UTILITY OF DRIED BLOOD SPOTS AS MATRICES FOR PHARMACOKINETIC STUDY AND METABOLOMIC PROFILING OF HUMAN DISEASE: THE EPILEPSY EXPERIENCE

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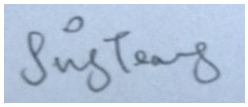
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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THESIS SUMMARY

Drug resistant epilepsy (DRE) is a neurological complication which affects approximately 20 - 40% of epilepsy subjects, causing their overall lower quality of life. Thus, it represents a condition which requires further understanding of its pathogenesis and optimization of its drug treatment for better management of the disease. In this regard, dried blood spot (DBS) appears as an attractive matrix for the study of pathogenesis of a disease as it can be conveniently collected from patients in a large population scale, and DBS can be utilized for selected biomarker profiling or drug quantitation assays. Up to date, there are only few but increasing numbers of studies in recent years on using DBS for metabolomic profiling and clinical monitoring of drug levels. In light of these, I endeavour to explore the application of DBS on epilepsy research in 2 main objectives in this thesis: (1) to investigate the feasibility of applying DBS in profiling epileptogenesis; and (2) to use DBS as matrices for drug monitoring.

For the first objective, DRE prevalence study to determine the severity and relevant risk factors was firstly carried out, followed by investigation of the reliability of DBS in metabolomic study. Using finger prick DBS samples, it was observed that the metabolomic profiles of drug responsive epilepsy subjects were distinguishable from those who were drug resistant ($Q^2 = 0.71$). From the metabolite profiles obtained through gas chromatography mass spectrometry (GC-MS), several metabolites were revealed to have different degree of abundance between these 2 groups of subjects. The metabolites were putatively identified as glutamine, aspartic acid, pyruvic acid, caprylic acid, serine, palmitic acid and oxalic acid.

For the second objective, clinical validation and population pharmacokinetic modeling using DBS derived concentrations (C_{dbs}) were conducted to examine their applicability. After the inclusion of individual hematocrit levels and antiepileptic drug (AED) red blood cell:plasma partition

ratio, AED concentrations derived using the newly developed DBS assay could accurately predict its plasma concentrations which were measured from plasma immunoassays (within 95% confidence interval of the means of two methods). To further enhance the clinical applicability of C_{dbs}, clearance of one of the AEDs was estimated through a population approach.

Findings from this thesis were to establish the utility of DBS for future larger or population-based clinical research, where sample acquisition can be done by epilepsy subjects at home. As evidenced by the comparable results between DBS and plasma, DBS could be a suitable matrix for both aspects of disease and drug monitoring. Once the relevant biomarkers of epilepsy are established, simultaneous monitoring along with AED concentrations would become feasible. This, in turn, will aid the management of epilepsy subjects by rendering monitoring of disease as well drug levels, just from one dried blood spot.

LIST OF PUBLICATION

Publication derived from this thesis:

1. Kong ST, Lin HS, Ching J, Ho PC. Evaluation of dried blood spots (DBS) as sample matrix for gas chromatography – mass spectrometry (GC-MS) based metabolomic profiling. *Analytical Chemistry* 2011;83:4313-4318

2. Kong ST, Lim SH, Chan E, Ho PC. Estimation and comparison of carbamazepine population pharmacokinetics using dried blood spot and plasma concentrations from people with epilepsy: the clinical implication. *The Journal of Clinical Pharmacology* 2014;54(2):225-233

3. Kong ST, Ho CS, Ho PC, Lim SH. Prevalence of drug resistant epilepsy in adult population in Singapore. *(manuscript submitted)*

4. Kong ST, Lee WB, Pasikanthi KK, Wang HYS, Ng YLS, Lim WHJ, Wong PS, Ho PC, Lim SH. Clinical validation of dried blood spot sampling for quantitation of carbamazepine, valproic acid and phenytoin using gas-chromatography mass spectrometry. *(manuscript submitted)*

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Co-author of other publication:

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2. Lee XL*, Kong ST*, Chan WSD, Chan EW, Tan WW, Ho PC. Developing a normogram for dose individualization of phenytoin in asian pediatric patients derived from population pharmacokinetic modelling of saturable pharmacokinetic profiles of the drug. *Therapeutic Drug Monitoring* 2013;35:54-62 (**equal contribution)*

3. Tan WW, Kong ST, Chan DW, Ho PC. A retrospective study on the usage of antiepileptic drugs in Asian children from 2000 to 2009 in the largest pediatric hospital in Singapore. *Pharmacoepidemiology and Drug Safety* 2012;21(10):1074-1080

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1. Kong ST, Lee WB, Pasikanthi KK, Wang HYS, Ng YLS, Lim WHJ, Wong PS, Ho PC, Lim SH. Comparison of dried blood spots and plasma valproic acid and carbamazepine levels. 10th European Congress on Epileptology, ICC London ExCeL, London, United Kingdom. 30 Sep – 4 Oct 2013

2. Kong ST, Lin HS, Ching J, Ho PC. Evaluation of dried blood spots (DBS) as sample matrix for gas chromatography – mass spectrometry (GC-MS) based metabolomic profiling. 6th PharmSci@Asia 2011 Symposium, Nanjing University, Nanjing, China. 25 – 26 May 2011

3. Lee XL, Kong ST, Chan Eli, Ho PC. Study of the saturable pharmacokinetic profiles of phenytoin in Asian paediatric patients in the presence or absence of co-administered drugs. 7th ANSC Scientific Symposium National University of Singapore, Singapore. 6 April 2011

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LIST OF ABBREVIATION

ADR	Adverse Drug Reactions
AED	Anti-epileptic Drug
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
Asp	Aspartate/Aspartic acid
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BDNF	Brain-derived Neurotrophic Factor
BFNC	Benign Familial Neonatal Convulsion s
BRE	Benign Rolandic Epilepsy
CBZ	Carbamazepine
C_{dbs}	Dried Blood Spot Concentration
CI	Confidence Interval
CL	Clearance
CLB	Clobazam
CMC	Comparative Medicine Center
C_{plasma}	Plasma Concentration
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CYP	Cytochrome
DBS	Dried Blood Spot
DD	Total Daily Dose per Unit Weight
DDD	Defined Daily Dose
DPS	Dried Plasma Spot
DRE	Drug Resistant Epilepsy
ECF	Extracellular Fluid
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
EI	Electron Impact
ESI	Electrospray Ionization
ESM	Ethosuximide
F	Bioavailability
GABA	γ-Aminobutyric acid
GC-MS	Gas Chromatography Mass Spectrometry
GEFS+	Generalized Epilepsy with Febrile Seizure plus
GGT	Gamma-glutamyltransferase
Gln	Glutamine
Glu	Glutamate/Glutamic acid
Hct	Hematocrit
HMDB	Human Metabolome Database
ICH	International Conference Harmonization
IEM	Inborn Error of Metabolism
IGE	Idiopathic Generalized Epilepsy
ILAE	International League against Epilepsy
IS	Infantile Spasm
JME	Juvenile Myoclonic Epilepsy
k_a	Absorption Constant

LC-MS	Liquid Chromatography Mass Spectrometry
LEV	Levetiracetam
LTG	Lamotrigine
MAE	Mean Absolute Error
Mb	Metabolomic
MCT	Medium Chain Triglyceride
ME	Mean Prediction Error
MetPA	Metabolomics Pathway Analysis
MPP	Mass Profiler Professional
MRI	Magnetic Resonance Imaging
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTS	Mesial Temporal Sclerosis
NIST	National Institute of Standards and Technology
NMDA	*N*-methyl *D*-aspartate
NMR	Nuclear Magnetic Resonance
NOAE	New-onset Absence Epilepsy
NUS	National University of Singapore
OBJ	Objective Function Value
OPLS-DA	Orthogonal Partial Least Square Discriminat Analysis
OR	Odds Ratio
P5P	Pyridoxal- 5-phosphate
PC	Principal Component
PCA	Principal Component Analysis
PD	Pharmacodynamic
Pgp	P-glyprotein
PHB	Phenobarbital
PHT	Phenytoin
PLS	Partial Least Square
PLS-DA	Partial Least Square Discriminat Analysis
PNPO	Pyridox(am)ine 5'-Phosphate Oxidase
PPK	Population Pharmacokinetic
PWE	People with Epilepsy
RBC	Red Blood Cell
R/P	Red Blood Cell to Plasma ratio
RMSE	Root Mean Squared Error
SER	Spontaneously Epileptic Rat
SPSS	Statistical Package for Social Sciences
TDM	Therapeutic Drug Monitoring
TGB	Tiagabine
TIC	Total Ion Chromatogram
TMCS	Trimethylchlorosilane
TPM	Topiramate
UPLC-oaToFMS	Ultra Performance Liquid chromatography and orthogonal acceleration time-of-flight mass spectrometry
V_d	Apparent Volume of Distribution
VGB	Vigabatrin
VIP	Variable Importance Plot
VPA	Valproic Acid

CHAPTER 1

Thesis Introduction

CHAPTER 1. THESIS INTRODUCTION

In this chapter, epilepsy and dried blood spot (DBS) will be concisely reviewed. Firstly, an overview of epilepsy and current knowledge on epileptogenesis will be discussed. Secondly, the role of currently available antiepileptic drugs (AED) in the treatment and metabolomics in the understanding of epilepsy will be evaluated. Thirdly, the significance of drug resistant epilepsy (DRE), associated risk factors, current hypothesized mechanisms of DRE and some prominent complications will be highlighted. This is followed by the utilities of DBS in clinical research and the advantages of DBS as sample matrices.

1.1 EPILEPSY

1.1.1 OVERVIEW OF EPILEPSY

Epilepsy is one of the most common chronic neurological disorders; and the onset of which is independent of age. It is characterized by recurrent or spontaneous seizures unprovoked by any immediately identifiable cause (1).

Seizure arises due to the imbalance in excitatory and inhibitory discharges from the neurons in the brain. It is defined as abnormal or excessive electrochemical activity in one part (focal) or a more diffuse origin (generalized) from the brain, which may manifest as changes in motor activity, sensation, behaviour, emotion or consciousness or a combination of these symptoms. Seizures that subside after the primary condition, e.g. alcohol withdrawal, has resolved are not considered to be epilepsy. In view of the complexity of its origin, epilepsy is observed to be a symptom of an underlying neurological disorder rather than as a disease per se (2).

The incidence of epilepsy in the United Kingdom (UK) is about 50 per 100,000 per annum, as surveyed in a study published in the year 2000 (3). The incidence is 25% in patients less than 15 years old, decreases in adulthood (51% for age ranging from 16 to 59 years) and rises again to 24% in patients older than 60 years (4). The prevalence for active epilepsy in the UK is 5-10 cases per 1,000 while in Singaporean men, the lifetime prevalence rate is about 4.9 cases per 1,000 (5). The annual incidence rate of childhood-onset epilepsy in Singapore is approximately 24 per 100,000 (6).

The diagnosis of epilepsy is clinically determined and comprised of seizure type, epilepsy syndrome and the original cause. Primarily, epilepsy is diagnosed based on the detailed description given by the patient and/or witness on the events before, during and after a seizure attack. This is then supplemented by findings from the electroencephalogram (EEG), magnetic resonance imaging (MRI) and computed tomography (CT) of the patient with known or suspected epilepsy. The classification of seizure type, however, is more controversial and depends on the (7):

1. Parts of the brain affected
2. Pattern of spread of epileptic discharges through the brain
3. Cause of epilepsy and
4. Age of the individual

The decision to treat has to be made after deliberate consideration in balancing the likelihood of further seizure attacks with the risk of adverse effects from the AEDs. According to established clinical guidelines, treatment should not be offered after a single seizure, unless the patient is determined to be at high risk for further seizures (2, 8, 9).

1.1.2 EPILEPTOGENESIS

Each type of epilepsy has its own set of cause and mechanism. Epileptogenesis is coined with reference to the developmental of changes from normal brain activity to repeated spontaneous seizures (10). Experts believe that epileptogenesis also constitutes pathway alterations responsible for pharmacoresistance (11). Current pharmacotherapy choices are mainly preventing symptomatic seizure expressions but do not interfere with generation or progression of epilepsy itself. Therefore, a complete understanding of epileptogenesis is believed to have significant clinical relevance in understanding as well as identifying new targets for intervention.

The revised terminologies for standardized description of etiology for epilepsy are divided into three; genetic, structural-metabolic and unknown (12). The etiology provides some insight into the epileptogenesis of the particular patient. Epilepsy of genetic etiology is considered for cases where seizures arise as a direct consequence of genetic defect such as juvenile myoclonic epilepsy (JME), channelopathies and neonatal epileptic encephalopathy caused by pyridox(am)ine 5'-phosphate oxidase (PNPO) gene mutation (12). Sometimes, genetic and protein expression abnormalities are linked (13). Structural-metabolic etiology encompasses structural or metabolic disorder of any parts of the brain (12). Some examples include cortical malformation, mesial temporal sclerosis and brain tumour. When the cause is unidentified, and could be either genetic or structural-metabolic, it will be classified as unknown etiology (12).

In recent years, several genes have been identified to be responsible in epileptogenesis. These genes encode for various receptors located at the surface of the neurons, ions channels and metabolism pathway. However, locus heterogeneity and variation in expressivity of the mutated genes have complicated our understanding on the influence of genetic mutations in epilepsy. Mutations in the SCN1A, SCN2A, SCN1B and GABRG2 genes

have been suggested to cause generalized epilepsy with febrile seizure plus (GEFS+), illustrating the problem of locus heterogeneity of the genes involved in epileptogenesis (14-17). On the other hand, a single gene mutation could result in different epilepsy phenotypes, reflecting variation in expressivity. For instance, mutations in SCN1A gene is linked to temporal lobe epilepsies, typical febrile seizures, febrile seizures plus and severe myoclonic epilepsy in infancy (18-22).

In benign familial neonatal convulsions (BFNC), single gene mutation at chromosome 20q13.3 and 8q24 is believed to be the cause. These mutations lead to loss of function of KCNQ2- and KCNQ3-voltage–gated potassium subunit channels, respectively. These channels are responsible for defying sustained depolarization and repetitive firing (23, 24). Loss of these channels, or a reduction of 20-25% of their functions, allows continuous depolarization that result in seizure (24). The patients could either have generalized tonic-clonic or focal seizures. Interestingly, BNFC is a syndrome that only appears in the first month of life without recurrence. It is speculated to result from a change in potassium channels expression genes during the brain development (25). To date, there are more than 80 subunit genes that encode for more than 24 major classes of potassium channels (23). Defects in one or two genes seemed to be replaceable by the functionalities of the others. In short, these findings suggest that there is no straightforward correlation between genotype-phenotype in epileptogenesis. It probably takes a certain combination of gene defects to contribute to different phenotypic characteristics of epilepsies (26).

Aside from genetic-related epileptogenesis, there is also environmental-acquired epileptogenesis. In structural-metabolic epilepsy such as brain trauma, stroke and brain infection, epilepsy occurs in a chronic way. In these cases, there is a fixed structural damage but only intermittent occurrences of seizures. Researchers hypothesized that the intact synaptic inhibition is enough to suppress the effect of persistent excitatory transmissions for most of the time (27). During acute epileptogenesis, it is

proposed that there is transient decrease in inhibitory synaptic transmission by γ-aminobutyric acid (GABA) and an increase in excitatory transmission by 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)-propanoic acid (AMPA) and *N*-methyl *D*-aspartate (NMDA) glutamate receptors, leading to sustained depolarizing potentials (28). In epilepsy patients, their seizure thresholds tend to be lower whereby a small increase in excitatory or decrease in inhibitory transmission is adequate to trigger seizure. Acute neuronal loss alone does not suffice to cause acute seizure (28), as seizure is likely to arise from a collective imbalances in neuronal transmission at the metabolite level. Therefore, it seems that epileptogenesis from various origins do converge at certain point and a closer look at the neurotransmitter expressions may provide a uniform explanation.

Generally, system biology begins with genes, which are translated into proteins whose function leads to the production of various sets of metabolites. In line with this, the genomics is the study of total DNA within a cell or organism. This could be done using DNA microarray where thousands of genes can be analysed simultaneously (29). Moreover, nucleotide polymorphism profiling has a role in pharmacogenomic study of individual responses to drugs. However, there is a lack of consensus surrounding microarray data interpretation .

Proteomics, on the other hand, is the study of all expressed proteins within a cell, tissue and organism with the aim to understand their functional relevance. It holds special place in biomarker discovery because proteins are most likely to be uniquitously affected in disease and disease response (30). Although much information can be gained from proteomics, it is limited by its huge domain size (> 100 000 proteins) and the inability to detect some of the low abundance proteins .

Metabolomics is the study of global metabolite profiles under a given set of condition (31). The metabolome is the final downstream product in a system biology and hence, its expression is amplified relative to transcriptome

and proteome (32). It is the closest to the phenotype of the biological system studied and can distinguish morphologically indistinguishable but phenotypically different condition. Despite being the smallest domain (approximately 5000 metabolites), it contains various biological molecules whose interactions are physically and chemically more complex (31). These complexities allowed metabolomics to reveal more informative observations. However, metabolomics can only provide a snapshot of the disease condition, it does not provide an answer to the cause of the disease. An overview of metabolomics in comparison to genomics and proteomics has been provided by Hollywood et al (33).

Metabolomics is an emerging scientific discipline that look at the association between disease and downstream pathophysiology, i.e. the metabolic profile in tissues and biofluids. The metabolic profile is a semi-quantitative measure of all low molecular weight compounds (e.g. nutrients, hormones, vitamins, neurotransmittors) in a given biological sample and represents fingerprint of the substrates, intermediates and by-products of physiological and pathological activities. As metabolite profiles are greatly influenced by genetic make-up and associated with enviromental factors such as lifestyle and diet, it offers a great opportunity to overcome the shortcomings imposed by genetic and proteomic studies.

The idea of profiling the metabolites in people with epilepsy (PWE) is partly coined from the fact that many hereditary epilepsies arise from inborn error of metabolism (IEM) (34-37). It is believed that any symptoms of altered enzymatic functions remain latent in the child with IEM, until the accumulation of the wrong product due to the defective enzymatic process is sufficient to trigger a seizure. This implies that variation in metabolite profiles have some direct effect in epileptogenesis, making it a possible tool for detecting biomarkers of epilepsy. For instance, in pyridoxal 5-phosphate (P5P)-sensitive seizures which affect infants of a few weeks-old, serum biochemistry analyses showed a metabolite profile of raised levels of glycine,

threonine and 3-methoxytyrosine while cerebrospinal fluid (CSF) biochemistry showed low concentrations of L-dopa, 5-hydroxy-tryptophan and 3-O-methyldopa (13). These metabolite fluctuations are now adopted as biomarkers for the diagnosis of P5P-sensitive seizures. The seizure ceases in neonates after the administration of pyridoxal-5-phosphate, either orally or intravenously. In other IEM cases where only transient clinical symptoms were observed, the function of the defective gene/s is replaced by others as development progresses, highlighting again, the inadequate attribution of genetic and proteomic factors to epileptogenesis.The standard treatment currently is to administer the defective enzymes or the resulting products/metabolites. In some cases, these products will restore their endogenous functions or levels and overall balances the activities in the brain to halt seizure (37). Therefore, it was hypothesized that the findings from metabolomic study would be an important adjunct to contemporary research in epilepsy.

Indeed, as shown by a recent study done in Chinese populations, five metabolism pathways were proposed to be involved in epilepsy (38). When compared to healthy subjects, serum levels of GABA, creatinine, L-threonine and L-tryptophan were lower while the levels of L-glutamate, glycine, glyceric acid, lactic acid, inositol and myristic acid were higher in the epilepsy group. These metabolites, individually (39-45), has been linked to seizure and their work provided the evidence that there is likely a complex network of metabolite disturbances in epilepsy which are different from healthy subjects.

It had been suggested that PWE who died unexpectedly or showed signs of hereditary disease should be thoroughly investigated in post-mortem. Interestingly, experts recommended that DBS should be acquired to aid diagnosis. DBS could be obtained during the first 18 hours after death and preserved indefinitely in liquid nitrogen (46). It is therefore more practical than the collection and storage of CSF or urine, which are the other common biological fluids used for biochemical analysis (34).

1.1.3 ANTI-EPILEPTIC DRUGS

Unlike other classes of drugs where they can be classified according to their pharmacological actions, AEDs are broadly categorized into older and newer generation. Specifically, there is 3 generation of AEDs if based on the period of their first introduction. Often, each AED has more than one mode of action but their detailed mechanisms of actions of AEDs are still largely unknown (47). Table 1 summarizes the current knowledge on AEDs actions in the treatment of seizure.

Table 1. Main mechanism of actions of old and new generation of antiepileptic drugs (adapted from (47))

Antiepileptic Drugs	Blockade of voltage-dependent sodium channels	Increase in brain or synaptic GABA levels	Selective potentiation of $GABA_A$-mediated responses	Direct facilitation of chloride ion influx	Blockade of calcium channels	Other actions
Older generation						
Benzodiazepines	-	-	++	-	-	-
Carbamazepine	++	?	-	-	+	+
Ethosuximide	-	-	-	-	++	-
Phenobarbital	-	+	+	++	?	+
Phenyotin	++	-	-	-	?	+
Valproic Acid	?	+	?	-	+	++
Newer generation						
Felbamate	++	+	+	-	+	+
Gabapentin	?	?	-	-	++	?
Lamotrigine	++	+	-	-	++	+
Levetiracetam	-	?	+	-	+	++
Oxcarbazepine	++	?	-	-	+	+
Pregabalin	-	-	-	-	++	-
Tiagabine	-	++	-	-	-	-
Topiramate	++	+	+	-	+	+
Vigabatrin	-	++	-	-	-	-
Zonisamide	++	?	-	-	++	+

++, primary action; +, secondary action; -, no action described; ?, controversial evidence; GABA, γ-aminobutyric acid

The first generation of AEDs, such as bromide, phenobarbital, phenytoin, primidone and ethosuximide, and second generation of AEDs, such as chlordiazepoxide, diazepam, carbamazepine, valproic acid, clonazepam and clobazam, are collectively referred to as older generation of AEDs. In this report, AEDs group will be loosely referred to as older or newer generation.

The older generation AEDs such as phenytoin, carbamazepine, valproic acid and phenobarbitone are well-established as the first choice of treatment for a variety of epilepsy syndromes. It is believed that employing AEDs with a different mechanism of action than the one ineffective previously or as a combination therapy may prove useful (48). Although a systematic review by Costa *et al*, 2011 suggested that no definitive conclusion can be drawn with regards to which new AEDs offer superiority as compared to the others in the treatment of refractory partial epilepsy (49), this may not be the case when involving both combinations of older and newer generation AEDs. A study specifically designed to answer this research question in a general PWE population is yet to be conducted. This finding may be useful since epilepsy has a long dynamic course of disease; it is likely that physicians will reuse a previously unsuccessful AED after due considerations (50).

The newer generation, specifically, the third generation of AEDs are also called 'targeted' AED since they were developed based on the rationality of mechanism of seizure occurrence. Examples of these drugs are vigabatrin (VGB) and tiagabine (TGB) which primarily block the metabolism of inhibitory neurotransmitter GABA by irreversibly inhibiting the GABA transaminases. The increased presence of GABA at presynaptic neuronal junctions may increase seizure threshold or aid in abating sustained epileptic firing. The advantage associated with this generation of drugs includes less drug-drug interactions. Whether or not they are less toxic than the older generation remains debatable as it is often dependent on specific drug-drug comparison itself rather than the group per se (51-53). With the more stringent requirements in clinical trials since the last two decades, most of them are getting approved as second line or adjunct treatment. Only in few cases, they could be used as first line for seizure treatment, such as lamotrigine for treatment of generalized seizures in women of child bearing potential.

Attempt to substitute the older generation AED has been so far, unsuccessful. Clinical efficacy of carbamazepine (CBZ) and valproic acid

(VPA) seems unsurpassed and remained the benchmark for newer generation AEDs. For example, VPA still outperformed lamotrigine (LTG) as the first line treatment for idiopathic generalized epilepsy (IGE), juvenile myoclonic epilepsy (JME) and new-onset absence epilepsy (NOAE) of childhood (51, 54, 55). Topiramate (TPM) and ethosuximide (ESM) was reported to be non inferior to VPA in the treatment of IGE and NOAE respectively, although the percentage of effective treatment outcome showed a trend favouring VPA (51, 55). The trial involving ESM, however, was only for 16 weeks and hence the result has to be interpreted cautiously. In untreated focal epilepsy, immediate-release CBZ was undefeated by gabapentin in efficacy but was likely to fail due to intolerability when compared to lamotrigine (51). When sustained-release CBZ was used, tolerability is comparable to LTG group while a trend of higher seizure free rates was observed in CBZ group (56).

There have also been efforts to develop antiepileptogenic drugs. Hitherto, the results had been disappointing (57-59). As with all new drugs, new antiepileptogenic agent will have to undergo preclinical and clinical evaluations. For preclinical studies in epilepsy, it has long been debated whether the current kindling and status epilepticus animal models are representative causes of human epileptogenesis (60, 61). Besides, evidence of antiepileptogenesis is difficult to be captured in human study. There seems to be a critical window between the time of brain insult to the treatment commencement and differing mechanisms depending on the type of brain insult, gender, age and comorbidities (62, 63). The treatment should begin between day 1 to 3 post-insult to halt epileptogenesis while the treatment duration should be at least 2 to 5 years to ensure optimum suppression of epileptogenesis (64). Thereafter, subjects should be followed up to determine sustained epilepsy prevention even after drug withdrawal. Overall, clinical trials are difficult and costly to conduct. Each etiology of epilepsy is likely to have its own set of epileptogenesis, further complicating both the trial design and findings generalization. It will probably take a long time before a new antiepileptogenic gets its approval. Due to the complicated preclinical and

clinical trial designs, biomarkers for human antiepileptogenesis are hence, favoured since the changes may occur relatively faster and can be objectively monitored (64-66). The currently researched independent biomarkers involved the microseizures and specific intracranial EEG spike patterns that present before seizure attacks (64-66). Further validation work is still required to establish them as useful surrogates to antiepileptogenesis.

On the other hand, some existing AEDs such as ESM and levetiracetam (LEV) are also considered as antiepileptogenic due to the observed persistent seizure suppression even after drug withdrawal in genetically modified spontaneously epileptic rats (SER) (67, 68). This finding is in contrast with seizure generation that occurs almost immediately following withdrawal of any antiseizure drugs. Nevertheless, the suboptimal efficacy observed with these agents suggested that improvement works in both preclinical and clinical trials are still required.

In view of the lengthy process for approval of antiepileptogenic drugs, first line treatment drugs such as VPA, CBZ and PHT will likely be the mainstay of treatment for years to come. VPA is licensed for use in a complete range of seizure types which include primary generalized seizures, absence and myoclonic seizures and focal seizures. CBZ is licensed for partial or focal seizures while PHT is used in a variety of cases spanning from tonic-clonic or partial or a combination of these two seizures, prevention of seizure post-neurosurgery or severe head injury to the emergency treatment to stop ongoing seizures. The use of these conventional AEDs is usually supported with plasma drug level monitoring as they have a narrow therapeutic index. Levels below the target therapeutic trough may result in inadequate control of the seizure attacks while levels above the target therapeutic peak will expose the patients to more adverse drug effects. Current plasma monitoring is very well-established with the therapeutic ranges of 50 – 100 µg/mL for VPA, 4 – 12 µg/mL for CBZ and 10 – 20 µg/mL for PHT.

The application of pharmacokinetic principles on antiepilepsy therapy is largely inspired by regulatory requirements because the serum concentrations of phenytoin and phenobrabital displayed strong correlation with their efficacy as well as side effects (69-75), highlighting the importance of PK estimations for AEDs. These similar findings had also propelled the need for comprehensive PK characterization of AEDs before drug registrations.

It is believed that only the free form of AEDs can penetrate the brain, and hence, is able to correlate better with clinical efficacy (76-78). However, monitoring of free drug concentrations are too costly to be adopted in routine practice and is only applied in cases deemed absolutely necessary by the clinicians. This method is also not readily available, further hampering its usefulness. The alternative measurements and correlation studies have include the use of CSF (79), tears (80), saliva (81) and DBS (82) instead of the conventional plasma.

Many population pharmacokinetic (PPK) studies were then performed on these conventional AEDs to better estimate the pharmacokinetic parameters (83-91). A precise estimation of the PK parameters will enhance the dosing efficiency, where the individualized dose instilled to the patient has a higher likelihood of reaching the therapeutic concentrations in a shorter duration while avoiding the unnecessary side effects.

1.1.4 DRUG RESISTANT EPILEPSY

The goal of treatment in PWE is to attain seizure freedom (2, 8, 9) and the mainstay of treatment modality is through daily use of AEDs. The most commonly prescribed AEDs around the world as well as in Singapore have been found to be of the older generation of AEDs, namely valproic acid, carbamazepine and phenytoin (92-95).

Approximately 20% to 40% of PWE will have drug resistant epilepsy (DRE) in their lifetime (96-101). Conventionally, the PWE are considered to have intractable or medically refractory epilepsy when they fail multiple antiepileptics drugs (AEDs). Studies conducted in the United States and European countries have defined intractability as failure to attain seizure freedom after the trials of 2 or 3 AEDs (101-105). In addition, these studies varied in their definition of seizure freedom. For example, Kwan and Brodie (101) defined seizure freedom as ≥ 1 year without a seizure occurrence from the date of last follow up, while Camfield and Camfield (105) utilized a more lenient criteria of < 1 seizure every 2 months in the last year of follow up. In view of the differing definitions, direct comparison of their findings is hindered. Moreover, findings from each study may not be representative of the epilepsy population in other settings, such as Singapore.

1.1.5 RISK FACTORS OF DRUG RESISTANT EPILEPSY

With the high occurrence rate of DRE, research has been attempted to identify some common predictors (100, 101, 106-108). It appears that a combination of characteristics is more likely to offer a better predictability of DRE as compared to any single factor. It has been reported that more than half of PWE will respond to their first AED prescribed. For those who did not achieve seizure freedom with their first AED trial, less than 20% will respond favorably to the following AEDs trial (107). Hence, poor response towards the first AED prescribed seems to be an important factor in predicting DRE (100, 107, 108).

In line with this suggestion, investigators from Scotland conducted a study to look for biomarkers that could be indicative of responsiveness among epilepsy patients towards their first AED (109). They recruited 125 newly diagnosed epilepsy patients who were then randomly assigned to lamotrigine, levetiracetam or topiramate group and prospectively followed for 12 months.

PWE who had no seizure since the start of treatment were classified as responders, continuous seizures despite on treatment were classified as non-responders and failed to reach endpoints were classified as unknown. The duration of 12 months seizure freedom was in agreement with the International League Against Epilepsy (ILAE) consensus, assuming the patients had the longest pre-treatment seizure interval of ≤ 4 months. Unfortunately, at the end of their study, no prominent serum biomarker was discovered. The authors attributed the lack of discrimination to their relatively small and equivocal clinical phenotype cohort (109). Moreover, the duration of 12 months may be too short for adjustments in *in vivo* system biology. It will be interesting to investigate the changes after a longer duration of treatment.

Another constantly cited predictor of DRE is the high number of seizures prior to treatment initiation (101, 104, 107). It is also generally perceived that epilepsy syndromes arising from a lesion in the brain are more difficult to be treated and hence, are more likely to render the PWE to be drug resistant (106, 107, 110). The age of epilepsy onset is also suggested to be another major predictor of DRE, with those presented at the age of ≥ 65 years tend to be less likely to develop DRE (111). Insofar, none of the studies conducted has considered the effect of Asian ethnicity in predicting DRE. It has been proposed that genetic makeup of PWE may influence their responses to certain AEDs such as carbamazepine (112, 113). Therefore, there may be a need to investigate the role of ethnicity, which indirectly implying geneticity, in predicting the treatment response of PWE, especially for multiracial countries like Singapore and Malaysia in Asia.

1.1.6 MECHANISMS OF DRUG RESISTANT EPILEPSY

Apart from discovering the risk factors associated with DRE, it is actually of more prominence to elucidate the pathogenesis of DRE in order to prevent its occurrence. A few hypotheses have been proposed.

Firstly, it has been shown that enhanced expression of multidrug transporters is associated with multidrug resistant epilepsy (114-116). It is generally believed that multidrug resistant epilepsy could be related to polymorphisms of the gene ABCB1, encoding P-glyprotein (Pgp) in the blood brain barrier (114, 117, 118). Pgp is an adenosine triphosphate (ATP)-driven efflux pump that is suggested to be over expressed in patients with DRE, and reduced the exposure of the brain to AEDs. In one study, ABCB1C3435T polymorphism was demonstrated to be significantly correlated with DRE in adult Caucasians and PWE with symptomatic etiology (currently termed as structural-metabolic), but not in children or PWE with idiopathic and cryptogenic etiology (119). The definition of drug resistant used were occurrence of at least 4 seizures in the preceding year and failure to more than 3 appropriate AEDs while drug responsive/seizure free were seizure freedom of at least 1 year (119). The result of a meta-analysis done using 19 studies, however, concluded otherwise (120). No association between ABCB1C3435T polymorphism and DRE was found (120). The difference could be due to the different DRE definitions utilized. The latter study's approach appeared to be more clinically relevant as it had adopted the latest ILAE definition of DRE (50). The absence of association between ABCB1C3435T polymorphism and DRE could be due to two factors: 1), not all AEDs are substrates for Pgp in the human brain; 2) Pgp transporters have yet to be proven to be able remove a significant amount of AEDs from the brain (121). Furthermore, there seems to be linear relationship between plasma AED levels with the brain AED levels. This finding opposed the characteristic of Pgp-mediated efflux because it should be a saturable system (122, 123). Hence, ABCB1 may not be the sole

gene involved in DRE. It is becoming increasingly plausible that epilepsies are multifactorial diseases involving both genetic and environmental factors.

Secondly, the altered sites for AEDs' actions could also be relevant in rendering lack of response to the drugs (116, 124). In drug resistant temporal lobe epilepsy, altered expression of $GABA_A$ receptor subtype had hindered the inhibitory actions by AEDs (125). Since most AEDs act by inhibiting the voltage-gated sodium channels, drug resistant epilepsy was also thought to be related to polymorphisms of SCN2A gene, which modified these channels to be non-receptive to AEDs (126). More research is definitely required to establish that these mutations occur in all DRE patients and the functions are affected significantly without compensation by others.

Thirdly, in neuroproteomics, the research approach probably arises from the development of animal models that best describe epilepsy (127). Interestingly, neuroproteins involved seem to be dependent on the origin of the epilepsy such as fronto-temporal or temporal lobe. The TARCs/ICAM5 ratio in patient plasma had been proposed to be a candidate biomarker for DRE in patients with fronto-temporal lobe epilepsy (128). In another study, transient inhibition of brain-derived neurotrophic factor (BDNF) receptor TrkB after status epilepticus in mice model through chemical-genetic approach had prevented epileptogenesis of temporal lobe epilepsy (129). This study highlighted the importance of TrkB kinase in promoting epileptogenesis post-seizure and that there is a critical window time that could prevent generation of epilepsy later on in life. Although the generalization of these proteins in a heterogeneous general population of PWE remains uncertain, they could facilitate development of targeted diagnostic and treatment approach.

In conclusion, research is still ongoing to determine which of these findings is the cause rather than the effect of long-term usage of AEDs and recurrent seizures. This uncertainty could be due to the heterogeneity of the DRE population itself. It becomes increasingly complicated when coupled with the differing definitions of DRE used in various studies. Nevertheless,

before any hypothesis is confirmed to be the trigger of DRE, the current ensemble of AEDs, or antiseizure, remains our best weapons against DRE. It is henceforth important to design ways to optimize their usages.

1.1.7 COMPLICATIONS FROM DRUG RESISTANT EPILEPSY

Despite the use of multiple AEDs, it is self-explanatory that DRE patients will still suffer multiple recurrent seizures. This high occurrence of seizures has been shown to be directly involved in causing deaths of DRE patients, at an increased rate of approximately 1.37 per 100 person-years (130-132). Aside from increased mortality, DRE individuals also have increased risk of disability from accidents, poor academic achievement, decreased employment and social isolation (133, 134). All these disadvantages have contributed towards the lower quality of life of PWE as compared to healthy patients (133, 135).

In contrast, no increased mortality was observed in seizure-free patient (136). The quality of life of PWE also improved meaningfully when their seizure occurrence is ≤ 1 per year, highlighting the significance of rendering seizure freedom in PWE. These substantial observations have led to the recommendations of seizure freedom, avoidance of AEDs related side effects and improvement in quality of life as the treatment goals in PWE in general.

To achieve these recommended goals, there is a complex interplay between the choices of drugs, access to mental care assistance and social encouragement in the form of job training as well as support from family members. Therefore, PWE have also to be equipped with self-financial ability or at least financial assistance from able bodies for those less fortunate. In the United States, the annual cost of epilepsy treatment is estimated to be US$5200 per patient (137). Although the estimated cost may not be applicable to local population in Singapore, it does provide insight into the financial

burden borne by PWE. This cost is expected to rise in those with DRE. Emphasis is hence required to be placed *on* appropriate and timely identification of DRE to prevent any complications from occurring or worsening, and overall, avoiding the rise in financial burden.

1.1.8 RECENT CONSENSUS

Recently, a joint task force of ILAE has recommended a detailed definition of DRE (50). In this definition, the PWE have to fail at least 2 appropriately chosen AEDs which have been prescribed at adequate dosages for a sufficiently long duration for the treatment of their epilepsy syndromes. There are no restrictions of the class of AEDs used but they must have been approved by the relevant drug control authority for the use in any particular syndrome. Seizure freedom is also standardized to at least 3 times the previous seizure free interval or 12 months, whichever is longer. With this conformed definition, it allows for standardization of DRE categorization across regions. By having similar backgrounds of DRE, relevant treatment options that have been proven useful in other population could then be adopted in our settings. PWE, especially our DRE patients can then be assured that they are being evidently treated. Hence, in summary, after considering the debilitating effects and complications brought by DRE, it is of utmost importance for us to identify our own population's rate of DRE and seek understanding in the drug resistant epileptogenesis in this group of patients, in order to design better treatment options in the future. Meanwhile, considering our current ensemble of AEDs is the best available treatment option, there is a need to optimize their usages.

1.2 DRIED BLOOD SPOT (DBS)

1.2.1 OVERVIEW OF DRIED BLOOD SPOT

Dried blood spot (DBS) is a sampling method popularized since 1960s by Dr Robert Guthrie. As implied by its name, drops of whole blood are spotted onto a collection paper and allowed to dry in room temperature. Generally, the first drop of blood from any needle prick has to be discarded due to the presence of interstitial fluid. Subsequent well-formed drops, accumulated at consistent and sufficient volume (~ 30 to 50 µL), are to be spotted directly onto the pre-printed circle on the collection paper. Only one clean attempt per circle is allowed with neither smearing nor direct contact between skin and paper. Production and quality of the collection paper, the most widely used Whatman 903®, is in compliance with the Good Manufacturing Practices (GMP) and Food and Drug Authority (FDA) Quality System Regulation 21 CFR Part 820. In addition, 903® is controlled for homogeneity of its cotton linters content with uniform thickness to ensure consistent flow rate, absorbency and purity of every blood spot. Each lot of 903® is a class II medical device and validated to meet Clinical and Laboratory Standards Institute (CLSI) LA4-A5 consensus standards which include blood spot diameter and paper weight. To minimize the risk of infection and enzymatic degradation of some blood components, a newer type of chemically-treated filter papers, FTA®DMPK-A and B, are introduced. However, clinically applicable methods developed using this filter paper is limited as compared to 903® (138, 139).

DBS was introduced for the screening of an IEM, phenylketonuria, in newborns (140). It has since been extensively researched for its other potential applications such as screening for folate deficiency (141), hyperthyroidism (142), prostate-specific antigen (PSA) (143), human immunodeficiency virus (HIV) (144, 145) and recently, metabolite profiling (146). With the development of tandem mass spectrometry, one blood spot can

be used to screen for a several IEMs in a single analytical step (147). DBS has also been adopted in the studies of drugs pharmacokinetics (148), therapeutic drug monitoring (149) and toxicokinetics (150).

Plasma or serum is the current gold standard for clinical analysis of disease and drug monitoring in view that it contains almost all analytes present in the blood. Its collection process involves venipuncture for whole blood, followed by centrifugation and separation of plasma composition or immediate freezing until analysis. Venipuncture itself can be challenging due to inaccessible vein and relatively high amount of blood sample required, especially in population such as neonates, children and elderly. The amount of blood withdrawn will increase with the number of tests and incompatibility with relevant vacuum containers for different tests.

DBS is formed from whole blood and theoretically should contain similar amount of analytes as plasma, if not more. The quantities, however, may differ, depending on its presence in capillary blood and stability on filter paper (146). The sample collection is relatively simple and noninvasive, involving a prick needle, similar to the one used by diabetic patients to measure their capillary blood glucose levels. This allows patient to collect the sample at home, especially in cases where set time point is crucial. Since DBS does not require centrifugation or isolation, once dried at room temperature, it can either be mailed to hospital laboratory or kept in fridge/away from direct heat source until his next visit (151). Analytes of various physicochemical properties has been reported to be stable upon drying for a long duration of time, ranging from 30 day to 15 years (152-154). It is also relatively easy to store and transport, with minimum hazard to the carrier as compared to plasma. Therefore, we decided to explore DBS potentials as sampling matrices for epilepsy research studies.

Nevertheless, many established clinical reference ranges were based on plasma levels and DBS levels are not directly comparable to serum or plasma. Hence, any attempt to replace plasma with DBS has to be accompanied with

correction factors for the DBS values (155). Alternatively, a new reference range for DBS measurements can be proposed (156). In contrast to plasma samples where the sample quantity could range from 30 µL to above, one disk of punched-out blood spot (≤ 6 mm diameter) contains ≤ 15 µL of blood. The amount of information that could be recovered remains uncertain. There is also a possibility that the filter paper may adsorb some analytes and cause lower extractions. Moreover, the compositions of capillary blood where DBS is derived from may be different from the venous blood. The analytes of interest are therefore, required to be tested for reproducibility and validated for consistency prior to using DBS as sampling matrices. Depending on the nature of desired analytes, varying extraction solvent polarities may theoretically increase the extraction efficiencies. With the advancement in sensitive and robust detection technology such as the mass spectrometry, there is an exponential increase in the amount of information that can be recovered from a small specimen. The potential of analysing a ≤ 15 µL of blood spot as sample matrix is worth exploring.

Research done using DBS are usually to detect and quantitate known analytes. It is rarely used for unknown analyte search such as biomarker search. Considering the limited experience most laboratory personnel have with DBS handling, it will be desirable for a simple and consistent analysis procedure development to facilitate its implementation across various laboratories.

In the subsequent section, we will discuss the utilities of DBS in both known and unknown analytes search and the rationales for us to adopt it in our study population.

1.2.2 DRIED BLOOD SPOT IN PHARMACOKINETIC STUDIES

DBS represents an alternative that overcomes many of current plasma/serum sampling problematic issues such as:

1. Stability of metabolites in plasma or blood samples after acquisition
2. The deeper needle penetration to obtain venous blood which could be unbearable for 'needle phobia' patients
3. The requirement of a phlebotomist to withdraw blood
4. The higher cost and cumbersome logistic considerations associated with for plasma and blood samples

As discussed in the earlier section, the small amount required for one DBS makes it attractive to preclinical as well as clinical pharmacokinetic studies where multiple time point of sampling and precise timing are required to elucidate the drug's PK characteristics. Similarly, it will facilitate sampling from neonates, premature infants and the critically ill patients as well as small animals such as mice or rats. In animal study, the lower amount of blood required per sampling may also translate into less number of animals required per study by reducing the need to sacrifice more animals. These advantages had enticed pharmaceutical companies such as Glaxo Smith Kline (GSK) to adopt DBS sampling in some of their preclinical pharmacokinetic and toxicokinetic studies (148).

Review paper by Li et al. outlined various drugs that had established its DBS monitoring (157). These drugs included antiretrovirals such as efavirenz, nevirapine, atazanavir, indinavir and ritonavir, barbiturates such as lorazepan, diazepam and nitrazepam, opiods such as morphine and cocaine, immunosuppressants such as cyclosporine and tacrolimus, antiepileptics such as topiramate and phenobarbital as well as common overdosed drugs such as acetaminophen. The type of filter paper use varies, with Whatman 903® being the most commonly used, and the size of spot also differs, ranging from 3 – 8

mm in diameter. Generally, the research aspect involves either assay development (148, 158, 159) or clinical validation with plasma/whole blood concentration comparisons (160-162). Clinically validated assays have the advantage of immediate applicability. Nevertheless, findings from either type of study demonstrated the suitability of DBS for PK study for drugs of varying properties.

Clinical validation involves concurrent quantitation of the analyte of interest using capillary DBS assay and the currently accepted standard of practise e.g. plasma immunoturbidimetric quantitation of antibiotics and whole quantitation of cyclosporine. Due to logistical issue, many studies collected whole blood from venipuncture, and prepared DBS using this same pool of blood (161-163) before centrifugation to collect its plasma for routine clinical measurement. This method facilitates the sample collection procedure (as patient will not be pricked twice) while allowing uniform blood spotting with pipetting tools. Although theoretically there could be some differences between capillary and venous concentrations, the differences for a majority of xenobiotics are not obvious, especially after the distribution phase (164-166).

1.2.3 DRIED BLOOD SPOT IN METABOLOMIC PROFILING

As for detection of disease related information, based on some of the research conducted so far, metabolites, proteins and genes seemed to be extractable from DBS. Generally the extraction procedures involve the usage of specific solvents to selectively recover the desired analyte. This solvent can be an additional solvent or a similar solvent used in blood/plasma extractions. For example, phosphate buffered saline (PBS), 0.5% Triton X-100 was used as washing solution to dissolve relevant genes and proteins at the start of HIV-1 proviral DNA extraction from DBS (145). After shaking, incubation and centrifugation, subsequent amplification steps are similar to the routine whole blood proviral DNA processing.

For a profiling study, such as biomarker/s search, a universal solvent is proposed to yield as many compounds from the DBS as possible (146). To be thorough, subsequent extraction/s using solvent of differing polarity may be considered to yield more compounds. A recent approach using dried biofluid spots from blood, plasma and urine concluded that biofluid spots can be an alternative for metabolite profiling. The number of ions/compounds detected could be significantly lower than their conventional plasma counterparts, but may be overcome by optimising the extraction procedures (146). The study, however, was done in ultra performance liquid chromatography and orthogonal acceleration time-of-flight mass spectrometry (UPLC-oaToFMS), with electrospray ionization (ESI) without metabolite putative identities. Although liquid chromatography (LC) provides better selectivity than immunoassays for different analytes, with the unknown metabolites, there is limited capability for immediate clinical interpretation and application.

Gas chromatography with mass spectrometric detection is generally more reproducible and robust due to its hard ionization nature as compared to LC-MS. This characteristic has allowed the construction of metabolites and analytes reference libraries such as the Fiehn, Wiley and National Institute of Standards and Technology (NIST) library of mass spectrum fingerprints which are unique to each individual compound. The putatively identified compound can then be compared with the pure standards to confirm its identity. For biomarker/s search, GC-MS presents an enticing method of analysis. Nonetheless, this advantage ought to be balanced with the more complex GC-MS sample preparation which involves derivatization as well as overall longer analytical run time (167, 168). If the identity search is the aim of study, GC-MS may offer a more direct result interpretation.

1.3 CHAPTER SUMMARY

This chapter has provided background on epilepsy, related treatment drugs and the current challenges in epilepsy management, which included drug resistant epilepsy as well as unavailability of effective disease modifying agents. The reported DBS usages in disease and treatment monitoring had also been discussed. In the following chapters, some of these challenges would be reiterated and related to the hypotheses of this thesis.

CHAPTER 2

Hypotheses & Objectives

CHAPTER 2. OBJECTIVES

2.1 RATIONALES

Thus far, some of the challenges observed in the field of epilepsy which have been discussed in Chapter 1 included:

1. Approximately 20% to 40% of PWE will have DRE in their lifetime. Risk factors for DRE may differ according to populations and ethnicity. DRE in Asians has not been extensively evaluated. In addition, differing definitions for DRE used in different studies make direct comparison of their findings difficult. As a result, findings from each study may not be representative of the epilepsy population in other settings, such as in Singapore.
2. Epilepsy is a multifactorial disease involving both genetic and environmental factors. The epileptogenesis of DRE is largely unknown, although there are a few hypotheses for DRE. Before any hypothesis is confirmed to be the trigger of DRE, and selective treatments for those triggers are developed, the current ensemble of AEDs, or antiseizure, remains our best armaments against DRE.
3. The time of sampling for epileptogenesis studies seems crucial in understanding of epileptogenesis as well as seizure occurrences. To illustrate, sampling should be obtained:
    a. Directly before or after seizure attacks for seizure biomarker search
    b. During interictal phase for biomarkers of epilepsy. Comparison should be made with healthy subjects
    c. During interictal phase for biomarker of drug resistant epilepsy. Comparison should be made with drug responsive subjects
    d. Before and after AED treatment for biomarker of AED responsiveness

e. Before and after developing epilepsy or before and after curing epilepsy for biomarker of epileptogenesis

4. Biomarkers for human antiepileptogenesis are favourable as the changes can be objectively and timely monitored (64-66). The currently researched independent biomarkers involved the microseizures and specific intracranial EEG spike patterns that are present before seizure attacks (64-66). Both biomarker detections required trained epileptogeneticist or EEG technicians and sophisticated technology that may not be readily available in many healthcare facilities
5. It remains uncertain whether there is a temporary decrease in plasma AEDs levels before or after a seizure attack as it is difficult for plasma sampling right after a seizure attack
6. Overall, clinical trials for antiepileptogenesis are difficult and costly to conduct. The currently available first line treatment drugs such as VPA, CBZ and PHT will likely be the mainstay of treatment for years to come. It is henceforth important to design ways to optimize their usages
7. Serum concentrations of the first line AEDs displayed strong correlation with their efficacy as well as side effects. Comprehensive PK characterizations of AEDs via relevant biofluids are part of regulatory requirements. Population pharmacokinetic (PPK) parameters estimation of these AEDs is one possible way to optimise their dosing efficiencies
8. Current plasma or serum sampling for both metabolomic profiling and PK study does not ease time point compliance. The lengthy and delicate sample acquisition and storage may result in missing the critical window period for acquisition. There is a need for alternative sampling method, preferably one which patients or carers can obtain by themselves, such as dried blood spot, where sampling can be done shortly after the episode of seizure.

2.2 HYPOTHESES

Based on the challenges and rationales observed, it is hypothesized that:

1. Drug resistant epilepsy (DRE) is a significant ongoing issue in Singapore population of people with epilepsy (PWE). It is of utmost importance for us to identify our own population's rate of DRE
2. Epileptogenesis of various origins, genetic, structural-metabolic and unknown, do converge at neurometabolite expressions immediately before triggering seizures. Metabolomic profiling during interictal phase will provide some useful insights into baseline metabolite perturbations involved in drug resistant epileptogenesis and dried blood spot (DBS) is a good surrogate to plasma sampling for this approach
3. DSB can also be used for therapeutic drug monitoring of AEDs. An accurate conversion factor for each AED could be obtained by comparing the DBS to plasma concentrations from clinical samples. The plasma and whole blood concentrations are hypothesized to be different because of the presence of red blood cells in whole blood and partition into erythrocytes or red blood cells. Once the conversion is validated, DBS sampling can be obtained by patient/care themselves during or post seizure.
4. Similar to plasma, DBS concentrations can be utilized to derive reliable population pharmacokinetic parameters. With this approach, it is hypothesized that there is a more direct dose-DBS concentrations relationship as compared to plasma concentrations.

2.3 OBJECTIVES

The overall objective of this thesis is to investigate the utilities of dried blood spots in metabolomic and pharmacokinetic studies related to epilepsy. This is in hope to facilitate implementation of DBS sampling method for population based research in the area of epilepsy, in particularly the pharmacotherapy and drug resistant cases. The specific objectives of each experiment designed are as follows:

1. To determine the drug resistant epilepsy prevalence study among people with epilepsy in a tertiary referral hospital in Singapore
    - In line with hypothesis 1, the findings will also provide insight of possible risk factors involved in developing DRE to gauge if metabolomics is a suitable platform. This study is described in Chapter 4
2. To evaluate the use of DBS for metabolites detection in GC-MS
    - This is conducted in part of hypothesis 2. DBS detected metabolites have to be firstly proven to be reproducible and stable. This work is presented in Chapter 5
3. To discern the interictal metabolomic profiles between drug resistant and drug responsive PWE using the DBS
    - In line with hypothesis 2, the putatively identified discriminating metabolites will be highlighted in Chapter 6
4. To obtain factors to correct DBS measured AED concentrations to its predicted plasma concentrations
    - For hypothesis 3, concurrent DBS and plasma quantitation of AEDs from PWE will be compared. A previously validated assay for simultaneous monitoring of 3 most commonly used AEDs in our population of PWE will be used. DBS concentrations of AEDs will be measured in GC-MS and correlated with its plasma concentrations measured in hospital laboratory. The role of red blood cells and hematocrit in

explaining the differences observed will also be investigated. Chapter 7 detailed the results obtained.

5. To characterize PPK parameter using DBS measured antiepileptic concentrations
    - This study is designed to test the hypothesis 4. DBS concentrations will be subjected to PPK modelling to gauge their usefulness in therapy optimization. The comparative findings with plasma derived PPK parameter will be discussed in Chapter 8

2.4 SCOPE

To facilitate future applicability, the reliability of DBS and relevant assays for both AEDs monitoring and metabolomic profiling of DRE will have to be demonstrated. Active PWE who are having venous sampling for therapeutic drug monitoring on the visit day will be recruited. As most of these patients are at the interictal phase, the metabolite perturbations observed are likely to represent the baseline levels of the disease state rather than causal of DRE.

The objective of the animal experiment presented in Chapter 5 was to establish that DBS is a suitable matrix for metabolomics study. Rats are homogenously bred and were chosen in favor of healthy subjects. This is because the definition of healthy subjects could be plagued by a few limitations: (1) What is the suitable age range of healthy subjects and where to locate as many of them within the study period? (2) For homogeneity, should they all be of one race, and which race should be chosen in favor of another, and if not, how to ascertain that the heterogeneity observed was not due to ethnicity differences? Although rats and humans are different species, their metabolite profiles should be comparable in contents as both of them are mammals. The experiment was designed to establish the consistency of DBS

as a platform for metabolomic study rather than establishing the set of metabolites that can be detected via DBS. It would be detrimental to the study if it was found later that many analytes were not easily extractable from the DBS. As outlined in that experiment, it is impractical to assess the recovery of every metabolite. In return, three analytes with differing physicochemical properties were tested. The results suggested that irrespective of the lipophilicity and hydrophilicity of the metabolites, they are extractable from DBS in consistent quantities. Extrapolated to metabolites in human blood, they also comprise compounds of various physicochemical properties and the consistent recoveries could be expected.

The temporal effects were considered in the animal study (Appendix II). As a whole, the related metabolome fluctuations were too subtle to be distinguished. Whether or not human samples would yield the same findings remains to be investigated and for the purpose of this research work, the timing of blood acquisition from subjects was confined to between 9 am and 12 pm only.

As for the prandial effects, metabolomics in nutrition is a new and unique field by itself and has to involve gut microbiome study in order to discuss its effects, which is beyond the scope of this study. Nevertheless, it will be good to include this as one of the potential covariate in future studies.

At the same time, to avoid pricking the same subject twice, the concentrations from venous plasma to venous dried blood spot will be compared. This method has the advantage of uniform blood spotting via pipetting, and hence, ensuring more precise measurements and direct in vivo correlations (161-163). Although theoretically there could be some differences between capillary and venous concentrations, the differences for a majority of xenobiotics are not obvious, especially after the distribution phase (164-166).

Although the sampling can be done concurrently, two different GC-MS assays will be used for analyses due to differing aims of research. In

metabolomic profiling, the aim is to search for potential biomarkers. Hence, to detect as many analytes as possible, exploratory GCMS approach was utilized whereby no optimization for any analyte is performed. In the AEDs PK study, the aim is for correlation between DBS and plasma concentrations of CBZ, VPA and PHT. Accurate quantitation is of more importance and hence, a targeted approach is utilized whereby the assay is optimized for quantitation of these specific analytes. Nevertheless, after affirmation of the target metabolites i.e. biomarkers, a combined GCMS assay for both the metabolites of interest as well as AEDs can be developed.

Then, PPK modelling will be attempted to estimate the pharmacokinetic parameters of CBZ using DBS concentrations. Since AEDs have narrow therapeutic indexes, the precise dose-whole blood concentration relationship elucidation is essential in ensuring maximum efficacy but minimum toxicity. PPK for VPA and PHT were not performed due to the relatively small number of available observations. Moreover, unfortunately, neurologists in Singapore do not routinely monitor the free drug levels of AEDs. Therefore, this aspect could not be included into the comparison and correlation investigations.

The findings from this study are hoped to ultimately convince the patients, clinicians and authorities that DBS sampling is reliable. Subsequently, in the future, researchers can embark on population-based research, where patient can collect DBS at designated timings without the need to travel to phlebotomists.

2.5 THESIS OUTLINE

With referral to the experiments outlined in the Objectives section, generally, there are two main research questions in this thesis; the utility of DBS in metabolomic profiling and the utility of DBS in pharmacokinetic study. In congruent with the research questions, the layout for the experiments conducted in this thesis write up is vertically-linked initially, followed by horizontal linking within each research question arm.

Since patient data collection and recruitment were done concurrently for most of the projects, their simplified descriptions are attempted in the Methodology chapter. Some of the technical analysis may have been missed, but will be explained in detail in their individual experimental chapters. For each study, a chapter introduction will be provided, followed by its methods, results, discussion and chapter conclusion. Thereafter, all these findings will be summarized and related to the objectives in the final Conclusion chapter. The limitations and future directions also will be provided in this chapter.

CHAPTER 3

Methodology

CHAPTER 3. METHODOLOGY

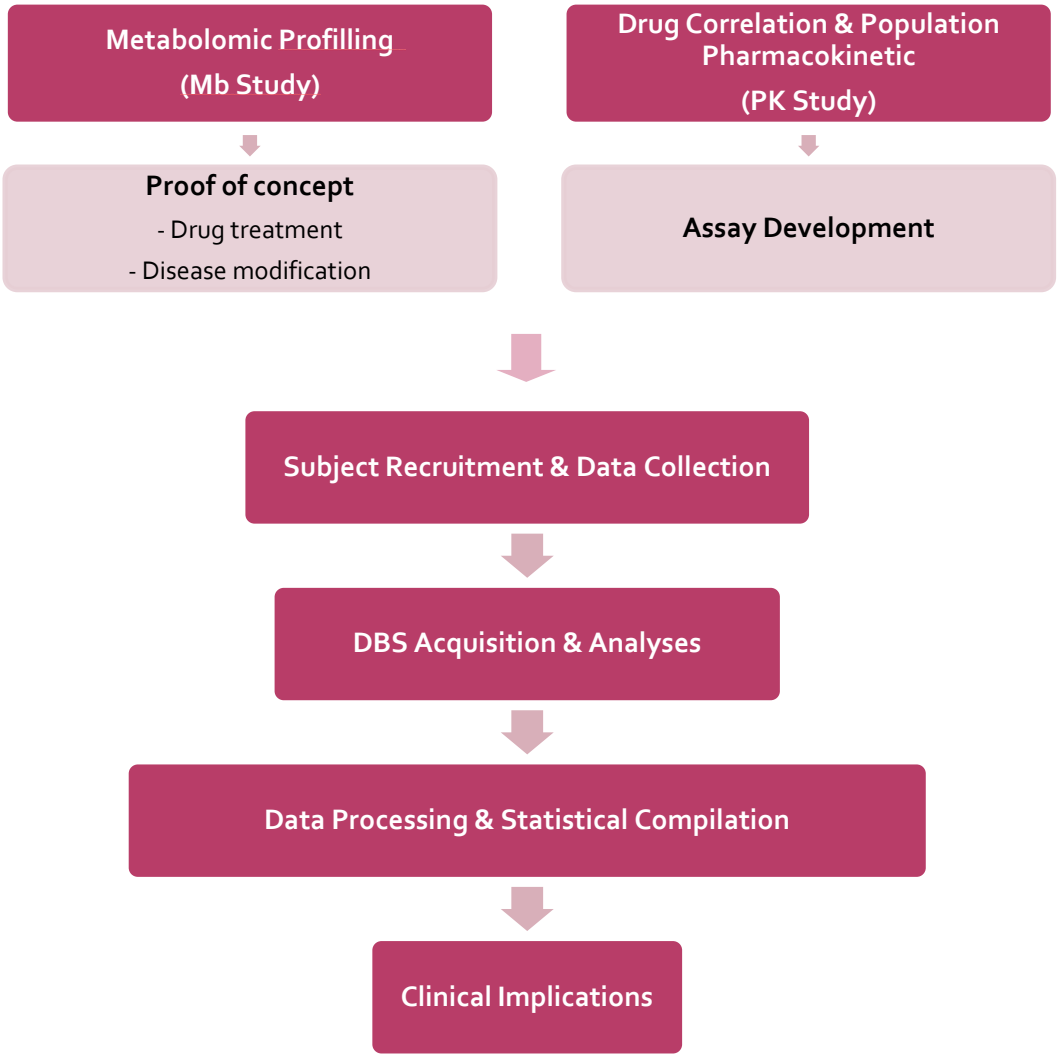

Figure 1. Simplified workflow of the entire thesis. After the initial assay validation, the two research arms converged at the subject recruitment and data collection. Two dried blood spots were obtained from each recruited subject; one for metabolomic profiling, one for pharmacokinetic study. The analyses differ and will be explained in Chapter 5 and 6 for metabolomic profiling and Chapter 7 for pharmacokinetic study. The acquired data will then undergo different processing algorithm for relevant statistical compilation before proposing their clinical implications.

This flow chart outlines the workflow of the entire thesis (Figure 1). An assay that can simultaneously quantitate the AEDs of interest from DBS was firstly developed and validated. To correlate this method of quantitation with the current standard immunoassay quantitation done in hospital laboratory, clinical samples were required. If DBS can quantitate the AEDs as accurate, the methods should be comparable to each other. Similar approach was adopted for metabolomic profiling. Before clinical samples were analyzed, 3 animal studies were designed to establish that DBS can indeed monitor a range of metabolites and detect *in vivo* metabolomic perturbations. These 3 models involved healthy rats, valproic acid treated rats as well as mice treated with p-glycoprotein inhibitor. The summarized results for the latter two animal studies are presented in Appendices I and II.

The patients' recruitment process for the two research arms was conducted simultaneously in Singapore General Hospital (SGH) from 1 October 2011 until 31 December 2012. Both studies were approved by SingHealth Institutional Review Board (IRB). Figure 2 illustrates the overall division of recruited patients according to the different study requirements.

After the end of recruitment process, DBS collected was analyzed. The results were later subjected to statistical compilations. Finally, their clinical implications were proposed.

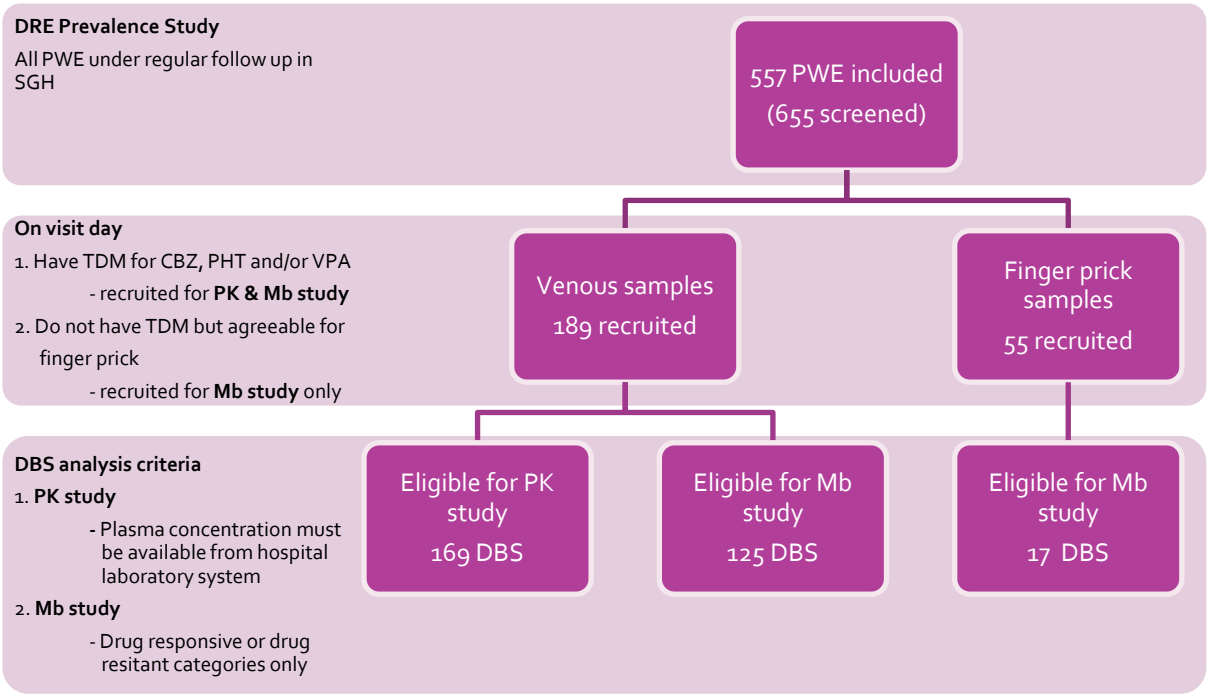

Figure 2. Schematic representation of the patient recruitment and data collection processes. A total of 655 people with epilepsy (PWE) visiting neurology clinic in Singapore General Hospital (SGH) was screened, 557 fulfilled the pre-determined set of criteria and were included in drug resistant prevalence (DRE) prevalence study. Out of this pool of PWE, 244 consented to participate and donate blood spots for our research. There were 189 PWE who had TDM on their visit day, and were included in both the pharmacokinetic (PK) and metabolomic (Mb) studies, while another 55 agreed to donate finger prick blood spots instead of venous blood in view that they do not require any monitoring on the visit day. After careful stratification, 169 DBS were included in PK study, while 142 DBS were included in Mb study.

CHAPTER 4

Prevalence of Drug Resistant Epilepsy in a Neurology Clinic of a Tertiary Referral Hospital in Singapore

CHAPTER 4. PREVALENCE OF DRUG RESISTANT EPILEPSY IN A TERTIARY REFERRAL HOSPITAL IN SINGAPORE

With reference to the first objective, the prevalence of DRE in local population and the associated risk factors are proposed to be determined. Findings from this study will aid in gauging whether metabolomic profiling is a suitable platform for biomarker search in this target group.

4.1 CHAPTER SUMMARY

The objective of this study is to determine the proportion of DRE in an adult PWE population in Singapore. The objective of this study is to determine the proportion of DRE in an adult PWE population in Singapore. All adult PWE attending the neurology specialist clinic of a tertiary referral hospital in Singapore were profiled for drug responses according to the International League against Epilepsy (ILAE) 2010 consensus on the definition for DRE. This is a retrospective cohort study. Data collected were demographics, characteristics of seizure and epilepsy, blood biochemistry levels, electroencephalogram and brain imaging findings, and medication histories. The types and dosages of AEDs used were retrieved from case notes. Routinely assessed treatment-related adverse effects were categorized in congruent to previous publication by Carreno et al, 2008 (169). The dates and number of seizures at the start, during and end of each treatment regimen as well as at the prevalence date were retrieved. They were later classified as daily, weekly, biweekly, in monthly intervals till 6-monthly, followed by yearly and rarely. Generally, the most frequent occurrence of seizure is chosen to represent the subject's seizure frequency. The prevalence rate of DRE in this population was 21.5%, while 40.9% of PWE were drug responsive/seizure

free at the point prevalence day (n=557). From multivariate analysis, structural-metabolic etiology [odds ratio (OR) 1.78, 95% confidence interval (CI) 1.003 – 3.148], mental retardation [OR 2.51, 95% CI 1.073–5.863], psychiatric illnesses [OR 3.349, 95% CI 1.181 – 9.501] and pre-treatment seizure frequency of more than once monthly [OR 2.775, 95% CI 1.190 – 6.469] were more likely to have DRE ($p \leq 0.05$). Even though the influence of Indian race on the risk of DRE was only found in the univariate analysis, it warrants investigation in a larger cohort. The findings may aid policy makers in designing treatment guidelines and allocating resources for PWE, in light of the situation that at any given time, 1 in 5 PWE had DRE. Based on the significant risk factors found in this study, genetic factor may not provide a holistic explanation for DRE and metabolomics could complement the knowledge in DRE pathogenesis by providing immediate causal or effect of frequent seizures in this group of subject.

4.2 CHAPTER INTRODUCTION

Epilepsy, and to a more severe extent, DRE brings about incapacitation to PWE. These unwanted effects include physical and mental disabilities which may lead to lower quality of lives (170, 171). It is hence, of prominence to identify these PWE to design better management strategy that best suit them. Research done so far has identified a few DRE predictors, namely symptomatic/cryptogenic syndromes and high number of seizures prior to treatment initiation (106, 172, 173). Their populations differ with either pharmacotherapy naïve adults or long term follow up children patients. These studies were done on Caucasians and Asian ethnicity may have been overlooked as a factor. Chinese PWE in Hong Kong were shown to have a 40% prevalence of DRE in their population and that poor control was associated with mental retardation and mesial temporal sclerosis, a finding that were parallel with others (98). Therefore, it will be interesting to investigate if

similar risk factors extend to long term follow up Asian PWE, which includes Malays and Indians.

Nevertheless, the definition of 'refractory' or 'resistant' was somewhat different among the studies, hindering direct comparisons. With the formation of a consensus for the definition of DRE by ILAE consensus (50), we aim to utilize this classification to determine the proportion of DRE patients in our PWE population and correlate patient specific characteristics with the seizure outcome. This is in hope to unravel pertinent risk factors for developing DRE.

4.3 METHODS

4.3.1 STUDY PATIENTS

A study sample of 288 PWE was required to estimate a 25% (relative precision of 5%) prevalence rate of DRE with 95% confidence interval (174). Therefore, a tertiary referral hospital in Singapore with a PWE population of approximately 600 was chosen as the study site. This hospital was chosen as it is a public institute with neurological department that caters for suspected and confirmed epilepsy cases, regardless of severity, from the whole Singapore population, including referrals from inpatient admission, primary care providers, other hospitals and overseas. These subjects were evaluated and treated locally by the same team of specialists, ensuring reliable registry and routine clinical assessment. Rarely, PWE chose to be seen in other institutions instead of this clinic due to proximity and shorter waiting time, with no direct connection to seizure control. Hence, despite being relatively accessible from the whole country, the study subjects were most likely representing the PWE population within the catchment area of Singapore General Hospital. The drug resistant epilepsy prevalence day was set on 31 December 2012. SingHealth

Institutional Review Board approval was obtained before study initiation (CIRB No: 2011/268/A).

4.3.2 CASE DEFINITION AND ASCERTAINMENT

Subjects were identified from hospital neurology-specialist-clinic appointment system as all neurology cases will be registered under this system. A list comprising of 632 epilepsy cases, indexed with identification number were retrieved. This study comprised of subjects above the age of 18 years as it was the starting age for follow up cases in this adult population, inclusive those discharged from paediatric follow-up elsewhere in Singapore. A final total of 557 subjects, being alive and reside within the catchment area were included after assessment by the attending neurologist.

Epilepsy is defined as the occurrence of two unprovoked seizures. It was part of the clinic's routine investigative procedures to incorporate biochemical tests, EEG and structural/perfusion/diffusion MRI or CT scan of the brain for each subject. The interpretation and revision of EEG was always done by the certified EEG technician and attending neurologist for EEG, while MRI and CT scan was by the radiologist and neurologist. Etiology of epilepsies was reported as genetic, structural-metabolic or unknown, while the seizures were focal, generalized or unknown, in accordance to the ILAE revised terminology (12). Classification was done retrospectively. Genetic epilepsy was revised from idiopathic epilepsy and represents epilepsies with presumed genetic origin such as dravet's syndrome and juvenile myoclonic epilepsy based on typical clinical, EEG and MRI findings. Structural-metabolic epilepsy described the epilepsies resulted from known structural lesions in the brain such as atrophy, cortical dysplasia, tumour or stroke, as evidenced by radiological imaging and metabolic disorder of the brain such as GABA transaminase deficiency. For epilepsy with underlying unidentified

abnormality, it will be categorized as unknown. In subjects with a few seizure types, seizure classification was based on the principal seizure type. For example, if the subject predominantly had generalized tonic-clonic seizure but complained of 2 fainting episodes for the past month, his seizure classification will remain as generalized seizure. Other generalized seizures most often seen in this study include absence, myoclonic and clonic. Subjects with simple/complex partial seizure and auras were redefined as focal seizures. Epileptic spasms and unclassified seizure types were referred as unknown.

In our neurology clinic, PWE were routinely assessed for clinical symptoms, seizure occurrences and frequencies, compliance, response and adverse drug reactions to antiepileptic drug treatments. Hence, the appropriateness of AED for the particular epileptic seizure type was assessed based on evidences (175-181) and guidelines (182-184). Data collected were inclusive of subjects' demographics, pretreatment seizures, baseline seizures prior to the latest AED intervention and current seizure frequencies. Seizure frequencies were recorded and classified into occurrence of at least one seizure in daily, weekly, biweekly and in monthly intervals till 6-monthly, followed by yearly and rarely. Generally, the most frequent occurrence of seizure is chosen to represent the subject's seizure frequency. For subjects with multiple visits over the study period, the clinical assessment from the latest visit before the end of study period was used for drug response determination. The following AEDs are available locally: carbamazepine, phenytoin, sodium valproate, phenobarbitone, primidone, clobazem, clonazepam, lamotrigine, levetiracetam, topiramate, gabapentin and pregabalin.

4.3.3 DRUG RESPONSE CLASSIFICATIONS

The recommendations by the ILAE were adopted to define DRE in our study population (50). Firstly, individual AED was assessed for subject's

treatment response (Level 1). Secondly, by summing all the Level 1 responses, an overall classification of either drug responsive, drug resistant or undefined will be determined (Level 2). Seizure freedom was standardized to at least 3 times the previous seizure free interval or 12 months, whichever was longer, in accordance to the ILAE consensus. Drug resistant was characterized by failure of at least two appropriate AEDs, which have been prescribed at adequate dosages for a sufficiently long duration for the treatment of their epilepsy syndromes. Therefore, a drug resistant PWE will have at least two Category 2 (treatment failure) outcomes from Level 1 categorization. Any deviation from these conditions will render the PWE to have 'Undefined' overall response to his AED/s therapy.

It was therefore, recognized that PWE responses to AEDs therapy at any particular period of life were not confined to either seizure free or drug resistant only. There could be multiple reasons that caused them to be in neither category. To further comprehend our PWE's well-being and better design management plans for the future, we attempted to identify some possible explanations. These include PWE's decision in pharmacotherapy such as choosing their preferred AED/s and the comfortable dosages. A pre-determined checklist with a total of 7 items was formulated, namely failure to one AED only, insufficient dose of either 1 or more AEDs, less than 3 times the previous seizure free interval, incompliant, new intervention for less than 6 months, one seizure occurrence after a seizure free period and absence of all required information (Table 4). In short, subject's response to his latest AEDs regimen in the 'Unknown' group can also be divided into two: (1) with ongoing seizure and (2) without seizure (refer Table 3). These items were designed to be mutually exclusive.

Clinically effective dosages are determined with reference to the World Health Organization (WHO)'s defined daily dose (DDD) (185). However, the individual maximum tolerated dose due to adverse drug reactions (ADRs) was prioritized over the DDD. We acknowledged that

attainment of therapeutic levels without ADR should not hinder the dosage increment in PWE with clinical seizure. In fact, dosage increment was observed in our study population despite sufficient TDM levels. Nevertheless, as TDM levels were used to approximate individual sufficient AED dosages in many clinical settings, we also consider that the dose was the clinically effective dose when therapeutic level was reached; and treatment failure if the PWE still had seizure (Level 1, Category 2).

4.3.4. STATISTICAL ANALYSIS

Demographics data collected such as age, gender, race, type of epilepsies and seizures, age of seizure onset, baseline seizure frequencies before treatment, types of AED used, concurrent illnesses, findings from brain imaging and EEG. SPSS version 21 was used for analyses. Chi-square test was utilized for categorical variables with counts more than 5 in one category, while Fisher's Exact test was considered when there were less than 5. Independent t-test was used for continuous variables. Statistical significance was determined when $p \leq 0.05$. To examine the risk factors of DRE, all covariates from both the seizure free and drug resistant groups of PWE were firstly subjected to univariate binomial logistic regression analyses. The significant covariates ($p < 0.05$) were then analyzed using multivariate binomial logistic regression analyses to estimate individual odds ratio. Co-linearity among the covariates was also examined through the correlation matrix.

4.4 RESULTS

4.4.1 STUDY POPULATION

As can be seen from Table 2, there were similar number of male and female PWE, 274 (49.2%) and 283 (50.8%), respectively. Our patient group had an average age of 42.7 years (range 12 – 90 years) and the mean duration of follow up was 12 years (range 1 – 28). Majority of the PWE was Chinese, 77.2%, while approximately 7.4% each were the Malays, Indians and other races. The most common etiology of epilepsy in this group of PWE was structural-metabolic, 51.2%, followed by unknown (previously defined as cryptogenic), 32.9% and genetic, 16%. Among the structural metabolic, 8.4% had a latest MRI finding that was reported to be normal while 34.7% had etiology originating from mesial temporal sclerosis, 10.5% from atrophic region of the brain and only 2.1% from dysplasia. However, it is noted that 11.9% of this group of PWE did not have an MRI of the brain. They had predominantly manifested focal seizures, 61.4%. The rest were classified as generalized seizures, 34.3% and unknown, 4.3%.

Table 2. Characteristics of study subjects			
Demographic Factors		**n**	**%**
Ethnic	Chinese	430	77.2
	Malay	43	7.7
	Indian	40	7.2
	Others (Sikhs, Filipinos, Burmese & Caucasians)	41	7.4
Gender	Male	274	49.2
	Female	283	50.8
Age (years)	Median 42 (Range 16 – 90 years old)	Mean 42.7	SD 13.99
MRI Findings	Normal	149	26.8
	MTS	103	18.5
	Others	134	24.1
	Not Available	171	30.7
Etiology	Genetic	89	16.0
	Structural-Metabolic	285	51.2
	Unknown	182	32.7
Seizure	Focal seizure	342	61.4
Type	Generalized seizure	191	34.3
	Unknown	24	4.3
No of AEDs	Monotherapy	263	47.2
	Polytherapy	288	51.7
	Not on any AED	6	1.1
ILAE Level 2	Drug Responsive	228	40.9
Classification	Drug Resistant	120	21.5
	Undefined	209	37.5
Age is presented as mean ± standard deviation (SD)			

The concurrent illnesses were rare and considerably diverged. With reference to previous reports on possible risk factors, mental deficiency were recorded in 47 PWE (8.4%) and any form of psychiatric illnesses such as depression and anxiety were documented in 33 PWE (5.9%). Due to small number of individual diagnosis, psychiatric illnesses were not further divided for analysis.

4.4.2 PREVALENCE OF DRUG RESPONSIVE AND DRUG RESISTANT EPILEPSY

A majority of subjects, 228 (40.9%), in this setting was seizure free or drug responsive, as defined by ILAE consensus. On the other hand, drug resistant epilepsy had a 21.5% prevalent rate in our study population. The age-specific prevalence, as presented in Figure 3, depicts the prevalence rates for both drug responsive and drug resistant. The DRE prevalence rate increased from 20% in the 20 – 29 years old group to the highest of 25.8% in the forties group and decreased thereafter, to approximately 5% in the elderly (≥ 70 years old). Chi-square cross tabulation analysis revealed that only PWE in the age group 60 to 69 years was significantly less likely to be drug resistant as compared to the other age groups ($p \leq 0.005$). The age of seizure onset was in fact, significantly younger in drug resistant subjects, median of 12.5 (range 0.3 – 54) years old, than drug responsive subjects, median of 18.0 (range 0.1 – 86) years old ($p < 0.001$).

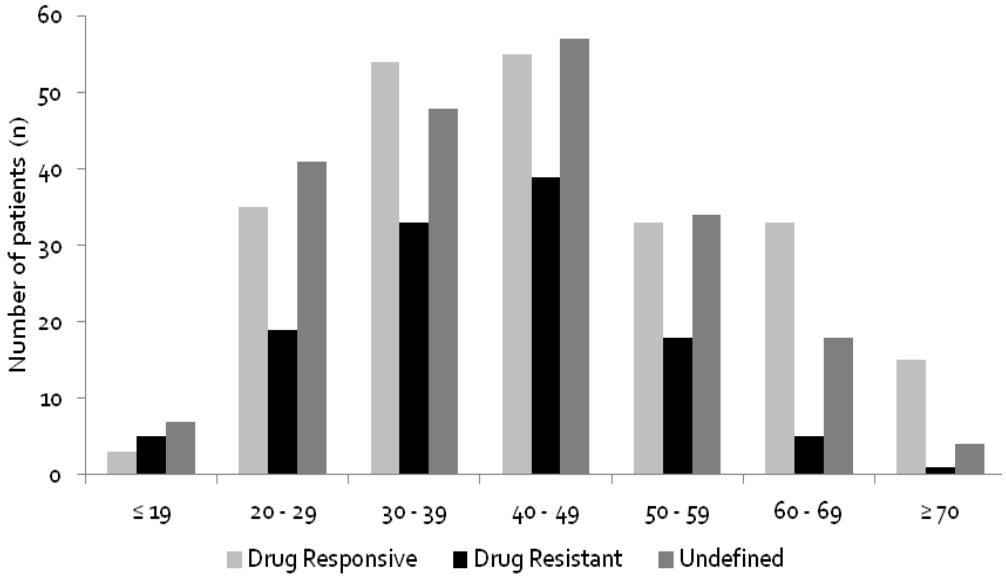

Figure 3. Age-specific prevalence of drug resistant epilepsy (n = 557)

4.4.3 RISK FACTORS ANALYSIS FOR DRUG RESISTANT EPILEPSY

From the initial univariate analysis, PWE who were Indians, mentally deficient or had concurrent psychiatric illnesses, structural-metabolic epilepsy, presence of mesial temporal sclerosis from brain MRI and a baseline seizure of at least once a month or a year, but not in between, were at increased risk of developing DRE ($p < 0.05$). Younger age of seizure onset also posed additional risk to developing DRE. After undergoing multivariate analysis, Indians, mesial temporal sclerosis and baseline yearly seizure were no longer significant risks ($p > 0.2$). The relevant odds ratio and p values are tabulated in Table 3.

Table 3. Univariate and multivariate logistic regression analyses of risk factors for drug resistant epilepsy when compared with those of drug responsive

	Univariate			Multivariate		
	OR	95% CI	*p value*	OR	95% CI	*p value*
Male	0.76	0.487 - 1.182	0.222			
Chinese	0.63	0.364 - 1.081	0.093			
Indian	3.73	1.345 - 10.363	0.011	2.083	0.653 - 6.643	0.215
Family History of Epilepsy	0.89	0.391 - 2.037	0.786			
Baseline seizure at diagnosis						
≥ 1 / month	5.30	3.139 - 8.958	< 0.001	2.775	1.190 - 6.469	0.018
≤ 1 / year	0.78	0.010 - 0.591	0.013	0.267	0.033 - 2.156	0.215
Brain imaging						
MTS	2.01	1.167 - 3.447	0.012	1.445	0.730 - 2.862	0.291
Normal	0.62	0.372 - 1.047	0.074			
ILAE Etiology Classification						
Genetic	0.62	0.323 - 1.193	0.153			
Structural-Metabolic	1.73	1.103 - 2.703	0.017	1.777	1.003 - 3.148	0.049
Unknown	0.00	NA	0.997			
ILAE Seizure Classification						
Focal seizure	0.68	0.434 - 1.063	0.091			
Generalized seizure	1.55	0.986 - 2.44	0.057			
Concurrent Illnesses						
Mentally challenged	4.43	2.067 - 9.489	<0.001	2.509	1.073 - 5.863	0.034
Psychiatric illnesses	3.34	1.344 - 8.305	0.009	3.349	1.181 - 9.501	0.023
Age of Seizure Onset	0.96	0.938 - 0.975	<0.001	0.96	0.940 - 0.981	< 0.001

(OR denotes Odds Ratio; CI denotes confidence interval; NA denotes not available)

4.4.4 PREVALENCE OF UNDEFINED CATEGORY OF PWE

Approximately 37.6% of PWE were classified as 'undefined'. After careful consideration of the reasons that were best suited to describe individual clinical progress, the accepted causes for our PWE to fail attaining seizure freedom were identified and presented in Table 4. From our observations, this group of PWE had, to the point of this study, been determined to have failed only one AED that was prescribed at the adequate dose for a sufficiently long period of time (40.2%). This group of subjects could still be on the same drug, have tried other AEDs but at inadequate doses or durations, or could have just

recently changed to another AED. However, failure was only considered in one AED. On another note, inadequate dose/s of current AED/s was observed in 65 subjects (31.1%), in which 25 of them were acknowledged by the attending neurologist to have declined dosage increments.

Table 4. Reasons for patients with epilepsy (PWE) to be categorized as 'Undefined'

Reasons for the 'Undefined'	**n (%)**
Failed one drug only	84 (40.2%)
Insufficient dose of either 1 or more AEDs	25 (12.0%)
Refusal by patient	40 (19.1%)
Titration phase	
Less than 3 times previous seizure free interval	18 (8.6%)
Incompliant	6 (2.9%)
New intervention (< 6 months)	4 (1.9%)
Only one seizure occurrence after a seizure free period	25 (12.0%)
Absence of all required information	1 (0.5%)

4.4.5 ADVERSE DRUG REACTIONS

In agreement with the ILAE recommendations, there is a clinical difference between seizure free patients who suffered considerable extent of adverse drug effects (ADRs) and those who did not. Therefore, the frequencies of adverse drug reactions were recorded for all patients and summarized in Figure 4. Subgroup analysis revealed statistically significant higher incidences of ADR in DRE subjects as compared to their seizure free counterparts, 23.3% and 12.9%, respectively (Fisher's Exact, $p < 0.001$).

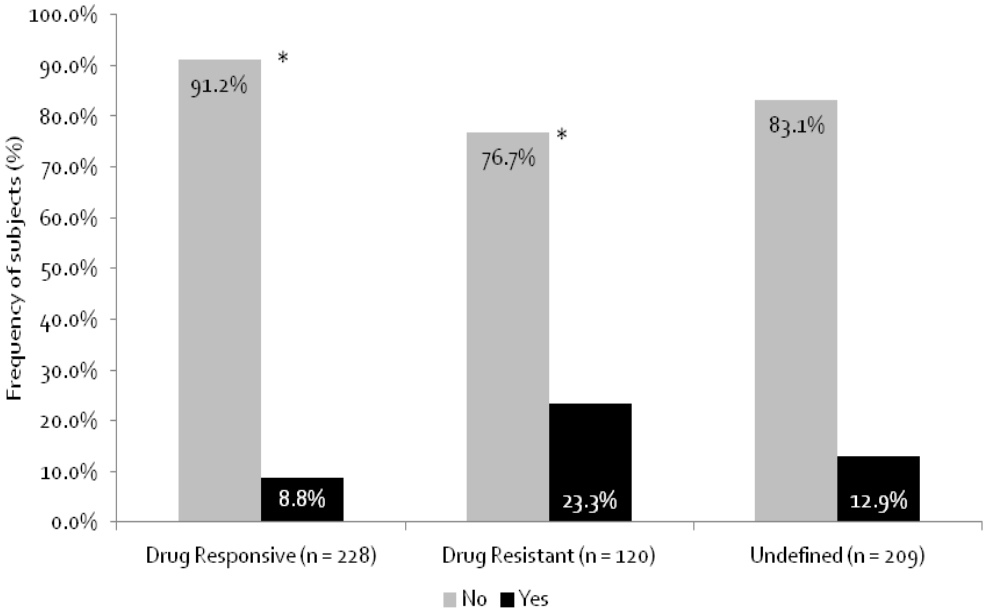

Figure 4. Occurrence of adverse drug reactions within each category of drug response

Table 5. Types of adverse reactions reported by people with epilepsy (PWE) and the frequencies of occurrences according to the drug response categories

Adverse Drug Reactions	Drug Responsive		Drug Resistant		Undefined	
	n	%	n	%	n	%
Sleepiness	1	5.0	5	17.9	2	7.4
Fatigue	0	0.0	2	7.1	1	3.7
Headache	0	0.0	1	3.6	0	0.0
Dizziness	2	10.0	4	14.3	4	14.8
Tremor	7	35.0	9	32.1	12	44.4
Weight Gain	1	5.0	3	10.7	2	7.4
Memory Problems	1	5.0	2	7.1	3	11.1
Difficulties with Speech	1	5.0	0	0.0	0	0.0
Double Vision/Blurred View	3	15.0	0	0.0	0	0.0
Irritability	0	0.0	1	3.6	0	0.0
Hair Loss	1	5.0	0	0.0	1	3.7
Gum Problems	1	5.0	1	3.6	0	0.0
Others	2	10.0	0	0.0	2	7.4
Total number of PWE	20		28		27	

As shown in Table 5, across all three categories of drug responsiveness, similar trends of type and frequency of ADRs were observed. For examples, tremor was the most commonly reported ADR, contributing 35.0%, 32.1% and

44.4% in drug responsive, drug resistant and 'undefined' groups, correspondingly. This was followed by dizziness, memory problems, sleepiness, weight gain, difficulty with speech, hair loss and gum problems. Their frequencies were reported to be approximately 10% each. Uniquely, blurred vision was complained by three subjects (15%) in the drug responsive category while irritability was complained by only one subject (3.6%) in the drug resistant category. The association of ADRs with the type of AEDs used was not examined due to insufficient power for correlation.

4.4.6 CHANGE IN SEIZURE FREQUENCIES

Despite not attaining seizure freedom, both the drug resistant and undefined group of PWE were evaluated for change in their seizure frequencies. This is, in part, to gauge the effectiveness of AEDs usage in our setting. Inidvidual subject's pre-treatment and current seizure occurrence classes were compared to reflect the change in seizure frequencies. As depicted in Figure 5, approximately half of the PWE in 'undefined' group and drug resistant groups had benefitted from AED/s therapy, reflected by a decline in their seizure frequencies. In contrast, 18.2% of the PWE from 'undefined' and 15.0% from drug resistant group suffered increased seizure frequencies while the rest was documented to have no changes. Previously, the pre-treatment median seizure frequencies were biweekly and monthly, for drug resistant and 'undefined' group, respectively. Currently, the median seizure frequencies were reduced to monthly in the drug resistant group and once every four months for the 'undefined' group of PWE.

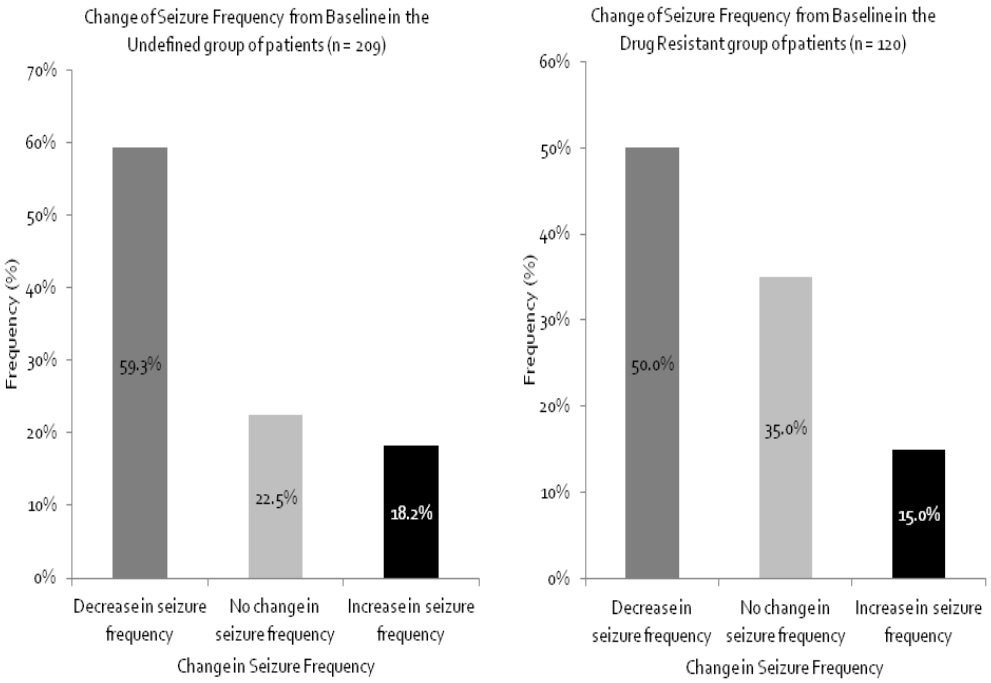

Figure 5. Change of seizure frequency from pre-treatment in the (left) Undefined and (right) Drug Resistant group

4.5 DISCUSSION

The demographics distribution of PWE was found to be comparable to a few studies done locally as well as overseas (5, 6, 96, 98, 99, 172, 173, 186, 187). The proportion of ethnicity was similar to those reported in an earlier Singapore study (5), with the additional of other races in this study. The previous study was conducted in Singaporean army men, and hence was naturally without 'other races'. Singapore is a well-known medical hub in the Asian region, and thus, not surprising that the current population includes some foreigners who seek medical services here. Although Chan et al reported a higher proportion of Malays (19.1%), their surveyed population was pediatrics, and included first afebrile seizure cases which was contrary to our adult population of all PWE.

The initial finding of significantly more Indians was drug resistant suggested that genetic make-up may have a role to play. Among Indians of South ancestry, a study conducted on ABCB1 gene which encodes P-glycoprotein transporter suggested that the association between the genes with refractory epilepsy could be random, simply due to the linkage of the gene to epilepsy itself, rather than resistant epilepsy specifically (188). Similarly, our finding could be random, considering the difference was no longer observed after adjustment made to the other parameters listed in Table 3. It will be interesting to investigate the true effect of ethnicity on rendering a PWE to be drug-responsive or resistant using a larger population.

Despite differences in definition, prevalence of DRE in this study conformed with those found in the French study, where the prevalence ranged from 15.6% to 22.5%, depending on the criteria of seizure occurrence; either once in 18 months or once in 12 months (96). It was also similar to the rate of intractable seizure in a Finnish paediatric population after 30 years of follow up (172), and 25% refractory epilepsy in a Glasgow study (97). Refractory epilepsy rate in Singaporean men was once reported to be 13% (189). However, this study was done in a carefully selected cohort, aged at 18 years old that had excluded PWE with incomplete medical records. Hence, it was possible that the refractory rate reported at that time was an underestimation. In contrast to the Taiwan and western countries findings which had documented 25% – 40% of adult PWE being refractory, drug resistant prevalence rate in this study was lower (98, 99, 101, 187). It was speculated to be a result of the recent conception of ‘undefined’ group, which was not available at the time when previous studies were conducted. Moreover, the more defined criteria for drug responsive and resistant categorization may have caused many PWE to be undefined. For example, there were approximately 18 PWE who had no seizure occurrence for at least 12 months after their last AED intervention was in the ‘undefined’ group (Table 3). If according to previous studies which defined seizure freedom as no seizure occurrence within the last 12 months, these PWE would be taken as ‘drug

responsive' (96, 99). Since the current definition is 3 times the previous seizure free interval, which could be a total of more than 12 months, seizure free time lapsed over only 12 months was regarded as insufficient. Therefore, they were categorized as 'undefined'. If these PWE is categorized as drug responsive, the percentage of drug responsive PWE will likely attain 51%, while there seems to be no changes in the proportion of DRE. Another typical example of undefined case was: subjects who had multiple seizures per week for years, but were not prescribed the DDD due to unspecified reasons. A closer look revealed that most of the current 'sub-optimal' dosages in these subjects were once effective in controlling individual epilepsy. Based on some of the previously discussed studies, these subjects could have been classified as drug resistant too. Therefore, in summary, a combination of these scenarios may contribute to the lower rates of drug responsive and resistant PWE, observed in this study based on the recent ILAE consensus.

As expected, polytherapy prevails in the drug resistant group. Studies had shown that for PWE who did not achieve seizure freedom with their first AED trial, less than 20% will respond favourably to the following AEDs trial (107). This group of subjects was believed to be inherently more difficult to treat (190). Additionally, long term follow up cases were included in this study, implying that substitutions and additions of AEDs, if deemed required, would have already been tried. Since the use of AED combination was superior to monotherapy for PWE with inadequate response to their first or second AED (191), more than 1 AED could have been given in attempts to abate seizure recurrences. This phenomenon was especially important when the PWE suffered multiple seizures despite being on a few ineffective therapies.

Relapse, as manifested by one seizure occurrences after a seizure free period could be attributed to incompliance upon realizing the possibility of AED withdrawal (172). Among the patients with relapses in our study, after examining the previous actions taken before the relapse occurred, nineteen (76%) of those with 1 seizure occurrence after a previous seizure free period

(more than 3 times their previous seizure free intervals), received no changes in their therapy on their last visit to the clinic, while only 12% (n=3) had their AED dosages reduced. Although frequencies of these actions slightly differed from those reported by Callaghan et al., it suggested that reduction in AED dosages might not trigger seizure (192). However, this finding should be interpreted cautiously as it was only a point prevalence rate that was reported to aid in defining our 'undefined' category subjects and not to determine the probability of relapse in our PWE population.

Prospective studies done in the Japan, UK, US and Europe in the last few decades, mostly utilizing treatment naïve PWE, had managed to unravel a few potential predictors for developing DRE. These factors were namely the presence of mesial temporal sclerosis or lesional region in the brain, high number of pre-treatment seizures, poor response towards the first AED prescribed, earlier age of seizure onset, presence of mental retardation and psychiatric illnesses (101, 106, 107, 110, 193). Our findings of risk factors were comparable to these previous works. PWE with structural-metabolic etiology, formerly termed as symptomatic, had been reported to negatively affect the likelihood for remission, a finding that echoed the other studies (96, 101, 106, 172). It was speculated to be harder to treat because the influential factor does not seem to originate from the focal epileptogenic zone (194). Seizures probably arise from a more complex network, which could be from any triggers or any parts of the brain.

Contrasting to a few earlier studies (98, 186), mesial temporal sclerosis (MTS) was not a risk factor in our population. It was noteworthy that most of the PWE in our center with operable lesion, inclusive of MTS had already undergone surgery. Many of these operated PWE had attained seizure freedom for more than 5 to 10 years and were already discharged from further follow up visits prior to this study initiation. There was a small number of PWE who chose not to have surgery (data not shown), even though they are good candidates for this intervention. These scenarios differed from other

populations where MTS was still intact at the point of their investigation. In a randomized trial done in the US, resective surgery combined with AEDs demonstrated a reduction in seizure occurrences when compared to using AEDs alone (odds ratio=∞; 95%CI 11.8 to ∞; $p < 0.001$) (195). This projected improvement in seizure control by a subgroup of operated subjects, coupled with a small fraction of subjects with more benign form of MTS, may account for the lack of contribution by MTS to DRE in this study, as demonstrated in multivariate regression.

Aside from MTS, younger age of seizure onset and higher frequencies of pre-treatment seizures were also associated with a higher risk of developing drug resistant (12, 99, 101, 172, 173, 187). These characteristics could be signs of a more resistant type of epilepsy (101, 196). Although the influence of age appears to be only marginally negative, odds ratio of 0.96, it is still a powerful predictor considering that with every one year of increase in seizure onset age, there is a 4% decrease in risk of becoming drug resistant.

In the chi square analysis, however, PWE who were in the sixty years of age (60 - 69 years old) demonstrated superior seizure freedom prevalence than the other age groups. Age of epilepsy onset had been suggested to be a major predictor of DRE, with those presented at the age of ≥ 65 years are less likely to develop DRE (111, 197). It could be inferred that the underlying pathogenesis of epilepsy varies by age group. Additionally, recognition of seizures in the elderly itself is challenging, especially by the non-medically trained family members. This could result in under-recognition and under-reporting of seizures recurrence in this age group. It is tempting to attribute the high frequency of remission to the dynamic nature of the disease itself (198-200), although evidence to support the optimal age at sixties, is lacking. An alternative explanation would be the changes in lifestyle. Experts suggested that as one matures, it is likely that one will become more conscious of well-being. Therefore, one become more compliant, emphasizes on sufficient rest and sleep, practices exercise such as tai-chi and yoga (which encourages

serenity) as well as avoids known triggers, most commonly documented in our setting; alcohol and long duration of computer use (201). Interestingly, sixty to sixty-five years old are the general retirement age range in Singapore. We speculate that lifestyle changes in this group of PWE could have improved their seizure control. In addition, the low prevalence rate of DRE did continue to subjects >70 years old, although the difference was not statistically significant, likely confounded by the small number of PWE in that age group (Figure 3).

Presence of psychiatric illnesses or mental retardation was known to increase the risk of unprovoked seizure (98, 172, 202-205). Our PWE with either one of these conditions were at 2 to 3 fold higher risk of persistent seizures. It is generally perceived that psychiatric disorders could further perturb the already imbalanced neuronal transmissions in the brains of PWE. This in turn may worsen the epileptogenicity and render it more difficult to be treated (99, 206). Likewise, this complexity of neuronal network interactions might occur in mental deficiency cases. Moreover, mentally deficient PWE could have difficulty in adhering to their prescribed regimen, unless they were accompanied by dedicated caretakers. It is noteworthy that there were approximately 5% of mentally deficient PWE in the drug responsive group. This outlined that although they are likely to have DRE, it is still possible for them to attain remission, as shown by Sillanpaa and Schmidt, 2006, where 35% of patients with mental retardation and symptomatic etiology may possibly achieve terminal remission (200).

4.6 CHAPTER CONCLUSION

Based on our findings, the recommendations from the ILAE global consensus for the definition of DRE are realistic and practical. Although the definitions used differed slightly with some earlier studies, the resulting

prevalence and risk factors determination still conforms to each other. The recent consensus provides a detailed description of our PWE group, without overrepresentation of either drug responsive or drug resistant. In view of the long and dynamic course of epilepsy, our clinicians adopted 'prescribe, wait and assess' strategy. This is clearly depicted from the 'undefined' PWE, where sufficient time is allowed to elapse before adjustment is made to their pharmacotherapy. By identifying the 'undefined', it allows our clinicians to include other aspects in their routine management of insufficient control PWE. To illustrate, in PWE who are satisfied with their current therapy but have not achieved seizure freedom, prompt counselling and referral to social support group may change their perception about receiving alternative therapies.

We acknowledged that this study is limited by several factors. Firstly, it was conducted only in one hospital – based referral center. It is generally perceived to have a higher proportion of drug resistant patients and the current prevalence may be an over estimation. Secondly, the retrospective natures of the study design, which is well-known to be restricted by the availability of data. Genetic testing results were not available for most of the PWE, as it is not routinely performed. However, due to the site being a neurology specialist clinic, most of the essential information required for accurate diagnosis and drug response categorization were readily available. Hence, it cannot be guaranteed that the documentation is as complete if studies were to be conducted in primary care settings. Nevertheless, our observations should be reflective of the usual clinical practices around the globe.

In conclusion, this study has managed to determine the prevalence rate of DRE in our population of PWE. At any one time, 1 in 5 PWE could have DRE. PWE with structural-metabolic etiology, concurrent psychiatric illnesses or mental deficiency and pretreatment seizure frequencies of more than once a month are at increased risk of developing DRE. Gender, ethnicity, focal seizures and presence of MTS do not seem to hinder seizure remission. Elderly PWE, in particular, those aged from 60 to 69, may have a higher

chance to be seizure free. This study could alert clinicians of the informative parameters that should be assessed and recorded during clinical consultations in order to determine PWE's treatment responses and offer timely alternatives. The findings may also aid policy makers in designing treatment guidelines and allocating resources around PWE.

With these findings, the first objective was achieved whereby DRE is still a significant on-going issue in PWE that deserves more attention. Moreover, genetic etiology and ethnicity did not emerge as prominent risk factors associated with DRE. Structural-metabolic etiology and psychiatric disorder/mental insufficiency which are known to have imbalances in neurometabolite transmitters were strongly linked to DRE occurrences. Metabolomics is hence, deemed as a rational approach. In the next chapter, the applicability of dried blood spot as sampling matrix for metabolomic study will be presented before the profiles between drug responsive and drug resistant subjects are determined.

CHAPTER 5

Evaluation of Dried Blood Spot as Sample Matrix for Gas Chromatography-Mass Spectrometry based Metabolomic Profiling

CHAPTER 5. EVALUATION OF DRIED BLOOD SPOTS AS SAMPLE MATRIX FOR GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS) BASED METABOLOMIC PROFILING

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5.1 CHAPTER ABSTRACT

We propose using DBS as sample matrix for GC-MS based metabolomic profiling for the benefits of higher sample stability, more convenient sample acquisition with DBS; higher analyte separation power, and readily analyte identification with GC-MS analyte libraries. To establish this proposition, the metabolomic profiles generated from DBS were compared with that obtained from the conventional whole blood and plasma matrices; and also with dried plasma spots (DPS) as another covariate control. Our findings indicated that whole blood produced the most number of detectable markers (866), whereas DPS yielded the least number (614). DBS and plasma matrix, on the other hand, produced the most similar numbers of detectable (695 vs 749) and identifiable markers (137 vs 147, matching with Fiehn library). From the analysis of the DBS and plasma metabolomic profiles, it was concluded that when L-lysine 2, iminodiacetic acid 2, DL-threo-beta-hydroxyaspartic acid, citric acid or adenosine-5-monophosphate 2 are not involved as markers, DBS could be a suitable substitute for plasma for metabolomic profiling.

5.2 CHAPTER INTRODUCTION

Metabolomics is a rapidly developing field and has brought about many contemporary discoveries, among which are metabolite-biomarkers for diseases. Some of these findings include biomarkers for Crohn's disease (207) and Onchocerciasis (208). The analytical tools commonly used in metabolites detection include nuclear magnetic resonance (NMR) spectroscopy, gas chromatography (GC), high performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS), and the more specialized Fourier transform infra-red (FTIR) spectroscopy. The analysis of the data obtained from the untargeted screening of metabolites has been eased with the advances in chemometric and bioinformatic methods. The clinical interpretation of the plethora data obtained from metabolic profiles of biological samples has also been enhanced. The usual biological samples used are plasma, urine, amniotic fluids, cerebrospinal fluid and tissue extracts, and more recently, dried blood spot.

DBS is a sampling method popularized since the 1960s, when it was introduced for the screening of a metabolic disease, phenylketonuria, in newborns. DBS has recently been extensively researched for its other potential such as screening for folate deficiency (141), hyperthyroidism (142) and metabolite profiling (146). DBS represents an alternative that overcomes many of current plasma metabolome problematic issues such as stability of metabolites in plasma or blood samples after acquisition and the invasive sampling technique involved.

Very recently, biofluid spotting has been shown to be a potential alternative to conventional plasma in metabolite profiling using UPLC oa-TOF-MS (146). In this regard, GC-MS has several advantages over UPLC-MS in offering high separating power and sensitivity, and generally better reproducibility of concentration and retention times (209). An introduction of chiral derivatization as part of sample preparation for GC-MS can help generating a new chiral center and render separation as well as identification

of the specific enantiomers from the endogenous metabolites (210, 211). There is also a readily available library created especially for the analysis of human metabolome e.g. the Fiehn library and Human Metabolome Database (HMDB). As GC-MS is one of the most widely used analytical tool in profiling of primary metabolites (212), it will be interesting to investigate the range of its detectable metabolites in different matrices for metabolomic profiling.

This has led us to our proposition of using GC-MS to explore the type of metabolites that might be identifiable when using DBS as sample matrix, and comparing with that generated from the conventional whole blood and plasma samples. To serve as a covariate for comparison, metabolomic profile in dried plasma spots was also investigated. We hypothesized that these findings might aid in refining the selection of sampling matrix that best fits the type of metabolomic studies.

5.3 MATERIALS & METHODS

5.3.1 CHEMICALS

Methanol of analytical grade was purchased from Prime Products Pte Ltd. (Singapore); and caffeine, palmitic acid 99% and cholesterol 99% were purchased from Sigma-Aldrich (St. Louis, MO). D,L α alanine was purchased from Merck & Co. (Whitehouse Station, NJ). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Thermo Scientific Pte. Ltd. (Waltham, MA). Deionized water was obtained from a Milli-Q system (Millipore, Boston, MA) and used throughout the experiment.

5.3.2 ANIMAL STUDY

The *in vivo* metabolic study was carried out according to the "Guidelines on the Care and Use of Animals for Scientific Purposes" (National Advisory Committee for Laboratory Animal Research, Singapore, 2004). The animal handling procedures of this study were reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS).

Sprague-Dawley rats (7-8 weeks old) were purchased from Comparative Medicine Center (CMC) of NUS. The rats were kept at a specific pathogen free animal facility (24°C, 60% relative humidity) at CMC and maintained on a 12-h light/dark cycle with free access to food and water. Twenty four hours before the metabolic study, a polyethylene tube (I.D. 0.58 mm, O.D. 0.965 mm, Becton Dickinson, Sparks, MD 21152, USA) was inserted into the right jugular vein under isoflurane anesthesia. This catheter was used for blood sampling at 9am on the study day. Blood samples (approximately 1 mL) from each of the 3 male rats were collected and kept in ice box until they were transferred back to laboratory for processing. Within 30 min of collection, each collected blood sample was aliquoted in triplicates of 25 µL into a 1.5 mL of Ependorff tube (Hamburg, Germany); and spotted onto 903® cards (903® Neonate Blood Collection Cards, Whatman GmbH, Dassel, Germany) respectively. The remaining blood sample was then centrifuged at 3,000 g (4 °C) for 10 min, the plasma collected was treated similarly as the blood sample (i.e., 25 µL of plasma aliquoted into 3 x 1.5 mL of Ependorff tube; and spotted in triplicates onto 903® cards). The spotted blood and plasma were first left dried in the fume hood for at least 3 hours, which was the observed minimum time required for complete dryness. During this drying period, the blood and plasma samples were stored at -80 °C. Then, they were processed together with the DBS and DPS samples 3 hours later.

5.3.3 SAMPLE PROCESSING

The processing of the dried blood spot (DBS), dried plasma spot (DPS), the blood and plasma samples were carried out as similarly as possible, to minimize bias. The DBS and DPS were punched and the cores (6 mm) obtained were placed inside the respective Ependorff tubes. Extraction of DBS and DPS was done using 160 µL of methanol (Prime Products Pte. Ltd.) with caffeine (Sigma-Aldrich Pte. Ltd.) as the internal standard at a concentration of 6.25 ng/mL and vortexed for 20 min. The blood and plasma samples were thawed once at 22°C and mixed well before extraction. 160 µL of cold methanol (-20°C) with caffeine (6.25 ng/mL) was used to precipitate protein and to extract both blood and plasma, which were then vortexed for 1 minute. The extracts were centrifuged at 20 800 *g*, 22°C for 10 min and 120 µL of the resulting supernatant would be transferred to 15 mL Kimble centrifuge glass tubes (Gerresheimer Co. Glass, Germany) for evaporation. First drying was done at 40°C under a stream of nitrogen. Thereafter, 100 µL of toluene was added, vortexed for 15 seconds and subjected to the similar condition for drying again. Toluene was used to ensure removal of water. Next, derivatization of the dried sample was accomplished using 100 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Thermo Scientific Pte. Ltd.). The derivatized samples were vortexed for 1 minute and incubated at 60°C for 1 hour. After being cooled to room temperature, the products of derivatization were centrifuged at 3500 *g*, 22°C for 20 min. Finally, 90 µL of this final supernatant was transferred into 200 µL conical base inert glass insert placed inside a 2 mL amber autosampler glass vial (Agilent Technologies, Germany).

5.3.4 STABILITY OF METABOLITES IN DBS

In metabolomic studies, there seems to be no generally accepted criteria for assessing the stability. Here, we adopted the approach by Zelena et al (213), that accepted a tolerance of 20% in variation in the analyte levels as stable for metabolomic analysis. Since we were interested in the detection of a wide range of metabolites rather than any specific metabolite, the detected responses of ten metabolites across the analytical time were randomly chosen to be compared. These metabolites could represent compounds of different physicochemical properties as reflected by their differences in retention times in the chromatographic separation. The stability of these ten metabolites in triplicates of DBSs stored at either 25°C or - 20°C at intervals of 0, 6, 24 and 48 hrs after complete drying were then evaluated.

5.3.5 RECOVERIES OF METABOLITES FROM DBS

It is impossible to evaluate recoveries of all metabolites detected. Therefore, the recoveries of three analytes which eluted at three distinct retention times; alanine (~7.00 min), palmitic acid (~18.45 min) and cholesterol (~27.00 min) were selected and investigated. Blood samples from the three rats were pooled and triplicates of quality control (QC) blood spots were spotted onto the 903® cards. Then, triplicates of DBS prepared with blood samples spiked with the three analytes at various concentrations were processed similarly as above. Since these analytes are naturally present in blood, the concentrations spiked were at least 50% higher than the normal blood concentrations i.e. 1 µg/mL and 100 µg/mL for alanine; 1 mg/mL and 5 mg/mL for palmitic acid; 1 mg/mL and 2 mg/mL for cholesterol. The standard solutions for the respective analytes were prepared in distilled water for alanine and in methanol for palmitic acid and cholesterol. Absolute recovery was calculated using the formula as follows:

$$\text{Absolute recovery} = \frac{\text{Response of analyte in the spiked DBS} - \text{Response of analyte in the QC DBS}}{\text{Response of analyte with the spiked amount in methanol/water}}$$

5.3.6 GC-MS SETTINGS

Analyses were done using GC-MS that comprised of 7890A GC System coupled to 5975 inert MSD with Triple-Axis Detector (Agilent Technologies) and in-built Fiehn method of analysis for metabolomic screening (214). The in-built settings included injector temperature at 250°C, split ratio of 10:1 at the starting temperature of 60°C held for 1 min, increased at 10°C per min to 325°C followed by a final hold for 10 min (total run time of 37.5 min). Ion source temperature was set to 250°C, while the scanning mass range was set as 50 to 600 *m/z*.

5.3.7 STATISTICAL SOFTWARE

Automated Mass Spectral Deconvolution and Identification System (AMDIS) 32 Version 2.66, 2008 was used to deconvolute the data. The minimum match factor of 40% was utilized while other deconvolution settings included adjacent peak subtraction of one, medium resolution of the peaks with high sensitivity and medium shape requirements. They were then matched against their putative identities with Fiehn library (with the addition of caffeine). The peak list data acquired were subjected to multivariate analyses by Mass Profiler Professional (MPP) Version B.02.00 (Agilent Technologies, Inc.). In the analysis of the MPP parameters, a retention time tolerance of 0.1 minute, with matching factor of at least 0.6 was adopted. Microsoft Office Excel 2007 was used for other compilation.

5.4 RESULTS

5.4.1 METABOLITES DETECTED FROM THE FOUR SAMPLE MATRICES

A typical total ion chromatogram (TIC) for each of the samples (DBS / Blood / DPS / Plasma) showed similarities in the initial detected peaks (Figure 6). After deconvolution, the total number of detected metabolites were 866 for blood with 268 (30.9%) of reproducible compounds, 749 for plasma with 237 (31.6%) of reproducible compounds, 695 for DBS with 196 (28.2%) of reproducible compounds and 614 for DPS with 178 (29%) of reproducible compounds. The reproducibility is defined as the presence of a compound in at least 2 out of 3 technical replicates in at least 2 of the 3 rats. Among the reproducible TICs, the numbers of identified compounds were 171, 147, 137 and 121 for the blood, plasma, DBS and DPS matrices, respectively.

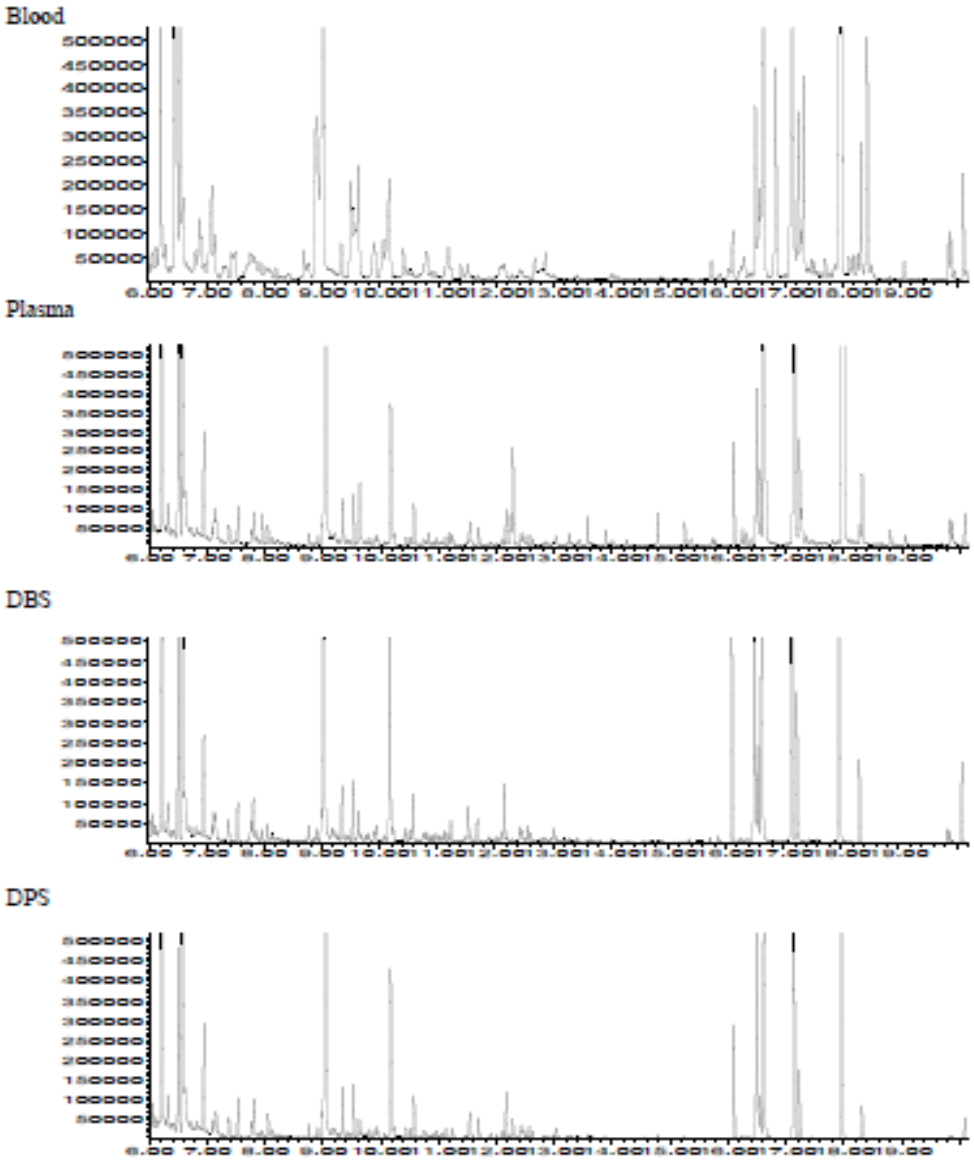

Figure 6. Typical total ion chromatogram (TIC) for the respective blood, plasma, dried blood spot (DBS) and dried plasma spot (DPS) matrices. The chromatograms are truncated at 20 min.

The deconvoluted data with their putative identity lists, normalized against the internal standard (caffeine) and adjusted to median across samples, were then subjected to principal component analysis (PCA). The data clustered differently according to the type of sample (Figure 7, left). Similar clustering was also seen with partial least square discrimination analysis (Figure 7, right) and hierarchical clustering (Figure 8).

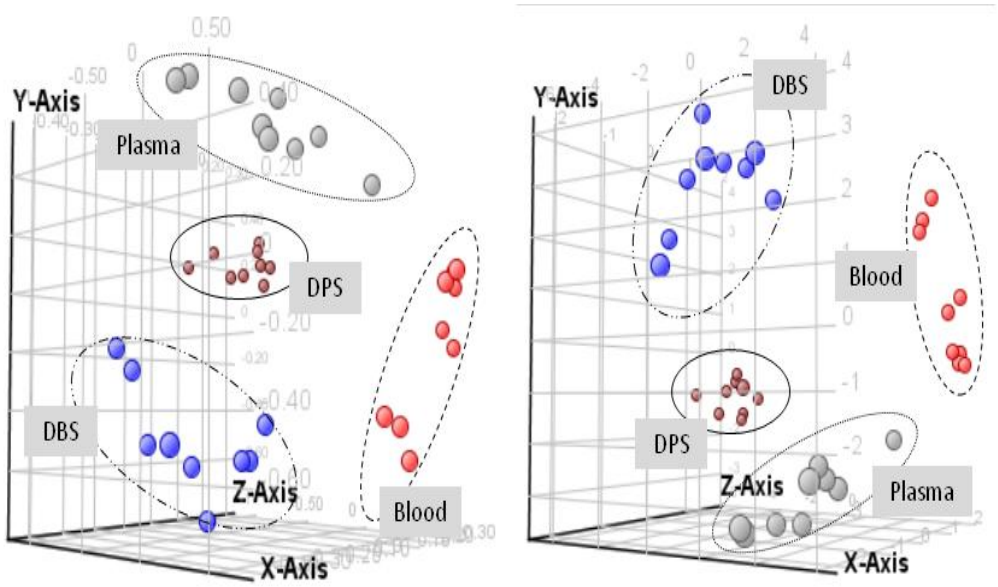

Figure 7. (left) Principal component analysis (PCA) and (right) partial least square (PLS) score plots for the 4 sample matrices: blood, plasma, dried blood spot (DBS) and dried plasma spot

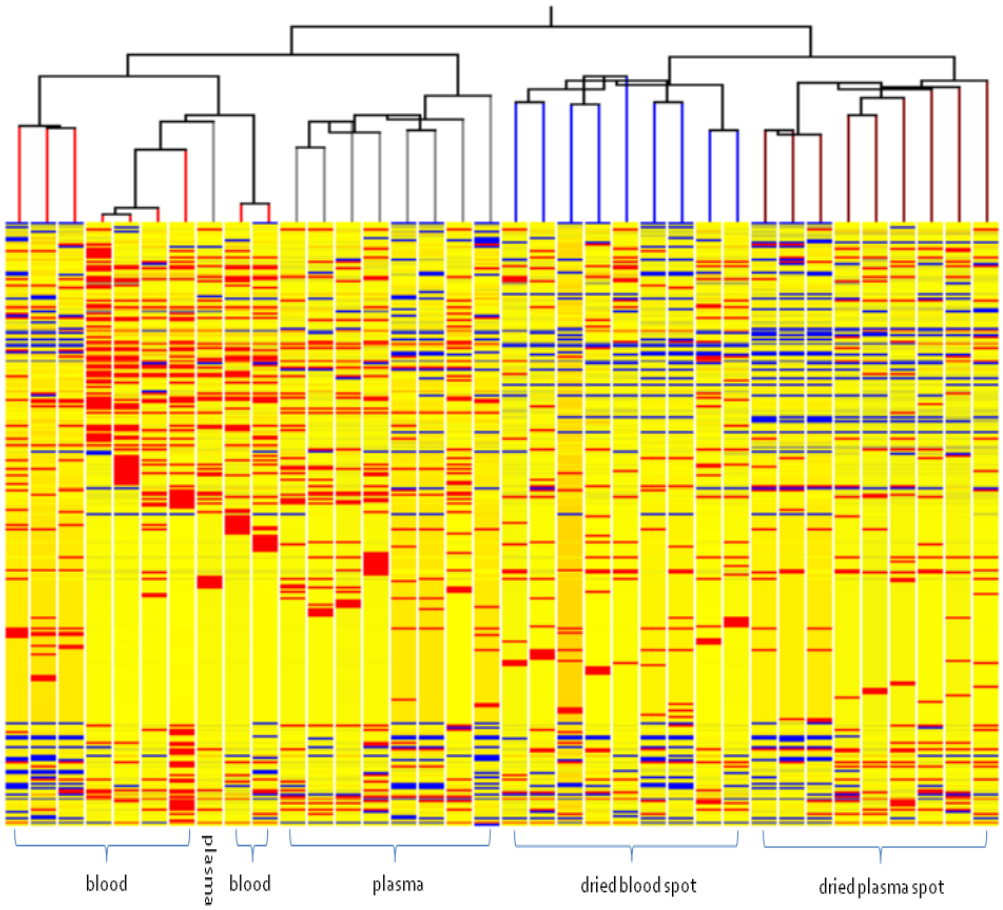

Figure 8. Hierachical clusters of the 4 sample matrices: blood, plasma, dried blood spot and dried plasma with 9 replicates each

Table 6. MPP derived discriminating compounds for the four sample matrices: DBS, blood, DPS, and plasma in number of fold change in relative to the internal standard
(L – 5 to 10 fold change; M – 10 to 15 fold change; H - more than 15 fold change; X-absent)

Compound	DBS	Blood	DPS	Plasma
urea	L	H	L	H
stearic acid	H	L	L	L
phosphoric acid	M	H	L	M
palmitic acid	H	L	L	L
oleic acid	H	H	X	H
methyl-beta-D-galactopyranoside	L	H	L	H
methyl linolenate	L	H	X	L
L-threonine 2	M	H	X	L
L-lysine 2	X	H	X	L
linoleic acid	H	H	L	H
L-glutamine 3	L	H	X	L
L-glutamic acid 3 (dehydrated)	L	H	L	L
L-alanine 1	L	H	X	L
L-(+) lactic acid	L	H	L	H
iminodiacetic acid 2	X	L	L	H
glycine	L	H	X	L
DL-threo-beta-hydroxyaspartic acid	X	L	X	H
citric acid	X	H	X	H
cholesterol	M	H	X	L
allo-inositol	L	H	X	H
adipamide 3	X	L	X	H
adenosine-5-monophosphate 2	X	H	X	X
79.0@21.1883	L	H	X	M
2-amino-2-methyl-1,3-propanediol 2	H	H	L	M
204.0@17.228256	L	H	X	M
204.0@16.245567	L	M	L	H

Generally, metabolites in the blood and plasma samples gave higher peak areas for similar compounds found in DBS and DPS. It is expected as the whole 25 µl was subjected to sample processing for the blood and plasma samples. However, for the DBS and DPS, 25 µl was spotted onto the 903® cards, and from which a disc of 6 mm in diameter that would contain less than 25 µl of sample was punched out from the middle of the spot for further sample processing. The compounds that were identified as the discriminating

metabolites among the 4 sample matrices are listed in Table 6, with most of them present abundantly in blood, but lesser or absent in DBS, DPS or plasma.

5.4.2 STABILITY OF METABOLITES IN DBS

Table 7. Average responses and standard deviations of 10 metabolites detected in DBS stored in freezer at (-20 °C) for 0, 6, 24 and 48 hr. The last column showed the relative standard deviation (RSD) of the average responses in the detection of the respective metabolites over the 48 hour period

Peak	Time (min)	0 hr	6 hr	24 hr	48 hr	RSD (%)
		Average±SD	Average±SD	Average±SD	Average±SD	
1	6.37	29.20±1.63	27.24±3.72	24.22±0.86	25.53±2.28	5.90
2	6.46	25.12±1.62	25.46±1.75	24.09±1.16	24.54±0.87	2.84
3	7.08	20.18±1.57	21.93±3.47	20.03±0.78	20.66±0.83	4.62
4	7.11	20.29±1.53	20.94±2.36	18.03±0.43	18.65±1.63	8.00
5	7.80	6.22±1.35	6.09±0.92	6.04±0.03	6.11±0.48	0.62
6	8.89	19.78±1.29	21.47±0.90	22.06±0.22	21.31±2.21	1.83
7	9.45	9.02±0.58	8.37±2.20	9.61±0.77	9.63±0.69	7.84
8	16.86	47.26±4.83	40.28±5.74	43.49±3.44	52.79±4.60	14.27
9	18.46	3.62±0.28	4.33±0.52	4.42±0.21	4.27±0.52	1.70
10	27.00	30.83±0.80	41.29±2.12	32.72±7.29	44.70±9.70	15.58

Table 8. Average responses and standard deviations of 10 metabolites detected in DBS stored at room temperature (25°C) for 0, 6, 24 and 48 hr. The last column showed the relative standard deviation (RSD) of the average response in the detection of the respective metabolites over the 48 hour period

Peak	Time (min)	0 hr	6 hr	24 hr	48 hr	RSD (%)
		Average±SD	Average±SD	Average±SD	Average±SD	
1	6.37	29.20±1.63	24.96±1.46	25.31±1.05	26.42±2.21	5.86
2	6.46	25.12±1.62	22.80±0.95	25.36±0.41	25.77±2.08	4.16
3	7.08	20.18±1.57	19.08±1.06	20.71±0.34	21.65±1.62	5.55
4	7.11	20.29±1.53	17.37±0.80	19.32±0.60	20.34±1.44	4.62
5	7.80	6.22±1.35	5.66±0.54	5.98±0.09	5.87±0.39	9.45
6	8.89	19.78±1.29	22.16±1.32	21.62±0.83	22.45±2.52	5.98
7	9.45	9.02±0.58	9.74±0.23	9.83±0.90	9.82±1.25	2.38
8	16.86	47.26±4.83	53.53±5.26	40.19±5.03	49.16±5.34	9.83
9	18.46	3.62±0.28	3.75±0.31	4.27±0.22	5.08±0.30	3.32
10	27.00	30.83±0.80	31.79±5.11	42.09±12.41	47.31±3.93	16.07

Tables 7 and 8 showed the detected responses of 10 selected metabolites in DBS stored at -20^{0}C and 25^{0}C respectively. The results indicated that the relative standard deviations of responses of each of these metabolites over the time period from 0 to 48 hrs were all less than 20% at both storage temperatures. These were within our tolerance acceptance range, suggesting stability of the metabolites in the matrix.

5.4.3 RECOVERIES OF METABOLITES FROM DBS

Table 9. The percentage recoveries from DBS of alanine, palmitic acid and cholesterol spiked at two different concentrations

Analyte	Concentration	Mean absolute recoveries ± SD (%)
Alanine	1 µg/mL	125.32 ± 0.37
	100 µg/mL	88.94 ±1.26
Palmitic acid	1 mg/mL	99.45 ± 1.92
	5 mg/mL	98.81 ± 6.15
Cholesterol	1 mg/mL	69.77 ±1.84
	2mg/mL	55.58 ±1.96

The differences in the mean absolute recovery between the 2 concentrations were less than 1% and 15% for palmitic acid and cholesterol respectively; but for alanine, the difference was more than 30% , probably due to the vast difference in the 2 concentration for this analyte (1 vs 100 µg/ml) (Table 9). Nevertheless, the recoveries of these metabolites at any one concentration remained consistent across the triplicates, as reflected by the small standard deviations (less than 2% for most cases).

5.5 DISCUSSION

Although direct comparison cannot be made in the detection ability among different metabolomic studies, especially when they use different instrument platforms, the number of identified peaks from plasma, DBS and DPS extracts in our study appeared to be comparable to that detected in serum

in the study by Begley et al. (Table 10) (215). The smaller number of detected peaks in our study as compared to those from UPLC-oa-TOF-MS could be due to multiple factors. Firstly, the metabolites detected through GC-MS include only low-polarity volatile metabolites such as fats and esters, and high-polarity metabolites of amino acids and organic acids converted into volatile derivatives (216). Hence, there is a chance that non-volatile compounds are not detected. Secondly, the derivatization itself may affect certain classes of metabolites and hamper the detection of compounds (217). The putative identities of compounds, however, could not be made in the study using UPLC coupled to oa-TOF-MS (146). This hindered our ability to discern the type of metabolites identified through different analytical tools. Nevertheless, within our own settings, blood, as expected, provided the most number of identified compounds while DPS yielded the least number.

Table 10. Comparison of the numbers of the detectable and identifiable markers using different sample matrices and analysis platforms among different studies

Study	**Michopolous et al, 2010** (146)		**Begley et al, 2009** (215)		**This study**	
Analytical tool	UPLC – ao-TOF-MS		GC-TOF		GC-MS	
Type of sample	Rat		Human		Rat	
Results	Number of detected ions* and peak**					
	*Plasma	954	**Serum	250	**Plasma	749
	*DBS	1239			**DBS	695
	*DPS	1039			**DPS	614
					**Blood	866
	Number of reproducible ions* and peak**					
	*Plasma	572	**Serum	250	**Plasma	237
	*DBS	768			**DBS	196
	*DPS	208			**DPS	178
					**Blood	268
	Number of identified targets					
	Plasma	N/A	Serum	130	Plasma	147
	DBS	N/A			DBS	137
	DPS	N/A			DPS	121
					Blood	171

The metabolites (as putatively identified by Fiehn Library in GCMS) lost after processing by blotting blood or plasma onto 903® cards could be due

to binding to the paper or lost on drying, which had been reported before (158). A better approach could be to dry the spotted biological fluids in an enclosed chamber with flowing inert gas to minimize degradation or loss due to oxidation. Nevertheless, in our experiment, upon drying, the 10 metabolites randomly selected across the analysis time appeared to be stable in DBS for up to 48 hours in our study. However, we cannot eliminate the possibility that some other metabolites may be degraded under the sample storage and process conditions. This scenario could also be applied to any sample matrix. Therefore, it is paramount to have all samples stored and processed under similar conditions, so that any difference in the metabolic profiles could be due to the experimental covariates rather than to the experimental artifacts.

The recoveries of metabolites from DBS were found to be consistent for the particular analyte at the specific concentration. However, for some metabolites as demonstrated by the example of alanine, the absolute recoveries could be different at different concentration levels. Since it is impossible to predict the concentration level of an individual analyte in the respective sample, we may have to accept this as a limitation in metabolomic study, and be aware that the difference in some of the metabolite levels could be due to difference in recoveries of analytes during sample extraction.

We have attained our aims whereby, in delineating the differentiating types of detectable metabolites (as matched against Fiehn Library) from the blood, plasma, DBS and DPS sample matrices. There are overlapping compounds between DBS, DPS and blood with plasma, suggesting that either one of these may be able to substitute the conventional plasma as the tool for metabolomic studies. Of note, DBS offers great advantages as it can be obtained easily as compared to the plasma; only one needle prick and an approximately 25 µL of blood volume is required as compared to the more invasive technique involved in acquiring the plasma sample. In comparison to blood, the dried blood spots are easier to be stored and transported. Moreover, once dried, the chances for the metabolites in DBS to degrade can be reduced,

as they will not be subjected to hydrolysis degradation. Generally, most chemicals are more stable in the dried form than in the aqueous form. In our study, there seemed to be more metabolites detected from the whole blood. This can be explained by the situation that both the whole blood and plasma samples were frozen shortly after collection; whereas the DBS and DPS were allowed to dry in air at room temperature for approximately 3 hrs. During this period, some naturally occurring enzymes in whole blood or plasma may hasten the degradation of certain metabolites. Alternatively, the lower numbers of metabolites recovered from DBS and DPS could be due to binding of some metabolites to the 903® cards and they were not readily extracted from the paper during sample processing.

5.6 CHAPTER CONCLUSION

This experiment is meant to be explore the use of DBS as sample matrix for GC-MS based metabolomic profiling. In our study, it was found that the number of detected metabolites in DBS was not much less than that from the plasma sample, which is a gold standard matrix in metabolomic studies. In our study, most of the discriminating compounds have a putative identity in the Fiehn library. Nevertheless, if there are any compounds identified to be promising biomarkers or new metabolites detected but without identity, the chromatogram could be matched against other libraries, such as HMDB or NIST for a wider search for identification.

Since most of the compounds detected in plasma, are also presenting in DBS extracts, DBS could be a substitute to plasma in metabolite profiling. However, caution has to be taken in studies where L-lysine 2, iminodiacetic acid 2, DL-threo-beta-hydroxyaspartic acid, citric acid or adenosine-5-monophosphate 2 is potential biomarker. As these metabolites are absent in DBS (Table 6), DBS will not be a useful proxy as a sample matrix for these

studies. It warrants investigating the cause for the loss of these metabolites from DBS and endeavoring to overcome these shortcomings with the objective to further increase the number of detectable compounds in DBS. That will make DBS an even more formidable tool for metabolomic studies.

Two animal studies were later developed with the aim to ascertain DBS capability to capture metabolite fluctuations *in vivo*. Modified approach using double solvent extraction for compounds in DBS was conducted to improve the extraction process. Simultaneously, individual detected compounds were examined for the presence of any of the L-lysine 2, iminodiacetic acid 2, DL-threo-beta-hydroxyaspartic acid, citric acid or adenosine-5-monophosphate 2 to establish DBS comparability to plasma. The results are presented in Appendices I and II.

CHAPTER 6

Use of Dried Blood Spot for Metabolomics Profiling of Response using Gas Chromatography-Mass Spectrometry in the Long-term Drug Treatment of Epileptic Patients

CHAPTER 6. USE OF DRIED BLOOD SPOT FOR METABOLOMIC PROFILING OF RESPONSE USING GAS CHROMATOGRAPHY MASS SPECTROMETRY IN THE LONG-TERM DRUG TREATMENT OF EPILEPTIC PATIENTS

Considering the comparability of the of the number of compounds detectable between plasma and DBS as well as the ability of DBS in discerning metabolite profiles from the previous animal models, use in our setting, we embarked on recruiting subjects for this study.

6.1 CHAPTER SUMMARY

Epilepsy from various origins, e.g. genetic or proteomic abnormality, seem to converge at the neurometabolite levels. In an attempt to understand the epileptogenesis of drug resistant subjects, we aim to investigate their metabolite profiles; how they may differ from drug responsive subjects. Local ethics committee approval was obtained. DBS was contributed via venipuncture or finger prick. GC-MS analysis, multivariate data processing and biological pathway visualization were applied for metabolomic profiling of drug responsive and drug resistant subjects. The profiles of 125 DBS samples, which were obtained from venipucture, the modelling failed to converge to privde a conclusive outcome using multivariate analyses. Only 17 DBS contributed via finger prick revealed clear distinction between these 2 groups of subjects. Seven discriminating metabolites and their related metabolism were highlighted in this study. These metabolites and pathways perturbations were comparable to those identified from earlier studies in epilepsy. Metabolomic profiles yielded from DBS of finger prick origin, appeared to be more informative attributable to its different concentration of some endogenous metabolites from the venous DBS. It may also be a good surrogate for CSF. Although DBS from venous blood did not unravel

significant metabolites in the profiles of drug resistant PWE, it could still be useful for targeted metabolite measurements. This work demonstrated the usefulness of DBS in understanding the altered pathology of a disease. A targeted assay has to be developed to quantitate the change in levels of the relevant metabolites to gauge the threshold above/below which, seizure occurrences become favourable.

6.2 CHAPTER INTRODUCTION

Gas chromatography with mass spectrometric detection is an effective analytical platform for metabolite profiling, as it is reproducible and robust due to the resultant consistent molecular ion fragmentation pattern from its electron impact (EI) ionization (218-220). This characteristic allows the construction of metabolites and analytes reference libraries such as the Fiehn, Wiley and National Institute of Standards and Technology (NIST) library of mass spectrum fingerprints for each individual compound.

Epilepsy has long been thought to be a disease which is closely related to genetics, probably related to the booming era of genomics in the 1960s. Research done had also showed that epilepsy could arise from various origins, e.g. genetic or proteomic abnormality. Despite the obvious differences, they seem to share similar physiological changes at the metabolite levels (221). The manifestation in acute epileptogenesis with increase in excitatory neurotransmitter, glutamate and decrease in inhibitory neurotransmitter, GABA, further rationalize the research in metabolomics (28). Although researchers did not unravel any biomarker for drug response towards newer antiepileptic drugs (AED) using metabonomic approach in one study (109), PWE were shown to have lower serum levels of GABA, creatinine, L-threonine and L-tryptophan and higher levels of L-glutamate, glycine, glyceric acid, lactic acid, inositol and myristic acid when compared to healthy subjects in another study (38). The researchers for the former study attributed the lack

of findings to the mixed clinical phenotypes in their sample population. Additionally, 12-months follow up may be a relatively short duration to capture significant metabolite changes. Biomarkers of specific drug response may be too subtle for detection.

As shown in the Chapter 4, approximately 1 in 5 PWE will have DRE. In an attempt to understand the epileptogenesis of this group of subjects, we aim to investigate their metabolite profiles; how they may differ from drug responsive subjects. In contrast to Al Zweiri et al study, PWE who are on chronic AEDs treatment will be recruited in our study (109). We hypothesized that irregardless of their etiologies, seizure types and AEDs, there should be similar metabolite perturbations in all DRE subjects.

6.3 METHODS

6.3.1 PATIENT RECRUITMENT

The target sample size was estimated to be 50 subjects per group after consultation with the biostatistician and with reference to the previous metabolomics studies (38, 109). Local ethics committee approval was obtained. Study period was from October 2011 to August 2012. PWE who had venous blood sampling between 9 am to 12 pm on their visit to the neurology specialist clinic of a tertiary referral hospital were approached for informed consent. Two drops of blood, ~30 µL each, from their withdrawn blood in EDTA tube were spotted onto 903® cards (903® Neonate Blood Collection Cards, Whatman GmbH, Dassel, Germany) and dried at room temperature, 25ºC for at least 3 hours. PWE who did not have any blood test but were agreeable for donation of two drops of blood via finger prick, were also included. The first drop from finger prick was discarded per protocol (222). After drying, DBS samples were kept at -80ºC until analysis. Clinical records and hospital information system were reviewed for PWE characteristics,

clinical history and biochemistry results. Drug response categorization was done in accordance to the recent International League Against Epilepsy (ILAE) recommendations (50). Only PWE who were categorized to be drug responsive (Category 1) and drug resistant (Category 2) were included in the final analyses.

6.3.2 DRIED BLOOD SPOT SAMPLES PROCESSING

A 6-mm diameter DBS from each PWE was punched and extracted with double solvents. First solvent used was 160 µL of HPLC grade methanol (Prime Products Pte Ltd, Singapore) premixed with internal standards (IS). Three IS were used for retention time comparison; D3-L-glutamic acid, D27–myristic acid and D5-L-tryptophan at 6.25 ng/mL each. Vigorous vortexing was done for 20 min on a large capacity mixer (Glas-Col, USA) before centrifugating at 6000 *g*. One hundred twenty microliter of the clear supernatant was transferred into 15 mL silanized glass tube (Gerresheimer Co. Glass, Germany). Similar process was repeated for the second solvent, chloroform (Fisher Scientific International Inc., Hampton, New Hampshire) before transferring 120 µL of the resulting supernatant into the same glass tube. After gentle mixing, the combined supernatant was dried under nitrogen at 10 psi, 30ºC for 15 min. For complete removal of water, 100 µL of toluene was added and then evaporated under similar condition. Thereafter, derivatization with 100 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Thermo Scientific Pte. Ltd., Waltham, Massachusetts, USA) and incubation at an optimum 70ºC for 1 hr were carried out. Subsequently, the clear mixture was cooled to room temperature and 80 µL was transferred into 200 µL conical base inert glass insert inside a 2 mL amber glass vial (Agilent Technologies, Santa Clara, California, USA). Quality control (QC) samples were derived from pooled DBS extracts within each batch. Each QC sample was assigned to be injected at the beginning,

during and ending of its batch of samples. Their replicates were averaged before pattern recognition analysis.

6.3.3 GC-MS SETTINGS

GC-MS that comprised of 7890A GC system coupled to 5975 inert MSD with triple-axis detector (Agilent Technologies Inc., USA) and its in-built Fiehn method for metabolomic analysis, retention time locked to D27-myristic acid, was used in this study (214). MSD ChemStation E.02.01.1177 was utilized for data acquisition and peak area computation. DB5ms (30 m × 0.25 mm × 0.25 µm) capillary column was purchased from Agilent Technologies J&W, Inc.

6.3.4 STATISTICAL ANALYSIS

Chromatogram deconvolution was achieved using Agilent MassHunter Workstation Software Qualitative Analysis version B.03.01. Compound identification was matched against Fiehn library of metabolites; with at least 40% mass spectra match. Individual AEDs and relevant peaks were added to the compound library for identity matching & eliminated from compound lists before analysis. Metabolites that were present in less than 60% of the samples were also removed. Missing values were replaced with half of the minimum value detected within its category and finally, peak areas were normalized to internal standards. Pattern recognition software used was SIMCA P+ 12.0.1. T-test and other statistical analyses were done in Microsoft Excel 2007®. Biological pathway analyses for significant metabolites were conducted using Metabolomics Pathway Analysis (MetPA) (223, 224). MetPA is a freely available web-based metabolomics tool for biologic pathway analysis visualization which covers 874 metabolic pathways and 11 organisms (224).

6.4 RESULTS

6.4.1 CLINICAL OUTCOME

At the end of study period, a total of 244 PWE were recruited. 189 DBS were obtained via venous samples while 55 were via finger prick. Only 142 DBS were included in the final analysis. The other 102 DBS were excluded as their clinical drug responses were categorized as Undetermined, Category 3. Out of 142 DBS, 58 belonged to drug resistant PWE (Category 2) while 84 belonged to drug responsive PWE (Category 1).

6.4.2 METABOLITE PROFILES

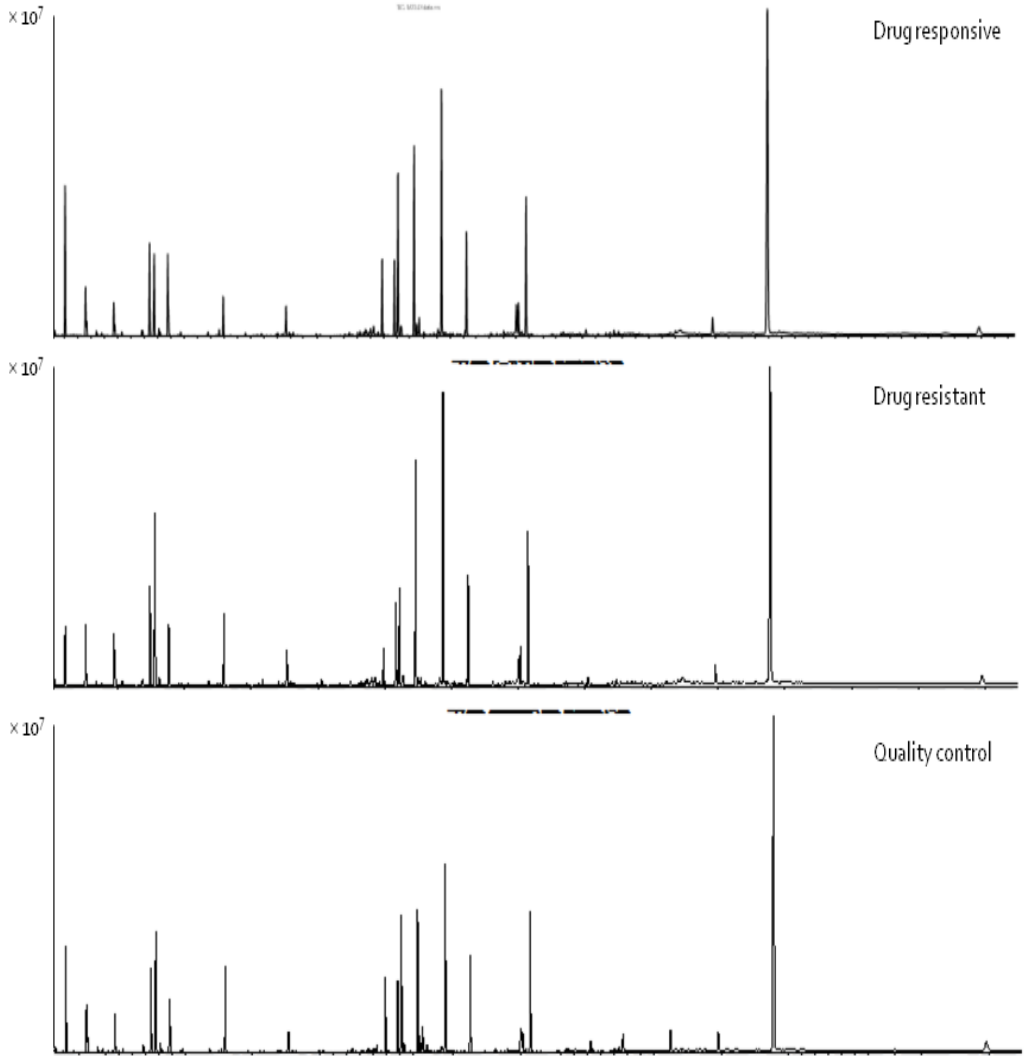

Figure 9. Typical total ion chromatogram (TIC) for the drug responsive epilepsy subjects, drug resistant epilepsy subjects and quality control samples

Typical total ion chromatograms (TIC) are illustrated in Figure 9. The compound lists, which contained the putatively identified metabolites, retention time and peak area, were generated from the deconvoluted chromatogram. After normalization to IS, the peak table lists were subjected to

principal component analysis (PCA) in SIMCA P+. As QC samples were derived from pooled patient samples which should be distinctive from the rest but similar among each other, they clustered together in the middle of the PCA scores plot (Figure 10). Manual observations and PLS-DA plots with additional classification information did not unravel distinctive clustering as a result of drug response category, gender, age, epilepsy type, AEDs used, number of AEDs used, duration of sample storage and day of analyses. Generally, for these cases, 'autofit' option for PLS-DA yielded at least 6 components to characterize the specified classes. Addition or deletion of any component/s resulted in worsened goodness-of-fit (R2Y) and goodness-of-prediction (Q2), and in some cases, reversal of Q2, which was indicative of failure to fit as well as data vulnerability to random variation.

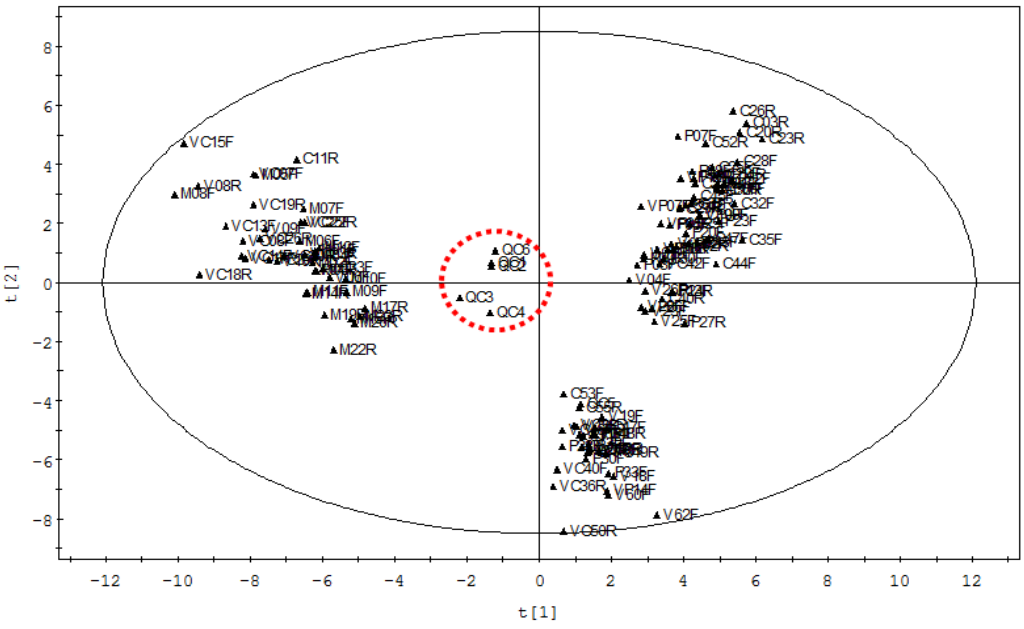

Figure 10. Principal component analysis (PCA) score plots for all subjects in this study (n = 142). Although quality control (QC) samples clustered together, there were no obvious clustering of the recruited subjects based on drug response categories, gender, type of antiepileptic drug used, age, time of sample storage nor day of GC-MS analysis

Subgroup analysis was later conducted to further attest the indiscrimination. Contrasting to the previous findings, PCA score plots for subgroup of finger prick PWE only, demonstrated clear clustering between drug responsive and drug resistant PWE (Figure 11). Principal component (PC) 1 accounted for 14.5% of the variance in the data while the successive PC2 accounted for 13.5%. Clear discrimination was achieved with 2 PC, loadings with higher principal components may cause over-fitting and were deemed unnecessary. Following data scaling to unit variance and inclusion of drug response category for discriminant analysis using Orthogonal PLS method, optimized separation was attained for metabolome from drug responsive and drug resistant subjects. The R2Y calculated was 0.989 while the Q2 was 0.742, indicating good fit and prediction. As can be seen from Figure 12, drug responsive subjects tend to cluster on the right while the drug resistant subjects clusters to the left.

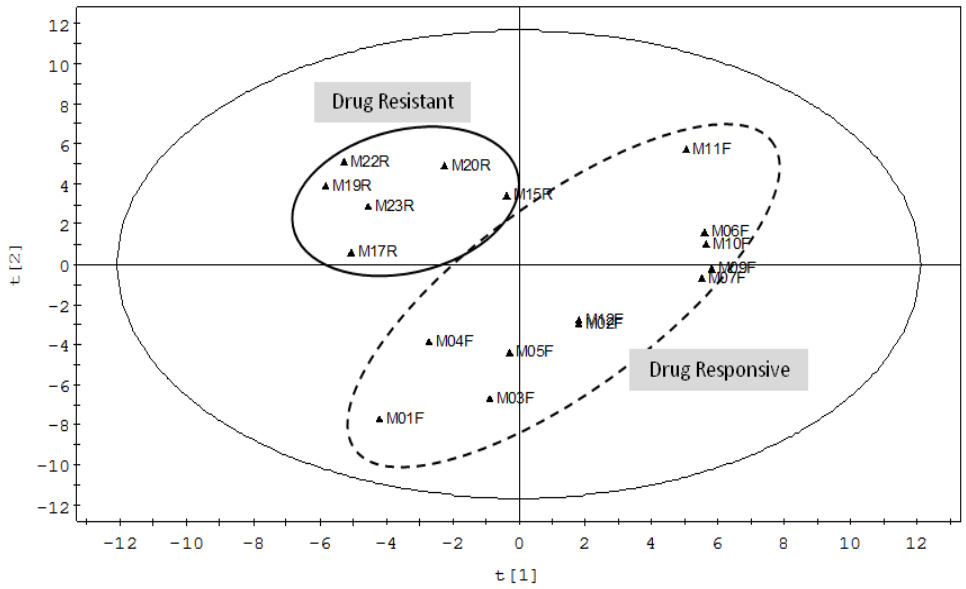

Figure 12. Principal component analysis (PCA) score plots for dried blood spot samples obtained through finger prick in this study (n = 17). Clustering was demonstrated for drug responsive and drug resistant subjects

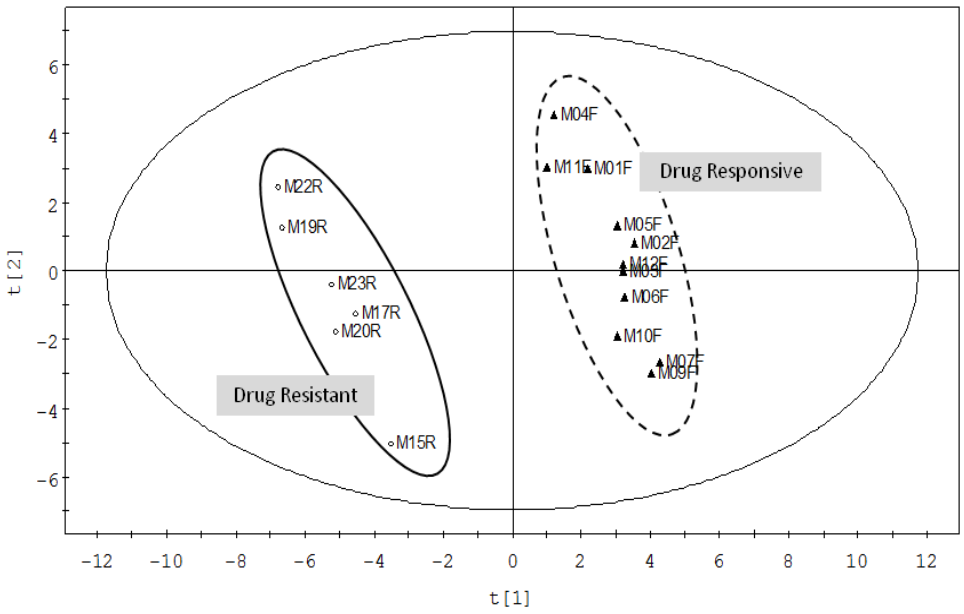

Figure 12. Orthogonal partial least square-discriminant analysis (OPLS-DA) score plots for dried blood spot samples obtained through finger prick in this study (n = 17). Clear discrimination was observed for drug responsive and drug resistant subjects, with goodness-of-fit, R2Y=0.989 and goodness-of-prediction, Q2=0.742

Based on their demographics which are presented in Table 11, no trend or bias is observed neither in the etiologies, seizure types, time from last seizures nor the AEDs used. Of note, only 4 out of 11 seizure free subjects were on polytherapy while all 6 of the DRE subjects were on polytherapy. Although not statistically significant, the potential clinical significance of this characteristic requires further investigation. A following response permutation test confirmed the reliability of this model predictive ability, with R2Y intercept at 0.769 and Q2 intercept at -0.169 (Figure 13). This suggested that the metabolome of DRE subjects was significantly different from their drug responsive counterparts.

Table 11. Demographics of subjects who had contributed finger prick samples in this study

Characteristics	**Drug Responsive (n=11)**	**Drug Resistant (n=6)**
Male, n (%)	5 (45%)	2 (33%)
Chinese, n (%)	10 (91%)	5 (83%)
Weight, Mean±SD (kg)	64.4 ± 9.0	74.7 ± 18.3
Age, Mean±SD (years)	46.0 ± 12.6	35.3 ± 18.5
Etiology, n (%)		
Structural-metabolic	7 (64%)	5 (83%)
Genetic	Nil	1 (17%)
Unknown	4 (36%)	Nil
Seizure Type, n (%)		
Focal	7 (64%)	4 (67%)
Generalized	4 (36%)	2 (33%)
Monotherapy, n (%)	7 (64%)	Nil
Polytherapy, n (%)	4 (36%)	6 (100%)
*Antiepileptic Drug/s, n		
Carbamazepine	7	4
Lamotrigine	4	3
Phenobarbitone	Nil	1
Valproate	1	2
Levetiracetam	1	1
Clobazam	1	3
Topiramate	Nil	2
Days from last seizure, Mean±SD (Range)	1978 ± 1247 (442 – 4350)	43 ± 49 (2 – 118)

*Total number of drugs does not coincide with number of subjects as some subjects were on more than 1 concurrent antiepileptic drugs; SD, standard deviation

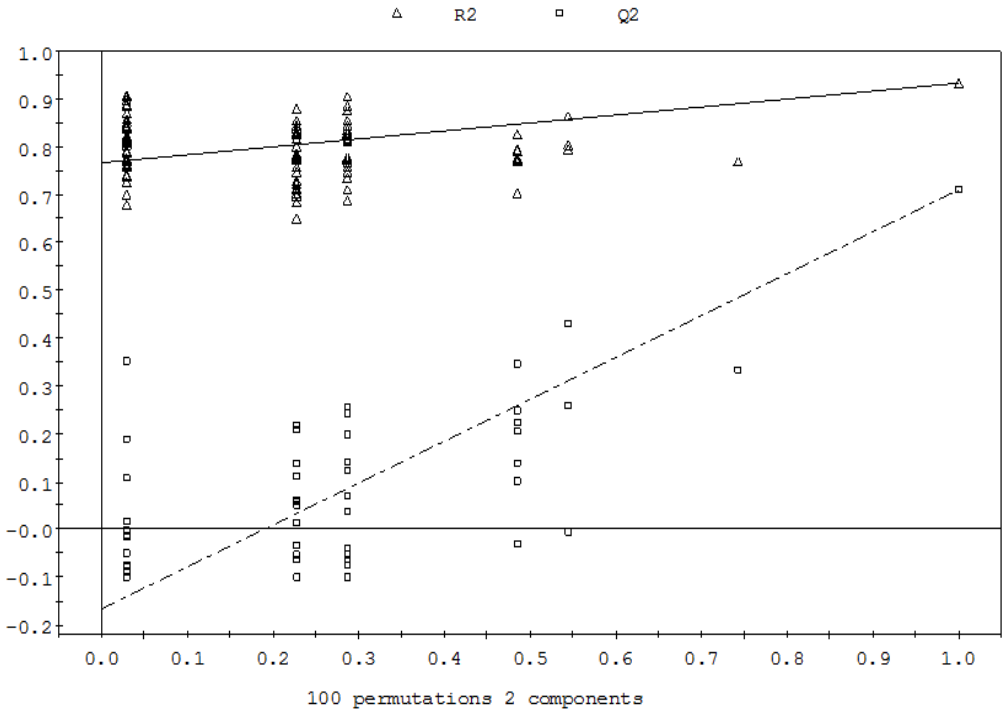

Figure 13. Response permutation test with 2 components revealed R2Y intercept at 0.769 and Q2 intercept at -0.169, indicating reliability of the model (n = 17).

Table 12. Discriminating metabolites from cross-validated PLS-DA list. Their levels were significantly different between the drug responsive and drug resistant subjects ($p < 0.05$)

Putatively Identified Metabolites	Difference in levels
L-glutamine	Higher in Drug Resistant
Pyruvic acid	Higher in Drug Resistant
Caprylic acid	Higher in Drug Resistant
L-serine	Higher in Drug Resistant
Palmitic acid	Lower in Drug Resistant
Oxalic acid	Higher in Drug Resistant
Aspartic acid	Higher in Drug Resistant

The variable importance plot (VIP) list of metabolites generated from the cross-validated PLS-DA was further examined using t-test for level differences between drug responsive and resistant subjects. A few significant putatively identified metabolites were highlighted here (Table 12). L-glutamine, pyruvic acid, caprylic acid, L-serine, oxalic acid and aspartic acid were revealed to be higher while palmitic acid was significantly lower in the drug resistant subjects.

6.4.3 BIOLOGICAL EXPLANATION

As can be seen from Figure 14, MetPA biological pathway visualization revealed that the discriminating endogenous metabolites are involved in a few metabolism pathways, namely, (A) alanine, aspartate and glutamate metabolism; (B) glycine, serine and threonine metabolism; (C) glyoxylate & dicarboxylate metabolism; (D) fatty acid biosynthesis; (E) cysteine & methionine metabolism; (F) aminoacyl-tRNA biosynthesis; (G) purine metabolism; (H) citrate cycle and (I) pyruvate metabolism. The x-axis is the p values compiled by MetPA using enrichment analysis, based on location of the metabolite in the biological pathway. The more upstream the metabolite is in the pathway, the higher impact value it will derive. The y-axis is plotted based on the degree of betweenness centrality. The more number of 'hits' i.e. metabolites affected in that biological pathway, the more important the pathway seems to be. Hence, generally, the nodes highlighted in this study (red/darker shades) were those with a combination of these two importances.

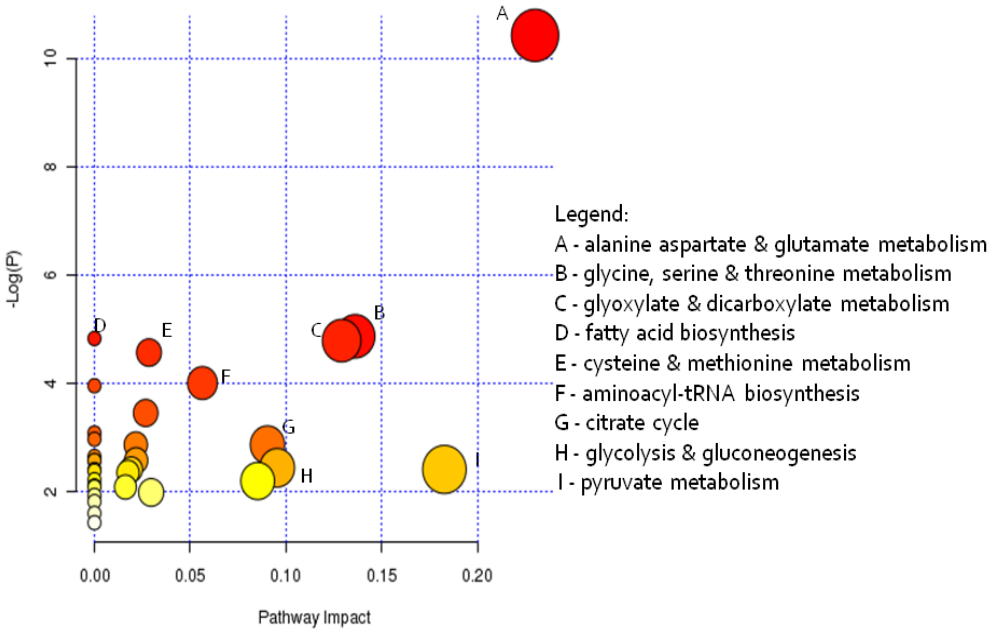

Figure 14. Visualization of pathway analysis result using MetPA. Only the most significant pathways were highlighted (those in darker shades)

Figure 15 depicts a simplified connectivity illustration between some of these pathways alongside the discriminating metabolites, which are shaded in grey boxes. Coefficients column plot of the respective metabolites is shown in Figure 16.

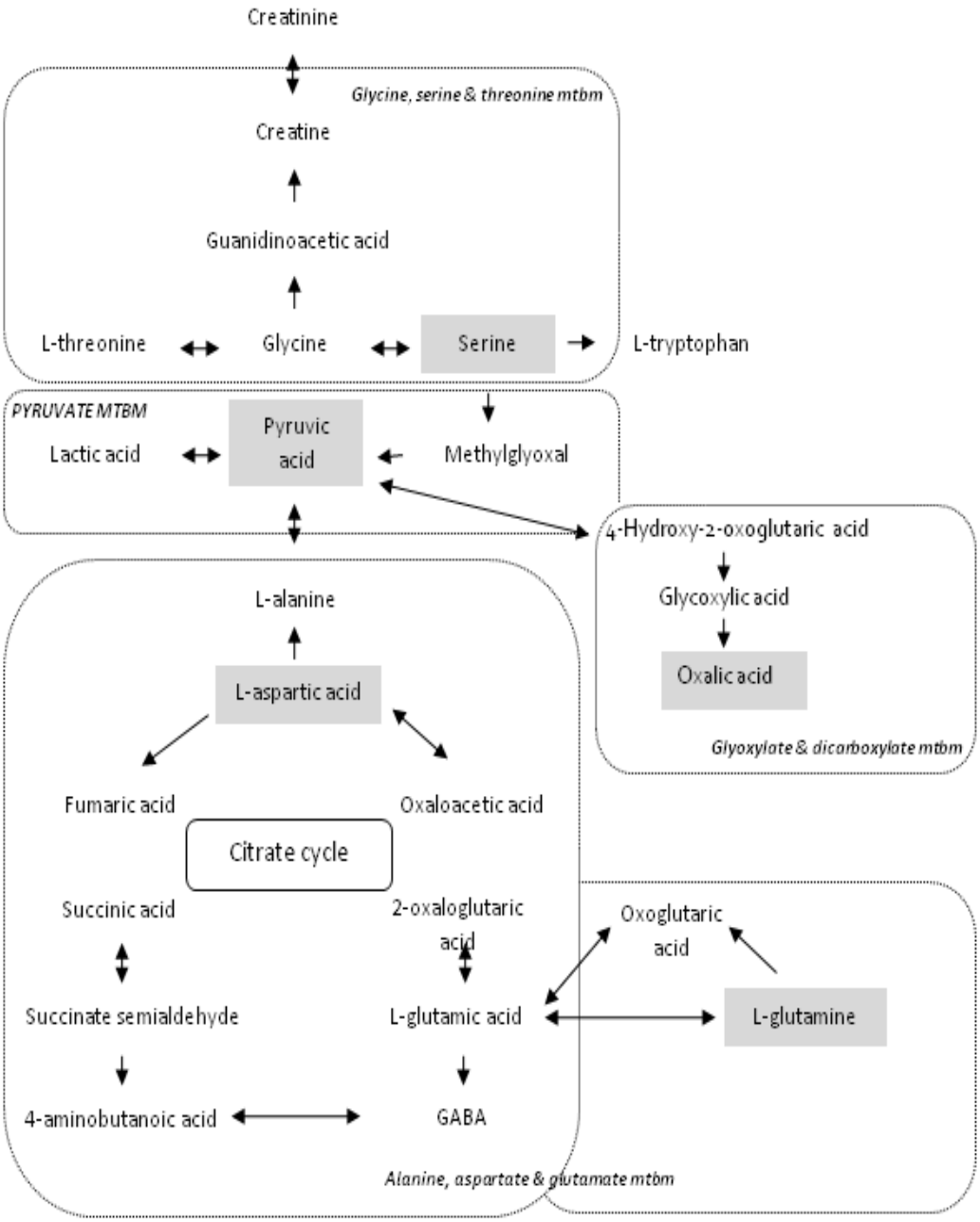

Figure 15. Simplified connectivity illustration between some of the biological pathways involved in discerning drug resistant epilepsy from drug responsive epilepsy. The grey shaded boxes represent the putatively identified metabolites detected in this study (modified from (38))

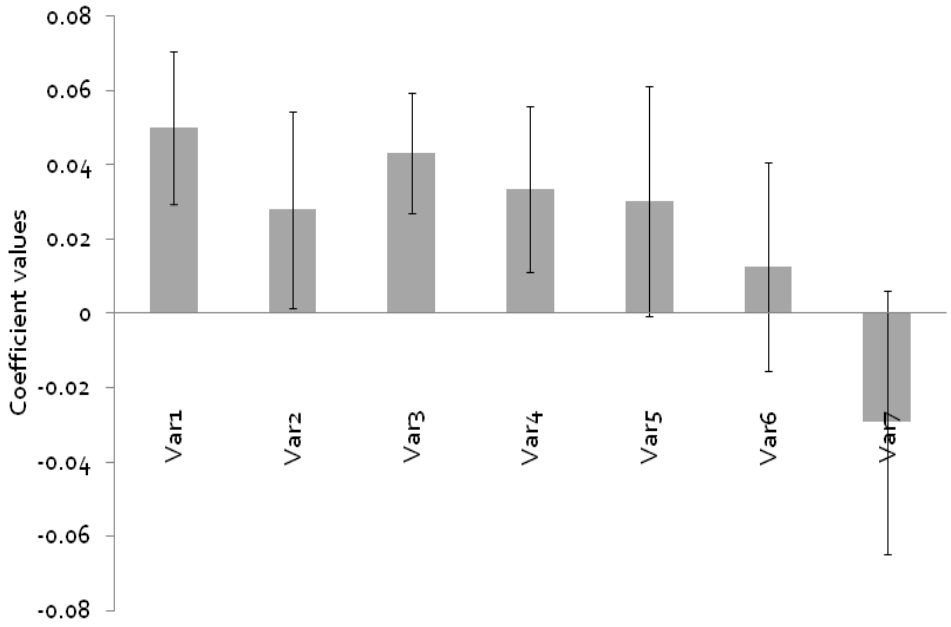

Figure 16. The coefficient plots from OPLS-DA generated using 17 finger prick DBS samples for drug resistant subjects. Only 7 most significant metabolites (total of 129 metabolites) are presented here. Var1, L-glutamine; Var 2, oxalic acid; Var 3, pyruvic acid; Var 4, caprylic acid; Var 5, L-serine; Var 6, aspartic acid and Var 7, palmitic acid

6.5 DISCUSSION

Methanol is used for extraction as it is deemed to be a universal solvent. It has a polarity index of 5.1, indicative of its inclination towards hydrophilicity. Water is avoided as it tends to rupture erythrocytes and dissolve the filter paper components, releasing pigments and paper debris into supernatant that interfere with metabolites analyses and detections in GC-MS. Furthermore, the filtering process usually involves laborious extractions that will reduce throughputs. To maximize the number of metabolites extracted, a second more lipophilic solvent, chloroform, which has a polarity index of 4.1 was used. Chloroform tends to favorably dissolve compounds which are slightly more hydrophobic. Indeed, combination of methanol and chloroform outperformed other combinations which included ethanol, propanol,

dichloromethane, hexane and acetonitrile and was ultimately chosen as the solvents of choice.

In terms of metabolite profiling, similar to the study utilizing venous plasma and newer generation of AEDs, venous blood did not detect discrimination between the metabolome of DRE and drug responsive PWE (109). PWE in this study were on the older generation of AED and their drug responses were synchronized to the latest recommendation from ILAE, which differed from the previous study. Yet, no biomarkers were identified, which could be due to the similar shortcoming of heterogeneity of the sample populations (109). In the subgroup analysis using only finger prick DBS, clear discrimination was observed despite the small number of subjects. This suggests that the endogenous metabolites and their levels in the capillary blood could be different from venous sources, which has been previously demonstrated (225, 226). Additionally, venous blood composition of endogenous metabolites varies depending on the origin e.g. femoral vein on the leg and basilic or median cubital vein on the hand. Generally, the concentrations of some metabolites especially amino acids are much lower in CSF as compared to venous plasma (227-229). Therefore, in comparison, capillary blood may be more useful for metabolomic profiling of DRE in PWE as it has been reported to resemble the arterial blood composition more than venous blood (230).

From the biological pathway analysis, alanine, aspartate and glutamate metabolism was found to be the most significant pathway identified. There were 3 distinguishing metabolites involved in this pathway which were detected in this study, namely glutamine, aspartic acid and pyruvic acid. All 3 metabolites were upregulated in the drug resistant subjects, presumably in the brain. Glutamine (Gln) is a precursor of the excitatory amino acids, glutamic acid (Glu) and aspartic acid (Asp), and the inhibitory amino acid, GABA in astrocytes. High Gln in astrocytes has been shown to trigger release of Glu to extracellular (231). On the other hand, small differences in extracellular Gln

levels or the resulting transmembrane electrochemical gradient can also activate an outward redistribution of Gln from astrocytes (232, 233). Accrual of Gln in EC causes depolarization and resulted in seizure. In addition, Gln also appears to directly affect neurotransmission by interacting with the NMDA class of Glu receptors, causing the ion channels to remain open for persistent transmission. This excess of Gln in extracellular could 'spillover' to the periphery arterial blood (227). Interestingly, the concentration of Gln in arterial blood is very close to extracellular fluid (ECF) and CSF (227, 233). This similarity was not observed between venous blood and CSF. Therefore, the concentrations of Gln in CSF and ECF are better represented by arterial blood than venous, and this finding may be true for some other metabolites as well (156). Since Asp is a product of Gln that exists in equilibrium with Glu, similar shift of its excess into periphery blood might have occurred. Alternatively, high intake of Gln from diet could also cause an increase in brain levels of Gln, and the subsequent consequences. Gln is known to be unstable and readily converts to 2-pyrrolidone-5-carboxylate, glutamic acid and carboxylic acid. The immediate drying on filter paper may have halted this conversion as opposed to venous collection, where the whole blood is left in the EDTA tube for a short duration before spotting. The fact that the DBS samples were acquired at inter-ictal phase could reflect a persistently high steady-state concentration of Gln in the brain of DRE subjects. Overall, these disturbances supported that glutaminergic and GABAergic transmissions are strongly associated not just with epilepsy, but also with drug resistant epilepsy.

Glu did show an increase trend in DRE subjects although the difference was not significant ($p = 0.12$). Despite being the discriminating metabolite when compared with healthy subjects (38), the insignificance elevation in DRE subjects could be attributable to the subtlety amongst PWE themselves. It is also interesting to note that at high concentrations, Glu will form glycine, which will be oxidized to ammonia, carbon dioxide and serine (234). In this study, instead of Glu, L-serine was found to be significantly higher in DRE subjects as compared to drug responsive subjects, prompting a

link to the direct result of high Glu oxidation. In contrast to the previous report where glycine and threonine were upregulated more than healthy subjects (38), L-serine seems to be more prominent in discriminating drug resistant from drug responsive subjects. However, all three metabolites are part of the glycine, serine and threonine metabolism pathway, another well-known perturbed pathway in epilepsy (38, 235, 236). Glycine is a preferred amino acid for storage in the epileptic site of the brain to shield against seizure (237). It acts as an inhibitory neurotransmitter. The inhibition of glycine action at the central nervous system was thought to be the main reason for strychnine-induced-convulsions (238). Serine has also been reported to have different concentrations between capillary and venous blood (156), highlighting again, the possible benefit of capillary samples in detecting metabolite disturbances, especially when the differences are subtle, such as amongst PWE.

In a related context, L-serine is an interchangeable product with pyruvic acid, which in this study, was also elevated in the drug resistant subjects. However, increased pyruvic acid concentrations could also result from perturbation of many other biological pathways such as alanie, aspartate and glutamine metabolism which was discussed earlier, glyoxylate and dicarboxylate metabolism, citrate cycle and pyruvate metabolism. It is an intermediate compound from the metabolisms of carbohydrates, proteins as well as fats. Its accumulation in tissues, especially the nervous structures is usually caused by impaired oxidation (239). PWE on AEDs has been reported to have folate and thiamine deficiencies (240). In the latter scenario, pyruvic acid oxidation is hindered and its accumulation has been reported (241, 242). On the other hand, pyruvic acid elevation could also indicate compensatory mechanism as it is known to exert cytoprotection against hydrogen peroxide (H_2O_2) toxicity in the central nervous system (243). In rats, pyruvic acid supplementation had prevented epilepsy-induced neuronal damage (244-246). This animal study was designed in conformance with natural neuroprotective mechanism where pyruvic acid is upregulated to quench the extra Glu in peripheral to increase the brain to blood ratio of Glu (244, 247). This will

increase efflux of Glu from brain, thus preventing excitotoxic neuronal cell damage caused by prolonged epileptic seizures (244, 246).

Caprylic acid is a medium chain triglyceride (MCT) which has biochemical advantage of fast oxidation. It forms the main component, 55%, of Huttenlocher's ketogenic diet (248, 249). There is evidence of carrier mediated transport of caprylic acid through the blood brain barrier that facilitates its permeation through the cerebral capillary bed (250). This feature is important for caprylic acid to directly exert anticonvulsant effect upon brain exposure (249). It has also been demonstrated to potentiate VPA antiseizure actions in pentylenetetrazole-induced seizure using Albino Swiss male mice (249). Perhaps, in PWE with frequent seizures, there is a tendency to store MCT as an attempt in defence against seizures. In turn, long chain fatty acids such as palmitic acid, may be produced less, indicating perturbation in fatty acids biosynthesis. Palmitic was shown to be lowered in the presence of higher dose Pgp inhibitor, LY335979, as a result of either less absorption, production or removal from intracellular. There seems to be no direct correlation between palmitic acid and seizure (251), although it has been reported to displace VPA from plasma protein rendering higher concentrations of free VPA (252). Since we did not measure free levels of VPA in drug responsive subjects, it remains unknown if this effect has indeed occurred. If it had occurred, the higher free fraction of VPA may be the contributory factor for better response observed in the seizure free subjects. Besides, chronic treatment VPA and CBZ are known to affect lipid profiles (253, 254). Nevertheless, the preference for the stronger lipid profile perturbation observed in DRE subject as compared to their drug responsive counterparts has yet to be determined.

Oxalic acid overproduction is linked with impaired metabolism of glycoxylic acid or ascorbic acid. Systemic oxalosis and primary hyperoxaluria in human is a rare, autosomal recessive disorder which is usually hereditary (255-257). It is also found in vegetables such as spinach and rhubard and fruits such as star fruits. High levels of oxalic acid have caused hypocalcemia-

induced neurotoxicity with symptoms including tetany, seizures and paraesthesia (258). Although it is unlikely that all drug resistant subjects have undiagnosed hyperoxaluria, it does seem that over-ingestion or over-production due to impaired metabolism have occurred in this group of subjects. Oxalic acid was found to be significantly elevated at 2.5 hr after administration of high dose VPA (refer Appendix II), suggesting that this could be an effect of long term treatment with antiepileptic drugs. Therefore, Oxalic acid elevation could be a potential biomarker for AEDs compliance assessment, where its low or non-elevated level can be associated with incompliance. Alternatively, the high levels of pyruvic acid, may have encouraged its transformation into 4-hydroxy-2-oxoglutaric acid and thereby, increasing oxalic acid production (Figure 15).

6.6 CHAPTER CONCLUSION

Although DBS and plasma concentrations may differ from brain extracellular and cerebrospinal fluid, the bi-directional transport of metabolites through blood brain barrier allows for estimation of abnormalities. Coupled with advances in biological pathways visualization, these platforms could aid in revealing collective changes in metabolite expressions.

To the best of our knowledge, this is the first study that demonstrated metabolite profile differences between drug responsive and drug resistant PWE using DBS obtained from finger prick. Hence, in this instance, DBS collected from finger prick may be suitable for metabolomic study of epilepsy.

The metabolite pathway perturbations observed in this study were comparable with those of other studies on epilepsy. What we have observed so far, the increase in glutamine, aspartic acid and serine indicated that alanine-aspartate-glutamate and glycine-serine-threonine metabolisms perturbations were more enhanced in drug resistant subjects. These disturbances were in line

with the imbalanced excitatory and inhibitory neurotransmissions that forms the foundation of seizure occurrences. Being a dynamic biological system, some auxiliary metabolites such as oxalic acid from glyoxylate-dicarboxylate metabolism pathway was also affected. It is difficult to attribute these perturbations to the effect of either long term seizure recurrences or antiepileptic drugs use. However, based on previous animal studies that our group has conducted, which are outlined in Appendix, there is a high possibility that alanine-aspartate-glutamate perturbations are both disease- and treatment-related. This was evidenced by the alteration observed in glutamic acid levels by Pgp inhibition (disease-modification) and VPA-treatment (treatment-modification) models. With reference to the Pgp-inhibition mice study (refers Appendix I), glycine-serine-threonine metabolism is suggested to be altered either as part of epileptogenesis or as consequences of frequent seizure occurrences rather than drug-induced.

It is also noted that among these complex interactions, *in vivo* compensatory mechanisms could have been in place, such as the pyruvic acid quenching and storage of more MCTs instead of long chain fatty acids. In view that seizure frequencies are still high among the drug resistant subjects, it is clear that these protective effects are neither sufficient nor timely. In fact, these presumed defence mechanisms could bring about detrimental effects if they are responsible to trigger even more production of excitatory metabolites/amino acids. Targeted treatment strategies which could help restore the balance; particularly aiming at these few biological pathways are deemed preferable in rendering a subject seizure free from drug resistant.

In reference to our study objectives, DBS from venous blood did not unravel significant metabolites in the profiles of drug resistant PWE. However, it could still be useful for targeted metabolite measurements. Metabolomic profiles yielded from DBS of finger prick origin, appeared to be more informative, attributable to some endogenous metabolite concentration differences from the venous DBS. It may also, to a certain extent, be a good

surrogate for CSF. A targeted assay has to be developed to quantitate the change in levels of these relevant metabolites to gauge the threshold above/below which, seizure occurrences become favorable.

Moving forward, there is a need to coincide the time of blood sampling with seizure occurrence to investigate if any of these biomarkers is the causal or at least indicative of an imminent seizure attack. If this pathophysiology could be delineated, it will aid delivery of timely antiseizure drug and in near future, antiepileptogenic drug.

CHAPTER 7

Clinical Validation of Dried Blood Spot sampling for Quantitation of Carbamazepine, Valproic Acid and Phenytoin using Gas Chromatography- Mass Spectrometry

CHAPTER 7. CLINICAL VALIDATION OF DRIED BLOOD SPOT SAMPLING FOR QUANTITATION OF CARBAMAZEPINE, VALPROIC ACID AND PHENYTOIN USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Using a previously validated assay for simultaneous monitoringof CBZ, PHT and VPA, we embarked on recruiting clinical samples for validation of DBS quantitation with the plasma turbidimetric immunoassay in hospital. This work was in line with Objective 4 where both methods can be compared for agreement. Appropriate conversion factors for each AED could then be tested and proposed.

7.1 CHAPTER SUMMARY

To facilitate faster therapeutic AEDs monitoring process by healthcare professionals for PWE, we (1) developed a GC-MS assay to concurrently measure three AEDs i.e. carbamazepine (CBZ), phenytoin (PHT) and valproic acid (VPA) concentrations using one dried blood spot (DBS), and (2) correlated DBS measured concentrations with their plasma levels. A hundred and sixty nine PWE on either mono- or polytherapy of CBZ, PHT or/and VPA were included. One DBS, containing ~15 µL of blood, was acquired for the simultaneous measurement of the drug levels using GC-MS. Simple Deming regressions were performed to correlate DBS levels obtained from GC-MS with plasma levels measured at the hospital laboratory. Statistical analyses were done using MedCalc® Version 12.6.1.0 and SPSS 21. DBS concentrations (C_{dbs}) were well-correlated to plasma concentrations (C_{plasma}): r=0.8381, 0.9305 and 0.8531 for CBZ, PHT and VPA, conversion formulas obtained were [0.89×C_{dbs}CBZ+1.00]µg/mL, [1.11×C_{dbs}PHT-1.00]µg/mL and [0.92×C_{dbs}VPA+12.48]µg/mL, respectively. After including RBC/plasma partition ratio and individual hematocrit levels in the estimation of C_{plasma} from C_{dbs}, better fit in identity between the observed and theoretical C_{plasma} of PHT

and VPA were demonstrated. Bland-Altman plots revealed good agreements between observed C_{dbs} and C_{plasma} of CBZ and between theoretical and observed C_{plasma}, of PHT and VPA where >93.0% of concentrations was within 95%CI (±2SD). The conversion factors for C_{dbs} were accurate in estimating the C_{plasma} of CBZ, PHT and VPA. Hence, DBS could be an alternative tool for drug monitoring in PWE who are on any of these drug/s. Future studies to establish the pharmacokinetic parameters such as clearance and apparent volume of distribution using C_{dbs} could be considered to improve the usefulness of measuring whole blood concentrations.

7.2 CHAPTER INTRODUCTION

It is well-known AEDs have narrow therapeutic indexes for maximum efficacy and minimum toxicity. Hence, routine plasma concentrations monitoring has been practised, especially during dose adjustments, compliance check and to rule out adverse drug reactions (259). Approximately 40% to 50% of PWE will require two or more AEDs at any one point of their therapy (94, 260, 261). Therefore, simultaneous monitoring of AEDs has always been an attractive alternative to the immunoturbimetric assay of each drug individually (262-264). The simultaneous assay of relevant AEDs is deemed to reduce workload and cost incurred by both patients and laboratories.

Among all the investigated sampling techniques which involve cerebrospinal fluid, tear and saliva (79-81, 265), DBS is convenient to collect and has better storage stability. These advantages have enabled patients to acquire their own sample at home and mail it to designated laboratory (149, 157). Moreover, acquisition of blood spot does not require professional phlebotomist and sample volume of DBS is small (<100 µL). The only caveat is patients' agreeability to be needle-pricked. Comparable phenytoin and phenobarbital concentrations from venous and capillary serum had been

previously demonstrated, indicating equivalence in accuracy between venous and capillary concentration (165). It seems likely that venous blood concentration of drug is comparable to capillary blood concentration after its distribution phase (266). Nevertheless, it remains debatable if DBS can be used to determine free drug concentrations.

Previous studies on concurrent monitoring of multiple AEDs from one DBS were mostly done in high performance liquid chromatography (HPLC) and included whole blood concentrations of AEDs such as carbamazepine, phenytoin, lamotrigine and barbiturates with limited clinical validation (262-264). Recently, a group in N. Ireland published a detailed HPLC ultraviolet method for concurrent determination of carbamazepine (CBZ) and its active metabolite carbamazepine-10,11 epoxide (CBZE), levetiracetam (LEV), lamotrigine (LTG) and phenobarbital (PHB) in paediatrics (267). However, there were no correlations made with plasma concentrations.

In our population of PWE, CBZ, sodium valproate (VPA) and phenytoin (PHT) are the most popular antiepileptic drugs (AEDs) used, either as mono or polytherapy (261). This has prompted us to investigate the applicability of monitoring all three AEDs using only one DBS. Considering the volatile nature of VPA, and previous successful quantitation of CBZ and PHT using gas GCMS (268), GCMS was ultimately chosen as the analytical tool for simultaneous determination these AEDs. Subsequently, to enhance the clinical applicability of this quantitation method, the DBS-measured concentrations will be validated against their corresponding plasma concentrations determined by the conventional immunoturbimetric assays in hospital laboratories. Most often, drug concentrations in whole blood will be affected by hematocrit levels, and hence, this effect will also be investigated.

7.3 MATERIALS & METHODS

7.3.1 PATIENT RECRUITMENT

Assuming constant analytical standard deviations, the sample size recommended for method validation is at least 41 per AED (range ratio = 2, α = 5%, power = 90%, standardized slope deviation of 4) (269, 270). PWE who were on either CBZ, VPA or/and PHT were recruited from October 2011 to August 2012 at neurology specialist clinic of a tertiary referral hospital. This study had obtained the local ethics committee approval. Only PWE who had routine plasma CBZ, VPA and/or PHT, blood and liver biochemistry monitoring on the day of visit were approached for informed consent prior to blood sampling. PWE characteristics and biochemistry results were retrieved from clinical records and hospital information system while the timings of last AED dose taken were obtained from PWE. Drug response categorization was done in accordance to the recent International League Against Epilepsy (ILAE) recommendations (50).

7.3.2 SAMPLING

Venous whole blood samples were collected in EDTA tubes. Two drops of blood from the withdrawn blood, ~30 µL each, were spotted onto 903® cards (903® Neonate Blood Collection Cards, Whatman GmbH, Dassel, Germany) and dried at room temperature, 25ºC for at least 3 hours. The rest of the whole blood was sent to hospital laboratory for plasma quantitations as per routine protocols. To maintain its direct comparability to the plasma quantitation, which is usually completed within 6-8 hours after collection, DBS samples were kept in -80ºC until the day of analysis.

7.3.3 PLASMA AEDS QUANTIFICATION

Hospital laboratory-based, particle enhanced turbidimetric inhibition immunoassays (Beckman Coulter Inc. Unicel DxC800, USA) with inter- and intra-assay coefficient of variation < 8%.

7.3.4 DBS SAMPLES PROCESSING

One 6-mm diameter DBS, containing approximately 15 µL of blood from each patient was extracted for simultaneous AEDs quantification using a previously validated GCMS assay. AEDs extraction was optimized using 500 µL of analytical grade (99%) acetonitrile (Prime Products Pte Ltd, Singapore):1 molar sodium hydroxide (JT Baker, Phillipsburg, NJ, USA) at a ratio of 24:1, v/v with 1 µg/mL 5-(p-methylphenyl)-5-phenylhydantoin (5MP) (Sigma Aldrich, St Louis, MO) as internal standard. The extraction procedure involved 1 min of vortexing and 5 min of sonication. Then, the mixture was centrifuged for 15 min at 6000 *g*. Four hundred microliters of supernatant was transferred into 15 mL Kimble glass tube (Gerresheimer Co. Glass, Germany) for evaporation under nitrogen gas for 15 min at 40ºC. After addition of 100 µL of toluene to the dried sample, second drying phase under similar condition was carried out. Subsequently, derivatization was attained using 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Thermo Scientific Pte. Ltd., Waltham, Massachusetts, USA) incubated at an optimum 70ºC for 50 min. Derivatised samples were cooled to room temperature and diluted with 50 µL of heptane before vortexing for 1 min. Finally, 80 µL of mixture was transferred into a 200 µL conical base inert glass insert placed in a 2 mL amber glass vial (Agilent Technologies, Santa Clara, California, USA).

7.3.5 GC-MS SETTINGS

The analytical assay was developed using GC-MS that comprised of GC 2010 Shimadzu GC coupled to a GCMS-QP2010 Plus quadrupole MS (Shimadzu Corporation, Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto, Japan). GCMSsolution (version 2.0), was utilized for data acquisition and peak area computation. DB5ms (30 m × 0.25 mm × 0.25 µm) supplied by Agilent Technologies J&W, Inc. was used as the capillary column Injector temperature was set at 250ºC while ion source at 220 ºC. Split ratio of 1:5 was applied and column flow was set at 1.9 mL/min. Column temperature began at 90 ºC with a 0.2 min hold time. Temperature was then ramped at 4 different rates: (1) 10 ºC /min to 120 ºC, held for 0.5 min (2) 65 ºC /min to 285 ºC, held for 0.5 min (3) 10 ºC/min to 291 ºC, held for 0.2 min and (4) 60 ºC/min to 300 ºC for a final hold time of 5 min. Selective ion monitoring (SIM) mode was used for detection of target analytes at their respective retention times and are tabulated in Table 13.

Table 13. Tabulation of selective ion monitoring (SIM) attributes and the ions monitored for the respective analytes

Analytes	Retention Time	Quantifying Ion	Qualifying Ions
Valproic Acid	3.453 min	201	129, 145
Phenytoin	7.068 min	281	165, 176, 253
Carbamazepine	7.166 min	193	194, 293
5-methylphenylhydantoin	7.245 min	267	290, 395

7.3.6 BIOANALYSIS

Calibration and quality control standards were prepared in blood and spotted onto the 903® cards at 30 µL each. One 6-mm diameter disc was punched out from each DBS and used for analysis. The assay was validated over a range of 0.5 – 120 µg/mL for all three AEDs. Accuracy ranged from

100 – 110% and imprecision was < 10%. The recoveries of analytes were relatively high (75% – 97%) and consistent (SD ≤ 5.7%). Although the inconsistency increased to 11% at the lower limit of quantitation for CBZ, it was still within the acceptable limit.

7.3.7 STATISTICAL ANALYSIS

DBS concentrations (C_{dbs}) and plasma concentrations (C_{plasma}) determined by respective methods were directly compared. Theoretical C_{plasma} was calculated using a formula which incorporates individual hematocrit values and red blood cell-to-plasma partition (RBC/plasma) ratio (271-274).

$$\text{Theoretical } C_{plasma} = \frac{C_{dbs}}{[1 - \text{Hct R/P}]}$$

where Hct is the individual hematocrit value and R/P is the RBC/plasma ratio of the AEDs. Since the RBC concentration is not routinely measured, individual specific R/P cannot be derived. To ease the clinical applicability, the R/P was fixed at literature values of 0.29 (275) and 0.43 for PHT (271) and 0.04 (276) and 0.20 (277) for VPA . The theoretical plasma concentration for CBZ was not calculated since its R/P was reported to be approximately 1, diminishing the effect of Hct (278).

Simple Deming regression was utilized to compare the methods and estimate the conversion factors. Paired sample t-test was used to assess the differences between methods. As the objective of this study is to correlate C_{dbs} to C_{plasma}, Bland-Altman plots were compiled using C_{plasma} and C_{plasma} predicted from C_{dbs}. Statistical analyses were done using SPSS 21® and MedCalc® Version 12.6.1.0. Outliers were confirmed using standardized score and removal was considered if the score exceeded 2.5.

7.4 RESULTS

7.4.1 PATIENTS

A total of 181 PWE were recruited but only 167 PWE, providing DBS were included in the final analyses. Fourteen PWE were excluded due to undetectable C_{plasma} (< 2 μg/mL), while 2 PWE were excluded due to standardized score more than 2.5 during outlier check. Characteristics of PWE within each AED group were tabulated in Table 14. There were equal proportions of male and female PWE who were on each AED. Some PWE contributed 2 AED concentrations, resulting in total DBSs of more than 167. No significant elevation in biochemistry results was noted. Median hematocrit of the venous samples was 41.3 (range 29.8 – 51.0). The average doses of CBZ, PHT and VPA used in this population were 870.5 ± 413.2 mg, 284.1 ± 71.3 mg and 934.0 ± 317.9 mg, respectively.

Table 14. Characteristics of the people with epilepsy (PWE) grouped according to the type of antiepileptic drug. Total recruited PWE were 183. Only 169 were included in the analysis. The remaining 14 subjects were excluded due to missing plasma levels from hospital laboratory system. (Note: Some recruited PWE contributed to the levels of two AEDs)

Characteristics	Valproic Acid (n = 90)	Phenytoin (n = 49)	Carbamazepine (n = 108)
Number of Eligible DBS	81	42	101
Number of Excluded DBS			
Undetectable plasma levels	8	6	7
Outlier	1	1	Nil
Number of Subjects	80	41	100
Male	46 (57.1%)	21 (51.2%)	48 (48%)
Age, Median (Range)	44.3 (19 - 78)	50.6 (18 - 72)	42.9 (20 - 78)
Ethnic, No. Subjects (%)			
Chinese	71 (87.7)	37 (90.2%)	89 (89%)
Malay	5 (6.2%)	3 (7.3%)	5 (5%)
Indian	3 (3.7%)	Nil	6 (6%)
Others	2 (2.5%)	1 (2.4%)	nil
Concurrent medications, No. Subjects (%)			
None	7 (8.3%)	22 (53.7%)	22 (22%)
Valproic Acid		10 (24.4%)	46 (46%)
Phenytoin	10 (11.9%)		nil
Carbamazapine	46 (54.8%)	Nil	
Other AEDs	21 (25.0%)	9 (21.9%)	32 (32%)
Blood Chemistry, Median (range)			
Hematocrit (%)	41.6 (30.7 - 51)	42.7 (33.3 - 49.2)	41.3 (29.8 - 49.7)
Hemoglobin (g/dL)	13.9 (9.8 - 16.8)	14.0 (10.5 - 16.4)	13.6 (9.2 - 16.8)
Liver Function Test	Median (range)		
Albumin (g/L)	40 (31 - 46)	41 (31 - 47)	41 (31 - 46)
ALT (U/L)	20 (8 - 109)	24 (12 - 79)	19 (9 - 43)
AST (U/L)	22 (12 - 59)	22 (16 - 78)	21 (12 - 59)
GGT (U/L)	53 (9 - 333)	84 (27 - 417)	50 (21 - 213)
Drug Monitoring (μg/mL), Mean (standard deviation)			
Mean plasma levels	57.2 (22.35)*	9.1 (4.67)*	8.4 (2.32)
Mean DBS levels	28.9 (14.67)*	6.3 (3.92)*	8.3 (2.56)
Mean predicted plasma levels	57.1 (22.34)	8.9 (5.06)	8.3 (2.56)
Average dose (mg), Mean (standard deviation)			
	870.5 (413.22)	934.0 (317.89)	284.1 (71.33)
ILAE Classification of Drug Response			
Drug Responsive	28 (34.6%)	21 (51.2%)	31 (31%)
Drug Resistant	30 (37.5%)	9 (22.0%)	38 (38%)
Undefined	23 (28.4%)	11 (26.8%)	31 (31%)

*Denotes significant difference

7.4.2 DBS AND PLASMA CONCENTRATIONS

Figure 17 illustrated the relationships between the C_{dbs} and C_{plasma} for the three AEDs, namely CBZ (Figure 17, top left), PHT (Figure 17, top right) and VPA (Figure 17, bottom). Good correlations were demonstrated for all

three AEDs, correlation coefficient, r = 0.8381, 0.9305 and 0.8531 for CBZ, PHT and VPA, respectively. C_{dbs} and C_{plasma} of CBZ were almost identical. In contrast, C_{dbs} of PHT and VPA were consistently lower than their corresponding C_{plasma}, averaging at 2.8 ± 1.9 µg/mL (29.7 ± 13.6%) and 28.3 ± 12.7 µg/mL (49.5 ± 22.3%), respectively ($p < 0.005$). Moreover, 95% CI for the slope of PHT and VPA did not cross the value 1, indicating there was at least a proportional increase between C_{plasma} and C_{dbs} of the two AEDs.

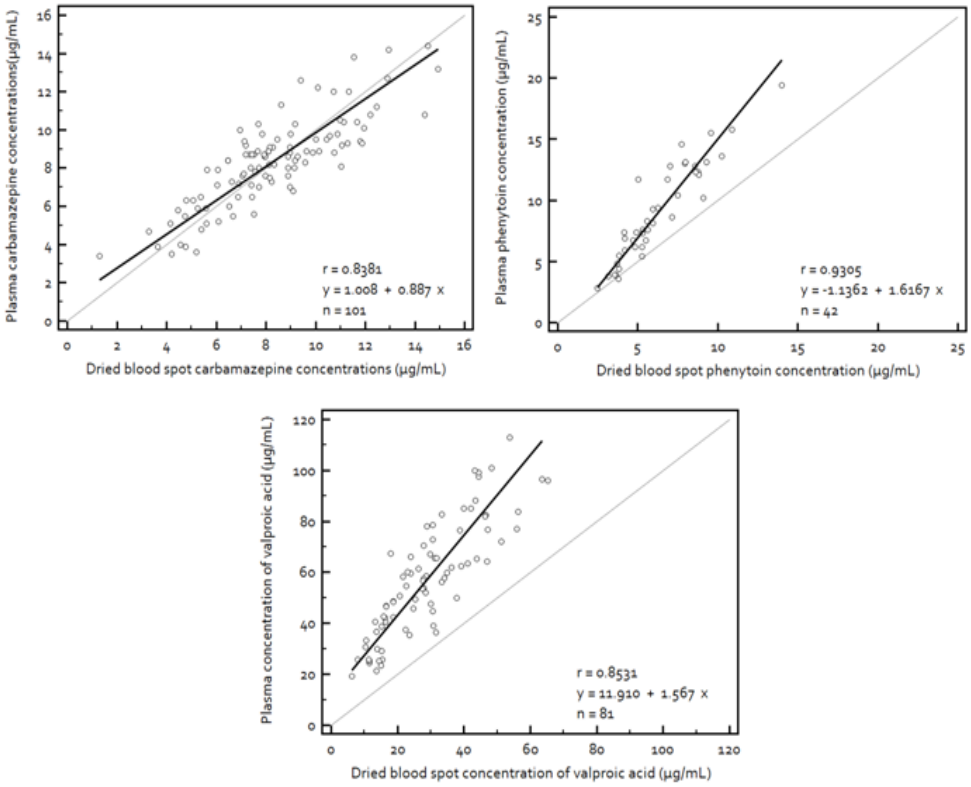

Figure 17. Plasma concentrations of (top left) carbamazepine (top right) phenytoin and (bottom) valproic acid regressed against their dried blood spot concentrations using Deming regression. The broken line is the line of unity while the continuous line is the line of regression. The (top left) slope is 0.84 (95% CI, 0.76 to 1.00) and the intercept is 1.00 (95% CI, 0.04 to 1.97) for carbamazepine, (top right) slope is 1.61 (95% CI, 1.39 to 1.84) and the intercept is -1.14 (95% CI, -2.40 to 0.12) for phenytoin and (bottom) slope is 1.57 (95% CI, 1.33 to 1.81) and the intercept is 11.91 (95% CI, 5.73 to 18.09) for valproic acid

When regressed against theoretical C_{plasma}, definite improvements in line of regression and distribution of points with marginal decrease in correlation coefficients for PHT and VPA were observed (Figure 18). The theoretical C_{plasma} of PHT was found to be comparable to its observed C_{plasma} (Figure 18, top). However, regression line drawn using theoretical C_{plasma} of PHT obtained from R/P of 0.29 showed a better fit with 95% CI of slope included 1 (Figure 18, top left) than the one obtained from R/P of 0.43 (Figure 18, top right). As for VPA, the observed C_{plasma} was still higher than both theoretical C_{plasma} calculated using R/P of 0.20 (Figure 18, bottom left) and 0.042 (Figure 18, bottom right), but at constant values of their intercepts.

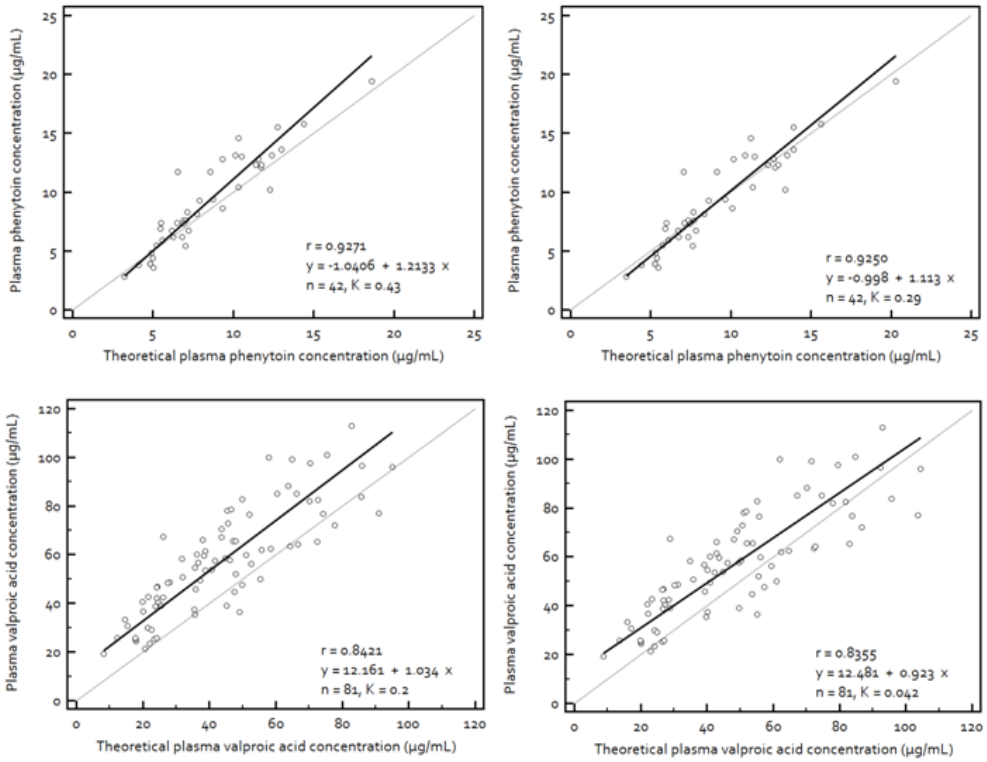

Figure 18. Plasma concentrations of (top) phenytoin and (bottom) valproic acid regressed against their theoretical plasma concentrations estimated from dried blood spot concentrations using Deming regression. [Theoretical plasma concentrations = Dried blood spot concentrations/1 - Hct × (1 – R/P)], where Hct is hematocrit and R/P is the RBC/plasma partition ratio. The broken line is the line of unity while the continuous line is the line of regression. The (top left) slope is 1.21 (95% CI, 1.04 to 1.38) and the intercept is -1.04 (95% CI, -2.32 to 0.24) for phenytoin with R/P=0.43, (top right) slope is 1.11 (95% CI, 0.95 to 1.27) and the intercept is -1.00 (95% CI, -2.28 to 0.29) for phenytoin with R/P=0.29, (bottom left) slope is 1.03 (95% CI, 0.87 to 1.20) and the intercept is 12.16 (95% CI, 5.95 to 18.37) for valproic acid with R/P=0.2 and (bottom right) slope is 0.92 (95% CI, 0.77 to 1.07) and the intercept is 12.48 (95% CI, 6.15 to 18.81) for valproic acid with R/P=0.042. *For the purpose of this figure, R/P is denoted as K

Figure 19 shows the Bland-Altman plots of all three AEDs using the respective predicted C_{plasma}. The concentration used for CBZ was C_{dbs} while for PHT and VPA, theoretical C_{plasma} was used in comparison with their observed C_{plasma}. Mean difference between the concentrations from proposed new method and conventional plasma immunoassay was -0.1 µg/mL for CBZ. For theoretical PHT C_{plasma} calculated using R/P = 0.43, the mean difference

was -0.7 µg/mL while with R/P = 0.29, the mean difference was zero. The difference was -13.7 and -8.1 µg/mL for VPA with R/P of 0.20 and 0.042, respectively. Most of the differences fell within the acceptable limit of ±1.96 SD for all three AEDs. The converted C_{dbs} reflected observed C_{plasma} well and these methods of approximating C_{plasma} for CBZ, PHT and VPA could be used interchangeably. The theoretical C_{plasma} of VPA estimated from R/P = 0.04 and PHT estimated from R/P = 0.29 yielded findings which were closer to the line of unity and were therefore, recommended for clinical use.

R/P

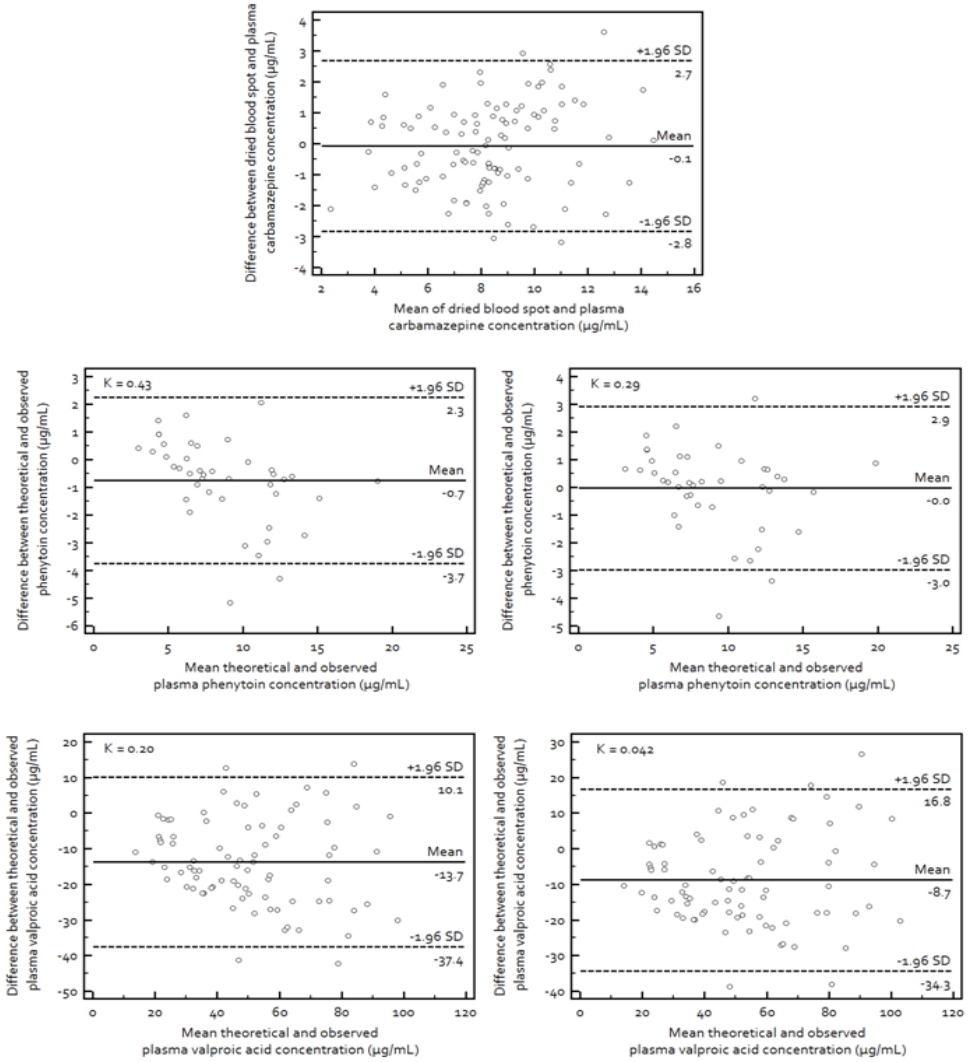

Figure 19. Bland Altman plots for plasma concentrations of (top) carbamazepine, (middle left) phenytoin, R/P=0.43, (middle right) phenytoin, R/P=0.29, (bottom left) valproic acid, R/P=0.20 and (bottom right) valproic acid, R/P=0.042. The broken lines represent the 95% CI (±1.96 SD) and the continuous line is the mean. *For the purpose of this figure, R/P is denoted as K

7.4.3 PLASMA CONCENTRATIONS FOR SEIZURE AND DRUG RESISTANT SUBJECTS

There were no significant differences between C_{dbs} in seizure free and drug resistant PWE for all three AEDs studied ($p > 0.05$). Similarly, C_{plasma} did not differ significantly between the two groups. Furthermore, the distributions and trends of C_{plasma} appeared diverse and alike between these two groups across all three AEDs studied (Figure 20). As illustrated, most of the concentrations measured for CBZ and VPA fell within the therapeutic ranges. In contrast, the measured concentrations of PHT mainly clustered at levels below the minimum recommended 10 µg/mL for both seizure free and drug resistant PWE.

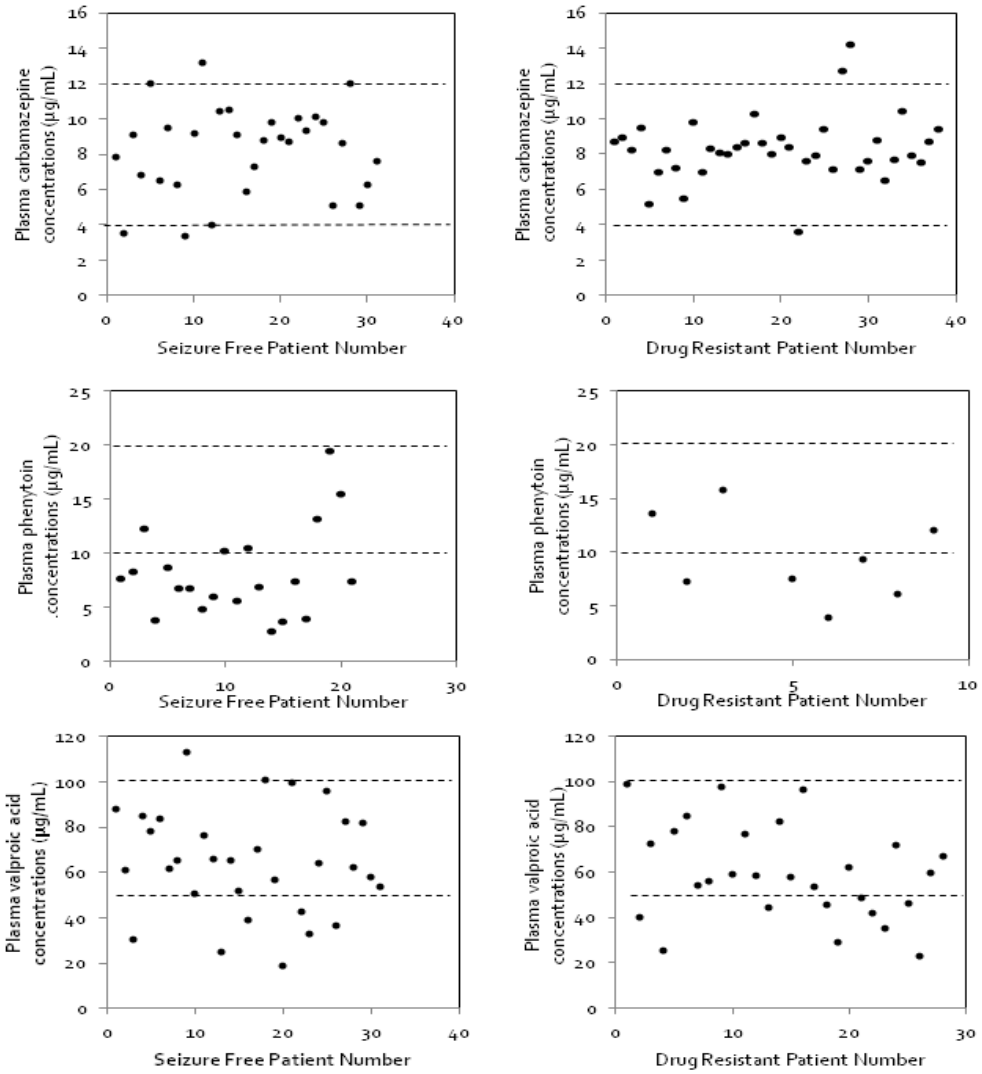

Figure 20. Observed plasma concentrations in seizure free (left panels) and drug resistant (right panels) people with epilepsy (PWE). Therapeutic ranges for individual AED is outlined by the broken lines in each figure. The average concentrations for seizure free versus drug resistant PWE were 8.21±2.47µg/mL versus 8.29±1.82µg/mL for carbamazepine, 8.13±4.18µg/mL versus 11.11±6.16µg/mL for phenytoin and 59.79±21.09µg/mL versus 64.62±23.46µg/mL ($p > 0.05$)

7.5 DISCUSSION

This group of PWE represents the typical population of PWE who would undergo routine plasma monitoring. In CBZ with presumed K of 1.06, the dilutional effect by RBC is minimal, if not none (272, 278). As CBZ partitioned significantly into the RBC compartment, the concentrations measured in whole blood were approximately the same as those that measured in plasma. This allowed a direct comparability between C_{dbs} and C_{plasma} of CBZ giving a conversion factor of about 0.9.

Contrasting to CBZ, C_{dbs} of VPA were constantly lower than its C_{plasma},. Lower C_{dbs} of VPA had previously been observed by Vermejiv and Edelbroek in their study, using HPLC for AED detection. They had found a conversion factor of 1.46 from C_{dbs} to C_{plasma}, which implied that C_{plasma} was 46% higher than C_{dbs} (279). The lower concentrations measured from DBS could be attributed to a combination of factors. Firstly, the dilutional effect by red blood cells (RBC). RBC accounted for 99% of cellular space of blood and its presence may serve to dilute the VPA concentrations compared to those measured in plasma alone (280). Secondly, for drugs with whole blood-to-plasma concentration ratio of less than (1 – Hct), such as VPA, they should not partition into RBC significantly (272). VPA whole blood-to-plasma concentration ratio in our study averaged at 0.51 while the (1 – Hct) averaged at 0.58. Furthermore, VPA had been shown to partition into RBC from plasma at ratios ranging from 0.04 to 0.20 (276, 277, 281). It is projected that only the unbound drugs can partition into RBCs (282, 283) and that this partition is consistent at varying concentrations (281, 284). Since most of our patients had plasma VPA concentrations within the therapeutic ranges, and normal albumin levels,, the K was presumably consistent (281). Hence, the relatively small amount in RBC compartment was likely unable to contribute any substantial value towards overall C_{dbs}. Thirdly, VPA, being lipophilic, could dissolve and detach from RBC during centrifugation of whole blood to obtain the plasma and resulted in the relatively higher concentrations observed in the plasma

(285, 286). Alternatively, despite extraction yield of > 80% (RSD < 6.0%) between 1 to 250 mg/L, (Table 15) the binding of VPA to 903® cards may have some effect in the lower C_{dbs}.

Table 15. Percentage of mean extraction recovery of analytes along with their respective residual standard deviation (RSD) at different concentrations in spiked blood. The consistent and high recovery (>70%) of the analytes allowed for reliable quantitative studies

Concentration (µg/mL)	Mean Recovery (RSD) in percentage (%)		
	Carbamazepine	Phenytoin	Valproic Acid
1	70.28 (11.10)	84.22 (8.21)	94.67 (4.64)
10	89.98 (1.73)	96.88 (4.76)	92.90 (1.13)
50	74.62 (3.67)	93.60 (3.00)	90.34 (5.69)
250	89.01 (1.57)	80.21 (1.02)	80.43 (4.10)

Although metabolism of VPA by enzymes present in RBC could have occured while the blood spot was being left to dry on the cards, enzymatic activity is thought to decrease at temperature lower than 37ºC, and this effect should be minimal (287).

There was definitive improvement found for theoretical C_{plasma} after incorporation of hematocrit and RBC/plasma partition ratio. Yet, a constant lesser theoretical C_{dbs} was calculated. The most probable explanations include the domination from RBC dilutional effect and VPA binding to 903® cards. In clinical practice, however, the potential effect of the difference in calculated C_{plasma} from C_{dbs} may not be obvious. An example of a worst case prediction would be for an actual C_{plasma} of 60 µg/mL, the predicted C_{plasma} could either be 48 or 72 µg/mL. At either concentration, physicians are likely to increase the dose if the PWE has uncontrolled seizure, which is a similar clinical decision to the result of 60 µg/mL. Therefore, it is unlikely that C_{dbs} would deter any major changes in VPA dosing regimen.

On the other hand, PHT readily partitions into and dissociates from the red blood cells, with a R/P reported to be approximately 0.29 (275) and 0.43 in healthy patients (271). Its whole blood-to-plasma concentration was approximately 1.33 in both studies, which was comparable to 1.14 and 1.23, before and after corrected for Hct and R/P, found in this study. PHT binds to hemoglobin in RBCs (288). At higher C_{plasma} of PHT, plasma protein binding sites start to be saturated and increases in R/P ratios were observed. This could be result from increase in unbound fraction of PHT that will readily partition into RBC (289). RBC had been shown to release PHT disproportionally in *in vivo* (275). At higher C_{plasma} where a presumably higher amount of PHT is transported by RBC, its unequal release may have been more prominent, especially during centrifugation and resulted in the higher concentrations measured from plasma. Therefore, an overall concentration from whole blood might provide an alternative explanation for scenarios such as uncontrolled seizure despite having therapeutic plasma PHT concentrations. Indeed, for drugs with high partition ratio into RBC such as neuroleptic drugs haloperidol, butaperazine and thioridazine, the RBC concentrations tend to have better association with treatment outcomes as compared to their plasma concentrations (290-292).

It is noteworthy that most of the PWE classified as drug-responsive by ILAE exhibited steady state C_{plasma} of below 10 µg/mL. One possible explanation could be these PWE were on decreasing doses of PHT due to long term seizure free. Hence, their levels were no longer maintained within therapeutic ranges. Incompliance to PHT regimen might be another contributing factor as incompliance upon realizing the possibility of AED withdrawal is not uncommon among the seizure free PWE (172). However, it might be unlikely that most of them were noncompliant simultaneously.

7.5.1 EFFECT OF HEMATOCRIT AND COMPOUND SPECIFIC RED BLOOD CELL-TO-PLASMA RATIOS

In this study, we had demonstrated that compound specific red blood cell-to-plasma binding and individual hematocrit level explained the difference in concentrations detected from DBS and plasma. It seems that the higher the drug partition into RBC, e.g. 1.06 for CBZ, the closer the C_{dbs} is to its C_{plasma}. Conversely, higher hematocrit levels will cause a lower C_{dbs}. DBS does seem to equate whole blood characteristics which were outlined in previous studies (272, 284). For lipophilic drugs such as AEDs, RBC is an important and very useful transporter with high capacity but low affinity to the drugs. RBC readily releases the drug it carries and equilibrates with surrounding tissues in capillary system. Although C_{plasma} is an optimum representation of tissue concentration, RBC concentration of drug may be able to function similarly. The constant ratios of RBC/plasma water over a wide range of AEDs concentrations proved that RBC is not a saturable system (275, 281). Therefore, RBC concentration of drug may be negligible at low concentration, but will gain importance as the concentration increases.

7.6 CHAPTER CONCLUSION

DBS concentration of AEDs was generally lower than the plasma concentrations, predominantly driven by the dilutional effect of RBC presence. The differences lessen with increasing RBC/plasma partition ratio of the drugs and decreasing hematocrit values. At clinically relevant blood and liver biochemistry variations, correcting the C_{dbs} to hematocrit and RBC/plasma partition improved the theoretical prediction of C_{plasma} for PHT and VPA. Nevertheless, this study did not cater for investigation of patient specific AEDs binding to red blood cells nor albumin. For the former effect, the R/P

was fixed at 2 diverse values for PHT and VPA to approximate the suitability as evidenced by improved graphical fit. Aside from recommended to be consistent among varying concentrations (275, 281), a constant value was used to ease clinical applicability. Since PWE recruited had levels that were within normal ranges, it may be assumed that at these ranges, the effects of AEDs binding to blood cells and albumin should not fluctuate significantly. In this study, DBS was obtained from venous source. Theoretically, there could be some differences between capillary and venous concentrations but the differences for a majority of xenobiotics are not obvious, especially after the distribution phase (164-166). At steady state drug concentrations, difference between capillary and venous should be insignificant and could be used interchangeably (166). In conclusion, the theoretical C_{plasma} can be estimated through the equations below for the respective AEDs:

$$C_{plasma}CBZ = (0.89 \times C_{dbs}CBZ) + 1.00\ \mu g/mL$$

$$C_{plasma}PHT = \left(1.11 \times \frac{C_{dbs}PHT}{1-(0.29 \times Hct)}\right) - 1.00\ \mu g/mL$$

$$C_{plasma}VPA = (0.92 \times \frac{C_{dbs}VPA}{1-(0.04 \times Hct)} + 12.48\ \mu g/mL$$

where Hct represent individual hematocrit value, C_{plasma} CBZ, C_{plasma} PHT and C_{plasma} VPA represent the plasma concentrations of CBZ, PHT and VPA, respectively while C_{dbs} CBZ, C_{dbs} PHT and C_{dbs} VPA represent the dried blood spot concentrations of CBZ, PHT and VPA, respectively For theoretical C_{plasma} of PHT and VPA, the conversion equation using R/P = 0.29 and R/P = 0.04, respectively were recommended due to its proximity to the line of unity.

In view of the good agreement between the theoretical C_{plasma} using DBS levels and observed C_{plasma} for all three AEDs, DBS is deemed suitable as an alternative to conventional plasma quantitations. Future studies that investigate the pharmacokinetic parameters such as clearance and apparent

volume of distribution using C_{dbs} and then correlate these concentrations to the treatment outcomes could be considered.

In the following chapter, the use of C_{dbs} in estimating the clearance of CBZ will be explored.

CHAPTER 8

Estimation & Comparison of Carbamazepine Population Pharmacokinetics using DBS & Plasma Concentrations from People with Epilepsy:

The Clinical Implication

CHAPTER 8. ESTIMATION AND COMPARISON OF CARBAMAZEPINE POPULATION PHARMACOKINETICS USING DRIED BLOOD SPOT AND PLASMA CONCENTRATIONS FROM PEOPLE WITH EPILEPSY: THE CLINICAL IMPLICATION

This work is published in *The Journal of Clinical Pharmacology* 2014;54(2):225-233.

8.1 CHAPTER SUMMARY

To clarify the potential of using DBS as a surrogate to plasma for TDM of CBZ, we compared the population pharmacokinetic (PPK) estimates from concurrent DBS and plasma levels. The dose-concentration relationship, estimated parameter and variability were determined. A total of 98 observations from 97 PWE were included in this study. Data was split into 3:1 ratio, with the larger dataset forming index group and the smaller set for evaluation. Non-linear mixed effects regression with one compartment, first order absorption and elimination model was utilized. Covariates were screened for inclusion into final model via forward stepwise addition and backward elimination method. Predictive performances of the final models were assessed for bias and precision. The typical clearance for CBZ was estimated to be 5.85 and 5.68 L/h from plasma and DBS concentrations, respectively. The final models for clearance estimates obtained from plasma concentrations (C_{plasma}) included total daily CBZ dose per unit weight (DD) and gender while from DBS concentrations (C_{dbs}) included only DD. The final models were both precise and non-bias. The developed PPK models had comparable estimates, errors and predictive performances. Our findings suggest that C_{plasma}

and C_{dbs} could be used interchangeably in the future for pharmacokinetic studies of CBZ.

8.2 CHAPTER INTRODUCTION

Carbamazepine (CBZ) is an established first line antiepileptic drug (AED) for the treatment of focal seizures by rendering neuronal cells more resistant towards excitation. It acts by stabilizing the inactive state of sodium channels and enhancing the inhibitory actions of gamma amino butyric acid receptors. CBZ is primarily metabolized by the CYP3A4 enzyme in the liver and less than 3% excreted unchanged in the urine. Doses are usually started at 200 – 400 mg/day and eventually increased to 800 – 1600 mg / day or 15 – 20 mg/kg/day. Higher doses may be required during maintenance phases due to either its inherent autoinduction or concurrent heteroinduction by other drugs (293-297). The period of induction varies between individuals, although complete induction has been proposed to occur between 3 – 5 weeks after the start of the interaction. Therapeutic plasma CBZ concentrations of 4 – 12 mg/L have been used as a gauge for rational dosing throughout its therapy.

Recently, DBS was proposed to be an alternative sampling matrix to conventional plasma CBZ monitoring (Chapter 7). The conversion factor from DBS concentrations to plasma concentrations was found to be approximately 0.9, indicating that CBZ partitions considerably into red blood cells (RBCs). Of note, there are studies that demonstrated superior relationships between whole blood concentrations and clinical outcomes as compared to their plasma concentrations for drugs that partition into RBCs at a significant amount (290-292). Considering that CBZ is the most used AED in our adult population of PWE, it has prompted further investigation to estimate the PK parameters using DBS concentrations of CBZ (261). Population pharmacokinetic modeling of DBS concentrations is hypothesized to provide a better explanation for the variations observed between dose given and measured

concentrations. This finding may facilitate future work in delineating RBCs concentration of CBZ and seizure treatment outcome relationship.

Thus far, compilations of pharmacokinetic properties of CBZ using plasma concentrations had shown somewhat differing covariates and estimates amongst various populations e.g. pediatrics (298), adults (90), elderly (299), Caucasian (300) and Chinese race (301). An estimation of CBZ pharmacokinetics (PK) in our own population of PWE is postulated to improve the understanding between dose and therapeutic outcomes during epilepsy treatment. In Singapore, study by Chan et al, showed that plasma CBZ CL is ~ 3.12 L/h for a 12-year-old child weighing at 35 kg and is concurrently taking phenobarbitone (85). In view that they had included mostly children and adolescent, with differing set of concurrent AEDs, its usefulness in a general adult population may be limited. Therefore, in this study, the population pharmacokinetic parameters in adult PWE were estimated. Simultaneously, the PPK estimates obtained from both plasma and DBS were assessed. Thereafter, the potential of DBS as a surrogate to plasma for TDM could be established.

8.3 METHODS

8.3.1 STUDY DESIGN

This is a cross sectional, retrospective cohort study approved by the local ethics committee and conducted in a Neurology Specialist Clinic of a tertiary referral hospital. The study was conducted in accordance to Declaration of Helsinki and the protocol was approved by the local ethics committee (CIRB2011/269/A). Prior to blood sampling, informed consent was obtained from PWE who had routine plasma CBZ monitoring on the day of visit. PWE characteristics such as demographics and concurrent medications, and biochemistry levels of blood profiles, renal function tests and catalytic

activities of liver enzymes were retrieved from clinical records and hospital information system. To ensure that the plasma levels were representative of the steady-state concentrations, only PWE who had constant therapeutic regimen for at least 3 months were included in the analysis. PWE who were suspected to be incompliant for the past 1 week before sampling were excluded. Hence, in conjunction with the constant therapeutic regimen, laboratory results were accepted if they were taken within 3 or more months (whichever in congruent with previous regimen change) before the samples were drawn. Blood panels were obtained on the same day of CBZ sampling. Missing values were later replaced with the median of all existing observations. PWE were interviewed for their usual time of dosing intake and last dose of CBZ administered. ILAE recommendations were utilized to categorize drug responsiveness of the PWE (50). Category 1, 2 and 3 represents PWE with drug responsive, drug resistant epilepsy and undefined treatment outcome, correspondingly.

8.3.2 SAMPLING AND ASSAY

Venous whole blood samples were collected in EDTA tubes for routine complete blood counts. Approximately two 30 µL drops from the withdrawn blood were spotted, onto 903® cards (903® Neonate Blood Collection Cards, Whatman GmbH, Dassel, Germany) and dried at room temperature, 25ºC for a minimum of 3 hr. Hence, technically the dried blood spot used in this study is dried EDTA blood spot. The rest of the blood was sent for laboratory measurement. Plasma quantitations were done in hospital laboratory, using particle enhanced turbidimetric inhibition immunoassays (Beckman Coulter Inc. Unicel DxC800, USA) with inter- and intra-assay coefficient of variation < 8%. The dried blood spots were punched at 6-mm diameter, extracted with alkalinized organic solvent The dried blood spots were punched at 6-mm diameter and extracted with alkalinized organic solvent comprised of 20 µL of

Sodium Hydroxide 1M (JT Baker, Phillipsburg, NJ, USA) in 480 µL of analytical grade acetonitrile (Prime Products Pte Ltd, Singapore). After vortexing for 1 min, sonicating for 5 min and centrifugating for 15 min at 6000 *g*, 400 µL of supernatant was transferred into silanized glass tube for drying under nitrogen at 40ºC. This is followed by addition of 100 µL of toluene for further drying with similar condition. Fifty microliters of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Thermo Scientific Pte. Ltd., Waltham, Massachusetts, USA) was added to the dried sample for derivatization under optimum temperature of 70ºC and duration of 50 mins. Finally, dilution was achieved using 50 µL of heptane before injected into gas chromatography mass spectrometry (Shimadzu Corporation, Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto, Japan) for CBZ quantitations in accordance to a validated assay. GC-MS settings included injector temperature of 250ºC, ion source at 220ºC, split ratio of 1:5 and column flow at 1.9 mL/min. The quantifying ion for CBZ was 193, while 194 and 293 were used as qualifying ion at 7.166 min. Capillary column was DB5ms (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies J&W, Inc.) and starting temperature was 90ºC. Temperature was ramped at 10ºC/min to 120 ºC, held for 0.5 min, followed by 65 ºC/min to 285 ºC, held for 0.5 min, 10 ºC/min to 291 ºC, held for 0.2 min and finally 60 ºC/min to 300 ºC, held for 5 min. The accuracy (within 15% of the actual value) and precision (%CV < 15%) of the assay for all measurements were in accordance to FDA guidance for industry on bioanalytical method validation (please also refer to Chapter 7, Materials and Methods; DBS samples processing and GC-MS settings for more details). T-test was utilized for comparison of continuous variables while chi-square was used for categorical variables. Statistical compilation and testing were done in Microsoft Excel 2007® and significance values were set at $p \leq 0.05$.

8.3.3 POPULATION PHARMACOKINETIC ANALYSES

Structural Basic Model

The non-linear mixed effects regression modelling software (WinNonMix Version 2.0; Pharsight, Corp, Mountain View, CA) was utilized to model the data. One compartment, first order absorption and elimination model was chosen to describe the concentration-time profiles of CBZ.(85, 90, 301, 302) First order estimation was employed. The data was randomly split into 3:1 ratio, with the larger data set as the index group for modelling and the smaller set as evaluation group. The bioavailabilty, F was not assumed nor estimated since majority of the observations were obtained at trough levels. Hence, clearance is denoted as CL/F while apparent volume of distribution is denoted as V_d/F throughout the study. At an average of one observation per patient, this data set does not allow for estimation of more than one parameter and hence only the CL/F was estimated. The V_d/F and absorption constant, k_a were fixed at literature values (90, 299, 301, 303). k_a was fixed based on controlled-release formulation because all subjects were taking this form of CBZ. The fixed values were varied systematically to at least 3 times lower and higher than the literature values to obtain a model with the lowest model objective function value (OBJ) as well as estimate errors with a plausible CL/F. Inter-individual and residual variabilities were modelled with either exponential, proportional or additive errors.

Covariate Model

Covariates were screened for influence on the individual estimated clearance using analysis of variance (ANOVA). The covariates tested included gender, age, age ≥ 60 years old, age of seizure onset, weight, ethnicity, current drug response class, total daily CBZ dose per kilogram of weight, concurrent therapy with valproic acid, the catalytic activities of alanine aminotransferase,

aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT); and the concentrations of albumin, total bilirubin, haemoglobin and hematocrit. Significant covariates ($p \leq 0.05$) were added either linearly or nonlinearly into the model via forward stepwise addition and backward elimination method. A covariate is considered significant if its inclusion into the basic model or deletion from the full regression model altered the OBJ $\geq$ 3.841 (χ^2, $p=0.05$, $df=1$). In addition, assessment of goodness-of-fit plots, reduction in the inter-individual variability, residual variability and plausible model parameter estimation were also considered. Confirmation of the final model parameters was assessed using first order conditional estimation (FOCE) method (304).

Evaluation

Predictive performance was assessed on the evaluation group The predicted and observed CBZ concentrations of the evaluation group were then compared. The mean prediction error (ME) was compiled to assess model biasnes while mean absolute prediction error (MAE) and root mean square error (RMSE) to assess model precision. A model is considered to be non bias when 95% confidence interval (CI) of ME included zero, and precise when MAE and RMSE have values close to zero.

8.4 RESULTS

8.4.1 SUBJECT SAMPLES

A total of 97 PWE who contributed 98 observations were included. The index group comprised of 72 observations from 71 PWE while the validation group consisted 26 observations from 26 PWE. There were 15 PWE (15.3%) with missing values for the liver biochemistries. Renal function was

unavailable in more than 80% of the PWE and therefore, was excluded as covariate for PPK modelling. As can be seen from Table 16, the characteristics of the PWE in both the index and evaluation groups were comparable ($p > 0.05$). There were equivalent proportions of sex in both groups with a seemingly higher representation of drug responsive subjects in the index as compared to the evaluation group. This difference, however, was not significant. The average age of the subjects was 42.7 ± 10.9 years old while the weight was 65.8 ± 15.1 kg. Valproic acid (VPA), clobazam (CLB) and levetiracetam (LEV) were most commonly used as adjunctive therapy in this population.

Table 16. Demographics of study population according to index and validation group

Characteristic	Index Group	Evaluation Group
No. of observations	72	26
Sex, n (%)		
Male	33 (46.5%)	12 (46.1%)
Female	38 (53.5%)	14 (53.9%)
Ethnic, n (%)		
Chinese	60 (84.5%)	27 (92.3%)
Malay	7 (9.9%)	0 (0%)
Indian	4 (5.6%)	2 (7.7%)
[£]Age (years)	42.7 ± 11.23 (22.3 - 77.8)	42.6 ± 10.60 (19.7 - 64.6)
[£]Weight (kg)	65.7 ± 19.01 (39.5 - 107.4)	66.2 ± 13.46 (40 - 96.1)
[£]CBZ dosages (mg/day)	936.1 ± 322.26 (200 - 1600)	924.1 ± 269.46 (400 - 1600)
[£]CBZe plasma concentrations (mg/L)	8.1 ± 2.23 (2.5 - 13.8)	8.4 ± (3.5 - 13.2)
[£]CBZ DBS concentrations (mg/L)	8.2 ± 2.40 (3.3 - 14.4)	8.3 ± (4.2 - 14.1)
Monotherapy with CBZ, n (%)	17 (23.9%)	4 ± (15.4%)
*Polytherapy, n (%)	54 (76.1%)	22 ± (84.6%)
Valproic Acid, n	31	13
Clobazam, n	17	6
Levetiracetam, n	13	5
Phenobarnitone, n	3	3
[£]Hematocrit, %	41.2 ± 4.48 (32.4 - 49.4)	41.1 ± 4.01 (29.8 - 47.4)
[£]Hemoglobin, U/L	13.6 ± 1.64 (9.5 - 16.8)	13.7 ± 1.52 (9.2 - 15.8)
[£]Albumin (g/L)	39.9 ± 3.17 (31 - 45)	40.5 ± 2.98 (33 - 46)
[£]Alkaline phosphatase (U/L)	72.8 ± 19.73 (36 - 137)	72.9 ± 17.35 (40 - 112)
[£]Alanine aminotransferase (U/L)	20.4 ± 6.60 (9 - 42)	21.7 ± 7.09 (13 - 43)
[£]Aspartate aminotransferase (U/L)	22.7 ± 6.78 (12 - 59)	21.3 ± 4.56 (14 - 34)
[£]Gamma-glutamyltransferase (U/L)	61.2 ± 33.66 (21 - 168)	68.0 ± 45.41 (21 - 213)
ILAE Classification of Drug Response(50)		
Drug Responsive, n (%)	25 (35.2%)	6 (23.1%)
Drug Resistant, n (%)	25 (35.2%)	11 (42.3%)
Undefined, n (%)	21 (29.6%)	9 (34.6%)

[£] Mean (range); *Subjects may be on more than 1 concomitant antiepileptic drugs; ILAE, International League Against Epilepsy; CBZ, carbamazepine

Generally, PWE who were in the DRE group documented higher frequency of side effects. There were a total of 15 PWE who complained of side effects from the AEDs they were on, with no specific attribution to CBZ use. Their C_{plasma} ranged from 6 – 12 mg/L and was within the therapeutic range. Among these cases, 6 (40%) had persistent tremor, 3 (20%) had occasional dizziness during the day, 3 (20%) had weight gain, 2 (13%) felt sleepy during the day and 1 (7%) complained of memory problems.

On the other hand, the observed C_{plasma} and C_{dbs} were plotted according to their corresponding drug response categories. As shown in Figure 21, PWE who were categorized as having DRE (Category 2), were observed to have C_{plasma} clustering around 7-9 mg/L. In comparison, those who were seizure free (Category 1) had a wider range of C_{plasma} (2.5 -13.8 mg/L). Despite on lower doses of CBZ (11.90 ± 4.82 mg/kg) than DRE subjects, (17.70 ± 6.21 mg/kg) *($p < 0.001$),* a few seizure free PWE had C_{plasma} which exceeded the therapeutic range. Interestingly, this scenario was not observed in C_{dbs}, where seizure free PWE had similar concentration distribution with their DRE counterparts. Their C_{dbs} lied within the plasma therapeutic ranges of CBZ.

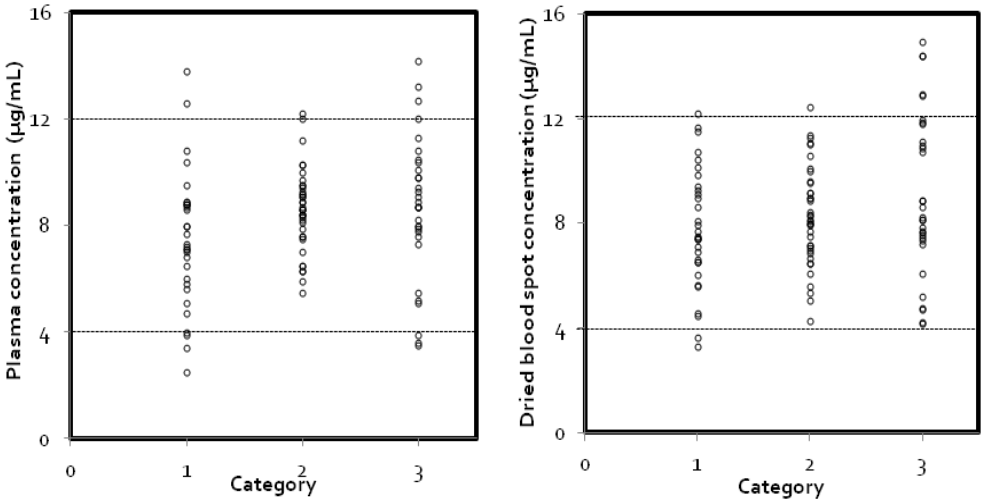

Figure 21. Observed carbamazepine concentration of (left) plasma and (right) dried blood spot in people with epilepsy grouped according to the drug response categories. Category 1, 2 and 3 represents drug responsive, drug resistant epilepsy and undefined category, respectively. The broken lines outline the therapeutic range

8.4.2 STRUCTURAL BASIC MODELS

Inter-individual variability was best described with exponential error while residual variability with additive error estimates. The typical CL/F values obtained from the population modelling were 5.88 L/hr (CV 5.16%, 95% CI 5.81 – 5.95) for C_{plasma} and 5.68 L/hr (CV 5.63%, 95% CI 5.53 – 5.77) for C_{dbs}. The fixed V_d/F was 50 L for plasma and 66 L for DBS structural PPK model. Interestingly, both models shared similar k_a values, 0.47 h^{-1} as well as the inter-individual and residual variability, 0.1% and 0.22 mg/L, respectively. The scatter plots of the weighted residual versus the individual and population predicted C_{plasma} did not show any bias issue with the structural models built (Figure 22).

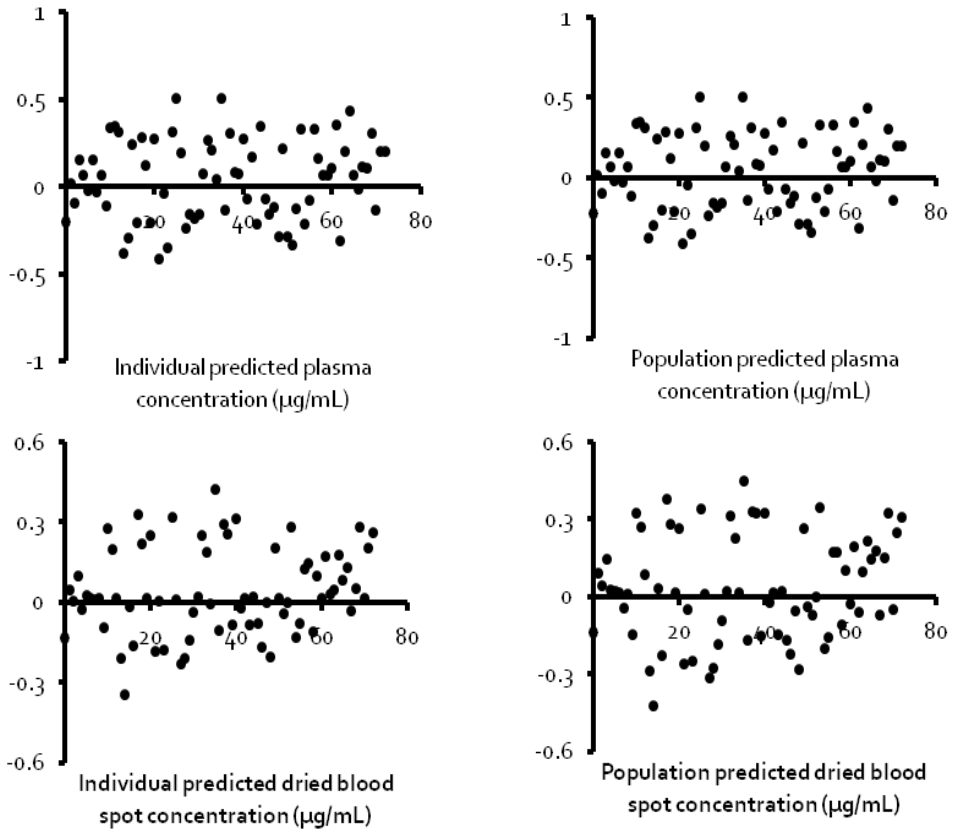

Figure 22. Weighted residual plots for predicted concentrations using individual and population estimates for the structural models of (top left & right) plasma and (bottom left & right) dried blood spot concentrations

8.4.3 COVARIATE MODELS

Plasma concentrations

Of all the potential covariates added to the model, only daily dose per body weight, male gender and concurrent therapy with VPA influenced the model significantly. The changes induced were tabulated in Table 17. A preliminary full model was generated with the covariates outlined in Equation 1.

Table 17. Summary of forward stepwise addition and backward elimination of covariates during final model building

Effect	OBJ	ΔOBJ	P value / Comment
Plasma clearance (CL)			
Base model	191.68		
Forward inclusion			
Weight (kg)	191.01	-0.67	NS
Age (years) *	210.74	19.06	Worsened
Age> 60	190.05	-1.63	NS
Total daily dose per unit weight (mg/kg)	170.18	-21.49	<0.001
Albumin (g/L)	191.39	-0.29	NS
Gender	185.47	-6.21	<0.05
Chinese	188.62	-3.06	NS
Valproic Acid	179.39	-12.28	<0.001
Seizure Free	190.45	-1.23	NS
Drug Resistant Epilepsy	189.85	-1.82	NS
Alkaline phosphatase (U/L)	190.43	-1.25	NS
Alanine aminotransferase (U/L)	190.45	-1.23	NS
Aspartate aminotransferase (U/L)	190.46	-1.22	NS
Full Model (preliminary)	150.25		
Backward Elimination			
Sex	164.03	13.78	<0.001
Total daily dose per unit weight (mg/kg)	173.94	23.69	<0.001
Valproic Acid	153.99	3.74	NS
Final Model	153.99		
Final Model FOCE	149.70		
Dried blood spot clearance			
Base model	219.60		
Forward inclusion			
Albumin (g/L)*	230.01	10.41	Worsened
Weight (kg)	219.58	-0.02	NS
Age (years)	218.23	-1.37	NS
Total daily dose per unit weight (mg/kg)	195.82	-23.78	<0.001
Sex	219.02	-0.57	NS
Final Model	195.82		
Final Model FOCE	193.72		

FOCE = first order conditional estimation; **OBJ** = objective function value; **ΔOBJ** = change in objective function value; **NS =** not significant

*denotes that the covariate inclusion into the model had worsen the estimated clearance and was hence, excluded

Equation1

$$CL/F_{plasma} = \theta \times (\frac{TOTAL\ DAILY\ DOSE}{WEIGHT \times 15})^{\theta_1} \times \theta_2^{SEX} \times \theta_3^{VPA}$$

where CL/F_{plasma} is the clearance (L/hr) estimated using C_{plasma} of CBZ, $\theta, \theta_1, \theta_2$ and θ_3 are the fixed effect parameters for the clearance intercept, total daily dose per weight (mg/kg), sex (female is denoted as 1 and male is denoted as 0) and concurrent therapy with VPA (presence of VPA is denoted as 1 and absence of VPA is denoted as 0). Backward elimination established the importance of total daily dose per weight and female gender in CBZ clearance. The inter-individual variability rose slightly to 0.20% while the residual variability was 0.86 mg/L. Therefore, the final model obtained is as represented by Equation 2.

Equation 2

$$CL/F_{plasma} = 5.984 \times (\frac{TOTAL\ DAILY\ DOSE}{WEIGHT \times 15})^{0.5199} \times 0.773^{SEX}$$

where CL/F_{plasma} is the clearance (L/hr) estimated using C_{plasma} of CBZ, total daily dose per weight (mg/kg) and sex (female is denoted as 1 and male is denoted as 0).

The parameters estimated from the final model conformed to those from FOCE algorithm. The differences of estimated were marginally higher in FOCE, but less than 1.7%. After systematic variation of estimates, the final fixed effects were optimized with k_a of 0.47/hr and V_d/F of 66 L.

Dried blood spot concentrations

Similar approach of forward stepwise inclusion revealed total daily dose per body weight to be the only covariate that improved clearance estimates in the final model generated by C_{dbs} (Equation 3). Backward elimination was not performed since there was only 1 significant covariate.

Equation 3

$$CL/F_{dbs} = 0.9842 \times (\frac{TOTAL\ DAILY\ DOSE}{WEIGHT})^{0.6152}$$

where CL/F_{dbs} is the clearance (L/hr) estimated using C_{dbs} of CBZ and the total daily dose per weight is in mg/kg. Inter-individual variability rose to 1.45% while residual variability was consistent at around 0.26 mg/L. Similar to the population estimates calculated using C_{plasma} of CBZ, estimates from the final model also conformed to that calculated from FOCE (< 2%). The objective function values, however, were higher in all the models generated using $C_{dbs.}$ The optimized fixed effect of k_a was 0.47/hr and V_d/F was 88 L for the final model.

8.4.4 EVALUATION

The prediction errors determined from basic and final models of CL/F_{plasma} (Equation 2) and CL/F_{dbs} (Equation 3) were similar. As can be seen from Table 18, the final models generated from C_{plasma} and C_{dbs} are unbiased (95% CI of ME included zero) and precise (MAE and RMSE are close to zero). These final models gave a 10% and 40% improvement in the predictions of C_{plasma} and C_{dbs}, respectively. The scatter plots of the predicted concentrations using the final models of C_{plasma} and C_{dbs} were shown in Figure 23.

Table 18. Comparison of prediction errors of estimation between basic and final population models constructed using plasma and dried blood spot concentrations of carbamazepine

Errors, mg/L (95% CI)	Plasma Concentrations		Dried blood spot concentrations	
	Basic Model	Final Model	Basic Model	Final Model
ME	-1.31 (-2.06 to -0.55)	-0.72 (-1.49 to 0.05)	0.20 (-1.38 to 1.78)	-0.64 (-1.59 to 0.30)
MAE	2.00 (1.55 to 2.46)	1.73 (1.27 to 2.18)	2.72 (1.55 to 3.88)	1.91 (1.28 to 2.54)
RMSE	2.32 (1.42 – 3.22)	2.09 (1.17 to 3.01)	4.03 (1.86 to 6.20)	2.50 (1.36 to 3.64)

***ME** = mean error; **MAE** = mean prediction error; **RMSE** = root mean square error

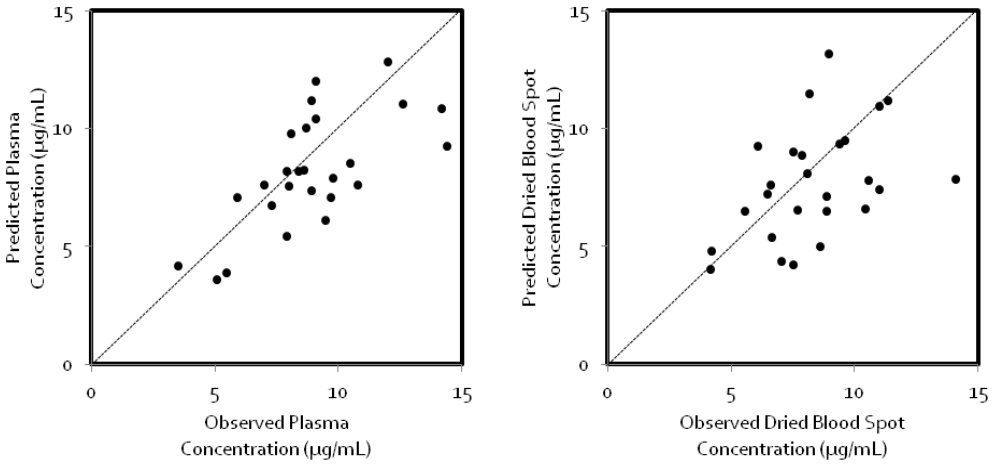

Figure 23. Observed concentration plotted against its predicted concentration by the final models of (left) plasma and (right) dried blood spot carbamazepine concentrations. The broken line represents the line of unity

8.5 DISCUSSION

In the present study, total daily dose per unit weight (DD) and gender influenced the CBZ CL/F_{plasma}. As the DD increases, there is a non-linear increase in CL/F_{plasma} and CL/F_{dbs}, a finding that echoed many others (Table 19) (301, 305, 306). The higher CL/F observed with higher CBZ doses could be a result of either lower bioavailability (F) or induction of hepatic metabolism (301, 305-308). In practice, doses are adjusted based on both clinical response as well as C_{plasma}. Therefore, it is likely that PWE receive

higher doses because their clearances of CBZ are higher. Indeed, this is supported by the observations of similar concentrations distribution among all the PWE although PWE in DRE category were receiving significantly higher doses of CBZ as compared to the rest. The inclusion of DD into our PPK models resulted in an improved overall fit, amidst the suggestion that this covariate could be bias since the model development itself was based on the dose input (298). Moreover, the observed correlation between DD and CL/F_{plasma} could have been induced by the process of TDM itself. Nevertheless, the improved fit with DD could reflect that in our setting, slower dissolution, incomplete absorption and the induction of hepatic metabolism with increasing doses of CBZ may better justify CL/F estimates than bias effect by the dose input (306, 307).

Table 19. A summary of characteristics and clearance estimates from different populations

Country	**No of subjects (No of observations)**	**DD (mg/kg/d) Mean±SD (Range)**	**Weight (kg) Mean±SD (Range)**	**Age (years) Mean±SD (Range)**	**Clearance (L/h)**	**Significant covariates**
USA (300)	113 (833)	NA	80.6±19.4	46.1±14.9	11.7 (CL_{un})	Caucasian
Serbia (305)	256 (423)	11.9±0.6	71.0±16.0	37.0±16.0	5.35	DD, TBW , PHB, VPA
Russia (299)	74 (NA)	Elderly: 5.9±3.3 Young: 9.1±4.7	Elderly: 77.0±11.6 Young: 69.0±2.3	Elderly: 62.9±6.18 Young: 34.1±7.80	Elderly: 3.08* Young: 4.43*	Age (>60 years old)
Singapore (85)	193 (302)	16.7±8.4	34.9±20.5	12.5±10.1	2.92*	TBW, Age, PHB
China (301)	585 (687)	9.9 (1.2-16.0)	53 (5-115)	23.3 (1.2-85.1)	5.71*	DD, TBW, PHT
China (309)	408 (459)	9.7±5.7	52.4±18.1	22.6±14.9	5.23*	TDD, TBW, VPA, PHT
USA (90)	829 (1834)	12.9 (1.3-42.7)	75 (28-159)	35 (17-89)	4.73*	TBW, Age (>70 years old), PHT, PHB/FBM,
France (310)	23 (~138)	19.8±5.7	53.4±11.9	21.0±5.4	5.60	NA
USA (311)	52 (57)	14.7±4.7	40.2±21.6	9.1±4.4	3.58	NA
UK (312)	48 (149)	9.7±4.4	60.8±14.4	27.8±13.0	8.41 (CL_{un})	NA
Egypt (313)	302 (302)	15.0±7.84	55.3±19.7	22.1±12.4	3.51	NA
Singapore (This study)	**71 (72)**	**14.2 ± 4.9**	**65.7 ± 19.01**	**42.7 ± 11.23**	**Plasma:5.89 DBS:5.68**	**Plasma: DD, Gender DBS: DD**

NA = not readily available from publication; **SD** = standard deviation; **CL_{un}** = clearance of unbound carbamazepine; **DD** = total daily dose per weight; **TDD** = total daily dose (mg); **TBW** = total body weight (kg); **PHB** = phenobarbitone; **VPA** = valproic acid; **PHT** = phenytoin; **FBM** = felbamate; **DBS** = dried blood spot

*clearance is an approximate as It is estimated based on a 65kg male, with CBZ dose of 15mg/kg, taking concurrent phenytoin/phenorbarbitone and aged 43 years old

On the other hand, male PWE had approximately 22.7% higher CL/F_{plasma} than their female counterparts, an observation that had been previously demonstrated (298, 314). It is suggested that estrogen in female could have inhibited the microsomal enzymes responsible in metabolizing CBZ and resulted in lower CL/F_{plasma}.(315) In addition, female PWE had a significantly higher total body weight than the male PWE (71.53 ± 14.64 kg versus 60.42 ± 15.0 kg). This had given rise to a greater DD in the male PWE. As both body weight and total daily dose per unit weight were generally associated with increased clearance (301, 305), a complex interplay between these few factors could have contributed to the increased CL/F_{plasma} in male PWE. Nevertheless, it is important to note that in the final PPK model built, the fixed effects of DD and SEX were not correlated, $r < -0.131$, indicating that they are independent of each other in this study.

In contrast, SEX was an insignificant covariate when estimating CL/F_{dbs}. In an earlier plasma and whole blood drug concentrations correlation study (Chapter 7), it was suggested that CBZ do reside in RBCs, although the magnitude and binding site/s are yet to be determined. In that study, despite being diluted by the presence of RBC in whole blood, C_{dbs} and C_{plasma} were comparable with an almost 1:1 conversion. As male PWE has higher RBC and hematocrit values, the additional amount of CBZ concentrated in RBC could have annulled any potential increment in CL/F_{dbs}. However, in view that the CL/F_{dbs} was estimated based on C_{dbs}, the subtle effect by RBC and its component was already incorporated, in part, during the model building. Therefore, independently, SEX does not seem to improve PPK of C_{dbs} model fitting. However, there is another school of thought whereby the observed significance of SEX as a covariate in CL/F_{plasma} estimation could be just an incidental finding. It was demonstrated that SEX do not withstand allometric scaling to size, which decoupled the effect of size and diminished the influence of SEX on drug clearance (316). For VPA, few studies documented

a definitive increase in CBZ CL/F_{plasma} of 7% to 23% as compared to those who were not taking VPA (306, 309). Here, CL/F_{plasma} seems to increase by 13% with the presence of VPA, but this increment failed to attain clinical significance after non-linear adjustment made to the model for DD and SEX. Aside from inhibition of microsomal enzymatic metabolism of CBZ, which leads to reduction in CL/F_{plasma}, VPA could also displace CBZ from its binding site of proteins, which if not metabolized, would lead to increment in CL/F_{plasma}. Depending on which effect is more prominent, CL/F_{plasma} of CBZ could either be higher, lower or unchanged.(317, 318) In this study, the effect could be unchanged. Researchers from a Chinese population had utilized a dichotomous approach for total daily VPA dose per unit weight as a covariate and concluded that significant change in CBZ CL/F_{plasma} was only demonstrated when the VPA dose exceeds 18mg/kg/d (309). The average total daily VPA dose per unit weight in this population was found to be 14.71 ± 7.12 mg/kg/d, which might be too low to impact CBZ pharmacokinetics. However, considering the complexity and projected magnitude of interactions between VPA and CBZ, it is still advisable to continue therapeutic monitoring when these two drugs are used concurrently, especially during dosing adjustments of either drug.

For CBZ elimination, it is primarily metabolized via epoxide hydrolase, forming an active metabolite CBZ-epoxide (CBZ-E). There are various reports on the effect of concurrent AEDs in altering CBZ CL/F_{plasma}, particularly the older generation drugs, which are well-documented to be either enzyme inducers or inhibitor (85, 90, 301, 305, 309). For example, PHT and PHB can induce CBZ biotransformation, resulting in a higher CBZ CL/F_{plasma}. The magnitude of biotransformation differs, with approximately 40 to 45% and 17 to 40% increase in CL/F_{plasma} when subject is on concurrent therapy with PHT (90, 301, 308) and PHB (85, 90), respectively. However, this study did not include any subject on PHT and only 6% were on PHB. It is likely a result of change in clinical practice where VPA, LEV and CLB are favoured over PHT and PHB as adjunctive therapy. The PPK model did not manage to identify

PHB influence on CBZ CL/F_{plasma}, probably due to the small number of related subjects.

Generally, DRE subjects received higher doses of CBZ than seizure free subjects and demonstrated an approximately 4% higher CL/F_{plasma} as compared to their counterparts. Nevertheless, this difference was not significant, a result comparable to an Egyptian study (313). These observations support the fact that PWE with harder to control seizure does require significantly higher amount of CBZ in order to attain therapeutic concentrations, and it may be related to the lower free fraction of CBZ. In contrast, similar trend of increment was not observed in whole blood clearance as calculated from C_{dbs}. It is hence speculated that in DRE subjects, there may be preference for CBZ to reside in RBC compartments, rendering them 'inactive' for elimination. This additional compartment may also explain the increased V_d/F required for C_{dbs} PPK model convergence. As only unbound CBZ will penetrate brain, distribution into RBCs may pose as hindrance for its action. This suggests that there may be a need for higher therapeutic range for PWE with harder to control seizures. However, caution is required against appearances of adverse drug reaction with higher CBZ concentrations.

Meanwhile, side effects occurrence within therapeutic ranges is not uncommon. Good correlations between plasma total and free CBZ concentrations and dose-related side effects, predominantly diplopia and nystagmus were previously demonstrated (319-321). In addition, intermittent occurrences of side effects are postulated to be related to diurnal fluctuations of CBZ C_{plasma} in between doses (320, 322). It is also noteworthy that unbound CBZ decreases with higher total CBZ concentrations (323) and hence, measurement of either one of the total or free CBZ might not suffice to explain the occurrences of side effects. On the other hand, previous works did not support correlation between the concentrations of CBZ active metabolite, CBZ-E (total and unbound) and side effects (319, 321). Theoretically, antibody immunoassay of CBZ quantitation could cross-react with CBZ-E,

resulting in overestimation of C_{plasma} of CBZ. A quick look at the CBZ concentrations of this group of PWE who complained of side effects revealed that their range was between 6 – 12 mg/L for C_{plasma} and 4.3 – 11 mg/L for C_{dbs}. In view that C_{dbs} measures only CBZ and not CBZ-E, the lower range of concentrations seems justifiable. Considering that at trough concentrations of CBZ, CBZ-E exists between 15% to 20% of the parent drug concentrations and cross-reaction was reported to be approximately 8% in Beckman system, it was likely that cross reaction in immunoassay observed here was minimum (324). Although tempted to correlate the C_{dbs} concentrations with side effects, it is uncertain at this point if C_{dbs} could provide a better correlation with the occurrences of these events as the sample size is too small. Generally, for PWE who are on normal release formulation of CBZ, lowering the dose but increasing frequencies of CBZ administration (to retain total effective daily doses of CBZ), may aid in reducing the degree of fluctuations in both C_{plasma} and C_{dbs}, and overall, minimise extend of side effects. Alternatively, they could be put on a trial of controlled release (CR) formulation. Thereafter, direct attribution of side effects to CBZ use must be affirmed before further changes in therapy can be proposed. Future PPK studies incorporating a larger population and concurrent measurement of plasma and DBS concentrations of CBZ, CBZ-E, unbound CBZ and CBZ-E should be more informative in delineating this relationship.

The population estimate of CL/F_{plasma} of approximately 5.89 L/h concurred with those found in previous studies, as summarized in Table 19. As majority (72%) of this population was on polytherapy, CL/F_{plasma} estimated are similar to the other polytherapy populations (90, 310, 313, 325). Interestingly, CL/F_{dbs} derived using similar PPK approach resulted in similar model specifications and identical parameter estimate with $CL/F_{plasma.}$ A typical CL/F_{dbs} in this population is 5.68 L/h. C_{dbs} of CBZ could be an alternative to C_{plasma} for PPK parameters estimation due to a few positive observations. Firstly, both the DBS and plasma models yielded similar inter-individual and residual variation in their respective PPK model. Of note, residual variability

error encompasses intra-individual, model misspecification as well as sampling and/or assay errors of pharmacokinetic parameters. This may imply that these variations, as a whole, are adequately and comparably addressed in both models, and that CL/F_{dbs} derived from C_{dbs} are as representative of the population as CL/F_{plasma} derived from C_{plasma}. Secondly, their final prediction models gave an almost identical precision estimates which further suggests that C_{dbs} and C_{plasma} could be used interchangeably for PPK studies. As such, the target therapeutic ranges for C_{dbs} are assumed to be similar to those of C_{plasma}, which is within 4 – 12 mg/L. However, for DRE subjects, there may be a need to establish a different effective range since the current range does not seem practical in controlling their seizures. Finally, there appears to be a more direct relationship between the dose and C_{dbs} since no other tested covariates showed significant influence. This may ease dosing adjustments in clinical practice if C_{dbs} is to be monitored routinely. To illustrate, for every1 mg/kg/d of increment in CBZ dose in a 65 kg individual, there will be a 0.44 L/h increase in clearance, assuming F is constant and there is no change in formulation. In a PWE with twice a day dosing, this increment, will result in a post 3-hr, post 8-hr and post 11.5-hr C_{dbs} increment of 1.77 mg/L, 1.51 mg/L and 1.24 mg/L, correspondingly. For C_{plasma} adjustment, however, the dose increase would be different for male and female PWE.

8.6 CHAPTER CONCLUSION

In summary, in this adult population of PWE, ethinicity, age and concurrent AEDs do not significantly alter their CBZ PK. For C_{dbs}, only the total daily dose per unit weight is the significant determinant of CBZ concentration. Similar to many PPK studies, the retrospective design of the study was the main limitation. The non-availability of data, especially the renal profiles prevented a more thorough investigation. Also, to ease sample collection and for direct comparison with venous plasma, DBS in this study was obtained from EDTA anti-coagulated venous whole blood. Unlike finger

prick DBS, this whole blood DBS is dried but not coagulated. Caution may be required for its similarity to finger prick DBS. Provided that the technique is correct, direct drop of blood onto filter paper would distribute uniformly through the filter paper, (326) minimizing coagulation effect. Usually the extraction of xenobiotic from blood dried on filter paper is related to hematocrit and blood spot volume (149, 327). In addition, researchers suggested that blotting onto filter paper rather than presence of EDTA is more likely the reason for different analyte yield (328). Of note, EDTA did not exert any stabilizing effect on ciclosporin extracted from DBS, although its influence on CBZ (329). In another study which also involved whole blood concentration of ciclosporin, the concentration from finger prick blood spot was similar to those from EDTA-anticoagulated venous blood, (160) outlining the comparability in recovery.

Nevertheless, it is demonstrated here, that for CBZ PPK studies, the use of DBS could be comparable to the use of plasma. Further studies involving more observations per individual and utilization of Bayesian feedback forecasting might improve both the estimation as well as prediction using C_{dbs}.

CHAPTER 9

Thesis Conclusion
&
Future Directions

CHAPTER 9. THESIS CONCLUSION & FUTURE DIRECTION

In Chapter 1, an overview of DRE, TDM of AEDs and DBS are presented. Some of the gaps and limitations of current knowledge on these three topics which are directly related to the study objectives are then discussed in Chapter 2.

In short, epilepsy is a dynamic disease where sampling time is crucial. As outlined in Chapter 2, Objectives, depending on the type of information required, sampling had to be timed appropriately to capture the most representative change in metabolite profiles of these patients. Same theory applies to TDM of some of the most effective and most commonly used AEDs such as CBZ, VPA and PHT where the drug concentration in the body at specific designated time could yield specific information. To illustrate, the concentration of AEDs directly after a seizure attack would be indicative of their relationship to disease control.

Dried blood spot usage has been expanding in recent years for various type of research. It is an attractive matrix as it can be collected by PWE themselves. With DBS, the accurate timing for sampling can be assured. The findings from this thesis can serve as a stepping stone for larger or population-based clinical research where patient does not need to travel to hospital for sample acquisition. The caveat is of course, proper training on eligible DBS is instilled on the potential sample collector.

To facilitate the implementation of DBS use in clinical practice and research, this thesis set out with 2 main objectives; (1) to investigate DBS feasibility in epilepsy and (2) its drug treatments monitoring.

For the first objective, a DRE prevalence study to determine the severity and relevant risk factors was firstly conducted, which is written in

Chapter 4. Then, the reliability and utility of DBS in metabolomic study by comparing its findings with those from plasma (Chapter 5) and in treatment modified animal models (Appendices 1 and 2) were examined. Finally, in Chapter 6, the results obtained from 142 DBS samples belonged to subject who was either drug responsive or drug resistant at the point of recruitment were presented.

Drug resistant epilepsy has been a longstanding complication among epilepsy patients. DRE prevalence and risk factors have always been topics of investigations. The last survey done in Singapore was on 1997, among the army cohort. With the development of a new consensus for definition of DRE, we embarked in characterizing the drug response of clinic population of PWE. As illustrated in Chapter 4, 1 in 5 PWE of the studied population had drug resistant, and this finding is comparable to the others. Genetic factors, as assessed by genetic aetiology and ethnicity, did not seem to directly contribute to a subject's drug resistant condition. However, as genetic testing was not routinely tested, specific genetic abnormalities was not correlated with the likelihood of having DRE. Based on the metabolite pathways that were affected from metabolomic profiling study discussed in Chapter 6, future work could incorporate genetic testing for diseases which were related to these pathways. For example, SNAT transporters mutation which could affect glutamine transport can be included for concurrent investigation with DRE metabolomic study. This can ascertain whether glutamine metabolism was affected during the drug resistant epileptogenesis or because of inherent genetic defects.

Neurological disease is best studied using CSF. In chapter 6, there is an incidental finding that capillary blood is more informative than venous blood in discerning drug resistant from drug responsive metabolomic profiles. It is probably attributable to capillary blood's resemblance to CSF in certain aspect. Verification, however, is best done with concurrent analysis of CSF samples. The analysis done in this study did provide some invaluable insights on the

advantage of DBS and capillary blood in study of epilepsy patients. As discussed in Chapter 6, direct spotting of blood drop onto 903® cards could have halted some possible enzymatic degradation of endogenous metabolites and improved the information retrieval from whole blood. Nevertheless, serum/plasma '–omics' studies does have the advantage of established standards and protocols that can be instilled to prevent enzymatic degradation and ensure consistency of analysis results, albeit the additional steps involved. In view of the complexity and ethical issues associated with CSF sampling acquisition, DBS from finger prick is suggested as a favourable surrogate for now.

For the second objective, clinical validation (Chapter 7) and population pharmacokinetic modelling (Chapter 8) using DBS derived concentrations were conducted to examine their immediate applicability in a clinical setting. A previously validated assay with proven recovery and accuracy of CBZ, PHT and VPA were utilized.

Therapeutic drug monitoring with DBS has always been proposed (157). The continuous development of validated assays for AEDs quantitation using DBS had substantiated its reliability and benefits over conventional plasma measurement. In Chapter 7, we had demonstrated the comparability of the current proposed DBS and GCMS method with conventional plasma immunoassays. For the first time, concurrent DBS quantitation of the respective CBZ, PHT and VPA were clinically validated in our population of PWE. As subjects were recruited based on their requirement of TDM, they should represent the general population who will undergo TDM routinely. However, none of the critically ill nor subjects who were just started on AED/s were included. Hence, the applicability of this DBS quantitation method remains unknown. It is likely that these groups of subjects are hemodynamically unstable and theoretical C_{plasma} may be overestimated, especially if DBS is acquired via finger prick. In spite of this, by investigating the degree of variation between the critically ill or newly started on AEDs

subjects with the typical PWE such as those recruited in this study, the conversion could accurately be estimated.

C_{plasma} of drugs with narrow therapeutic indexes; low concentrations associated with therapeutic failure while high concentrations lead to adverse drug reactions, are usually employed in compilation of pharmacokinetic parameter estimates such as clearance and volume of distribution. These estimates are important to approximate both maintenance and loading doses of relevant AEDs which can attain and sustain the desired target concentrations within the fastest time and for the longest duration. Using C_{dbs} of CBZ, AED with the highest RBC/plasma ratio among the 3 AEDs studied in Chapter 6, the PPK parameter estimation was found to be comparable to those derived from C_{plasma}. The methods and results were explained in Chapter 8. Therefore, in terms of PK related monitoring and research work, DBS could perform as well as plasma. Further PPK compilation using C_{dbs} of PHT and VPA, drugs with R/P < 1, would be able to reaffirm DBS feasibility and probably a new set of target DBD therapeutic ranges could be proposed.

In relation to research direction, further study which investigates the metabolomic profile difference between finger prick and venous DBS from the same epilepsy individual can be considered. If permissible, simultaneous cerebrospinal dried biofluid spot profiling will be ideal. Quantitation of the discriminating metabolites would provide valuable evidence to finger prick DBS in the study and monitoring of epileptogenesis. These findings will help answer the hypothesis that capillary DBS is significantly different from venous's and delineate the similarity between capillary DBS and CSF.

To further comprehend the drug resistant epileptogenesis, future work should include genomic and proteomic profiling to ascertain the inherent or acquired mechanism of the disease. As outlined earlier, genetic and transcription abnormalities related to the affected pathways discussed in Chapter 6 could be referred. Since this study involved only one time point observation, future research should consider multiple sampling points from the

same subject; at seizure free, drug resistant and also in between this period to detect potential biomarker that marks the transition phases.

Although TDM is usually considered at steady state trough concentrations, attempt ought to be made to establish correction factors between finger prick DBS with venous plasma during the dose titration phase of CBZ, PHT and VPA. Again, multiple sampling is encouraged. Furthermore, critically ill patients should be included as this group of patients is projected to have elevated whole blood levels that must be adjusted against its venous plasma levels to facilitate dosage adjustments. Similar to our research approach in Chapter 8, these DBS levels can be subjected to non-linear mixed effects modelling to approximate its pharmacokinetic parameters and contribution to seizure control.

In summary, epilepsy is a disease which requires further understanding in its pathogenesis and optimization of its treatment drugs. As evidenced by results in Chapter 6, 7 and 8, DBS could be a suitable matrix for both aspects. Once the relevant biomarkers of epilepsy are established, simultaneous monitoring of them together with AEDs could be developed. This, in turn, will aid the management of epilepsy patients by rendering monitoring of disease as well drugs, one dried blood spot away.

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APPENDICES

APPENDIX I. DRIED BLOOD SPOT USE IN METABOLIC PROFILING AFTER PHARMACOLOGICAL INHIBITION OF THE P-GP EXPRESSION WITH LY335979

1.1 Objectives

P-glycoprotein transporters over-expression has been linked to pharmacoresistance in epilepsy as well as other diseases such as cancer. As Pgp also transports endogenous metabolites, its over-expression will impact the metabolites transport as well. These endogenous perturbations are assumed to be similar to a 'diseased' state. The currently available Pgp functional assays detect Pgp efflux activities using chemoluminiscent contrast, which are quantitated based on fold changes. Perhaps, there are certain metabolite level changes which are associated with its increased or decreased activity. This metabolite has the potential to be a quantitative biomarker of Pgp expressions. Hence, we had designed an *in vivo* investigation involving the inhibition of this transporter. It is hypothesized to provide insight of some of these biomarkers, which could be indicative of the severity of the 'disease'. Since we proposed the use of DBS for metabolite, we will explore the utility of dried blood spot for detection of metabolic profiling changes caused by P-glycoprotein transporters inhibition.

1.2 Methods

A total of 18 C57BL/6 male mice were equally divided into 3 groups. They were aged between 10 - 12 weeks and weighed an average of 25.5 g (24-28 g). A selective P-glycoprotein inhibitor, LY335979 (zosuquidar hydrochloride) was dissolved with miminum DMSO, further diluted with water. Three different doses were given to capture the dose-concentration

relationship, with higher concentration of LY335979 representing greater inhibition of Pgp efflux activities (Table A1.1).

Table A1.1 Different treatment groups of LY335979

Groups	Group 1	Group 2	Group 3
LY335979 dose	100mg/kg	50mg/kg	10mg/kg
Total mice at the end of study	5	6	5
	(one died on second day of treatment)		(one was excluded due to abnormal behavior)

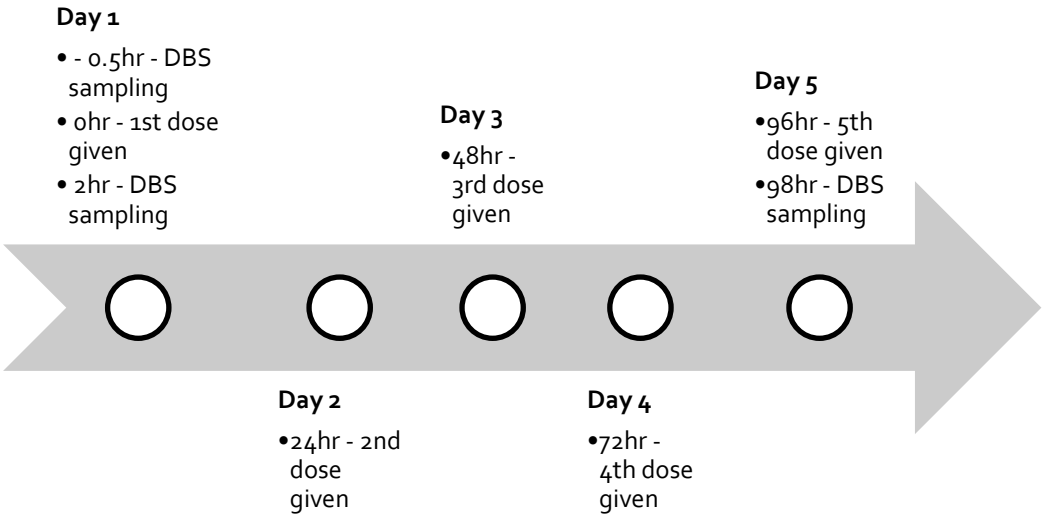


Figure A1.1. Timeline illustration for the dried blood spot sampling and treatment of LY335979 over 5 days. On day 1, 30 mins (-0.5hr) before first dose is given, 2 dried blood spots were obtained from all the mice via venipuncture. Similar dried blood spot acquisition was repeated at 2hr post-first dose treatment (2hr) and 2hr post-fifth dose treatment (98hr)

All mice were treated with LY335979 at 9 am daily for 5 consecutive days via oral gavage. Dried blood spot were obtained via venipuncture of facial vein by sterile lancet. At least 2 drops of blood, ~ 30 µL, were obtained from each mouse at 0 hr, 2 hr and 98 hr post treatment (Figure A1.1). The mice were fasted for at least 2 hours before and 2 hours after treatment to ensure maximum absorption of drugs. On the first and last day of treatment, the mice were fasted overnight. Overnight fasting is to allow for measurement of baseline endogenous metabolites.

1.2.1 Sample processing and GC-MS GC-MS settings for metabolomics

DBS samples processing, GC-MS settings and multivariate data processing were similar to those described in Chapter 6. Extraction of DBS was done twice, on separate occasion, first using 160 µL of methanol and subsequently using 160 µL of chloroform, with D27–myristic acid as the internal standard at concentration of 6.25 ng/mL and vortexed for 20 mins. The total methanol and chloroform extracts were centrifuged at 6 000 *g*, 22°C for 10 mins and 240 µL of the resulting supernatant would be transferred to 15 mL Kimble centrifuge glass tubes (Gerresheimer Co. Glass, Germany) for evaporation. Drying was done at 30°C under a stream of nitrogen. Thereafter, 100 µL of toluene was added, vortexed for 15 seconds and subjected to the similar condition for drying again. Toluene was used to ensure removal of water. Next, derivatization of the dried sample was accomplished using 100 µL MSTFA with 1% TMCS. The derivatized samples were vortexed for 1 min and incubated at 60°C for 1 hour. After being cooled to room temperature, 80 µL of this final supernatant was transferred into a 200 µL conical base inert glass insert placed inside a 2 mL amber autosampler glass vial (Agilent Technologies, Germany).

Analyses were done using GC-MS that comprised of 7890A GC System coupled to 5975 inert MSD with Triple-Axis Detector (Agilent Technologies) and an in-built Fiehn method of analysis for metabolomic screening.[2] The in-built settings include injector temperature at 250°C, split ratio of 10:1 at the starting temperature of 60°C held for 1 min, increased at 10°C per min to 325°C followed by a final hold for 10 mins (total run time of 37.5 mins). Ion source temperature was set to 250°C, while the scanning mass range was set as 50 to 600 *m/z*.

1.3 Results & Conclusion

A total of 325 metabolites were detected from all the mice. 113 were removed due to presence in less than 25% of the samples. As mice are assumed to be homogenous, lower presence were deemed acceptable for an exploratory analysis. In the end, 212 metabolites were subjected to multivariate analysis using SIMCA P+ v12.0. Significant discriminating metabolites which were obtained through the cross-validated PLS-DA model using post-98hr of Pgp inhibition were tabulated in Table A1.2. At post-2hr of Pgp inhibition, metabolite perturbations were not significantly altered between the 2 groups.

Table A1.2. Significant discriminating metabolites from dried blood spot obtained post-98hr of daily P-glycoprotein inhibition with LY335979

No	Putatively identified metabolites	Level differences
1	Oleic acid	Higher with increasing inhibition
2	Myristic acid	Higher with increasing inhibition
3	L-glutamic acid	Lower with increasing inhibition
4	L-threonine	Higher with increasing inhibition
5	L-methionine	Lower with increasing inhibition
6	L-tyrosine	Higher with increasing inhibition
7	L-serine	Higher with increasing inhibition
8	4-pyridoxic acid	Higher with increasing inhibition
9	Palmitic acid	Lower with increasing inhibition

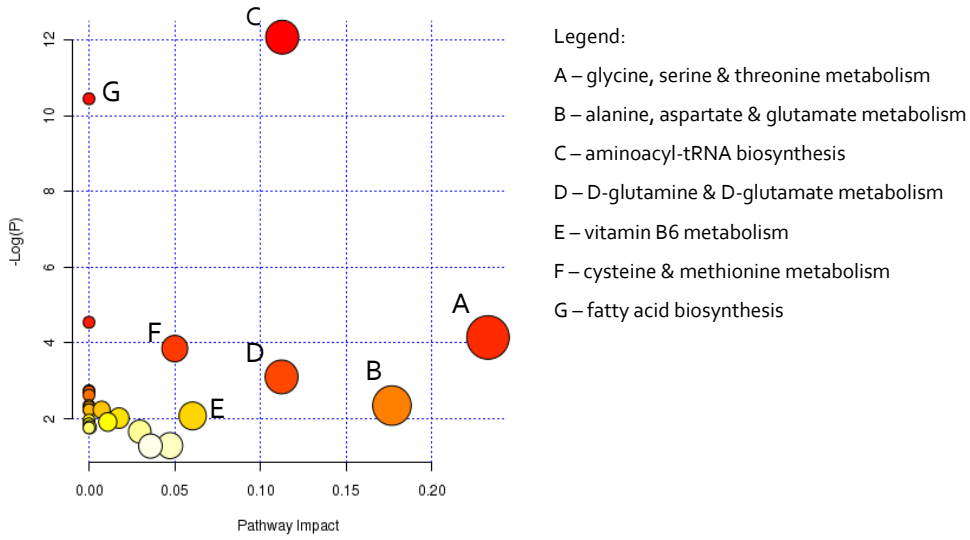


Figure A1.2. Visualization of biological pathway analysis using MetPA. Only the few most significant pathways were highlighted (those in darker shades)

Biological pathway visualization using the ten significant endogenous metabolites revealed that glycine-serine-threonine metabolism was the most significant pathway affected by Pgp inhibition (Figure A1.2). Alanine-aspartate-glutamate metabolism, aminoacyl-tRNA biosynthesis, glutamine-glutamate metabolism, vitamin B6 metabolism, cysteine-methione metabolism and fatty acid metabolism were also affected after selective inhibition of Pgp transporter with LY335979. Pgp transporters are present abundantly in liver, gut, renal and brain for the transport of xenotbiotics as well as endogenous metabolites. Therefore, the metabolite perturbations observed could be the effect of inhibition in any of or all these organs in the mice. These perturbations were assumed to represent disease-related metabolites disturbances. Although it has been shown to act by selectively inhibiting Pgp transporters (1), we cannot rule out the possibility of disturbances due to the presence of LY335979 itself. However, LY335979 has yet to be proven to react enzymatically *in vivo*, and is thus far, unlikely to cause metabolite perturbation via other routes (1). Nevertheless, in a chemical-induced Pgp inhibition mice model, the perturbations were detectable in DBS, as early as

post-98 hr of inhibition. DBS is hence, deemed suitable for metabolomic profiling of changes in a disease course.

In addition, a look at the detectable compounds in DBS revealed the presence of L-lysine-2, iminodiacetic acid, DL-threo-beta-hydroxyaspartic acid, citric acid and adipamide which were not extracted from DBS in our previous study (2). The use of double solvents for extraction helped to recover these metabolites from DBS.

APPENDIX II. DRIED BLOOD SPOT USE FOR QUANTITATION OF VALPROIC ACID DRUG LEVELS AND ITS ASSOCIATED METABOLOMIC CHANGES

2.1 Objectives

Valproic acid has been postulated to be able to selectively enhance post synaptic brain gamma-aminobutyric acid (GABA) responses, directly acting on neuronal membranes, reduce aspartate excitatory transmission and inhibit reuptake of GABA into the glia and nerve endings (3-5).

To minimise inherent metabolic changes due to epilepsy disease itself, normal healthy rats were used. The dose of VPA used in this study was the lowest in the dosing range employed in previous studies for the investigation of its toxicities (6-8). This is in hope to capture snapshots of the immediate and delayed metabolite changes that may shed insights into the metabolic pathways involved in its pharmacological actions, and generally the metabolomic changes induced by drug treatment.

Similarly, DBS use was coupled with GC-MS analytical platform. Since the amount of blood required was very little, we extended the DBS applicability to simultaneously quantify a targeted analyte concentration, which was VPA.

2.2 Methods

2.2.1 Animal study

The *in vivo* metabolic study was carried out according to the 'Guidelines on the Care and Use of Animals for Scientific Purposes' (National Advisory Committee for Laboratory Animal Research, Singapore, 2004). The

animal handling procedures of this study were reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS).

Sprague-Dawley rats (7-8 weeks old, ♂: 10) were purchased from Comparative Medicine Center (CMC) of NUS. The rats were kept at a specific pathogen free animal facility (24°C, 60% relative humidity) at CMC and maintained on a 12-h light/dark cycle with free access to food and water. Twenty four hours before the metabolic study, a polyethylene tube (I.D. 0.58 mm, O.D. 0.965 mm, Becton Dickinson, Sparks, MD 21152, USA) was inserted into the right jugular vein under isoflurane anesthesia. This catheter was used for drug injection and blood sampling at 9am on the study day.

On the study day, 5 rats were injected with VPA at a dose of 200mg/kg body weight (treatment group). The other 5 rats were given sodium chloride 0.9% as placebo (control group). Prior to VPA injection, blood samples (approximately 300 µL) from each of the 10 male rats and 10 female rats were collected and kept in ice box until they were processed. After the VPA injection, blood samples were collected at 0.5 hr, 2.5 hr, 5 hr, 8 hr and 24 hr respectively from the 10 rats. The 6 time points were estimated to represent the metabolite fluctuations in a day. Within 30 mins of collection, blood sample was spotted in duplicates of 25 µL onto 903® cards and were left dried in the fume hood for at least 3 hours, which was the observed minimum time required for complete dryness. After drying, the DBS were punched and the cores (6 mm) obtained were placed inside their respective Ependorff tubes. All the acquired DBS were stored at -80°C until processing for analysis.

2.2.2 Sample processing and GC-MS settings for metabolomics

Please refer to section Appendix 1. SIMCA P+ v12 was utilized for multivariate analysis.

2.2.3 Sample processing and GC-MS settings for valproic acid quantitation

The processing and GC-MS settings were similar to Chapter 7. Extraction of VPA from DBS was done using 480 µL of acetonitrile and 20 µL of Sodium Hydroxide 1N, vortexed for 1 min and followed by sonication for 5 mins. The resulting extracts were centrifuged at 3500 *g*, 22°C for 15 mins. Four hundred µL of supernatant were transferred into 15 mL Kimble centrifuge glass tubes for drying under a stream of nitrogen at 30°C, followed by removal of water using toluene and drying under nitrogen again. Derivatization was imposed on the dried samples using 50 µL MSTFA with 1% TMCS and incubation at 70°C for an optimum time of 50 mins. Dilution with 50 µL of hexane was carried out and vortexed for a min before 80 µL of the final volume is transferred into 200 µL conical base inert glass insert placed inside a 2 mL amber autosampler glass vial.

An assay for the quantitation of VPA concentrations extracted from DBS was previously optimized in accordance to International Conference of Harmonization (ICH) guideline. The settings included an injection temperature of 200°C, ion source temperature of 220°C, split ratio of 1:5, at the starting temperature of 90°C for 0.2 min with initial gradient ramp at 10°C/min to 120°C and held for 0.5 min. The second ramp imposed was 65°C/min up to 285°C and held for another 0.5 min. The temperature ramp was then reduced to 10°C/min to reach a temperature of 291°C and held for 0.2 min before continuing at 60°C/min to 300°C with a final hold at 300°C for 5 min. Selective ion monitoring (SIM) mode was applied for identifier ion of 201 and qualifying ions of 145 and 129. Calibration curve was established with 9 concentrations ranging from 0.5 µg/mL to 5 mg/mL.

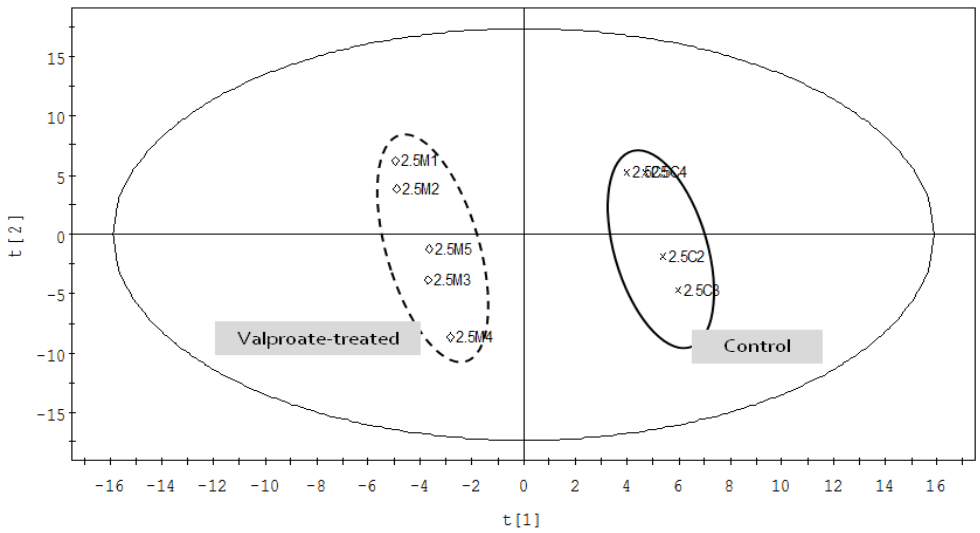
2.2.4 Pharmacokinetic Analysis

Concentration-time profiles of VPA after intravenous bolus injection were assessed using non compartmental model (WinNonlin Professional, Version 5.1, Pharsight Corporation, Mountain View, CA).

2.3 Results and Conclusions

2.3.1 Metabolomics

Peak area changes from baseline were utilized for multivariate analysis. Each rat served as their own control, where the changes of metabolites at 30 min, 150 min, 300 min, 480 min and 1440 min were modeled for differences between the control and VPA-treated rats. Clear clustering and reliable prediction between the 2 groups of rats were only observed at 150 min post VPA treatment (Figure A2.1). The comparison of the metabolite profiles at other timings did not yield reliable classification groups.



FigureA2.1. Partial least square-discriminant analysis score plots of metabolite profiles of control and valproate-treated rats at post-2.5hr treatment. Clear discrimination was observed

The discriminating metabolites after t-test analysis were tabulated in Table A2.1 while the biological pathway analysis was shown in Figure A2.2.

Table A2.1. Significant discriminating metabolites from dried blood spot obtained post-2.5hr of intravenous bolus administration of 200mg/kg of valproic acid

No	Putatively identified metabolites	Level differences
1	DL-3,4-dihydroxyphenyl glycol	Higher in VPA-treated Group
2	phenylacetaldehyde	Higher in Control Group
3	cholesterol	Higher in VPA-treated Group
4	3-hydroxycinnamic acid	Higher in Control Group
5	D-erythrose-4-phosphate	Higher in Control Group
6	L-glutamic acid	Higher in VPA-treated Group
7	oxalic acid	Higher in VPA-treated Group
8	2-aminoethanethiol	Higher in Control Group

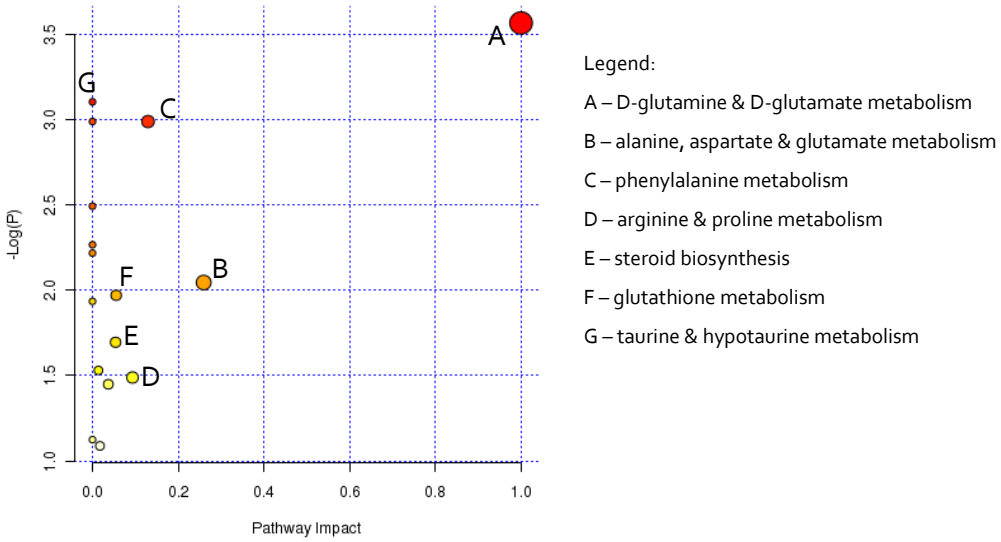


Figure A2.2. Visualization of biological pathway analysis using MetPA. Only the few most significant pathways were highlighted (those in darker shades)

2.3.2 Quantitation of VPA

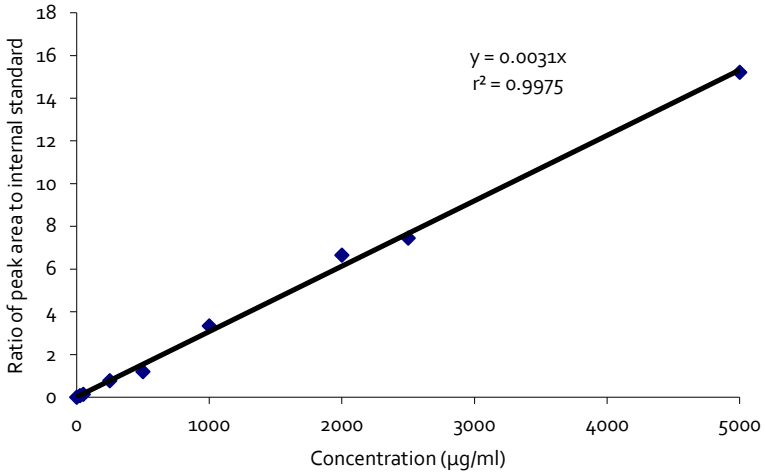


Figure A2.3. Calibration curve for valproic acid concentrations extracted from dried blood spot samples ranging from 0.5 µg/ml to 5000 µg/ml. The correlation factor, r^2 = 0.9975

Linearity of VPA concentration was defined using seven calibration standard solutions ranging from 0.5 µg/mL to 5 mg/mL (Figure A2.3). Linear regression analysis was used to establish the calibration curve which had a correlation coefficient (r^2) of greater than 0.99. Two quality control (QC) samples at concentrations of 2 mg/L and 50 µg/mL were used. Their experimentally determined values were within 12% of the theoretical values and were consistent throughout the triplicates with coefficient of variation (CV%) of less than 5%.

The average concentrations measured from all VPA-treated rats were illustrated in Figure A2.4. Generally, with the exception of 0.5hr post-VPA treatment, they had displayed very similar pharmacokinetic profiles, most notably a seemingly recirculation of VPA at the 5th hr post VPA-treatment (9, 10).

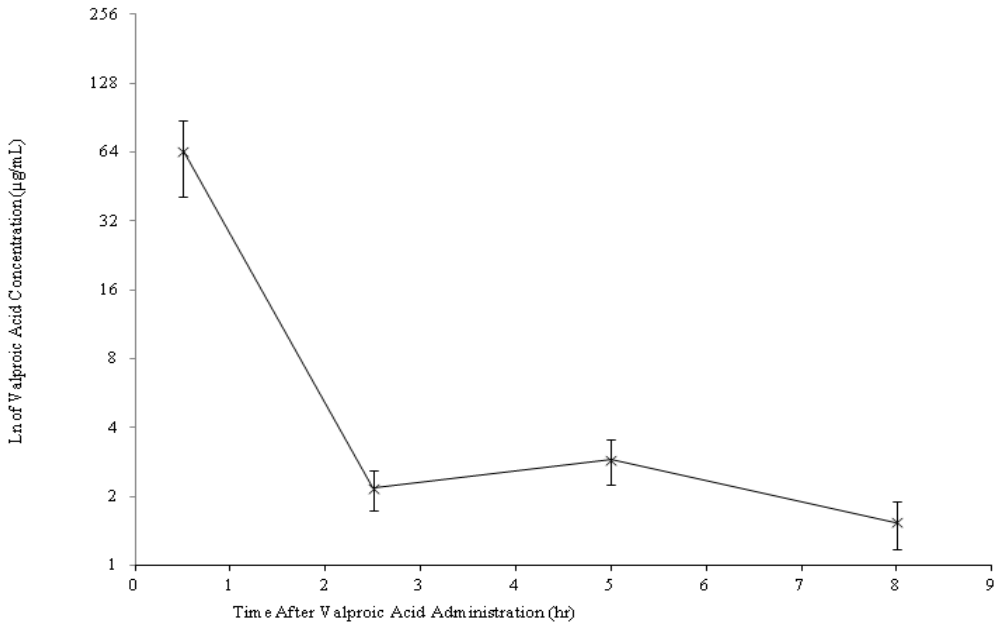


Figure A2.4. The concentrations of VPA in rats after intravenous (I.V.)

Pearson correlation tests found no significant correlations ($p > 0.05$, $-0.4 < r < 0.4$) between the individual metabolite with VPA concentrations (data not shown). Multiple linear regression analysis also did not reveal any important metabolite that can account for the measured VPA concentrations as well as the inter-individual variations observed.

Concentration-time profiles of VPA after intravenous (I.V.) injection revealed a terminal elimination half life (HL Lambda Z) of 1.71 ± 0.18 hr and mean residence time (MRT) of 1.14 ± 0.28 hr using a non-compartmental model. The pharmacokinetic parameters obtained were listed in Table A2.2. These profiles are comparable to those found from other studies (11, 12). Plasma/whole blood concentrations ratio of VPA had been demonstrated to be 1.46 ± 0.03 (mean ± standard error) (9), therefore it is not surprising to see some discrepancies, with generally higher levels obtained from plasma calculated values.

Table A2.2. Pharmacokinetic parameters estimated following intravenous bolus administration of valproic acid at the dose of 200 mg/kg. Mean ± SD	
Pharmacokinetic parameters	**Male**
Area under the curve (AUC), µg hr/mL	146.51 ± 48.24
Clearance (CL), L/hr	0.41 ± 0.12
Volume of Distribution (V_{ss}), L	0.55 ± 0.22
HL Lambda Z, hr	1.71 ± 0.18
Mean Residence Time (MRT), hr	1.14 ± 0.28

In summary, this study has shown that it is possible to use DBS for both *in vivo* non-targeted metabolomic as well as targeted analytes studies.

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APPENDIX III. INSTITUITIONAL REVIEW BOARD APPROVAL LETTERS

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CIRB Ref: 2011/268/A

25 April 2011

Prof Lim Shih Hui
Department of Neurology
Singapore General Hospital

Dear Prof Lim

SINGHEALTH CENTRALISED INSTITUTIONAL REVIEW BOARD (CIRB) APPROVAL

Study Title: **Characterization of Clinical Response to Antiepileptic Drug Treatment Among People with Epilepsy and Analysis of their Metabolic Profiles using Dried Blood Spot**

We are pleased to inform you that the SingHealth CIRB A has approved the above research project to be conducted in Singapore General Hospital.

The documents reviewed are:

a) CIRB / DSRB Application Form dated 18 Apr 2011
b) Participant Information Sheet and Consent Form: Version 1 dated 11 Aug 2010
c) Data Collection Form dated 31 Jul 2010

The SingHealth CIRB operates in accordance with the ICH/ Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirement(s).

The approval period is from **25 April 2011 to 24 April 2012**. The reference number for this study is CIRB Ref: 2011/268/A. Please use this reference number for all future correspondence.

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CIRB Ref: 2011/269/A

25 April 2011

Prof Lim Shih Hui
Department of Neurology
Singapore General Hospital

Dear Prof Lim

SINGHEALTH CENTRALISED INSTITUTIONAL REVIEW BOARD (CIRB) APPROVAL

Study Title: Clinical Validation of Dried Blood Spot as a Sample Collection Technique in Therapeutic Drug Monitoring of Phenytoin, Sodium Valproate and Carbamazepine

We are pleased to inform you that the SingHealth CIRB A has approved the above research project to be conducted in Singapore General Hospital.

The documents reviewed are:

a) CIRB / DSRB Application Form dated 18 Apr 2011
b) Participant Information Sheet and Consent Form: Version 1 dated 11 Aug 2010
c) Data Collection Form dated 31 Jul 2010

The SingHealth CIRB operates in accordance with the ICH/ Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirement(s).

The approval period is from **25 April 2011 to 24 April 2012**. The reference number for this study is CIRB Ref: 2011/269/A. Please use this reference number for all future correspondence.