4 hours, 6 hours and 8 hours from the cannula. The total amount of blood taken will be approximately 30mls.

Visit Schedule Variations

If you are unable to attend appointments on the dates outlined above then you will be allowed to reschedule them up to 48 hours before the specified date.

Withdrawal Visit

If you decide to stop the study before the end you will need to attend for a final visit. We will check your pulse, blood pressure and temperature.

What are the possible side effects/risks of taking part?

You may have side effects whilst taking part in this study. However, we will monitor everyone in the study for side effects. If you are worried about your symptoms then you can contact the study team at any time. You will be given contact numbers of the study team.

Carbamazepine side effects

Dizziness, ataxia (loss of co-ordination), double vision, drowsiness, and nausea and vomiting may occur with single doses in about 10% of people. However, these are usually short lasting. Other side effects which have been reported with carbamazepine include low platelets, fluid retention, dry mouth, elevated liver enzymes and skin rashes.

Abnormal blood test results

If any blood tests taken during your participation in the study are abnormal then we will invite you back to the clinical research facility to explain the results to you. We will also ensure that you are referred to the appropriate clinician for further management.

Other possible risks

When you give blood you may feel faint or experience bruising or tenderness at the site of the needle puncture. You will be required to remain in hospital for 8 hours during study visits 1, 3, 4 and 5 which may cause inconvenience.

What are the benefits of taking part?

There will be no direct benefits to you by taking part in the study. However, in the long-term, the information we get from this study may help improve the treatment of people with carbamazepine**.**

What happens if something goes wrong?

If you are unhappy with any aspect of the study then complaints should be made to the chief investigator in the first instance. There are no special compensation arrangements. If you are harmed because of negligence then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you.

Will my taking part in this study be kept confidential?

All the information collected about you during the course of the study will be kept strictly confidential. Any information about you which leaves the clinic will have your name and address removed so that you cannot be recognised from it. All samples will be coded and anonymised. All identifying details (e.g. name, address, date of birth) will be removed, but the sample will remain linked to data collected from this study (e.g. weight, age). Your medical records may be inspected by research staff for the purposes of checking that the information we have is accurate and complete. We will seek your consent to notify your GP about your participation in this study.

What will happen to my samples?

Your blood and urine samples will be analysed for levels of carbamazepine and its breakdown products. We will look at genes in your DNA samples to see how this determines the way your body handles carbamazepine. **If during the study you lose capacity then any samples we have collected will still be analysed but no further samples will be taken.**

What happens when the research study stops?

You will be asked if we can store the samples from this study for use in other research projects. The samples will be kept in a locked freezer in the University of Liverpool. ALL THESE SAMPLES WILL BE CODED (but will be linked to details that we will collect about your drug levels). We will keep your sample until all of it is used up. The samples will be considered a gift to the University of Liverpool, which will act as a custodian of all the samples obtained as part of this project. Rarely, a small amount of your sample may be provided to other researchers in the UK or other parts of the world. However, it is important to remember that this will only be identified by a unique code, and your name will not be given out.

We are asking for your permission to store your samples and linked details for these future studies. Your samples will not be sold for profit. (It is not the purpose of this study to produce commercial gain, and in any case, any commercial value in

the future will only come from findings in groups of people rather than from samples from a single person).

Once the studies are completed your anonymised data will be kept for a minimum of 15 years.

What will happen to the results of the study?

We will combine all the results from the subjects taking part in the study, and publish any important results in medical journals. Results may also be presented at scientific meetings. No individuals will be specifically identified in any publication.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the area that is being studied. We will share this information with you right away if it is important to your health. If you have any concerns about it you should discuss this with the research team.

Who is funding the research?

This study is funded by the Medical Research Council.

Who has reviewed the study?

This study has been reviewed by the National Research Ethics Service Committee North West – Haydock

Reimbursement

You will be reimbursed a total of £200 on completion of the study. **In addition, travel arrangements will also be reimbursed. If required travel arrangements can be organised with the Walton Centre NHS Foundation Trust**. If you withdraw before completion of the study you will be reimbursed for the time you participated. You will be reimbursed £40 for each visit. Withdrawal of consent or violation of the protocol may, however, result in partial or complete forfeiture of compensation. Protocol violation is defined as the disobeying of instructions communicated verbally or in writing. You will be asked to provide details of your

bank account so this payment can be made. You will be responsible for paying tax on any payment received.

Involvement of your General Practitioner

With your permission, your general practitioner will be informed that you are taking part in the study and may be contacted to confirm your past medical history. Your signature on the consent form will authorise the study team to do this.

For further information on this study please contact:

Dr Vincent Yip, MRC Clinical Fellow in Clinical Pharmacology and Therapeutics, Department of Molecular and Clinical Pharmacology, The Wolfson Centre for Personalised Medicine, The University of Liverpool, L69 3GL

0151 795 5407

vyip@liv.ac.uk

Study Investigator _____

Tel Number _____

E-mail _____

Summary of Study Visits

	Day 1	Day 2	Day 14	Day 28	Day 42
	Visit 1	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**
Informed consent	X				
Interview	X	X	X	X	X
Medication review	X	X	X	X	X
Physical examination	X				
Cannula	X		X	X	X
Safety blood tests	X				
Blood tests for genetic variation	X				
Carbamazepine blood test	X	X	X	X	X
24 hour urine sample		X	X	X	X
Adverse event monitoring	X	X	X	X	X

Thank you for reading this. This sheet (and a copy of the consent form) should be retained by you if you decide to participate.

A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN EPILEPSY PATIENTS

Participant Consent Form (Autoinduction Group)

The volunteer should complete the whole of this sheet himself/herself

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Participant identification number: _____

Name of Researcher: _____

Contact Details for Research Team: _____

PART 1 Please initial boxes

1. I confirm that I have read and understand the information sheet dated _____for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I agree to give blood samples for research in this project. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw at any time without giving any reason, without medical care or legal rights being affected. ☐

3. In the (unlikely) event that I lose capacity to consent during the study I understand that I will be withdrawn from the study. The samples that I have provided for the study up to this point will be retained and used for research as outlined in the study information sheet. ☐

4. I understand that once the study has ended the samples will be fully anonymised and once gifted to the University of Liverpool cannot be withdrawn. ☐

5. I understand that relevant sections of my study records and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I understand that I will not benefit financially if this research leads to the development of a new medical test. ☐

6. I agree to my GP being informed of my participation in the study and I understand that the GP may be contacted to confirm my past medical history. ☐

7. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

8. I agree to take part in the above study ☐

10. I understand that I will receive up to £280 for participating in this study. ☐

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

PART 2

9. **Consent for storage and use in possible future research projects**

I agree that the samples I have given and the information gathered about me can be stored by the University of Liverpool for possible use in future projects as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than the University of Liverpool Pharmacology department who ran the first project. ☐

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

Thank you for agreeing to participate in this research.

Prof A Marson, Department of Molecular and Clinical Pharmacology, Block A: Wolfson Centre for Personalised Medicine, University of Liverpool, Liverpool L69 3GL

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes

A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN EPILEPSY PATIENTS

Participation Information Sheet (Maintenance Group)

Invitation

You are being invited to participate in a research study **because you are prescribed carbamazepine by your doctor**. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read this sheet carefully and discuss it with relatives, friends and the study team. You may also wish to discuss this with your GP. Ask us about anything that is not clear and take time to decide whether or not you wish to take part.

What is the purpose of the study?

This study is looking at the formation of breakdown products produced by the body following carbamazepine ingestion and how genetic variation affects this process. Carbamazepine is a medication that is used to treat epilepsy, trigeminal neuralgia and some psychiatric conditions. We know that some patients develop adverse reactions, which are termed hypersensitivity reactions, to carbamazepine. These hypersensitivity reactions include skin rash, fever and lethargy, and usually occur with multiple doses. At the moment we do not know what causes these hypersensitivity reactions but we believe that they are caused by the way the body handles carbamazepine and forms breakdown products. These breakdown products (also called metabolites) then cause hypersensitivity reactions in genetically susceptible patients. The main purpose of this study is to find out how genetic variation can affect the formation of carbamazepine metabolites.

Do I have to take part?

It is up to you to decide whether or not to take part in the study. Participation is entirely voluntary. If you do decide to participate you will be given this information sheet to keep and be asked to sign a consent form. You will receive a copy of this signed consent form to keep. If you decide to take part you are still free to

withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

You will be given sufficient time to consider whether or not you wanted to take part. If you agree to take part in the study you will be required to donate 2 blood samples and one urine sample.

Visit 1

If you agree to take part in the study immediately then we will collect a blood and urine sample on straight after your clinic appointment. The total amount of blood taken will be approximately 20mls.

Subsequent Visits

You may be asked to donate further blood samples for this study if you are attending the Walton Centre for other clinic appointments. We will ask your permission and if it is within 1 year of your first blood sample donation then we will not need to obtain consent again.

What are the possible side effects/risks of taking part?

When you give blood you may feel faint or experience bruising or tenderness at the site of the needle puncture. However, these risks will be minimised because only trained health professionals will be permitted to take your blood.

What are the benefits of taking part?

There **will be no** direct benefits to you by taking part in the study. However, in the long-term, the information we get from this study may help improve the treatment of people with carbamazepine**.**

What happens if something goes wrong?

If you are unhappy with any aspect of the study then complaints should be made to the chief investigator in the first instance. There are no special compensation

arrangements. If you are harmed because of negligence then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you.

Will my taking part in this study be kept confidential?

All the information collected about you during the course of the study will be kept strictly confidential. Any information about you which leaves the clinic will have your name and address removed so that you cannot be recognised from it. All samples will be coded and anonymised. All identifying details (e.g. name, address, date of birth) will be removed, but the sample will remain linked to data collected from this study (e.g. weight, age). Your medical records may be inspected by research staff for the purposes of checking that the information we have is accurate and complete. We will seek your consent to notify your GP about your participation in this study.

What will happen to my samples?

Your blood and urine samples will be analysed for levels of carbamazepine and its breakdown products. We will look at genes in your DNA samples to see how this determines the way your body handles carbamazepine. **If during the study you lose capacity then any samples we have collected will still be analysed but no further samples will be taken.**

What happens when the research study stops?

You will be asked if we can store the samples from this study for use in other research projects. The samples will be kept in a locked freezer in the University of Liverpool. ALL THESE SAMPLES WILL BE CODED (but will be linked to details that we will collect about your drug levels). We will keep your sample until all of it is used up. The samples will be considered a gift to the University of Liverpool, which will act as a custodian of all the samples obtained as part of this project. Rarely, a small amount of your sample may be provided to other researchers in the UK or other

parts of the world. However, it is important to remember that this will only be identified by a unique code, and your name will not be given out.

We are asking for your permission to store your samples and linked details for these future studies. Your samples will not be sold for profit. (It is not the purpose of this study to produce commercial gain, and in any case, any commercial value in the future will only come from findings in groups of people rather than from samples from a single person).

Once the studies are completed your anonymised data will be kept for a minimum of 15 years.

What will happen to the results of the study?

We will combine all the results from the subjects taking part in the study, and publish any important results in medical journals. Results may also be presented at scientific meetings. No individuals will be specifically identified in any publication.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the area that is being studied. We will share this information with you right away if it is important to your health. If you have any concerns about it you should discuss this with the research team.

Who is funding the research?

This study is funded by the Medical Research Council.

Who has reviewed the study?

This study has been reviewed by the National Research Ethics Service Committee North West – Haydock

Involvement of your General Practitioner

With your permission, your general practitioner will be informed that you are taking part in the study and may be contacted to confirm your past medical history. Your signature on the consent form will authorise the study team to do this.

For further information on this study please contact:

Dr Vincent Yip, MRC Clinical Fellow in Clinical Pharmacology and Therapeutics, Department of Molecular and Clinical Pharmacology, The Wolfson Centre for Personalised Medicine, The University of Liverpool, L69 3GL

0151 795 5407

vyip@liv.ac.uk

Study Investigator _____

Tel Number _____

E-mail _____

Summary of Study Visits

	Visit 1	**Subsequent Visits**
Informed consent	X	Not necessary to re-consent if within 1 year
Interview	X	X
Medication review	X	X
Physical examination	X	X
Blood tests for genetic variation	X	X
Carbamazepine blood test	X	X
Spot urine sample	X	X

Thank you for reading this. This sheet (and a copy of the consent form) should be retained by you if you decide to participate.

A PHARMACOKINETIC INVESTIGATION INTO THE FORMATION OF CARBAMAZEPINE METABOLITES AND CARBAMAZEPINE-PROTEIN CONJUGATES IN EPILEPSY PATIENTS

Participant Consent Form (Maintenance Group)

The volunteer should complete the whole of this sheet himself/herself

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Participant identification number: _____

Name of Researcher: _____

Contact Details for Research Team: _____

PART 1 Please initial boxes

1. I confirm that I have read and understand the information sheet dated _____ for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I agree to give blood samples for research in this project. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw at any time without giving any reason, without medical care or legal rights being affected. ☐

3. In the (unlikely) event that I lose capacity to consent during the study I understand that I will be withdrawn from the study. The samples that I have provided for the study up to this point will be retained and used for research as outlined in the study information sheet. ☐

4. I understand that once the study has ended the samples will be fully anonymised and once gifted to the University of Liverpool cannot be withdrawn. ☐

5. I understand that relevant sections of my study records and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

6. I understand that I will not benefit financially if this research leads to the development of a new medical test. ☐

7. I agree to my GP being informed of my participation in the study and I understand that the GP may be contacted to confirm my past medical history. ☐

8. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

9. I agree to take part in the above study ☐

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

Please see overleaf for part 2 of the consent form.

PART 2

10. **Consent for storage and use in possible future research projects**

I agree that the samples I have given and the information gathered about me can be stored by the University of Liverpool for possible use in future projects as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than the University of Liverpool Pharmacology Department who ran the first project. ☐

_____ Name of patient (BLOCK CAPITALS)	_____ Date	_____ Signature
_____ Name of person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Name of Researcher	_____ Date	_____ Signature

Thank you for agreeing to participate in this research.

Prof A Marson, Department of Molecular and Clinical Pharmacology, Block A: Wolfson Centre for Personalised Medicine, University of Liverpool, Liverpool L69 3GL

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes

Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
HRA NRES Centre - Manchester
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7827
Facsimile: 0161 625 7299

11 September 2013

Dr Vincent Yip
The Wolfson Centre for Personalised Medicine
Block A: Waterhouse Buildings
1-5 Brownlow Street
L69 3GL

Dear Dr Yip

Study title:	**A Pharmacokinetic Investigation into the Formation of Carbamazepine Metabolites and Carbamazepine-Protein Conjugates in Epilepsy Patients**
REC reference:	**13/NW/0503**
Protocol number:	**UoL000969**
EudraCT number:	**2013-002743-28**
IRAS project ID:	**127979**

Thank you for your letter of 09 September 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Assistant Co-ordinator Ms Josephine Foxall Dant, nrescommittee.northwest-haydock@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter from Dr Vincent Yip		19 June 2013
GP/Consultant Information Sheets GP Information Letter	1	30 April 2013
Investigator CV Professor Anthony Guy Marson		06 May 2013
Investigator CV Dr Vincent Lai Ming Yip		
Other: Summary of Product Characteristics		
Participant Consent Form: Auto-Induction Group	1	23 April 2013
Participant Consent Form: Maintenance Group	1	23 April 2013
Participant Information Sheet: Maintenance	2	02 September 2013
Participant Information Sheet: Autoinduction	2	02 September 2013

Protocol	2	02 September 2013
REC application 1279/468665/1/226		21 June 2013
Response to Request for Further Information from Dr Vincent Yip		09 September 2013

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/NW/0503	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Ms Josephine Foxall Dant
Assistant Co-ordinator

E-mail: nrescommittee.northwest-haydock@nhs.net

Enclosures: "After ethical review – guidance for researchers" [SL-AR1]

Copy to: Alex Astor
University of Liverpool

Mr Dave Watling
The Walton Centre NHS Foundation Trust

Professor Tony Marson
University of Liverpool

HYPERSENSITIVITY STUDY – A MECHANISTIC INVESTIGATION INTO DRUG AND CHEMICAL INDUCED HYPERSENSITIVITY

PATIENT INFORMATION SHEET

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. If there is anything that is not clear, or if you would like more information, please ask. This is a voluntary project, and if, when you have heard about the study, you would prefer not to take part, your decision will be accepted without question and will not affect the standard of care you receive.

What is the purpose of the study?

The purpose of this study is to investigate drug induced hypersensitivity reactions. Most diseases and/or disorders require treatment by drugs, which may either cure the disease or reduce its symptoms. Unfortunately, drugs and chemicals can also cause side effects such as hypersensitivity reactions. These are unexpected reactions produced by the normal immune system, for example allergies. These reactions may be uncomfortable, damaging, or occasionally fatal. This study aims to look at the metabolism (breakdown in the human body) of certain drugs in order to gain appreciation of the quality and intensity of drug specific immune responses. It is hoped that this will pave the way for increased drug safety.

Why have I been chosen?

You have been asked to participate in this study because you have experienced a hypersensitive reaction to one of the drugs we are investigating. Although, this drug is normally considered to be safe a small number of patients have been found to have developed a hypersensitive reaction.

Do I have to take part?

It is up to you to decide whether or not to take part in this study. If you do decide to take part you will be given a copy of this information and be asked to sign a consent form. You are free to withdraw from this study at anytime without giving a reason. The standard of your care will not be affected in any way.

What will happen to me if I take part?

If you do decide to take part in this study it will not affect your usual clinical care in any way. That is; you will continue to take all current medications or continue with any planned treatments, as perscribed by your clinician/GP. If you do decide to take part you will be seen by a research nurse or other health professional who will:

1) Take some details including your height, weight, date of birth, past medical history, current medications, alcohol and smoking history.
2) Details will be obtained from your case notes regarding the drug(s) that caused the reaction(s), other drugs that were taken at that time and also details of the reaction that you suffered.
3) A blood sample will be collected (40mL – approximately 4 tablespoons). This sample will be analysed in order to further understand why an individual may respond is such a way.

After this initial visit you may be recalled for further visits. At any additional visit a research nurse or other health profession will verbally confirm that you consent to continue in the study, and a blood sample (up to 60mL) will be collected.
You may be recalled several times over the duration of the study, up to a total of 180mL per year may be collected for a maximum of 3 years. If after 3 years it is thought the data from your blood sample(s) warrant further investigation you may be approached for further blood donation, this will not exceed 180mL per year.
If you have to attend hospital or clinic especially for the study, we will pay you reasonable travel expenses.

What are the possible disadvantages and risks of taking part?

There may be some minor but short-lasting discomfort from having a blood test. Taking part in the study **will not affect** your current treatment, **nor will it affect** your ability to obtain insurance for health purposes.

What are the possible benefits of taking part?

It is unlikely that the study will be of direct benefit to you. However the study results will in the future hopefully aid patients who have had a hypersensitive reaction to drugs.

What will happen to my blood test?

Your blood sample will be stored at the University of Liverpool. A number of experiments will be performed on your sample including body cell and protein based experiments to determine how you respond to the drug which caused your hypersensitivity reaction.

It is important to note that all blood samples received by the University will be identified by a code number only. All coded sample will be held, securely, within the Department of Pharmacology within the University of Liverpool. Once the study has been completed we will irreversibly anonymise your clinical details and your blood sample(s), therefore, it will not be possible to trace your sample back to you. After anonymisation, it will also not be possible to withdraw your blood sample(s). Once anonymised, your sample(s) may be used for other research, but as this cannot be traced back to you, it will have no impact on your clinical care. Further approval will be sought from the ethics committee for any future studies.

Your blood sample(s) will be considered a gift to the University of Liverpool, which will act as custodian for all samples obtained as part of this project.

In the short-term, it is unlikely that the sample(s) will be of any commercial value to the University or the hospital. However, it is possible that there may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to the findings in a group of patients rather

than from samples from a single patient. You will not be paid for taking part in this study, nor will you derive financial benefit from future discoveries.

Will my taking part in this study be kept confidential?

As stated above, all samples will be coded and anonymised. Any information obtained from your blood sample(s) will be kept strictly confidential and will not be disclosed to anyone. All information collected about you during the course of the research will also remain strictly confidential. Any information about you that leaves the research centers taking part in this study will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Results from the project will be published in leading, international medical journals.

Who is organising and funding the research?

The study has been designed as a collaborative project between the University of Liverpool and the Royal Liverpool and Broadgreen University Hospital NHS trust, and has been funded by the Medical Research Council.

Who has reviewed the study?

The study has been reviewed by the North West – North Research Ethics Committee.

Contact for further information

If you need further information or are worried about any aspect of the study, please do not hesitate to contact the research nurse working on the study, **[NAME]**, on [**TELEPHONE NUMBER]**

If you are unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure by contacting your local NHS Trust on (TELEPHONE NUMBER)

THANK YOU FOR READING THIS INFORMATION LEAFLET

HYPERSENSITIVITY STUDY – A MECHANISTIC INVESTIGATION INTO DRUG AND CHEMICAL INDUCED HYPERSENSITIVITY

CONSENT FORM

PLEASE INITIAL EACH BOX BEFORE SIGNING THE FORM

1	I confirm that I have read and understood the information sheet dated…… Version number……for the above study.	
2	I have had the opportunity to discuss the research and ask questions.	
3	I understand that my participation is voluntary and that I may withdraw at any time without giving a reason up until the time that my biological sample can still be identified, without my medical care or legal rights being affected.	
4	I understand that relevant sections of my medical notes and data collected during the study, may be looked at by authorised responsible individuals from regulatory authorities and/or from the NHS Trust, where it is relevant to my taking part in the research. I give permission for these individuals/bodies to have access to my records to access information relevant to this study	
5	I agree to have a blood sample(s) taken for tests that determine how people react to drugs	
6	I agree for my sample(s) to be stored for future use in this research study	
7	I agree to gift my blood sample(s) for future research and further tests as there are more scientific advances and understand that my sample(s) will be anonymised and it will not be possible to trace the sample(s) back to me	
8	I understand that the results will not be added to my medical records	
9	I give permission for a copy of my consent form to be sent to the Sponsor of this study	

10 I agree to participate in the study ☐

Name of Patient:	Date:	Signature:
	[DD/MM/YYYY]	
Name of person taking consent:	Date:	Signature:
	[DD/MM/YYYY]	

NHS
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30 August 2012 Reissued 12 October 2012 with revised document list

Dear Professor Pirmohamed

Study title:	**Hypersensitivity Study: A Mechanistic Investigation into Drug and Chemical Induced Hypersensitivity Reactions**
REC reference:	**12/NW/0525**
Protocol number:	**N/A**

Thank you for your letter of 20 August 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Advertisement: Poster	1	04 July 2012
Covering Letter		04 July 2012
Covering Letter	1	20 August 2012
GP/Consultant Information Sheets	1	04 July 2012
Investigator CV		23 February 2010
Letter of invitation to participant	1	20 June 2012
Letter from Sponsor		12 June 2012
Participant Consent Form: Cohort Study	2	10 August 2012
Participant Consent Form: Generic Case	2	10 August 2012
Participant Consent Form: Generic Control	2	10 August 2012
Participant Information Sheet: Cohort Study	2	10 August 2012
Participant Information Sheet: Generic Case	2	10 August 2012
Participant Information Sheet: Generic Control	2	10 August 2012
Protocol	2	10 August 2012
REC application	3.4	04 July 2012
Response to Request for Further Information	1	20 August 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators

- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NW/0525	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Peter Klimiuk
Chair

This letter has been signed electronically. If you require a wet ink version please request one from the Committee Co-ordinator by email and it will be sent in the post.

Enclosures: "After ethical review – guidance for researchers" SL-AR2

Copy to: Mrs Anita Hanson, Lead Research Nurse, The Wolfson Centre for Personalised Medicine Anita.Hanson@liverpool.ac.uk

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Ms Heather Rogers, R&D office for Royal Liverpool & Broadgreen University Hospitals NHS Trust heather.rogers@rlbuht.nhs.uk

Figure 9.1 Differential equations describing the semi-mechanistic CBZ metabolite model

```
$MODEL

      COMPARTMENT=(CBZ_DEP)   ;1 PO CBZ depot
      COMPARTMENT=(CBZ_CENT)   ;2 CBZ plasma
      COMPARTMENT=(CBZE)   ;3 CBZE plasma
      COMPARTMENT=(CBZ_DiOH)   ;4 CBZ_DiOH plasma
      COMPARTMENT=(CBZ_2OH)    ;5 CBZ_2OH
      COMPARTMENT=(CBZ_3OH)    ;6 CBZ_3OH

$PK
;metabolite formation clearances
TVCLtot=THETA(1)
CLtot=TVCLtot*EXP(ETA(1))

TVFM1=THETA(2)
FM1=TVFM1*EXP(ETA(2))

TVFM2=THETA(3)
FM2=TVFM2*EXP(ETA(3))

;metabolite elimination clearances
TVCL2=THETA(4)
CL2=TVCL2*EXP(ETA(4))

TVCL2E=THETA(5)
CL2E=TVCL2E*EXP(ETA(5))

TVCL3E=THETA(6)
CL3E=TVCL3E*EXP(ETA(6))

TVCL4E=THETA(7)
CL4E=TVCL4E*EXP(ETA(7))

TVKA_star=THETA(8)
KA_star=TVKA_star*EXP(ETA(8))

TVV1=THETA(9)
V1=TVV1*EXP(ETA(9))

TVV2=THETA(10)
V2=TVV2*EXP(ETA(10))

TVV3=THETA(11)
V3=TVV3*EXP(ETA(11))
```

```
;TVKEL_rest=THETA(10)
;KEL_rest==TVV3*EXP(ETA(10))

;reparameterisation

;if you want to constrain the volumes
;V2=V1/V2_diff
;V3=V2/V3_diff

KEL=CLtot/V1
KA=KEL+KA_star ; fix KA to be faster than KEL

KEL1=FM1*KEL ; constrained to 70% total clearance based on prior paper
KEL2=CL2/V2

KEL3=FM2*KEL
KEL4=(1-FM1-FM2)*KEL

;KEL_rest=CL_rest/V1 ; assumed 100% routes accounted for

;KEL1E dropped i.e. 100% CBZE is converted to CBZ-DiOH
KEL2E=CL2E/V3
KEL3E=CL3E/V2 ; assume approx. V2 = V4 = V5 as physchem is similar
KEL4E=CL4E/V2 ; assume elimination rate is equal to the other mono-hydroxy

;scaling
S2=V1 ; CBZ parent
S3=V2 ; CBZE
S4=V3 ; CBZ-DiOH
S5=V2 ; CBZ-2-OH
S6=V2 ; CBZ-3-OH

D1=THETA(12)*EXP(ETA(12))

$DES

DADT(1) = -KA*A(1)
DADT(2)=KA*A(1)-KEL1*A(2)-KEL3*A(2)-KEL4*A(2);CBZ plasma
DADT(3)=KEL1*A(2)-KEL2*A(3); CBZE plasma
DADT(4)=KEL2*A(3)-KEL2E*A(4); DiOH plasma
DADT(5)=KEL3*A(2)-KEL3E*A(5) ;2-OH plasma
DADT(6)=KEL4*A(2)-KEL4E*A(6) ; 3-OH plasma
```

Table 9.1 Gene expression profile in patients with positive response on LTT to CBZ incubated with CBZ

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
CCL8	3.031	5.91E-03	chemokine (C-C motif) ligand 8	cytokine
RSAD2	2.825	3.04E-02	radical S-adenosyl methionine domain containing 2	enzyme
IFIT3	2.419	4.45E-02	interferon-induced protein with tetratricopeptide repeats 3	other
CMPK2	2.007	2.69E-02	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	kinase
USP18	1.882	3.32E-02	ubiquitin specific peptidase 18	peptidase
DDX60	1.710	2.30E-02	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	enzyme
HERC6	1.684	4.92E-02	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	enzyme
PLSCR1	1.662	2.71E-02	phospholipid scramblase 1	enzyme
EIF2AK2	1.576	4.23E-02	eukaryotic translation initiation factor 2-alpha kinase 2	kinase
RTP4	1.503	2.94E-02	receptor (chemosensory) transporter protein 4	other
SAMD9L	1.494	4.57E-02	sterile alpha motif domain containing 9-like	other
PARP9	1.424	3.52E-02	poly (ADP-ribose) polymerase family, member 9	enzyme
MX2	1.310	4.13E-02	MX dynamin-like GTPase 2	enzyme
LAMP3	1.303	2.34E-02	lysosomal-associated membrane protein 3	other
PNPT1	1.261	2.76E-02	polyribonucleotide nucleotidyltransferase 1	enzyme
USP41	1.259	4.98E-02	ubiquitin specific peptidase 41	other
TRIM25	1.164	4.82E-02	tripartite motif containing 25	transcription regulator
DDX60L	1.146	3.64E-02	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	other
IFI35	1.142	4.60E-02	interferon-induced protein 35	other
DTX3L	1.100	3.87E-02	deltex 3 like, E3 ubiquitin ligase	enzyme
PARP12	1.091	4.08E-02	poly (ADP-ribose) polymerase family, member 12	other
OR56B1	1.051	2.82E-02	olfactory receptor, family 56, subfamily B, member 1	G-protein coupled

				receptor
SAMD9	1.005	3.52E-02	sterile alpha motif domain containing 9	other
MIR181B1	-1.031	3.13E-02	microRNA 181a-1	microRNA

Table 9.2 Gene expression profile in patients with positive response on LTT to CBZ and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
IL1B	7.992	2.22E-03	interleukin 1, beta	cytokine
IL1A	7.161	1.16E-02	interleukin 1, alpha	cytokine
CCL20	6.991	1.70E-02	chemokine (C-C motif) ligand 20	cytokine
PTGS2	6.781	2.88E-02	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	enzyme
CXCL5	6.114	7.38E-03	chemokine (C-X-C motif) ligand 5	cytokine
CXCL8	5.849	1.96E-03	chemokine (C-X-C motif) ligand 8	cytokine
CXCL1	5.668	1.72E-02	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	cytokine
TNFAIP6	4.977	1.78E-02	tumor necrosis factor, alpha-induced protein 6	other
IL6	4.856	2.00E-02	interleukin 6	cytokine
CXCL2	3.486	1.73E-02	chemokine (C-X-C motif) ligand 2	cytokine
NAMPT	3.458	6.92E-03	nicotinamide phosphoribosyltransferase	cytokine
MMP14	3.334	3.87E-02	matrix metallopeptidase 14 (membrane-inserted)	peptidase
NAMPTL	3.301	1.73E-02	nicotinamide phosphoribosyltransferase pseudogene 1	other
PLAUR	3.207	3.10E-02	plasminogen activator, urokinase receptor	transmembrane receptor
EREG	2.976	3.49E-02	epiregulin	growth factor

RN7SKP283	2.945	3.33E-02		other
CXCL3	2.931	2.78E-02	chemokine (C-X-C motif) ligand 3	cytokine
TREM1	2.709	4.84E-02	triggering receptor expressed on myeloid cells 1	transmembrane receptor
ITGB8	2.659	2.02E-02	integrin, beta 8	other
GPR84	2.624	3.87E-02	G protein-coupled receptor 84	G-protein coupled receptor
IL2RA	2.566	7.06E-03	interleukin 2 receptor, alpha	transmembrane receptor
SOD2	2.461	1.43E-02	superoxide dismutase 2, mitochondrial	enzyme
C3	2.335	3.46E-02		chemical - endogenous mammalian
FLT1	2.329	4.63E-02	fms-related tyrosine kinase 1	kinase
SOCS3	2.143	5.41E-03	suppressor of cytokine signaling 3	phosphatase
ACSL1	2.132	2.75E-02	acyl-CoA synthetase long-chain family member 1	enzyme
TLR2	2.112	3.85E-02	toll-like receptor 2	transmembrane receptor
LOC541472	1.977	4.77E-02	uncharacterized LOC541472	other
SERPINB2	1.969	3.66E-02	serpin peptidase inhibitor, clade B (ovalbumin), member 2	other
MT1G	1.928	3.65E-02	metallothionein 1G	other
RGS1	1.925	6.58E-03	regulator of G-protein signaling 1	other
ETS2	1.842	3.07E-02	v-ets avian erythroblastosis virus E26 oncogene homolog 2	transcription regulator
MT1H	1.825	3.68E-02	metallothionein 1H	other
CCL22	1.720	4.21E-02	chemokine (C-C motif) ligand 22	cytokine
PFKFB3	1.697	3.14E-02	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	kinase

SLC41A2	1.682	4.97E-02	solute carrier family 41 (magnesium transporter), member 2	transporter
NFKBIA	1.665	1.57E-03	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	transcription regulator
NFKBIZ	1.637	1.15E-04	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	transcription regulator
RNF144B	1.525	4.08E-02	ring finger protein 144B	enzyme
SAMSN1	1.519	4.86E-02	SAM domain, SH3 domain and nuclear localization signals 1	other
SLC7A5	1.375	3.91E-02	solute carrier family 7 (amino acid transporter light chain, L system), member 5	transporter
BHLHE40	1.368	3.23E-02	basic helix-loop-helix family, member e40	transcription regulator
TNIP1	1.238	2.82E-02	TNFAIP3 interacting protein 1	other
MT1M	1.237	2.34E-02	metallothionein 1M	other
PIM1	1.212	3.07E-02	Pim-1 proto-oncogene, serine/threonine kinase	kinase
CHST15	1.209	3.11E-02	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	enzyme
BCL6	1.197	3.06E-02	B-cell CLL/lymphoma 6	transcription regulator
MT1B	1.131	3.70E-02	metallothionein 1B	other
NFKB1	1.097	5.51E-03	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	transcription regulator
JUNB	1.093	4.26E-02	jun B proto-oncogene	transcription regulator
HDGFRP3	1.078	5.75E-03	hepatoma-derived growth factor, related protein 3	other
CTAGE6	1.070	1.25E-02	CTAGE family, member 4	other
HIF1A	1.055	2.79E-03	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	transcription regulator
MT1E	1.029	4.21E-02	metallothionein 1E	other

LDHA	1.014	3.63E-02	lactate dehydrogenase A	enzyme
FAIM3	-1.012	4.63E-02	Fc fragment of IgM receptor	other
MIR16-2	-1.014	4.58E-04	microRNA 15a	microRNA
AK5	-1.016	2.75E-02	adenylate kinase 5	kinase
TRAV8-3	-1.029	1.33E-02	T cell receptor alpha variable 8-3	other
RNU7-125P	-1.073	2.96E-03		other
MIR3671	-1.082	4.45E-03	microRNA 3671	microRNA
CD84	-1.114	1.05E-02	CD84 molecule	other
C1orf162	-1.237	2.32E-02	chromosome 1 open reading frame 162	transporter
MIR181B1	-1.244	1.21E-02	microRNA 181a-1	microRNA
CD52	-1.256	5.10E-03	CD52 molecule	other
MIR580	-1.269	8.05E-04	microRNA 580	microRNA
MPEG1	-1.485	4.38E-02	macrophage expressed 1	other
FABP5	-1.504	3.78E-02	fatty acid binding protein 5 (psoriasis-associated)	transporter
FABP5P1	-2.261	3.38E-02	fatty acid binding protein 5 pseudogene 1	other
FABP5P7	-2.330	4.44E-02	fatty acid binding protein 5 pseudogene 7	other
CD36	-2.712	4.22E-02	CD36 molecule (thrombospondin receptor)	transmembrane receptor
FABP4	-5.016	1.64E-02	fatty acid binding protein 4, adipocyte	transporter

Table 9.3 Gene expression profile in subjects with negative response to LTT with CBZ and incubated with CBZ

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
MIR4795	-1.068	9.34E-04	microRNA 4795	microRNA

Table 9.4 Gene expression profile in subjects with negative response to LTT with CBZ and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
MRC1	4.596	1.61E-02	mannose receptor, C type 1	transmembrane receptor
STEAP4	4.386	9.66E-04	STEAP family member 4	enzyme
CXCL10	4.028	3.51E-02	chemokine (C-X-C motif) ligand 10	cytokine
ANKRD22	2.752	4.42E-03	ankyrin repeat domain 22	transcription regulator
CXCL9	2.672	2.60E-03	chemokine (C-X-C motif) ligand 9	cytokine
IL2RA	2.562	7.14E-03	interleukin 2 receptor, alpha	transmembrane receptor
CXCL11	2.416	3.95E-02	chemokine (C-X-C motif) ligand 11	cytokine
CCL8	2.190	3.39E-02	chemokine (C-C motif) ligand 8	cytokine
ENPP2	2.049	1.73E-03	ectonucleotide pyrophosphatase/phosphodiesterase 2	enzyme
SLAMF8	1.963	1.04E-02	SLAM family member 8	other
IGSF6	1.939	3.36E-02	immunoglobulin superfamily, member 6	transmembrane receptor

CD80	1.856	3.14E-03	CD80 molecule	transmembrane receptor
GBP1P1	1.775	2.27E-02	guanylate binding protein 1, interferon-inducible pseudogene 1	other
WARS	1.751	3.49E-03	tryptophanyl-tRNA synthetase	enzyme
MYOF	1.738	1.95E-02	myoferlin	other
CD274	1.734	3.26E-02	CD274 molecule	enzyme
FBP1	1.709	3.56E-02	fructose-1,6-bisphosphatase 1	phosphatase
CXCR2P1	1.640	1.13E-02	chemokine (C-X-C motif) receptor 2 pseudogene 1	other
PSME2P2	1.597	7.08E-03	proteasome activator subunit 2 pseudogene 2	other
SUCNR1	1.576	7.21E-04	succinate receptor 1	G-protein coupled receptor
GBP4	1.562	4.70E-02	guanylate binding protein 4	enzyme
FCER2	1.558	4.79E-02	Fc fragment of IgE, low affinity II, receptor for (CD23)	transmembrane receptor
PSTPIP2	1.505	4.75E-03	proline-serine-threonine phosphatase interacting protein 2	other
BCL2	1.499	7.72E-03	B-cell CLL/lymphoma 2	transporter
SNX10	1.483	1.93E-03	sorting nexin 10	transporter
SPRED1	1.479	7.59E-03	sprouty-related, EVH1 domain containing 1	other
KMO	1.474	3.25E-02	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	enzyme
ARHGAP31	1.461	8.18E-03	Rho GTPase activating protein 31	other
PSME2	1.431	6.40E-03	proteasome activator subunit 2	peptidase
EMP1	1.404	2.92E-02	epithelial membrane protein 1	other
TXN	1.352	2.05E-02	thioredoxin	enzyme
CLEC6A	1.298	1.38E-02	C-type lectin domain family 6, member A	transmembrane receptor
DRAM1	1.295	3.62E-02	DNA-damage regulated autophagy modulator 1	other
PIM1	1.289	2.30E-02	Pim-1 proto-oncogene, serine/threonine kinase	kinase

CTSC	1.284	3.37E-02	cathepsin C	peptidase
CLEC4A	1.238	2.55E-02	C-type lectin domain family 4, member A	transmembrane receptor
FLVCR2	1.237	4.19E-02	feline leukemia virus subgroup C cellular receptor family, member 2	transporter
PDCD1LG2	1.234	3.38E-02	programmed cell death 1 ligand 2	enzyme
CCL13	1.212	1.68E-03	chemokine (C-C motif) ligand 13	cytokine
CISH	1.207	5.44E-03	cytokine inducible SH2-containing protein	other
PRRG4	1.189	1.97E-02	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	other
CD209	1.176	2.77E-02	CD209 molecule	other
TNFAIP2	1.172	3.21E-02	tumor necrosis factor, alpha-induced protein 2	other
ARNTL2	1.162	1.66E-02	aryl hydrocarbon receptor nuclear translocator-like 2	transcription regulator
ITGAM	1.161	1.20E-02	integrin, alpha M (complement component 3 receptor 3 subunit)	transmembrane receptor
MTHFD2	1.153	1.69E-02	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	enzyme
MB21D2	1.136	1.65E-02	Mab-21 domain containing 2	other
LMNB1	1.109	2.74E-02	lamin B1	other
BST2	1.106	3.16E-02	bone marrow stromal cell antigen 2	other
PTGER2	1.093	1.24E-02	prostaglandin E receptor 2 (subtype EP2), 53kDa	G-protein coupled receptor
MOB3B	1.090	7.83E-03	MOB kinase activator 3B	other
P2RY14	1.029	2.06E-02	purinergic receptor P2Y, G-protein coupled, 14	G-protein coupled receptor
NFKBIA	1.011	3.14E-02	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	transcription regulator
MIR663A	-1.023	3.63E-03	microRNA 663a	microRNA

NFXL1	-1.083	1.04E-02	nuclear transcription factor, X-box binding-like 1	transcription regulator
MIR4766	-1.093	5.31E-04	microRNA 4766	microRNA
MIR548AL	-1.094	8.00E-03	microRNA 570	microRNA
SEPP1	-1.715	2.81E-03	selenoprotein P, plasma, 1	other

Table 9.5 Gene expression profile in patients with positive response on LTT to CBZE incubated with CBZE

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
COX8A	1.133	1.56E-02	cytochrome c oxidase subunit VIIIA (ubiquitous)	enzyme
RNU6-267P	-1.004	2.37E-02		other
RNU6-162P	-1.024	2.44E-02		other
MIR4441	-1.030	3.00E-03	microRNA 4441	microRNA
RNU7-38P	-1.033	4.97E-02	RNA, U7 small nuclear 38 pseudogene	other
RNU7-123P	-1.063	4.41E-05	RNU7-123P snRNA	other
AF178030.2	-1.129	3.05E-03	trichorhinophalangeal syndrome I	transcription regulator
PTGES	-1.147	2.86E-02	prostaglandin E synthase	enzyme
RNA5SP237	-1.169	8.15E-03	RNA, 5S ribosomal pseudogene 237	other
CHST15	-1.176	3.52E-02	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	enzyme
BACH1-IT2	-1.182	2.13E-02	BACH1 intronic transcript 2	other
NAMPT	-2.367	4.74E-02	nicotinamide phosphoribosyltransferase	cytokine
RN7SKP283	-2.735	4.56E-02		other
PTGS2	-6.122	4.50E-02	prostaglandin-endoperoxide synthase 2	enzyme

Table 9.6 Gene expression profile in patients with positive response on LTT to CBZE incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
STAT1	1.870	1.64E-02	signal transducer and activator of transcription 1, 91kDa	transcription regulator
RNU4-1	1.098	3.01E-02	RNA, U4 small nuclear 1	other
RNU6-151P	-1.036	2.13E-02	RNU6-151P snRNA	other
LOC644090	-1.043	3.55E-02	uncharacterized LOC644090	other
AF178030.2	-1.140	2.84E-03	trichorhinophalangeal syndrome I	transcription regulator
MIR4307	-1.241	5.22E-03	microRNA 4307	microRNA
RN7SKP283	-2.706	4.77E-02		other

Table 9.7 Gene expression profile in patients with negative response on LTT to CBZE incubated with CBZE

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
MIR302F	1.493	2.65E-03	microRNA 302c	microRNA
MIR4317	1.272	1.79E-02	microRNA 4317	microRNA
DOCK4-AS1	1.204	4.37E-03	DOCK4 antisense RNA 1	other
RNU6-1006P	1.182	3.28E-02	RNU6-1006P snRNA	other
RNA5SP201	1.060	6.20E-05	RNA, 5S ribosomal pseudogene 201	other
MIR4308	1.052	1.03E-02	microRNA 4308	microRNA
MIR548M	1.044	2.58E-02	microRNA 570	microRNA
RNU6-1176P	1.031	2.58E-02	RNU6-1176P snRNA	other
RNU6-395P	1.004	4.10E-02	RNU6-395P snRNA	other
MYCBP2-AS2	-1.027	4.18E-02	MYCBP2 antisense RNA 2	other
RNU6-1045P	-1.044	4.69E-02		other
MIR1302-5	-1.153	1.31E-02	microRNA 1302-5	microRNA
MIR4307	-1.214	3.76E-02	microRNA 4307	microRNA
MIR15B	-1.216	3.78E-02	microRNA 15a	microRNA
OR11H12	-1.224	3.66E-05	olfactory receptor, family 11, subfamily H, member 12	other
MIR4679-1	-1.261	3.19E-02	microRNA 4679-2	microRNA
RNU5E-3P	-1.318	9.77E-03	RNA, U5E small nuclear 3, pseudogene	other
MIR548C	-1.498	2.94E-02	microRNA 570	microRNA

Table 9.8 Gene expression profile in patients with negative response on LTT to CBZE incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Entrez Gene Name	Type(s)
MIR627	1.574	5.83E-03	microRNA 627	microRNA
MIR302F	1.572	1.83E-03	microRNA 302c	microRNA
RNU6-1289P	1.265	9.55E-03	RNU6-1289P snRNA	other
DCSTAMP	1.098	8.13E-03	dendrocyte expressed seven transmembrane protein	other
MIR3145	1.094	2.41E-02	microRNA 3145	microRNA
PCDH9-AS3	1.079	2.34E-03	PCDH9 antisense RNA 3	other
RNU6-699P	1.059	9.13E-03	RNU6-699P snRNA	other
RNU6-989P	1.056	1.12E-03	RNU6-989P snRNA	other
LINC00392	1.035	1.21E-04	long intergenic non-protein coding RNA 392	other
MIR3910-1	1.023	1.54E-02	microRNA 3910-2	microRNA
MIR548M	1.012	2.99E-02	microRNA 570	microRNA
CTD-2201E9.1	1.006	1.98E-05	uncharacterized LOC101929338	other
PFN1P2	-1.015	6.30E-03	profilin 1 pseudogene 2	other
LOC100287934	-1.029	2.65E-02	uncharacterized LOC100287934	other
RNU6-396P	-1.046	4.42E-02	RNU6-396P snRNA	other
MIR125B2	-1.080	7.94E-04	microRNA 100	microRNA
MIR4719	-1.116	2.27E-02	microRNA 4719	microRNA
RNU6-1214P	-1.122	6.46E-03		other
TAS2R7	-1.137	5.39E-04	taste receptor, type 2, member 7	G-protein coupled receptor
MIR548O	-1.243	2.86E-05	microRNA 548o	microRNA
RNU7-147P	-1.254	1.29E-02		other
RNU5E-3P	-1.265	1.24E-02	RNA, U5E small nuclear 3, pseudogene	other

MIR15B	-1.439	1.69E-02	microRNA 15a	microRNA
LOC644172	-1.562	4.98E-05	mitogen-activated protein kinase 8 interacting protein 1 pseudogene	other
MIR1255A	-1.865	2.06E-04	microRNA 1255b-1	microRNA
IGHD3-22	-2.503	3.40E-02	immunoglobulin heavy diversity 3-22	other

Table 9.9 CBZ/CBZE-specific mRNA normalised expression levels (Normalised according to GAPDH)

	RSAD2	PARP9	PARP12	PTGES	IFIT3	COX8A	IFI35	USP18	USP41
RLH001 CBZ 0	1.65	1.85	1.75	1.11	1.59	2.08	1.39	1.33	1.32
RLH001 CBZ 25	2.32	2.18	2.01	1.07	2.16	2.17	1.66	1.85	1.65
RLH001 CBZ TT	2.05	2.12	1.95	1.13	1.94	2.09	1.51	1.53	1.46
RLH002 CBZ 0	2.01	1.94	1.84	1.11	1.90	2.10	1.51	1.51	1.43
RLH002 CBZ 25	2.10	2.03	1.89	1.11	1.96	2.26	1.56	1.58	1.51
RLH002 CBZ TT	2.06	2.00	1.90	1.09	1.90	2.32	1.56	1.57	1.48
RLH007 CBZ 0	1.71	1.76	1.73	1.09	1.69	2.23	1.44	1.35	1.30
RLH007 CBZ 25	1.99	1.90	1.82	1.11	1.94	2.14	1.55	1.47	1.39
RLH007 CBZ TT	1.67	1.73	1.64	1.39	1.66	2.00	1.38	1.29	1.25
RLH007 CBZE 0	1.44	1.71	1.69	1.43	1.49	2.27	1.38	1.29	1.27
RLH007 CBZE 25	1.88	1.90	1.84	1.11	1.80	2.29	1.50	1.47	1.41
RLH007 CBZE TT	2.05	1.96	1.89	1.11	1.99	2.34	1.56	1.52	1.44
RLH010 CBZE 0	1.39	1.71	1.67	1.11	1.40	2.27	1.36	1.25	1.23
RLH010 CBZE 25	1.47	1.76	1.67	1.14	1.53	2.16	1.40	1.29	1.27
RLH010 CBZE TT	1.36	1.69	1.64	1.07	1.42	2.30	1.37	1.22	1.18
WAL004 CBZ 0	2.35	2.09	1.97	1.08	2.15	2.23	1.68	1.69	1.56
WAL004 CBZ 50	2.03	1.92	1.82	1.10	1.93	2.27	1.57	1.49	1.40
WAL004 CBZ TT	2.69	2.35	2.17	1.12	2.55	2.36	2.00	2.03	1.83
WAL004 CBZE 0	1.32	1.73	1.65	1.21	1.38	1.96	1.33	1.18	1.20
WAL004 CBZE 25	1.55	1.81	1.71	1.14	1.43	2.40	1.42	1.25	1.25
WAL004 CBZE TT	1.54	1.93	1.82	1.28	1.51	2.27	1.48	1.26	1.26

Table 9.10 Significant gene sets identified by GSEA (FDR<0.05) in subjects with positive response on LTT to CBZ incubated with CBZ (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	Size	NES	FDR (q value)
CHEMOKINE_RECEPTOR_BINDING	41	2.89	0.00
CHEMOKINE_ACTIVITY	40	2.87	0.00
G_PROTEIN_COUPLED_RECEPTOR_BINDING	51	2.86	0.00
CYTOKINE_ACTIVITY	108	2.76	0.00
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	77	2.74	0.00
LOCOMOTORY_BEHAVIOR	89	2.66	0.00
RESPONSE_TO_VIRUS	42	2.56	0.00
BEHAVIOR	139	2.54	0.00
RESPONSE_TO_EXTERNAL_STIMULUS	296	2.35	0.00
INFLAMMATORY_RESPONSE	124	2.34	0.00
RNA_BINDING	240	2.30	0.00
RESPONSE_TO_CHEMICAL_STIMULUS	297	2.26	0.00
RIBONUCLEOPROTEIN_COMPLEX	135	2.24	0.00
RESPONSE_TO_OTHER_ORGANISM	73	2.24	0.00
CELL_CELL_SIGNALING	386	2.20	0.00
JAK_STAT_CASCADE	30	2.16	0.00
TRANSLATION	167	2.16	0.00
EXTRACELLULAR_SPACE	230	2.16	0.00
RESPONSE_TO_BIOTIC_STIMULUS	109	2.13	0.00
DEFENSE_RESPONSE	253	2.12	0.00
EXOCYTOSIS	23	2.12	0.00
NUCLEOLUS	115	2.10	0.00

VIRAL_GENOME_REPLICATION	18	2.08	0.00
RESPONSE_TO_WOUNDING	182	2.08	0.00
PROTEASOME_COMPLEX	22	2.06	0.00
RECEPTOR_BINDING	357	2.05	0.00
RIBOSOME	35	2.01	0.01
CATION_HOMEOSTASIS	101	1.98	0.01
CELLULAR_CATION_HOMEOSTASIS	99	1.98	0.01
VIRAL_INFECTIOUS_CYCLE	28	1.96	0.02
ION_HOMEOSTASIS	121	1.96	0.02
EXTRACELLULAR_REGION_PART	320	1.96	0.02
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	80	1.95	0.02
ORGANELLE_LUMEN	428	1.95	0.02
MEMBRANE_ENCLOSED_LUMEN	428	1.94	0.02
NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	148	1.92	0.03
COLLAGEN_BINDING	14	1.89	0.04
PROTEIN_FOLDING	55	1.89	0.04
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	45	1.88	0.04
CHEMICAL_HOMEOSTASIS	147	1.89	0.04
CELLULAR_HOMEOSTASIS	138	1.89	0.04
STRUCTURAL_MOLECULE_ACTIVITY	232	1.89	0.04
METALLOENDOPEPTIDASE_ACTIVITY	26	1.88	0.04
MULTI_ORGANISM_PROCESS	153	1.88	0.04
DEFENSE_RESPONSE_TO_VIRUS	8	1.87	0.04
CASPASE_ACTIVATION	26	1.87	0.04
PROTEIN_TARGETING_TO_MEMBRANE	11	1.86	0.04
CELLULAR_BIOSYNTHETIC_PROCESS	297	1.86	0.04

PROTEIN_KINASE_REGULATOR_ACTIVITY	36	1.85	0.04
VIRAL_REPRODUCTIVE_PROCESS	32	1.85	0.04
REGULATION_OF_CELL_PROLIFERATION	296	1.85	0.04
ENDOPLASMIC_RETICULUM_LUMEN	13	1.84	0.05
APOPTOSIS_GO	417	1.84	0.05
PROGRAMMED_CELL_DEATH	418	1.84	0.05
NUCLEAR_PART	537	1.84	0.05
VIRAL_REPRODUCTION	37	1.83	0.05
IMMUNE_RESPONSE	217	1.83	0.05
REGULATION_OF_APOPTOSIS	330	1.83	0.05
REGULATION_OF_CYCLIN_DEPENDENT_PROTEIN_KINASE_ACTIVITY	42	1.83	0.05
REGULATION_OF_PROGRAMMED_CELL_DEATH	331	1.82	0.05
HOMEOSTATIC_PROCESS	196	1.82	0.05
TRNA_METABOLIC_PROCESS	19	1.82	0.05

Table 9.11 Significant gene sets identified by GSEA (FDR<0.05) in subjects with negative response on LTT to CBZ incubated with CBZ (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	77	2.30	0

Table 9.12 Significant gene sets identified by GSEA (FDR<0.05) in subjects with positive response on LTT to CBZ incubated with TT (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
CYTOKINE_ACTIVITY	108	2.93	0.00
EXTRACELLULAR_SPACE	230	2.84	0.00
EXTRACELLULAR_REGION_PART	320	2.82	0.00
G_PROTEIN_COUPLED_RECEPTOR_BINDING	51	2.75	0.00
CHEMOKINE_RECEPTOR_BINDING	41	2.75	0.00
CHEMOKINE_ACTIVITY	40	2.69	0.00
RESPONSE_TO_EXTERNAL_STIMULUS	296	2.68	0.00
INFLAMMATORY_RESPONSE	124	2.62	0.00
LOCOMOTORY_BEHAVIOR	89	2.61	0.00
NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	148	2.60	0.00
RECEPTOR_BINDING	357	2.58	0.00
RESPONSE_TO_CHEMICAL_STIMULUS	297	2.54	0.00
BEHAVIOR	139	2.54	0.00
RESPONSE_TO_WOUNDING	182	2.54	0.00
REGULATION_OF_CELL_PROLIFERATION	296	2.50	0.00
EXTRACELLULAR_REGION	424	2.50	0.00
CELL_CELL_SIGNALING	386	2.50	0.00
RESPONSE_TO_VIRUS	42	2.48	0.00
NEGATIVE_REGULATION_OF_APOPTOSIS	146	2.40	0.00
NEGATIVE_REGULATION_OF_DEVELOPMENTAL_PROCESS	192	2.41	0.00
PATTERN_BINDING	44	2.42	0.00
DEFENSE_RESPONSE	253	2.42	0.00

RESPONSE_TO_STRESS	484	2.35	0.00
APOPTOSIS_GO	417	2.33	0.00
PROGRAMMED_CELL_DEATH	418	2.33	0.00
NEGATIVE_REGULATION_OF_PROGRAMMED_CELL_DEATH	147	2.32	0.00
CELL_PROLIFERATION_GO_0008283	483	2.32	0.00
POLYSACCHARIDE_BINDING	35	2.32	0.00
GLYCOSAMINOGLYCAN_BINDING	33	2.31	0.00
REGULATION_OF_PROGRAMMED_CELL_DEATH	331	2.30	0.00
REGULATION_OF_APOPTOSIS	330	2.28	0.00
POSITIVE_REGULATION_OF_CELL_PROLIFERATION	145	2.27	0.00
JAK_STAT_CASCADE	30	2.26	0.00
NEGATIVE_REGULATION_OF_CELLULAR_PROCESS	615	2.23	0.00
METALLOENDOPEPTIDASE_ACTIVITY	26	2.22	0.00
VIRAL_GENOME_REPLICATION	18	2.21	0.00
NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS	645	2.21	0.00
REGULATION_OF_BLOOD_PRESSURE	18	2.20	0.01
RESPONSE_TO_OTHER_ORGANISM	73	2.20	0.01
RESPONSE_TO_BIOTIC_STIMULUS	109	2.20	0.01
REGULATION_OF_DEVELOPMENTAL_PROCESS	426	2.19	0.01
ANTI_APOPTOSIS	114	2.17	0.01
CELL_DEVELOPMENT	559	2.17	0.01
CARBOHYDRATE_TRANSPORT	18	2.16	0.01
METALLOPEPTIDASE_ACTIVITY	48	2.14	0.01
NEGATIVE_REGULATION_OF_CYTOKINE_BIOSYNTHETIC_PROCESS	11	2.10	0.02
HOMEOSTASIS_OF_NUMBER_OF_CELLS	19	2.10	0.02
VIRAL_INFECTIOUS_CYCLE	28	2.09	0.02

NEGATIVE_REGULATION_OF_CELLULAR_BIOSYNTHETIC_PROCESS	26	2.07	0.03
NEGATIVE_REGULATION_OF_BIOSYNTHETIC_PROCESS	27	2.06	0.03
NEGATIVE_REGULATION_OF_TRANSLATION	21	2.05	0.03
CYTOKINE_PRODUCTION	66	2.05	0.03
CYTOKINE_METABOLIC_PROCESS	38	2.04	0.04
RESPONSE_TO_HYPOXIA	26	2.04	0.04
VIRAL_REPRODUCTIVE_PROCESS	32	2.03	0.04
INTEGRIN_COMPLEX	19	2.03	0.04
MONOSACCHARIDE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	10	2.03	0.04
HOMEOSTATIC_PROCESS	196	2.03	0.04
NEGATIVE_REGULATION_OF_CELL_DIFFERENTIATION	27	2.02	0.04

Table 9.13 Significant gene sets identified by GSEA (FDR<0.05) in subjects with negative response on LTT to CBZ incubated with TT (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
CYTOKINE_ACTIVITY	108	3.01	0.00
G_PROTEIN_COUPLED_RECEPTOR_BINDING	51	2.96	0.00
LOCOMOTORY_BEHAVIOR	89	2.91	0.00
CHEMOKINE_RECEPTOR_BINDING	41	2.89	0.00
BEHAVIOR	139	2.88	0.00
CHEMOKINE_ACTIVITY	40	2.86	0.00
INFLAMMATORY_RESPONSE	124	2.83	0.00
RESPONSE_TO_EXTERNAL_STIMULUS	296	2.77	0.00
IMMUNE_RESPONSE	217	2.76	0.00
RESPONSE_TO_WOUNDING	182	2.72	0.00
DEFENSE_RESPONSE	253	2.72	0.00
RESPONSE_TO_CHEMICAL_STIMULUS	297	2.67	0.00
CELL_CELL_SIGNALING	386	2.66	0.00
IMMUNE_SYSTEM_PROCESS	307	2.61	0.00
RECEPTOR_BINDING	357	2.60	0.00
CELLULAR_CATION_HOMEOSTASIS	99	2.49	0.00
CARBOHYDRATE_BINDING	70	2.47	0.00
CATION_HOMEOSTASIS	101	2.45	0.00
RESPONSE_TO_VIRUS	42	2.42	0.00
NEGATIVE_REGULATION_OF_APOPTOSIS	146	2.41	0.00
ION_HOMEOSTASIS	121	2.41	0.00
RESPONSE_TO_BIOTIC_STIMULUS	109	2.40	0.00

PATTERN_BINDING	44	2.40	0.00
APOPTOSIS_GO	417	2.38	0.00
NEGATIVE_REGULATION_OF_PROGRAMMED_CELL_DEATH	147	2.38	0.00
CHEMICAL_HOMEOSTASIS	147	2.37	0.00
PROGRAMMED_CELL_DEATH	418	2.36	0.00
REGULATION_OF_PROGRAMMED_CELL_DEATH	331	2.36	0.00
REGULATION_OF_APOPTOSIS	330	2.35	0.00
JAK_STAT_CASCADE	30	2.32	0.00
CELLULAR_HOMEOSTASIS	138	2.33	0.00
EXTRACELLULAR_SPACE	230	2.33	0.00
TRANSLATION	167	2.29	0.00
EXTRACELLULAR_REGION_PART	320	2.30	0.00
VIRAL_REPRODUCTIVE_PROCESS	32	2.30	0.00
ANTI_APOPTOSIS	114	2.31	0.00
SIGNAL_TRANSDUCTION	1547	2.26	0.00
VIRAL_INFECTIOUS_CYCLE	28	2.27	0.00
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	77	2.28	0.00
RESPONSE_TO_STRESS	484	2.28	0.00
NEGATIVE_REGULATION_OF_DEVELOPMENTAL_PROCESS	192	2.28	0.00
VIRAL_GENOME_REPLICATION	18	2.25	0.00
VIRAL_REPRODUCTION	37	2.24	0.00
REGULATION_OF_DEVELOPMENTAL_PROCESS	426	2.23	0.00
PROTEASOME_COMPLEX	22	2.21	0.00
CELL_DEVELOPMENT	559	2.23	0.00
SOLUBLE_FRACTION	153	2.21	0.00
HOMEOSTATIC_PROCESS	196	2.21	0.00

ELECTRON_CARRIER_ACTIVITY	75	2.22	0.00
SUGAR_BINDING	33	2.22	0.00
RESPONSE_TO_OTHER_ORGANISM	73	2.20	0.00
PROTEIN_FOLDING	55	2.20	0.00
CELL_PROLIFERATION_GO_0008283	483	2.19	0.00
REGULATION_OF_CELL_PROLIFERATION	296	2.18	0.00
CELLULAR_BIOSYNTHETIC_PROCESS	297	2.16	0.00
CYTOPLASM	1982	2.16	0.00
INTRACELLULAR_TRANSPORT	271	2.16	0.00
PYROPHOSPHATASE_ACTIVITY	211	2.15	0.00
HUMORAL_IMMUNE_RESPONSE	32	2.15	0.00
EXTRACELLULAR_REGION	424	2.15	0.00
RECEPTOR_SIGNALING_PROTEIN_ACTIVITY	81	2.15	0.00
MULTI_ORGANISM_PROCESS	153	2.14	0.00
CELL_FRACTION	471	2.14	0.00
HYDROLASE_ACTIVITY_ACTING_ON_ACID_ANHYDRIDES	213	2.12	0.00
CELLULAR_PROTEIN_METABOLIC_PROCESS	1059	2.12	0.00
ESTABLISHMENT_OF_CELLULAR_LOCALIZATION	340	2.11	0.00
RIBONUCLEOPROTEIN_COMPLEX	135	2.11	0.00
ENDOPLASMIC_RETICULUM_LUMEN	13	2.11	0.00
GLYCOSAMINOGLYCAN_BINDING	33	2.11	0.00
CELLULAR_MACROMOLECULE_METABOLIC_PROCESS	1072	2.10	0.00
PROTEIN_METABOLIC_PROCESS	1164	2.09	0.00
POSITIVE_REGULATION_OF_CELL_PROLIFERATION	145	2.07	0.01
ENDOPLASMIC_RETICULUM	275	2.07	0.01
CELLULAR_LOCALIZATION	357	2.06	0.01

PROTEIN_KINASE_CASCADE	278	2.06	0.01
SERINE_TYPE_ENDOPEPTIDASE_INHIBITOR_ACTIVITY	22	2.06	0.01
MACROMOLECULE_BIOSYNTHETIC_PROCESS	298	2.06	0.01
MEMBRANE_ORGANIZATION_AND_BIOGENESIS	132	2.05	0.01
NUCLEOSIDE_TRIPHOSPHATASE_ACTIVITY	199	2.05	0.01
REGULATION_OF_PROTEIN_MODIFICATION_PROCESS	43	2.05	0.01
ENVELOPE	153	2.05	0.01
POLYSACCHARIDE_BINDING	35	2.05	0.01
NUCLEOLUS	115	2.05	0.01
RESPONSE_TO_ORGANIC_SUBSTANCE	29	2.05	0.01
HYDROLASE_ACTIVITY_ACTING_ON_CARBON NITROGEN_NOT_PEPTIDEBONDSIN_CYCLIC_AMIDINES	16	2.05	0.01
REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	89	2.05	0.01
OXIDOREDUCTASE_ACTIVITY	274	2.04	0.01
I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	107	2.04	0.01
ORGANELLE_MEMBRANE	280	2.04	0.01
UNFOLDED_PROTEIN_BINDING	41	2.04	0.01
RESPONSE_TO_CARBOHYDRATE_STIMULUS	11	2.04	0.01
NEGATIVE_REGULATION_OF_CELLULAR_PROCESS	615	2.04	0.01
POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	83	2.04	0.01
ENDOPLASMIC_RETICULUM_PART	91	2.03	0.01
MONOSACCHARIDE_BINDING	11	2.03	0.01
ENZYME_REGULATOR_ACTIVITY	304	2.03	0.01
BIOSYNTHETIC_PROCESS	435	2.02	0.01
MACROMOLECULAR_COMPLEX	886	2.01	0.01
ORGANELLE_ENVELOPE	153	2.01	0.01

RNA_BINDING	240	2.01	0.01
GTP_BINDING	42	2.01	0.01
HETEROCYCLE_METABOLIC_PROCESS	23	2.00	0.01
REGULATION_OF_BIOLOGICAL_QUALITY	392	2.00	0.01
NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS	645	2.00	0.01
TRNA_METABOLIC_PROCESS	19	2.00	0.01
GUANYL_NUCLEOTIDE_BINDING	43	1.99	0.01
PEPTIDYL_TYROSINE_MODIFICATION	28	1.99	0.01
MICROVILLUS	10	1.99	0.01
MITOCHONDRIAL_PART	129	1.99	0.01
VESICLE_MEDIATED_TRANSPORT	190	1.98	0.01
CYTOPLASMIC_PART	1292	1.98	0.01
MYELOID_CELL_DIFFERENTIATION	35	1.98	0.01
PHAGOCYTOSIS	17	1.97	0.01
MEMBRANE	1860	1.97	0.01
POSITIVE_REGULATION_OF_SIGNAL_TRANSDUCTION	120	1.97	0.01
NUCLEAR_ENVELOPE	67	1.97	0.01
PEPTIDYL_TYROSINE_PHOSPHORYLATION	26	1.97	0.01
CELLULAR_DEFENSE_RESPONSE	54	1.96	0.01
PROTEOLYSIS	184	1.95	0.01
RESPONSE_TO_TOXIN	10	1.95	0.01
POST_TRANSLATIONAL_PROTEIN_MODIFICATION	458	1.95	0.01
MITOCHONDRIAL_OUTER_MEMBRANE	18	1.95	0.01
IMMUNE_EFFECTOR_PROCESS	32	1.95	0.01
TRANSPORT	760	1.95	0.01
INTRACELLULAR_PROTEIN_TRANSPORT	139	1.95	0.01

NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	148	1.95	0.01
PROTEIN_TRANSPORT	150	1.94	0.02
PHOSPHORIC_ESTER_HYDROLASE_ACTIVITY	147	1.94	0.02
INACTIVATION_OF_MAPK_ACTIVITY	12	1.94	0.02
GTPASE_ACTIVITY	91	1.94	0.02
RUFFLE	30	1.94	0.02
BIOPOLYMER_MODIFICATION	620	1.93	0.02
PLASMA_MEMBRANE	1326	1.93	0.02
PROTON_TRANSPORTING_TWO_SECTOR_ATPASE_COMPLEX	14	1.93	0.02
RIBOSOME	35	1.93	0.02
RECEPTOR_ACTIVITY	553	1.93	0.02
ORGANELLE_OUTER_MEMBRANE	23	1.93	0.02
PROTEIN_MODIFICATION_PROCESS	602	1.93	0.02
PROTEIN_COMPLEX	764	1.93	0.02
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	45	1.93	0.02
MONOOXYGENASE_ACTIVITY	30	1.93	0.02
REGULATION_OF_CELLULAR_PROTEIN_METABOLIC_PROCESS	151	1.92	0.02
NUCLEAR_MEMBRANE_PART	40	1.92	0.02
POSITIVE_REGULATION_OF_PROTEIN_MODIFICATION_PROCESS	28	1.92	0.02
POSITIVE_REGULATION_OF_CELLULAR_PROCESS	639	1.91	0.02
PROTEIN_AMINO_ACID_PHOSPHORYLATION	266	1.91	0.02
NUCLEAR_MEMBRANE	48	1.91	0.02
POSITIVE_REGULATION_OF_BIOLOGICAL_PROCESS	676	1.91	0.02
ESTABLISHMENT_OF_LOCALIZATION	831	1.91	0.02
RAS_GTPASE_BINDING	25	1.91	0.02
ENDOMEMBRANE_SYSTEM	206	1.91	0.02

PHOSPHORYLATION	297	1.91	0.02
PROTEIN_KINASE_REGULATOR_ACTIVITY	36	1.91	0.02
INTRACELLULAR_NON_MEMBRANE_BOUND_ORGANELLE	579	1.90	0.02
NON_MEMBRANE_BOUND_ORGANELLE	579	1.90	0.02
ENZYME_BINDING	168	1.90	0.02
MITOCHONDRIAL_MEMBRANE	77	1.89	0.02
INTRACELLULAR_SIGNALING_CASCADE	631	1.89	0.02
CYTOKINE_BINDING	43	1.89	0.02
PEPTIDYL_AMINO_ACID_MODIFICATION	60	1.89	0.02
RHO_GTPASE_ACTIVATOR_ACTIVITY	17	1.88	0.02
INTRACELLULAR_ORGANELLE_PART	1110	1.88	0.02
MITOCHONDRION	314	1.88	0.02
CELL_PROJECTION	104	1.88	0.02
PROTEIN_KINASE_ACTIVITY	271	1.88	0.02
MITOCHONDRIAL_ENVELOPE	87	1.88	0.02
MESODERM_DEVELOPMENT	20	1.88	0.02
SECRETORY_PATHWAY	82	1.88	0.02
ORGANELLE_PART	1115	1.88	0.02
SMALL_GTPASE_BINDING	32	1.87	0.02
REGULATION_OF_PROTEIN_METABOLIC_PROCESS	160	1.87	0.02
REGULATION_OF_CELL_MIGRATION	27	1.87	0.02
MEMBRANE_PART	1562	1.87	0.02
CELLULAR_COMPONENT_DISASSEMBLY	31	1.86	0.03
PHOSPHORIC_MONOESTER_HYDROLASE_ACTIVITY	106	1.86	0.03
DEFENSE_RESPONSE_TO_VIRUS	8	1.85	0.03
PROTEASE_INHIBITOR_ACTIVITY	35	1.85	0.03

ENZYME_INHIBITOR_ACTIVITY	110	1.85	0.03
MACROMOLECULE_LOCALIZATION	223	1.85	0.03
INTERLEUKIN_BINDING	21	1.85	0.03
LEADING_EDGE	45	1.84	0.03
NUCLEAR_PORE	30	1.84	0.03
REGULATION_OF_PHOSPHORYLATION	47	1.83	0.03
ATPASE_ACTIVITY	107	1.83	0.03
INTEGRAL_TO_PLASMA_MEMBRANE	906	1.83	0.03
PATTERN_RECOGNITION_RECEPTOR_ACTIVITY	10	1.83	0.03
ESTABLISHMENT_OF_PROTEIN_LOCALIZATION	182	1.83	0.03
VACUOLAR_MEMBRANE	10	1.83	0.03
REGULATION_OF_PROTEIN_AMINO_ACID_PHOSPHORYLATION	29	1.83	0.03
MITOCHONDRIAL_TRANSPORT	20	1.83	0.03
INTEGRAL_TO_MEMBRANE	1243	1.83	0.03
INTRINSIC_TO_MEMBRANE	1260	1.82	0.03
OUTER_MEMBRANE	24	1.82	0.04
ISOMERASE_ACTIVITY	34	1.81	0.04
NUCLEOLAR_PART	18	1.81	0.04
COFACTOR_METABOLIC_PROCESS	50	1.81	0.04
INTRINSIC_TO_PLASMA_MEMBRANE	919	1.82	0.04
REGULATION_OF_PEPTIDYL_TYROSINE_PHOSPHORYLATION	17	1.81	0.04
PHOSPHOTRANSFERASE_ACTIVITY_ALCOHOL_GROUP_AS_ACCEPTOR	317	1.81	0.04
NEGATIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	15	1.82	0.04
MEMBRANE_FRACTION	325	1.81	0.04
SIGNAL_SEQUENCE_BINDING	15	1.81	0.04
TYROSINE_PHOSPHORYLATION_OF_STAT_PROTEIN	12	1.81	0.04

TRANSLATION_INITIATION_FACTOR_ACTIVITY	22	1.81	0.04
INTEGRIN_COMPLEX	19	1.81	0.04
PROTEIN_LOCALIZATION	203	1.81	0.04
PROTEIN_AMINO_ACID_DEPHOSPHORYLATION	60	1.81	0.04
LIGASE_ACTIVITY_FORMING_CARBON_OXYGEN_BONDS	14	1.80	0.04
PROTEIN_DOMAIN_SPECIFIC_BINDING	70	1.80	0.04
MONOVALENT_INORGANIC_CATION_TRANSMEMBRANE TRANSPORTER_ACTIVITY	30	1.80	0.04
CATION_BINDING	204	1.80	0.04
POSITIVE_REGULATION_OF_PEPTIDYL_TYROSINE_PHOSPHORYLATION	12	1.80	0.04
CYTOSKELETON_DEPENDENT_INTRACELLULAR_TRANSPORT	24	1.80	0.04
REGULATION_OF_VIRAL_REPRODUCTION	11	1.79	0.04
PROTEIN_TYROSINE_PHOSPHATASE_ACTIVITY	53	1.79	0.04
POSITIVE_REGULATION_OF_PHOSPHATE_METABOLIC_PROCESS	27	1.79	0.04
AMINE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	40	1.79	0.04
TRANSLATIONAL_INITIATION	35	1.79	0.04
HEPARIN_BINDING	22	1.79	0.04
PORE_COMPLEX	35	1.78	0.04
SECRETION_BY_CELL	113	1.78	0.04
REPRODUCTIVE_PROCESS	149	1.78	0.04
KINASE_ACTIVITY	347	1.78	0.04
POSITIVE_REGULATION_OF_PROTEIN_METABOLIC_PROCESS	70	1.78	0.04
TRANSFERASE_ACTIVITY_TRANSFERRING_PHOSPHORUS CONTAINING_GROUPS	399	1.79	0.04
GTPASE_BINDING	33	1.78	0.04
L_AMINO_ACID_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	17	1.78	0.04

BIOPOLYMER_METABOLIC_PROCESS	1595	1.78	0.04
MUSCLE_DEVELOPMENT	88	1.78	0.04
ION_BINDING	261	1.78	0.04
PERIPHERAL_NERVOUS_SYSTEM_DEVELOPMENT	11	1.78	0.04
ORGANELLAR_SMALL_RIBOSOMAL_SUBUNIT	10	1.77	0.04
POSITIVE_REGULATION_OF_CELLULAR_PROTEIN_METABOLIC_PROCESS	68	1.77	0.04
KINASE_REGULATOR_ACTIVITY	43	1.77	0.04
ORGANELLE_LUMEN	428	1.77	0.04
MEMBRANE_ENCLOSED_LUMEN	428	1.77	0.05
STRUCTURAL_MOLECULE_ACTIVITY	232	1.77	0.05
CALCIUM_ION_TRANSPORT	26	1.76	0.05
REGULATION_OF_PH	12	1.76	0.05
VACUOLAR_PART	11	1.76	0.05
CYTOSOL	192	1.76	0.05
MONOVALENT_INORGANIC_CATION_HOMEOSTASIS	13	1.76	0.05
ATP_DEPENDENT_HELICASE_ACTIVITY	26	1.76	0.05
NEGATIVE_REGULATION_OF_CELL_MIGRATION	15	1.75	0.05
FATTY_ACID_BIOSYNTHETIC_PROCESS	14	1.75	0.05
PLASMA_MEMBRANE_PART	1076	1.75	0.05

Table 9.14 Significant gene sets identified by GSEA (FDR<0.05) in subjects with positive response on LTT to CBZE incubated with CBZE (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	77	3.05	0.00
RNA_BINDING	240	2.95	0.00
RIBONUCLEOPROTEIN_COMPLEX	135	2.77	0.00
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	80	2.63	0.00
NUCLEOLUS	115	2.63	0.00
RNA_PROCESSING	164	2.62	0.00
MITOCHONDRION	314	2.56	0.00
ORGANELLE_MEMBRANE	280	2.56	0.00
MITOCHONDRIAL_INNER_MEMBRANE	58	2.54	0.00
INTRACELLULAR_ORGANELLE_PART	1110	2.51	0.00
PROTEIN_RNA_COMPLEX_ASSEMBLY	63	2.50	0.00
ORGANELLE_INNER_MEMBRANE	66	2.49	0.00
ORGANELLE_PART	1115	2.48	0.00
COFACTOR_METABOLIC_PROCESS	50	2.47	0.00
MITOCHONDRIAL_PART	129	2.46	0.00
MEMBRANE_ENCLOSED_LUMEN	428	2.44	0.00
ENDOPLASMIC_RETICULUM_PART	91	2.42	0.00
ORGANELLE_LUMEN	428	2.41	0.00
MITOCHONDRIAL_ENVELOPE	87	2.40	0.00
NUCLEAR_PART	537	2.38	0.00
MACROMOLECULAR_COMPLEX	886	2.37	0.00
RIBOSOME	35	2.35	0.00

NUCLEAR_ENVELOPE_ENDOPLASMIC_RETICULUM_NETWORK	88	2.35	0.00
MITOCHONDRIAL_MEMBRANE	77	2.35	0.00
ENDOPLASMIC_RETICULUM_MEMBRANE	80	2.34	0.00
NUCLEAR_LUMEN	361	2.34	0.00
TRANSLATIONAL_INITIATION	35	2.33	0.00
MRNA_PROCESSING_GO_0006397	70	2.32	0.00
CYTOKINE_ACTIVITY	108	-2.33	0.00
EXTRACELLULAR_REGION_PART	320	-2.33	0.00
PATTERN_BINDING	44	-2.34	0.00
EXTRACELLULAR_SPACE	230	-2.36	0.00
CYTOPLASMIC_PART	1292	2.22	0.00
COENZYME_METABOLIC_PROCESS	35	2.23	0.00
MITOCHONDRIAL_MEMBRANE_PART	47	2.23	0.00
RNA_SPLICING	88	2.24	0.00
HELICASE_ACTIVITY	48	2.25	0.00
SPLICEOSOME	50	2.26	0.00
MRNA_METABOLIC_PROCESS	81	2.26	0.00
ORGANELLE_ENVELOPE	153	2.28	0.00
PROTEASOME_COMPLEX	22	2.28	0.00
GOLGI_VESICLE_TRANSPORT	48	2.28	0.00
ENVELOPE	153	2.29	0.00
REGULATION_OF_TRANSLATIONAL_INITIATION	29	2.20	0.00
PROTON_TRANSPORTING_TWO_SECTOR_ATPASE_COMPLEX	14	2.20	0.00
DNA_DIRECTED_RNA_POLYMERASEII_HOLOENZYME	62	2.21	0.00
TRANSLATION_REGULATOR_ACTIVITY	37	2.18	0.00
TRANSLATION_FACTOR_ACTIVITY_NUCLEIC_ACID_BINDING	35	2.18	0.00

STRUCTURE_SPECIFIC_DNA_BINDING	50	2.19	0.00
LYSOSOME	56	2.17	0.00
STRUCTURAL_MOLECULE_ACTIVITY	232	2.17	0.00
CYSTEINE_TYPE_ENDOPEPTIDASE_ACTIVITY	34	2.16	0.00
DNA_REPAIR	119	2.15	0.00
LYTIC_VACUOLE	56	2.13	0.00
PROTEIN_COMPLEX	764	2.13	0.00
GLYCEROPHOSPHOLIPID_BIOSYNTHETIC_PROCESS	28	2.12	0.00
HYDROGEN_ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	24	2.11	0.00
TRANSLATION_INITIATION_FACTOR_ACTIVITY	22	2.12	0.00
SINGLE_STRANDED_DNA_BINDING	32	2.11	0.00
MITOCHONDRIAL_RESPIRATORY_CHAIN	21	2.11	0.00
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	17	2.11	0.00
VACUOLE	64	2.10	0.00
MONOOXYGENASE_ACTIVITY	30	2.10	0.00
SMALL_NUCLEAR_RIBONUCLEOPROTEIN_COMPLEX	22	2.10	0.00
ENDOMEMBRANE_SYSTEM	206	2.09	0.00
ORGANELLAR_RIBOSOME	21	2.08	0.00
CYTOPLASM	1982	2.09	0.00
RNA_HELICASE_ACTIVITY	23	2.08	0.00
PROTEIN_SERINE_THREONINE_PHOSPHATASE_ACTIVITY	22	2.09	0.00
MITOCHONDRIAL_RIBOSOME	21	2.08	0.00
MITOCHONDRIAL_LUMEN	43	2.06	0.00
MITOCHONDRIAL_MATRIX	43	2.06	0.00
SUGAR_BINDING	33	2.04	0.00
RIBOSOMAL_SUBUNIT	19	2.04	0.00

MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	45	2.05	0.00
CYSTEINE_TYPE_PEPTIDASE_ACTIVITY	46	2.03	0.00
PHOSPHOINOSITIDE_BIOSYNTHETIC_PROCESS	23	2.03	0.00
RNA_SPLICINGVIA_TRANSESTERIFICATION_REACTIONS	34	2.03	0.00
NUCLEOLAR_PART	18	2.02	0.00
CELLULAR_RESPIRATION	18	2.02	0.00
INTRACELLULAR_NON_MEMBRANE_BOUND_ORGANELLE	579	2.02	0.00
ENDONUCLEASE_ACTIVITY_GO_0016893	11	2.01	0.00
NON_MEMBRANE_BOUND_ORGANELLE	579	2.01	0.00
TRICARBOXYLIC_ACID_CYCLE_INTERMEDIATE_METABOLIC_PROCESS	11	2.00	0.00
UNFOLDED_PROTEIN_BINDING	41	2.00	0.00
PERINUCLEAR_REGION_OF_CYTOPLASM	51	2.00	0.00
RNA_DEPENDENT_ATPASE_ACTIVITY	17	1.98	0.00
NUCLEUS	1313	1.98	0.00
TRANSLATION	167	1.98	0.00
ENDODEOXYRIBONUCLEASE_ACTIVITY	11	1.97	0.00
NUCLEASE_ACTIVITY	53	1.96	0.01
LIPID_BINDING	83	1.96	0.01
GLYCEROPHOSPHOLIPID_METABOLIC_PROCESS	42	1.96	0.01
ATP_DEPENDENT_RNA_HELICASE_ACTIVITY	16	1.96	0.01
RRNA_PROCESSING	14	1.96	0.01
GLYCOSAMINOGLYCAN_BINDING	33	-2.16	0.01
EXONUCLEASE_ACTIVITY	18	1.95	0.01
G_PROTEIN_COUPLED_RECEPTOR_BINDING	51	-2.14	0.01
MONOVALENT_INORGANIC_CATION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	30	1.95	0.01
INTRINSIC_TO_ENDOPLASMIC_RETICULUM_MEMBRANE	23	1.95	0.01

INTEGRAL_TO_ENDOPLASMIC_RETICULUM_MEMBRANE	23	1.95	0.01
AEROBIC_RESPIRATION	14	1.94	0.01
LIGASE_ACTIVITY_FORMING_CARBON_OXYGEN_BONDS	14	1.94	0.01
OLIGOSACCHARYL_TRANSFERASE_COMPLEX	9	1.94	0.01
POLYSACCHARIDE_BINDING	35	-2.14	0.01
RRNA_METABOLIC_PROCESS	15	1.93	0.01
ATP_DEPENDENT_HELICASE_ACTIVITY	26	1.93	0.01
INTRACELLULAR_TRANSPORT	271	1.93	0.01
NEGATIVE_REGULATION_OF_DNA_METABOLIC_PROCESS	17	1.93	0.01
PROTEIN_FOLDING	55	1.93	0.01
COATED_MEMBRANE	16	1.92	0.01
PROTEIN_EXPORT_FROM_NUCLEUS	12	1.92	0.01
RNA_SPLICING_FACTOR_ACTIVITYTRANSESTERIFICATION_MECHANISM	19	1.92	0.01
INTRACELLULAR_PROTEIN_TRANSPORT	139	1.92	0.01
MEMBRANE_COAT	16	1.92	0.01
TRNA_METABOLIC_PROCESS	19	1.91	0.01
NUCLEAR_PORE	30	1.92	0.01
ESTABLISHMENT_OF_VESICLE_LOCALIZATION	8	1.92	0.01
SECRETORY_PATHWAY	82	1.92	0.01
NUCLEOPLASM_PART	198	1.91	0.01
ELECTRON_CARRIER_ACTIVITY	75	1.92	0.01
PHOSPHOINOSITIDE_METABOLIC_PROCESS	30	1.91	0.01
CHEMOKINE_ACTIVITY	40	-2.11	0.01
NUCLEOPLASM	260	1.90	0.01
CELLULAR_COMPONENT_ASSEMBLY	278	1.90	0.01
MICROTUBULE_ORGANIZING_CENTER	63	1.90	0.01

MACROMOLECULAR_COMPLEX_ASSEMBLY	260	1.90	0.01
CHEMOKINE_RECEPTOR_BINDING	41	-2.09	0.01
MACROMOLECULE_BIOSYNTHETIC_PROCESS	298	1.90	0.01
ELECTRON_TRANSPORT_GO_0006118	47	1.90	0.01
INFLAMMATORY_RESPONSE	124	-2.10	0.01
DOUBLE_STRANDED_DNA_BINDING	29	1.89	0.01
NUCLEAR_MEMBRANE_PART	40	1.89	0.01
NUCLEAR_MEMBRANE	48	1.89	0.01
MITOCHONDRIAL_SMALL_RIBOSOMAL_SUBUNIT	10	1.89	0.01
VESICLE_COAT	15	1.88	0.01
PROTEIN_AMINO_ACID_N_LINKED_GLYCOSYLATION	28	1.88	0.01
VESICLE_LOCALIZATION	9	1.88	0.01
RESPONSE_TO_DNA_DAMAGE_STIMULUS	152	1.88	0.01
ORGANELLE_LOCALIZATION	23	1.87	0.01
CENTROSOME	54	1.87	0.01
NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS	1175	1.87	0.01
ORGANELLAR_SMALL_RIBOSOMAL_SUBUNIT	10	1.87	0.01
CELLULAR_PROTEIN_COMPLEX_DISASSEMBLY	12	1.87	0.01
COATED_VESICLE_MEMBRANE	16	1.87	0.01
ENDOPLASMIC_RETICULUM_LUMEN	13	1.87	0.01
VESICLE_MEMBRANE	28	1.87	0.01
SMALL_RIBOSOMAL_SUBUNIT	10	1.87	0.01
ENDOPLASMIC_RETICULUM	275	1.86	0.01
METALLOEXOPEPTIDASE_ACTIVITY	12	1.86	0.01
CELLULAR_LOCALIZATION	357	1.86	0.01
DEOXYRIBONUCLEASE_ACTIVITY	20	1.86	0.01

DAMAGED_DNA_BINDING	21	1.85	0.01
EXTRACELLULAR_REGION	424	-2.06	0.01
LIGASE_ACTIVITY	92	1.85	0.01
MICROBODY	47	1.85	0.01
DNA_HELICASE_ACTIVITY	24	1.85	0.01
COFACTOR_BIOSYNTHETIC_PROCESS	20	1.84	0.01
HEME_METABOLIC_PROCESS	10	1.84	0.01
CYTOPLASMIC_VESICLE_MEMBRANE	26	1.84	0.01
TRANSCRIPTION_FACTOR_TFIID_COMPLEX	12	1.84	0.01
REGULATION_OF_CELLULAR_PH	10	1.83	0.02
RESPONSE_TO_WOUNDING	182	-2.03	0.02
ESTABLISHMENT_OF_CELLULAR_LOCALIZATION	340	1.83	0.02
RNA_METABOLIC_PROCESS	793	1.83	0.02
OXIDOREDUCTASE_ACTIVITY_GO_0016616	54	1.83	0.02
T_CELL_ACTIVATION	41	1.83	0.02
CYTOCHROME_C_OXIDASE_ACTIVITY	12	1.83	0.02
RESPONSE_TO_EXTERNAL_STIMULUS	296	-2.03	0.02
REGULATION_OF_HOMEOSTATIC_PROCESS	13	1.82	0.02
BIOSYNTHETIC_PROCESS	435	1.82	0.02
CELLULAR_PROTEIN_CATABOLIC_PROCESS	56	1.82	0.02
PEROXISOME	47	1.82	0.02
IMMUNOLOGICAL_SYNAPSE	11	1.82	0.02
LOCOMOTORY_BEHAVIOR	89	-2.04	0.02
UBIQUITIN_LIGASE_COMPLEX	24	1.82	0.02
PROTEIN_TRANSPORT	150	1.82	0.02
POSITIVE_REGULATION_OF_T_CELL_ACTIVATION	20	1.82	0.02

GOLGI_ASSOCIATED_VESICLE	26	1.81	0.02
COENZYME_BINDING	16	1.81	0.02
APOPTOTIC_MITOCHONDRIAL_CHANGES	11	1.82	0.02
MEMBRANE_LIPID_METABOLIC_PROCESS	95	1.81	0.02
CYTOPLASMIC_VESICLE_PART	26	1.81	0.02
NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	148	-2.01	0.02
RESPONSE_TO_HYPOXIA	26	-2.02	0.02
DNA_RECOMBINATION	45	1.81	0.02
CHROMATIN_ASSEMBLY_OR_DISASSEMBLY	23	1.80	0.02
INTRA_GOLGI_VESICLE_MEDIATED_TRANSPORT	12	1.80	0.02
OXIDOREDUCTASE_ACTIVITY	274	1.80	0.02
CYTOKINE_METABOLIC_PROCESS	38	-2.00	0.02
ONE_CARBON_COMPOUND_METABOLIC_PROCESS	25	1.80	0.02
DNA_METABOLIC_PROCESS	245	1.80	0.02
PROTEIN_N_TERMINUS_BINDING	38	1.80	0.02
CELLULAR_PROTEIN_COMPLEX_ASSEMBLY	31	1.79	0.02
POSITIVE_REGULATION_OF_LYMPHOCYTE_ACTIVATION	23	1.80	0.02
ESTABLISHMENT_OF_ORGANELLE_LOCALIZATION	16	1.79	0.02
DNA_REPLICATION	97	1.79	0.02
PYRIMIDINE_NUCLEOTIDE_METABOLIC_PROCESS	9	1.78	0.02
MEDIATOR_COMPLEX	16	1.78	0.02
REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	89	1.78	0.02
BEHAVIOR	139	-1.98	0.02
NUCLEOBASENUCLEOSIDENUCLEOTIDE_KINASE_ACTIVITY	23	1.78	0.02
BIOPOLYMER_METABOLIC_PROCESS	1595	1.77	0.02
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_CH_OH_GROUP_OF_DONORS	60	1.77	0.02

RNA_POLYMERASE_II_TRANSCRIPTION_MEDIATOR_ACTIVITY	11	1.77	0.02
REGULATION_OF_PH	12	1.77	0.02
PROTEIN_TARGETING_TO_MEMBRANE	11	1.77	0.02
RIBONUCLEOPROTEIN_BINDING	11	1.77	0.02
PHOSPHOLIPID_BIOSYNTHETIC_PROCESS	37	1.77	0.02
EUKARYOTIC_TRANSLATION_INITIATION_FACTOR_3_COMPLEX	10	1.77	0.02
3_5_EXONUCLEASE_ACTIVITY	12	1.77	0.02
PROTEIN_AMINO_ACID_LIPIDATION	21	1.77	0.02
REGULATION_OF_BLOOD_PRESSURE	18	-1.98	0.02
CHROMATIN_ASSEMBLY	14	1.77	0.02
NADH_DEHYDROGENASE_COMPLEX	12	1.76	0.02
INTEGRIN_COMPLEX	19	-1.98	0.02
MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_I	12	1.76	0.03
TRNA_PROCESSING	10	1.76	0.03
ATPASE_ACTIVITY	107	1.76	0.03
MONOSACCHARIDE_BINDING	11	1.75	0.03
ER_TO_GOLGI_VESICLE_MEDIATED_TRANSPORT	18	1.75	0.03
LIPOPROTEIN_METABOLIC_PROCESS	30	1.75	0.03
POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	83	1.75	0.03
BASE_EXCISION_REPAIR	16	1.75	0.03
LIGASE_ACTIVITY_FORMING_CARBON_NITROGEN_BONDS	65	1.75	0.03
RESPIRATORY_CHAIN_COMPLEX_I	12	1.75	0.03
THIOLESTER_HYDROLASE_ACTIVITY	15	1.74	0.03
MANNOSYLTRANSFERASE_ACTIVITY	10	1.74	0.03
ORGANELLE_ORGANIZATION_AND_BIOGENESIS	443	1.74	0.03
PROTEIN_DNA_COMPLEX_ASSEMBLY	44	1.74	0.03

CELLULAR_CATABOLIC_PROCESS	204	1.73	0.03
POST_GOLGI_VESICLE_MEDIATED_TRANSPORT	14	1.73	0.03
CHROMOSOME	112	1.73	0.03
SPHINGOID_METABOLIC_PROCESS	11	1.73	0.03
CATABOLIC_PROCESS	217	1.73	0.03
ACID_AMINO_ACID_LIGASE_ACTIVITY	56	1.72	0.03
CELLULAR_MACROMOLECULE_CATABOLIC_PROCESS	100	1.72	0.03
MICROTUBULE_ORGANIZING_CENTER_ORGANIZATION_AND_BIOGENESIS	15	1.73	0.03
CELLULAR_MONOVALENT_INORGANIC_CATION_HOMEOSTASIS	11	1.72	0.03
CERAMIDE_METABOLIC_PROCESS	10	1.72	0.03
GENERAL_RNA_POLYMERASE_II_TRANSCRIPTION_FACTOR_ACTIVITY	29	1.72	0.03
DOUBLE_STRAND_BREAK_REPAIR	22	1.72	0.03
RESPONSE_TO_ENDOGENOUS_STIMULUS	188	1.71	0.03
ANTIOXIDANT_ACTIVITY	16	1.71	0.03
PROTEIN_CATABOLIC_PROCESS	67	1.71	0.03
CENTROSOME_ORGANIZATION_AND_BIOGENESIS	14	1.71	0.03
REGULATION_OF_GENE_EXPRESSION_EPIGENETIC	28	1.71	0.03
CHROMOSOMAL_PART	90	1.71	0.03
LIPOPROTEIN_BIOSYNTHETIC_PROCESS	23	1.71	0.04
PYROPHOSPHATASE_ACTIVITY	211	1.70	0.04
REGULATION_OF_CELL_PROLIFERATION	296	-1.92	0.04
PIGMENT_METABOLIC_PROCESS	16	1.70	0.04
PATTERN_RECOGNITION_RECEPTOR_ACTIVITY	10	-1.93	0.04
HYDROLASE_ACTIVITY_ACTING_ON_ACID_ANHYDRIDES	213	1.70	0.04
OXIDOREDUCTASE_ACTIVITY_GO_0016706	10	-1.93	0.04
TUBULIN_BINDING	43	1.69	0.04

AMINO_ACID_DERIVATIVE_METABOLIC_PROCESS	21	1.69	0.04
MEMBRANE_LIPID_BIOSYNTHETIC_PROCESS	46	1.69	0.04
BIOPOLYMER_CATABOLIC_PROCESS	114	1.69	0.04
PORE_COMPLEX	35	1.69	0.04
PHOSPHOLIPID_BINDING	45	1.69	0.04
COFACTOR_CATABOLIC_PROCESS	10	1.68	0.04
DNA_DEPENDENT_ATPASE_ACTIVITY	21	1.68	0.04
MEMBRANE_FUSION	28	1.68	0.04
BIOGENIC_AMINE_METABOLIC_PROCESS	14	1.68	0.04
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_CH_GROUP_OF_DONORS	23	1.67	0.04
APOPTOTIC_PROGRAM	57	1.67	0.04
RIBONUCLEASE_ACTIVITY	25	1.67	0.04
SPLICEOSOME_ASSEMBLY	21	1.67	0.04
MACROMOLECULE_LOCALIZATION	223	1.66	0.05
POSITIVE_REGULATION_OF_CELL_PROLIFERATION	145	-1.89	0.05
CELLULAR_BIOSYNTHETIC_PROCESS	297	1.66	0.05
OXIDOREDUCTASE_ACTIVITY_GO_0016705	36	-1.87	0.05
PROTEIN_TARGETING	106	1.66	0.05
CYTOKINE_PRODUCTION	66	-1.87	0.05
CYTOKINE_BIOSYNTHETIC_PROCESS	37	-1.88	0.05
CARBOHYDRATE_TRANSPORT	18	-1.88	0.05
RESPONSE_TO_TOXIN	10	1.66	0.05
MICROTUBULE_ORGANIZING_CENTER_PART	18	1.66	0.05
MITOTIC_CELL_CYCLE_CHECKPOINT	20	1.66	0.05
POSITIVE_REGULATION_OF_DNA_METABOLIC_PROCESS	11	-1.89	0.05
GENE_SILENCING	10	1.65	0.05

CELLULAR_LIPID_CATABOLIC_PROCESS	32	1.65	0.05
TRANSCRIPTION_COACTIVATOR_ACTIVITY	118	1.65	0.05
CELLULAR_MACROMOLECULE_METABOLIC_PROCESS	1072	1.65	0.05
PROTEIN_METABOLIC_PROCESS	1164	1.65	0.05
LATE_ENDOSOME	9	1.65	0.05
MONOVALENT_INORGANIC_CATION_HOMEOSTASIS	13	1.65	0.05
NUCLEAR_EXPORT	32	1.65	0.05
HEME_BIOSYNTHETIC_PROCESS	9	1.65	0.05
RNA_3END_PROCESSING	9	1.65	0.05
CELLULAR_PROTEIN_METABOLIC_PROCESS	1059	1.65	0.05
MONOSACCHARIDE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	10	-1.89	0.05
DNA_REPLICATION_INITIATION	14	1.65	0.05

Table 9.15 Significant gene sets identified by GSEA (FDR<0.05) in subjects with positive response on LTT to CBZE incubated with TT (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	77	3.00	0.00
RNA_BINDING	240	2.87	0.00
RIBONUCLEOPROTEIN_COMPLEX	135	2.78	0.00
ORGANELLE_MEMBRANE	280	2.67	0.00
RNA_PROCESSING	164	2.66	0.00
ORGANELLE_PART	1115	2.61	0.00
INTRACELLULAR_ORGANELLE_PART	1110	2.60	0.00
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	80	2.59	0.00
ENDOPLASMIC_RETICULUM_PART	91	2.56	0.00
NUCLEOLUS	115	2.49	0.00
ORGANELLE_ENVELOPE	153	2.46	0.00
NUCLEAR_PART	537	2.45	0.00
ENDOPLASMIC_RETICULUM_MEMBRANE	80	2.45	0.00
PROTEIN_RNA_COMPLEX_ASSEMBLY	63	2.44	0.00
MACROMOLECULAR_COMPLEX	886	2.44	0.00
MRNA_METABOLIC_PROCESS	81	2.43	0.00
MITOCHONDRIAL_PART	129	2.43	0.00
ENVELOPE	153	2.43	0.00
RIBOSOME	35	2.42	0.00
NUCLEAR_ENVELOPE_ENDOPLASMIC_RETICULUM_NETWORK	88	2.41	0.00
PROTEASOME_COMPLEX	22	2.39	0.00
MITOCHONDRION	314	2.38	0.00

MITOCHONDRIAL_INNER_MEMBRANE	58	2.37	0.00
CYSTEINE_TYPE_ENDOPEPTIDASE_ACTIVITY	34	2.28	0.00
RNA_SPLICING	88	2.29	0.00
SPLICEOSOME	50	2.29	0.00
CYSTEINE_TYPE_PEPTIDASE_ACTIVITY	46	2.31	0.00
MRNA_PROCESSING_GO_0006397	70	2.31	0.00
MITOCHONDRIAL_ENVELOPE	87	2.35	0.00
ORGANELLE_INNER_MEMBRANE	66	2.35	0.00
ORGANELLE_LUMEN	428	2.35	0.00
MEMBRANE_ENCLOSED_LUMEN	428	2.36	0.00
VACUOLE	64	2.25	0.00
NUCLEAR_LUMEN	361	2.25	0.00
NUCLEAR_MEMBRANE	48	2.26	0.00
SECRETORY_PATHWAY	82	2.26	0.00
ENDOMEMBRANE_SYSTEM	206	2.27	0.00
MITOCHONDRIAL_MEMBRANE	77	2.27	0.00
SMALL_NUCLEAR_RIBONUCLEOPROTEIN_COMPLEX	22	2.21	0.00
LYSOSOME	56	2.21	0.00
COFACTOR_METABOLIC_PROCESS	50	2.21	0.00
PROTEIN_COMPLEX	764	2.21	0.00
CYTOPLASM	1982	2.22	0.00
TRANSLATIONAL_INITIATION	35	2.22	0.00
NUCLEUS	1313	2.22	0.00
CYTOPLASMIC_PART	1292	2.22	0.00
TRANSLATION_REGULATOR_ACTIVITY	37	2.22	0.00
DNA_REPAIR	119	2.23	0.00

PROTON_TRANSPORTING_TWO_SECTOR_ATPASE_COMPLEX	14	2.24	0.00
LYTIC_VACUOLE	56	2.20	0.00
TRANSLATION_FACTOR_ACTIVITY_NUCLEIC_ACID_BINDING	35	2.20	0.00
REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	89	2.18	0.00
DNA_DIRECTED_RNA_POLYMERASEII_HOLOENZYME	62	2.18	0.00
REGULATION_OF_TRANSLATIONAL_INITIATION	29	2.19	0.00
INTRACELLULAR_NON_MEMBRANE_BOUND_ORGANELLE	579	2.18	0.00
GOLGI_VESICLE_TRANSPORT	48	2.18	0.00
NON_MEMBRANE_BOUND_ORGANELLE	579	2.17	0.00
TRANSLATION_INITIATION_FACTOR_ACTIVITY	22	2.16	0.00
STRUCTURAL_MOLECULE_ACTIVITY	232	2.16	0.00
HELICASE_ACTIVITY	48	2.13	0.00
INTRACELLULAR_TRANSPORT	271	2.13	0.00
CELLULAR_LOCALIZATION	357	2.13	0.00
GLYCEROPHOSPHOLIPID_BIOSYNTHETIC_PROCESS	28	2.14	0.00
MITOCHONDRIAL_RESPIRATORY_CHAIN	21	2.15	0.00
RNA_DEPENDENT_ATPASE_ACTIVITY	17	2.12	0.00
ENDOPLASMIC_RETICULUM	275	2.12	0.00
TRICARBOXYLIC_ACID_CYCLE_INTERMEDIATE_METABOLIC_PROCESS	11	2.11	0.00
PROTEIN_SERINE_THREONINE_PHOSPHATASE_ACTIVITY	22	2.12	0.00
ENDOPLASMIC_RETICULUM_LUMEN	13	2.11	0.00
INTEGRAL_TO_ENDOPLASMIC_RETICULUM_MEMBRANE	23	2.11	0.00
NUCLEAR_PORE	30	2.11	0.00
ESTABLISHMENT_OF_CELLULAR_LOCALIZATION	340	2.11	0.00
TRANSLATION	167	2.11	0.00
GTP_BINDING	42	2.11	0.00

POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	83	2.10	0.00
TRNA_METABOLIC_PROCESS	19	2.09	0.00
RNA_HELICASE_ACTIVITY	23	2.09	0.00
MITOCHONDRIAL_MEMBRANE_PART	47	2.08	0.00
PHOSPHOINOSITIDE_BIOSYNTHETIC_PROCESS	23	2.08	0.00
INTRINSIC_TO_ENDOPLASMIC_RETICULUM_MEMBRANE	23	2.07	0.00
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	45	2.07	0.00
ATP_DEPENDENT_RNA_HELICASE_ACTIVITY	16	2.06	0.00
UNFOLDED_PROTEIN_BINDING	41	2.07	0.00
PHOSPHOINOSITIDE_METABOLIC_PROCESS	30	2.06	0.00
RNA_SPLICINGVIA_TRANSESTERIFICATION_REACTIONS	34	2.05	0.00
VIRAL_REPRODUCTION	37	2.04	0.00
GUANYL_NUCLEOTIDE_BINDING	43	2.04	0.00
RNA_METABOLIC_PROCESS	793	2.04	0.00
CYTOKINE_BINDING	43	2.04	0.00
ATP_DEPENDENT_HELICASE_ACTIVITY	26	2.04	0.00
INTRACELLULAR_PROTEIN_TRANSPORT	139	2.03	0.00
VESICLE_COAT	15	2.03	0.00
PATTERN_BINDING	44	-2.19	0.00
TRNA_PROCESSING	10	2.03	0.00
CELLULAR_COMPONENT_ASSEMBLY	278	2.02	0.00
NUCLEAR_MEMBRANE_PART	40	2.02	0.00
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	17	2.01	0.00
RESPONSE_TO_DNA_DAMAGE_STIMULUS	152	2.01	0.00
MACROMOLECULAR_COMPLEX_ASSEMBLY	260	2.01	0.00
JAK_STAT_CASCADE	30	2.00	0.00

GLYCEROPHOSPHOLIPID_METABOLIC_PROCESS	42	1.99	0.00
MACROMOLECULE_BIOSYNTHETIC_PROCESS	298	1.99	0.00
STRUCTURE_SPECIFIC_DNA_BINDING	50	1.99	0.00
INTRA_GOLGI_VESICLE_MEDIATED_TRANSPORT	12	1.98	0.01
BIOPOLYMER_METABOLIC_PROCESS	1595	1.98	0.01
COATED_MEMBRANE	16	1.98	0.01
NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS	1175	1.98	0.01
IMMUNE_SYSTEM_PROCESS	307	1.97	0.01
PROTEIN_TRANSPORT	150	1.98	0.01
ORGANELLE_ORGANIZATION_AND_BIOGENESIS	443	1.97	0.01
COENZYME_METABOLIC_PROCESS	35	1.97	0.01
SINGLE_STRANDED_DNA_BINDING	32	1.97	0.01
PORE_COMPLEX	35	1.97	0.01
IMMUNOLOGICAL_SYNAPSE	11	1.97	0.01
EXTRACELLULAR_SPACE	230	-2.08	0.01
RNA_POLYMERASE_ACTIVITY	15	1.96	0.01
INTERLEUKIN_BINDING	21	1.96	0.01
MITOCHONDRIAL_LUMEN	43	1.96	0.01
MEMBRANE_COAT	16	1.95	0.01
RNA_SPLICING_FACTOR_ACTIVITYTRANSESTERIFICATION_MECHANISM	19	1.95	0.01
PROTEIN_EXPORT_FROM_NUCLEUS	12	1.95	0.01
GLYCOSAMINOGLYCAN_BINDING	33	-2.09	0.01
NUCLEAR_EXPORT	32	1.94	0.01
RESPONSE_TO_VIRUS	42	1.94	0.01
IMMUNE_RESPONSE	217	1.94	0.01
PEPTIDYL_AMINO_ACID_MODIFICATION	60	1.93	0.01

EXTRACELLULAR_REGION_PART	320	-2.06	0.01
POLYSACCHARIDE_BINDING	35	-2.09	0.01
AEROBIC_RESPIRATION	14	1.93	0.01
MITOCHONDRIAL_MATRIX	43	1.92	0.01
RIBOSOMAL_SUBUNIT	19	1.92	0.01
ESTABLISHMENT_OF_ORGANELLE_LOCALIZATION	16	1.92	0.01
LATE_ENDOSOME	9	1.91	0.01
REGULATION_OF_PH	12	1.91	0.01
CELLULAR_RESPIRATION	18	1.90	0.01
REGULATION_OF_HOMEOSTATIC_PROCESS	13	1.90	0.01
PYROPHOSPHATASE_ACTIVITY	211	1.90	0.01
VIRAL_REPRODUCTIVE_PROCESS	32	1.90	0.01
REGULATION_OF_CELLULAR_PH	10	1.90	0.01
EXONUCLEASE_ACTIVITY	18	1.90	0.01
NUCLEOPLASM_PART	198	1.90	0.01
NUCLEOPLASM	260	1.89	0.01
MONOVALENT_INORGANIC_CATION_HOMEOSTASIS	13	1.89	0.01
CELLULAR_MONOVALENT_INORGANIC_CATION_HOMEOSTASIS	11	1.89	0.01
COATED_VESICLE_MEMBRANE	16	1.89	0.01
HYDROLASE_ACTIVITY_ACTING_ON_ACID_ANHYDRIDES	213	1.89	0.01
VESICLE_MEMBRANE	28	1.89	0.01
CYTOPLASMIC_VESICLE_PART	26	1.89	0.01
CELLULAR_DEFENSE_RESPONSE	54	1.88	0.01
HETEROGENEOUS_NUCLEAR_RIBONUCLEOPROTEIN_COMPLEX	12	1.88	0.01
UBIQUITIN_LIGASE_COMPLEX	24	1.87	0.01
INTERLEUKIN_RECEPTOR_ACTIVITY	16	1.87	0.01

PEPTIDYL_TYROSINE_PHOSPHORYLATION	26	1.87	0.01
MITOCHONDRIAL_RIBOSOME	21	1.87	0.01
PIGMENT_METABOLIC_PROCESS	16	1.87	0.01
CYTOPLASMIC_VESICLE_MEMBRANE	26	1.87	0.01
CHROMOSOME	112	1.87	0.01
VACUOLAR_PART	11	1.86	0.01
RESPONSE_TO_ENDOGENOUS_STIMULUS	188	1.86	0.01
RESPONSE_TO_BIOTIC_STIMULUS	109	1.86	0.01
DOUBLE_STRAND_BREAK_REPAIR	22	1.85	0.02
MEMBRANE_LIPID_METABOLIC_PROCESS	95	1.85	0.02
PERINUCLEAR_REGION_OF_CYTOPLASM	51	1.85	0.02
ESTABLISHMENT_OF_VESICLE_LOCALIZATION	8	1.85	0.02
LIPID_BINDING	83	1.85	0.02
LIGASE_ACTIVITY_FORMING_CARBON_OXYGEN_BONDS	14	1.85	0.02
ORGANELLAR_RIBOSOME	21	1.85	0.02
CATION_HOMEOSTASIS	101	1.85	0.02
CELLULAR_CATION_HOMEOSTASIS	99	1.84	0.02
COENZYME_BINDING	16	1.84	0.02
GOLGI_ASSOCIATED_VESICLE	26	1.84	0.02
MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_I	12	1.84	0.02
CHROMOSOMEPERICENTRIC_REGION	28	1.84	0.02
NUCLEOTIDE_EXCISION_REPAIR	20	1.84	0.02
NUCLEOSIDE_TRIPHOSPHATASE_ACTIVITY	199	1.84	0.02
VESICLE_MEDIATED_TRANSPORT	190	1.83	0.02
PHOSPHORIC_DIESTER_HYDROLASE_ACTIVITY	40	1.83	0.02
PHOSPHOLIPASE_ACTIVITY	41	1.83	0.02

DOUBLE_STRANDED_RNA_BINDING	15	1.83	0.02
PROTEIN_METABOLIC_PROCESS	1164	1.82	0.02
CELLULAR_MACROMOLECULE_METABOLIC_PROCESS	1072	1.82	0.02
DOUBLE_STRANDED_DNA_BINDING	29	1.82	0.02
PATTERN_RECOGNITION_RECEPTOR_ACTIVITY	10	-1.99	0.02
EUKARYOTIC_TRANSLATION_INITIATION_FACTOR_3_COMPLEX	10	1.81	0.02
RRNA_PROCESSING	14	1.81	0.02
DNA_DAMAGE_RESPONSESIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	12	1.81	0.02
I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	107	1.81	0.02
RNA_EXPORT_FROM_NUCLEUS	19	1.81	0.02
ATPASE_ACTIVITY	107	1.81	0.02
HYDROGEN_ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	24	1.81	0.02
BIOSYNTHETIC_PROCESS	435	1.81	0.02
ORGANELLE_LOCALIZATION	23	1.81	0.02
CHROMATIN_ASSEMBLY_OR_DISASSEMBLY	23	1.81	0.02
MACROMOLECULE_LOCALIZATION	223	1.80	0.02
RESPIRATORY_CHAIN_COMPLEX_I	12	1.80	0.02
MICROTUBULE_CYTOSKELETON	140	1.80	0.02
ADAPTIVE_IMMUNE_RESPONSE_GO_0002460	21	1.80	0.02
MULTI_ORGANISM_PROCESS	153	1.80	0.02
CHROMOSOMAL_PART	90	1.80	0.02
PROTEIN_FOLDING	55	1.80	0.02
PROTEIN_CATABOLIC_PROCESS	67	1.79	0.02
HEME_METABOLIC_PROCESS	10	1.79	0.02
ACTIN_BINDING	72	1.79	0.02
INTERACTION_WITH_HOST	16	1.79	0.02

ENDOSOME	62	1.79	0.02
TRANSFERASE_ACTIVITY_TRANSFERRING_GROUPS_OTHER_THAN_AMINO_ACYL_GROUPS	45	1.79	0.02
CELLULAR_PROTEIN_CATABOLIC_PROCESS	56	1.79	0.02
LYSOSOMAL_MEMBRANE	9	1.79	0.02
3_5_EXONUCLEASE_ACTIVITY	12	1.79	0.02
CELLULAR_HOMEOSTASIS	138	1.79	0.02
PROTEIN_PHOSPHATASE_TYPE_2A_REGULATOR_ACTIVITY	12	1.79	0.02
MICROBODY	47	1.79	0.02
CELLULAR_PROTEIN_METABOLIC_PROCESS	1059	1.79	0.02
MONOSACCHARIDE_BINDING	11	1.79	0.02
ADAPTIVE_IMMUNE_RESPONSE	22	1.78	0.03
EXOCYTOSIS	23	1.78	0.03
ION_HOMEOSTASIS	121	1.78	0.03
TRANSCRIPTION_ACTIVATOR_ACTIVITY	167	1.77	0.03
SPINDLE	38	1.77	0.03
PROTEIN_TARGETING	106	1.77	0.03
TRANSCRIPTION_FACTOR_TFIID_COMPLEX	12	1.77	0.03
TRANSCRIPTION_COACTIVATOR_ACTIVITY	118	1.77	0.03
PEROXISOME	47	1.77	0.03
DAMAGED_DNA_BINDING	21	1.77	0.03
INOSITOL_OR_PHOSPHATIDYLINOSITOL_PHOSPHATASE_ACTIVITY	11	1.77	0.03
PROTEIN_TARGETING_TO_MEMBRANE	11	1.77	0.03
NUCLEAR_ENVELOPE	67	1.77	0.03
OXIDOREDUCTASE_ACTIVITY_GO_0016616	54	1.77	0.03
NADH_DEHYDROGENASE_COMPLEX	12	1.76	0.03
VESICLE_LOCALIZATION	9	1.76	0.03

RESPONSE_TO_OTHER_ORGANISM	73	1.76	0.03
RRNA_METABOLIC_PROCESS	15	1.76	0.03
METALLOEXOPEPTIDASE_ACTIVITY	12	1.75	0.03
VACUOLAR_MEMBRANE	10	1.76	0.03
CELLULAR_BIOSYNTHETIC_PROCESS	297	1.75	0.03
N_TERMINAL_PROTEIN_AMINO_ACID_MODIFICATION	10	1.75	0.03
HISTONE_ACETYLTRANSFERASE_ACTIVITY	15	1.75	0.03
LIGASE_ACTIVITY	92	1.75	0.03
CELLULAR_PROTEIN_COMPLEX_ASSEMBLY	31	1.75	0.03
RIBONUCLEOPROTEIN_BINDING	11	1.75	0.03
CYTOSKELETAL_PART	220	1.75	0.03
T_CELL_ACTIVATION	41	1.75	0.03
OLIGOSACCHARYL_TRANSFERASE_COMPLEX	9	1.74	0.03
RESPONSE_TO_HYPOXIA	26	-1.96	0.03
PROTEIN_DNA_COMPLEX_ASSEMBLY	44	1.74	0.03
ENDODEOXYRIBONUCLEASE_ACTIVITY	11	1.74	0.03
DNA_METABOLIC_PROCESS	245	1.74	0.03
TYROSINE_PHOSPHORYLATION_OF_STAT_PROTEIN	12	1.74	0.03
RECEPTOR_MEDIATED_ENDOCYTOSIS	33	1.74	0.03
LIPASE_ACTIVITY	49	1.74	0.03
SIGNAL_SEQUENCE_BINDING	15	1.74	0.03
ACTIN_FILAMENT_BINDING	22	1.73	0.03
COATED_VESICLE	44	1.73	0.03
PROTEIN_AMINO_ACID_N_LINKED_GLYCOSYLATION	28	1.73	0.03
GROWTH_FACTOR_BINDING	29	1.73	0.03
PROTEIN_LOCALIZATION	203	1.73	0.03

NUCLEOLAR_PART	18	1.72	0.04
DNA_RECOMBINATION	45	1.72	0.04
MRNA_BINDING	21	1.72	0.04
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_CH_OH_GROUP_OF_DONORS	60	1.72	0.04
MICROTUBULE_BASED_PROCESS	75	1.72	0.04
NEUTRAL_AMINO_ACID_TRANSPORT	10	1.72	0.04
COFACTOR_BIOSYNTHETIC_PROCESS	20	1.72	0.04
MEMBRANE_ORGANIZATION_AND_BIOGENESIS	132	1.72	0.04
CHROMOSOME_ORGANIZATION_AND_BIOGENESIS	116	1.72	0.04
PML_BODY	13	1.71	0.04
PIGMENT_BIOSYNTHETIC_PROCESS	15	1.71	0.04
PROTEIN_AMINO_ACID_LIPIDATION	21	1.71	0.04
NEGATIVE_REGULATION_OF_CELLULAR_COMPONENT_ORGANIZATION_AND_BIOGENESIS	26	1.71	0.04
ONE_CARBON_COMPOUND_METABOLIC_PROCESS	25	1.71	0.04
DEOXYRIBONUCLEASE_ACTIVITY	20	1.71	0.04
U12_DEPENDENT_SPLICEOSOME	10	1.71	0.04
MITOTIC_CELL_CYCLE_CHECKPOINT	20	1.71	0.04
BASE_EXCISION_REPAIR	16	1.71	0.04
ESTABLISHMENT_OF_PROTEIN_LOCALIZATION	182	1.71	0.04
DNA_BINDING	566	1.70	0.04
PHOSPHOLIPID_METABOLIC_PROCESS	70	1.70	0.04
KINETOCHORE	22	1.70	0.04
MICROTUBULE_ORGANIZING_CENTER	63	1.70	0.04
ENZYME_REGULATOR_ACTIVITY	304	1.70	0.04
CALCIUM_MEDIATED_SIGNALING	13	1.70	0.04
REGULATION_OF_IMMUNE_EFFECTOR_PROCESS	13	1.70	0.04

BIOPOLYMER_CATABOLIC_PROCESS	114	1.69	0.04
LEUKOCYTE_ACTIVATION	64	1.69	0.04
IMMUNE_EFFECTOR_PROCESS	32	1.69	0.04
INACTIVATION_OF_MAPK_ACTIVITY	12	1.69	0.04
CYTOCHROME_C_OXIDASE_ACTIVITY	12	1.69	0.04
RNA_POLYMERASE_COMPLEX	17	1.69	0.04
TUBULIN_BINDING	43	1.69	0.04
INSOLUBLE_FRACTION	15	1.68	0.04
PHOSPHOLIPASE_C_ACTIVITY	15	1.68	0.04
MONOSACCHARIDE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	10	-1.91	0.04
ACTIN_FILAMENT	16	1.68	0.04
LYMPHOCYTE_ACTIVATION	57	1.68	0.04
ELECTRON_TRANSPORT_GO_0006118	47	1.68	0.04
ER_TO_GOLGI_VESICLE_MEDIATED_TRANSPORT	18	1.68	0.04
CARBOXYLIC_ACID_METABOLIC_PROCESS	172	1.68	0.04
SMALL_RIBOSOMAL_SUBUNIT	10	1.68	0.04
DNA_HELICASE_ACTIVITY	24	1.68	0.05
REGULATION_OF_GENE_EXPRESSION_EPIGENETIC	28	1.68	0.05
OXIDOREDUCTASE_ACTIVITY_GO_0016705	36	-1.92	0.05
SECRETION_BY_CELL	113	1.67	0.05
VIRAL_INFECTIOUS_CYCLE	28	1.67	0.05
NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	148	-1.90	0.05
SUGAR_BINDING	33	1.67	0.05
COFACTOR_BINDING	22	1.67	0.05
CHROMATIN_REMODELING_COMPLEX	15	1.67	0.05
APOPTOTIC_MITOCHONDRIAL_CHANGES	11	1.66	0.05

APOPTOTIC_PROGRAM	57	1.66	0.05
ENDOPEPTIDASE_ACTIVITY	109	1.66	0.05
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_CH_GROUP_OF_DONORS	23	1.66	0.05
ENDONUCLEASE_ACTIVITY_GO_0016893	11	1.66	0.05
RECEPTOR_SIGNALING_PROTEIN_ACTIVITY	81	1.66	0.05
MANNOSYLTRANSFERASE_ACTIVITY	10	1.66	0.05
GTPASE_REGULATOR_ACTIVITY	119	1.66	0.05

Table 9.16 Significant gene sets identified by GSEA (FDR<0.05) in subjects with negative response on LTT to CBZE incubated with TT (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
CHEMOKINE_RECEPTOR_BINDING	41	2.43	0.00
CHEMOKINE_ACTIVITY	40	2.42	0.00
G_PROTEIN_COUPLED_RECEPTOR_BINDING	51	2.41	0.00
LOCOMOTORY_BEHAVIOR	89	2.18	0.00
CYTOKINE_ACTIVITY	108	1.98	0.04
CELLULAR_CATION_HOMEOSTASIS	99	1.96	0.04
PATTERN_BINDING	44	1.94	0.05

Table 9.17 CBZ/CBZE-specific gene sets identified by GSEA (FDR<0.05) (size: number of genes in gene set, NES: normalised enrichment score, FDR: false discovery rate)

Gene Set (Functional Category)	SIZE	NES	FDR.q.val
NUCLEASE_ACTIVITY	53	1.96	0.01
NEGATIVE_REGULATION_OF_DNA_METABOLIC_PROCESS	17	1.93	0.01
COLLAGEN_BINDING	14	1.89	0.04
MITOCHONDRIAL_SMALL_RIBOSOMAL_SUBUNIT	10	1.89	0.01
CENTROSOME	54	1.87	0.01
CELLULAR_PROTEIN_COMPLEX_DISASSEMBLY	12	1.87	0.01
CASPASE_ACTIVATION	26	1.87	0.04
REGULATION_OF_CYCLIN_DEPENDENT_PROTEIN_KINASE_ACTIVITY	42	1.83	0.05
POSITIVE_REGULATION_OF_T_CELL_ACTIVATION	20	1.82	0.02
POSITIVE_REGULATION_OF_LYMPHOCYTE_ACTIVATION	23	1.80	0.02
PROTEIN_N_TERMINUS_BINDING	38	1.80	0.02
DNA_REPLICATION	97	1.79	0.02
PYRIMIDINE_NUCLEOTIDE_METABOLIC_PROCESS	9	1.78	0.02
MEDIATOR_COMPLEX	16	1.78	0.02
NUCLEOBASENUCLEOSIDENUCLEOTIDE_KINASE_ACTIVITY	23	1.78	0.02
PHOSPHOLIPID_BIOSYNTHETIC_PROCESS	37	1.77	0.02
RNA_POLYMERASE_II_TRANSCRIPTION_MEDIATOR_ACTIVITY	11	1.77	0.02
CHROMATIN_ASSEMBLY	14	1.77	0.02
LIGASE_ACTIVITY_FORMING_CARBON_NITROGEN_BONDS	65	1.75	0.03
LIPOPROTEIN_METABOLIC_PROCESS	30	1.75	0.03
THIOLESTER_HYDROLASE_ACTIVITY	15	1.74	0.03
POST_GOLGI_VESICLE_MEDIATED_TRANSPORT	14	1.73	0.03

CELLULAR_CATABOLIC_PROCESS	204	1.73	0.03
CATABOLIC_PROCESS	217	1.73	0.03
SPHINGOID_METABOLIC_PROCESS	11	1.73	0.03
MICROTUBULE_ORGANIZING_CENTER_ORGANIZATION_AND_BIOGENESIS	15	1.73	0.03
CELLULAR_MACROMOLECULE_CATABOLIC_PROCESS	100	1.72	0.03
ACID_AMINO_ACID_LIGASE_ACTIVITY	56	1.72	0.03
CERAMIDE_METABOLIC_PROCESS	10	1.72	0.03
GENERAL_RNA_POLYMERASE_II_TRANSCRIPTION_FACTOR_ACTIVITY	29	1.72	0.03
ANTIOXIDANT_ACTIVITY	16	1.71	0.03
CENTROSOME_ORGANIZATION_AND_BIOGENESIS	14	1.71	0.03
LIPOPROTEIN_BIOSYNTHETIC_PROCESS	23	1.71	0.04
AMINO_ACID_DERIVATIVE_METABOLIC_PROCESS	21	1.69	0.04
MEMBRANE_LIPID_BIOSYNTHETIC_PROCESS	46	1.69	0.04
PHOSPHOLIPID_BINDING	45	1.69	0.04
COFACTOR_CATABOLIC_PROCESS	10	1.68	0.04
DNA_DEPENDENT_ATPASE_ACTIVITY	21	1.68	0.04
MEMBRANE_FUSION	28	1.68	0.04
BIOGENIC_AMINE_METABOLIC_PROCESS	14	1.68	0.04
RIBONUCLEASE_ACTIVITY	25	1.67	0.04
SPLICEOSOME_ASSEMBLY	21	1.67	0.04
MICROTUBULE_ORGANIZING_CENTER_PART	18	1.66	0.05
GENE_SILENCING	10	1.65	0.05
HEME_BIOSYNTHETIC_PROCESS	9	1.65	0.05
CELLULAR_LIPID_CATABOLIC_PROCESS	32	1.65	0.05
DNA_REPLICATION_INITIATION	14	1.65	0.05
RNA_3END_PROCESSING	9	1.65	0.05

CYTOKINE_BIOSYNTHETIC_PROCESS	37	-1.88	0.05
POSITIVE_REGULATION_OF_DNA_METABOLIC_PROCESS	11	-1.89	0.05
OXIDOREDUCTASE_ACTIVITY_GO_0016706	10	-1.93	0.04

Table 9.18 MicroRNA expression profile in patients with positive response on LTT to CBZ incubated with CBZ

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-mir-711	2.18	2.76E-02	Cytoplasm	microRNA
hsa-miR-4430	-1.005	3.16E-02	Other	microRNA
hsa-miR-637	-1.023	2.60E-02	Cytoplasm	microRNA
hsa-miR-7112-5p	-1.111	1.87E-02	Cytoplasm	mature microRNA
hsa-miR-6840-3p	-1.111	3.05E-02	Cytoplasm	mature microRNA
hsa-miR-27a-3p	-1.171	1.33E-02	Cytoplasm	mature microRNA
hsa-miR-181c-5p	-1.198	2.99E-02	Cytoplasm	mature microRNA
hsa-miR-6861-5p	-1.257	2.17E-02	Cytoplasm	mature microRNA
hsa-miR-6847-5p	-1.262	4.24E-03	Cytoplasm	mature microRNA
hsa-miR-3619-5p	-1.311	1.63E-02	Cytoplasm	mature microRNA
hsa-miR-1224-5p	-1.347	1.49E-02	Cytoplasm	mature microRNA
hsa-miR-30c-5p	-1.369	3.68E-02	Cytoplasm	mature microRNA
hsa-miR-29c-3p	-1.421	2.70E-02	Cytoplasm	mature microRNA
hsa-miR-30e-3p	-1.53	4.04E-04	Cytoplasm	mature microRNA
hsa-miR-501-3p	-1.585	3.20E-02	Cytoplasm	mature microRNA
hsa-miR-128-3p	-1.685	2.82E-02	Cytoplasm	mature microRNA
hsa-miR-28-5p	-1.724	3.52E-02	Cytoplasm	mature microRNA
hsa-miR-6723-5p	-1.812	9.05E-03	Cytoplasm	mature microRNA
hsa-miR-6124	-1.83	1.71E-02	Other	microRNA
hsa-miR-574-3p	-1.863	2.98E-02	Cytoplasm	mature microRNA
hsa-miR-92b-3p	-1.92	3.19E-03	Cytoplasm	mature microRNA
hsa-miR-30e-5p	-1.99	1.95E-02	Cytoplasm	mature microRNA
hsa-miR-27b-3p	-2.023	5.29E-03	Cytoplasm	mature microRNA
hsa-miR-21-5p	-2.139	1.75E-02	Cytoplasm	mature microRNA
hsa-let-7d-3p	-2.221	2.49E-02	Cytoplasm	mature microRNA
hsa-miR-199a-3p	-2.36	3.24E-02	Cytoplasm	mature microRNA
hsa-miR-199b-3p	-2.36	3.24E-02	Cytoplasm	mature microRNA

Table 9.19 MicroRNA expression profile in patients with positive response on LTT to CBZ and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-6887-5p	1.065	3.05E-02	Cytoplasm	mature microRNA
hsa-miR-6840-3p	-1.01	4.61E-02	Cytoplasm	mature microRNA
hsa-miR-4788	-1.072	5.63E-03	Cytoplasm	microRNA
hsa-miR-6847-5p	-1.109	9.64E-03	Cytoplasm	mature microRNA
hsa-miR-30e-3p	-1.166	3.40E-03	Cytoplasm	mature microRNA
hsa-miR-6768-5p	-1.288	3.79E-02	Cytoplasm	mature microRNA
hsa-miR-29c-3p	-1.301	4.02E-02	Cytoplasm	mature microRNA
hsa-miR-363-3p	-1.431	4.17E-02	Cytoplasm	mature microRNA
hsa-miR-128-3p	-1.478	4.99E-02	Cytoplasm	mature microRNA
hsa-miR-1224-5p	-1.516	7.48E-03	Cytoplasm	mature microRNA
hsa-miR-6861-5p	-1.568	6.16E-03	Cytoplasm	mature microRNA
hsa-miR-152-3p	-1.61	3.53E-02	Cytoplasm	mature microRNA
hsa-miR-501-3p	-1.763	1.91E-02	Cytoplasm	mature microRNA

Table 9.20 MicroRNA expression profile in subjects with negative response to LTT with CBZ and incubated with CBZ

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-455-3p	1.779	4.58E-02	Cytoplasm	mature microRNA
hsa-miR-8060	1.549	6.84E-03	Other	microRNA
hsa-miR-4717-3p	1.309	2.39E-03	Cytoplasm	mature microRNA
hsa-miR-6815-5p	-1.040	1.30E-02	Cytoplasm	mature microRNA
hsa-miR-1292-5p	-1.054	1.49E-02	Cytoplasm	mature microRNA
hsa-miR-7112-5p	-1.085	2.10E-02	Cytoplasm	mature microRNA
hsa-miR-433-3p	-1.087	1.96E-02	Cytoplasm	mature microRNA
hsa-miR-6829-5p	-1.126	8.97E-03	Cytoplasm	mature microRNA
hsa-miR-212-3p	-1.199	3.51E-03	Cytoplasm	mature microRNA
hsa-miR-30e-3p	-1.232	2.30E-03	Cytoplasm	mature microRNA
hsa-miR-675-5p	-1.367	1.07E-02	Cytoplasm	mature microRNA
hsa-miR-128-3p	-1.524	4.40E-02	Cytoplasm	mature microRNA
hsa-miR-7111-5p	-1.786	4.69E-02	Cytoplasm	mature microRNA
hsa-miR-642a-3p	-1.800	6.31E-04	Cytoplasm	mature microRNA

Table 9.21 MicroRNA expression profile in subjects with negative response to LTT with CBZ and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-6732-5p	1.251	3.15E-03	Cytoplasm	mature microRNA
hsa-miR-4499	1.002	1.70E-02	Cytoplasm	microRNA
hsa-miR-6772-5p	-1.069	7.21E-04	Cytoplasm	mature microRNA
hsa-mir-142	-1.078	2.76E-02	Cytoplasm	microRNA
hsa-miR-211-3p	-1.089	1.81E-03	Other	mature microRNA
hsa-miR-675-5p	-1.091	3.39E-02	Cytoplasm	mature microRNA
SNORD126	-1.096	2.99E-03	Other	other
hsa-miR-182-5p	-1.181	7.06E-03	Cytoplasm	mature microRNA
hsa-miR-193b-5p	-1.201	8.92E-03	Cytoplasm	mature microRNA
hsa-miR-30a-5p	-1.244	5.74E-03	Cytoplasm	mature microRNA
hsa-miR-7112-5p	-1.254	9.52E-03	Cytoplasm	mature microRNA
hsa-miR-6829-5p	-1.3	3.54E-03	Cytoplasm	mature microRNA
hsa-miR-8073	-1.321	3.70E-02	Other	microRNA
hsa-miR-25-5p	-1.338	4.33E-02	Cytoplasm	mature microRNA
hsa-miR-29c-3p	-1.372	3.18E-02	Cytoplasm	mature microRNA
hsa-mir-484	-1.449	4.62E-02	Cytoplasm	microRNA
hsa-miR-2392	-1.465	4.06E-02	Cytoplasm	microRNA
hsa-miR-941	-1.473	2.73E-02	Cytoplasm	mature microRNA
hsa-miR-193b-3p	-1.531	1.96E-02	Cytoplasm	mature microRNA
hsa-miR-6795-5p	-1.554	1.17E-02	Cytoplasm	mature microRNA
hsa-miR-363-3p	-1.559	2.85E-02	Cytoplasm	mature microRNA
hsa-miR-874-3p	-1.628	2.60E-02	Cytoplasm	mature microRNA
hsa-miR-1307-3p	-1.728	3.39E-02	Cytoplasm	mature microRNA
hsa-miR-484	-1.785	2.00E-02	Cytoplasm	microRNA
hsa-miR-130a-3p	-1.795	2.49E-03	Cytoplasm	mature microRNA
hsa-miR-128-3p	-1.808	2.00E-02	Cytoplasm	mature microRNA
hsa-miR-1231	-1.813	4.35E-02	Cytoplasm	microRNA
hsa-miR-1247-3p	-1.886	1.43E-02	Other	mature microRNA
hsa-miR-30e-3p	-2.206	1.11E-05	Cytoplasm	mature microRNA
hsa-miR-3651	-2.22	1.53E-02	Cytoplasm	microRNA
hsa-miR-1909-3p	-2.28	2.91E-02	Cytoplasm	mature microRNA
hsa-miR-7977	-2.289	1.31E-02	Other	microRNA
hsa-miR-1260b	-2.456	1.70E-03	Cytoplasm	microRNA
hsa-miR-7641	-3.421	1.42E-02	Cytoplasm	mature microRNA

Table 9.22 MicroRNA expression profile in patients with positive response on LTT to CBZE incubated with CBZE

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-28-3p	1.665	4.77E-02	Cytoplasm	mature microRNA
hsa-miR-505-5p	1.638	2.17E-03	Cytoplasm	mature microRNA
hsa-miR-6782-5p	1.631	2.23E-02	Cytoplasm	mature microRNA
hsa-miR-200c-3p	1.482	4.91E-02	Cytoplasm	mature microRNA
hsa-miR-6124	1.480	4.62E-02	Other	microRNA
hsa-miR-3648	1.429	4.78E-02	Cytoplasm	mature microRNA
hsa-miR-486-3p	1.365	4.05E-02	Cytoplasm	mature microRNA
hsa-miR-6785-5p	1.292	9.74E-03	Cytoplasm	mature microRNA
hsa-miR-744-5p	1.008	3.59E-02	Cytoplasm	mature microRNA
hsa-miR-6780a-5p	-1.012	1.10E-02	Cytoplasm	mature microRNA
hsa-miR-1224-3p	-1.089	5.15E-03	Cytoplasm	mature microRNA
hsa-miR-4750-3p	-1.579	1.98E-02	Cytoplasm	mature microRNA
hsa-miR-1281	-1.777	2.38E-02	Cytoplasm	microRNA

Table 9.23 MicroRNA expression profile in patients with positive response on LTT to CBZE and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-150-3p	2.452	1.63E-03	Cytoplasm	mature microRNA
hsa-miR-4651	2.356	3.29E-02	Cytoplasm	microRNA
hsa-miR-1307-3p	2.280	7.82E-03	Cytoplasm	mature microRNA
hsa-miR-339-3p	1.967	2.36E-02	Cytoplasm	mature microRNA
hsa-miR-671-5p	1.903	4.09E-03	Cytoplasm	mature microRNA
hsa-miR-3188	1.796	4.39E-02	Cytoplasm	microRNA
hsa-miR-4734	1.756	4.86E-02	Cytoplasm	microRNA
hsa-miR-92a-1-5p	1.744	3.19E-02	Cytoplasm	mature microRNA
hsa-miR-28-3p	1.733	4.04E-02	Cytoplasm	mature microRNA
hsa-miR-6821-5p	1.691	1.79E-02	Cytoplasm	mature microRNA
hsa-miR-6724-5p	1.685	4.05E-02	Cytoplasm	mature microRNA
hsa-miR-4758-5p	1.670	2.22E-02	Cytoplasm	mature microRNA
hsa-miR-505-5p	1.661	1.96E-03	Cytoplasm	mature microRNA
hsa-miR-25-5p	1.650	1.60E-02	Cytoplasm	mature microRNA
hsa-miR-6789-5p	1.647	4.70E-02	Cytoplasm	mature microRNA
hsa-mir-3656	1.612	1.42E-02	Cytoplasm	microRNA
hsa-miR-642a-3p	1.572	1.86E-03	Cytoplasm	mature microRNA
hsa-miR-3648	1.571	3.18E-02	Cytoplasm	mature microRNA
hsa-miR-200c-3p	1.537	4.23E-02	Cytoplasm	mature microRNA
hsa-miR-6785-5p	1.442	4.90E-03	Cytoplasm	mature microRNA
hsa-miR-6850-5p	1.422	2.17E-02	Cytoplasm	mature microRNA
hsa-miR-378a-3p	1.419	3.76E-02	Cytoplasm	mature microRNA
hsa-miR-6723-5p	1.293	4.85E-02	Cytoplasm	mature microRNA

hsa-miR-6510-5p	1.197	4.94E-02	Cytoplasm	mature microRNA
hsa-miR-3679-5p	1.193	2.78E-02	Cytoplasm	mature microRNA
hsa-miR-423-3p	1.169	4.56E-02	Cytoplasm	mature microRNA
hsa-miR-744-5p	1.141	1.99E-02	Cytoplasm	mature microRNA
hsa-miR-4667-5p	1.101	8.69E-04	Cytoplasm	mature microRNA
hsa-mir-4537	1.095	2.30E-02	Cytoplasm	microRNA
hsa-miR-181b-5p	1.020	2.56E-02	Cytoplasm	mature microRNA
hsa-miR-7855-5p	-1.056	2.51E-02	Cytoplasm	mature microRNA
hsa-miR-6790-3p	-1.082	7.47E-03	Cytoplasm	mature microRNA
hsa-mir-320e	-1.100	4.59E-02	Cytoplasm	microRNA
hsa-let-7f-5p	-1.241	3.98E-02	Cytoplasm	mature microRNA
hsa-mir-6776	-1.295	2.53E-02	Other	microRNA
hsa-miR-378h	-1.472	2.83E-02	Cytoplasm	microRNA
hsa-miR-4706	-1.769	1.39E-02	Cytoplasm	microRNA
hsa-miR-4722-3p	-2.080	2.34E-02	Cytoplasm	mature microRNA
hsa-miR-3613-5p	-2.840	2.70E-02	Cytoplasm	mature microRNA
hsa-miR-4720-5p	-3.936	2.14E-02	Cytoplasm	mature microRNA

Table 9.24 MicroRNA expression profile in subjects with negative response to LTT with CBZE and incubated with CBZE

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-miR-548a-3p	3.052	2.44E-04	Cytoplasm	mature microRNA
hsa-miR-4433-3p	2.726	9.12E-04	Cytoplasm	mature microRNA
hsa-miR-6831-5p	2.707	5.84E-03	Cytoplasm	mature microRNA
hsa-miR-328-3p	2.508	4.75E-02	Cytoplasm	mature microRNA
hsa-miR-130b-3p	2.496	3.05E-02	Cytoplasm	mature microRNA
hsa-miR-197-5p	2.174	1.08E-02	Cytoplasm	mature microRNA
hsa-miR-4758-5p	2.064	4.12E-02	Cytoplasm	mature microRNA
hsa-miR-532-3p	1.921	1.55E-02	Cytoplasm	mature microRNA
hsa-miR-18a-5p	1.727	4.75E-02	Cytoplasm	mature microRNA
hsa-miR-378c	1.667	7.12E-03	Cytoplasm	microRNA
hsa-miR-130a-3p	1.563	3.98E-02	Cytoplasm	mature microRNA
hsa-miR-6133	1.476	3.87E-02	Other	microRNA
hsa-miR-6769a-5p	1.405	3.07E-02	Cytoplasm	mature microRNA
hsa-miR-6076	1.264	4.67E-02	Other	microRNA
hsa-miR-3154	1.207	2.95E-02	Cytoplasm	microRNA
hsa-mir-4454	1.039	4.69E-03	Cytoplasm	microRNA
hsa-miR-1976	1.013	1.09E-02	Cytoplasm	microRNA
hsa-mir-4648	-1.013	3.37E-02	Cytoplasm	microRNA
hsa-miR-4783-3p	-1.033	2.01E-02	Cytoplasm	mature microRNA
hsa-miR-6753-3p	-1.035	4.82E-02	Cytoplasm	mature microRNA
hsa-mir-4296	-1.047	7.87E-03	Cytoplasm	microRNA
hsa-miR-6784-3p	-1.080	5.62E-03	Cytoplasm	mature microRNA

hsa-miR-3194-5p	-1.106	9.34E-03	Cytoplasm	mature microRNA
hsa-miR-6735-5p	-1.114	2.82E-02	Cytoplasm	mature microRNA
hsa-miR-6890-5p	-1.126	3.60E-02	Cytoplasm	mature microRNA
hsa-miR-6746-3p	-1.148	2.09E-02	Cytoplasm	mature microRNA
hsa-mir-6887	-1.315	1.45E-02	Other	microRNA
hsa-miR-1273h-5p	-1.491	1.38E-02	Cytoplasm	mature microRNA
hsa-miR-181c-5p	-1.558	4.32E-02	Cytoplasm	mature microRNA
hsa-miR-4462	-2.244	1.64E-02	Cytoplasm	microRNA
hsa-miR-6782-5p	-2.683	9.82E-03	Cytoplasm	mature microRNA

Table 9.25 MicroRNA expression profile in subjects with negative response to LTT with CBZE and incubated with tetanus toxoid

ID	Exp Log Ratio	Exp p-value	Location	Type(s)
hsa-let-7d-3p	3.622	1.17E-02	Cytoplasm	mature microRNA
hsa-miR-328-3p	2.786	3.01E-02	Cytoplasm	mature microRNA
hsa-miR-18a-5p	2.494	7.30E-03	Cytoplasm	mature microRNA
hsa-miR-8089	2.088	4.92E-02	Other	microRNA
hsa-miR-4668-5p	2.072	3.08E-02	Cytoplasm	mature microRNA
hsa-miR-130a-3p	1.767	2.26E-02	Cytoplasm	mature microRNA
hsa-miR-30e-3p	1.657	3.29E-03	Cytoplasm	mature microRNA
hsa-miR-422a	1.566	1.37E-02	Cytoplasm	microRNA
hsa-miR-6133	1.520	3.40E-02	Other	microRNA
hsa-miR-6769a-5p	1.466	2.51E-02	Cytoplasm	mature microRNA
hsa-miR-3147	1.395	4.68E-02	Cytoplasm	microRNA
hsa-miR-4265	1.342	1.93E-03	Cytoplasm	microRNA
hsa-miR-4667-3p	1.216	2.48E-03	Cytoplasm	mature microRNA
hsa-miR-4433-5p	1.158	1.01E-02	Other	mature microRNA
hsa-miR-4767	1.156	1.20E-02	Cytoplasm	microRNA
hsa-miR-380-3p	1.032	4.76E-03	Cytoplasm	mature microRNA
hsa-miR-4687-5p	1.000	1.38E-02	Cytoplasm	mature microRNA
hsa-mir-18b	-1.006	9.45E-03	Cytoplasm	microRNA
hsa-mir-4474	-1.055	1.26E-03	Cytoplasm	microRNA
hsa-miR-127-3p	-1.266	3.30E-02	Cytoplasm	mature microRNA
hsa-miR-6730-3p	-1.296	1.81E-03	Cytoplasm	mature microRNA
hsa-miR-877-5p	-1.356	2.34E-02	Cytoplasm	mature microRNA
hsa-miR-1273h-5p	-1.410	1.86E-02	Cytoplasm	mature microRNA
hsa-miR-1268a	-1.526	4.42E-02	Cytoplasm	microRNA
hsa-mir-4417	-1.584	1.48E-02	Cytoplasm	microRNA
hsa-miR-181c-5p	-1.771	2.42E-02	Cytoplasm	mature microRNA
hsa-miR-3162-5p	-1.984	1.36E-02	Cytoplasm	mature microRNA
hsa-miR-8071	-2.622	1.55E-02	Cytoplasm	mature microRNA

Table 9.26 CBZ/CBZE-specific miRNA identified by comparison analyses in IPA

miRNA	Exp Log Ratio	Exp P-Val	Drug
hsa-miR-548f-3p	3.052	2.44E-04	CBZE
hsa-miR-711	2.184	2.76E-02	CBZ
hsa-miR-197-5p	2.174	1.08E-02	CBZE
hsa-miR-532-3p	1.921	1.55E-02	CBZE
hsa-miR-455-3p	1.779	4.58E-02	CBZ
hsa-MIR378C	1.667	7.12E-03	CBZE
hsa-miR-6782-5p	1.631	2.23E-02	CBZE
hsa-MIR8060	1.549	6.84E-03	CBZ
hsa-miR-486-3p	1.365	4.05E-02	CBZE
hsa-miR-4717-3p	1.309	2.39E-03	CBZ
hsa-MIR6076	1.264	4.67E-02	CBZE
hsa-miR-3154	1.207	2.95E-02	CBZE
hsa-MIR4454	1.039	4.69E-03	CBZE
hsa-miR-1976	1.013	1.09E-02	CBZE
hsa-miR-4430	-1.005	3.16E-02	CBZ
hsa-mir-4648	-1.013	3.37E-02	CBZE
hsa-miR-637	-1.023	2.60E-02	CBZ
hsa-miR-4783-3p	-1.033	2.01E-02	CBZE
hsa-miR-5106	-1.035	4.82E-02	CBZE
hsa-miR-6815-5p	-1.040	1.30E-02	CBZ
hsa-mir-4296	-1.047	7.87E-03	CBZE
hsa-miR-1247-3p	-1.054	1.49E-02	CBZ
hsa-miR-6862-3p	-1.08	5.62E-03	CBZE
hsa-miR-433-3p	-1.087	1.96E-02	CBZ
hsa-miR-1224-3p	-1.089	5.15E-03	CBZE
hsa-miR-3194-5p	-1.106	9.34E-03	CBZE
hsa-miR-4436b-3p	-1.114	2.82E-02	CBZE
hsa-miR-6890-5p	-1.126	3.60E-02	CBZE
hsa-miR-6746-3p	-1.148	2.09E-02	CBZE
hsa-miR-132-3p	-1.199	3.51E-03	CBZ
hsa-miR-214-3p	-1.311	1.63E-02	CBZ
hsa-mir-6887	-1.315	1.45E-02	CBZE
hsa-miR-345-5p	-1.579	1.98E-02	CBZE
hsa-miR-708-5p	-1.724	3.52E-02	CBZ
hsa-miR-1281	-1.777	2.38E-02	CBZE
hsa-miR-4723-5p	-1.786	4.69E-02	CBZ
hsa-miR-6124	-1.830	1.71E-02	CBZ
hsa-miR-574-3p	-1.863	2.98E-02	CBZ
hsa-miR-27a-3p	-2.023	5.29E-03	CBZ
hsa-miR-21-5p	-2.139	1.75E-02	CBZ
hsa-miR-4462	-2.244	1.64E-02	CBZE
hsa-miR-199a-3p	-2.360	3.24E-02	CBZ

Table 9.27 CBZ/CBZE-specific miRNA normalised expression levels (normalised according to HSA-miR-92a-3p)

	Hsa-miR-433-3p	Hsa-miR-3194-5p	Hsa-miR-455-3p	Hsa-miR-4723-5p	Hsa-miR-345-5p
RLH001 CBZ 0	0.147	0.081	0.460	0.087	0.157
RLH001 CBZ 25	0.095	0.126	0.584	0.094	0.156
RLH001 CBZ TT	0.062	0.094	0.555	0.084	0.213
RLH002 CBZ 0	0.160	0.130	0.448	0.073	0.242
RLH002 CBZ 25	0.070	0.074	0.630	0.051	0.142
RLH002 CBZ TT	0.132	0.152	0.630	0.078	0.195
RLH007 CBZ 0	0.098	0.078	0.619	0.069	0.211
RLH007 CBZ 25	0.040	0.078	0.591	0.096	0.118
RLH007 CBZ TT	0.111	0.096	0.522	0.100	0.241
RLH007 CBZE 0	0.074	0.113	0.533	0.067	0.175
RLH007 CBZE 25	0.069	0.142	0.461	0.119	0.144
RLH007 CBZE TT	0.073	0.085	0.533	0.109	0.212
RLH010 CBZE 0	0.120	0.157	0.606	0.094	0.103
RLH010 CBZE 25	0.076	0.061	0.720	0.114	0.143
RLH010 CBZE TT	0.106	0.097	0.579	0.074	0.199
WAL004 CBZ 0	0.186	0.117	0.458	0.083	0.363
WAL004 CBZ 50	0.173	0.106	0.601	0.046	0.236
WAL004 CBZ TT	0.105	0.117	0.584	0.103	0.348
WAL004 CBZE 0	0.063	0.113	0.755	0.067	0.089
WAL004 CBZE 25	0.097	0.086	0.585	0.075	0.331
WAL004 CBZE TT	0.187	0.137	0.597	0.055	0.181