Pharmacokinetic studies of drug absorption into human skin

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By

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To my parents, wife and my daughter Lean

Statement of originality

This is entirely my own work and all the quotations, illustrations and source materials have been appropriately acknowledged.

Faisal Al-Otaibi, February 2010

Abstract

Optimum therapeutic outcomes require not only proper drug selection but also effective drug delivery and monitoring. The aim of this thesis was to A) study drug delivery through the skin with a liquid formulated to promote absorption, B) develop and validate methods to analyze the drug in the samples obtained, C) assess appropriate methods to measure the transdermal delivery of drug, and D) apply to pharmacodynamics.

The stability of a rectal formulation of diazepam, Diastat®, and a quality control of a topical form, TDS® diazepam, were studied using high performance liquid chromatography (HPLC) with ultraviolet absorption detection (UV). It was found that diazepam at 10 mg/mL was stable in solution at various temperatures for at least 4 weeks. A pharmacokinetic study of diazepam delivery from the TDS® delivery system was compared with delivery of the drug following rectal administration of Diastat® in 12 healthy volunteers. The TDS® diazepam was evaluated for safety and no adverse effects or events were observed. The preparation was found to be able to deliver diazepam systemically in humans, the confidence interval (CI) of the ratios for C_{max} and AUC of diazepam from the two formulations A (TDS®): B (rectal) were not contained within the bioequivalence limit 80–125%, C_{max} (0–72h): 7.3–14% and $AUC_{0\text{-}72h}$: 20–38%. In addition, the 90% CI of desmethyldiazepam (A:B) ratio were not contained within the bioequivalence limit, C_{max} (0–72h): 38–54% and $AUC_{0\text{-}72h}$: 33–58%. Although not bioequivalent to Diastat® these finding suggest that skin may be an alternative method of diazepam delivery but further developments and studies would be required.

The development and validation of fast, high throughput methods to evaluate tetracaine from skin tape samples was another challenge. Sensitive and reliable capillary electrophoresis with UV and HPLC-UV methods were developed and validated to measure tetracaine in skin using tape samples from volunteers given 1 mL Ametop gel (4% w/w of tetracaine) to support a pharmacokinetic drug delivery study of Ametop. The results from these validation studies demonstrated an equal ability of the two methods to measure tetracaine concentrations reproducibly and accurately. The Bland Altman test was in a range of ± 1.96 SD from the mean (SD = ± 8.02, Mean = 2.23), and percentage

error (± 20%.), which show an acceptable difference. The assays were found to possess both the sensitivity and specificity necessary to measure the analyte in the skin tape stripping at the concentrations range in these tapes.

Finally, observation of appropriate methods to measure the transdermal drug *in vivo* techniques, such as microdialysis (MD) and tape stripping (TS) have been employed by plotting a concentration time profile to investigate the capability of measuring tetracaine (pharmacokinetics) in local tissue, instead of measuring tetracaine by conventional systemic measurements. The results showed that the tetracaine C_{max} concentration was higher in the stratum corneum compared with the major metabolites of tetracaine, 4-butylaminobenzoic acid (BABA) by 3 and 10 times in MD and plasma, respectively. TS samples reached the maximum concentration quicker than BABA in dialysate and plasma samples ($p = 0.002$). The median t_{max} was higher in plasma (IQR -53minutes, 95% CI: -30– -105) compared with tape samples. The AUC and C_{max} for tetracaine were higher in TS compared with BABA in MD and plasma (Mean $AUC_{0\text{-}4h}$: 88582, 55594 and 13208 nM.min: Mean C_{max} (0–4h), 850, 459, 110 nM, respectively).

In addition, the AUC and C_{max} values demonstrated that data from the TS study showed less variability compared with the data from plasma. The most variable data were for MD (CV%; $AUC_{0\text{-}4h}$, 24, 63, and 85%: C_{max} (0–4h), 42, 60, 80%, respectively). AUC and C_{max} (Bartlett's test, $p = 0.004$ for AUC; and Levene's test, $p = 0.042$, and 0.028, respectively)

This thesis has demonstrated that 1) diazepam was successfully delivered through the skin into the systemic circulation by the TDS® system, 2) novel methods have been developed for the measurement of tetracaine and its metabolite, and 3) the methods have been successfully applied to three different sample types employed in pharmacokinetic studies.

Publications

Article

1. Al-Otaibi F, Tucker AT, Johnston A, Perrett D. 2009. Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis. Biomed Chromatogr 23 (5): 488–491.

Abstracts

1. Al-Otaibi F, Johnston A, Tucker AT, Lee T, Langford R, Ratcliffe S, Kirby K, Alam C. A Randomized, Single-Dose, Two-Period, Cross-Over Pharmacokinetic Study to Compare TDS®-Diazepam with Rectal Diazepam in Healthy Adult Subjects. British Pharmacological Society Winter Meeting Brighton, United Kingdom 17th–20th December 2007.

2. Al-Otaibi F, Tucker AT, Perrett D, Johnston A. Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis. William Harvey Day, London, United Kingdom 14th October 2008.

3. Al-Otaibi F, Tucker AT, Perrett D, Johnston A. Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis. British Pharmacological Society Winter Meeting Brighton, United Kingdom 16th–18th December 2008.

4. Al-Otaibi F, Johnston A, Tucker AT, Lee T, Langford R, Ratcliffe S, Kirby K, Alam C. Systemic measurement of TDS®-Diazepam, compared with Rectal Diazepam, a pharmacokinetic study in Healthy Adult Subjects. Saudi International Conference 2009, University of Surrey, Guildford, Surrey, United Kingdom 5th–6th June 2009.

5. Al-Otaibi F, Tucker AT, Perrett D, Johnston A. Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis.

Saudi International Conference 2009, University of Surrey, Guildford, Surrey, United Kingdom 5th–6th June 2009.

6. Al-Otaibi F, Ghazaly EA, Tucker AT, Perrett D, Johnston A. Development of high performance liquid chromatography method for analysis of tetracaine for a tape stripping pharmacokinetic study: a comparison with capillary zone electrophoresis (CE) Saudi International Conference 2009, University of Surrey, Guildford, Surrey, United Kingdom 5th–6th June 2009.

7. Al-Otaibi F, Johnston A, Tucker AT, Lee T, Langford R, Ratcliffe S, Kirby K, Alam C. Systemic measurement of TDS®-Diazepam, compared with Rectal Diazepam, a pharmacokinetic study in Healthy Adult Subjects. 11th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology held in Montréal, Québec, Canada, October 3–8, 2009.

8. Al-Otaibi F, Tucker AT, Perrett D, Johnston A. Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis. 11th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology held in Montréal, Québec, Canada, October 3–8, 2009.

9. Al-Otaibi F, Ghazaly EA, Tucker AT, Perrett D, Johnston A. Development of high performance liquid chromatography method for analysis of tetracaine for a tape stripping pharmacokinetic study: a comparison with capillary zone electrophoresis (CE). 11th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology held in Montréal, Québec, Canada, October 3–8, 2009.

10. Al-Otaibi F, Ghazaly EA, Tucker AT, Perrett D, Johnston A. Development of high performance liquid chromatography method for analysis of tetracaine for a tape stripping pharmacokinetic study: a comparison with capillary zone electrophoresis (CE). British Pharmacological Society Winter Meeting London, United Kingdom 15th–17th December 2009.

11. Al-Otaibi F, Tucker AT, Lee T, Collier D, Johnston A. Comparative pharmacokinetics of tetracaine using microdialysis, tape stripping, and systemic measurement. British Pharmacological Society Winter Meeting London, United Kingdom 15th–17th December 2009.

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Table of Contents

Abbreviations

µg	Micro gram
µL	Micro litre
µm	Micro metre
AMPE	Absolute mean percentage error
ANOVA	Analysis of variance
AUC	Area under the curve
A.Conc	Average concentration
BABA	4-butyl amino benzoic acid
BBB	Blood brain barrier
BGE	Background electrolyte
BMI	Body mass index
BP	Blood pressure
Ca	Calcium
Cal	Calibration
CE	Capillary electrophoresis
CI	Confidence interval
cm	Centimetre
cm^2	Centimetre square
C_{max}	Maximum plasma concentration
CNS	Central nervous system
Conc	Concentration
COREC	Central office for research ethics committees
CPMP	Committee for proprietary medicinal products
CRF	Case report form
CTA	Clinical trial authorisation
CTIMPs	Clinical trials of investigational medicinal products
CV%	Coefficient of variation (percentage)
CYP	Cytochrome P450
CZE	Capillary zone electrophoresis

DAD	Dio array detection
DMEA	Di methyl ethanolamine
DPK	Dermatopharmacokinetic
ECF	Extra cellular fluid
EMEA	European medicines evaluation agency
EMLA	Eutectic mixture of local anaesthetics
EOF	Electro osmotic flow
EU	European union
FDA	Food and drug administration
g	Gram
GABA	Gama amino butyric acid
GC	Gas chromatography
GCP	Good clinical practice
GIT	Gastro intestinal tract
GLP	Good laparatory practice
GMP	Good manufacturing practice
h	Hour
HCl	Hydrochloride
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatography
IM	Intra muscular
IS	Internal standard
IV	Intra venous
kg	Kilogram
kV	Kilovolt
L	Litre
LC	Liquid chromatography
LLOD	Lower limit of detection
LLOQ	Low level of quantification
M	Molar
m^2	Square meter

Max	Maximum
MD	Microdialysis
MPE	Mean percentage error
MeOH	Methano/water
mg	Milligram
MHRA	Medicines and healthcare products regulatory agency
MHz	Megahertz
MI	Myocardial infraction
mL	Millilitre
mm	Millimetre
MS	Mass spectrometric
m/z	Mass to charge ratio
ng	Nano gram
nm	Nano meter
nM	Nano molar
Na	Sodium
NaOH	Sodium hydroxide
OTC	Over the counter
PAGE	Polyacrylamide gel electrophoresis
PFP	Pentafluorophenyl
PK	Pharmacokinetics
Q1	First quartile
Q3	Third quartile
QC	Quality control
r	The correlation coefficient
RCF	Relative centrifugal force
REC	Research ethics committee
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of the mean
SND(Z)	Standard normal distribution

$t_{1/2}$	Plasma concentration half life
TDS®	Proprietary transdermal drug delivery system
TEWL	Transepidermal water loss
t_{lag}	Lag time
t_{max}	Time passed since administration at which the maximum plasma concentration occurs
TS	Tape stripping
UK	United Kingdom
ULOQ	Upper limit of quantity
UV	Ultraviolet
US&USA	United States of America
v/v	Volume/volume
VAS	Visual analogue score
VRS	Verbal rating score
w/w	Weight/weight

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Chapter 1 Introduction

1.1 The human skin

The skin (*integumentum commune*) or cutaneous membrane is the largest organ in the human body, providing approximately 10% of body mass, covering an average area of 1.8–2 m^2 of an average adult person. The skin acts as an important barrier between an organism's internal and external environment.

It has proved to be an efficient and complicated tissue with metabolic, immunologic, and sensory functions. Several fundamental processes are maintained by the skin including body temperature, excretion, preservation of body fluids and tissues, maintenance and control of fluid and electrolyte balance within the body. It also acts as a defence barrier, and mechanical functions include mechanical and chemical protection; protection against ultraviolet radiation (UV), protection against organisms such as fungi, bacteria, virus, and sensory perception (e.g. pain, heat, sexual sensation), and vitamin D and B synthesis. In addition, the skin may include metabolic functions related to other endogenous and exogenous substrates.

In addition to protection and defence, the skin is also an important pathway for the entry of drugs and other substances (Schuplein and Blank, 1971, Katz and Poulsen, 1971, Kligman, 1983). Certain types of substances have the capability to penetrate the layers of the skin, which gives an alternative therapeutic route of drug administration in humans. This is known as transdermal drug delivery (through the skin), an alternative to traditional routes including oral, intravascular, intramuscular, subcutaneous, and sublingual. For this kind of delivery, and in order to recognize how drugs permeate the skin, a basic knowledge of the structure of the skin is important.

The structure of human skin is composed of three main layers. Going from outside to inside the body there are the epidermis, the dermis, and the hypodermis (subcutaneous adipose tissue or underlying subdermal tissue). Figure 1.1 shows the skin's three main layers.

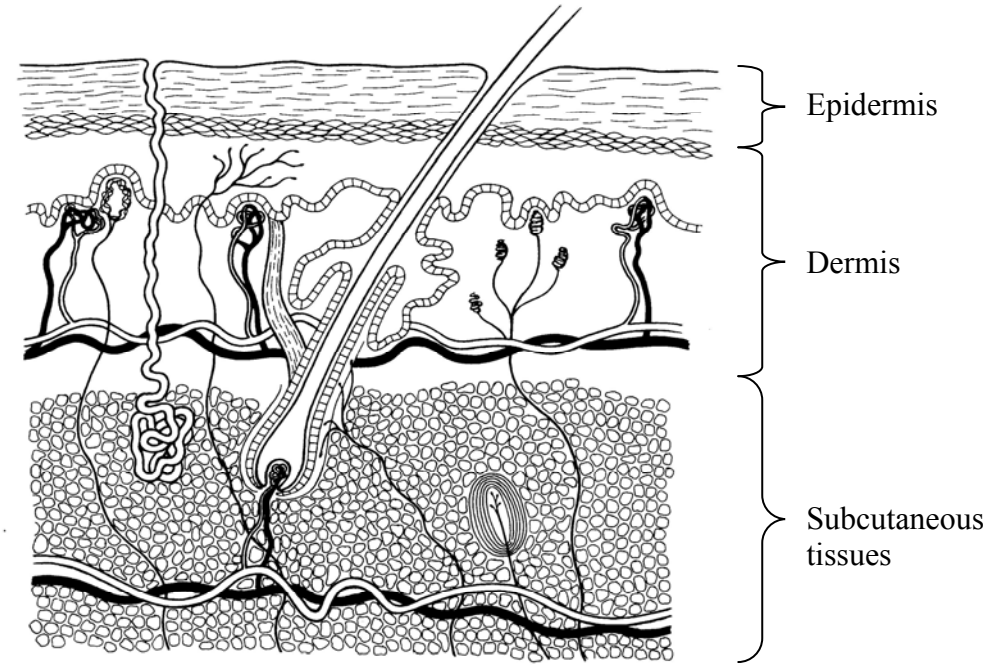


Figure 1.1 Cross section diagram of human skin showing the three main layers (modified from Barts medical image library at Queen Mary)

1.1.1 Epidermis

The epidermis: Greek *epi* = upon (above, or top), and *derm* = skin, hence, epidermis is the outermost layer of the skin, consisting of a complex proliferation layer membrane. This layer varies in thickness from approximately 0.06 mm on the eyelids to about 0.8 mm on the palms of the hand and the soles of the feet. The epidermis does not contain blood vessels. However nutrients and waste compounds must penetrate through the dermal and epidermal layer, in order to maintain the health and integrity of the skin. Similarly, molecules permeating through the epidermis may penetrate through the dermal and epidermal layer, and finally into the systemic circulation. Throughout adult life, proliferation persists. There are four physiological separate layers which, from the inside to outside the body, are: 1) the stratum germinativum, 2) the stratum spinosum, 3) the stratum granulosum, and 4) the stratum corneum. A fifth layer, the stratum lucidum (Figure 1.2), is sometimes described but is usually considered to be part of the lower layer of the stratum corneum.

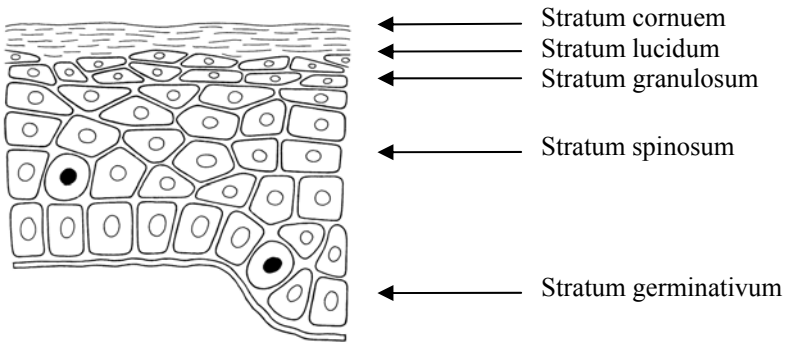


Figure 1.2 Cross section diagram of epidermis from human skin showing the five main layers (modified from Barts medical image library at Queen Mary).

1) *The stratum germinativum*

The stratum germinativum is also known as the basal stratum or, more commonly, the basal layer. The cells of the basal layer are like the cells of other tissues in the body; they contain the typical organelles such as mitochondria and ribosomes, and the cells are metabolically active. The basal layer is the only layer that contains cells known as keratinocytes. These cells which produce keratin, hence their name, also undergo cell division in the epidermis. After the cell reproduces via mitosis, one of these cells is retained in the basal layer whereas the second cell migrates to the surface, through the epidermis and onto the skin surface. The keratinocytes of the basal layer are connected to the basilar membrane, known as dermal and epidermal membrane, by hemidesmosomes. These act as protein basis for the lower layer cell. A defect in the bond between the basal cells and the basic membrane results in dissociation of the skin, such as in some blistering conditions. Within the basal stratum and the next cell layer the stratum spinosum, keratinocytes are connected through desmosomes, which are a particular protein involved in cell connection.

In addition to the keratinocytes, the basal stratum contains other specific cell types called melanocytes. This cell type synthesises melanin which originates from tyrosine. The melanin is responsible for the pigmentation of the skin. Melanin can be found in two forms, as eumelanin, the more abundant, which is brown or black colour, while the less abundant is phaeomelanin which is red or yellow. Melanin granule synthesis in the

melanocytes, tend to be a mixture of both forms. Melanocytes create a contact with neighbour keratinocytes through dendritic connections, and this permits the melanin granules to cross through the melanocytes to the keratinocytes. The melanocytes are arranged in the body and appear to be in different amounts depending on the area exposed to the light. Chronic exposure such as in the face results in more melanocytes than in the less exposed areas such as the chest. Melanin affords a protection within the skin against dangerous light, such as UV radiation, and free radicals. Thus every human except albinos have an equivalent melanocyte quantity regardless of skin colour. However people with darker skin have more active and efficient melanocytes.

Langerhans cells also originate within the basal stratum. They were discovered and named by P. Langerhans (Kobayashi and Hoshino, 1979) in 1868 in gold chloride stained preparations of skin (Kobayashi and Hoshino, 1979, Norikatsu and Akira, 2004). Their lineage or function remained unknown until the 1960s when the markers were discovered. These include surface I-A molecules, membrane associated enzymatic activities to hydrolyze extracellular ATP, intracellular organelles, and more specific markers, such as CD1a, E-cadherin, and langerin. Langerhans cells are dendritic and can attach and connect to keratinocytes. Langerhans cells originate from bone marrow and are the most important antigen present in the cell surface of the skin. Although, Langerhans cells are unable to phagocytose, they have the antigens on the surface. Nevertheless the possibly contact of skin with many different antigens, results in allergic reactions, inflammation and itching, which is the mechanism of defence by the Langerhans cells.

Another important and functional cell in the skin is called the Merkel cell, which is one of the dedicated cell types that can be found within the basal layer. The cells can be found in numerous numbers in the more touch sensitive sites of the body, such as fingertips, lips, and nipple. This is due to the nerve endings which have a sensational characteristic when in contact with another body.

2) *The stratum spinosum*

The spinous layer, or prickle cell layer are other names for the stratum spinosum, the cells that originate on the top of the basal layer. Basal stratum and spinosum layers together form the malpighian layers. The stratum spinosum consists of keratinocytes that are modified in structure from monolateral to multilateral cells. Additionally, in the spinous layer the keratinocytes start to differentiate and synthesise keratins that combine to form tonofilaments.

Similarly, desmosomes, connecting the cell membranes to the next keratinocytes, which are formed from combined keratins called tonofilaments, have another function of keeping a distance between the cells.

3) *The stratum granulosum*

The stratum granulosum is the next layer to the spinous layer, hence referred to as the granular layer. In this layer the keratinocytes persist in the synthesis of keratin and differentiate, but begin to flatten. The layer thickness of stratum granulosum contains fewer cells. Additionally the cell components such as the nucleic acids, and organelles (e.g. mitochondria, etc), begin to be nonviable due to enzymatic degradation in the stratum granulosum.

The keratin established inside the cell by keratohyalin granules is composed of cysteine-rich proteins, loricrin, and profillagrin. Additionally, a membrane covering the granules is produced, most likely in the endoplasmic reticulum and Golgi apparatus. The membrane has the originator of the intercellular lipid lamellae in the stratum corneum. Consequently, the production of the lamellar granules from the cells into the intercellular spaces results in the movement of the cells into the top layer of the stratum granulosum.

4) *The stratum corneum*

The stratum corneum (or horny layer) is the upper (outside) part of the epidermis, which is frequently used as a separate part in transdermal drug delivery studies. Stratum corneum forms the finishing cell product after cell differentiation in the epidermal. This horny layer consist of 10 to 15 cell layers, with a total thickness of 20–40 μm, around 10 μm thick when dry, although it may swell to several times this thickness when wet. The stratum corneum varies in thickness depending on the physiological function of the organ; the thickness is greatest on the palms of the hand, and also in the soles of the feet, while it is thinner on the lips. The stratum corneum is composed of dead, non nucleate keratinized cells implanted in a lipid media. The stratum corneum plays an important role in the regulation and protection of functions within the body. The regulatory function is focused mainly on water and electrolyte balance in the body, by excreting sweat. The protecting mechanism is focused mainly in the stratum corneum, preventing of the entrance of dangerous material, including germs to the body. The stratum corneum has been portrayed as the 'brick and mortar' model (Thomas and Paul, 1995, Barry and Williams, 1995), the keratinized cells, "the bricks", are embedded in a mortar of lipid bilayer (Michaels et al., 1975, Elias, 1981, Thomas and Paul, 1995, Barry and Williams, 1995). The keratinocytes (coenocytes) are multilateral. It takes 14 days for a daughter cell in the basal layer to differentiate into the stratum corneum, and further 14 days to remain in the stratum corneum before it is separated. The nature of the stratum corneum protection is dependent upon its unique components; 5% lipid and protein in the cell membrane, 10% lipid, protein, and mucopolysaccharides in the intracellular layer, and 85% lipid, fibrous and non fibrous proteins. These are primarily cellular with only approximately 10% of the components being extracellular (Wilkes et al., 1973). Furthermore, under normal conditions (e.g. normal humidity), the water content of the stratum corneum is between 15–20% of dry weight (Roberts and Walker, 1993). Water has a significant role in maintaining the integrity of the stratum corneum barrier, and facilitates the activity of some enzymes for hydrolysis within the stratum corneum, while the humidity in the atmosphere can influence the activities of enzymes involved in the exfoliation process. Furthermore, water in keratinocytes controls the enzyme which is

responsible for the production of the natural moisturising factor (NMF). Additionally water enables the stratum corneum to be smooth and elastic, avoiding break down by involuntary and physical attack. Hydration of the stratum corneum resulted in an increase in its permeability to several solutes (Roberts and Walker, 1993).

The stratum corneum is composed of a particular lipid combination. These lipid components of the multiple layers are important in the regulation of most drug permeation through the tissue. Thus, much work has been dedicated to establishing the lipid composition of the intercellular space (Elias, 1981, Elias, 1983, Wertz et al., 1985). The amount of the lipid matter in the stratum corneum obviously differs from individual to individual and also within the body area (Lampe et al., 1983), and particularly between genders. The lipid matter is arranged in multiple bilayers which are composed of fatty acids, cholesterol, cholesterol sulphate, and ceramides.

5) *The stratum lucidum*

The stratum lucidum is the lower layer from the stratum corneum. In this layer the nucleus is fragmented, resulting in an increase in keratin within the cell, followed by structural changes which have flattened cells. The stratum lucidum is most likely to be seen visibly in the comparatively thick skin area of the body, such as the palms of the hand and the soles of the feet. Often transdermal studies tend to view the stratum lucidum as part of the stratum corneum and combine it in one layer.

1.1.2 Dermis

The dermis, also known as corium, is normally 3–5 mm thick and is the most important constituent of human skin. Corium consists of a complex network of connective tissue, containing collagen, reticulin fibres to offer tensile strength and elastin fibre to offer elasticity of the tissue, and is embedded in a complex matrix of polysaccharide , proteins, enzymes, and other substances (Wilkes et al., 1973). In most elderly people the elasticity and flexibility of the skin disappears resulting in skin wrinkling.

The dermis contains water, thus a lower barrier can be observed in the dermis layer rather than the epidermis layer. For instance most polar transdermal drugs can be delivered easily through this layer, whereas highly lipophilic drugs can barely be delivered. In addition, the dermis contains blood vessels, lymph nodes, nerve endings, pilosebaceous components (hair follicles and sebaceous glands), and sweat glands (eccrine and apocrine).

Water is a liquid media that is superior for transport in the presence of an extensive blood supply. Blood vessels are vital for delivering oxygen, nutrition, other molecules to the cells and tissue, the removal of waste and toxic products, and the regulation of body temperature. In addition blood is crucial in stopping the bleeding of a wound, due the presence of platelets, which breakdown and aggregate, and is also necessary for the healing and repairing of wounds. However, high blood flow is critical in bleeding, easy for transport such as for transdermal drug delivery to maintain the concentration of compound between applied drug on the skin and the tissue. Hence the permeation of transdermal drug is dependent on the concentration of the substance in the tissue. The driving force follows Fick's law (Michaels et al., 1975, Barry and Williams, 1995), from high concentration to low concentration, and may be expressed mathematically by Equation 1.1:

$$q = D\,\frac{A}{h}\,K\,(C_0 - C_i)$$

Equation 1.1 Passive diffusion.

Where q is the flux of the substance (μg/cm^2/h), D the diffusion coefficient (cm^2/h), A the area (cm^2), h the thickness of the stratum corneum (cm), K the partition coefficient, C_0 the concentration of substance in the first layer of the membrane at the skin surface, and C_i the concentration of the substance inside the skin (μg/cm^3).

In the dermis the body has a unique defensive system (lymphatic systems) that may reach the epidermis layer. The lymphatic mechanism is involved in immunological and

regulatory effects, defender and eliminator against microbes and toxic materials. Additionally, it may remove substances that have permeated through the skin layer into the dermis. Thus, it may help in drug delivery, and the regulation of interstitial pressure. In the dermis blood flow is involved in the elimination of small molecules such as tetracaine and lidocaine. While the lymphatic system is involved in the elimination of large molecules such as interferon (Cross and Roberts, 1999).

Moreover, the dermis also contains hair follicles, sebaceous glands, and sweat glands. These can be seen on the surface of the human skin, and have been described in detail (Katz and Poulsen, 1971). Hair follicles are normally found on the entire surface of the human skin except the lips, palm of the hand, and the soles of the feet. The hair density is varied between adult, children, and genders. Hair is linked with the secretion of sebum from sebaceous glands which are found mainly in the face, chest, and shoulder of human skin. This oily substance (free fatty acids, triglycerides, waxes) is required to lubricate and maintain the hair and skin surface at approximately pH 5. Human skin with a pH under 5 is beneficial and has a greater barrier function in comparison with skin at a pH above 5 (Lambers et al., 2006). Ebling has described sebaceous glands in detail (Ebling, 1977). Sweat glands (eccrine glands and apocrine glands) are also found in the dermis and secrete sweat when stimulated by heat, fever, stress, in order to regulate the body temperature. Eccrine glands can be found everywhere on the surface of human body, with most located in the forehead, palms of the hands, and soles of the feet. Apocrine glands are developed and activated at puberty and are limited to a particular part of the body such as armpits, nipples, and genital regions.

Hair follicles and sweat glands offer a possible route of delivery for transdermal drugs, secluded from the stratum corneum barrier. The route can be described as transapendages or through appendages, which may help large polar molecules to cross more easily to the stratum corneum. Nevertheless, most drugs permeate through these appendages in low amounts, while the main pathway of transport is through the rigid barrier of the stratum corneum in the skin surface. The potential pathways of this route are discussed in detail, section 1.4 transport pathways (page 54).

1.1.3 Subcutaneous

The subcutaneous fat tissue, or hypodermis, is the bottom layer of human skin. This layer is the partition layer between the epidermal-dermal layers and the fundamental body components. This fatty layer varies in order of thickness in the human body, and is lacking in areas such as eyelids.

The fatty tissue in this layer mostly provides a protective mechanism against physical shock, and in the retention of body heat. The adipose tissue layer carries blood vessels and nerves to the skin, and also keeps polar molecules in the skin for longer time, acting as a reservoir and supplier.

1.2 Drug delivery

Drug delivery is an important field for researchers, pharmaceutical companies, and clinical practice. It has recently attracted research interest. Traditionally, once a drug is released from its dosage form, it must pass through several barriers before it arrives at the site of action. This entire barrier consists of membranes, varying in thickness. Permeation or trans-membrane movement, is the process called diffusion, with the rate of diffusion depending essentially on the size of the concentration gradient across the membrane, the nature of the membrane surface through which the dissolved molecules must pass, and the thickness of the membrane (Heilmann, 1984). This can be affected by many factors; physiological mechanisms, such as cellular organisation, efflux, and/or chemical and enzymatic degradation such as the first pass effect, physicochemical properties of the drug molecule itself such as polarity, hydrophilicity, pH, stability, pathological mechanism e.g. disease state, as well as the patient himself. Indeed the diverse forms of drug delivery routes help us in the following:

1) Deliver drug to a specific site of action to provide a more safe (minimize the area of the site of action) and effective drug (specific site), e.g. chemotherapy.

2) Avoid factors affecting drug bioavailability including:

a) Disease states such as; ulcer, epilepsy.

b) Chemical degradation such as; stomach acidity.

c) Enzymatic degradation such as; first pass metabolism, e.g. propranolol is affected by the liver "first pass effect".

3) Increase patient compliance. by:

a) Avoiding the complexity of the drug regimen that is related to the frequency of administration, which may result in missing doses especially in elderly people. An excellent example of avoiding the complexity of dose is by using patches e.g. nicotine patch 7 mg/24 hours is better than taking a sublingual tablet 2mg/hour (Silagy et al., 2000). Another example is clonidine, available as a tablet taken by mouth and also as a patch applied to the skin. The tablet usually is taken two or three times a day at evenly spaced intervals, but the patch is applied to the skin every 7 days (Martindale, 1996d).

b) Choosing a convenient available route of administration e.g. oral is better than IV in people with injection phobia.

c) Administer the drug to its specific site of action which may prevent or minimize the unwanted effects, and may enable a reduction in the length of treatment required.

4) Minimize contamination, diseases, infections, and other possible health hazards that may have potentiated/happened by the injection route, e.g. IV, IM, etc.

5) Helpful with narrow therapeutic index drugs, e.g. digoxin in the treatment of congestive heart failure, or warfarin to prevent blood clotting.

6) Pharmacoeconomic benefits, involve controlling the cost of drug by choosing the right dosage form for the right disease or administering the drug directly to the

site of action to minimize side effects or adverse events which may need another treatment.

Routes of administration can broadly be divided into:

- Enteral; oral, gastric feeding tube, rectal, etc.
- Parenteral; intravenous bolus or infusion, intramuscular, subcutaneous implant, etc.
- Topical/transdermal; inhalation, buccal mucosa, intranasal, epicutaneous (on the skin), vaginal (female), etc.

These three major routes of drug administration are the ways in which a drug can be delivered to the site of action dependant on its physicochemical properties and/or physiological and chemical factors.

1.2.1 Enteral drug delivery

From the above three major routes, the enteral route, especially the oral route, is the most common, least invasive, easiest, and most convenient for the patient. Once the drug enters the alimentary tract, it can be absorbed from the oral cavity, sublingual, stomach, small intestine, and large intestine. With regards to the others sections the small intestine is the most important part in the alimentary tract. The epithelial coating of the small intestine consists of a mono layer of cells known as enterocytes. This layer contains many villi and microvilli attached together by a network of blood and lymphatic vessels enabling the absorption of digested food, drugs, etc. The villi of the upper intestine have a large surface area (~ 200 m^2) to enhance absorption of substances. The absorption occurs mainly in the upper part of small intestine (*in the proximal jejunum*), and so the speed of gastric emptying determines the speed at which the drug reaches its site of absorption. However, some patients cannot swallow tablets and/or capsules, including the elderly and the very young person. Other factors, such as malabsorption of a drug, may occur due to disease in the gastro intestinal tract (GIT), or be a result of the patient's age.

In contrast, the physicochemical properties of the drug play an important role in the absorption from the GIT. Thus, in the presence of food, drug absorption may be delayed

or reduced. Drugs such as tetracycline, which are highly ionized, can form a complex with Ca^{2+} ions in the membrane, and Ca^{2+} ions present in food e.g. milk leading to a reduction in their rate of absorption. As food can reduce or delay absorption it can also increase drug absorption because the splanchnic blood flow is increases during eating.

Furthermore, drug metabolism changes the bioavailability of drug. It can be defined as the chemical alteration of the drug or compound by the metabolic pathway in the body, yielding a drug metabolite which may be inactive, active, or a toxic compound in relation to the parent compound. Drug metabolism occurs in the gut lumen, gut wall, liver, the lung and some happens elsewhere. In the gut lumen, benzylpenicillin (BNF, 2009), and insulin (Kenny, 1960), are both almost completely inactivated by gastric acid and proteolytic enzymes, respectively. Drugs such as amoxicillin are partially inactivated. In the gut wall isoprenaline is sulphated (Back and Rogers, 1987), and many drugs are metabolized by enzymes such as monoamine oxidase, and CYP3A4. However, the liver is the main site of drug pre-systemic metabolism or first pass effect, which converts propranolol to 4-hydroxypropranolol, a pharmacologically inactive metabolite, thus explaining the large difference between effective oral and intravenous doses of propranolol. This can be overcome by using an oral dose higher than the effective dose administered by the intravenous route, which may be useful, but can also enhance the possibility of side effects occurring. In some cases, e.g. lidocaine and insulin, metabolism is so extensive that it renders oral therapy impossible with conventional oral formulations. In such cases the drug must be given by other routes.

The rectal route with drainage via the inferior rectal veins directly into the systemic circulation, offers a comparable alternative route. However, it is not common in the United Kingdom (UK), least preferred by some patients (Kogan et al., 2002), and is only used for certain types of drugs when the other alternative routes are not available, for example to control seizure, anxiolytic, analgesia (Pannuti et al., 1982, Beebe et al., 1992, Kogan et al., 2002). In children, this route is used more commonly to achieve a topical effect on the rectum and colon, such as a prednisolone enema to treat ulcerative colitis, or to minimize adverse effects occurring in the upper gut by indomethacin, which is more likely to cause gastric ulceration if given orally. Others such as Fleet Enema (Sodium

biphosphate, Sodium phosphate), are used as a laxative for the relief of constipation, and to clean the bowel before surgery.

Other routes such as a gastric feeding tube can be given only if the patient is admitted to the hospital and in an appropriate condition. In these cases, drugs must be administered by alternative means including injection, inhalation, and transportation through the skin.

1.2.2 Parenteral drug delivery

Injection is usually a patient's least favoured route of drug administration. Drug delivery by injection is affects the whole body, rather than reaching a specified site of action. This may result in an ineffective dose and possible side effects/events to other organs in the body. Also there is a need to avoid needle delivery risks, such as infectious diseases of microbials, viruses, and other contamination. Inconvenience and even pain are additionally a problem. While it may be a minor problem in adults, young people especially children, experience a far greater pain which can develop into "needle phobia", an intense fear of needles that triggers immediate anxiety. This also affects some adults. However local anaesthetics are normally used to reduce the pain by inducing a loss of feeling (numbness) in skin and mucous membranes. Many researchers have searched for a local anaesthetic method that is needleless and pain free via topical application, e.g. EMLA (Eutectic Mixture of Local Anaesthetics containing 2.5% each of lidocaine/prilocaine) cream and Ametop (4% w/w tetracaine base). But unfortunately the slow onset times (EMLA, 1 hour, Ametop, 30 – 45 minutes) remain a deterrent to widespread acceptance with the need to organise clinic, ward and operating theatre routines accordingly. Additionally, these methods are of little benefit in acute situations. Other examples to get a painless injection include the needleless injection and micro-needle, but due to its difficult use, improper handling of the system, leading to the loss of drugs entering the body, it is more complicated, and requires a trained administrator which increases the cost.

1.2.3 Which drug delivery is appropriate?

The individuals most opposed to intravenous treatment generally are children. This produces several challenges for clinician as children often are unwilling to swallow tablets and/or capsules, they refuse injection therapy, and they do not like the taste of some medicines (Harold, 2007).

A good example for children is cited in this recent case study: a nine year old boy was complaining of headache, back pain and generalized arthralgia/myalgia. The boy had severe bone pain on his forearms, wrists and both hands. He received continuous subcutaneous or transdermal fentanyl at 75μg/hour, hydromorphone 3 mg and lorazepam when required, gabapentin and naproxen as analgesic, ondansetron and nabilone for nausea, polyethylene glycol and docusate as laxative. The treatment reduced all pain except for the bone pain in the arms. Clinical teams then attempted to choose a bisphosphonate category drug for this untreated pain. However the selection of an IV route was opposed by the boy, claiming he was uncomfortable because of his awful history in hospital. An oral route was then chosen, but due to his previous gastric outlet obstruction, this was not a suitable method. Additionally, he refused the rectal route. Hence clinical teams then decided to use the subcutaneous route, which was an acceptable and useful treatment for him (Harold, 2007).

1.2.4 Transdermal drug delivery

Optimum therapeutic outcomes require proper drug selection and effective drug delivery. Many effective drug delivery methods have been reviewed and discussed above; one of these being transdermal drug delivery. Transdermal drug delivery is an alternative choice for therapeutic agents, not only for systemic drug delivery, with avoidance of hepatic first-pass metabolism and increase of patient's comfort compared with the oral and parental route, respectively, but particularly for targeted local drug delivery to the skin, and sometimes it is the only route that can be given, as described before in the previous section (page 48).

The human skin is a readily accessible surface for drug delivery. However, to consider the cutaneous route viable, sufficient amounts are required in local tissue. In the last 60 years or so, many terms have been used to describe transdermal drug (substance) delivery, e.g. penetration of a substance from the outside of the skin through the skin and into the systemic circulation, or the passage of a substance from the entire surface of the skin through its complex layers into the blood circulation. Rothman described this as percutaneous absorption as mentioned by Cleary (Cleary et al., 1984). Some describe it as the administration of therapeutic agents through intact skin in sufficient quantities for systemic or local effects. Others have used different terms such as sorption, persorption, permeation, and penetration (Cleary et al., 1984). All of these relate to the passively driven force of transfer; some terms such as sorption, have other conflicting senses. Thus the best definition of transdermal drug delivery can be described as: the absorption through the skin that involves passive diffusion through the outer and middle structure of the skin until the systemic circulation is attained (Schuplein and Blank, 1971, Cleary et al., 1984) or local effects are produced, and the likelihood of unwanted side effects or toxic effects may occur.

Transdermal drugs are available today as topically applied creams, ointment, patches, and gel. Many studies, including those on transdermal drug delivery have been required to verify what causes the skin to have these barrier properties which prevent substance permeation. In 1924, Rein claimed a negative charge existed; impermeable to anions and that electrostatically traps cations, it was a barrier layer in the dermis, assuming transepidermal absorption through the cell (Katz and Poulsen, 1971). In 1953 Blank (Blank, 1953) adapted this by eliminating the stratum corneum layers from the surface of the skin, and found that water was lost rapidly from the skin, and considerably more after elimination of the stratum corneum. In 1965–1971 Scheuplein and colleagues (Schuplein, 1965, Scheuplein, 1967, Schuplein and Blank, 1971) demonstrated that transdermal permeation was limited by the stratum corneum via a passive process.

Michaels and colleagues determined that the diffusion coefficient of ideal drugs in the stratum corneum, resulted in significant permeability of some drugs (Michaels et al., 1975). This demonstration assisted in the development of active transdermal patches in

the 1970s. The initial first patch was approved by the FDA in 1979. It delivered scopolamine over three days to treat motion sickness (Shaw and Chandrasekaran, 1978). The next patch was then approved containing nitroglycerin for cardiovascular disease (Dasta and Geraets, 1982), followed by other patches containing clonidine for hypertension, estradiol as hormone replacement therapy, and nicotine used as a method to quit smoking.

Drug delivery through the skin offers advantages over other pathways including: avoiding the hepatic first pass effect, avoiding the physiological environment and chemical or metabolic degradation in GIT such as changing pH, luminal micro flora, etc. It has an advantage that the treatment can be interrupted or eliminated when necessary (Godwin and Michniak, 1999). Improved patient compliance is especially notable for patches that require only once weekly application e.g. once a week transdermal estradiol of adhesive patch appears to be an acceptable means of hormone replacement therapy compared with the twice a week patch (Harrison et al., 1997).

The continuous permeation of drug through the skin allows for more steady blood concentrations. Although IV infusion at constant rate achieves this objective, it is more invasive than transdermal drug delivery. In addition, the slow rise in plasma concentration for transdermal drug delivery can reduce the risk of side effects.

Generally drug absorption into the skin occurs by passive diffusion, and because of the barriers imposed by the skin, this process occurs very slowly. The rate of drug transport across the stratum corneum follows Fick`s law of diffusion (Michaels et al., 1975, Barry and Williams, 1995), which depends on the aqueous solubility, oil/water partition coefficient, concentration in the formulation vehicle, molecular size and shape, the surface area of the skin to be exposed, thickness of the stratum corneum, etc. Since, the stratum corneum is thickest in the palm of the hand and soles of the feet, but thinnest in the lips, armpits, genital, and scalp regions of the body, choosing the site of application is very important for this type of drug delivery.

Furthermore a drug may need assistance to enhance its penetration through the skin. These methods can be categorized as physical and chemical methods of enhancement.

Physical methods including: iontophoresis which applies a small amount of electric current to drive polar and neutral molecules through the skin (Banga et al., 1999, Denet et al., 2003). In contrast, electroporation applies a high voltage for a very short time which enhances the permeability of the skin (Banga et al., 1999, Denet et al., 2003). Phonophoresis or sonophoresis is the use of ultrasound within the therapeutic range of 0.5–5 MHz to enhance the delivery of topically applied drugs (James et al., 1993). Chemical methods include the use of chemical penetration enhancers and development of prodrugs with proper physicochemical properties appropriate for transdermal delivery. See section 1.5 (page 57) for more details.

1.3 Skin metabolism

Skin metabolism is a protective function in the epidermis and dermis, making the second penetration barrier to drug permeation through the skin possible. The biochemical barrier makes drug delivery through the skin more difficult in addition to the first barrier "the epidermis" or mainly "the stratum corneum". The metabolism of skin has been well documented with many different organs in the body, which have the capability to metabolize certain drugs/substances. Among these organs, the liver can still be considered the most important organ for metabolism of drugs/substances in the body (Pannatier et al., 1978, Tauber and Rost, 1987). The levels of some enzyme activities in the skin are relatively low when compared with the liver (Pannatier et al., 1978, Noonan and Wester, 1985, Noonan and Wester, 1989, Sartorelli et al., 2000). However skin metabolism is important, due to its large surface area which is approximately 1.8–2 m^2, and the constancy to environmental facts. This is especially true for the epidermis, which constitutes part of the major interface between the body and the environment. Thus, the role of the skin cannot be overlooked. In addition, studying the metabolism in the skin is imperative, and useful information can be obtained which can improve knowledge regarding health hazards. A study by Yourick (Yourick and Bronaugh, 2000), in predicting the extent of 2-nitro-p-phenylenediamine (2NPPD) and/or 2NPPD metabolite, may be used for future safety assessment purposes in systemic absorption relative to a dermal exposure.

The enzymatic activity of human skin is mostly estimated, and by assuming that these enzymes are constrained in the epidermal layer, the real activities range from 80 to 240% of those in the liver (Noonan and Wester, 1989), and sometimes exceed that of the corresponding hepatic enzyme (Martin et al., 1987). Thus the enzymatic activity taking place in the multiple layers of the skin imposes a great influence on the efficacy and safety of transdermal drug delivery (Noonan and Wester, 1985, Bando et al., 1997, Sartorelli et al., 2000). The effectiveness of skin metabolism is typically in the prevention of systemic toxicity of transdermal delivery drugs, such as the metabolism of benzoyl peroxide, which yields a benzoic acid metabolite in the skin (Nacht et al., 1981, Sartorelli et al., 2000). In contrast the process is also helpful and useful in the case of prodrugs, which are designed for the purpose of enhancing percutaneous penetration.

Skin metabolism can be affected by a number of factors, including, physicochemical properties, the amount of drug applied, the concentration of drug, the area applied, the duration of time to be applied, and the level of metabolizing enzymes which is affected by age, sex, race, skin condition, enzyme inducers and inhibitors, and duration of the applied drug to the enzymes. In addition, there is a predictable difference in simultaneous skin transport and metabolism between species, especially in esterase activity (Tauber and Rost, 1987, Steinstrasser and Merkle, 1995, Rittirod et al., 1999).

Skin metabolism has at least two broad enzymatic categories/reactions; phase I (oxidation, reduction, and hydrolysis), and phase II (conjugation) biotransformation reactions (Pannatier et al., 1978, Martin et al., 1987, Tauber and Rost, 1987, Boehnleine et al., 1994, Sartorelli et al., 2000).

The metabolism enzymes available in the skin are similar to the most important metabolism enzymes in the liver (Sartorelli et al., 2000), and other organs (Pannatier et al., 1978). Phase I enzymes like alcohol and aldehyde dehydrogenase can be found in the human skin. These enzymes are involved in the metabolism of a variety of endogenous and exogenous alcohol and aldehyde substances in the body to prevent systemic toxicity, cytotoxicity, mutagenicity and carcinogenicity (Cheung et al., 1999). Table 1.1 shows the most important metabolizing pathways. In phase I oxidation/reduction/hydrolysis of the

substance is mediated by cytochrome P450 isoenzymes to be finally conjugated in phase II. Phase II generally converts lipophilic drugs to hydrophilic derivatives, to be easily excreted from the body. However, such metabolism results in the detoxification of compounds. Thus, if the chemical had not been detoxified, and was highly reactive, it might accumulate inside the skin layers which could result in binding to nuclei/protein in cells, which could cause morphological changes in the gene, resulting in genetic cancer, or dermatitis. Hence, the balance between the two pathways (phase I and phase II) of skin metabolism has been mediated to avoid any toxicity (Smith et al., 2003).

Table 1.1 Some cutaneous xenobiotic metabolising pathways (Sartorelli et al., 2000).

Phase I	Phase II
Oxidation	Glucuronidation
Hydroxylation	Sulfation
Deamination	Glutathione conjugation
Dealkylation	Acetylation
Epoxidation	Amino acid conjugation
Aldehyde oxidation	Methylation
Alcohol oxidation	
Reduction	
Azo reduction	
Nitroxide reduction	
Quinone reduction	
Hydrolysis	
Carboxylester hydrolysis	
Sulfate ester hydrolysis	
Phosphate ester hydrolysis	
Peptide hydrolysis	
Epoxide hydrolation	

Finally, skin metabolism is hard to determine *in vivo* as the analyzed sample may contain other metabolites not related to skin metabolism. Thus, the bioavailability of a metabolized drug by the skin can be practically estimated (Nakashima et al., 1987), and the appropriate method to analyze endogenous and exogenous samples in the extracellular space of skin tissues is by using microdialysis (MD).

1.4 Pathways of transport

As discussed before, the skin has multiple barriers which prevent the easy permeation of drugs. However the topical drug product (emulsion, gel, liquid formulation, etc) applied on the skin and released from the formulation, is thought to permeate through the skin tissue by a passive diffusion process through viable epidermis. The process usually is thought to obey Fick's laws of diffusion, and the drug is delivered then to the site of its action, or reaches the systematic circulation. For example drugs/substances with a low molecular weight (<500 g/mol) and with a log P of > 1 are very likely to be able to penetrate the lipid in the stratum corneum effectively (Smith et al., 2003). Nevertheless, to complete this process, there are three possible pathways of transport across the skin which have been recognized by Schuplein (Schuplein, 1965) Figure 1.3 and Figure 1.4.

1. Transcellular permeation, through the cells.
2. Intercellular permeation, between the cells.
3. Transappendageal permeation, via the hair follicles, sebaceous and sweat glands.

From the three main pathways by which drugs can cross the skin and reach the systemic circulation, the first two pathways require further diffusion (passive diffusion) through the rest of the epidermis and dermis (Figure 1.4). The first pathway, “transcellular permeation” was initially considered to be the most likely, since most of the volume is comprized of cells, *in vivo* experiments have indicated that solutes which are more lipid soluble and do not alter the epidermis follow intracellular permeation (Albery and Hadgraft, 1979) and that the intercellular route may provide the major pathway for transdermally delivered drugs. Transcellular permeation is a direct route by which the

drugs cross the skin by directly passing through both the phospholipid membranes and the cytoplasm of the dead keratinocytes that constitute the stratum corneum (Figure 1.4). Although this is the path of shortest distance, the drugs encounter significant resistance to permeation. This is because the drugs must cross the lipophilic membrane (stratum corneum) of each cell, then the hydrophilic cellular contents (viable epidermis) containing keratin, and then the hydrous layer (dermis) one more time (McCarley and Bunge, 2001), which makes multiple transfers between these cells and the intercellular domains the only way. Thus more hydrophilic drugs are unable to permeate through the stratum corneum, and more lipophilic drugs tend to be retained in the stratum corneum (Naik et al., 2000). Due to the hydrophilic properties of the dermis few drugs have the properties to cross via this pathway.

The second pathway is more common; in this pathway the drugs cross the skin by passing through the small spaces between the cells of the skin, making the route more complicated (Figure 1.3). Although the thickness of the stratum corneum is only about 20–40 μm, the actual diffusional path of most molecules crossing the skin is in the order of 300–500 μm (Hadgraft, 2001).

The third pathway allows diffusion leakage into the epidermis and direct permeation into the dermis. The appendages may be important at short diffusion times and for polar molecules, but are usually considered to be of minor importance because the appendages occupy only a very small proportion of the total skin surface, approximately 0.1% (Schuplein, 1967).

For drugs penetrating directly across the intact stratum corneum, the mechanism of drug entry may be transcellular and/or intercellular and/or transappendage. The mechanism of drug entry is affected by many factors, including the duration of time the drug is applied, physiochemical properties of the drug (such as pKa, protein binding, size of the molecule, stability, solubility, partition coefficient), physiological factors (such as integrity and thickness of stratum corneum, amount of sweat glands and follicles, skin humidity, metabolism), and chemical factors (such as vehicle effects, enhancer, content).

In addition, penetration can be improved by developing/amending methods, techniques, and/or systems used to enhance, increase, facilitate, the absorption process of the drug in the viable epidermis. These methods are discussed in detail in section 1.5 (page 57). A good example is the use of penetration enhancers such as water, alcohol, etc.

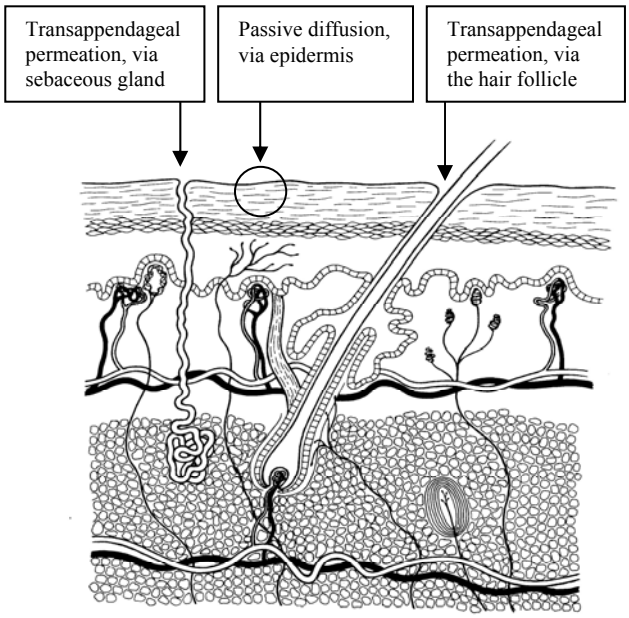


Figure 1.3 Cross section of the human skin showing the three main pathways of drug transport through the skin (modified from Barts medical image library at Queen Mary).

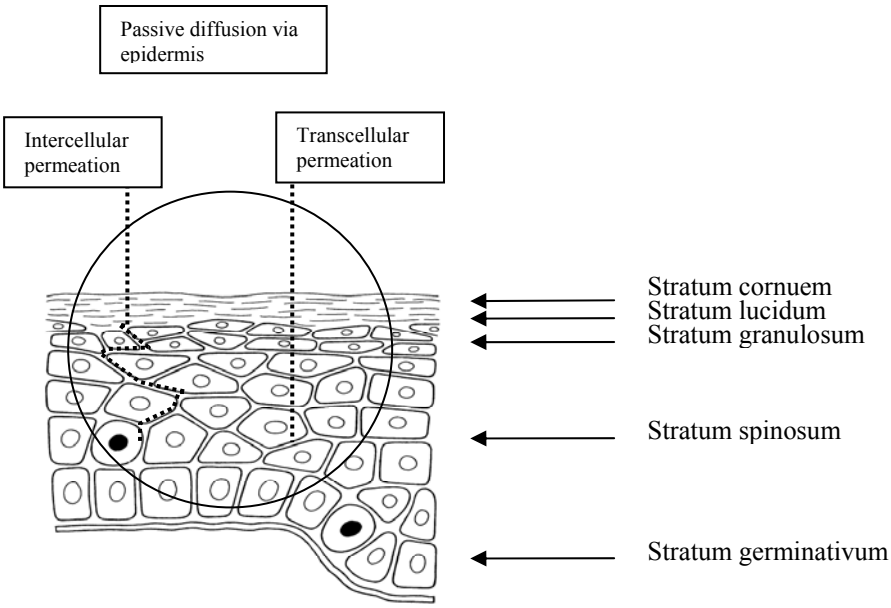


Figure 1.4 Enlargement of the human epidermis showing the "passive diffusion via epidermis" of the transcellular and intercellular routes through the skin layers (modified from Barts medical image library at Queen Mary).

1.5 The physiology and properties of the barrier of the stratum corneum

The primary physiological barrier to drug absorption across the skin is the stratum corneum (SC) (Marzulli, 1962, Katz and Poulsen, 1971, Michaels et al., 1975, Barry, 1983a, Kligman, 1983, Noonan and Wester, 1985, Noonan and Wester, 1989), the 20–40 μm thick matrix, forming the outer layer of skin. It consists of dehydrated keratinocytes, anucleate cells, which are separated from the dermis by a thin underlying membrane, and is frequently studied by the researcher as a separate membrane. The stratum corneum prevents any harmful material from entering from the outside to inside which may cause inflammation. Thus in inflammatory skin diseases the stratum corneum is affected, which may allow harmful material to enter, causing unwanted effects. Removing the stratum corneum, either by removing the stratum corneum using tape stripping, or extracting the lipids from the horny layer, will result in a stratum corneum similar to that seen in inflammatory skin diseases. Morgan and colleagues demonstrated that removing the

stratum corneum by tape stripping enhanced penciclovir and acyclovir absorption by 1300 and 440 fold respectively. The results of this study confirmed that the stratum corneum is the main barrier in the absorption for hydrophilic drugs (Morgan et al., 2003). Another study using the same method of tape stripping showed that the absorption of EMLA (Eutectic Mixture of Local Anaesthetics containing 2.5% each of lidocaine/prilocaine) cream was accelerated by removal of the stratum corneum, and the pain assessment for 15 minutes was less in a patient whose stratum corneum had been removed compared with patient whose stratum corneum was intact (Singer et al., 1998). Any substance that penetrates the stratum corneum is subjected to metabolism in the viable epidermis which is the major site of metabolism in the skin (Noonan and Wester, 1985, Noonan and Wester, 1989). Therefore the ideal drug needs to penetrate the stratum corneum to be absorbed quickly, in sufficient quantity, and thus be available to exert its local or systemic effect.

Many researchers have currently developed systems and methods, and the future research studies may promise to enhance the absorption through the stratum corneum. This includes the drug/vehicle interaction (e.g. eutectic system in EMLA cream, metabolism of prodrugs), vesicles and particles (e.g. as liposomes and analogues), stratum corneum modification (e.g. chemical enhancers, hydration), stratum corneum bypass (e.g. laser ablation, micro-needles, follicular delivery), and electrically assisted methods (ultrasound, iontophoresis, etc) (Barry, 2001). The modification of the stratum corneum by using penetration enhancers (or accelerants) is more popular and widely used in transdermal drug delivery of topical steroids (Barry, 1983b). The penetration enhancers are substances used to temporarily diminish the stratum corneum, such as water, hydrocarbons, fatty acids, esters and alcohols, azone and its derivatives, sulphoxides and analogues, pyrrolidones, surfactants (anionic, cationic and nonionic), amides, epidermal enzymes, and lipid synthesis inhibitors (Barry, 2001). Penetration enhancers are substances that enhance the penetration of transdermal drugs, and are defined as penetration enhancers, absorption promoters, or accelerants. Barry and Williams (Barry, 1983b, Barry and Williams, 1995) described the attributes of the ideal penetration enhancer as the following;

1. Pharmacologically non effective, with no action at receptor sites, and/or anywhere in the body.

2. Nontoxic, not irritating, and not allergenic, in acute or chronic cases.

3. Onset of action should be rapid, and duration of activity should be predictable and suitable for the drug used.

4. Stratum corneum should immediately and fully recover to its normal barrier property after removal of the enhancer.

5. The barrier function of the skin should be reduced in one direction only. Endogenous materials should not be lost to the environment by diffusion out of the skin.

6. The enhancer should be chemically and physically compatible with all drugs and adjutants to be formulated in topical preparations and devices.

7. Spread well on the skin, with a suitable skin "feel".

8. Easy to be formulated into transdermal preparations, transdermal procedure, and skin patches.

9. Tasteless, odourless, colourless, and non expensive.

10. Should be an excellent solvent for the drug.

In addition to the attributes of the ideal penetration enhancer described by Barry and Williams, Kang et. al. said that there should be;

11. There is no interaction between skin and enhancer, unless the stratum corneum barrier properties are retained. Chemical enhancers selectively interact with the lipid components of stratum corneum (Kang et al., 2006).

Penetration enhancers may act by one or more of three main mechanisms (Barry and Williams, 1995):

- ◊ Disruption of the highly ordered lipid structure of the stratum corneum,
- ◊ Interaction with intracellular protein, and
- ◊ Improved partitioning of a drug, co-enhancer or solvent into the stratum corneum.

1.6 Other topical delivery

As discussed before, the stratum corneum (SC) is the major barrier preventing drugs/substances from penetrating through the skin. However other topical drug delivery can be used avoiding the main barrier (stratum corneum) in the skin, through the mucosal membrane. Where there is no SC available to prevent the absorption of topically applied drugs, which enables the drug to avoid the barrier and penetrate easily through the skin to reach the systemic circulation, or exert its effect locally. This includes, the mouth (sublingual or buccal), respiratory tract (nasal mucosa, hypopharynx, lungs), and vagina. In comparison, the respiratory tract provides a large area of absorption, the nasal region is rich with blood vessels, and the lungs contain bronchi which divide into bronchioles and then into alveoli surrounded by blood vessels. An example of drugs taken by this route, via inhalation, is salbutamol for the treatment of asthma, or through the nasal mucosa such as nasal sprays, nasal drops, etc. Such a type of delivery is limited for multi-usage as it may lead to disruption of nasal blood vessels and bleeding may occur. The mouth is rich with blood vessels, so drugs delivered into mouth via sublingual or buccal mucosa will have a fast absorption rate e.g. isosorbide dinitrate is absorbed quickly, which is important in a case of acute myocardial infarction (MI), where a fast onset of action is required. However sometimes this is not useful such as in acute seizure where the oral route is dangerous, and hazardous. Furthermore, females can suffer from infection and

other conditions in the genital organs, and a vaginal topical route can be used to treat the affected area. However this route is limited to certain diseases.

1.7 TDS® delivery system

One of the main projects in this thesis, is to study the TDS® drug delivery system (Transdermal Technologies Inc., Florida, USA), which is a unique liquid formulation applied locally via a metered pump dispenser, not requiring a patch or other appliance for precise delivery of the drug dose systemically and locally without causing any damage to the skin. The novel proprietary transdermal technology has been developed for use in pharmaceutical, cosmetic and over the counter (OTC) products. The TDS® consists of nutritional and/or nontoxic substances. The system is a stable solution principally containing water, ethanol, and propylene glycol, in addition to cationic surfactants (quaternary ammonium compounds) and non-ionic surfactants (long chain fatty acids). The solution is formulated to be a spray and the volatile compound can be evaporated quickly, leaving the drug in a concentrated solution over the skin, and resulting in a rapid absorption into the stratum corneum. Due to the reversible alteration in the fluid of the lipid tissue in the stratum corneum, the drug is then released from the stratum corneum over time. TDS® formulations have been developed for transdermal administration, combining a rapid onset of action with a convenient and patient friendly method of administration, and containing substance to preserve the health of skin integrity.

The TDS® systems have been evaluated by the Institute for In Vitro Sciences in Gaithersburg, Maryland USA for primary skin irritation, sensitivity, and toxicity. All materials are tested within non-irritating limits. Unless the outer layer (stratum corneum) of the skin is disrupted or removed by any kind of physical or chemical action, drug molecules can be delivered by the TDS® system reliably through the skin. It is capable of delivers drugs with a molecular weight less than 500 daltons, such as lipophobic, non-polar molecules, and in doses adequate to achieve therapeutic concentrations.

Furthermore, the technology is used and available on the market and it is established in OTC medication such as the self administered analgesic, Penetran Plus®. Also the TDS®

system has been studied for two popular drugs, lidocaine and testosterone. The TDS® lidocaine (Tucker et al., 2006) and TDS® testosterone (Chik et al., 2006) have been tested in 100 and 12 subjects, respectively, without any side effect/events observed during and after the study finished, resulting in a positive result of the two treatments.

1.8 Pharmacokinetic studies

The word pharmacokinetic (PK) appears to have been first utilized in 1953 by Friedrich Hartmut Dost (Dost, 1953). Pharmacokinetics (in Greek: "pharmacon" meaning drug, and "kinetikos" meaning putting in motion) is a branch of pharmacology devoted to measuring the outcome of the substances that are administered to a living organism. Pharmacokinetics is defined as the movement of drug into, through, and out of the body as the metabolite/unchanged substance, expressed as the time course of its absorption, distribution, metabolism and excretion (ADME).

Absorption is the entry of a substance into the body; resulting in its bioavailability in the blood stream. Blood then passes and disperses or disseminates the substance throughout the fluids and tissues of the body into the cells. However the drug may transform into its metabolite before it reaches the receptor site, the phenomenon called metabolism or biotransformation. The drug then can be eliminated from the body by a process known as excretion. However in rare cases, some drugs irreversibly accumulate or stay longer than usual in the body tissue. The measurement of drug concentration using pharmacokinetics as a mathematical pattern has been expanded to include statistical data analysis. This can define and answer most difficult questions which may arise from analyzed data.

Pharmacokinetics is often studied in conjunction with pharmacodynamics, to achieve its useful potential. While pharmacokinetics explains what the body does to the drug, it also determines the onset of action or lag time, duration of action, and efficiency of a drug's effect. Pharmacodynamics describes what a drug does to the body, drug-receptor interaction, which involves receptor binding, post-receptor effects, and chemical interactions. The concentration of drug in plasma is often assumed to behave linearly or at least log linearly with drug effects. This means there is enough drug concentration at

the specific receptor target to provide a therapeutic response. However, the absence of equilibrium between plasma and receptor, the presence of metabolites, drug interaction and drug tolerance may be complicated when choosing an appropriate dosage regimen.

The pharmacokinetics of a drug depend on patient related factors as well as on the drug's chemical properties. Some patient related factors (e.g., age, gender, genetic) can be used to predict the pharmacologic response of populations. For example, the half life of some drugs, especially those that require both metabolism and excretion, may be longer in the elderly. Other factors are related to individual physiology, such as the effects of some individual factors (e.g., disease status, dehydration, obesity), that can be reasonably predicted, but other factors are idiosyncratic and thus have unpredictable effects. Because of individual differences, drug administration must be based on each patient's needs traditionally, by empirically adjusting dosage until the therapeutic objective is met. This approach is frequently inadequate because it can delay optimal response or result in adverse effects. Knowledge of pharmacokinetic principles helps prescribers adjust dosage more accurately and rapidly.

1.9 Clinical trials

A clinical trial (also called clinical research or clinical study) is a research study in human volunteers (healthy or patients, dependent upon the study purpose) to answer specific questions.

Clinical trials are conducted in phases (phase I, phase II, phase III, and phase IV–post marketing), each phase having a different purpose and questions to help the researchers get the answers and information they need. In phase I trials, researchers test an investigational drug or formulation in a small number of subjects, usually healthy subjects in the first instance, where it is possible to identify side effects, efficacy, pharmacological activity, and determine the safe dosage range. Most clinical trials in phase I include both pharmacokinetics and pharmacodynamics. The study data and information obtained with successful drug or dosage form from phase I can be interpreted and used to design phase II trials.

In phase II trials, the investigational drug is given to a large number of patients. The objective of phase II is to examine the efficacy and evaluate further the safety of the investigational drug in patients. The drug is given initially in a low dose and is gradually increased to monitor the tolerability, safety, and identify the suitable dose, dosage form, and the side effects of the treatment. All the study data and information obtained from phase I and phase II, with a potentially successful drug with a sufficient measure of effectiveness and acceptable side effects can be interpreted and used to design phase III studies.

In phase III trials, the investigational drug is given to a larger number of patients, usually hundreds to thousands, to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the investigational drug to be used safely. Phase IV trials are usually achieved after the drug has been marketed. Post marketing studies define further information and any unanswered questions from phase III, including the different effects due to drug benefits, risks, use, and include factors such as gender, ethnicity, etc.

1.9.1 Good clinical practice (GCP)

Good clinical practice is a global ethical and methodological quality standard for designing, conducting, performing, monitoring, auditing, recording, analyzing and reporting of clinical trials, and to confirm that the study information and reported results are convincing, accurate, and precise. Also reliability and privacy of the study subjects are protected [EMEA, ICH GCP 1.24] (EMEA, 2002).

The EU Clinical Trials Directive (2001/20/EC) appeared in law on the 1st of May 2004 and has been incorporated into UK law by the medicines for human use (Clinical Trials) regulations 2004. The medicines for human use (Clinical Trials) Amendment regulations 2006 were placed before parliament on 20 July 2006 and became law on 29 August 2006 for clinical trials. The amendment regulations implement the EU Directive (2005/28/EC) on good clinical practice and also include further requirements relating to agreements for expenses/fees; informing the licensing authority of serious breaches of GCP and/or the protocol; and the expansion of the breach notices (warning notices) organisation.

Together the medicines for human use (Clinical Trials) regulations 2004 and the medicines for human use (Clinical Trials) amendment regulations 2006 and clinical trials regulations were complied with throughout this project.

1.9.2 Good laboratory practice (GLP)

Good laboratory practice (GLP) is a dedicated standard laboratory framework by which the studies are designed, conducted, monitored, recorded, documented, and archived (MHRA, 2007). The study is designed to be able to report data to avoid any risk, hazard, and harmful material to individuals, users, or effect the environment. Thus GLP is applicable to chemical industries, pharmaceuticals, agrochemicals, cosmetics, vitamins and supplements, and food additives. The safety assessment is obtained before a study is conducted to ensure the study is regulated and compliance is verified.

1.9.3 Good manufacturing practice (GMP)

Good manufacturing practice (GMP) is a standard of quality assurance exercise, conducted and employed to certify that the developed chemical products are consistently formed and qualified for their proposed use, which is required by the regulatory/authorities (MHRA, 2008).

1.10 Objectives of the project

The purpose of this project was to study the pharmacokinetics of transdermal drug delivery in healthy humans. The studies were performed to 1) study transdermal drug delivery of diazepam, currently available in different routes of delivery, 2) develop and validate methods to analyze tetracaine in skin samples, and 3) assess appropriate methods to measure the transdermal delivery of drug.

The aim of this work was to:

1. Conduct and investigate pharmacokinetic studies in healthy human volunteers for the development and assistance of drug delivery through the skin. The TDS® diazepam is a liquid combined formulation using a pump dispenser to deliver diazepam through the skin into the systemic circulation. The pharmacokinetics and skin metabolism of diazepam obtained from this study will be investigated for further development of the TDS® system and diazepam skin delivery.

2. Develop, optimize, and validate bioanalytical methods for analysis of drugs in biological matrixes such as plasma, dialysate from microdialysis, and skin tape samples. Investigate the quantitative performance of developed CE and HPLC, to analyzed tetracaine in skin using tape samples obtained from healthy volunteers given tetracaine as a part of pharmacokinetic drug studies, in order to find a rapid, simple, inexpensive method to use to measure transdermal drugs.

3. Investigate the correlation of methods used to measure the pharmacokinetic profile of topically applied or transdermal drugs between skin stripping, microdialysis, and conventional systemic measurement in blood. The results obtained from this study may find a use in determine pharmacokinetic, toxicokinetic, forensic measurements of topically applied drugs.

Chapter 2 Study comparison of rectal and dermal diazepam

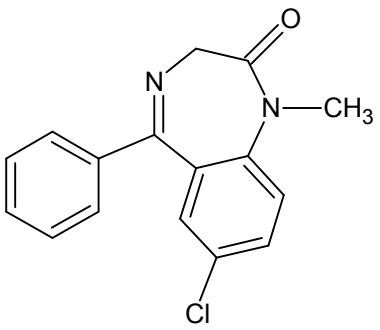
2.1 Introduction

Benzodiazepines exert their effects by interacting with the endogenous γ-aminobutyric acid (GABA) molecule, at a specific subunit on the $GABA_A$ receptors, in the brain. $GABA_A$ is an inhibitory receptor site, which when activated, causes an inhibition of neurotransmission. Positive allosteric binding modulates the activity of $GABA_A$ receptors, resulting in hyperpolarisation of post synaptic membranes, due to the presence of the negative chloride ion. Mild inhibition of neuronal activity exerts an anxiolytic effect in patients, while moderate to high inhibition induces sedation and sleep, and may cause death. The anticonvulsant effect of diazepam and other benzodiazepines may be due to the binding affinity to sodium (Na) channels rather than benzodiazepine receptors. This is seen in the slow recovery of Na channels from inactivation.

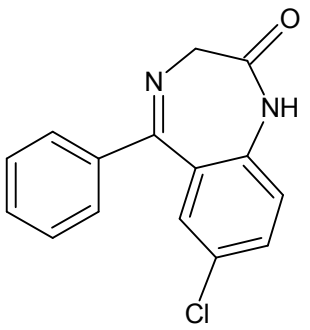
The most widely used drugs for treatment in acute seizures are benzodiazepines, such as diazepam, clonazepam, with no benzodiazepine having been confirmed to be better than another (Treiman, 1989, Rey et al., 1999). Although, diazepam is superior in the management of status epilepticus, lorazepam is preferable for the initial management of status epilepticus (Treiman, 1989).

The physicochemical properties of benzodiazepines such as lipid solubility, and protein binding, facilitate their penetration through the blood brain barrier (BBB) and cerebrospinal fluid, which results in rapid onset of action. The penetration mainly occurs by passive diffusion (Pardridge, 1995), according to Fick's law. The lipid characteristic of diazepam enhances its solubility to penetrate the BBB and to target GABA receptors, and together with its metabolites exert its pharmacological action.

2.1.1 Diazepam



Diazepam



Desmethyldiazepam

Figure 2.1 Chemical structures of diazepam and desmethyldiazepam.

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is a psychotropic drug which is a benzodiazepine derivative, from the class of 1, 4-benzodiazepines (Figure 2.1). Diazepam can be used as a sedative and hypnotic in the management of short term insomnia, a muscle relaxant and anticonvulsant in the management and control of muscle spasm and alcohol withdrawal syndrome, an anxiolytic, to suppress tension and agitation, and it also has amnesic properties. It has a long duration of action. Its actions are mediated by enhancement of GABA activity.

Diazepam binds to specific receptors in the central nervous system (CNS) and particular peripheral organs. The benzodiazepine receptors in the CNS have a close functional connection with the receptors of the GABA-ergic transmitter system. After binding to the benzodiazepine receptor, diazepam enhances the inhibitory effect of GABA-ergic transmission.

Diazepam may be administered orally, intravenously (IV), intramuscularly (IM), or rectally. Oral diazepam has a rapid absorption and fast onset of action, especially in the fasting state, which confers a rapid and nearly complete absorption (Ochs et al., 1982). Oral dose of diazepam results in a plasma concentration in the range of 20 to 1010 µg/L,

and the metabolite desmethyldiazepam in the range of 60 to 1770 µg/L (Rutherford et al., 1978, Greenblatt et al., 1981), with peak plasma levels achieved between 30 to 120 minutes. In fact, the oral route is the most frequent for therapeutic use, accidental poisonings and abuse. It is inappropriate to administer diazepam orally during seizures. While the IM route may currently be the only practical approach, it has no apparent pharmacokinetic advantage over the oral route. Serum plasma concentrations of diazepam in 6 healthy subjects after administration of diazepam 20 mg IV, IM, and orally were 1600, 290, 490 µg/L respectively, t_{max} values were 15, 60, 30 minutes respectively (Martindale, 1982b). The time for diazepam to exert a pharmacological response is 15 minutes to 1 hour for IV and IM routes of administration (Martindale, 1982b). In patients with active seizures, the IV route may be used for cessation of seizures because it has a rapid onset of action; yet this method is by no means without risk, hence facilities for protecting the airway and reversing respiratory depression with mechanical ventilation should be at hand. Venous thrombophlebitis can be reduced by using an emulsion (Diazemuls®), but at the expense of a lower plasma concentration than for IV (Valium®) (Fee et al., 1984). This could not be confirmed by Naylor and Burlingham (Naylor and Burlingham, 1985). Similarly, the rectal route can be used to treat acute repetitive seizures. It can be administered relatively easily and safely by non-medical personnel; however, suppositories generally have less predictable bioavailability as a result of slow and erratic absorption. Administration of rectal solutions and gels have their own problems which include leakage of the active ingredient through the cracks as recently found in the tips of the 10 and 20 mg Diastat® AcuDial® gel prefilled syringes, the consequence of which was an FDA alert. Leakage of gel/solution may result in reduced delivery of active diazepam, and thus less than that required for effective treatment of acute repetitive seizures, ultimately increasing the incidence of more serious problems, including status epilepticus.

Thus, looking for an alternative route, such as transdermal delivery, is desired. Transdermal delivery has the advantage of maintaining a constant blood level of drug, that might be equivalent to the intravenous infusion route (Schwarz et al., 1995). Several studies have been conducted to enhance diazepam penetration through the skin. The potential of transdermal diazepam has been previously studied *in vitro*. Although,

diazepam penetrated human and hairless mouse skin, in twin chamber diffusion cells (Table 2.1), it was not effective in penetrating human skin in the Franz cell technique which was similar to the topical administration to human skin (Koch et al., 1987). Nevertheless, the stratum corneum is a remarkably efficient barrier. Thus attempts to use chemical enhancers or physical methods might be effective to assist delivery of diazepam through the skin into the systemic circulation. By using submicron emulsions *in vivo*, the transdermal delivery of diazepam was effective and equivalent to the level of parenteral delivery (Schwarz et al., 1995).

Table 2.1 Diffusion rate, permeability coefficient, and diffusion coefficients for human skin with twin-chambered diffusion cells (Koch et al., 1987).

Tissue I.D NO.	Flux ($\mu g/cm^2/h$)	Permeability Coefficient (10^{-2} cm/h)	Lag Time (hours)	Diffusion Coefficient (10^{-6} cm^2/h)
1-Trial 1	0.29	1.2	0.64	0.76
Trial 2	0.30	1.0	0.24	2.01
Trial 3	0.24	0.8	0.19	3.31
2-Trial 1	0.28	0.9	0.13	1.73
3-Trial 1	0.42	1.4	0.43	1.12
Trial 2	0.38	1.2	0.35	1.37

Twin chambered (TC) cells have a 0.79-cm^2 surface area for diffusion.

The lipophilic properties of diazepam allow the molecules to penetrate both the BBB and the placenta, resulting in wide distribution throughout the body after administration. Furthermore, diazepam is highly protein bound to human plasma albumin (about 98.5%) and desmethyldiazepam also appears to bind the same protein (Divoll and Greenblatt, 1981). This is explains the accumulation of diazepam in the adipose tissue and muscle after diazepam absorption and distribution, because it is able to build up in the body tissues, particularly in the fat tissue. Thus the concentrations of diazepam may exceed the normal therapeutic level.

Diazepam is metabolized in the liver via cytochrome P450 enzyme, resulting in at least three pharmacologically active metabolites, with half life ranging between 1–5 days. The most important pharmacologically active metabolite of diazepam is desmethyldiazepam, also known as nordiazepam (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one) (Figure 2.1). Other active metabolites include temazepam and oxazepam (Martindale, 1996c). These metabolites are conjugated with glucuronide, and are excreted primarily in the urine and in the breast milk. Because of these active metabolites, the serum/plasma values of diazepam alone are not useful in predicting the effects of the drug.

In this study a novel transdermal drug delivery system was developed (TDS®, Transdermal Technologies Inc, Florida, USA) to deliver diazepam through intact human skin. The Transdermal Drug Delivery System is a liquid formulation that can be combined with a drug entity to form a novel and more convenient pharmaceutical dosage form (spray form), to enhance drug delivery through the skin. This dosage form would provide a fast, simple, easy, and effective method, without leaving any residue. Early studies have shown that the TDS® is capable of transporting drug molecules across human skin (Hadley et al., 1998). Recent studies of the TDS® for lidocaine and testosterone administration have reported no side effects, and resulted in a) acceptable anaesthesia 5 minutes post application of TDS® lidocaine (Tucker et al., 2006), and b) TDS® testosterone was bioequivalent to AndroGel® (Chik et al., 2006).

2.2 Study aims

This study had two main objectives. The primary objective was to determine the pharmacokinetic profile of a topically applied formulation of diazepam (TDS® diazepam) administered by pump dispenser spray to healthy adult subjects, in comparison with rectal diazepam.

2.3 Materials and methods

2.3.1 Study approval

The study was approved by St Thomas Hospital Research Ethics Committee; EudraCT Number: 2006-002609-29. This committee is recognized by United Kingdom of Ethics Committee Authority under the medicine for human use (Clinical Trial Regulation) 2004, and is authorized to carry out the ethical review for clinical trials of investigations of medical products.

2.3.2 Good clinical practice

This study was conducted in accordance with all stipulations of the protocol and the Medicines for Human Use (Clinical Trials Regulations) 2004; The European Clinical Trials Directive (EC2001/20) transposed into UK law, and in accordance with the Declaration of Helsinki.

2.3.3 Subjects

This study involved 12 (3 male and 9 female) Caucasian healthy volunteers aged 18 to 50 years for a period of 49 days.

2.4 Assay procedures

2.4.1 Drug assays

After obtaining approval for the study from the Ethics Committee, the two dosage forms of diazepam were taken to the Analytical Unit of the Department of Cardiac and Vascular Sciences, St George's, University of London, to test the stability of the diazepam (Diastat®), and the quality control of the new dosage form TDS® diazepam, which was carried out as agreed with Analytical Services International Ltd.

2.4.1.1 Chemicals and reagents

The reference product was rectal diazepam Diastat®, AcuDial™, diazepam rectal gel (http://www.diastat.com/) and the TDS® was prepared by Transdermal Technologies Inc., Florida, USA. Diazepam was added to TDS® to give TDS® diazepam (five depressions of the pump spray contained 10 mg diazepam; batch number CT2007007) which was prepared and supplied by the Barts Pharmacy Production Department in The Royal London Hospital.

The Agilent 1100 series HPLC was from Agilent Technologies UK Ltd., West Lothian, UK. The Supelcosil LC-18-DB, 5µm, 15cm column was obtained from Sigma Aldrich, Poole, Dorset, England. All HPLC grade solvents were obtained from Rathburn Chemicals Limited, Walkerburn, Scotland. All AnalaR grade reagents were obtained from Merck (BDH) Limited, Poole, Dorset, England. De-ionized water was obtained from the St George's – University of London de-ionized water supply, operating a system installed by ELGA Limited, High Wycombe, England. The polypropylene storage and dilution tubes were obtained from Sarstedt Ltd, Beaumont Leys, Leicester, England.

2.4.1.2 Stability of diazepam in Diastat® and TDS® diazepam formulations at various temperatures

In this study, an HPLC/UV assay was performed to measure the stability of diazepam solutions at various temperatures. Two solutions were supplied, both containing diazepam at 10 mg/mL. In addition, four rectal preparations (Diastat®), and one TDS® diazepam were analyzed for diazepam. To prevent evaporation changes affecting the measurements, the diazepam solutions provided were aliquoted into sealed tubes. These tubes were then stored at approximately 4ºC, 20ºC and 35ºC. The concentration of these aliquots from each temperature was measured over a period of 33 days, with nine separate measurements made. On each occasion the concentration of the diazepam in the aliquots stored at 4ºC, 20ºC and 35ºC was measured by HPLC/UV using calibration standards prepared from a stock stored at –20ºC.

The reagents and consumables were acetonitrile for HPLC, phosphoric acid AnalaR, de-ionized water (ELGA), prazepam, and diazepam.

2.4.1.2.1 Internal standard

Prazepam was used as internal standard, and prepared by adding 0.5mL of prazepam (10mg/mL in acetonitrile) in 50mL acetonitrile, and completed to 100mL with de-ionised water.

2.4.1.2.2 Diazepam rectal preparation storage

The Diastat® samples were stored at approximately 4°C prior to analysis.

2.4.1.2.3 Diazepam solution storage (Diastat®)

All solutions were stored at approximately 4°C prior to analysis. The samples were allowed to reach room temperature (nominally 20°C) and then aliquoted and stored at approximately 4ºC, 20ºC and 35ºC.

Over a period of 33 days one aliquot from each solution at each temperature was assayed by HPLC/UV.

2.4.1.2.4 Sample preparation (Diastat®)

All dispensers were adjusted to deliver a 10 mg dose, one 10 mg dose from each dispenser was dispensed into a 100 mL volumetric flask, and the flasks were made up to 100 mL with 50% acetonitrile in de-ionized water and placed in an ultrasonic bath for 15 minutes. Then 10 mL of each solution was diluted to 20 mL with 50% acetonitrile, then 500 µL of each solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard (25µg/mL). The tubes were capped and mixed by inversion.

2.4.1.2.5 Calibrator preparation (Diastat®)

500 µL of each calibration solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard. The tubes were capped and mixed by inversion. An aliquot from each tube was transferred into labelled auto sampler tubes. 10 µL of each aliquot was injected onto the HPLC/UV.

2.4.1.2.6 Sample preparation (diazepam solution)

One aliquot from each solution at each temperature was allowed to reach room temperature. 50 µL of each solution was pipetted into a labelled polypropylene tube.

10mL of sample diluent (50% acetonitrile in de-ionized water) was added (1:201 dilution). 500 µL of each solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard. The tubes were capped and mixed by inversion. An aliquot from each tube was transferred into labelled auto sampler tubes. 10µL of each aliquot was injected onto the HPLC/UV.

2.4.1.2.7 Calibrator preparation (diazepam solution)

500 µL of each calibration solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard. The tubes were capped and mixed by inversion. An aliquot from each tube was transferred into labelled auto sampler tubes. 10 µL of each aliquot was injected onto the HPLC/UV.

2.4.1.2.8 Diazepam solution storage (TDS® diazepam)

The solution was stored at approximately 4°C prior to analysis. The sample was allowed to reach room temperature (nominally 20°C) before analysis by HPLC/UV.

2.4.1.2.9 Sample preparation (TDS® diazepam solution)

The solution was allowed to reach room temperature. Four separate 50 µL aliquots of the solution (10 mg/mL) were pipetted into four labelled polypropylene tubes. 10 mL of

sample diluent (50% acetonitrile in de-ionized water) was added (1:201 dilution) to each tube. 500 µL of each solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard. The tubes were capped and mixed by inversion. An aliquot from each tube was transferred into labelled auto sampler tubes. 10 µL of each aliquot was injected onto the HPLC/UV.

2.4.1.2.10 Calibrator preparation (diazepam solution)

500 µL of each calibration solution was mixed with 500 µL 50% acetonitrile in de-ionized water containing prazepam as an internal standard. The tubes were capped and mixed by inversion. An aliquot from each tube was transferred into labelled auto sampler tubes. 10 µL of each aliquot was injected onto the HPLC/UV.

2.4.1.3 Calibration samples

A stock solution containing diazepam was prepared by pipetting 1 mL of diazepam solution (10mg/mL) into a 10 mL volumetric flask and making up to the mark with 50% acetonitrile in de-ionized water to produce a sub-stock.

5 mL of the stock solution was pipetted into a 50 mL volumetric flask and made up to the mark with 50% acetonitrile in de-ionized water to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with 50% acetonitile in de-ionized water as tabulated in Table 2.2.

Table 2.2 Calibration solutions preparation from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/mL)	Volume of sub stock (mL)
7	10	0.00	0.00
6	10	10.0	1.00
5	10	20.0	2.00
4	10	40.0	4.00
3	10	60.0	6.00
2	10	80.0	8.00
Cal 1 = Sub stock		100.0	

The calibration solutions were stored at approximately 4ºC.

2.4.1.4 Instrumentation

Solvent delivery was achieved using an Agilent series 1100 pump set at 1.0 mL/minute. Sample injection was performed by using an Agilent series 1100 auto injector. Chromatography was on a Supelcosil LC-18-DB, 5 µm, 15 cm x 4.6 mm column maintained at 50ºC with an Agilent series 1100 column oven. Detection was by an Agilent series 1100 UV detector set at 245 nm.

A Windows XP computer running AZUR version 4.6 was used to record the output from the detector and perform integration of peak areas. The AZUR software was supplied by Jasco, England.

2.5 Overall study design

This was a randomized, single-dose, two-period, cross-over phase I (pharmacokinetic) study. Subjects were randomized in a 1:1 ratio to receive either a 10 mg dose of transdermal diazepam followed by a 10 mg dose of rectal diazepam, or *vice-versa*. The study consisted of two administration days (separated by 14 days) with a further safety

visit seven days after the second administration. This safety visit was done by telephone. Subjects went in the study for a maximum of 49 days, including screening.

2.6 Screening

Subjects attended the study site for screening, within 28 days of the first treatment day. They had had the nature of the study, the procedures and the risks fully explained, and were given time to consider, and the opportunity to ask any questions they had. Before any screening procedures occurred they signed an Informed Consent Form. At this time, a 20 mL blood sample was taken for routine biochemical and haematological screening, and a urine specimen was obtained for pregnancy testing, urinalysis and drug screening (SureStep™, Inverness Medical International, Cranfield, England). Only people who met the inclusion and exclusion criteria were allowed to participate, referred to Appendix 5.

2.7 Admission and procedure

On each of the two treatment days, subjects were admitted to the Study Centre, in The Clinical Microvascular Unit, St Bartholomew's Hospital at about 07:30 (AM) in the morning. After confirming the suitability of the subject, blood pressure, radial pulse, respiratory rate, and body temperature were measured after subjects had rested for 10 minutes. Then, a 20 G cannula was placed in a large antecubital vein for blood collection for each treatment day. A 20 mL blood sample was taken for routine biochemical and haematological screening. In the study plan subjects were randomized in a 1:1 manner (Table 2.3) to receive either a 10 mg dose of transdermal diazepam followed by a 10 mg dose of rectal diazepam, or *vice-versa* (separated by 14 days). However due to the time taken in the preparation of TDS® diazepam, the subjects could not be given the formulations in random order. Therefore, the subjects were divided into two groups, the first Saturday from the first week for group A to receive rectal diazepam, and the second Saturday from the second week for group B to receive rectal diazepam. Serial 5 mL blood samples were collected at 0, 15 min, 30 min, 45 min, 1, 1½, 2, 2½, 3, 4, 5, 6, 8, 10 and 12 hour time points, after topical application of TDS® diazepam or rectal administration of

diazepam. The first 1–2 mL of each subsequent collection was discarded as this represented residual blood and saline from within the cannula. Following each blood collection the cannula was flushed with 7 mL sterile saline to preserve patency.

After collection the sample was transferred to K_3EDTA containing tubes, mixed then centrifuged at 3000 g for 10 minutes. The plasma was transferred to labelled tubes (cryo-vials) and stored at 4º C. At the end of the study day the plasma samples were transported and stored at –20º C until the whole study finished. After that, all samples were analyzed.

Table 2.3 Randomization (R), single dose treatment, two-period, cross over with 14 days washout period.

Subjects No (R000)	Treatment Day 1	Washout period Day	Treatment Day 2
R001	RDZ 10 mg	14 days	TDS® DZ10 mg
R002	RDZ 10 mg	14 days	TDS® DZ10 mg
R003	RDZ 10 mg	14 days	TDS® DZ10 mg
R004	RDZ 10 mg	14 days	TDS® DZ10 mg
R005	RDZ 10 mg	14 days	TDS® DZ10 mg
R006	RDZ 10 mg	14 days	TDS® DZ10 mg
R007	TDS® DZ10 mg	14 days	RDZ 10 mg
R008	TDS® DZ10 mg	14 days	RDZ 10 mg
R009	TDS® DZ10 mg	14 days	RDZ 10 mg
R010	TDS® DZ10 mg	14 days	RDZ 10 mg
R011	TDS® DZ10 mg	14 days	RDZ 10 mg
R012	TDS® DZ10 mg	14 days	RDZ 10 mg

RDZ: Rectal diazepam, TDS®DZ: Transdermal drug delivery system-diazepam

The TDS® diazepam dose was sprayed topically to the upper chest and gently rubbed into the skin. Hypoallergenic surgical gloves were worn (prior to the administered dose) to prevent self dosing. Rectal diazepam was administered according to routine clinical practice following how to administer and dispose of Diastat®, AcuDial™ (http://www.diastat.com/).

A digital photograph was taken of the application site prior to administration and after 30 minutes (for TDS® diazepam dosing), and at the final visit.

Providing subjects were considered fit for discharge from the study centre, they were allowed to depart after the final blood sample for pharmacokinetic testing had been taken (after 12 hours).

Upon return to the Study Centre at pre-defined times, 5 mL of blood was collected at the 24, 32, 48 and 72 hours time points; vital signs were also recorded at these time-points. At the final visit, 25 mL blood was collected from which 20 mL of blood was taken for routine biochemical and haematological screening.

2.8 Study restriction

2.8.1 Concomitant therapy

The subjects were not allowed to use any medications (prescribed or over-the-counter including herbal remedies, but excluding oral contraceptives) judged to be clinically relevant by the principal investigator during the 14 days preceding the study, and during the course of the study (until the final visit).

Any concomitant medication and significant non drug therapies were recorded in the CRF.

2.9 Treatments

The investigational medicinal product in this phase I study was TDS® diazepam (10 mg diazepam per application, requiring five sprays from a metered-dose pump dispenser). The reference product was rectal diazepam (one DiaStat® AcuDial™ containing 10 mg diazepam). Study medication was supplied by the Barts Pharmacy Production Department in packages containing sufficient rectal diazepam and diazepam for transdermal use for the entire study.

The topical (transdermal) application was applied to the upper chest by rubbing the skin; DiaStat® diazepam was administered rectally with a check for leakage 5 minutes after dosing.

2.10 Safety and tolerability

Adverse events were recorded throughout the study, at all visits and during any telephone calls. The skin at the application site was assessed at each clinic visit by the investigator. Any dermal irritation was scored according to the OECD Guideline for Testing of Chemicals No. 404, adopted 17th July, 1992: "Acute Dermal Irritation/Corrosion (Table 2.4 and Table 2.5) (OECD/OCDE, 1992). Details on medical history and concomitant illnesses were recorded on the day of screening by conducting a medical interview of the subject or subject's relative (s) and/or review of the subject's medical records. Any changes observed or reported during the study were recorded. A brief physical examination was performed at screening and at the final visit. Vital signs were recorded at every visit, both pre and post-administration at defined time-points. Clinical laboratory tests were performed at screening, and on the day of each diazepam administration. Clinical and digital photographic assessment of the TDS® diazepam skin application site occurred before and after dosing and at follow up when the subject returned to the study centre for the final visit. The photograph did not identify the subjects visually. Clinic assessments of vital signs and physical examinations were done.

Drug accountability records for treatment compliance were checked by the monitor during site visits and at the completion of the trial.

Table 2.4 Erythema and eschar formation, the OECD guideline for testing of chemicals.

Erythema and eschar formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

Table 2.5 Oedema formation, the OECD guideline for testing of chemicals.

Oedema Formation	Score
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm, extending beyond area of exposure)	4

2.11 Plasma analysis

A high performance liquid chromatographic assay, with mass spectrometric detection, (HPLC/MS) for the measurement of diazepam and metabolites (desmethyldiazepam, temazepam, oxazepam) in human plasma samples from healthy volunteers given diazepam as part of a clinical study (pharmacokinetic study) was used. The assays were performed at the Analytical Unit, St George's, University of London, using a liquid/liquid extraction method.

2.11.1 Instrumentation

Solvent delivery was achieved using a Perkin Elmer series 200 pump set at 1mL/minute. Sample injection was performed by using a Perkin Elmer series 200 auto injector. Chromatography was on a Supelcosil LC-18-DB, 5µm, 15cm, 4.6 mm column (serial number 62200-04) maintained at 50ºC with a Perkin Elmer series 200 column oven. Detection was by Applied Biosystems SCIEX API2000 Mass spectrometer. The mobile phase consisted of formic acid 1mL/L (85%), and methanol 75%, isocratic.

A Windows NT computer running Analyst software (current version) was used to control the HPLC/MS, record the output from the detector, perform integration of peak areas and calculate the diazepam and metabolites concentrations. The Analyst software was supplied by Applied Biosystems, England.

MS Settings: A SCIEX API4000 triple quadrupole mass spectrometer equipped with a heated nebulizer interface was used to introduce the sample into the mass spectrometer. Nitrogen was used as the collision gas. The NM20ZA high purity nitrogen and air generators were supplied by Peak Scientific Instruments.

2.11.2 Good laboratory practice

This study was conducted in accordance with United Kingdom Statutory Instrument 1999 No 3106 as amended by SI 2004 No. 994, The Good Laboratory Practice (Codified Amendments Etc) Regulations, The Good Laboratory Practice Regulations, Department of Health, London, and the OECD Principles on Good Laboratory Practice (revised 1997, Issued Jan 1998) ENV/MC/CHEM (98) 17. The final report fully and accurately reflects the raw data generated during the conduct of the study.

The study was carried out as agreed with Analytical Services International Ltd. and documented in the Analytical Unit Study Plan number.

2.11.3 Chemicals and reagents

The SCIEX API2000 equipment was obtained from Applied Biosystems, England. The high purity air and nitrogen generators were obtained from Peak Scientific Instruments Ltd., Renfrew, Scotland. All AnalaR grade reagents and Hipersolv solvents were obtained from Merck (BDH) Limited, Poole, Dorset, England. The Supelcosil LC-18-DB, 5µm, 15cm, 4.6 mm column (serial number 62200-04) was obtained from Merck (BDH) Limited, Poole, Dorset, England. De-ionized water was obtained from St George's, University of London, de-ionized water supply, operating a system installed by ELGA Limited, High Wycombe, England. Drug-free human plasma was obtained from Biological Specialty Corporation, USA. The polypropylene dilution tubes were obtained from Sarstedt Ltd, Beaumont Leys, Leicester, England. The auto-sampler tubes were obtained from NLG Analytical, Adelphi Mill, Bollington, Cheshire, England.

Diazepam, desmethyldiazepam, temazepam and oxazepam, for calibrator and control sample preparation, were obtained as 1mg/mL calibrated solutions in methanol supplied by Cerilliant and purchased from LGC Promochem, UK. Diazepam-d5, desmethyldiazepam-d5, temazepam-d5 and oxazepam-d5, for use as internal standards, were obtained as 0.1mg/mL calibrated solutions in methanol supplied by Cerilliant (certifications referred in Appendix 7) and purchased from LGC Promochem, UK.

2.11.4 Quality assurance statement

The study described in this report has been subject to Quality Assurance evaluation by the Analytical Unit's independent Quality Assurance Officer. The study was inspected for the items, and at the intervals, specified below. The findings of each inspection were reported to the study director and analytical unit management.

The study report inspection was designed to confirm that, as far as can be reasonably established, the methods described and the results incorporated in this study are a true and accurate reflection of the raw data.

2.11.5 Calibration curve criteria

The calibration curves contained seven, non-zero, calibrators, containing diazepam, desmethyldiazepam, temazepam and oxazepam, assayed in duplicate. Nominal values for all analytes were 5, 10, 25, 100, 250, 500 and 1000 µg/L Two analyte-free samples were analyzed, one with internal standard and one without internal standard; neither were included when fitting the calibration line.

For the calibration curve to be acceptable, the correlation coefficient (r) between concentration and peak area ratio, with respect to the internal standard, must be equivalent to, or better than, 0.98.

The following conditions were also met in developing a calibration curve:

- no more than 20% deviation of the LLOQ from nominal concentration
- no more than 15% deviation of standards, other than LLOQ, from nominal concentration

At least 66% of the non-zero standards must meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.
Using these criteria, all calibration curves were well within the ranges allowed.

2.11.6 Accuracy

For run acceptance there should be no more than 15% deviation of the controls from their nominal concentrations. At least 66% of the controls must meet the above criteria. Using these criteria, accuracy for the above quality control samples were well within the ranges allowed, as shown under accuracy.

2.11.7 Calibration

A combined stock solution containing diazepam, desmethyldiazepam, temazepam and oxazepam was prepared by pipetting 1 mL of each stock solution into a 10 mL volumetric flask and making up to the mark with 25% methanol in de-ionized water to produce a sub-stock.

1 mL of the combined stock solution was pipetted into a 100 mL volumetric flask and made up to the mark with 25% methanol in de-ionized water to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with 25% methanol in de-ionized water

Table 2.6 Calibration solution preparation from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
8	10	0.00	0.00
7	10	5.0	0.05
6	10	10.0	0.10
5	10	25.0	0.25
4	10	100.0	1.00
3	10	250.0	2.50
2	10	500.0	5.00
Cal 1 = Sub stock		1000.0	

The calibration solutions were stored at approximately 4ºC.

2.11.8 Quality control samples

A combined stock solution containing diazepam, desmethyldiazepam, temazepam and oxazepam was prepared by pipetting 1 mL of each stock solution into a 10 mL volumetric flask and making up to the mark with 25% methanol in de-ionized water to produce a sub-stock.

0.25 mL of the stock solution was pipetted into a 10 mL volumetric flask and made up to the mark with analyte free human plasma to produce a sub-stock (QC 4). Then working controls were prepared by diluting the sub stock (QC 4) with human plasma as in Table 2.7.

Table 2.7 Quality control preparation from sub-stock (QC4).

QC No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
1	25	20.0	0.20
2	25	200.0	2.00
3	10	750.0	3.00
QC 4 = Sub stock		2500.0	

The control solutions were stored frozen at approximately –20ºC.

2.11.9 Internal standard solution

A combined stock solution containing diazepam-d5, desmethyldiazepam-d5, temazepam-d5 and oxazepam-d5 was prepared by pipetting 1 mL of each stock solution into a 200 mL volumetric flask and making up to the mark with de-ionized water.

2.11.10 Extraction buffer

Phosphate buffer (0.5 M, pH 7) containing approx. 5 mg/100 mL Orange G was prepared by dissolving 26g of di-sodium hydrogen phosphate, and 14g potassium dihydrogen phosphate in 500mL volumetric flask containing de-ionised water, and Orange G approx 5mg/100mL. The Orange G was used to make the interface between the aqueous layer and the solvent layer easier to visualize.

2.11.11 Extraction calibrators

0.1 mL of calibration solution, 0.1 mL blank plasma, 0.05 mL internal standard, 0.25 mL extraction buffer and 2 mL ethylacetate were pipetted into a 4.5 mL polypropylene tube.

2.11.12 Control samples

0.1 mL of control sample, 0.1 mL 25% methanol in de-ionized water, 0.05 mL internal standard, 0.25 mL extraction buffer and 2 mL ethylacetate were pipetted into a 4.5 mL polypropylene tube (Flow Chart 2.1).

The tubes were capped, mixed (30 minutes, minimum) and then centrifuged (1509–2054 g Relative Centrifugal Force (RCF), for 5 minutes, minimum). The organic phase (upper layer) from each tube was transferred to a 4.5 mL polypropylene tube and evaporated to dryness in the SpeedVac. The dried extracts were reconstituted in 250 µL 25% methanol, mixed for 1 minute and transferred to auto sampler vials. 50–100 µL of each extract was injected onto the analytical column.

2.11.13 Test substances

Diazepam, desmethyldiazepam, temazepam and oxazepam, for calibrator and control sample preparation, were obtained as 1 mg/mL calibrated solutions in methanol supplied by Cerilliant and purchased from LGC Promochem, UK.

Diazepam-d5, desmethyldiazepam-d5, temazepam-d5 and oxazepam-d5, for use as internal standards, were obtained as 0.1 mg/mL calibrated solutions in methanol supplied by Cerilliant and purchased from LGC Promochem, UK.

2.11.14 Calibrator and control matrices

Calibrators were prepared in 25% methanol and spiked into plasma prior to extraction.

Controls were prepared in diazepam, desmethyldiazepam, temazepam and oxazepam-free plasma.

2.11.15 Quality control

The three quality control samples were dispersed throughout the analytical run, with one quality control sample run in duplicate after every 8–15 study samples (e.g. calibration curve, the low quality control samples, 8–15 study samples, the medium quality control,

8–15 study samples, the high quality control sample, 8–15 study samples, the low quality control sample etc.). The nominal values for low, medium and high control samples were 20, 200 and 750 µg/L, respectively. A minimum of two sets of quality control samples at each level were included in each full analytical run. Appropriate controls were included in shorter runs.

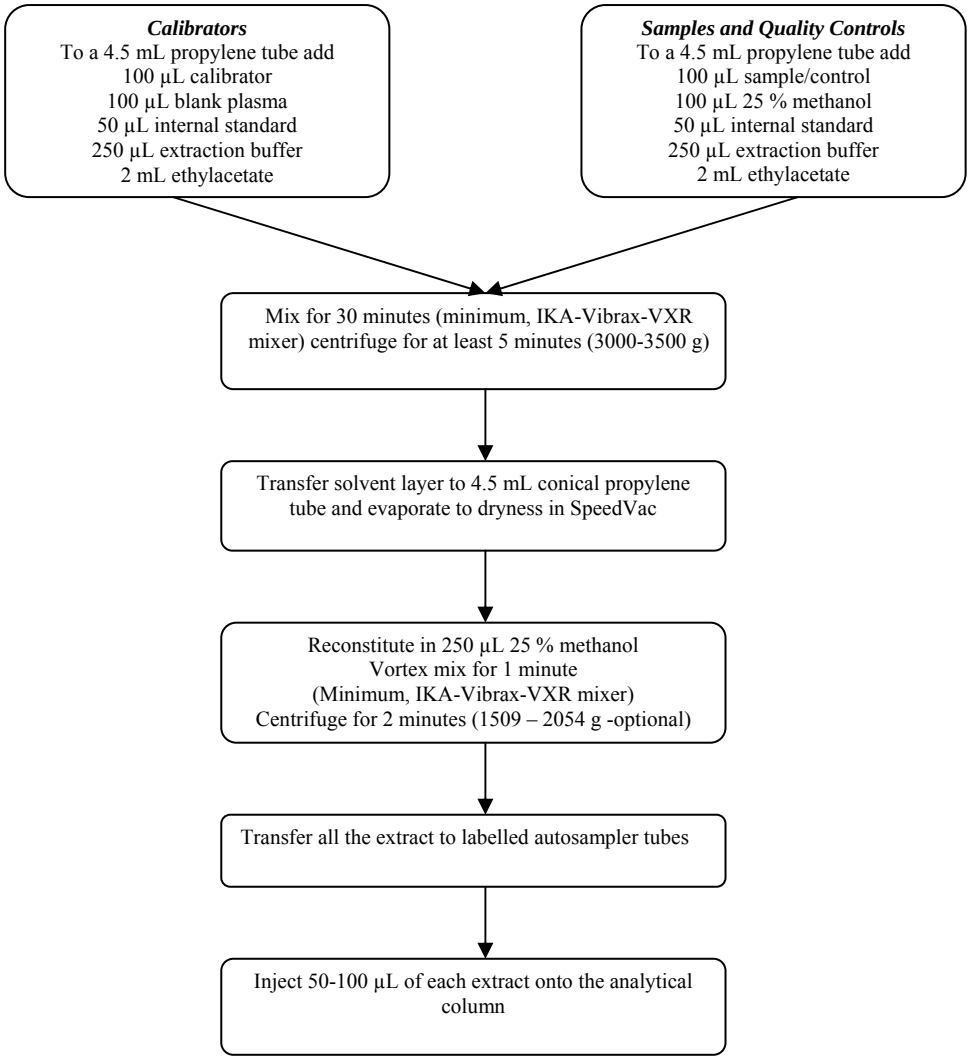
The concentration of both the control samples and the patient samples were calculated by using the peak areas of each analyte with respect to the peak of the appropriate internal standard. The following conditions were met for run acceptance:

- No more than 15% deviation of controls from their nominal concentration

At least 66% of the controls must meet the above criteria.

Flow Chart 2.1 Schematic diagram of diazepam extraction procedures.

Method details



Internal standard:
0.5 µg/mL diazepam-d5
0.5 µg/mL desmethyldiazepam-d5
0.5 µg/mL temazepam-d5
0.5 µg/mL oxazepam-d5
Extraction buffer:
Phosphate buffer (0.5 M, pH 7) containing approx. 5 mg/100 mL Orange G to make the interface between the aqueous layer and the solvent layer easier to visualise

HPLC conditions:
Column: Any suitable 15 cm x 4.6 mm C 18 column
Mobile phase:
Methanol 75 % (acceptable range: 65 %-85 %)
De –ionised water to 100 %
Formic acid (1mL/L)

Flow rate: 1.0 mL/minute (acceptable range: 0.8 – 1.2 mL/minute)

MS/MS setting: A SCIEX triple quadrupole mass spectrometer equipped with a turbo-ion spray (heated electro-spray, ESI) source was used to introduce the sample into the mass spectrometer. Nitrogen was used as the collision gas. The mode of operation was multiple reaction mode (MRM)

Sample ID	**Initial ion (m/z)**	**Product ion (m/z)**	**Ionisation mode**
Diazepam	285±0.5	193±0.5	positive
Desmethyldiazepam	271±0.5	140±0.5	positive
Temazepam	301±0.5	255±0.5	positive
Oxazepam	287±0.5	241±0.5	positive

2.11.16 Validation of an HPLC/MS assay to measure diazepam and metabolites in human plasma

2.11.16.1 Calculation of results

Unless stated otherwise, all the results shown were calculated using a $1/x^2$ weighted regression. The peak area ratio, regression coefficient and the slope of the calibration line etc. were calculated from the peak area data by the Analyst program.

2.11.16.2 Precision

Precision was assessed using three quality control samples with nominal diazepam and metabolite values of 20, 200 and 750 μg/L.

2.11.16.3 Within-assay reproducibility

The three quality control samples were, initially, each extracted six times in one batch. Subsequently, the three quality control samples were each extracted six times in two additional batches. On each occasion a separate calibration curve was extracted.

The lower limit of accurate quantification was set at the value of the lowest calibrator and the upper limit of accurate quantification was set at the value of the highest calibrator. The repeatability at these levels was investigated by extracting the lowest and highest calibrator six times in three separate assays.

Table 2.8, Table 2.9, Table 2.10, and Table 2.11 shows the within assay reproducibility of diazepam, desmethyldiazepam, temazepam, and oxzepam, respectively.

Table 2.8 The within assay reproducibility of three batches of diazepam.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Batch 1, mean (n=6)	5.18	20.12	198.67	787.01	1036.21
Standard Dev.	0.25	1.99	9.67	54.44	40.43
%CV	4.91	9.93	4.87	6.92	3.90
Accuracy	103.54	100.60	99.33	104.94	103.62
Batch 2, mean (n=6)	5.07	19.97	197.48	759.57	979.42
Standard Dev.	0.53	1.99	7.28	29.47	43.15
%CV	10.37	9.95	3.69	3.88	4.41
Accuracy	101.40	99.85	98.74	101.28	97.94
Batch 3, mean (n=6)	5.25	19.31	202.53	797.33	976.78
Standard Dev.	0.41	0.60	4.64	29.37	53.31
%CV	7.89	3.13	2.297	3.687	5.46
Accuracy	105.07	96.55	101.26	106.31	97.68

Table 2.9 The within assay reproducibility of three batches of desmethyldiazepam.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Batch 1, mean (n=6)	5.27	20.46	194.87	734.88	993.18
Standard Dev.	0.24	1.68	10.65	41.29	45.98
%CV	4.60	8.21	5.46	5.62	4.63
Accuracy	105.46	102.30	97.43	97.98	99.32
Batch 2, mean (n=6)	4.67	19.63	203.97	757.28	973.20
Standard Dev.	0.24	1.42	7.08	14.19	29.29
%CV	5.04	7.23	3.47	1.87	3.01
Accuracy	93.39	98.17	101.99	100.97	97.32
Batch 3, mean (n=6)	4.99	19.99	203.03	788.50	1000.65
Standard Dev.	0.31	0.76	5.30	19.14	25.943
%CV	6.19	3.83	2.61	2.43	2.59
Accuracy	99.80	99.95	101.51	105.13	100.06

Table 2.10 The within assay reproducibility of three batches of temazepam.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Batch 1, mean (n=6)	5.22	20.00	192.97	732.99	982.56
Standard Dev.	0.37	2.23	7.83	52.99	48.54
%CV	7.17	11.15	4.06	7.23	4.94
Accuracy	104.37	100.00	96.48	97.73	98.26
Batch 2, mean (n=6)	5.27	18.62	195.93	736.16	947.58
Standard Dev.	0.46	1.06	8.03	24.36	34.24
%CV	8.67	5.71	4.09	3.31	3.61
Accuracy	105.41	93.12	97.96	98.15	94.76
Batch 3, mean (n=6)	4.54	18.99	203.26	769.91	977.60
Standard Dev.	0.13	0.47	3.80	12.95	35.87
%CV	2.92	2.45	1.87	1.68	3.67
Accuracy	90.88	94.94	101.63	102.65	97.76

Table 2.11 The within assay reproducibility of three batches of Oxazepam.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Batch 1, mean (n=6)	5.10	19.72	194.91	731.49	991.56
Standard Dev.	0.42	1.54	12.26	47.82	59.55
%CV	8.16	7.83	6.29	6.54	6.01
Accuracy	102.07	98.62	97.45	97.53	99.16
Batch 2, mean (n=6)	4.77	19.99	201.73	738.89	973.84
Standard Dev.	0.20	1.29	5.72	14.45	31.68
%CV	4.22	6.44	2.83	1.96	3.25
Accuracy	95.37	99.95	100.86	98.52	97.38
Batch 3, mean (n=6)	4.73	20.03	196.26	741.45	959.86
Standard Dev.	0.55	0.81	3.23	13.76	29.92
%CV	11.53	4.03	1.65	1.86	3.12
Accuracy	94.58	100.17	98.13	98.86	95.99

2.11.16.4 Between-assay repeatability

For each of the three assays mentioned above the mean concentration from each assay was used to calculate the between assay reproducibility. Table 2.12, Table 2.13, Table 2.14, and Table 2.15 shows the between assay repeatability of diazepam, desmethyldiazepam, temazepam, and oxzepam, respectivel

Table 2.12 The between assay reproducibility of three batches of diazepam.

	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Mean (n=3)	5.17	19.80	199.56	781.30	997.47
Standard Dev.	0.09	0.43	2.64	19.52	33.58
%CV	1.78	2.18	1.32	2.50	3.37
Accuracy	103.34	99.00	99.78	104.17	99.75

Table 2.13 The between assay reproducibility of three batches of desmethyldiazepam.

	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Mean (n=3)	4.98	20.03	200.62	760.22	989.01
Standard Dev.	0.30	0.41	5.01	26.93	14.19
%CV	6.06	2.07	2.49	3.54	1.43
Accuracy	99.55	100.14	100.31	101.36	98.90

Table 2.14 The between assay reproducibility of three batches of temazepam.

	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Mean (n=3)	5.01	19.20	197.39	746.35	969.24
Standard Dev.	0.41	0.71	5.30	20.46	18.93
%CV	8.09	3.71	2.68	2.74	1.95
Accuracy	100.22	96.02	98.69	99.51	96.92

Table 2.15 The between assay reproducibility of three batches of oxazepam.

	LLOQ	QC1	QC2	QC3	ULOQ
Expected Conc. (µg/L)	5.00	20.00	200.00	750.00	1000.00
Mean (n=3)	4.87	19.92	197.63	737.28	975.09
Standard Dev.	0.21	0.17	3.61	5.17	15.89
%CV	4.23	0.84	1.83	0.70	1.63
Accuracy	97.34	99.58	98.82	98.30	97.51

2.11.16.5 Accuracy

The criteria for the acceptance of the quality control samples were that the mean measured values should be within ±15% of the expected value. Using these criteria, accuracy for the above quality control samples were well within the ranges allowed, as shown under accuracy, above.

2.11.16.6 Recovery

Absolute recovery of diazepam and metabolites was tested at the same nominal concentrations as the quality control samples. Absolute recovery of the internal standards was tested at a nominal concentration of 250µg/L.

2.11.16.6.1 Extracted control sample

100µL of each aqueous control sample, 100µL blank human plasma and 50µL internal standard solution was processed as per the method.

2.11.16.6.2 Non-extracted control sample

100µL of each aqueous control sample was mixed with 50µL internal standard solution and 100µL 25% methanol.

Table 2.16 Diazepam, and metabolites recovery after extraction (Ext) compared to non extracted samples.

Diazepam						
	100% QC1	Ext QC1	100% QC2	Ext QC2	100% QC3	Ext QC3
Mean (n=4)	20.01	7.56	198.91	68.76	753.78	234.56
Recovery		37.8%		34.4%		31.3%
Desmethyldiazepam						
	100% QC1	Ext QC1	100% QC2	Ext QC2	100% QC3	Ext QC3
Mean (n=4)	20.01	17.92	198.82	171.32	754.09	650.02
Recovery		89.6%		85.7%		86.7%
Temazepam						
	100% QC1	Ext QC1	100% QC2	Ext QC2	100% QC3	Ext QC3
Mean (n=4)	19.97	14.06	203.50	138.34	737.86	514.55
Recovery		70.3%		69.2%		68.6%
Oxazepam						
	100% QC1	Ext QC1	100% QC2	Ext QC2	100% QC3	Ext QC3
Mean (n=4)	19.99	16.82	201.79	183.07	743.80	711.39
Recovery		84.1%		91.5%		94.9%

2.11.16.7 Auto sampler stability

In order to assess the stability of the sample extracts a single aliquot of each of the three quality control samples were extracted. This extract was then injected seven times over a

period of approximately 29 hours. The auto sampler was operated at ambient temperature, approximately 20.6ºC (maximum temperature 21.5ºC, minimum temperature 19.0ºC), Table 2.17, Table 2.18, Table 2.19, and Table 2.20 shows the stability of diazepam, desmethyldiazepam, temazepam, and oxazepam, respectively.

Table 2.17 The auto sampler stability of diazepam after extraction, over a period of approximately 29 hours.

	QC1	QC2	QC3	Elapsed time
Data	(µg/L)	(µg/L)	(µg/L)	(Hrs)
1	19.10	195.63	746.70	0
2	18.43	196.20	741.79	3
3	21.75	202.07	754.19	6
4	18.01	188.91	752.14	12
5	19.01	199.88	725.06	21
6	19.50	197.66	729.16	26
7	19.28	194.10	718.04	29

Table 2.18 The auto sampler stability of desmethyldiazepam after extraction, over a period of approximately 29 hours

	QC1	QC2	QC3	Elapsed time
Data	(µg/L)	(µg/L)	(µg/L)	(Hrs)
1	20.13	188.46	715.13	0
2	19.30	183.82	696.02	3
3	19.63	184.38	713.90	6
4	19.37	183.94	720.901	12
5	19.92	185.32	705.65	21
6	21.02	186.96	704.42	26
7	18.72	183.56	715.98	29

Table 2.19 The auto sampler stability of temazepam after extraction, over a period of approximately 29 hours

	QC1	QC2	QC3	Elapsed time
Data	(µg/L)	(µg/L)	(µg/L)	(Hrs)
1	18.11	206.69	741.99	0
2	21.40	205.11	785.17	3
3	22.41	211.38	740.88	6
4	21.18	200.59	772.09	12
5	22.04	200.88	726.37	21
6	22.38	203.29	807.22	26
7	17.96	187.37	718.19	29

Table 2.20 The auto sampler stability of oxazepam after extraction, over a period of approximately 29 hours

	QC1	QC2	QC3	Elapsed time
Data	(µg/L)	(µg/L)	(µg/L)	(Hrs)
1	21.90	196.95	674.17	0
2	17.34	196.98	744.13	3
3	19.29	184.62	680.75	6
4	17.29	186.54	688.28	12
5	14.36	184.02	750.37	21
6	21.17	199.49	752.25	26
7	21.36	197.46	763.35	29

2.11.16.8 Dilution accuracy

Assay linearity, with a view to dilution of samples producing results above the ULOQ, was assessed by the preparation of an out of range control sample. Diazepam and metabolites were added to human plasma to produce a sample with a nominal concentration of 2500µg/L (QC4). This sample was diluted 1 in 10 and 1 in 25 using

human plasma. Each dilution was assayed in quadruplicate as shown in Table 2.21, Table 2.22, Table 2.23, and Table 2.24.

Table 2.21 The dilution accuracy of diazepam concentration samples.

Data	QC4 Diluted 1:10	QC4 Diluted 1:25
Expected Conc.	2500.00	2500.00
Mean (n=4)	2489.61	2617.26
Standard Dev.	75.98	43.25
%CV	3.05	1.65
Accuracy	99.58	104.69

Table 2.22 The dilution accuracy of desmethyldiazepam concentration samples.

Data	QC4 Diluted 1:10	QC4 Diluted 1:25
Expected Conc.	2500.00	2500.00
Mean (n=4)	2492.99	2467.73
Standard Dev.	36.47	44.65
%CV	1.46	1.81
Accuracy	99.72	98.71

Table 2.23 The dilution accuracy of temazepam concentration samples.

Data	QC4 Diluted 1:10	QC4 Diluted 1:25
Expected Conc.	2500.00	2500.00
Mean (n=4)	2549.79	2485.79
Standard Dev.	50.91	46.95
%CV	1.99	1.89
Accuracy	101.99	99.43

Table 2.24 The dilution accuracy of oxazepam concentration samples.

Data	QC4 Diluted 1:10	QC4 Diluted 1:25
Expected Conc.	2500.00	2500.00
Mean (n=4)	2495.72	2532.12
Standard Dev.	20.64	13.36
%CV	0.83	0.53
Accuracy	99.83	101.28

2.11.16.9 Stability

In order to assess the stability of the analytes in human plasma the three quality control samples were stored either at room temperature (temperature range 19.5ºC to 22.5ºC) for approximately 24 hours or at approximately 4ºC (temperature range 4.0ºC to 6.6ºC) for approximately 24 hours. These samples were then extracted as per the method on page 88 , and Flow Chart 2.1.

Table 2.25 Stability of diazepam in human plasma at room and 4 ºC temperature over 24 hours.

Stability data	Data	QC1	QC2	QC3
Expected Conc. (µg/L)		20.00	200.00	750.00
Time 0	Mean (n=6)	20.12	198.67	787.01
Room temperature	Mean (n=4)	18.42	203.98	759.06
4°C	Mean (n=4)	17.85	198.59	761.48

Table 2.26 Stability of desmethyldiazepam in human plasma at room and 4 ºC temperature over 24 hours.

Stability data	Data	QC1	QC2	QC3
Expected Conc. (µg/L)		20.00	200.00	750.00
Time 0	Mean (n=6)	20.46	194.87	734.88
Room temperature	Mean (n=4)	19.83	206.76	741.16
4°C	Mean (n=4)	20.98	205.69	760.79

Table 2.27 Stability of temazepam in human plasma at room and 4 ºC temperature over 24 hours.

Stability data	Data	QC1	QC2	QC3
Expected Conc. (µg/L)		20.00	200.00	750.00
Time 0	Mean (n=6)	20.00	192.97	732.99
Room temperature	Mean (n=4)	18.51	198.66	702.68
4°C	Mean (n=4)	19.07	201.17	748.32

Table 2.28 Stability of oxazepam in human plasma at room and 4 ºC temperature over 24 hours.

Stability data	Data	QC1	QC2	QC3
Expected Conc. (µg/L)		20.00	200.00	750.00
Time 0	Mean (n=6)	19.72	194.91	731.49
Room temperature	Mean (n=4)	19.92	203.76	726.61
4°C	Mean (n=4)	20.29	198.38	750.40

2.11.16.10 Freeze / thaw stability

In order to assess the stability of the analytes in human plasma samples during three freeze/ thaw cycles aliquots of each of the three quality control samples were stored frozen. These samples were then removed from the freezer, allowed to defrost and then extracted as per the method on page 88, and Flow Chart 2.1. The control samples were then re-frozen. A total of three freeze / thaw cycles were performed. The maximum and minimum temperatures recorded were -14.3ºC and -26.5ºC respectively.

Table 2.29 Stability of diazepam in human plasma during three freeze/ thaw cycles.

	Data	QC1	QC2	QC3
	Expected Conc.	20.00	200.00	750.00
Time 0 stability data	Mean (n=6)	20.12	198.67	787.01
Freeze / thaw stability - Cycle 1	Mean (n=4)	18.98	201.37	774.95
Freeze / thaw stability - Cycle 2	Mean (n=4)	17.91	191.75	730.93
Freeze / thaw stability - Cycle 3	Mean (n=4)	18.29	195.131	759.25

Table 2.30 Stability of desmethyldiazepam in human plasma during three freeze/ thaw cycles.

	Data	QC1	QC2	QC3
	Expected Conc.	20.00	200.00	750.00
Time 0 stability data	Mean (n=6)	20.46	194.87	734.88
Freeze / thaw stability - Cycle 1	Mean (n=4)	20.69	210.70	762.91
Freeze / thaw stability - Cycle 2	Mean (n=4)	19.28	191.21	729.68
Freeze / thaw stability - Cycle 3	Mean (n=4)	18.76	193.99	740.42

Table 2.31 Stability of temazepam in human plasma during three freeze/ thaw cycles.

	Data	QC1	QC2	QC3
	Expected Conc.	20.00	200.00	750.00
Time 0 stability data	Mean (n=6)	20.00	192.97	732.99
Freeze / thaw stability - Cycle 1	Mean (n=4)	18.28	200.36	749.54
Freeze / thaw stability - Cycle 2	Mean (n=4)	19.61	194.03	744.87
Freeze / thaw stability - Cycle 3	Mean (n=4)	18.307	192.06	726.35

Table 2.32 Stability of oxazepam in human plasma during three freeze/ thaw cycles.

	Data	QC1	QC2	QC3
	Expected Conc.	20.00	200.00	750.00
Time 0 stability data	Mean (n=6)	19.72	194.91	731.49
Freeze / thaw stability - Cycle 1	Mean (n=4)	19.19	198.88	742.32
Freeze / thaw stability - Cycle 2	Mean (n=4)	19.51	188.26	741.91
Freeze / thaw stability - Cycle 3	Mean (n=4)	17.84	188.41	722.29

2.11.16.11 Matrix effects

Matrix effects on the measurement of diazepam and metabolites were tested at the same nominal concentration as the middle quality control sample (QC2). A matrix effect on the measurement of the internal standards was tested at a nominal concentration of 250µg/L.

A spiking solution containing diazepam, desmethyldiazepam, temazepam, oxazepam and the internal standards were prepared by mixing 1mL of the aqueous QC2 (recovery QC2), 0.5mL of the internal standard solution and 1mL of 25% methanol.

2.11.16.11.1 Extracted control sample

In order to assess the possibility of matrix effects (ion suppression) six different plasma samples were extracted without internal standard, as per the method. To each of the dried extract from the six samples 250µL of the spiking solution was added.

2.11.16.11.2 Non-extracted reference sample:

The reference solution was injected without further processing.

Table 2.33 Matrix effects on the measurement of diazepam and metabolites.

	Diazepam		Desmethyldiazepam		Temazepam		Oxazepam	
Data	100% QC2	Ext. QC2	100% QC2	Ext QC2	100% QC2	Ext QC2	100% QC2	Ext QC2
µg/L	200.0 (n=4)	200.0 (n=6)	200.0 (n=4)	200.0 (n=6)	200.0 (n=4)	200.0 (n=6)	200.0 (n=4)	200.0 (n=6)
Mean	200.0	205.7	200.0	197.5	200.0	196.1	200.0	191.9
SDev.	1.50	3.69	3.97	3.94	2.35	5.21	2.03	4.87
%CV	0.75	1.80	1.98	1.99	1.177	2.66	1.02	2.53
Accuracy	100.0	102.9	100.0	98.8	100.0	98.1	100.00	95.9

2.11.16.12 Validation result

The results from this validation study demonstrate the ability of this method to perform the assay for diazepam and metabolites reproducibly and accurately, and to establish suitable internal quality control systems for the assay. The assay was designed to provide the best sensitivity for the metabolites as these are expected to be present at lower concentration than the primary analyte (diazepam). The LLOQ for diazepam and the metabolites was 5µg/L with signal to ratio above 10. The separations were 2.66, 2.81, 3.17, and 3.38 minutes for oxazepam, temazepam, desmethyldiazepam, and diazepam, respectively. Under these conditions, the recovery of all three metabolites was greater than 70%, but the recovery for diazepam was only approximately 35%. This assay was still found to possess both the sensitivity and specificity necessary to measure the analyte in human plasma samples at the concentrations attained during clinical therapeutic use of the drug.

2.11.17 Data analysis

Pharmacokinetic parameters and the statistical analysis were performed using Microsoft Excel 2007. C_{max}, t_{max}, t_{lag} were determined directly from the individual plasma concentration time points. The AUC was calculated using the linear trapezoidal method.

The difference between treatments for $AUC_{0\text{-}72h}$ and C_{max} were analyzed after logarithmic transformation using analysis of variance (ANOVA) for cross over studies. Additionally to 90% confidence intervals (CI), the data were summarized by medians, maximum, and minimum values.

The secondary pharmacokinetic variables included: an estimation of $t_{1/2}$ was made; bioavailability of TDS® diazepam relative to rectal diazepam (A:B) was calculated (90% CI for the ratio of population Least Squares geometric means [TDS/rectal] for key pharmacokinetic variables), also for B:A. The elimination half-life of diazepam from the transdermal and rectal routes was compared using non transformed data and parametric techniques.

Bioequivalence testing was based upon the 90% CI for the ratio of population mean between two treatments. This is equivalent to the corresponding two one-sided test procedures, with the null hypothesis of bioequivalence at the 5% significance level (Schuirmann, 1987). Formulations were considered bioequivalence if the 90% CI of the ratio, test to reference (A:B), was contained within 80 to 125% (Nation and Sansom, 1994, FDA, 2001b, EMEA, 2001).

2.12 Results

2.12.1 Analytical result

The results from this study demonstrate the ability of the HPLC-UV method to separate diazepam and the internal standard prazepam reproducibly and accurately at retention time of 5.01, and 8.60 minutes, respectively. The LLOQ was set at 10 µg/mL as the lower calibration criteria. Figure 2.2 illustrate chromatogram obtained from extracted rectal gel containing 10mg/mL diazepam per 2 g gel.

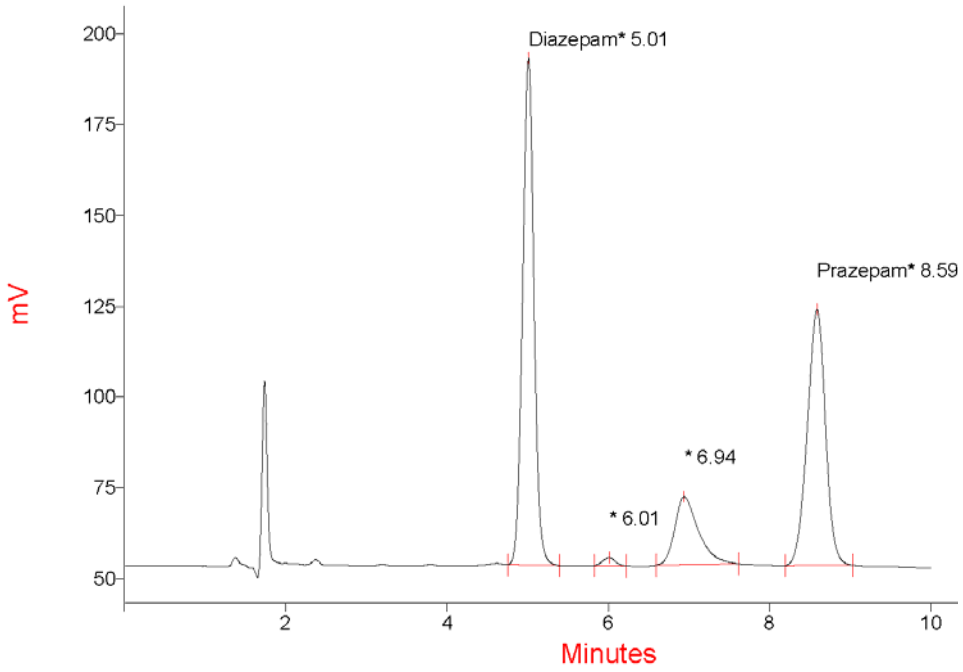


Figure 2.2 Chromatograph obtained from extracted rectal gel (diazepam 50 µg/mL) spiked with IS of 25 µg/mL prazepam, displayed at 245 nm.

2.12.2 Stability test of rectal diazepam (Diastat®)

The HPLC results from this study demonstrated that the diazepam in the solutions supplied was stable at temperatures up to 35ºC for at least 33 days (Figure 2.3, Figure 2.4), Table 2.34 show the temperature stability test data.

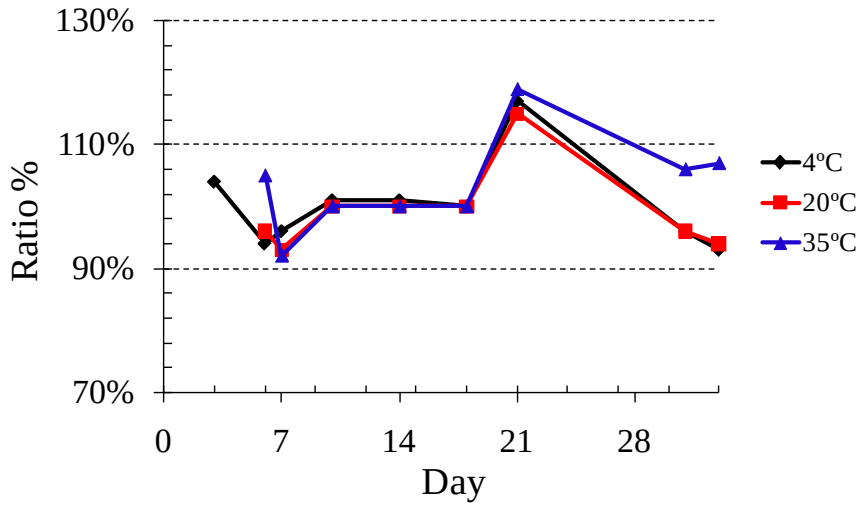


Figure 2.3 Stability test for diazepam solution (1) at temperatures of 4, 20, and 35 ºC over a period of 33 days.

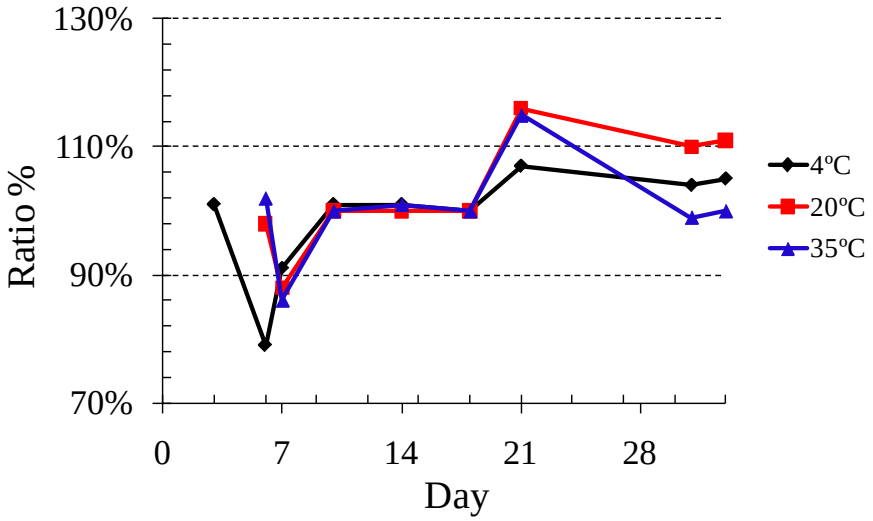


Figure 2.4 Stability test for diazepam solution (2) at temperatures of 4, 20, and 35 ºC over a period of 33 days.

Table 2.34 The temperature stability test data for diazepam.

Temperature	Mean Temp	Minimum Temp	Maximum Temp
4ºC	3.5ºC	2.0ºC	9.5ºC
20ºC	19.5ºC	18.0ºC	22.0ºC
35ºC	34.5ºC	29.5ºC	36.0ºC

The results from this study also demonstrated the ability of the Analytical Unit to perform the assay for diazepam reproducibly and accurately, and to establish suitable internal quality control systems for the assay.

2.12.3 Quality control of TDS® diazepam

The HPLC results from this study demonstrated that the diazepam in the solutions supplied is within the acceptable range (90–110%) and had passed QC.

2.12.4 Dose calculation

2.12.4.1 Rectal diazepam

Statistical analysis and calculation of the mean, standard deviation (SD), coefficient of variation percentage (CV%), and standard normal distribution (SND, or z) of the data were analyzed (Table 2.35 and Table 2.36), and showed that:

The minimum and maximum doses of diazepam were 9.48 and 10.04 mg, respectively, with an average dose of 9.84 mg, after an application of rectal gel of diazepam to the healthy volunteer, the CV% was 2%. The doses came from a minimum and maximum of 1.97 and 2.17 g of gel, respectively, with an average of 2.13 g gel, CV% was 3%.

Table 2.35 Ten samples showing gel weight containing 10 mg diazepam (see Table 2.36 for the average concentration).

No of Sample	Flask (g)	Flask+ Gel (g)	Gel (g)	z [a] (SND)
B01	63.32	65.46	2.15	0.28
B02	49.05	51.21	2.17	0.60
B03	61.21	63.31	2.09	-0.61
B04	64.10	66.26	2.16	0.43
B05	63.83	65.98	2.15	0.42
B06	56.14	58.29	2.15	0.32
B07	62.61	64.76	2.15	0.33
B08	61.52	63.48	1.97	-2.68
B09	54.64	56.79	2.15	0.38
B10	55.06	57.22	2.16	0.53
Mean			2.13	
SD [b]			0.061	
CV% [c]			3%	
Median			2.15	
Q1 [d]			2.15	
Q3 [e]			2.16	

z [a] Standard Normal Distribution (SND), SD [b]: Standard Deviation, CV% [c]: Coefficient of Variation Percentage, Q1 [d]: First Quartile, Q3 [e]: Third Quartile

Table 2.36 Ten samples showing the average concentration (mg/L) of diazepam in about 2 g gel.

NO Sample	Conc[a] (mg/L) (1)	Conc[a] (mg/L) (2)	Time (minutes)	AvergConc[b] (mg/L)	z[c] (SND)	Dose (mg)
1	98.0	96.8	10	97.4	-0.65	9.74
2	98.1	98.9	20	98.5	0.041	9.85
3	98.0	91.6	30	94.8	-2.30	9.48
4	99.5	97.7	40	98.6	0.104	9.86
5	98.5	102.0	50	100.25	1.147	10.03
6	98.2	98.4	60	98.3	-0.085	9.83
7	101.1	99.6	70	100.35	1.21	10.04
8	98.0	98.1	80	98.05	-0.24	9.81
9	100.3	98.6	90	99.45	0.64	9.95
10	98.1	99.2	100	98.65	0.14	9.87
Mean				98.44		
SD[d]				1.58		
CV%[e]				2%		
Median				98.55		
Q1[f]				98.11		
Q3[g]				99.25		

Conc[a]: Concentration, AvergConc[b]: Average Concentration, z[c]: Standard Normal Distribution (SND), SD [d]: Standard Deviation, CV% [e]: Coefficient of Variation Percentage, Q1 [f]: First Quartile, Q3 [g]: Third Quartile.

2.12.4.2 TDS® diazepam

Summary of analysis data of the mean, standard deviation (SD), and coefficient of variation percentage (CV%) are shown in Table 2.38:

The result shows that minimum and maximum doses of diazepam were 10.12 and 10.22 mg/mL, respectively, with an average dose of 10.17 mg/mL, after an application of diazepam (TDS®) drug, by applying 5 sprays to the skin.

Table 2.37 Working calibration concentration of diazepam.

No.#	Sample	Peak area diazepam	Peak area ratio	Results (µg/mL)
1	100µg/mL Calibrator	2202.13	0.62	101.11
2	80µg/mL Calibrator	1955.81	0.49	80.34
3	60µg/mL Calibrator	1441.37	0.36	59.81
4	40µg/mL Calibrator	978.17	0.24	39.31
5	20µg/mL Calibrator	509.91	0.12	20.08
6	10µg/mL Calibrator	247.59	0.06	10.01

Table 2.38 Diazepam concentration in sample solution.

No.#	Sample	Peak area diazepam	Peak area ratio	Results (µg/mL)
1	Dilution 1	1185.99	0.310	10224.05
2	Dilution 2	1189.83	0.310	10219.28
3	Dilution 3	1177.97	0.307	10121.83
4	Dilution 4	1241.13	0.307	10125.87
			Mean	10172.76
			SD	56.53
			CV%	1%
			Median	10172.58
			Maximum	10224.05
			Minimum	10121.83

2.13 Plasma assay

2.13.1 Analyte mass transitions

Table 2.39 show diazepam, metabolites, and internal standard mass transitions.

Table 2.39 Analyte mass transitions, showing Q1 Mass and Q3 Mass.

Analyte name	Initial ion (m/z), Q1 Mass (amu)	Product ion (m/z) Q3 Mass (amu)	Ionisation mode (TIC)
Diazepam	285.1	193.3	Positive
Desmethyldiazepam	271.1	140.2	Positive
Temazepam	301.1	255.3	Positive
Oxazepam	287.1	241.2	Positive
Diazepam-d5	290.2	198.2	Positive
Desmethyldiazepam-d5	276.1	140.1	Positive
Temazepam-d5	306.2	260.3	Positive
Oxazepam-d5	292.1	246.3	Positive

2.13.2 Quality control data results

Table 2.40, Table 2.41, Table 2.42, and Table 2.43 show a summary of statistical analysis of the mean, standard deviation, and coefficient of variation percentage, from the QC results obtained during the study of diazepam, desmethyldiazepam, temazepam, and oxazepam.

Table 2.40 Three quality control (QC) measurements of diazepam.

Data	QC 1	QC 2	QC 3
Expected Conc (µg/L).	20.00	200.00	750.00
N	32 of 32	32 of 32	32 of 32
Mean (µg/L)	20.51	205.52	753.01
Low (µg/L)	18.46	182.04	688.79
High (µg/L)	22.80	227.18	818.83
SD	1.27	8.83	26.98
CV%	6.21	4.29	3.58
Accuracy	102.53	102.76	100.40

Table 2.41 Three quality control (QC) measurements of desmethyldiazepam.

Data	QC 1	QC 2	QC 3
Expected Conc (µg/L).	20.00	200.00	750.00
N	32 of 32	32 of 32	32 of 32
Mean (µg/L)	20.78	203.69	742.40
Low (µg/L)	17.71	189.66	704.72
High (µg/L)	22.62	225.72	832.38
SD	1.21	8.82	26.94
CV%	5.82	4.33	3.63
Accuracy	103.91	101.84	98.99

Table 2.42 Three quality control (QC) measurements of temazepam.

Data	QC 1	QC 2	QC 3
Expected Conc (µg/L).	20.00	200.00	750.00
N	31 of 32	32 of 32	32 of 32
Mean (µg/L)	21.17	212.89	765.23
Low (µg/L)	18.72	194.90	700.88
High (µg/L)	22.81	227.82	840.33
SD	1.24	9.23	31.53
CV%	5.85	4.34	4.12
Accuracy	105.84	106.44	102.03

Table 2.43 Three quality control (QC) measurements of oxazepam.

Data	QC 1	QC 2	QC 3
Expected Conc (µg/L).	20.00	200.00	750.00
N	32 of 32	32 of 32	32 of 32
Mean (µg/L)	20.69	205.48	753.62
Low (µg/L)	18.29	185.29	685.36
High (µg/L)	22.82	224.47	809.22
SD	1.22	9.35	29.84
CV%	5.90	4.55	3.96
Accuracy	103.43	102.74	100.48

2.14 Overall study results

Twelve healthy males and females (Table 2.44) out of thirteen recruited subjects completed the protocol in this study. Subject number S01 who did not meet the inclusion and exclusion criteria was not included. No side effects/events were observed in any subjects in this study. Skin digital photographs of subjects have been taken, prior to, 30 minutes after, and one week after the diazepam dose. In Figure 2.5 (A: pre- dose, B: 30 minutes post-dose, and C: one week post-dose) the images show that there was no sign of irritation, itching, inflammation, swelling, or other dermatological problems. The diagnoses were assessed by a physician for all subjects. All of the plasma samples were analyzed for diazepam and its metabolites (desmethyldiazepam, temazepam, and oxazepam) by high performance liquid chromatography assay, with mass spectrometric detection, (HPLC/MS). The assay was capable of detecting diazepam and desmethyldiazepam at concentrations above 5 µg/L, but not for temazepam and oxazepam.

Table 2.44 Demographic data for 12 subjects in the rectal and TDS® diazepam study.

Subject	Age (years)	Sex	Height (m)	Weight (kg)	Body Mass Index (kg/m^2)
S02	41	M	1.81	85	26
S03	25	M	1.81	114	35
S04	25	F	1.77	67	21
S05	19	F	1.68	58	21
S06	22	F	1.76	62	20
S07	24	F	1.58	50	20
S08	33	F	1.70	67	23
S09	22	F	1.63	55	21
S10	32	F	1.55	58	24
S11	25	F	1.74	78	26
S12	25	F	1.66	78	28
S13	47	M	1.69	68	24

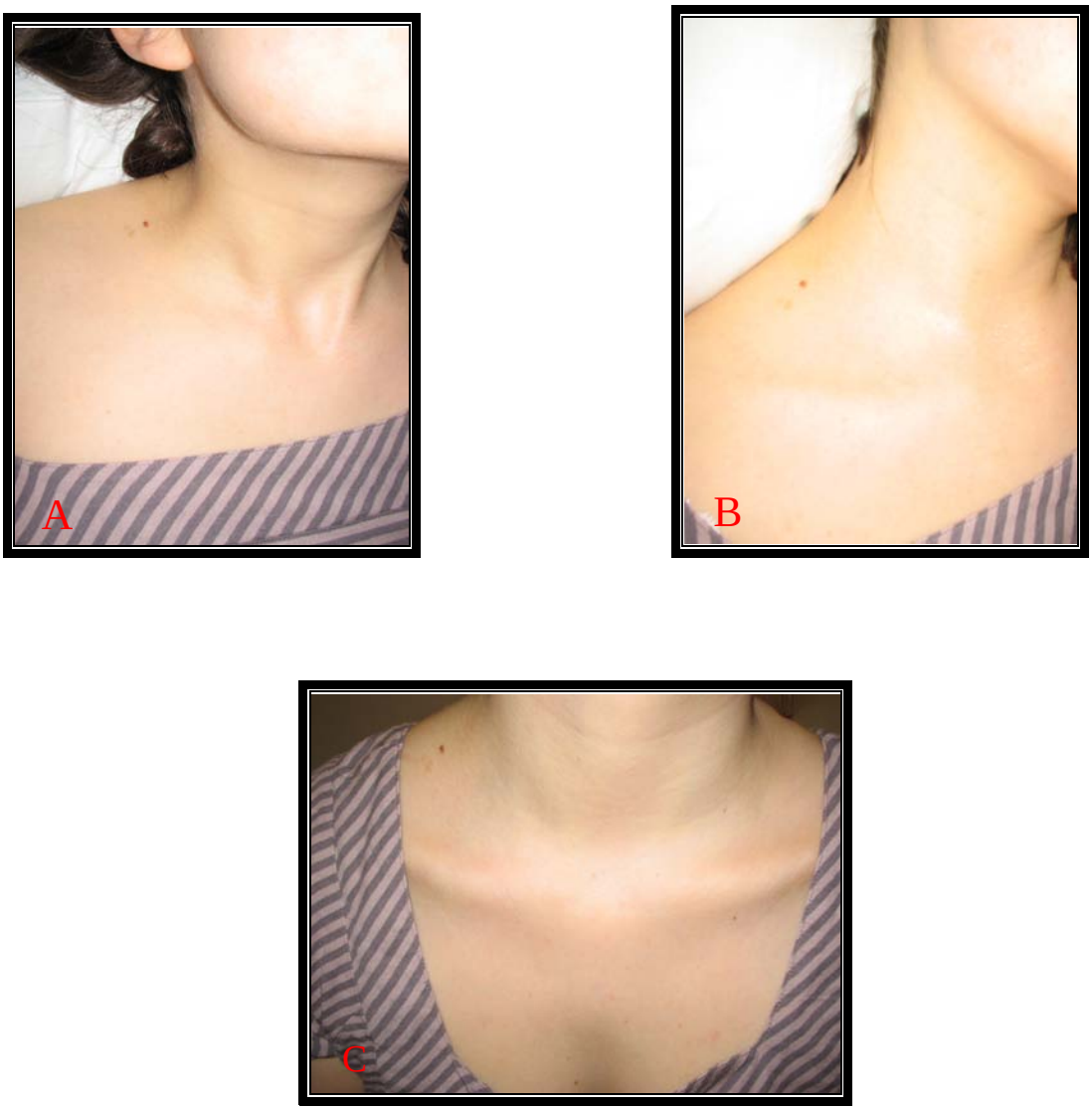


Figure 2.5 Application of TDS® diazepam on the chest shows no marks in A (pre-dose), B (30 minutes post-dose), and C (one week post-dose) at the site area.

Figure 2.6 and Figure 2.7 show the plot of the mean plasma concentration in 12 subjects, linear and logarithmic axis respectively, for diazepam after both treatments. Figure 2.8, Figure 2.9, Figure 2.10, Figure 2.11 show the plot of 12 subjects with the mean line for rectal and TDS® diazepam, respectively. In Figure 2.11, diazepam was detected after 24 hours; this may be due to diazepam metabolism. The comparison of plasma concentration of diazepam (µg/L) versus time (h), shows that diazepam concentration in plasma was higher after rectal diazepam (Diastat®) compared to TDS® diazepam.

In addition, Figure 2.12 and Figure 2.13 show the plots of the mean plasma concentration in 12 subjects, linear and logarithmic axis respectively, for desmethyldiazepam, after both treatments. Figure 2.14 and Figure 2.15, show the plots of desmethyldiazepam linear and logarithmic axis, respectively, from 12 subjects, with the mean value, following rectal 10 mg doses of diazepam. Figure 2.16 and Figure 2.17 show the plots of desmethyldiazepam linear and logarithmic axis, respectively, in 12 subjects, with the mean value, following TDS® 10 mg doses of diazepam. Figure 2.18 and Figure 2.19 show the DotPlots of the plasma C_{max} concentration for diazepam and desmethyldiazepam, respectively.

Furthermore, the pharmacokinetic parameters of diazepam and desmethyldiazepam, AUC, $C_{max,}$ and t_{max} are summarized in Table 2.47 and Table 2.48. The AUC, C_{max}, and t_{max} values were calculated for 0–72 hours. The geometric mean, mean, the 25th quartile, median, 75th quartile of the $AUC_{0\text{-}72h}$, and C_{max} were higher following application of rectal diazepam (Diastat®). The t_{lag} was 0.25 h for rectal diazepam, and less than 0.75 h in the TDS® 10 mg dose of diazepam.

From these results, the 90% CI on the relative difference of diazepam ratio (Table 2.45) for the $AUC_{0\text{-}72h}$ (up to 43.30%) and the C_{max} (0–72h) (up to 22.40%) between TDS® diazepam (A) and rectal diazepam (Diastat®) (B) were not contained within the bioequivalence limit (80–125%), C_{max} (0–72h): 7.3–10.1–14% and $AUC_{0\text{-}72h}$: 19.7–27.2–37.6% (Figure 2.20).

In addition the CI values obtained for the desmethyldiazeapam ratio are summarized in Figure 2.21 show that the $AUC_{0\text{-}72h}$ (up to 81.10%) and the C_{max}(0–72h) (up to 79.9%) between TDS® diazepam (A) and rectal diazepam (Diastat®) (B) were not contained within the bioequivalence limit (80–125%), C_{max} (0–72h): 37.6%–45%–53.8% and $AUC_{0\text{-}72h}$: 33.4%–44.0%–57.8% (Figure 2.21). In subject number nine (S09), C_{max} and AUC ratio were 79.9% and 79.2%, respectively, and in subject number four (S04) C_{max} and AUC ratio were 65.9% and 81.1%, respectively.

Table 2.40 summarized the QC sample result obtained during the analysis of diazepam by HPLC/MS. From the concentration obtained, mean, low, high standard deviation,

coefficients of variation (CV%), and accuracy, the CV for imprecision and inaccuracy were all below 15%.

Table 2.45 Bioequivalence parameters for TDS® diazepam (test formulation, A) versus rectal diazepam (reference formulation, B).

	-90% CI	Point Estimate %	+90% CI
t_{max}	1.63	2.13	6.75
C_{max} (A:B)	7.3	10.1	14.0
C_{max} (B:A)	715.8	990.1	1367.7
$AUC_{0\text{-}72h}$ (A:B)	19.7	27.2	37.6
$AUC_{0\text{-}72h}$ (B:A)	266.1	367.6	507.8

Table 2.46 Bioequivalence parameters for desmethtyldiazepam, TDS® diazepam (test formulation, A) versus rectal diazepam (reference formulation, B).

	-90% CI	Point Estimate %	+90% CI
t_{max}	-20	-4	12
C_{max} (A:B)	37.6	45.0	53.8
C_{max} (B:A)	186.0	222.4	266.0
$AUC_{0\text{-}72h}$ (A:B)	33.4	44.0	57.8
$AUC_{0\text{-}72h}$ (B:A)	172.9	227.5	299.4

Table 2.47 Derived diazepam pharmacokinetic parameters for rectal and TDS® diazepam (10mg).

Subject	Rectal $t_{½}$ (h)	Rectal t_{max} (h)	TDS t_{max} (h)	TDS - Rectal Difference (h)	Rectal C_{max} (µg/L)	TDS C_{max} (µg/L)	TDS/Rectal Ratio	Rectal AUC_{0-72h} (µg/L.h)	TDS AUC_{0-72h} (µg/L.h)	TDS/Rectal Ratio
S02	73.2	0.50	3.00	2.50	308.7	37.0	12.0%	4401.5	1218.6	27.7%
S03	53.3	1.00	4.00	3.00	375.4	21.0	5.6%	4318.8	1103.0	25.5%
S04	57.4	0.75	2.00	1.25	286.8	33.8	11.8%	5321.4	1695.1	31.9%
S05	147.0	0.50			309.7			2737.2		
S06	53.6	0.50	12.00	11.50	198.7	27.6	13.9%	4403.5	1608.8	36.5%
S07	32.4	0.50	1.50	1.00	349.2	26.5	7.6%	5503.8	495.5	9.0%
S08	63.3	0.50	24.00	23.50	254.3	7.3	2.9%	4500.3	380.0	8.4%
S09	50.5	1.00	3.00	2.00	343.3	63.4	18.5%	4253.8	1842.0	43.3%
S10	396.0	0.50	2.50	2.00	359.2	23.6	6.6%	4083.8	1238.1	30.3%
S11	22.3	0.75	2.00	1.25	138.3	17.0	12.3%	1518.9	651.1	42.9%
S12	39.4	0.75	2.50	1.75	297.8	66.8	22.4%	3900.1	1584.4	40.6%
S13	28.9	1.00	3.00	2.00	378.7	53.1	14.0%	4358.8	1837.9	42.2%
Geomean	59.2				289.7	29.1	10.1%	3929.3	1104.5	27.2%
Mean	84.8				300.0	34.3	11.6%	4108.5	1241.3	30.8%
SD	103.2				72.9	19.2	5.7%	1066.1	533.1	12.5%
CV	121.7%				24.3%	56.0%	49.6%	25.9%	42.9%	40.8%
Minimum	22.3	0.50	1.50	1.00	138.3	7.3	2.9%	1518.9	380.0	8.4%
25th	37.7	0.50	2.25	1.50	278.7	22.3	7.1%	4037.9	877.1	26.6%
Median	53.4	0.63	3.00	2.00	309.2	27.6	12.0%	4338.8	1238.1	31.9%
75th	65.8	0.81	3.50	2.75	351.7	45.1	14.0%	4427.7	1652.0	41.4%
Maximum	396.0	1.00	24.00	23.50	378.7	66.8	22.4%	5503.8	1842.0	43.3%

Table 2.48 Derived desmethyldiazepam pharmacokinetic parameters for rectal and TDS® diazepam (10mg).

Subject	Rectal t_{max} (h)	TDS t_{max} (h)	TDS - Rectal Difference (h)	Rectal C_{max} (µg/L)	TDS C_{max} (µg/L)	TDS/Rectal Ratio	Rectal AUC_{0-72} (µg/L.h)	TDS AUC_{0-72} (µg/L.h)	TDS/Rectal Ratio
S02	72	72	0.00	27.6	15.0	54.3%	1440.6	899.3	62.4%
S03	72	72	0.00	22.9	13.5	59.0%	1302.6	806.9	61.9%
S04	72	72	0.00	38.7	25.5	65.9%	1932.4	1567.0	81.1%
S05	24	32	8.00	23.7	6.8	28.7%	754.8	132.6	17.6%
S06	72	72	0.00	41.1	19.7	47.9%	1848.6	1031.3	55.8%
S07	48	24	-24.00	57.6	15.2	26.4%	3537.8	578.7	16.4%
S08	72	1.5	-70.50	41.0	11.6	28.3%	2194.8	726.8	33.1%
S09	72	32	-40.00	31.8	25.4	79.9%	1875.2	1485.9	79.2%
S10	72	32	-40.00	37.6	15.6	41.5%	1686.5	1002.2	59.4%
S11	24	48	24.00	20.7	8.7	42.0%	1275.8	429.8	33.7%
S12	48	72	24.00	36.4	17.7	48.6%	2103.5	992.6	47.2%
S13	24	48	24.00	32.5	15.1	46.5%	1939.7	810.5	41.8%
Geomean				33.0	14.8	45.0%	1714.9	753.8	44%
Mean				34.3	15.8	47.4%	1824.4	872.0	49%
SD				10.2	5.7	16.0%	678.4	401.7	21%
CV				29.8%	36.2%	33.7%	37.2%	46.1%	43.6%
Minimum	24.00	1.50	-70.50	20.7	6.8	26.4%	754.8	132.6	16.8%
25th	42.00	32.00	-28.00	26.6	13.0	38.3%	1406.1	689.7	33.5%
Median	72.00	48.00	0.00	34.5	15.2	47.2%	1861.9	854.9	51.5%
75th	72.00	72.00	12.00	39.3	18.2	55.5%	1980.7	1009.5	62.1%
Maximum	72.00	72.00	24.00	57.6	25.5	79.9%	3537.8	1567.0	81.1%

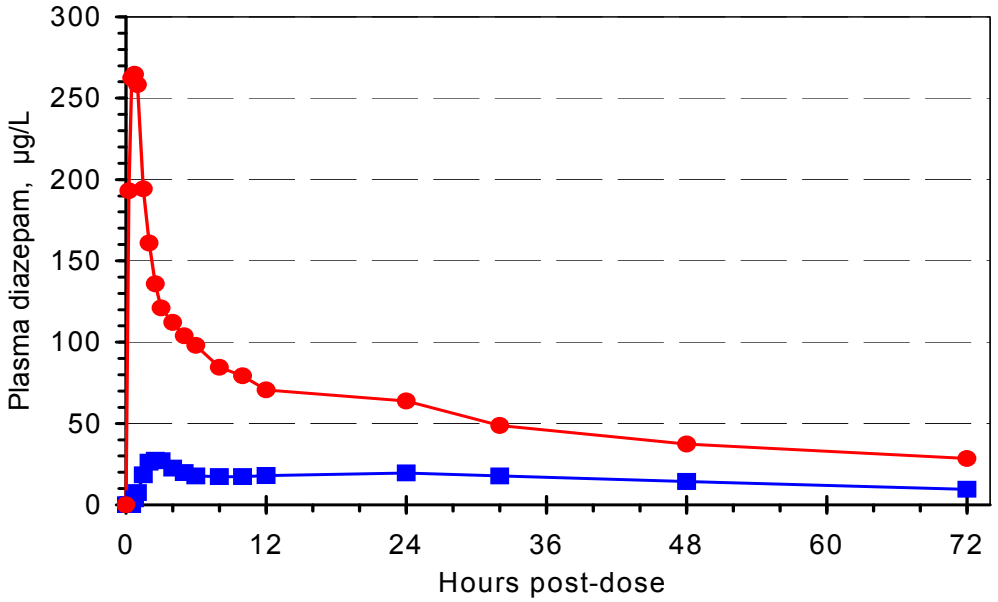


Figure 2.6 Mean plasma diazepam concentration versus time in 12 subjects following a 10 mg dose rectally (filled red circles) and dermally by TDS® diazepam (filled blue squares), linear concentration axis.

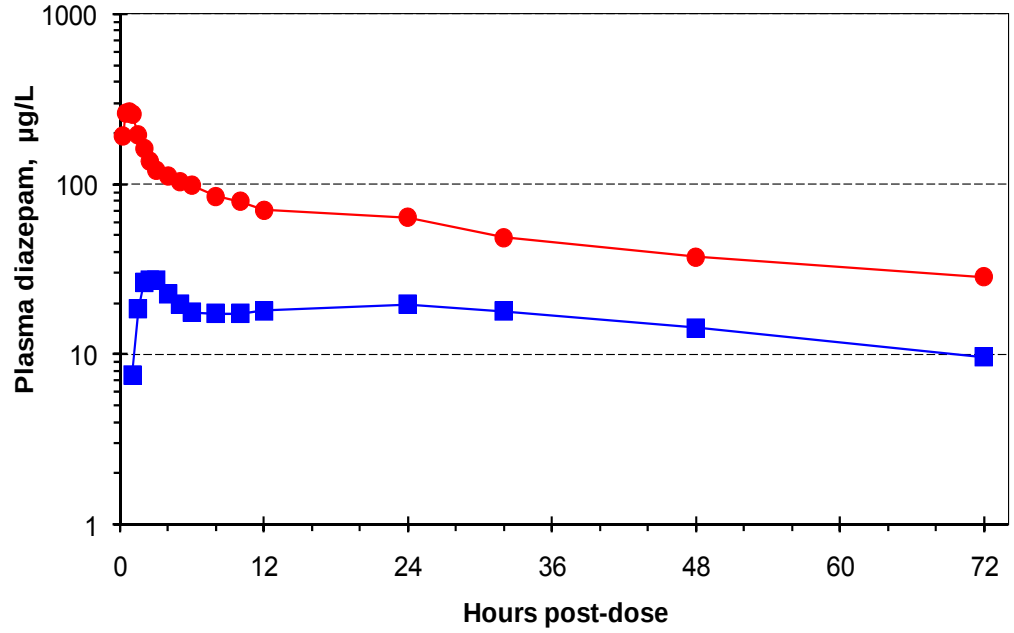


Figure 2.7 Mean plasma diazepam concentration versus time in 12 subjects following a 10 mg dose rectally (filled red circles) and dermally by TDS® diazepam (filled blue squares), logarithmic concentration axis.

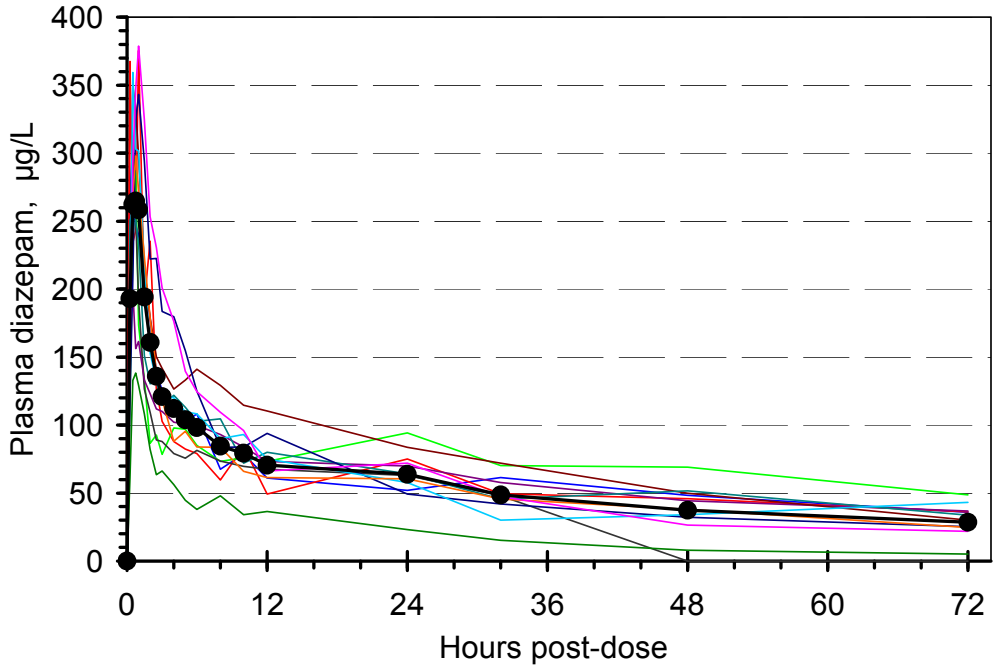


Figure 2.8 Plasma diazepam concentration versus time in 12 subjects following a 10 mg dose rectally. Mean values are represented by a heavy line with filled circles, linear concentration axis.

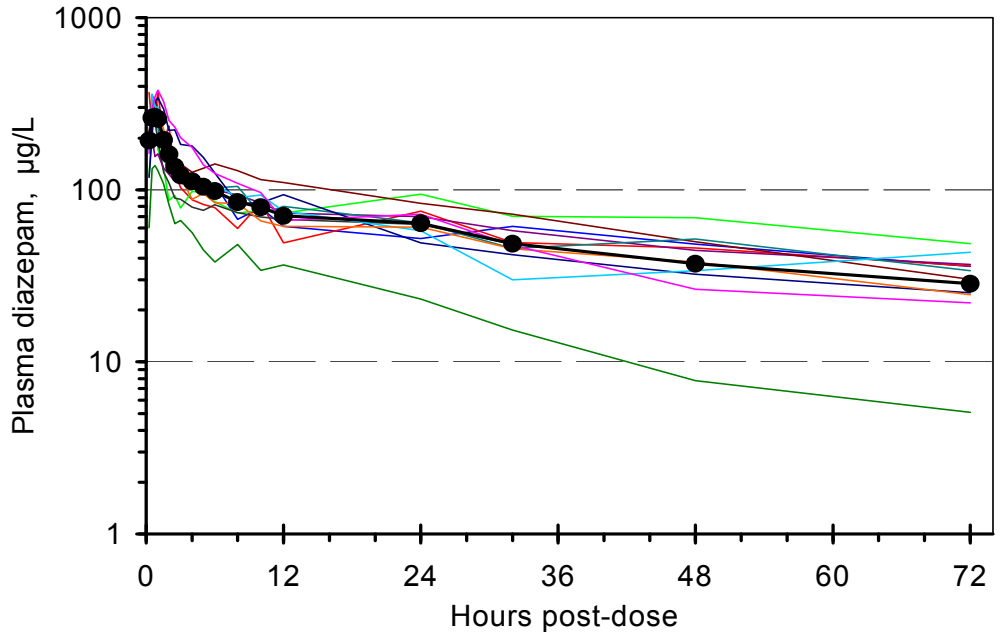


Figure 2.9 Plasma diazepam concentration versus time in 12 subjects following a 10 mg dose rectally. Mean values are represented by a heavy line with filled circles, logarithmic concentration axis.

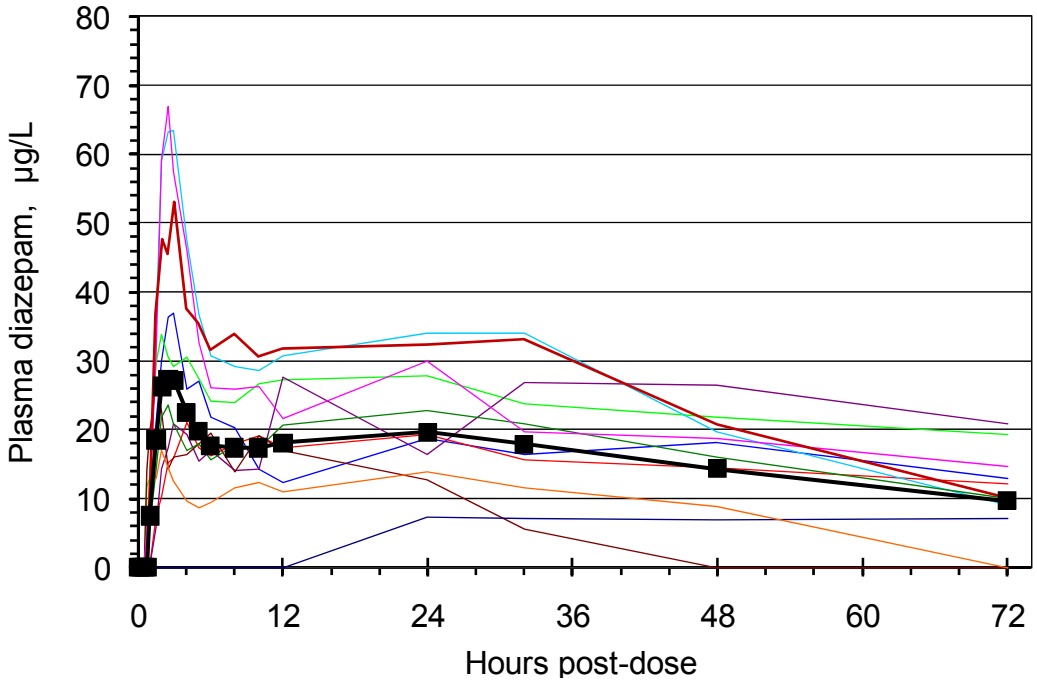


Figure 2.10 Plasma diazepam concentration versus time in 12 subjects following a 10 mg dose of TDS® diazepam. Mean values are represented by a heavy line with filled squares, linear concentration axis.

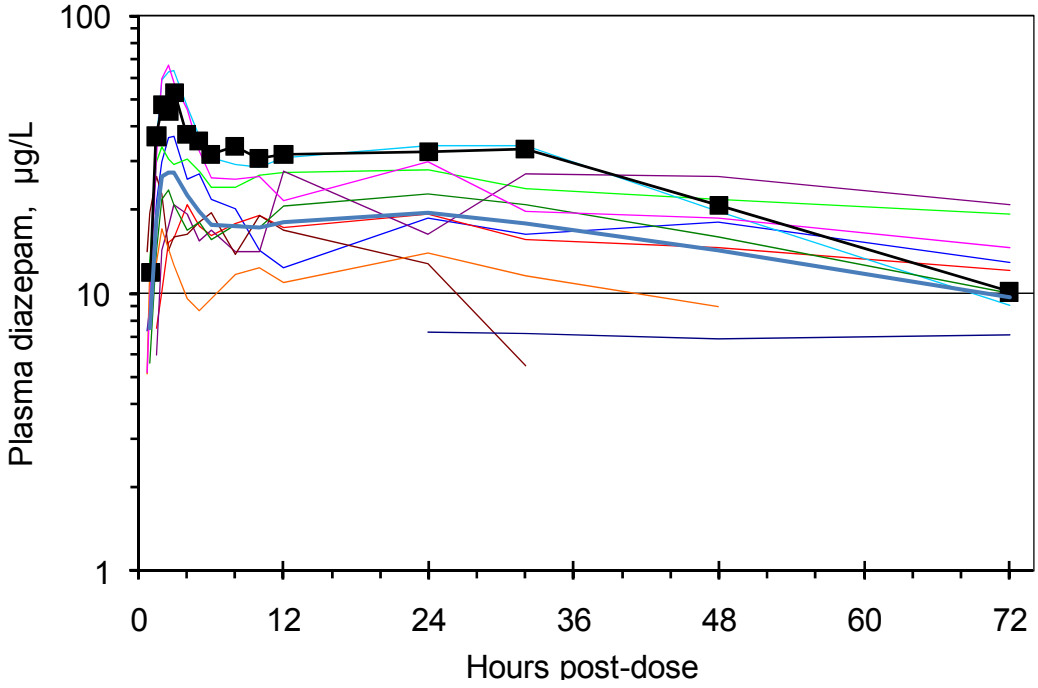


Figure 2.11 Plasma diazepam concentration versus time in 12 subjects following a 10 mg dose of TDS® diazepam. Mean values are represented by a heavy line with filled squares, logarithmic concentration axis.

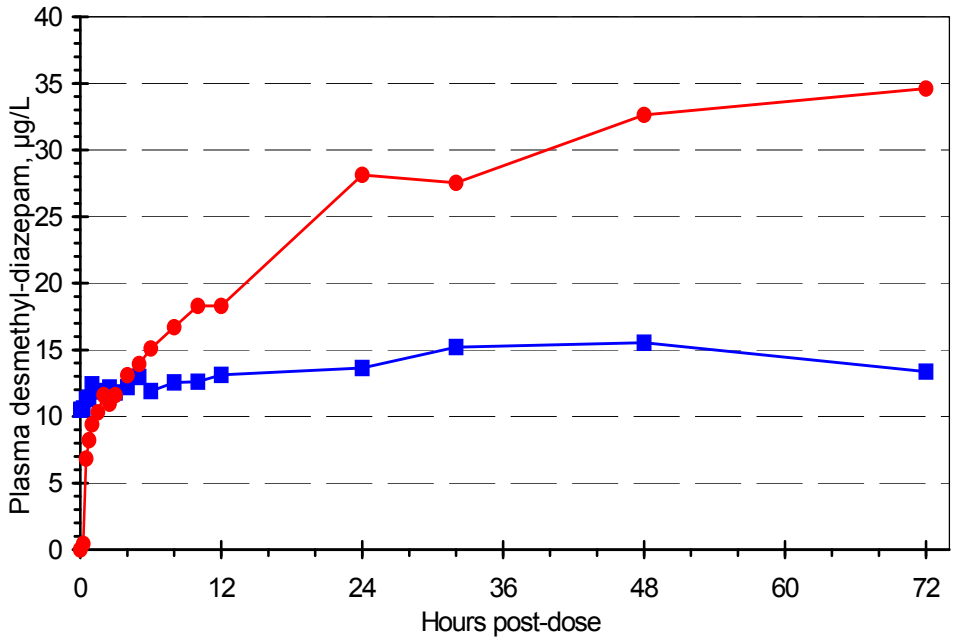


Figure 2.12 Mean plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose rectally (filled red circles) and dermally by TDS® diazepam (filled blue squares), linear concentration axis.

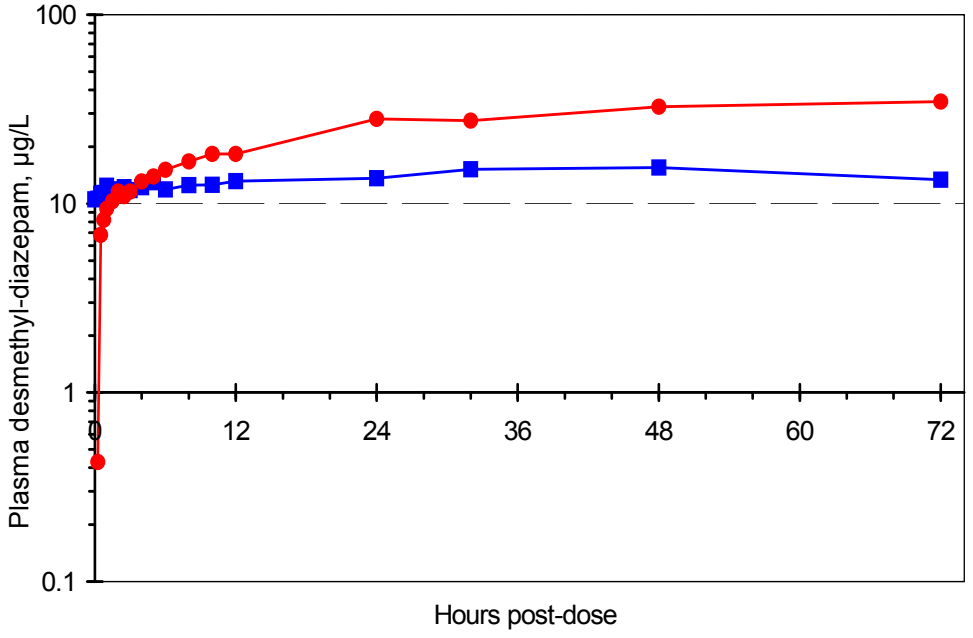


Figure 2.13 Mean plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose rectally (filled red circles) and dermally by TDS® diazepam (filled blue squares), logarithmic concentration axis.

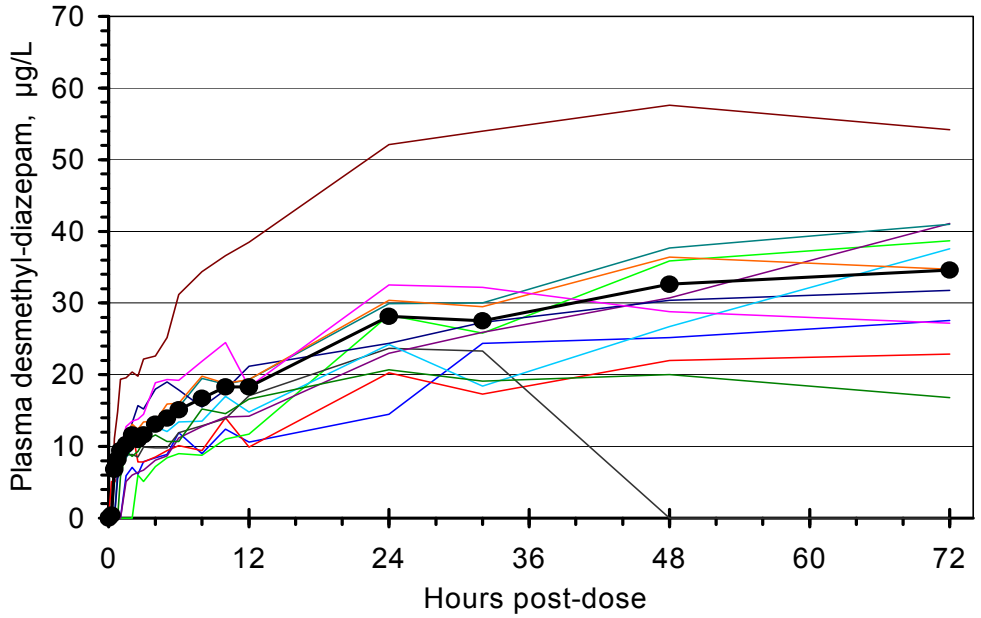


Figure 2.14 Plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose rectally. Mean values are represented by a heavy line with filled circles, linear concentration axis.

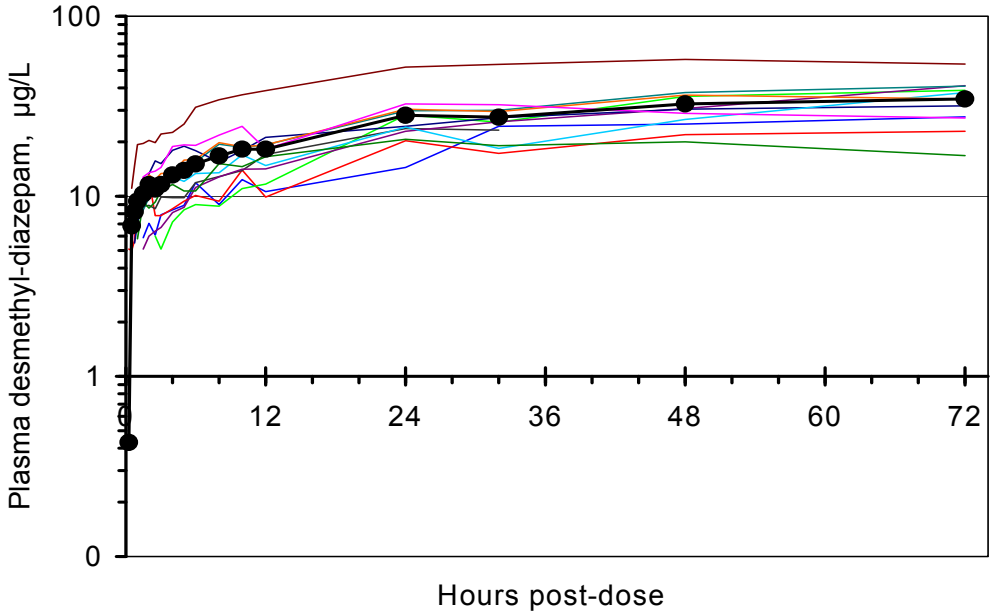


Figure 2.15 Plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose rectally. Mean values are represented by a heavy line with filled circles, logarithmic concentration axis.

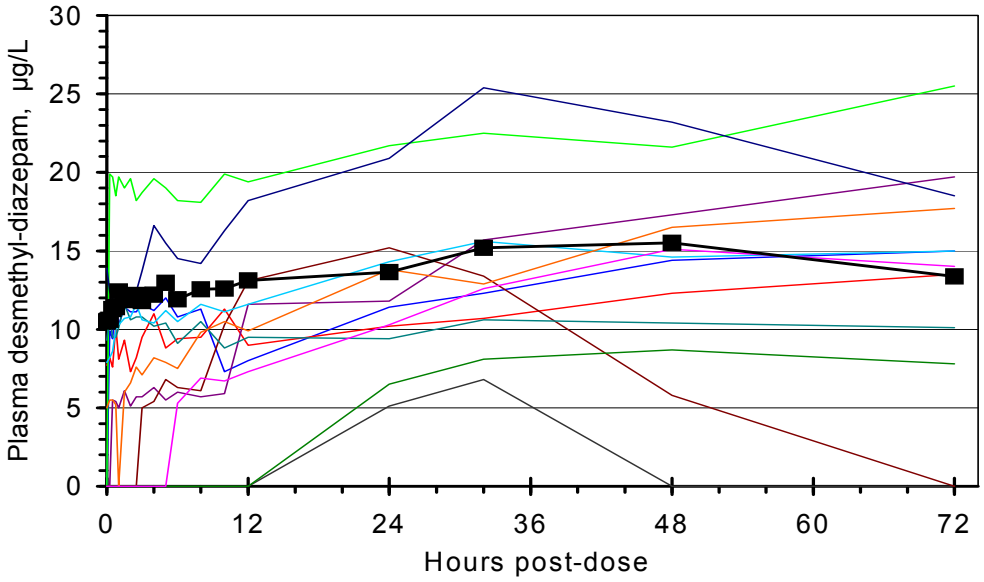


Figure 2.16 Plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose of TDS® diazepam. Mean values are represented by a heavy line with filled circles, linear concentration axis.

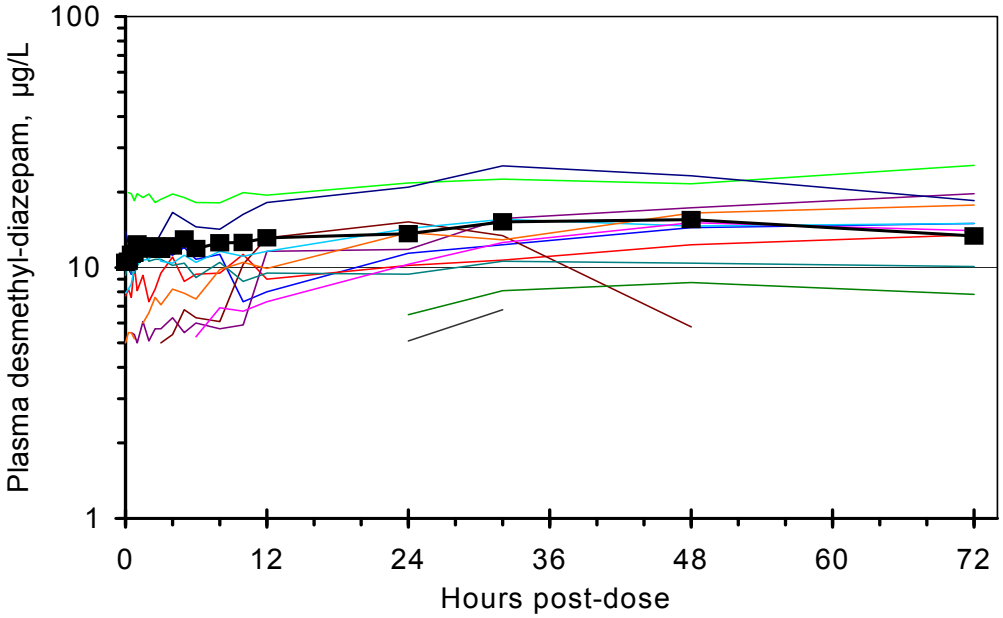


Figure 2.17 Plasma desmethyldiazepam concentration versus time in 12 subjects following a 10 mg dose of TDS® diazepam. Mean values are represented by a heavy line with filled circles, logarithmic concentration axis.

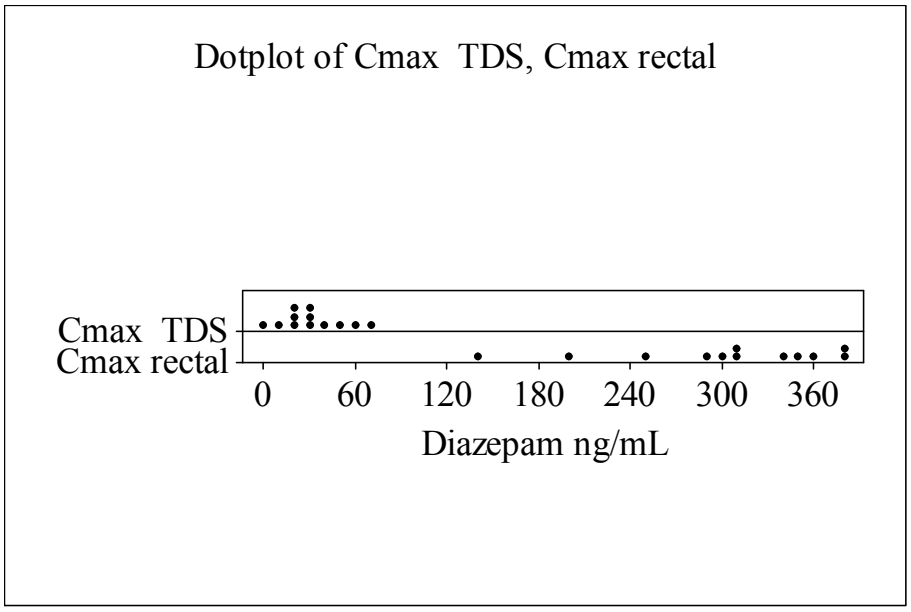


Figure 2.18 Plasma diazepam C_{max} concentrations following rectal dose and TDS® dose in 12 subjects, Dotplot.

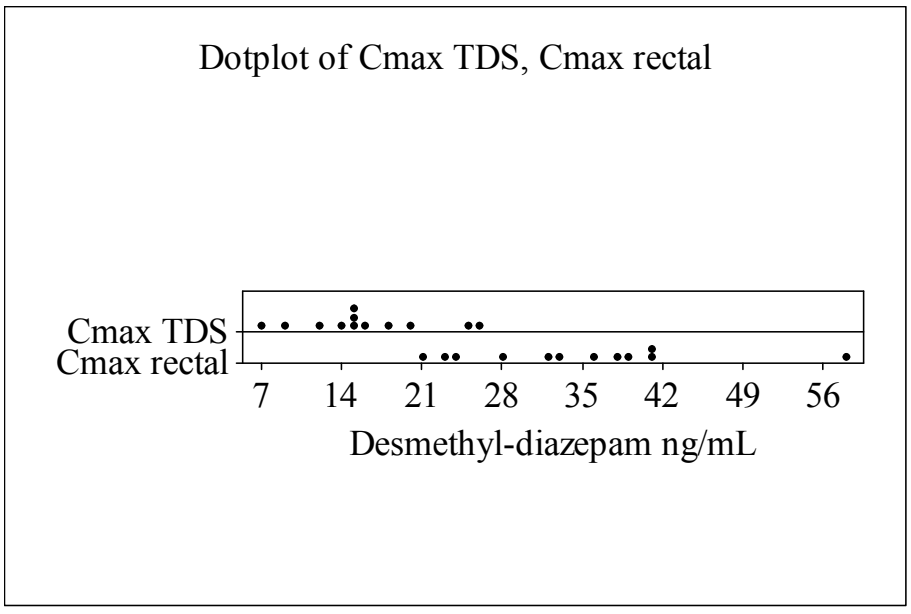


Figure 2.19 Plasma desmethyldiazepam C_{max} concentrations following rectal dose and TDS® dose in 12 subjects, Dotplot.

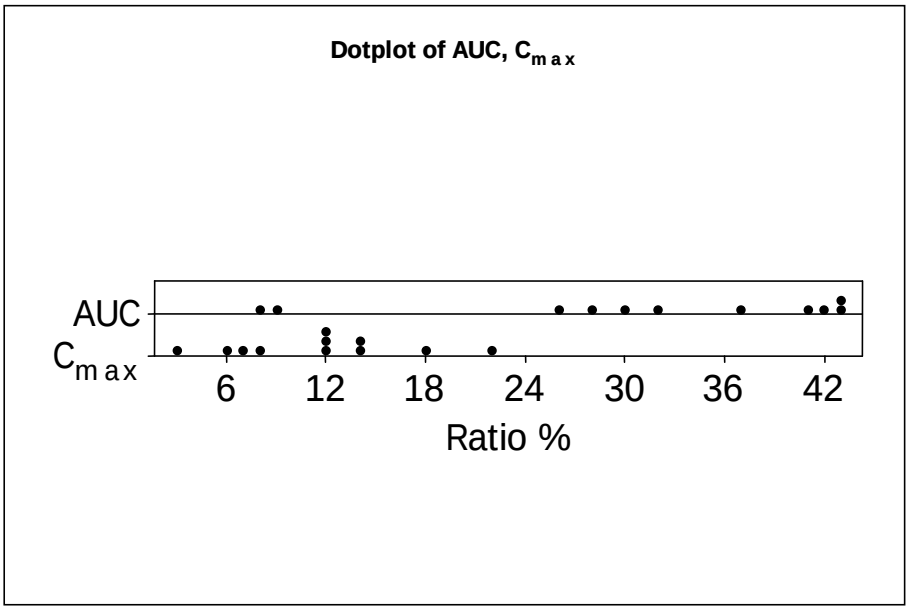


Figure 2.20 Pharmacokinetic parameters of diazepam $AUC_{0\text{-}72h}$, C_{max}, ratio percentage TDS/Rectal in 12 subjects, Dotplot.

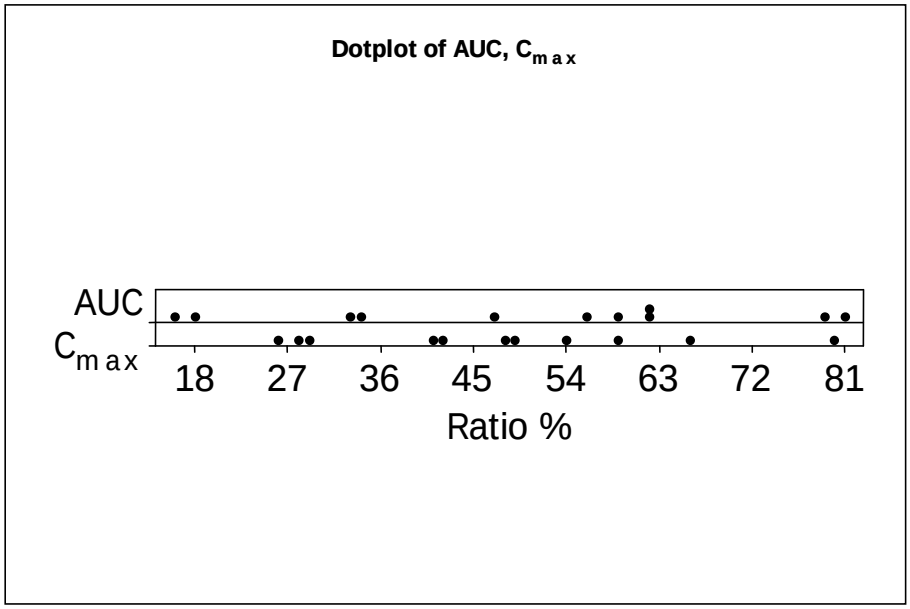


Figure 2.21 Pharmacokinetic parameters of desmethyldiazepam $AUC_{0\text{-}72h}$, C_{max}, ratio percentage TDS/Rectal in 12 subjects, Dotplot.

2.15 Discussion

No serious or unexpected adverse events were reported or observed during the study. The drug formulations and protocol requirements were well tolerated by all subjects. The TDS® diazepam preparation was shown to be able to deliver diazepam systemically in human subjects. However, the maximum concentrations of diazepam and desmethyldiazepam following TDS® diazepam administration were found to be lower than those found for rectal diazepam (Diastat), the mean ratio was 11.6 and 47.4%, respectively. In this study, the results of the AUC and C_{max} between the two treatments show that TDS® diazepam (A) and rectal diazepam (Diastat®) (B) were not contained within the bioequivalence limit (80–125%), C_{max} (0–72h): 7.3 to 14% and $AUC_{0\text{-}72h}$: 19.7 to 37.6%. The plasma concentrations of all subjects administered TDS® diazepam were all below the anticonvulsant plasma concentrations of 150–350 µg/L.

The plasma concentration of diazepam needed to stop convulsions is not well established, although a range of 150–350 µg/L has been suggested (Agurell et al., 1975), which was supported by Knudsen with a range from 200–300 µg/L (Knudsen, 1977). Additionally, the recurrence of seizures in two children was observed in Agurell et al's study, even though the anticonvulsion plasma level was of the order of 150 to 200 µg/L (Agurell et al., 1975). A study by Ogutu et al (Ogutu et al., 2002) following rectal administration of diazepam not only failed to terminate all of the convulsions but also reported variable plasma drug concentrations over time (112–1953 µg/L, 0.17–36h) compared with the IV group (402–1507 µg/L, 0.42–3.13 h). The pharmacokinetic characteristics have also been studied for oral diazepam, which were highly variable even in a relatively homogeneous population (Greenblatt et al., 1989).

Similarly, in this study, Diastat® only achieved the suggested effective plasma concentration (138.3–378.7 µg/L) in the range 150–350 µg/L in 11 of 12 subjects; probably due to variability in diazepam metabolism between individuals, which could explain the results of the Ogutu et al. study. Desmethyldiazepam was not quantified in all children who received diazepam IV but it was measured in three children who received rectal diazepam. The pharmacokinetics of a particular dose of diazepam seem

to vary widely between subjects, an observation noted by Ogutu et al. (Ogutu et al., 2002); "One child had a plasma diazepam concentration below 200 µg/L 5 min following IV administration. Even after a repeat dose of diazepam 30 min later, plasma diazepam concentrations in this child declined rapidly from 414 µg/L at 5 minutes after the repeat dose to less than 200 µg/L within 20 min. Two children who received diazepam p.r. also failed to achieve a plasma concentration of 200 µg/L. Despite not achieving this target, all three children stopped convulsing, although convulsions recurred within 30 min in each child."

Due to the presence of active metabolites, the serum/plasma concentration of diazepam alone is not useful in predicting the effects of the drug. Diazepam and its metabolites were analyzed, and only diazepam and desmethyldiazepam were assayed in all subjects for both treatments, (i.e. diazepam was metabolized in both dosage forms). Desmethyldiazepam is an active sedative, which is excreted by the kidneys. In fact a study by Nicholson and co-workers has reported that desmethyldiazepam (10 mg) can improve sleep compared to clorazepate (15 mg) (Nicholson et al., 1976). Furthermore, in one subject in the TDS® treatment group, no diazepam was observed; however desmethyldiazepam was present. This may be due to the rapid skin metabolism of diazepam, or accumulation of diazepam in subdermal adipose tissue.

Future development of this novel system will focus on staged adjustments to the TDS® formulation, dose and/or concentration of the TDS® diazepam system, in order to attain the therapeutic concentration range with the aim of developing a more convenient alternative to rectal or intravenous diazepam treatments. Additionally, we may need to consider further the influence of metabolism in the skin of diazepam. There are numerous enzymes beneath the stratum corneum viable skin (Noonan and Wester, 1985). The entire skin-to-liver metabolism ratio has been suggested to be 0.8–2.4 for different enzyme systems (Noonan and Wester, 1989) for slowly penetrating compounds. Such compounds possessing lipophilic characteristics like diazepam might be expected to be metabolized more completely. The presence of desmethyldiazepam concentrations in the first time point after the administration of the TDS® system in five

subjects suggested that desmethyldiazepam had a half life of more than 14 days in this study.

2.16 Conclusions

The TDS® diazepam preparation was shown to deliver diazepam systemically in human subjects. The concentrations of diazepam and desmethyldiazepam following TDS® diazepam administration were found to be lower and not bioequivalent to the rectal diazepam (Diastat®). Poor absorption and accumulation of diazepam in the skin might be the main reason for its low bioavailability in the circulation. Additionally, the presence of various metabolic enzymes in the skin may cause a decline of diazepam concentration.

This suggests that in future studies concerned with the bioequivalence, absorption, and bioavailability of topically applied drugs, skin metabolism of the drug needs to be considered.

Chapter 3 CE and HPLC method development for the analysis of tetracaine tape stripping samples

3.1 Introduction

Analysis of biological matrices such as blood, plasma, serum, and urine, to quantify and determine drugs and their metabolites *in vivo* is used to evaluate and interpret bioequivalence, pharmacokinetic (PK), toxicokinetic, and forensic studies. Sensitivity and selectivity are necessary for accurate, precise, reproducible and stable methods in preclinical and clinical pharmacology. Typically rapid and simple methods are required in clinical PK studies because of high numbers of samples to assay and low cost benefit.

The development of analytical methods using the guidance and principles of validation approved by the FDA is directly related to the quality and the performance of these studies. FDA guidance and principle (FDA, 2001a) information generally applies to bioanalytical procedures such as gas chromatography (GC), high performance liquid chromatography (HPLC) or liquid chromatography (LC), and combined GC and HPLC with mass spectrometry (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS performed for the quantitative determination of drugs and their metabolites in biological matrices such as blood, plasma, serum, or urine. Also it can be applied to other bioanalytical methods such as capillary electrophoresis (CE), as well as other biological matrices such as skin samples.

The guidance and principles of the validation process of analytical methods needs to be explored before addressing the methods themselves; for example instruments and any software being used must be qualified or validated according to standard operating procedures. Then the methods which apply to sample preparation, method development, validation and application can be addressed.

Analytical methods are employed to separate a mixture of unknown compounds into individual compounds that can be identified and determined, together with the purity or concentration of compounds. In clinical practice precise values typically are expected.

However the sensitivity and selectivity of analytical methods usually depends on the analyzed compound/sample quantity. Thus selecting the appropriate analytical method is important, so that a rapid and simple method is developed.

In this study HPLC and CE with ultraviolet (UV) absorbance detection have been selected for comparison, in the separation and quantitation of tetracaine and procaine, after application of tetracaine gel locally. Tetracaine was obtained by a tape sampling technique. HPLC with UV detection has been used for determination and quantification of tetracaine in plasma samples (Menon and Norris, 1981, Mazumdar et al., 1991, Rauf et al., 2002), and UV absorbance was capable of detecting tetracaine and its metabolite. Also HPLC has been compared with CE, in different fluids such as the determination of cetirizine dihydrochloride in human plasma (Kowalski and Plenis, 2007), the determination of mycophenolic acid in human plasma (Carlucci et al., 2007), the determination of carvedilol in serum (Clohs and McErlane, 2003), the determination of diazepam in pharmaceutical tablets (Aurora Prado et al., 2005), the measurement of histamine in seafood (Muscarella et al., 2005), the determination of flavonoids in Achillea mellefolium (Kocevar et al., 2008), and the determination of xyloglucan structures in blackcurrants (Hilz et al., 2006). All of them concluded that CE was preferable to HPLC.

3.1.1 Beyond cocaine

The synthetic local anaesthetics are structurally related to cocaine. Cocaine was the first alkaloid extracted from the coca plant leaves (*Erythroxylum coca*) (Jesus and Angel, 2003). The leaves were used as a stimulant and adopted many years ago as part of traditional native South American and Indian cultures.

In 1860, a collection of coca leaves was sent to the chemist Albert Niemann, in his German laboratory, to extract and isolate the active compound, which was named cocaine. In 1865 Lossen determined the correct molecular formula $C_{17}H_{21}NO_4$. In 1898, the structure of cocaine was drawn by the chemist Richard Willstatter (Jesus and Angel, 2003). This was of significant importance to researchers and chemists, so that they could

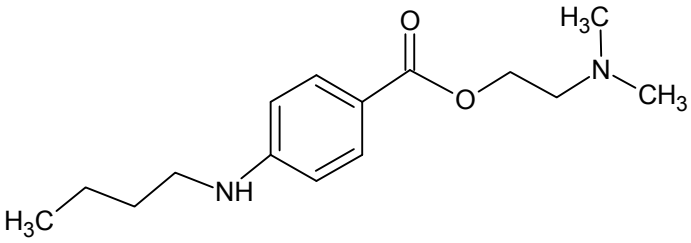
modify the structure of cocaine and synthesise other similar compounds with local anaesthetic properties.

In 1884, Koller and Brettauer revealed that the hydrochloride salt form of cocaine had beneficial anaesthetic and mydriatic effects when a topical solution was applied to the eye. This result encouraged a common intention to use the drug as a local anaesthetic. However its unwanted side effects including high toxicity and dependence forced the chemist to look for alternative anaesthetic drugs.

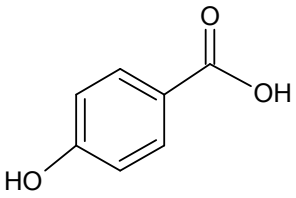
Since 1884, chemists have attempted to discover and develop a novel local anaesthetic with reduced side effects. It was not until 1904 that the German chemist, Alfred Einhorn developed 4-aminobenzoic derivatives. His first active drug from cocaine was synthesized in 1905, and named novocaine, which is now known as procaine (Jesus and Angel, 2003). Procaine was found to be more safe, but less effective, and produced some allergic reactions. This led to the discovery of other anaesthetic drugs such as tetracaine in 1930 (Biscoping and Bachmann-Mennenga, 2000), and lidocaine in1943 (Jesus and Angel, 2003).

Local anaesthetics are drugs used clinically to reversibly block the conduction of impulses in the nerve ends, leading to loss of sensation (numbness) in the applied area, such as local anaesthetics on the skin. Local anaesthetics can be classified by their chemical structure into either an amide group such as lidocaine, mepicaine, prilocaine, ropivacaine, or an ester group such as cocaine, procaine, and tetracaine.

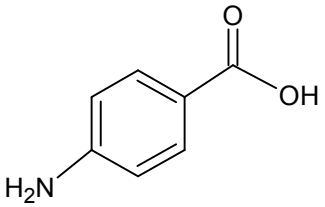
3.1.2 Tetracaine



Tetracaine



4-hydroxybenzoic acid



4-aminobenzoic acid

Figure 3.1 Chemical structures of tetracaine, 4-hydroxybenzoic acid and 4-aminobenzoic acid.

Tetracaine is a white odourless, hygroscopic, crystalline powder, with slightly bitter numbing taste. Soluble 1:7.5 in water, 1:40 alcohol, 1:30 chloroform, and insoluble in ether, or acetone (Martindale, 1982a). The dissociation constant (pK_a) is 8.39, and the partition coefficient Log P (octanol/water) is 3.5.

Tetracaine, also known as amethocaine $C_{15}H_{24}N_2O_2$.HCl (2-dimethylaminoethyl 4-butylaminobenzoate), is a potent local anaesthetic of the amino ester group, and is used for topical anaesthesia in ophthalmology, spinal anaesthesia and nerve block. It is also the first alternative anaesthetic for nasal septoplasty since cocaine (Drivas et al., 2007). Tetracaine has been incorporated into a mucosa adhesive polymer film to reduce the pain of oral wounds as a consequence of radiation and antineoplastic drugs.

Tetracaine can be found as the hydrochloride in solutions, creams, gels, and as the base in ointments. The topical formulation of tetracaine gel as a local anaesthetic was developed in the early 1990s, enabling a more rapid and long duration of action than the mixture of

lidocaine and prilocaine known as EMLA cream (McCafferty et al., 1989, Woolfson et al., 1990 , Martindale, 1996b, Bishai et al., 1999, Boyd and Jacobs, 2001). The onset of action is 30 to 45 minutes, while EMLA cream is about 1 hour, without any risk of methemoglobinaemia. Nichani et al. (Nichani et al., 2008) mentioned that Ametop and EMLA were equally effective when applied for the same time, and used as local anaesthetics during grommet insertion. Although, the ear has a thin layer, this needs to be studied further because Ametop gel may require less time than EMLA to exert its effect during grommet insertion, which reflects the fact that Ametop has a more rapid onset of action than EMLA in skin, and Ametop is superior to EMLA cream (Browne et al., 1999, Choy et al., 1999, Arrowsmith and Campbell, 2000).

Additionally, *in vivo*, Ametop has a local vasodilatation effect, which results in an increase in blood flow by decreasing the microvessel tone and activity close to the local tissue of the skin (Wiles et al., 2008). This may be an advantage when it is used prior to central venous catheter placement, or when a difficulty occurs whilst attempting to obtain IV access or blood, especially in newborns.

The pharmacokinetics of tetracaine can be illustrated by the administration of tetracaine into humans via any available route, such as the application of Ametop gel into the skin. However, the bioavailability of tetracaine or its metabolite in plasma from the application of Ametop gel is dependent on the absorption of tetracaine from the skin, which is mainly related to the duration and the area of application. However this may differ from subject to subject, is also affected by the area where it is applied, and the thickness of the dermis. Thus tetracaine may continuously be released from storage sites in the skin (Mazumdar et al., 1991).

The pharmacodynamics and toxicity of tetracaine are related to the concentration of the drug in plasma. In addition, the CNS toxicity of local anaesthetics proportionally depends on the speed of absorption, and individual variability (Scott, 1986). When tetracaine is applied locally to healthy subjects, a plasma concentration of up to 200 µg/L produces no signs or symptoms of toxicity because of the slow release of the drug from the dermis (Mazumdar et al., 1991). Additional side effects of local tetracaine gel may include

transient erythema, itching and swelling of the epidermis at the local site, and rarely blistering.

The determination of tetracaine in biological samples has been performed mainly in plasma. Several methods are available such as HPLC (Menon and Norris, 1981, Mazumdar et al., 1991), HPLC with multiwavelength detector (Rauf et al., 2002). Nevertheless human plasma contains proteins and endogenous compounds that may interfere with the chromatographic system, causing an increase in the pressure due to blockage of the column. There are several reported ways to extract tetracaine from plasma e.g. organic extraction or solid phase extraction, but these are time consuming. Thus the use of coupled columns (RAM-columns) as alternative methods, or micellar liquid chromatography (MLC) have been proposed (Escuder et al., 2001). In addition GC-MS (Kudo et al., 2001, Hino et al., 2002), direct ultraviolet spectrophotometry and colorimetry (Robert and Albert, 2006), liquid-liquid-liquid microextraction (LLLME) coupled with HPLC (Zhaohui et al., 2006), and raman spectroscopy (Dennis et al., 2004) can also be used.

However tetracaine is unstable in plasma, and can rapidly hydrolyze to 4-butyl aminobenzoic acid (BABA) (Keiko et al., 2001, Hino et al., 2002), and dimethylethanolamine (DMEA) (Rauf et al., 2002). Therefore to determine tetracaine alone is not enough, and BABA must also be determined. BABA is stable for several months in blood when stored at –20 C° (Keiko et al., 2001). In a study involving ten children (aged 1–5 years), tetracaine cream 4% was applied to each hand; only BABA and not tetracaine was detected in the serum (van Kan et al., 1997), and also after injection for spinal anaesthesia (Kudo et al., 2001). In another study involving ten subjects, tetracaine was detected in plasma in only three, when 2g of tetracaine cream 5% had been applied to the dorsum of the hand (Mazumdar et al., 1991). However the degradation of tetracaine by human plasma can be inhibited by the addition of a cholinesterase inhibitor such as physostigmine (Rauf et al., 2002).

The proposed study will use samples from tape stripping of the skin in healthy volunteers. The amount of the drugs in tape samples is greater than the amount in plasma

samples of topically applied drugs (Bareggi et al., 1998). Thus using simple and rapid techniques such as CE is possible. CE is a simple and fast technique to determine tetracaine in skin using tape stripping samples, since high sensitive techniques such as LC-MS are not required to detect such concentrations of drugs and UV absorption is sufficient to detect such concentrations, and has a low cost compared with MS detection. In addition using HPLC techniques is possible and can be compared with CE to find out more appropriate and reliable methods.

3.2 Capillary electrophoresis (CE)

Capillary electrophoresis, also known as capillary zone electrophoresis (CZE) is an alternative to the chromatographic techniques for drug analysis. It was developed in the 1980s and expanded in the 1990s. It uses narrow-bore (20–200 μm internal diameter) capillaries, and it has become a complementary separation technique which can separate a variety of compounds using an electric field, with most CE being carried out in as aqueous medium. The separation is based on the differences in electrophoretic mobility of buffer analytes. Weak acids/bases, analyte charge is dependent on pH and pKa, but strongly charged solutes like strong acids and bases are preferred. For fully dissociated ions, the ionic volume can be changed by the choice of buffer counter-ion or ion pairs. The separation resembles a cross between traditional polyacrylamide gel electrophoresis (PAGE) and modern HPLC. In addition CE can be interfaced with a UV detector (CE/UV) and mass spectrometer (CE/MS).

The basic component of CE is relatively simple (Figure 3.2). All that is required is a fused-silica capillary with an optical viewing window, high voltage, controllable temperature, two electrodes, an aqueous buffer and a detector such as UV.

The fused silica capillary in CE is made of a polyimide coated tube. The polyimide is removed to open a window to allow UV spectrophotometric detection through the capillary and measurement of the light absorption of compound (s). The new capillary must be flushed before it can be used to ensure a fully and uniform charge is on its surface. This is done by flushing the capillary for 20 min at a temperature of 40 ºC with 1

molar (M) sodium hydroxide, then returning the temperature to 25 ºC and flushing it again with 0.1 M sodium hydroxide for 10 minutes, 5 minutes with water, and 10 minutes with buffer. Sometimes it needs to be flushed as described between method runs if there is a change in migration time, resolution, or a noisy baseline. The fused silica capillary forms a hydrodynamic connection between the sample and the buffer solution by placing the capillary ends in the buffer solution to fill the capillary with buffer solution. Then the sample is introduced by dipping the end of the capillary in the sample solution. A small amount of the solution is taken for analysis so that a symmetric shape peak can be read by the detector.

Most CE is carried out in an aqueous medium, thus the compound should be soluble in water, and dissociate to suitable ions.

A wide range of different background electrolytes (BGE) can be employed in CE. The BGE salt is most effective within one or two pH units of its pKa, for example, phosphate is used around pH 2.5 and pH 7, borate around pH 9. The typical buffer concentration is 50–100 mM. In addition various BGE additives can be used to change the selectivity of the separation.

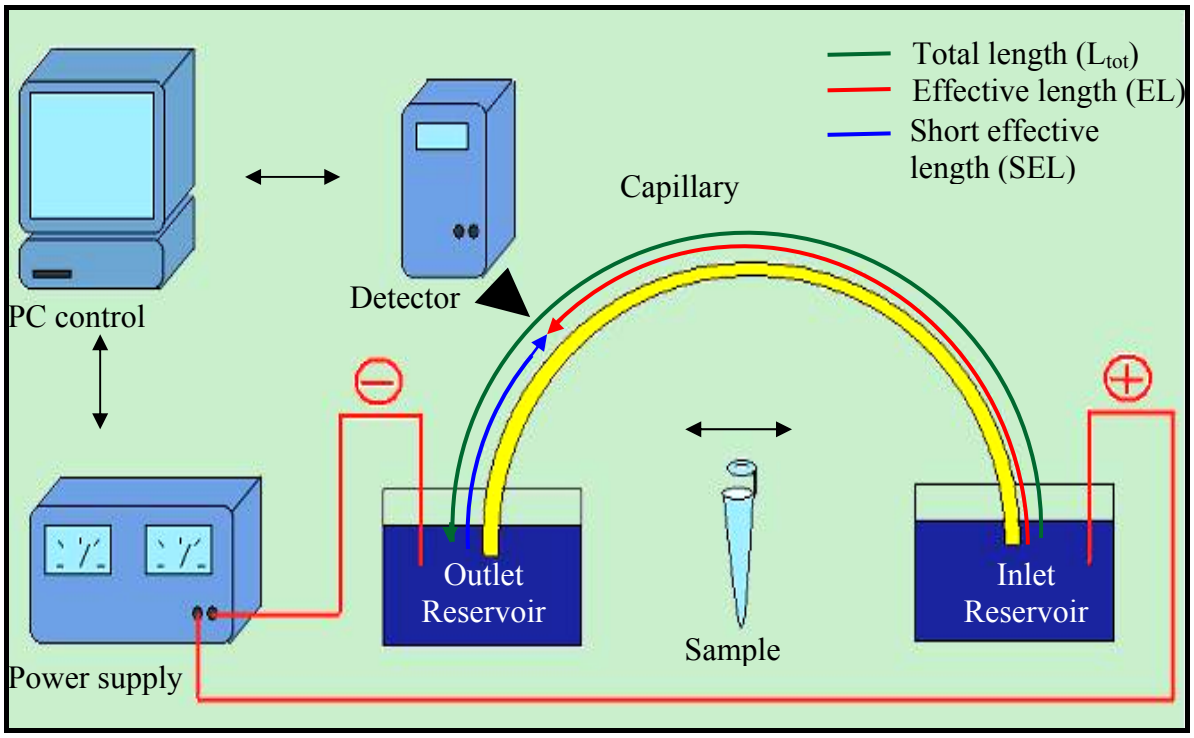


Figure 3.2 Basic components of a capillary electropherograph system.

The mechanism of separation of compounds by CE is charged species travel at different velocities (v) (Equation 3.1) depending upon their mobility (μ_a), which is determined by dividing the velocity by the electric field. This is dependent on the buffer composition, pH, and temperature:

$$v = \frac{I_{eff}}{t_m} = \frac{I_{eff}\ L_{tot}}{t_m\ v} = \mu_a \times E$$

Equation 3.1 Ion velocity formula in capillary electrophoresis

Where:

v = Ion velocity (cm/s)

μ_a = Apparent electrophoretic mobility (cm^2/Vs)

E = Electrical field strength (V/cm)

t_m = Migration time (s)

v = Applied voltage (v)

I_{eff}/ L_{tot} = Effective/total capillary length (cm)

Electro-osmotic-flow

One of the most important phenomenons in CE is the electro-osmotic-flow (EOF). EOF is a result of the surface charge on the capillary wall. The silanol groups in the fused silica are ionized when in contact with the buffer, and this ionisation is controlled by the pH of the buffer.

The EOF is defined by the formula in Equation 3.2:

$$v_{eo} = \frac{\in \zeta}{4\pi\eta} \times E$$

Equation 3.2 The formula for electro-osmotic-flow on the capillary wall.

Where $\in$ is the dielectric constant, η is the viscosity of the buffer (cp), and ζ is the zeta potential (mv).

3.2.1 Use of short effective length capillaries in CE

Effective length (l_{tot}) of the capillary can be measured as the distance from the injection end to the detector, ranging from 40 to 75 cm. However, an effective length of 8.5 cm can be employed for short-end injection techniques. This is has advantage of reducing analysis time including the migration time, as well as improving peak efficiency and sensitivity. The minimum capillary length from the inlet to the detector should be 25 cm, and the minimum total length 33.5 cm. For reversed mode the shortest effective length should be 8.5 cm from the outlet end of the capillary to the detector. This is can be applied by changing method parameter only such as changing the positive voltage to negative, applying a vacuum instead of pressure. Figure 3.2 shows the basic components of CE and the application of reversed mode from the outlet to the detector.

3.3 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is the most widely used analytical technique for the separation of mixed components, qualitative/quantitative analysis, or preparation of components of interest. HPLC is applied in the analytical separation of drugs in preclinical, clinical pharmacology, and others e.g. therapeutic drug monitoring, to help the drug development process. HPLC is used to separate a mixture of substances by pumping liquid (mobile phase) together with the analyte which is introduced in small volume at high pressure through the column (stationary phase). In the column the analyte (s) is/are resolved into their components as they are retarded by physical interaction between the substance being analyzed (analyte) and the stationary phase. The nature of the substance or the analyte, the column, temperature of the column, and the mobile phase reveal the amount of retardation, and the time at which the analyte elutes from the column after the component in the analyte interacts with the stationary phase in the column and a delay in elution occurs. Reducing the retention time speeds up the method, which may help in the high demand on an analytical laboratory.

A typical HPLC instrument includes a high pressure pump, injector, column, detector, and data recorder or system. Different types of high pressure pump, injector, column, detector, and data recorder or system depend on the analyte and application. The high pressure pump is needed to force the analyte together with the mobile phase through the stationary phase, and for large particles less pressure is required to maintain flow rate stability. The flow rate can range from 0.01 to 10 mL/min with maximum pressure up to 5000 psi (345 bar, 340 atm). Many methods have been attempted to overcome the pressure limitation of HPLC, via increasing the temperature (Lestremau et al., 2006), using a monolithic column (Ishizuka et al., 2002). A pressure drop over the column of only about 400 bar can be achieved with conventional methods in HPLC (Lestremau et al., 2006). The alternative method is the introduction of ultra performance liquid chromatography (UPLC), which capable of providing liquid flow at pressure up to 15,000 psi (about 1000 bar) (Plumb et al., 2004). Additionally, a degassing system can be considered e.g. helium purging, vacuum degassing.

The column is stainless steel, 5–25 cm long, 2–5 mm internal diameter, 1–5 μm particle size, and 50,000–90,000 plates/m. It is usually packed with silica particles, although there are different packing materials or phases, for different separation modes in HPLC. The types of HPLC modes available include partition, adsorption (liquid-solid), ion exchange, size exclusion or gel. Partition chromatography is used mainly for polar or non polar analytes, with low molecular weight (MW < 3000), using bonded stationary phases.

In the normal mode, the stationary phase is silica or chemically modified silica by polar groups such as cyano (most polar), diol, and amino (least polar), and the solvent is of low polarity e.g. hexane, methylene chloride or a mixture of these. Therefore least polar compounds are eluted first, and polar compounds will be retained by the polar stationary phase.

Reversed phase chromatography is the most commonly used mode of separation in HPLC. A polar solvent e.g. water, methanol, or a mixture of both, is used to elute analyte from silica gel modified by bonding non-polar groups such as long hydrocarbon chains. Therefore polar compounds are not retained and non-polar compounds will be retained on the hydrocarbon chain because of Van der Waal's forces. A high polarity mobile phase will elute polar analytes faster, but with less resolution.

Recently, hydrophilic interaction liquid chromatography (HILIC), also called aqueous normal phase, has been introduced. Compounds not well retained in reversed phase because of their high polarity, such as amino acids, may separate on a HILIC phase. HILIC chromatography phases are classified for neutral polar or ionic surface into unbonded silica silanol, amino bonded, amide bonded, and zwitterion bonded.

Other separation modes include size exclusion chromatography, also called gel permeation chromatography, which is used for proteins and polymers, and the separation depends on the size of the molecules. Large molecules excluded from pores and not retained elute first. Ion exchange chromatography, the retention is based on the attraction between the analyte ions and the charged bond in the stationary phase, e.g. aminoacids can be separated on a cation exchange column.

Once the compounds are separated, they are carried out by the mobile phase to a detector. Different kinds of detectors, including ultra violet (UV), fluorometric (fluorescence), electrochemical (ECD), mass spectrometry (MS), can be attached to HPLC depending on the structure and properties of the analyzed analyte or compound.

The most commonly used detector in HPLC is the UV/Visible detector. The UV/Visible detector has a wavelength range from 190-600 nm, and is selective and highly sensitive at the maximum absorption wavelength of the molecule. UV absorbance depends on the UV wavelength and the functional group in the chemical structure of the compound analyzed.

Fluorometric detection is a highly selective and sensitive method, as long as the analyte fluoresces.

Mass spectrometry (MS) is the mass to charge ratio (m/z). There are numerous types of ionization techniques, such as electrospray, atmospheric pressure chemical ionization, electron impact. MS measures the molecular weight as a mass weight of compound which is useful in providing both qualitative and quantitative identification with high sensitivity and more selectivity compared to other LC detectors. The MS combines with the HPLC to form LC/MS, LC/MS/MS.

3.3.1 Column performance

The aim in chromatography techniques is to resolve analyte peaks in a short separation time. Thus, understanding the measurement of resolution is imperative. Therefore resolution is the separation of two peaks in terms of ratio in the time difference (t_r) to the sum of peak widths (w) as shown in Equation 3.6, the higher the ratio, the higher the resolution (R_s).

Good column performance is essential for providing a Gaussian symmetrical peak shape in chromatography, and requires measurements of the following parameters; column efficiency (HETP), column permeability (kv0), retention or capacity factor (k), and selectivity (α).

HETP is a measure of column efficiency; which is described as the column length over the number of theoretical plates. The theoretical plate is a separated layer within the column which separate equilibrations of the analyzed between the stationary and mobile phase, and reflected by the column permeability. Column permeability or specific permeability is defined as the resistance of the column to permit the flow, high flow resulting in low pressure, which depends on the packing materials of the column. Selectivity is employed for measurement of relative retention of two substances being separated;

$$\alpha = \frac{t_{r2}}{t_{r1}}$$

Equation 3.3 Selectivity formula.

Where t_{r1} and t_{r2} are the retention time of peaks 1 and 2, respectively, and k_1 and k_2 are the corresponding capacity factors.

However for a mixture of substances capacity factors should be applied. The retention factor is a useful value for the column performance; Figure 3.3 shows the retention parameters.

$$k = \frac{t_R - t_0}{t_0}$$

Equation 3.4 Capacity factor formula.

k: capacity factor

t_R: the time between the injection point and the maximum detector response called the retention time.

t_0: the time required for an analyte not retained by the stationary phase to pass through the column.

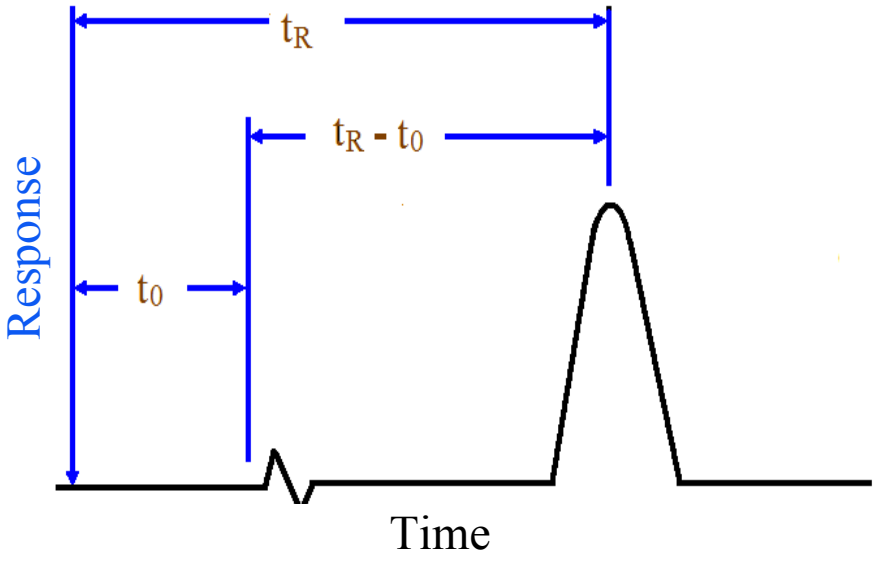


Figure 3.3 Schematic of a simple chromatogram.

3.3.2 Data processing in HPLC

The data processing is qualitative and/or quantitative, or molecular weight distribution. After analysis the compound can be identified from its retention time, off line UV spectrum, on flow emission spectrum, or multichannel dio-array detection (DAD). The amount of compound can be measured from calibration standards.

3.4 Method development of CE and HPLC

The analytical method must be adequately accurate, precise, and sensitive to determine the actual amount of drug or substance in the body. At least three phases of method development in most chromatography techniques must be applied to ensure valid and precise methods were applied. These include optimized selectivity for target compounds, the selection of the mobile and stationary phase in HPLC or buffer in CE. After optimizing selectivity, stability of the selective compound needs to be reproducible for either migration time in CE or retention time in HPLC and resolution. The last phase is to optimize sensitivity and quantitative reproducibility.

The overall concept in chromatographic techniques is to maximize peak resolution in the shortest time. Thus understanding and applying method parameters is essential, to optimize separation efficiency by measuring the resolution factor (R_s) of the different peaks migration or retention time (t_m or t_r), and the total peak widths (W_1+W_2). R_s is proportional to differences in time and inversely to the widths, with higher R_s value resulting in higher resolution, and efficient separation.

R_s for CE:

$$R_s = \frac{t_{m2} - t_{m1}}{W_1 + W_2}$$

Equation 3.5 Resolution factor in CE.

R_s for HPLC:

$$R_s = \frac{t_{r2} - t_{r1}}{W_1 + W_2}$$

Equation 3.6 Resolution factor in HPLC.

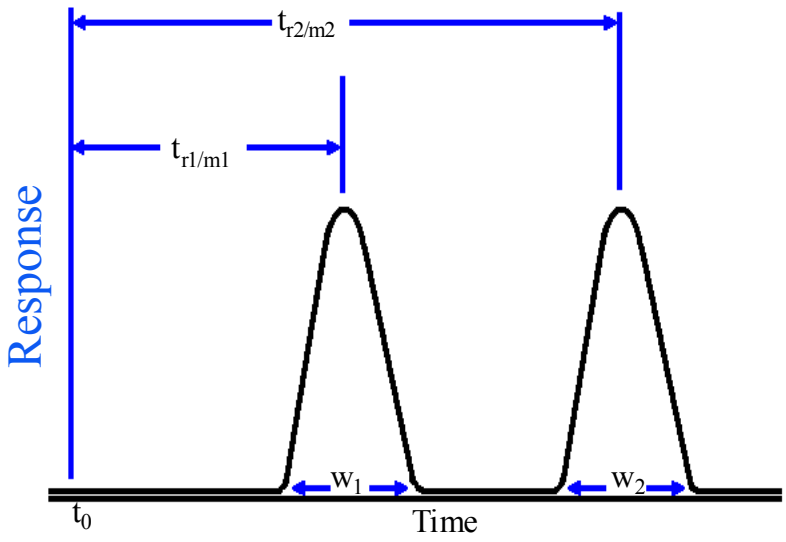


Figure 3.4 Chromatography model to determine peak resolution.

3.5 Description of the study

The validation of CE and HPLC assays to determine tetracaine in skin using tape stripping samples during clinical use of the drug.

3.6 Objectives

The main objective of this study was to develop, optimize, and validate a rapid and simple method of analysis, to determine tetracaine in skin using tape samples from 12 healthy volunteers given tetracaine as a part of a pharmacokinetic drug delivery study.

The second objective was to investigate the quantitative performance of the developed CE and HPLC methods and compare the two results.

3.7 Materials and methods

3.7.1 General chemicals

All chemicals listed below were supplied by Sigma Aldrich Company, Poole, UK;

1) Tetracaine hydrochloride (HCl) (Lot no 065k1501) (99% purity) for calibrator and control sample preparation was obtained as a white powder.

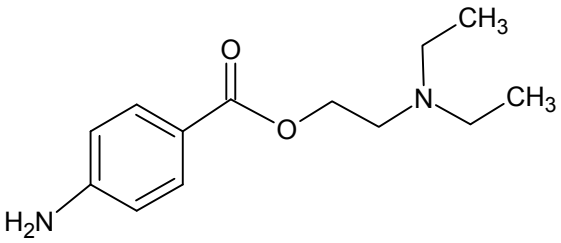
2) Procaine hydrochloride (HCl) (Lot no 114k0569) (100% purity) for use as internal standard, was obtained as a white powder.

3) 4-Butylaminobenzoic acid (Lot no 02514BJ) (97% purity), was obtained as white/yellowish powder.

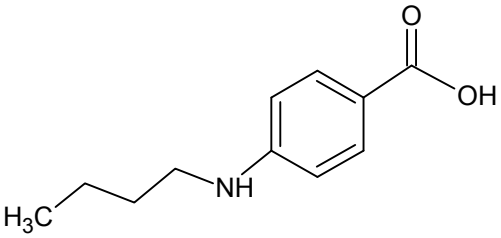
4) N, N-Dimethylethanolamine (Lot no 02514BJ) (97% purity) was obtained as redistilled, ≥ 99.5%.

5) Methyl 4-hydroxybenzoate (Lot no 016K00602) (99% purity) was obtained as a white powder.

6) Propyl 4-hydroxybenzoate (Lot no 016K0699) (100% purity) has been obtained as a white powder.



Procaine



4-butylaminobenzoic acid

Figure 3.5 Chemical structures of procaine and 4-butylaminobenzoic acid.

3.7.2 Adhesive tape

The adhesive tape used in this study was 19mm width, Tesa 4205 PV5, Beiersdorf, Hamburg Germany, supplied by TESA UK.

3.7.3 Procaine (Internal Standard)

The stock solution of procaine was prepared by dissolving 356.5mg procaine hydrochloride in 1 L de-ionized water. The concentration of the stock solution with respect to procaine was (after correcting for the salt and assuming purity of 100%) 300μg/mL.

The stock solutions for tetracaine calibration and quality control were stored at approximately –20ºC.

3.7.4 Method I

To develop, optimize, and validate a CE method to determine tetracaine in skin tape samples, obtained from healthy subjects.

3.7.4.1 Materials

The concentrations of tetracaine in skin from tape stripping samples were determined by CE. All HPLC grade solvents were obtained from Rathburn Chemicals Limited, Walkerburn, Scotland. All AnalaR grade reagents were obtained from Merck (BDH) Limited, Poole, UK.

3.7.4.2 CE instrumentation and capillaries

The analyses were carried out on an Agilent ^{3D}CE G1600AX capillary electropherograph capable of employing up to 30 kV, controlled by a 3D-CE Chemstation Rev.B.02.01[244] (Agilent, West Lothian, UK) for acquisition and handling of data. Fused silica capillaries, 485mm total length with a window at 85mm from one end ×50 μm i.d. (Composite Metal Services, Ilkley, UK) were conditioned on first use by flushing with 1M NaOH (BDH, Poole, UK) at > 950 mbar, 40 °C for 20 min. Pre-conditioning on injection was a 3 min flush with 0.1M NaOH (BDH, Poole, UK), then 3 min flush with background electrolyte (BGE). In the final separation a potential of –25kV was employed. The capillary was thermostated at 25.0 °C. The injections of the samples into the system were carried out hydrodynamically for 10 seconds at –50 mbar. Detection was by photodiode-array over 195–360 nm, but 312 nm with bandwidth 10nm was used for quantitation since 312 nm is the maximum for tetracaine. The run time was 2 min. Both BGE vials were replenished every 12 injections. All samples and standards in the auto-sampler were kept at ambient temperature.

3.7.4.3 Background electrolyte (Buffer)

0.1 M Tris-phosphate at pH 2.5 was used as the aqueous background electrolyte. The buffer was prepared by diluting 20 mL phosphoric acid (0.5 M) with 60 mL de-ionized water followed by adding 15 mL of 0.5 M Tris to obtain a solution at pH 2.5. This solution was made up to 100 mL with de-ionized water. Then the pH was checked and adjusted if necessary.

3.7.4.4 Method development

3.7.4.4.1 Overall description of method development

Flow Chart 3.1 describes method development process for CE.

3.7.4.4.2 UV absorbance

The UV detector was set at 312, and 210 nm absorption wavelengths (λ_{max}).

3.7.4.4.3 Optimization of buffer pH

The influence of pH of 0.1 M Tris-phosphate BGE was studied over the range 2 to 3.5.

3.7.4.4.4 Optimization of temperature

Precise temperature control is important. Thus various temperatures were studied: 15, 20, 25, 30 °C.

3.7.4.4.5 Optimization of buffer concentration

Different buffer concentrations at constant pH in the range of 0.05 to 0.2 M were investigated.

3.7.4.4.6 Optimization of voltage

The optimum voltage was determined by performing runs at increasing voltage until deterioration in resolution was noted.

3.7.4.4.7 Optimization of injection time

Injections time were studied starting from 1 to 65 seconds.

3.7.4.4.8 Sensitivity

The lower limit of detection was determined as the concentration of tetracaine that had a peak height three times higher than the baseline noise. The lower limit of quantification was defined as the lowest calibration concentration of tetracaine.

3.7.4.4.9 Specificity

Specificity or selectivity is the ability to measure the analyte of interest free of interference from other components in the sample, interference from the tape, from the buffer, drug excipients, and drug metabolites.

3.7.4.5 Recovery

Absolute recovery of tetracaine was determined using tape samples spiked with tetracaine at the same nominal concentration as the calibration curve samples using Equation 3.7 Peak area ratio measurements from extracted samples were compared with the peak areas ratio from direct solvent injection of the test compounds. Mean and standard deviations were calculated from at least six measurements at each level. Two samples of solution and six samples of tapes were loaded with 50, 100, 200, 400, 600, 800, 1000, 1200µg of tetracaine, and 1500µg procaine as internal standard.

$$\text{Recovery} = \frac{\text{A}}{\text{B}} \times 100$$

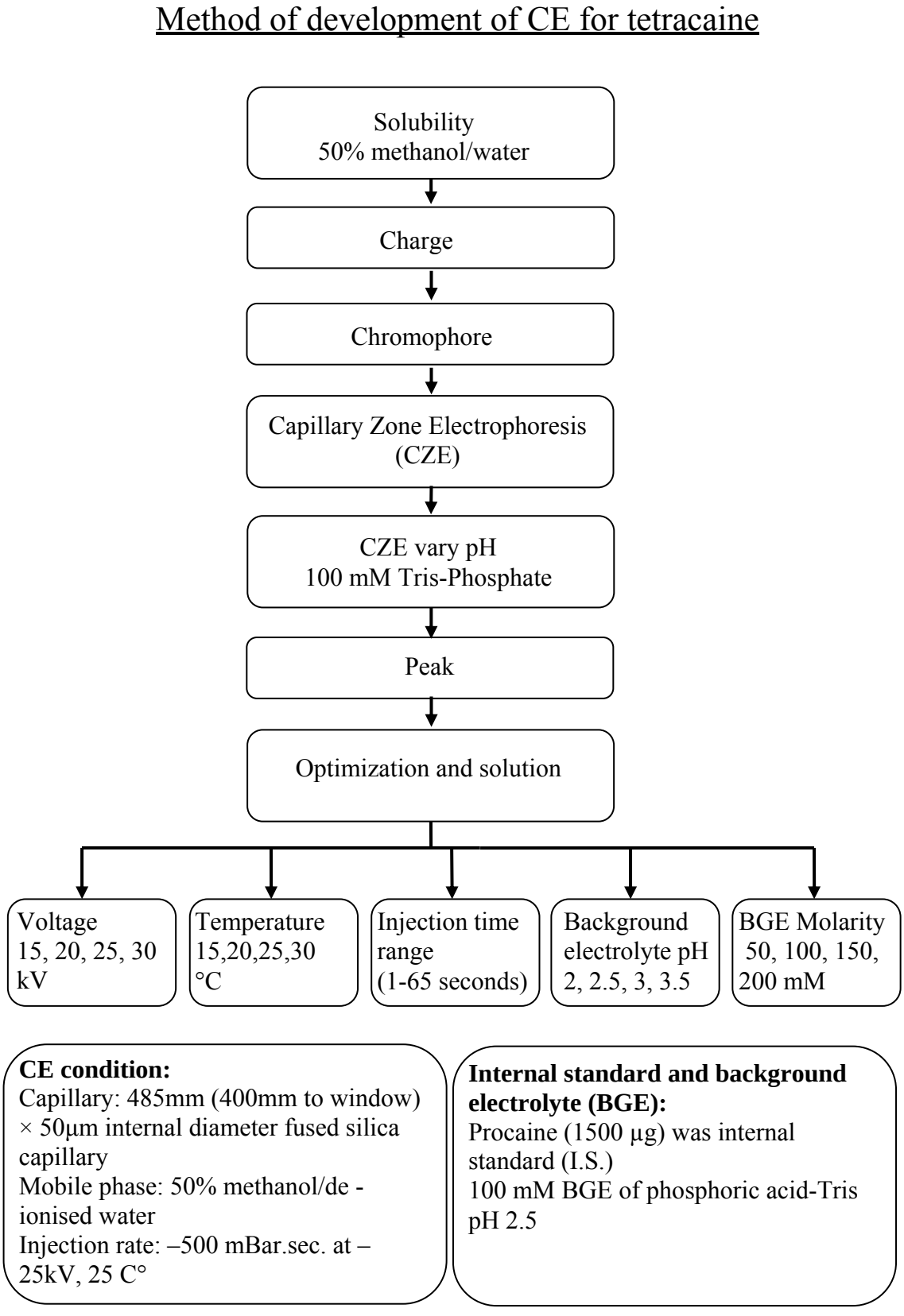
Equation 3.7 Absolute recovery formula.

Where, A is the peak area ratio of tetracaine in the tape, and B the peak area ratio of tetracaine in 50% methanol/water.

3.7.4.6 Stability

The stability of tetracaine was determined using tape samples spiked with tetracaine at the same nominal concentration as the quality control samples. The stability of the samples was measured before and after extraction. Both samples were kept at ambient temperature for 24 hours and then stored at –20 ºC for at least 3 weeks. Samples were loaded with 100 (Q1), 250 (Q2), 1000 (Q3), µg of tetracaine, and 1500µg procaine as internal standard.

Flow Chart 3.1 Method of development of capillary electrophoresis for tetracaine.



3.7.4.7 Assay procedures for tetracaine in CE

3.7.4.7.1 Calibrators and quality control samples

Stock solutions for tetracaine (5mg/mL) were prepared in 50% methanol/water and for procaine 300µg/mL in water (Internal Standard (I.S.)). All stock solutions were stored at –20ºC. All calibrators and quality control samples were prepared by appropriate dilution of the stock.

3.7.4.7.2 Tetracaine calibrator

The stock solution of tetracaine was prepared by dissolving 287.29 mg of tetracaine hydrochloride in 50mL of 50% v/v aqueous MeOH. The concentration of the stock solution with respect to tetracaine was (after correcting for the salt and assuming a purity of 100%) 5.0mg/mL.

3.7.4.7.3 Tetracaine quality control (QC)

The stock solution of tetracaine was prepared by dissolving 143.65 mg of tetracaine hydrochloride in 25mL of 50% v/v aqueous MeOH. The concentration of the stock solution with respect to tetracaine was (after correcting for the salt and assuming a purity of 100%) 5.0mg/mL.

3.7.4.7.4 Working standard solutions

Calibrator

50 mL of the tetracaine stock standard solution was pipetted into a 100mL volumetric flask and made up to the mark with 50% v/v aqueous MeOH to produce a sub-stock (Cal 10). Working calibration solutions were prepared by diluting the sub stock (Cal 10) with 50% v/v aqueous MeOH as tabulated in Table 3.1.

Table 3.1 Working calibration concentrations with related dilutions in 50% v/v aqueous MeOH.

Cal No.	Volume of sub stock	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration
	(mL)	(mL)	(mL)	(µg/mL)
1	0.05	24.95	25.0	5
2	0.25	24.75	25.0	25
3	0.50	24.50	25.0	50
4	2.0	23.0	25.0	200
5	4.0	21.0	25.0	400
6	6.0	19.0	25.0	600
7	8.0	17.0	25.0	800
8	10.0	15.0	25.0	1000
9	12.0	13.0	25.0	1200
10 (Sub stock)	50.0 mL stock solution	50.0	100	2500

Calibrator 1 and calibrator 9 were used as lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ), respectively.

Quality control (QC) samples

12.50 mL of the tetracaine stock solution was pipetted into a 25mL volumetric flask and made up to the mark with 50% v/v aqueous MeOH to produce a sub-stock (QC 4, 2.50 mg/mL). Working controls were prepared by diluting the sub stock with 50% v/v aqueous MeOH as tabulated in Table 3.2.

Table 3.2 Working quality control concentrations with related dilutions in 50% v/v aqueous MeOH.

Cal No.	Volume of sub stock	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration
	(mL)	(mL)	(mL)	(µg/mL)
1 (Low)	1.0	24.0	25	100
2 (Medium)	2.5	22.50	25	250
3 (High)	10.0	15.0	25	1000
4 (sub stock)	12.5mL stock solution	12.5	25	2500

3.7.4.8 Calculating Inaccuracy and imprecision

Inaccuracy was tested by the determination of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. Each control sample contained tetracaine. The nominal values for the low, medium and high control samples were 100, 250 and 1000µg respectively. The nominal values for the ULOQ and LLOQ were the same nominal concentration as the highest and the lowest calibration standards, respectively.

Assay imprecision was measured both within and between batch by the analysis of three control samples, the LLOQ and the ULOQ. Within-batch and between-batch imprecision was calculated by the nested analysis of variance (ANOVA) using internationally agreed methods (ISO-5725, 1994), calculated and plotted using Excel 2007 and Minitab 15 © 2006 Minitab Inc.

3.7.5 Method II

To develop, optimize, and validate a HPLC method to determine tetracaine in skin tape samples, obtained from healthy subjects.

3.7.5.1 Overall description of the method

This study describes a HPLC method for the determination of tetracaine in tape samples obtained from tape stripping of human skin using procaine as an internal standard. Analytes were separated on a Luna PFP (2), 3µm, 150mm x 4.6 mm column at ambient temperature, the mobile phase consisted of 10 mM KH_2PO_4 at pH 2.5, and methanol (35:65, v:v). Tetracaine and the internal standard were monitored by ultraviolet absorption at 312 nm. The overall method was validated and used in the analysis of tetracaine/procaine ratio in 106 skin tape samples. The tetracaine/procaine ratio in these skin tape samples were previously measured by the developed and validated CE method, as described and discussed in this chapter.

3.7.5.2 Materials

As described previously in method I, section 3.7.4.1, but the system was HPLC.

3.7.5.2.1 Chromatographic conditions and instrumentation

1) The experiment was carried out using a Shimadzu LC-6A pump, Gilson 231 autosampler fitted with a 200 µL injector loop, Gilson diluter 401. The system was coupled to a Shimadzu SPD-6A UV/Vis detector.

2) The separation was carried out using a Luna PFP (2) column, 3µm, 150mm x 4.60 mm from Phenomenex, Macclesfield, U.K. The analytes were eluted using isocratic elution with a flow rate of 1 mL/min with KH_2PO_4 buffer (pH 2.5) and methanol (35:65, v:v), and were monitored by ultraviolet absorption at 312 nm.

3) The data acquisition system consisted of a data interface (HP 35900 interface) and HP Chemstation, version A.04.01. The software was operated under Windows 95 environment.

3.7.5.3 Sensitivity

As described previously in method I, section 3.7.4.4.8.

3.7.5.4 Specificity

As described previously in method I, section 3.7.4.4.9.

3.7.5.5 Recovery

Recovery of tetracaine was calculated by dividing the peak area ratio of the standard samples spiked with tetracaine by the peak area ratio of the same tetracaine concentration in 50% MeOH.

Recovery was calculated using Equation 3.7, where A is: the peak area ratio of the tetracaine in tape sample and B is peak area ratio of the same tetracaine concentration in 50% v/v aqueous MeOH.

3.7.5.6 Stability

The stability of tetracaine was determined using tape samples spiked with tetracaine at the concentration of 10 µg/mL stock solutions. Aliquots from this solution were injected into the HPLC after each freeze / thaw cycle stored at –20 ºC.

3.7.5.7 Assay procedures of tetracaine for HPLC

3.7.5.7.1 Calibrators and quality control samples

For tetracaine calibration and tetracaine quality control the concentrations of 5.0mg/mL and 2.5mg/mL have been used, respectively, and for procaine as internal standard the concentration was the same as in method I. (All after correcting for the salt and assuming a purity of 100%).

3.7.5.7.2 Working standard solutions

Calibrator

25 mL of the tetracaine stock standard solution was pipetted into a 100mL volumetric flask and made up to the mark with 50% v/v aqueous MeOH to produce a stock solution (1.25 mg/mL). Working calibration solutions were prepared by diluting the sub stock (Cal 9) with 50% v/v standard as tabulated in Table 3.3.

Table 3.3 Working calibration concentrations with related dilutions in 50% v/v aqueous MeOH.

Cal No.	Volume of sub stock	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration
	(mL)	(mL)	(mL)	(µg/mL)
1	0.006	24.994	25.0	0.3
2	0.02	24.980	25.0	1
3	0.06	24.940	25.0	3
4	0.2	24.800	25.0	10
5	0.6	24.400	25.0	30
6	2	23.000	25.0	100
7	6	19.000	25.0	300
8	20	5.000	25.0	1000
9 (Sub stock)	25.0 mL stock solution	75.0	100	1250

Calibrator 1 and calibrator 8 were used as lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ), respectively.

Quality control (QC) samples

12.50 mL of the tetracaine stock solution was pipetted into a 25mL volumetric flask and made up to the mark with 50% methanol to produce a sub-stock (QC 4, 1.25 mg/mL). Working controls were prepared by diluting the sub stock with standard zero as tabulated in Table 3.4.

Table 3.4 Working quality control concentrations with related dilutions in 50% v/v aqueous MeOH.

Cal No.	Volume of sub stock	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration
	(mL)	(mL)	(mL)	(µg/mL)
1 (Low)	0.1	24.9	25	5
2 (Medium)	1	24.0	25	50
3 (high)	10.0	15.0	25	500
4 (sub stock)	12.5mL stock solution	12.5	25	1250

3.7.5.8 Calculating inaccuracy and imprecision

Inaccuracy was tested by the determination of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. Each control sample contained tetracaine. The nominal values for the low, medium and high control samples were 5, 50 and 500µg respectively. The nominal values for the ULOQ and LLOQ were the same nominal concentration as the highest and the lowest calibration standards, respectively.

Assay imprecision was measured both within and between batch by the analysis of three control samples, the LLOQ and the ULOQ. Within-batch and between-batch imprecision, regression coefficient and the slope of the calibration line were calculated from the peak area ratio by excel 2007, using internationally agreed methods (ISO-5725, 1994).

3.7.6 Method III

To develop and optimize a CE method to determine BABA in skin tape samples, obtained from healthy subjects.

3.7.6.1 Materials

As described in the previous method (method I), section 3.7.4.1

3.7.6.1.1 CE instrumentation and capillaries

The analyses were carried out to determine BABA in skin tape samples obtained from healthy subjects. The method was run in the same system and conditioning applied as tetracaine method I, except that fused silica capillaries, 485mm total length with a window at 85 mm from one end ×50μm i.d. were used. In the final separation a potential of –27kV was employed. The capillary was thermostated at 20.0°C. The injections of the samples into the system were carried out hydrodynamically for 7 seconds at –50mbar. Detection was by photodiode-array over 195–360nm, but 285 nm with bandwidth 10nm was used for quantitation since 285 nm is the maximum for BABA. The run time was 1.50min. Both BGE vials were replenished every 12 injections. All samples and standards in the auto-sampler were kept at ambient temperature.

3.7.6.2 Background electrolyte (Buffer)

30 mM disodium tetraborate at pH 8.0 was used as the aqueous background electrolyte. The buffer was prepared by dissolving 1.14 g disodium tetraborate in 100mL de-ionized water. Then the pH was checked using a pH meter and adjusted, if necessary.

3.7.6.3 Method development

3.7.6.3.1 UV wave length

The UV detector was set at 285 and 312 nm absorption wave length (λ_{max}) as 285, and 312 nm were the highly selective wave lengths for BABA, and tetracaine, respectively.

3.7.6.3.2 Optimization of buffer pH

The influence of pH of 30 mM disodium tetraborate BGE was studied over the range 7 to 10.50.

3.7.6.3.3 Optimization of temperature

Precise temperature control is important, thus various temperatures were studied from 10, 15, 20, 25, 30 °C.

3.7.6.3.4 Optimization of buffer concentration

Different buffer concentrations at constant pH in the range of 20 to 50 mM were investigated.

3.7.6.3.5 Optimization of voltage

The optimum voltage was determined by performing runs at increasing voltage until deterioration in resolution was noted.

3.7.6.3.6 Optimization of injection time

Injections times were studied starting from 1 to 12 seconds.

3.7.6.4 Sensitivity

As described previously in method I, section 3.7.4.4.8.

3.7.6.5 Specificity

As described previously in method I, section3.7.4.4.9.

3.7.6.6 Recovery

As described in method I , but samples of solution and samples of tape were loaded with 10, 25, 50, 100, 250, 500, 750, 1000 μg for BABA, and tetracaine, and 1500μg for procaine as the internal standard.

3.7.6.7 Stability of samples

The stability of BABA was determined using tape samples spiked with BABA at the same nominal concentrations as the QC1, QC2, and QC3 samples, as described in method I.

3.7.6.8 Stability of sample injection

The stability of the injection samples, after adding 30mM disodium tetraborate (BGE) was measured every day for at least 10 days for refrigerated samples, and for at least 6 hours for samples at ambient and 37 ºC temperatures at the nominal concentrations of BABA (1000 μg), tetracaine (1000 μg), and procaine (1500 μg).

3.7.6.9 Calibrators and quality control samples

Stock solutions, for 4-butylaminobenzoic acid (BABA) (5mg/mL) were prepared in 50% methanol/water and for procaine 300μg/mL in water (Internal Standard (I.S.)). All stock solutions were stored at -20ºC. All calibrators and quality control samples were prepared by appropriate dilution of the stock.

3.7.6.9.1 BABA Calibrator

The stock solution of BABA was prepared by dissolving 515.4 mg of 4-butylaminobenzoic acid (BABA) in 50 mL of 99.9% v/v aqueous methanol, and then water was added to prepared 50% v/v aqueous MeOH. The concentration of the stock solution with respect to BABA was (after assuming a purity of 100%) 5.0 mg/mL.

3.7.6.9.2 BABA Quality Control (QC)

The stock solution of BABA was prepared by dissolving 257.7 mg of 4-butylaminobenzoic acid (BABA) in 25 mL of 99.9% v/v aqueous methanol, and then water was added to prepared 50% v/v aqueous MeOH. The concentration of the stock solution with respect to BABA was (after assuming a purity of 100%) 5.0 mg/mL.

Procaine was prepared as mentioned in method I. The stock solutions for tetracaine calibrator and quality control were stored at approximately -20ºC.

3.7.6.9.3 Working standard solutions

Calibrator

Working calibration solutions were prepared by diluting the stock solution with 50% v/v aqueous MeOH as tabulated in Table 3.5.

Table 3.5 Working calibration concentrations with related dilutions in 50% v/v aqueous MeOH

Cal No.	Volume of stock solution BABA	Volume of stock solution TC	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration of BABA & TC
	(mL)	(mL)	(mL)	(mL)	(µg/mL)
1	0.375	0.15	24.475	25.0	75
2	0.75	0.30	23.95	25.0	150
3	3.75	1.50	19.75	25.0	750
4	7.50	3	14.50	25.0	1500
5	11.25	4.5	9.25	25.0	2250
6	15.0	6	4.0	25.0	3000
Stock solutions: BABA 5000 µg/mL and tetracaine (TC)12500 µg/mL					

Calibrator 1 and calibrator 6 were used as upper limit of quantitation (ULOQ) and lower limit of quantitation (LLOQ), respectively.

3.7.6.9.4 Quality Control (QC) samples

Working controls were prepared by diluting the stock solution with 50% v/v aqueous MeOH as tabulated in Table 3.6.

Table 3.6 Working quality control concentrations with related dilutions in 50% v/v aqueous MeOH

Cal No.	Volume stock solution BABA	Volume of sub stock TC	Volume of 50% v/v aqueous MeOH	Total volume	Nominal concentration Of BABA & TC
	(mL)	(mL)	(mL)	(mL)	(µg/mL)
1 (Low)	1.5	0.75	22.75	25	300
2 (Medium)	6.0	3	16	25	1200
3 (high)	12.0	6	7	25	2400
Stock solutions: BABA 5000 µg/mL and tetracaine (TC)10000 µg/mL					

All the samples were placed in tape as described in section3.7.7 (page, 171), and left to dry at ambient temperature and transferee to polypropylene tubes, which then extracted from the tape as explained later in section 3.7.8 (page, 171), the samples were then drawn and placed into 250 auto injector vial, which is diluted before injection in the CE as 1:3 sample, 1:3 background electrolyte, and 1:3 distilled water.

The final concentration of calibrations were nominally 25, 50, 250, 500, 750, 1000 µg for BABA, and tetracaine. The 25 and 1000µg being the low limit of quantity (LLOQ) and the upper limit of quantity (ULOQ) respectively, plus 500 µg I.S. and quality control samples were nominally 100, 400, 800µg BABA, and tetracaine plus 500µg I.S.

3.7.6.10 Calculation of results

The statistical analysis and graphical presentation was carried out using Excel 2007, Window Vista.

3.7.7 Preparation of tape samples

For the purpose of validation and assay calibration, a length of self adhesive tape (Tesa 4205 PV5) was cut into 10 samples so that each tape was approximately 3 × 2cm. 100µL volume of standard or quality control solution were loaded on the adhesive part of each tape. The solution was distributed approximately evenly on each tape. The samples were left to dry naturally at ambient temperature. This took, approximately 30 minutes. The tapes were then transferred into 10mL polypropylene tubes and capped. The tubes were stored at –20°C until analysis. The tape samples used in this study were obtained from healthy volunteers by applying 3×2 cm tape on their skin gently with the same pressure, and then using forceps to strip off, with constant movement. Each volunteer has repeated the procedure 10 times to provide 10 samples at each skin site. All samples obtained were transferred to 10mL polypropylene tubes, for each site one tube for every volunteer, and stored frozen at –20º C until analysis.

3.7.8 Extraction procedure

5mL of 100% methanol was dispensed into the tube containing standard/QC tape or samples. The contents were mixed for 45 minutes using a shaker. 5mL of internal standard solution containing 300µg/mL procaine in water was added to the tube to make a 50% v/v aqueous MeOH solution. The contents were mixed again by a shaker for another 15 minutes. 200µL of the solution was transferred into a 250µL auto injector vial for CE analysis, and same amount for HPLC. In the analysis of BABA, samples were diluted before injection as 1:3 sample, 1:3 BGE, 1:3 water.

3.8 Validation procedures and results

3.8.1 Results of method I

3.8.1.1 Method development

Figure 3.6 and Figure 3.7 show that the UV absorbance of tetracaine was optimum at 312 nm wave length (λ_{max}). Table 3.7 shows the stable and optimum buffer pH was 2.5,

precise and stable temperature was 25 °C, optimum buffer concentration (M) at constant pH was 0.1 M Tris-phosphate, and when voltage was changed, the deterioration in resolution was noted at –25KV. Additionally Figure 3.8 and Figure 3.9 show the response was linear to injection time of 20s, however 10 s was selected.

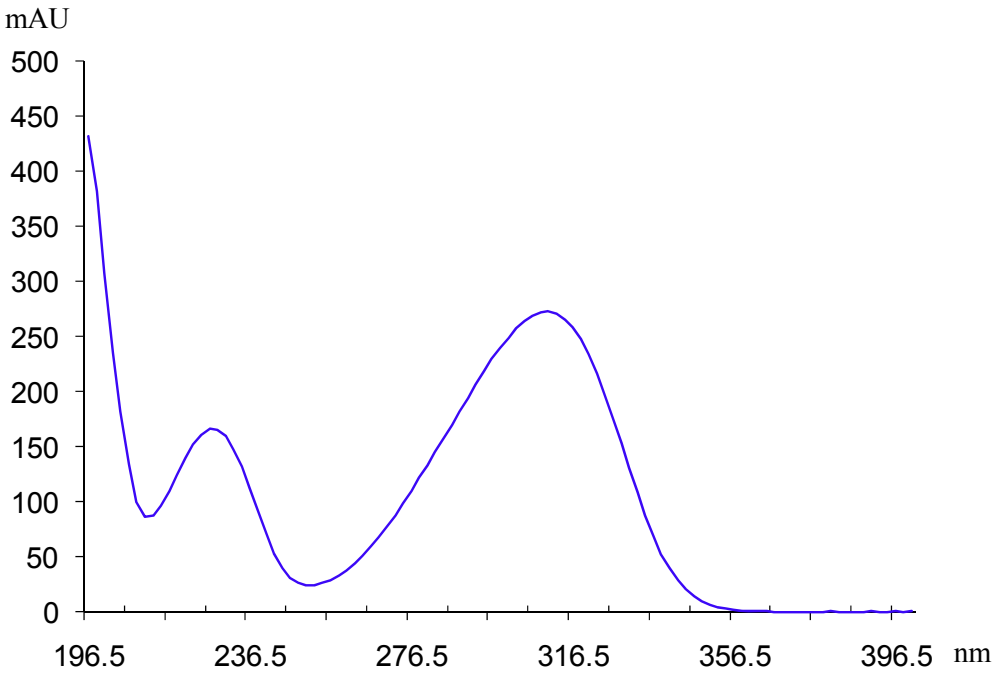


Figure 3.6 UV spectra of tetracaine.

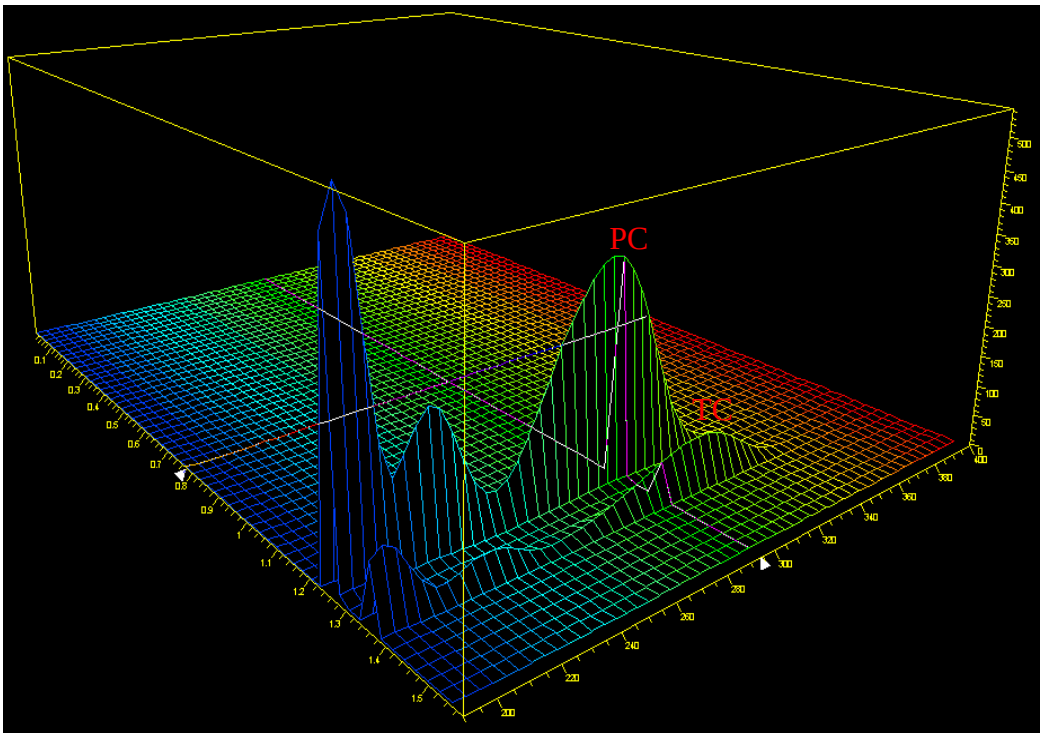


Figure 3.7 3-D spectra of procaine (PC) and tetracaine (TC) peaks displayed from a capillary electropherograph controlled by 3D-CE Chemstation Rev. B.02.01 [244].

Table 3.7 Capillary electrophoresis method development of buffer molarity and pH, and instrument temperature and voltage.

Molarity of buffer

M	Mig time (min) TC	Mig time (min) PC	Difference time (min)	PC width second	TC width second	Rs
0.05	1.25	1.16	0.10	1.19	1.29	4.59
0.10	1.40	1.28	0.12	1.18	1.22	5.86
0.15	1.33	1.23	0.09	1.03	1.00	5.57
0.20	1.27	1.18	0.09	0.96	0.94	5.48

pH of buffer

pH	Mig time (min) TC	Mig time (min) PC	Difference time (min)	PC width second	TC width second	Rs
2	1.07	1.00	0.07	0.92	0.86	4.52
2.5	1.30	1.19	0.11	1.09	1.15	5.80
3	1.49	1.33	0.16	1.41	1.42	6.79
3.5	1.36	1.21	0.15	1.40	1.42	6.17

Temperature of instrument

Temp °C	Mig time (min) TC	Mig time (min) PC	Difference time (min)	PC width second	TC width second	Rs
20	1.39	1.27	0.12	1.16	1.15	6.08
25	1.24	1.14	0.10	1.14	1.15	5.41
30	1.15	1.06	0.09	1.07	1.22	4.66

Voltage of instrument

kV	Mig time (min) TC	Mig time (min) PC	Difference time (min)	PC width second	TC width second	Rs
–15	2.37	2.18	0.19	2.34	2.47	4.73
–20	1.71	1.57	0.14	1.60	1.65	5.18
–25	1.30	1.19	0.11	1.09	1.15	5.80
–30	0.99	0.91	0.08	0.80	0.86	6.01

M: Molarity, Mig: migration, min: minute, TC: tetracaine, PC: procaine, Rs: resolution, kV: kilovolt.

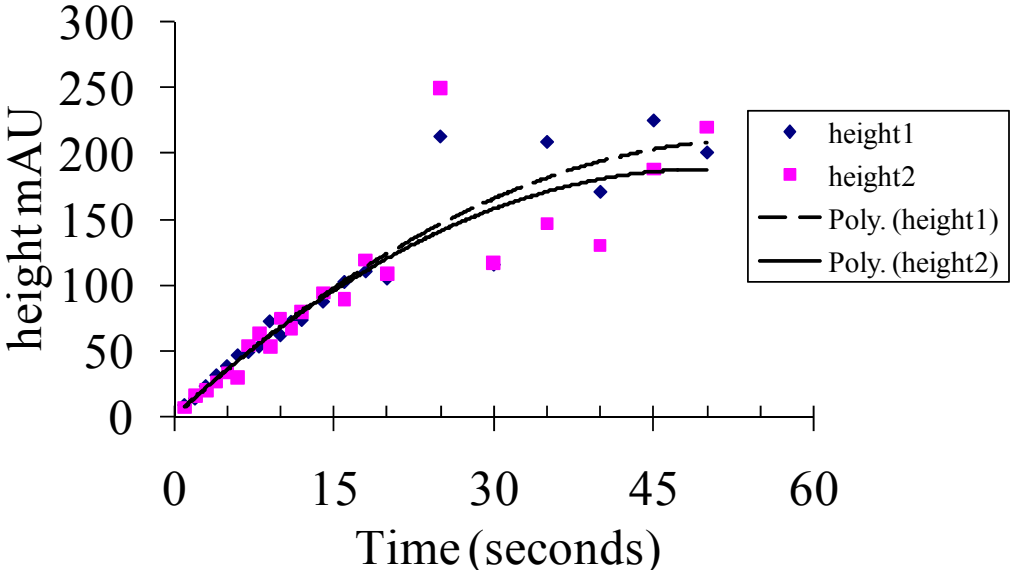


Figure 3.8 Peak height of tetracaine at two wave lengths, 210 (height 1), 312 (height 2) nm, and over injection time in seconds.

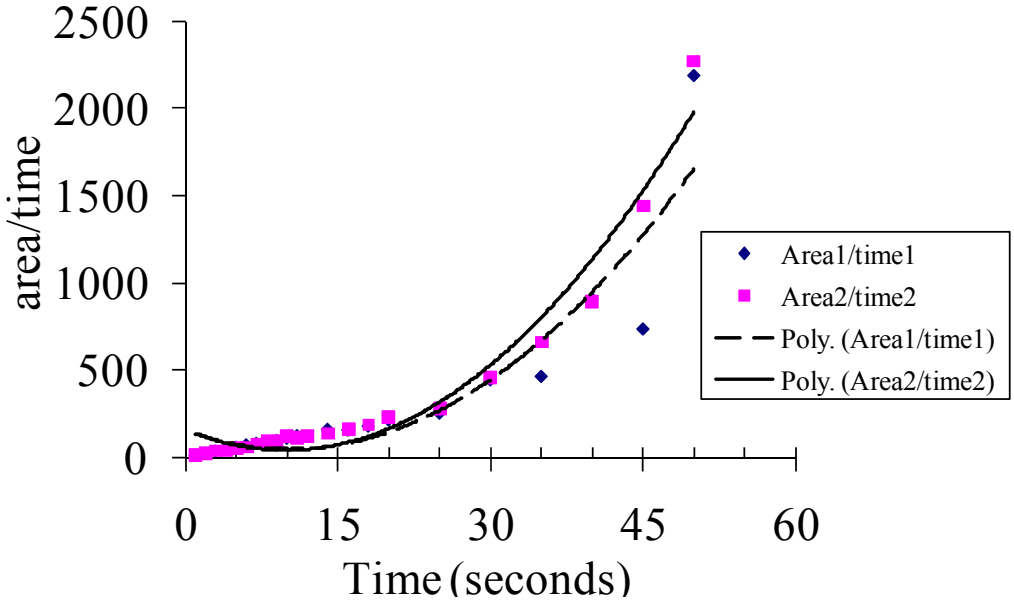


Figure 3.9 Peak area/time of tetracaine at two wave lengths, 210 (Area 1/time1), 312 (Area 2/time2) nm, and over injection time in seconds.

3.8.1.2 Sensitivity

The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) for tetracaine were 100 µg/L and 2.5 µg/mL, respectively.

3.8.1.3 Specificity

Six samples of blank tape and six samples of tape spiked with tetracaine were prepared and carried through the extraction. The concentration of tetracaine used was 2.5 µg/mL (LLOQ) and the internal standard was 150 µg/mL. Tetracaine was selective in λ_{max} of 312 nm (Figure 3.6 and Figure 3.7), and with no significant interfering peaks were found at the migration time of tetracaine or procaine. The signal-to-noise ratio at the LLOQ for both drugs was greater than 3. Figure 3.10 shows the electropherogram obtained from blank tape spiked with IS of 1500 µg procaine, while Figure 3.11, Figure 3.12, Figure 3.13, and Figure 3.14 show the electropherograms of tape placed with 50 µg tetracaine, Ametop gel 50 µg solution, tetracaine solution, and one of the tape samples from the study, respectively, with added IS of 1500 µg procaine.

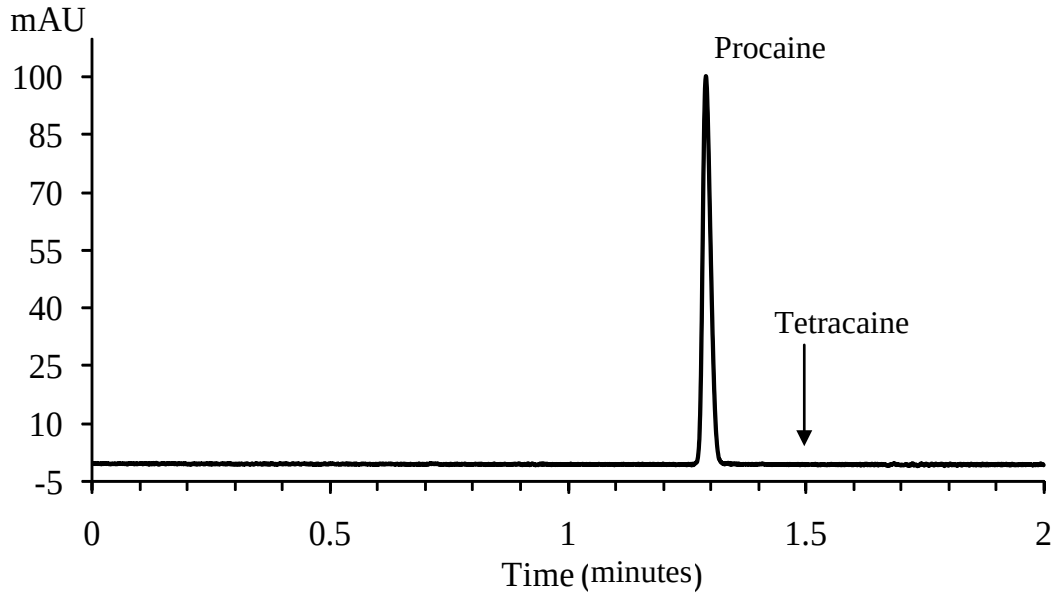


Figure 3.10 Electropherogram obtained from extracted tape sample spiked with IS of 1500 µg procaine, displayed at 312 nm.

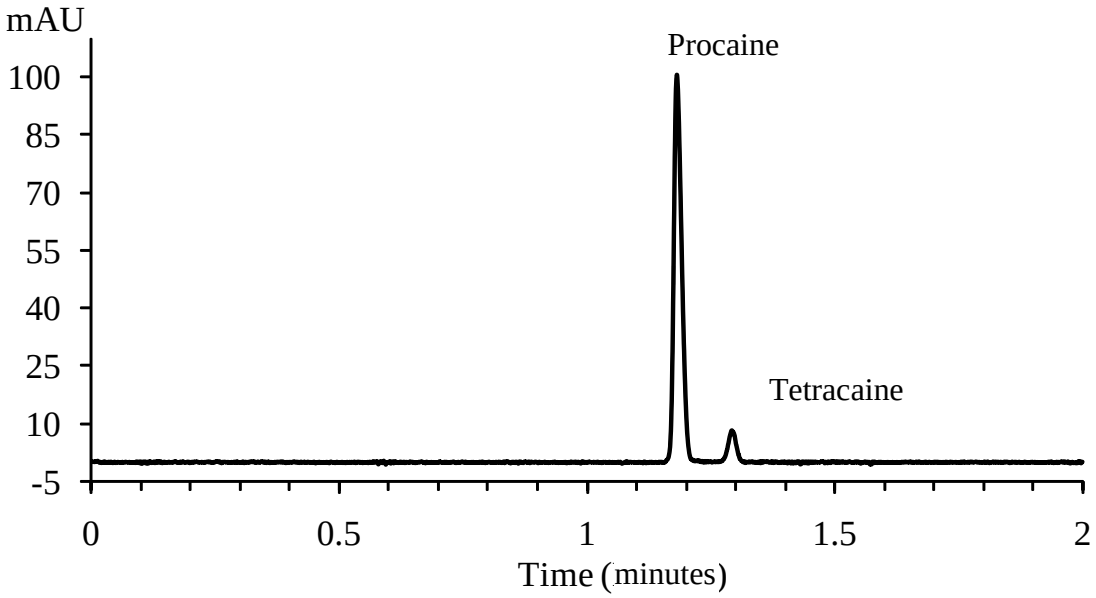


Figure 3.11 Electropherogram obtained from extracted tape sample spiked with 50 µg tetracaine with added IS of 1500 µg procaine, displayed at 312 nm.

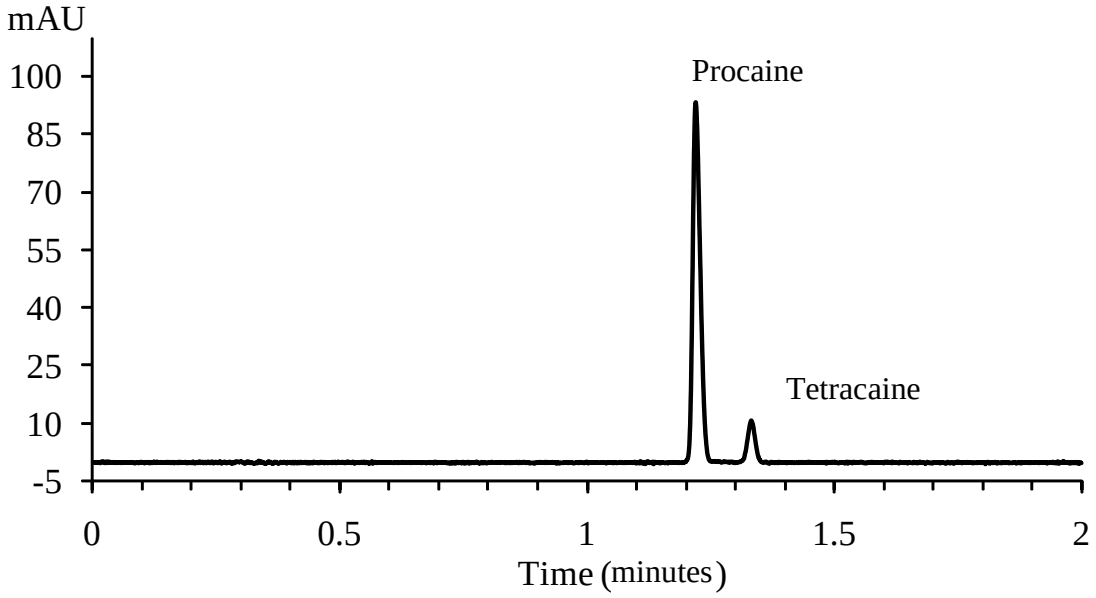


Figure 3.12 Electropherogram obtained from Ametop gel solution containing 50 µg tetracaine with added IS of 1500 µg procaine, displayed at 312 nm.

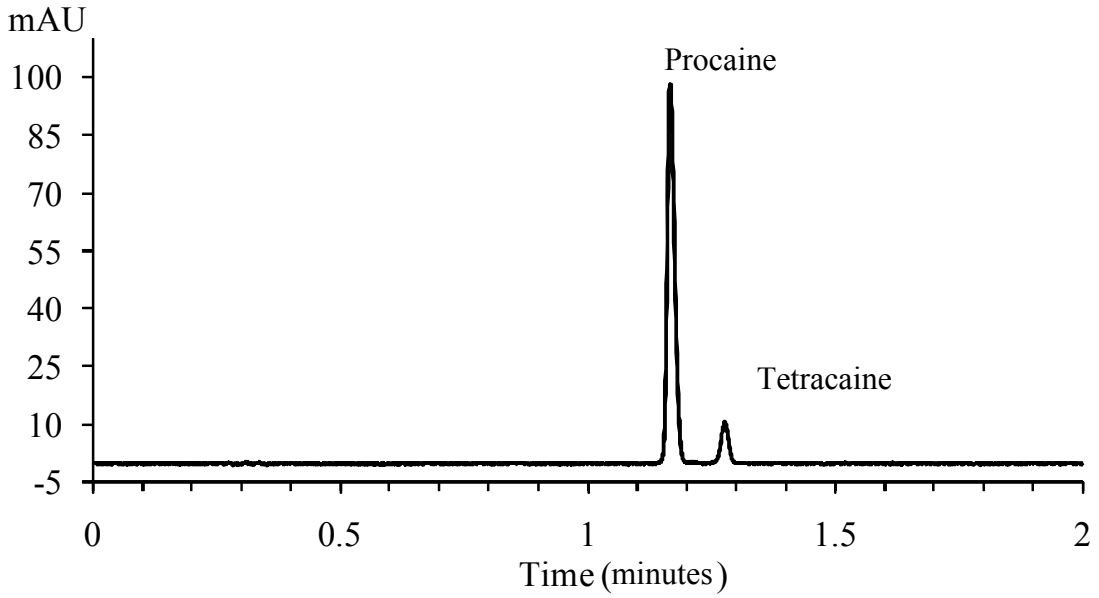


Figure 3.13 Electropherogram obtained from tetracaine solution containing 50 µg tetracaine with added IS of 1500 µg procaine, displayed at 312 nm.

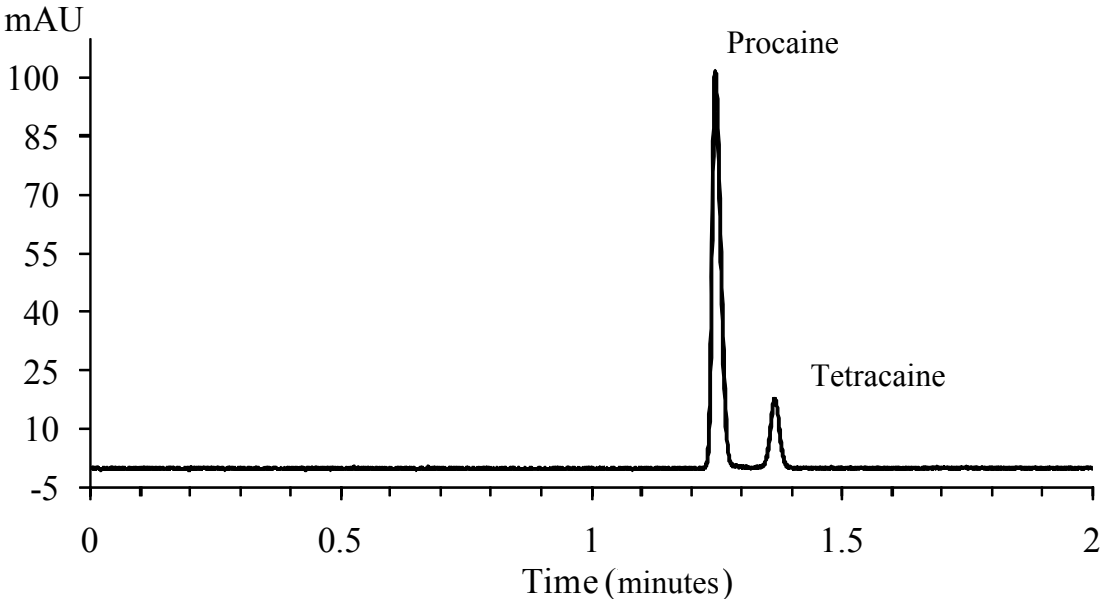


Figure 3.14 Electropherogram obtained from tape sample at 4 hours post dose with added IS of 1500 µg procaine, displayed at 312 nm.

3.8.1.4 Calibration curve/linearity

Figure 3.15 shows a typical calibration line containing seven non-zero calibrators assayed. Nominal values were 50, 200, 400, 600, 800, 1000, 1200 µg tetracaine and 1500 µg procaine. Six batches of calibration curves were plotted using the area ratio of tetracaine to IS vs known concentration of tetracaine. All the results were calculated using a y =AX + B linear regression (Table 3.8).

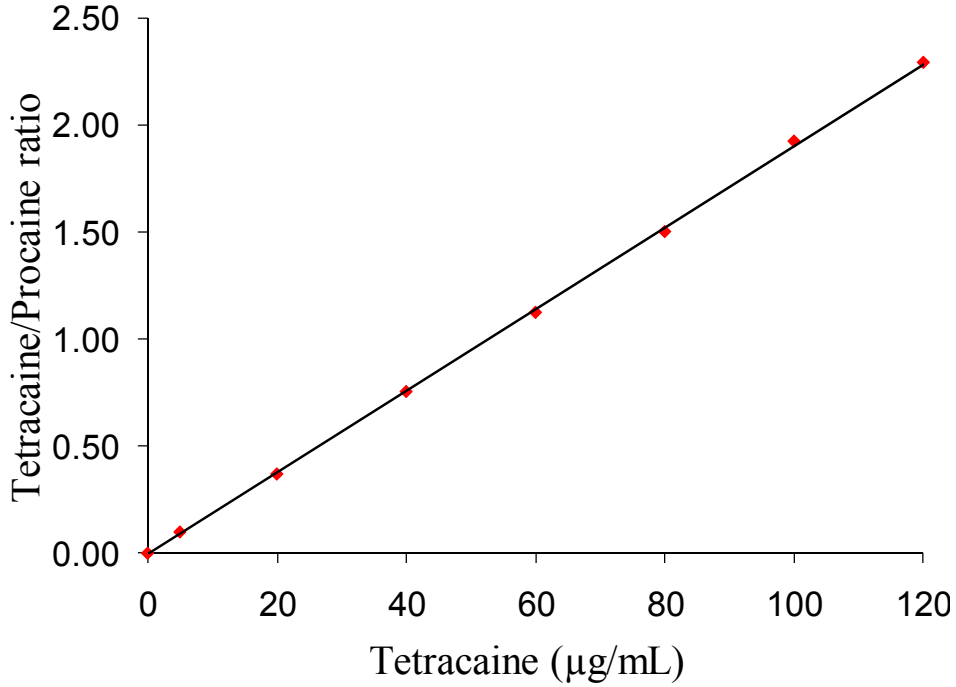


Figure 3.15 Typical calibration curve and linearity of tetracaine (CE).

Table 3.8 Calibration line parameters for 5 separate runs.

Batch	Slope(A)	Intercept(B)	r^2
1	0.0194	-0.0192	0.9992
2	0.0195	0.0099	0.9993
3	0.0194	0.0155	0.9986
4	0.0191	0.0070	0.9998
5	0.0198	0.0041	0.9997

The regression coefficients for all the calibration curves obtained were greater than 0.99.

3.8.1.5 Inaccuracy and imprecision

The within and between batch, and the total variability obtained from the ANOVA are summarized in Table 3.9. The percentage inaccuracy for all the quality control samples including LLOQ and ULOQ was below 11%.

Within assay reproducibility

For within batch and between batch imprecision the LLOQ and ULOQ and the three control samples were each assayed six times in three separate assays. Each assay had an individual calibration curve. The coefficient of variation (CV) for imprecision for all the quality control samples including LLOQ and ULOQ was below 2.5%.

Between assay repeatability

For each of the three assays mentioned above, the mean concentration for each assay was used to calculate the between assay reproducibility. The CV for imprecision for all the quality control samples including LLOQ and ULOQ was below 12.1%.

Within and between batch precision was calculated using internationally agreed methods (FDA, 2001a).

Table 3.9 The within and between batch and the total variability obtained from the nested analysis of variance (ANOVA).

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal conc (µg/mL)	5.00	10.00	25.00	100.00	120.00
Mean; (µg/mL) n = 18	5.53	10.27	27.35	106.36	121.00
Inaccuracy (%)	10.58	2.77	9.42	6.36	0.83
SDw	0.052	0.25	0.31	2.06	1.86
SDb	0.56	1.24	1.61	8.99	3.27
SDt	0.56	1.27	1.64	9.23	3.76
CVw (%)	0.94	2.47	1.13	1.94	1.54
CVb (%)	10.13	12.06	5.89	8.46	2.70
CVt (%)	10.18	12.31	6.00	8.68	3.10

w= within batch; b = between batch; t = total

3.8.1.6 Recovery

The absolute recovery of tetracaine from tape samples compared with tetracaine solution ranged from 97 to 104%.

3.8.1.7 Stability

Stability was assessed both in solution (50% v/v aqueous MeOH) after extraction, and the stability of tetracaine before extraction, during three freeze cycles of 10 (Q1), 25 (Q2), and 100 µg/mL (Q3) stock solutions. Aliquots from these solutions were injected into the CE after each freeze / thaw cycle. Figure 3.16 and Figure 3.17 show that tetracaine 10, 25, 100 µg/mL were stable in tape prior to and after extraction, respectively.

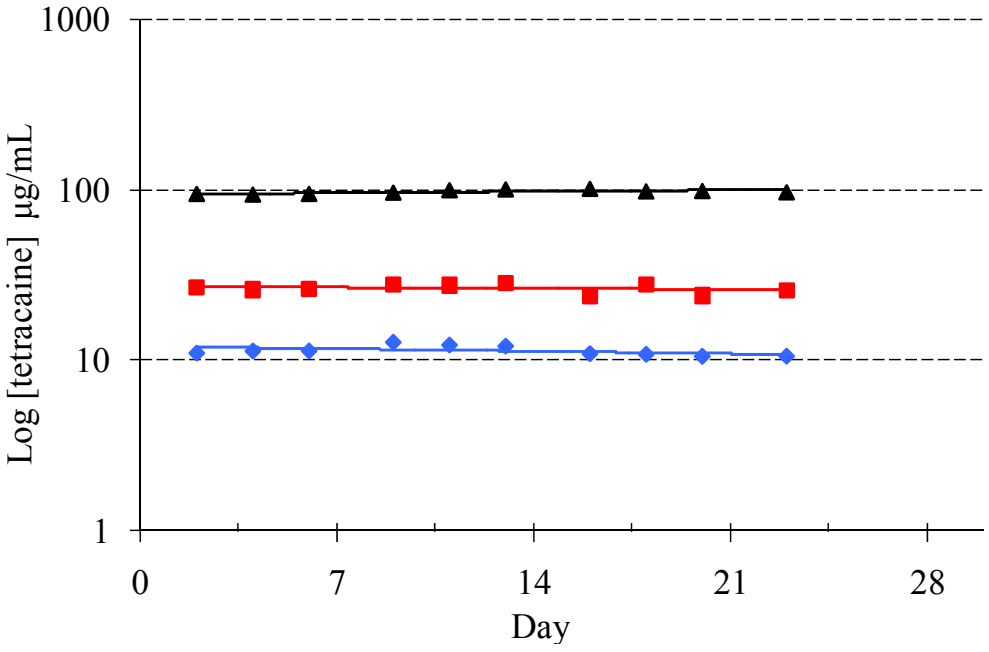


Figure 3.16 Stability measurements of three quality controls, for tetracaine, in tape, following three freeze cycles before extraction.

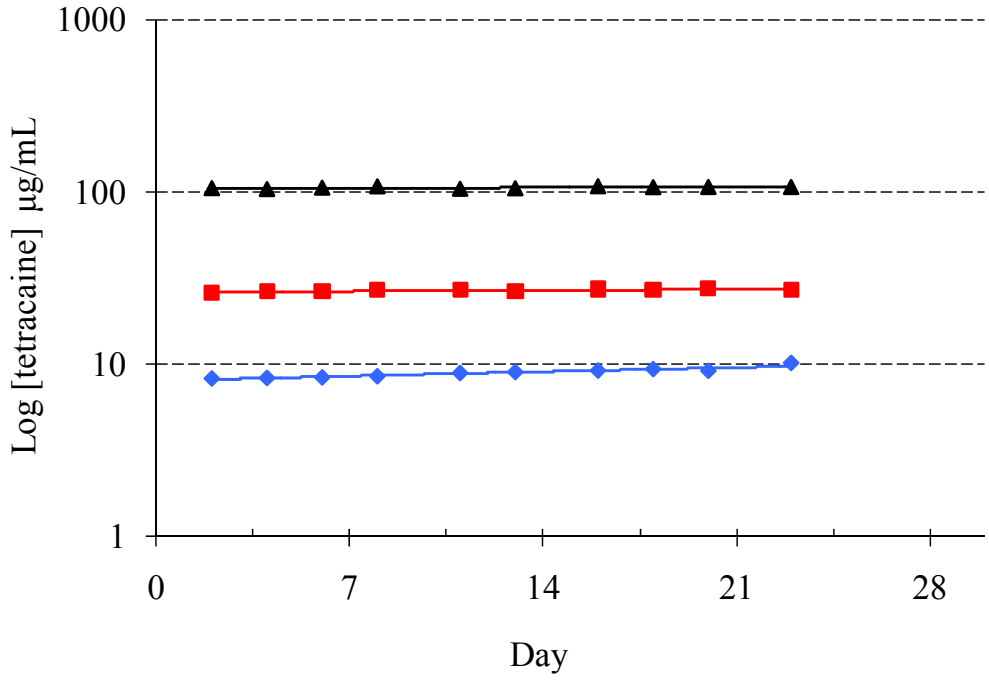


Figure 3.17 Stability measurements of three quality controls, for tetracaine, in tape, following three freeze cycles, after extraction.

3.8.2 Results of method II

3.8.2.1 Sensitivity

The lower limit of detection (LLOD) for tetracaine was 3 µg/L. The lower limit of quantification (LLOQ) was 30 µg/L.

3.8.2.2 Specificity

Figure 3.18 shows a chromatograph of a time zero healthy volunteer sample with no significant interfering peaks at the retention time of procaine (150 µg/mL) or tetracaine, compared with Figure 3.19, 10µg/mL tetracaine, and Figure 3.21, 1 hour post-treatment chromatograph, at λ_{max} of 312 nm.

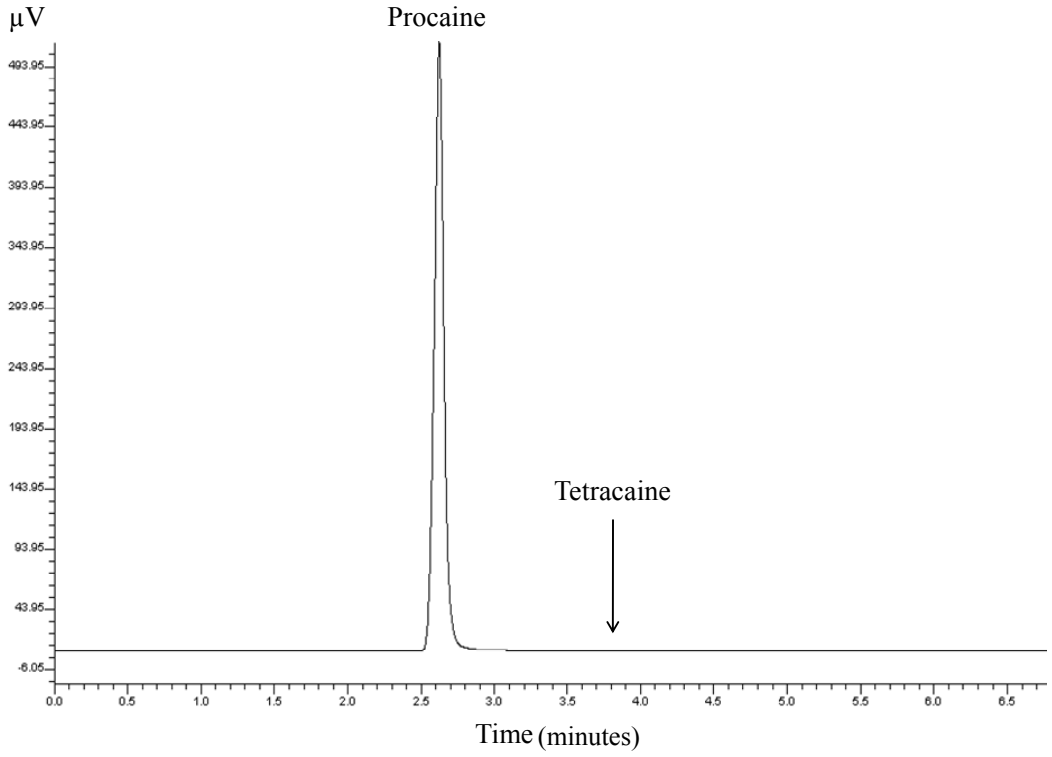


Figure 3.18 Chromatogram obtained from extracted tape of time zero healthy volunteer sample spiked with IS of 1500 µg procaine, displayed at 312 nm.

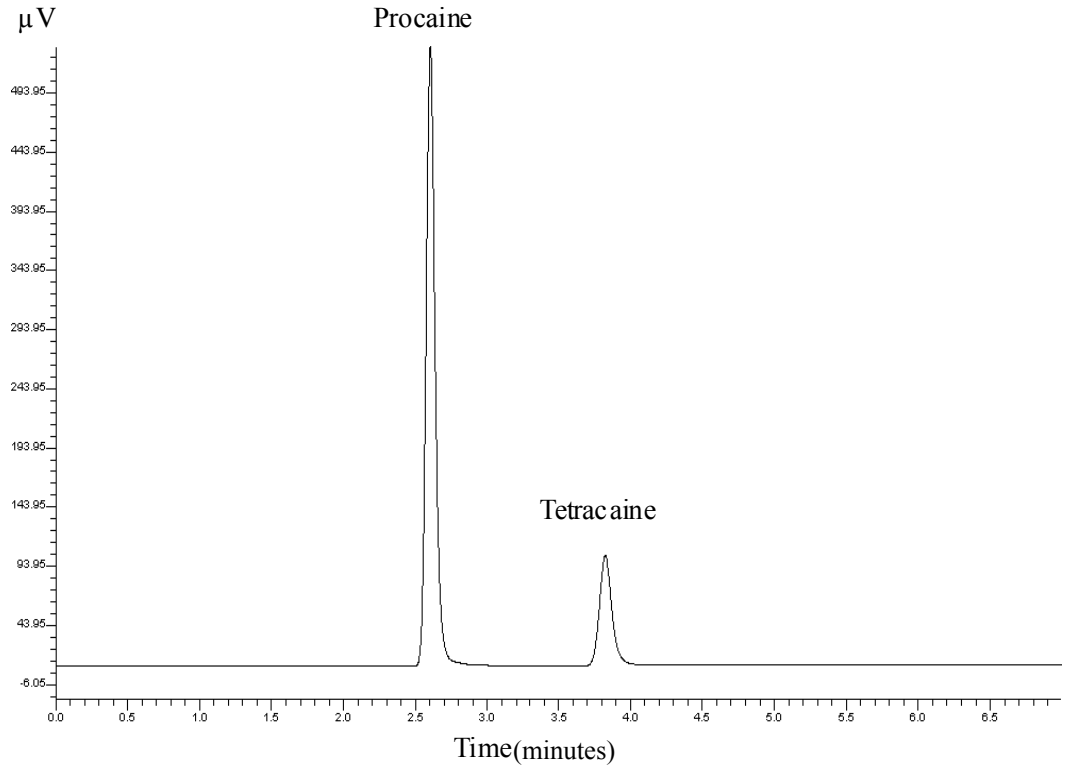


Figure 3.19 Chromatogram obtained from extracted tape of 10µg tetracaine spiked with IS of 1500 µg procaine, displayed at 312 nm.

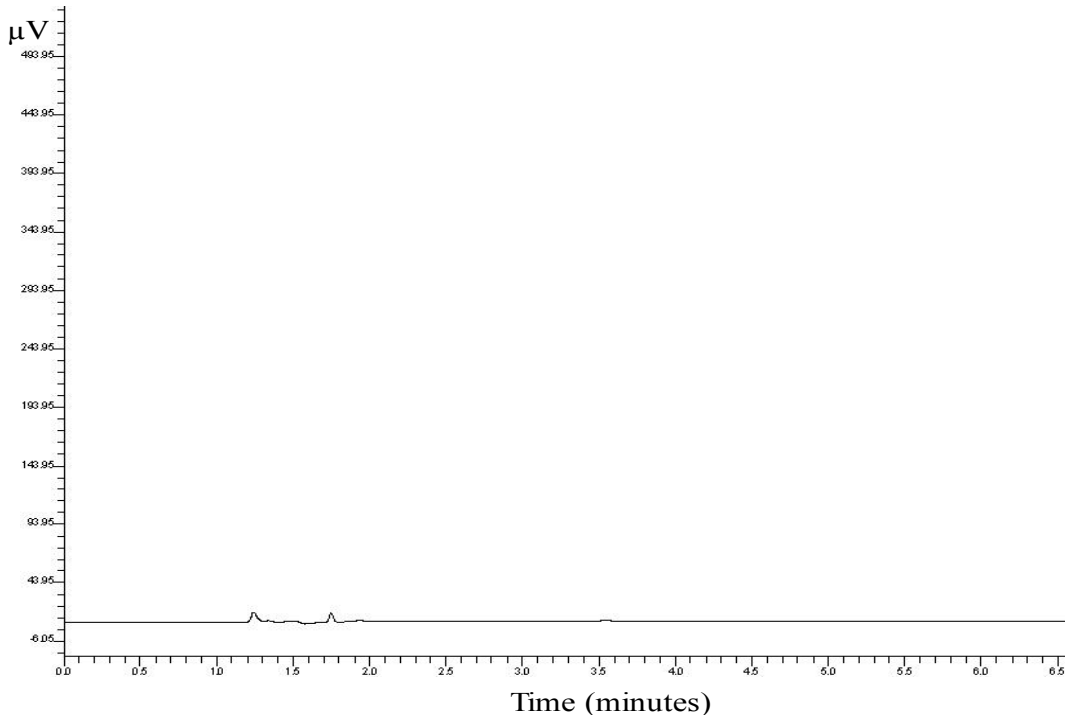


Figure 3.20 Chromatogram obtained from blank tape, displayed at 312 nm.

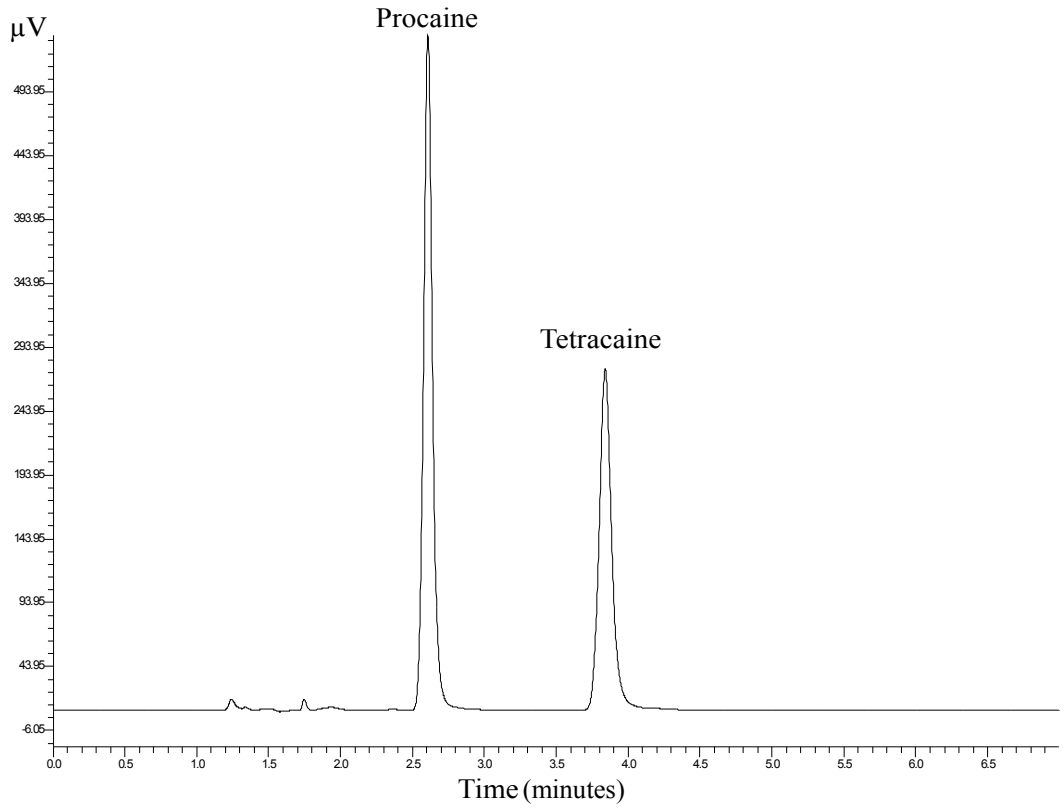


Figure 3.21 Chromatogram obtained from extracted tape of 1 hour post-treatment spiked with IS of 1500 μg procaine, displayed at 312 nm.

3.8.2.3 Calibration curve/linearity

Figure 3.22 shows the calibration line containing the eight non-zero calibrators assayed. Nominal values were 0.3, 1, 3, 10, 30, 100, 300, 1000 μg tetracaine and 1500 μg procaine. Six batches of calibration curve were plotted using the area ratio of tetracaine to IS vs known concentration of tetracaine. All the results were calculated using a $1/x^2$ weighted quadratic regression. Linear regression of slope, intercept, r^2 are shown in Table 3.10.

Table 3.10 Calibration line parameters for 5 separate runs.

Batch	Slope(A)	Intercept(B)	r^2
1	0.0240	0.0002	0.9999
2	0.0239	0.0002	0.9999
3	0.0244	0.0005	0.9999
4	0.0240	0.0004	0.9999
5	0.0242	0.0004	0.9999

The regression coefficients for all the calibration curves obtained were greater than 0.99.

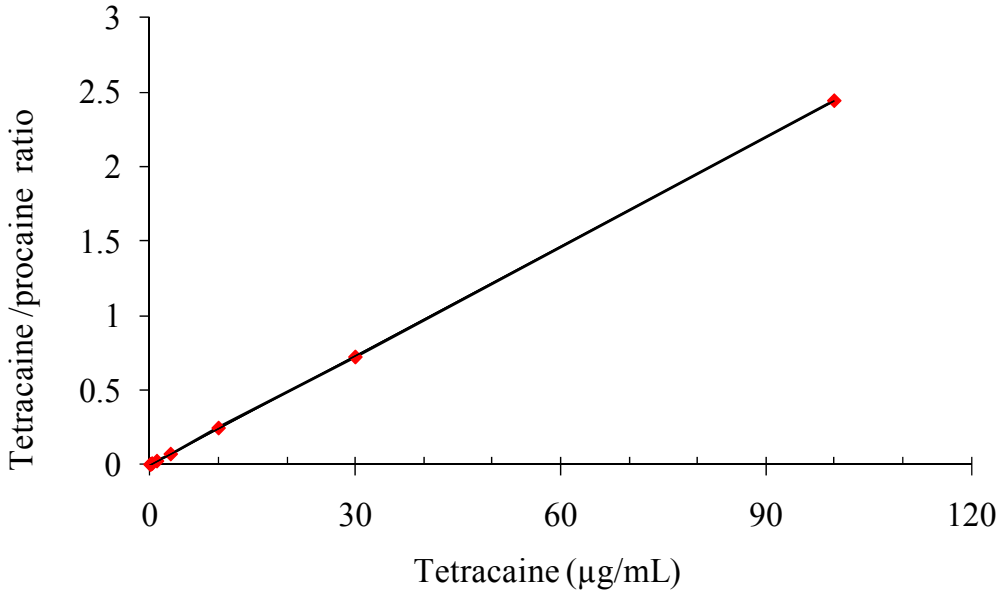


Figure 3.22 Typical calibration curve and linearity of tetracaine (HPLC).

3.8.2.4 Accuracy and precision

Accuracy was tested by determination of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. Each control sample contained tetracaine. The nominal values for the low, medium and high control samples were 5, 50 and 500 µg respectively. The nominal values for the ULOQ and LLOQ were the same nominal concentration as the highest and the lowest calibration standards, respectively.

Assay precision was measured both within and between batch by the analysis of three control samples, the LLOQ and the ULOQ.

3.8.2.4.1 Within assay reproducibility

For within and between batch precision the LLOQ and ULOQ and the three control samples were each assayed six times during one assay. The CV for imprecision and the percentage inaccuracy for all the quality control samples including LLOQ and ULOQ was below 10 and 5%, respectively.

Table 3.11 The within batch imprecision and inaccuracy of three quality controls together with LLOQ and ULOQ.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Nominal Conc (µg/mL)	0.03	0.50	5.0	50.0	100.0
Mean	0.03	0.51	4.86	47.19	95.97
SD	0.001	0.002	0.019	0.105	2.614
CV%	4.02	0.41	0.40	0.22	2.73
Inaccuracy %	3.40	2.65	2.83	5.63	4.25

3.8.2.4.2 Between assay repeatability

The mean concentration from each assay was used to calculate the between assay reproducibility. The CV for imprecision and percentage inaccuracy for all the quality control samples including LLOQ and ULOQ was below 7 and 5%, respectively.

Table 3.12 The between batch imprecision and inaccuracy of three quality controls together with LLOQ and ULOQ.

Data	LLOQ	QC1	QC2	QC3	ULOQ
Nominal Conc (µg/mL)	0.03	0.50	5.0	50.0	100.0
n =	6	6	6	6	6
Mean	0.03	0.51	5.03	48.46	92.29
SD	0.001	0.004	0.083	0.492	0.986
CV%	3.66	0.85	1.65	1.02	1.07
Inaccuracy %	4.38	1.61	1.41	3.46	7.71

Within and between batch precision was calculated using internationally agreed methods (FDA, 2001a).

3.8.2.5 Stability

The CV% of 100 µg/mL tetracaine was less than 2%, when injected into the HPLC after each freeze / thaw cycle.

Table 3.13 Three freeze/ thaw cycles of the 100 µg/mL stock solutions.

Data	Standard 100 µg/mL
Time 0	100.78
Freeze / thaw week 1	101.2
Freeze / thaw week 2	99.79
Freeze / thaw week 3	103.23
CV%	1.42

3.8.2.6 Recovery

The absolute recovery of tetracaine ranged from 79 to 104%. The result was obtained by dividing the peak height for specific tetracaine concentrations in methanol: water, 50:50 solutions and the peak height of the same concentration in skin tape stripping soaked in methanol:water, 50:50 from time zero (sample 0).

3.9 Method comparison

Tape samples obtained from patients' skin were successfully analyzed by two different analytical techniques, short end CE and HPLC. An internal standard was used to minimize injection volume variability, and error result from volatile methanol in sample. The two methods of measurement were compared to find out if there was any difference related to the analysis. The analyzed data were calculated and plotted for both methods, Figure 3.23 shows different percentage errors were in a range of ± 20%. and Figure 3.24 shows Bland–Altman analysis was in a range of ± 1.96 SD from the mean, and Table 3.14 shows the mean, standard deviation, and standard error of the mean percentage error (MPE) and the absolute mean percentage error (AMPE) between the HPLC and the CE measurements.

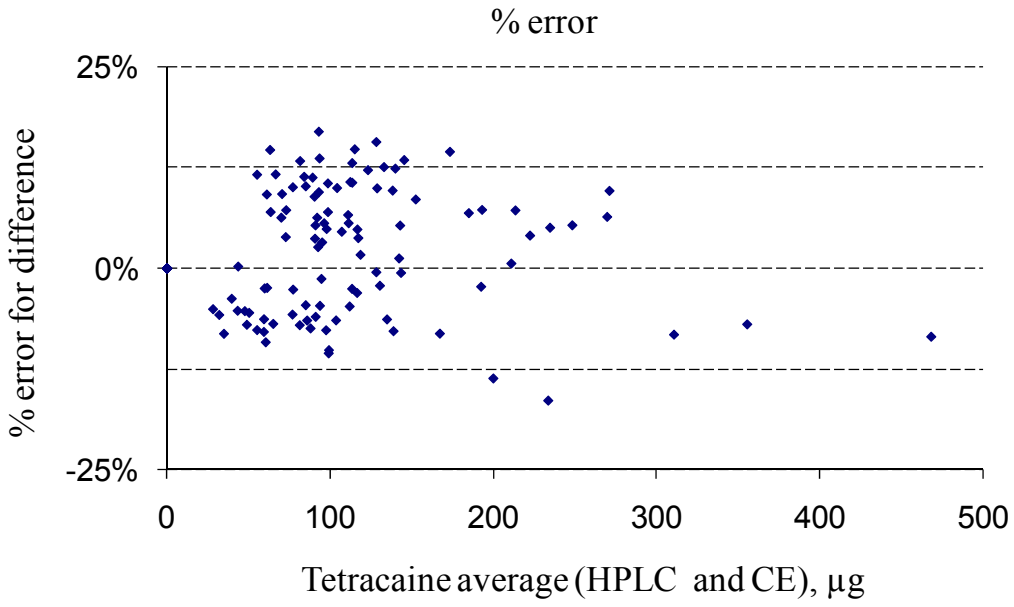


Figure 3.23 The percentage difference error between HPLC and CE methods in the analysis of tetracaine tape stripping samples (n=102).

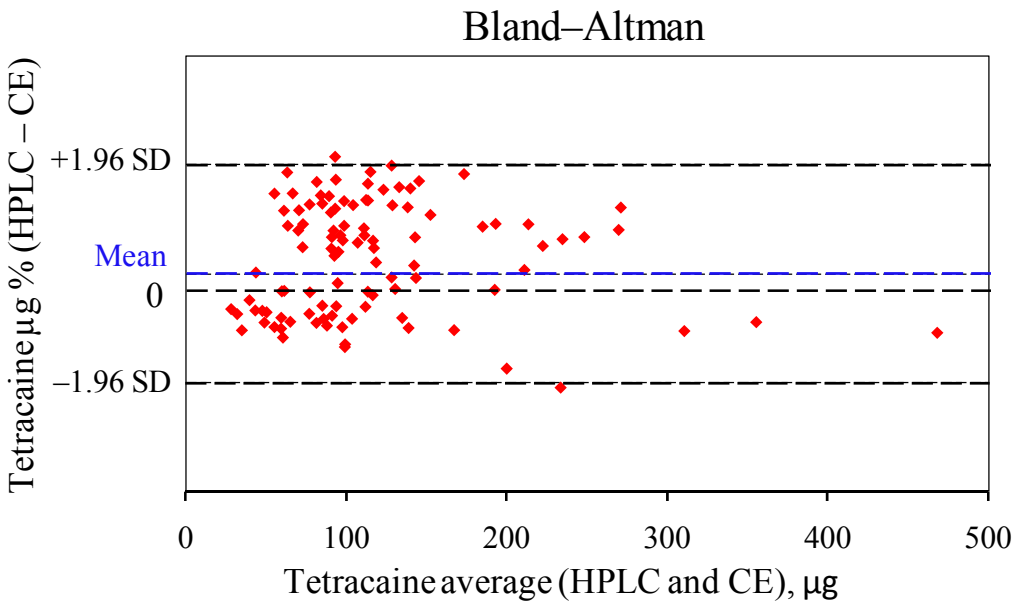


Figure 3.24 Bland–Altman analysis: correlation of HPLC and CE in the analysis of tetracaine tape stripping samples, (SD = ±8.018, Mean = 2.231, n=102).

Table 3.14 The mean percentage error and the absolute mean percentage error between the HPLC and the CE measurements.

Data	MPE	AMPE
Mean	2.23	7.86
SD	8.08	4.63
n	102	102
SEM	0.79	0.46

3.10 Results of method III

3.10.1 Method development

Figure 3.25 shows the UV absorbance of BABA was optimum at 285 nm wave length (λ_{max}). The optimum buffer pH was 8, precise and stable temperature was 20°C, optimum buffer concentration (M) at constant pH was 30 mM disodium tetraborate BGE, and when voltage was applied to the instrument, deterioration in resolution was noted at –

27KV. Additionally the maximum injection time reached was 10s, however 7s was selected, which gave a better peak shape and good resolution.

3.10.2 Sensitivity

The lower limit of detection (LLOD) for BABA was 1 µg/mL. The lower limit of quantification (LLOQ) was 2.5 µg/mL.

3.10.3 Specificity

Six samples of blank tape and six samples of tape spiked with BABA were prepared and taken through the extraction. The amount of BABA used was 2.5 µg (LLOQ) and the internal standard was 1500 µg procaine. BABA was selective at λ_{max} of 285 nm (Figure 3.25), and no significant interfering peaks were found at the migration time of BABA or procaine (Figure 3.26). The signal-to-noise ratio for both drugs was greater than 3. Figure 3.27 shows the electropherogram obtained from blank tape spiked with 100 µg BABA and added IS of 1500 µg procaine, while Figure 3.28 and Figure 3.29 show the electropherograms obtained from a patient sample without, and with added 100 µg BABA, respectively.

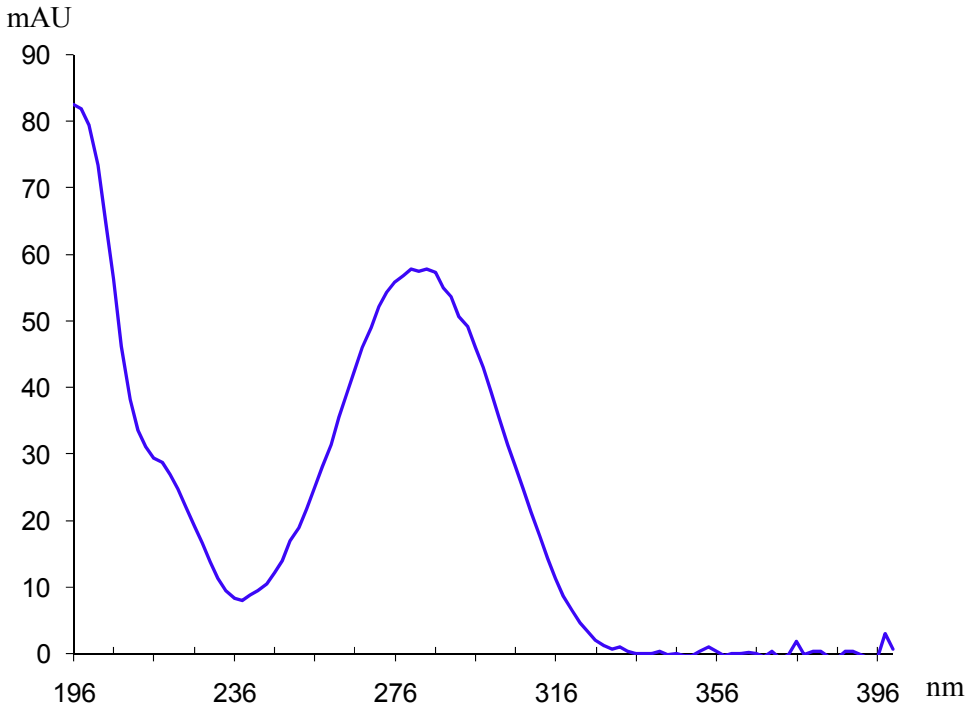


Figure 3.25 UV spectra showing that BABA was selective at 285 nm.

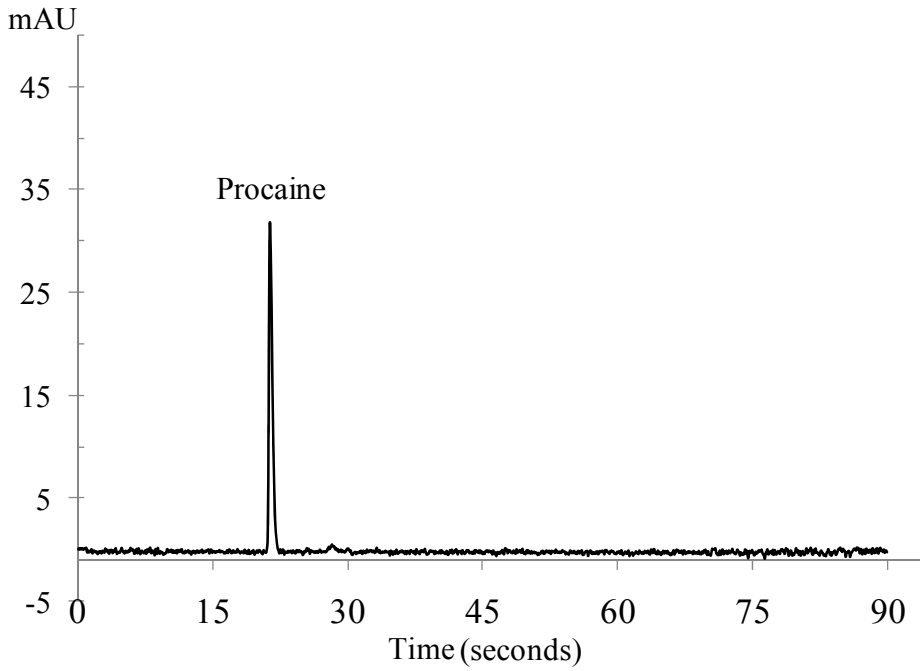


Figure 3.26 Electropherogram obtained from extracted tape sample spiked with IS of 1500 µg procaine, displayed at 285 nm.

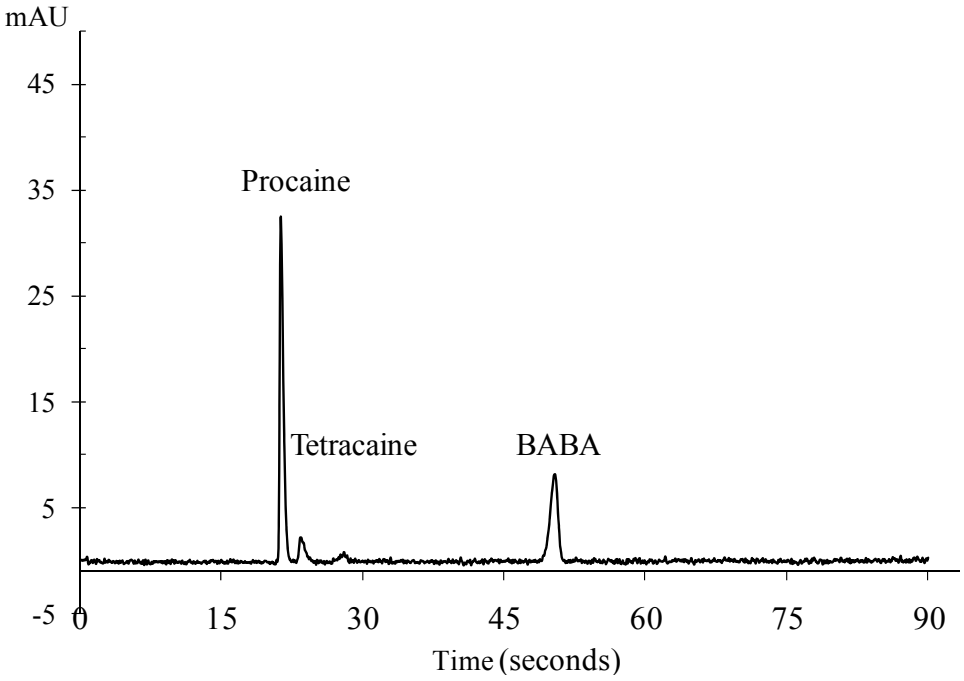


Figure 3.27 Electropherogram obtained from tape sample spiked with 100 µg BABA and 100 µg tetracaine with added IS of 1500 µg procaine, displayed at 285 nm.

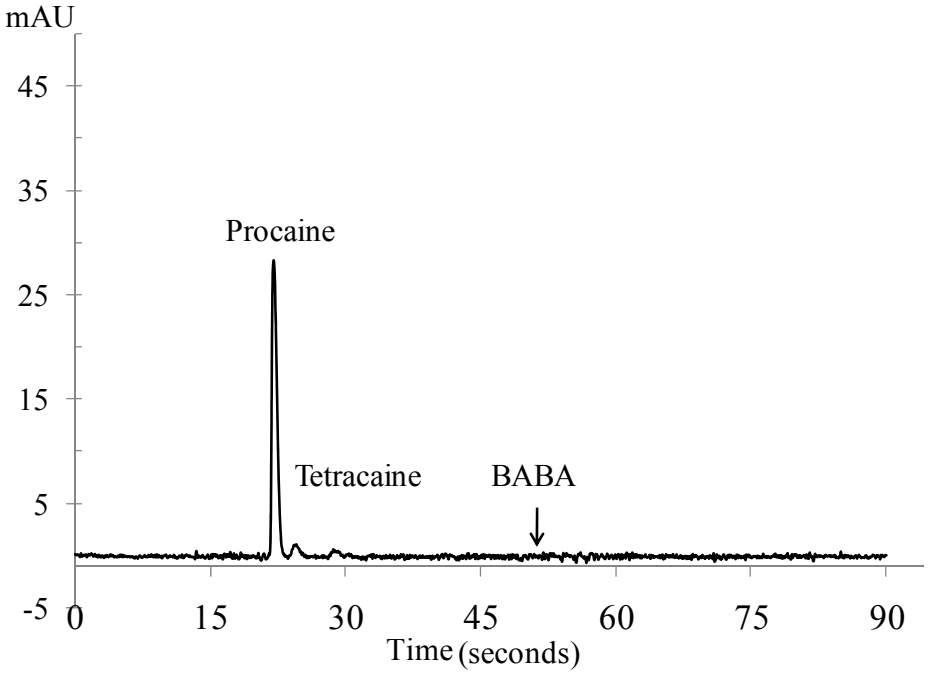


Figure 3.28 Electropherogram obtained from tape sample 1 hour post dose with added IS of 1500 µg procaine, displayed at 285 nm.

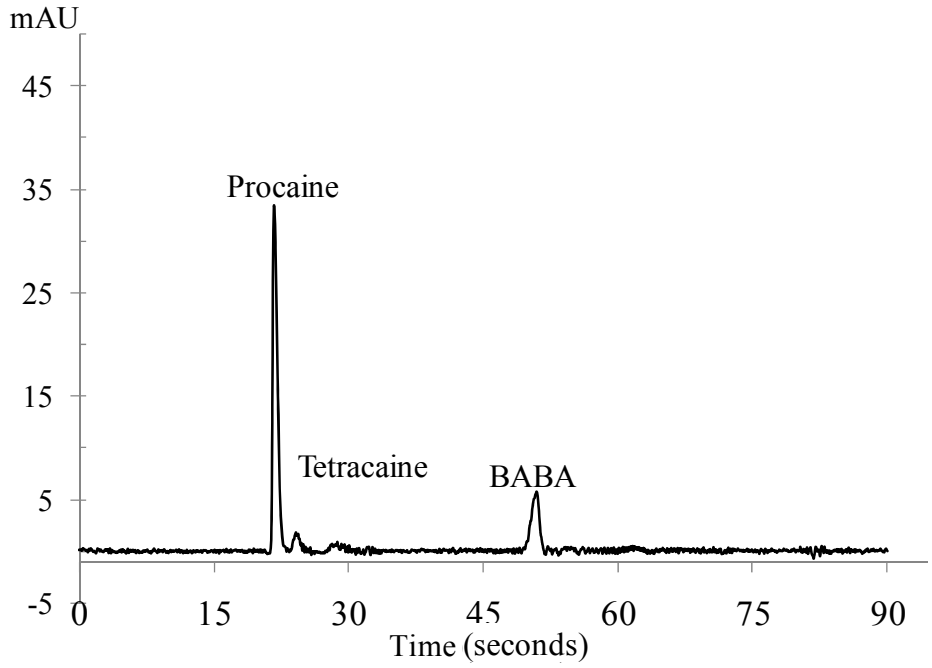


Figure 3.29 Electropherogram obtained from tape sample 1 hour post dose with added 100 μg BABA, and IS of 1500 μg procaine, displayed at 285 nm.

3.10.4 Recovery

The absolute recovery of BABA spiked in tape compared with BABA solution ranged from 89 to 124%.

3.10.5 Stability of samples

The CV% of QC1, QC2, and QC3 of BABA were less than 7%, when injected into the CE after each freeze / thaw cycle.

Table 3.15 Three freeze/ thaw cycles of the QC1, QC2, and QC3 of BABA.

Data	Standard 80 µg/mL	Standard 40 µg/mL	Standard 10 µg/mL
Freeze / thaw week 1	81.95	41.21	8.93
Freeze / thaw week 2	78.30	40.65	9.88
Freeze / thaw week 3	75.26	43.55	8.83
Mean	78.51	41.81	9.21
SD	3.35	1.54	0.58
CV%	4.26	3.68	6.27

3.10.6 Stability of sample injection

The stability of injection samples after adding 30mM BGE, was measured for procaine, tetracaine, and BABA. BABA was stable during the analysis at ambient and 37 ºC temperatures and after at least 10 days refrigeration. Procaine and tetracaine were hydrolyzed at ambient and 37 ºC temperatures, and even when the solutions were refrigerated. The amount of hydrolysis increased with time and as temperature increased. For procaine the hydrolysis was 2.84%, and 11.37%, and for tetracaine the hydrolysis was 2.14%, and 5.80%, at ambient and 37 ºC temperatures, respectively.

3.11 Discussion

The quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic, toxicokinetic, and forensic studies. The huge number of samples generated by these studies necessitates the development of rapid, simple, and valid, analytical methods. Thus the validation of these methods by the latest guidance and principles of validation approved by for example, the FDA, is important and critical.

In this study CE and HPLC methods were developed, optimized and validated. The first technique was a short end injection (reverse) CE method. Applying a short end injection with -ve voltage resulted in tetracaine and procaine being fully separated. However, an attempt to reduce the migration time even further by reducing the capillary length to 35 cm from the original 48.5 cm, thereby increasing the potential gradient, resulted in tetracaine and procaine not being fully separated and was only about 30 seconds faster. The short migration times obtained for procaine (1.25 min) and tetracaine (1.36 min) are an advantage of CE compared with the more than 5 min retention time for tetracaine using HPLC (Mazumdar et al., 1991), and the HPLC method developed here on a pentafluorophenyl (PFP) column. The new HPLC method resulted in a good separation with excellent resolution and minimum tailing. However it is difficult to rationalise the precise separation mechanism on a PFP column. PFP is known to exhibit both normal and reversed phase characteristics (Marin and Barbas, 2006). The HPLC on a PFP column used in this study gave retention times of 2.6 and 3.8 min for procaine and tetracaine, respectively. All the validation results met the international requirements as outlined by the FDA's 2001 bioanalytical method validation guidelines (FDA, 2001a).

CE and HPLC methods with UV detection have been compared for the determination of tape samples obtained from healthy volunteers, given tetracaine as part of a pharmacokinetic study. UV detection was chosen to be used for its simplicity and low cost. Although the HPLC method performed slightly better in terms of sensitivity, both methods in this study measured all samples obtained without any problems, including the lower concentrations obtained in the tape samples. The data obtained were within ±1.96 SD, and 17% error of the difference. Nevertheless the purpose of this study was to develop a fast and simple analytical method, to determine tetracaine in skin using tape samples from 12 healthy volunteers given tetracaine as part of a pharmacokinetic drug delivery study. Although the imprecision was higher, the lower operation costs and volume of electrolyte used, and faster running time made CE preferable to HPLC for this kind of study. This result was confirmed by different authors in a comparison between the two methods (Clohs and McErlane, 2003, Aurora Prado et al., 2005, Muscarella et al., 2005, Kowalski and Plenis, 2007, Carlucci et al., 2007, Kocevar et al., 2008). Sometimes

HPLC was not useful (Hilz et al., 2006), due to the physicochemical properties of the analyte resulting in poor resolution and reproducibility (Bexheti et al., 2006).

In addition BABA has been analyzed by another method using the same samples obtained in this study. The CE method was a very short end injection (reversed). Applying a short end injection (reversed) by –ve voltage resulted in BABA, tetracaine, and procaine being fully separated in very short times 0.85, 0.40 and 0.35 minutes, respectively. Applying this method to the samples resulted in negative value for BABA. This is suggested that the samples did not contain BABA, but tetracaine and procaine were separated. However after adding BGE, tetracaine and procaine were not stable resulting in drug hydrolysis, leading to BABA formation from tetracaine, and an unknown compound from procaine. Procaine and tetracaine were hydrolyzed at ambient and 37 ºC temperature, and even when refrigerated. The hydrolysis increased with time and with temperature increase, for procaine the hydrolysis was 2.8%, and 11.4%, and for tetracaine the hydrolysis was 2.14%, and 5.80%, at ambient and 37 ºC temperature, respectively. The pH was 7.8 close to blood pH. This may promote drug hydrolysis, and as suggested by Iglesias-Martinez (Iglesias-Martinez et al., 2006), who found that procaine hydrolyzed in alkaline media. Tetracaine belongs to the ester group of anaesthetics, metabolized in the plasma by pseudocholinesterase enzyme, resulting in the production of BABA.

3.12 Conclusions

A simple and rapid short end direction (reversed) CE method using CZE with UV detection, and a HPLC method using UV detection to determine tetracaine in skin using tape samples have been developed and validated for separation and quantification. Both methods have simple tape extraction procedures of tetracaine. The accuracy and selectivity of the two methods allowed the measurement of tetracaine in all samples obtained from a skin tape stripping study in healthy subjects. The results are shown in chapter 4. The separation was fast for both methods, especially for short end direction (reversed) CE. Although the imprecision was higher, the lower cost and volume of

solvent used and faster running time made CE preferable over HPLC for this kind of study.

In addition BABA was separated by a rapid and simple method. However, no BABA was detected (less than 2.5 μg/mL) in TS sample from subjects.

Chapter 4 Comparison of tape stripping, microdialysis, and systemic measurement for pharmacokinetic studies

4.1 Introduction

Pharmacokinetics (PK) consists of a drug concentration against time profile. The PK of most drugs are characterized by measurement of plasma concentrations instead of local tissue concentration. Today many drugs are available to use. They are available in different dosage forms, such as oral, intravenous (IV), intramuscular (IM) formulations. Once absorbed from the gastrointestinal tract (GIT), an oral drug is available systemically, before it can be distributed to the site of action. There are many factors that may affect a drug's therapeutic effect such as drug dosage form, absorption, distribution, metabolism, mechanism of action, and elimination. However, systemic measurement of a drug is not useful for all drugs. Topical drug products, such as antibacterial, antifungal, local anaesthetics, topical corticosteroids, act locally. These pharmaceutical drugs are designed to target a specific site in the body, so there is a need to ensure that an adequate amount of drug is distributed in the target tissue. When drug is applied to the skin, the absorption occurs near the site of action. Although it is not clear if it can reach the tissue or other compartments, the concentration of these drugs is mainly localized in the local tissue. The plasma concentration of the drug can be measured but this may not reflect the actual amount of drug available in the local tissue. Although they may be useful in the evaluating the toxicity of the compound (Kudo et al., 2001), plasma measurements may be misleading in the design and development of topically applied drugs. Hence, conventional drug measurement using systemic blood samples has proved inappropriate for this purpose (Benfeldt et al., 2007). When assessing the pharmacokinetics of these kinds of drugs appropriate methods must be applied. Thus, *in vivo* and *in vitro* measurement of a topically applied drug should be obtained by a method that evaluates directly local tissue concentration against time, probably in the local interstitial (tissue) fluid.

The study of drug permeation into the skin is quite simple. Useful information has been obtained via *in vitro* experiments using animal or human skin obtained from a skin bank or by donation from patients after surgery. The human or animal skin is usually used as a membrane to study the skin as a barrier, and to measure the drug diffusion through the skin, using two compartment cell diffusion. Several types of diffusion cell are available, and the most commonly used cell type is the Franz type diffusion cell (Franz, 1975). The Franz cell apparatus used in testing with human skin more closely resembles a topical administration to human skin (Koch et al., 1987). Nevertheless, the data and the results obtained from the preclinical study, especially in transdermal drug delivery, may not be interpreted to represent *in vivo* experiments. The skin obtained may lose its integrity, the barrier may disrupt, and blood supply and metabolic enzymes may not available. In addition, human skin is not always available or not in sufficient quantity. While using animal skin may be possible, the animal model is different to human skin.

To obtain clinical information about a drug in the skin, relevant *in vivo* methods must be applied, especially in a pharmacokinetic study. Many techniques are available for direct assessment of pharmacokinetic profiles in local tissue such as tape stripping (TS) or dermatopharmacokinetic (DPK) (Pershing et al., 1994, Schwarb et al., 1999, Weigmann et al., 1999b, Weigmann et al., 1999a), microdialysis (MD) (Muller et al., 1995, Lorentzen et al., 1996, Fang et al., 1999), or combined microdialysis with other techniques, such as positron emission tomography (PET) (Langer et al., 2005). Microdialysis can be used to evaluate electroporation and transcutaneous sampling (ETS) (Sammeta et al., 2009). Other techniques such as magnetic resonance imaging, skin biopsies, skin blister fluid sampling, confocal microscopy, pharmacodynamic methods and suction blisters to assess skin drug levels following systemic administration, have been explored for measuring bioavailability of topical drug products (Shah, 2001).

Until now the clinical experience in the application of microdialysis and tape stripping has been limited, with little clinical information on the appropriate methods for the measurement of highly lipid soluble and protein bound drugs. Tetracaine is a highly lipophilic and protein bound compound that penetrates the skin and is available in the local tissue for several hours. Thus TS and MD methods were used to determine the

concentration of tetracaine and its metabolite in the different compartments rather than plasma. Both techniques were compared with the conventional systemic method.

4.2 Microdialysis

Microdialysis (MD) is a relatively non-invasive technique, used in clinical and preclinical studies including pathophysiology, pharmacology, and physiology. MD is applied for continuous sampling of endogenous and exogenous substances in the extra cellular fluid (ECF) of the tissue at normal conditions. Also MD can be used to deliver a drug to specific target tissues (Hocht et al., 2007). Microdialysis was developed in 1958 (Kalant, 1958), when Kalant modified the dialysis procedure of Axelrod and Zaffaroni (Axelrod and Zaffaroni, 1954). It was used in neuroscience early on in the 1960s, when scientists were trying to correlate between neurochemistry and behaviour (Groth, 1996). In 1966, Bito et al. (Bito et al., 1966) illustrated that a semi permeable membrane can be used to sample the amino acids and electrolytes in animal brains. The results of using MD in animals encouraged researchers to use it in humans. In 1987, MD was used to understand the characterization of the intracellular space, by measuring glucose concentrations (Lonnroth et al., 1987). Successful results supported the introduction of MD in other experiments, such as:

- The evaluation and interpretation of a drug's concentration and its metabolites, and the investigation of physiological function in the human body, induced by chemicals (Schmelz et al., 1997, Clough, 1999, Morgan et al., 2006).
- The investigation of physiological function in animals (Ungerstedt et al., 2009, Zhu et al., 2009, Wang et al., 2009).
- Using MD for the measurement of the neurotransmitters GABA and glutamate in rat following stimulation with potassium (Buck et al., 2009).
- Using MD to evaluate preferred alternative drug route of administration (Feng et al., 2009).

- Using MD for the measurement of drug distribution (Chang et al., 2009).
- In an *in vivo* study, a MD sampling method was connected directly to chromatography techniques (Johansen et al., 1997), for continuous analysis of the pharmacokinetic time profile of unbound baicalin in rat blood and brain (Huang et al., 2008).

MD is a simple technique to obtain tissue drug concentrations. For this reason it is increasingly being used in drug development and research. The system can be used *in vitro* and *in vivo* for the measurement of drug and/or metabolite concentrations in tissues and organs. This can give useful information to investigate tissue penetration of drugs into a variety of tissues (Chang et al., 2009, Hegemann et al., 1995a, Boelsma et al., 2000b), to identify that adequate drug concentrations are obtained (Feng et al., 2009, Muller et al., 1995), to investigate the physiological function such as neurotransmitters (Buck et al., 2009), and to deliver drugs to target organs or tissues (Hocht et al., 2007).

Compared with intramuscular (IM) and subcutaneous injections, MD causes minimum pain and is easy to perform. MD has several research benefits, starting with continuous sampling, cost-benefit, time, and precise data. To date the MD technique has been used in three possible ways, as an alternative to blood sampling, to collect organ specific data, or as a combination of both ways.

MD can be used in different organs and tissues, hence, different types of MD probes are available for use, including concentric, linear, loop, and side by side (Johansen et al., 1997, Garrisona et al., 2002 , Klimowicz et al., 2004). All catheter probes except linear have a concentric design. Figure 4.1 shows the concentric probe connected to a MD apparatus pump, which was used in this study. The MD apparatus pumps the perfused fluid into the dialysis tube where the exchange of molecules takes place between the extracellular fluid and the Ringer solution, similar to the blood elements exchange (Figure 4.2). MD catheters have been used to examine different human organs, including adipose tissue, brain, breast, bone, lung, gastrointestinal tract, heart, muscle, and subcutaneous tissue. However, the most common organ is the skin, with the most frequently used probe being the linear probe due to its simplicity and low cost

(Kreilgaard, 2002). The probe is implanted either in the dermis or in the subcutis (Anderson, 2006), in parallel. MD has been shown to give a proven estimate of the skin penetration of a substance (Hegemann et al., 1995b, Muller et al., 1997, Benfeldt et al., 1999, Barbour et al., 2009). Boelsma et al. (Boelsma et al., 2000a) have shown that methyl nicotinate can be detected after 1 minute exposure to a concentration of 25 mM, with an increase in the level when the concentration and/or when the application time was increased. Another study showed the benefit of MD to investigate the use of microemulsion formulations in dermal drug delivery (Kreilgaard, 2001). A recent study investigated the response to *in vivo* cytokines generation following an intradermal injection of an allergen (Geraldine et al., 2007). Some other studies used MD to administer a drug singly and in a combination for a continuous dose (Morgan et al., 2006).

4.2.1 The principle of microdialysis

The basic principle of MD has been extensively reviewed, and described in detail (Kreilgaard, 2002, Muller, 2002, Joukhadar and Muller, 2005, Chaurasia et al., 2007, Schmidt et al., 2008). In brief, the principle of MD is to implant a tubular semipermeable dialysis membrane "hollow fibre" into the tissue, which mimics the passive function and movement of molecules into and out of a capillary blood vessel, into a living tissue, similar to that used in an artificial kidney (Anderson, 2006) (Figure 4.2). The hollow fibre is typically 0.2–0.6 mm in diameter, and about 8μm thick. The membrane is made with a very fragile fibre which can easily be damaged. The possibility that a MD probe can be assembled with a puncture needle before insertion of the introducer to the skin has been discussed (Anderson, 2006).

The tubular semipermeable dialysis membrane is connected to a MD pump (a precise syringe drive pump) and constantly perfused with tissue compatible sterile buffer similar to the physiological fluid (the perfusate), at flow rate between 0.1–5 μL/minute (Figure 4.1). Now that the probe is implanted into the tissue, the perfusate diffuses into and out of the semipermeable dialysis membrane to equilibrate with the ECF of the tissue surrounding the membrane. The probe functions as an artificial membrane in the skin.

Molecules diffuse across the membrane from the tissue to the probe and vice versa according to Fick's law of simple diffusion. The exchange of substances occurs due to the concentration gradients, a process called passive diffusion, in this process the degree of equilibration is subject to Fick's second law of diffusion (Stahle, 2000).

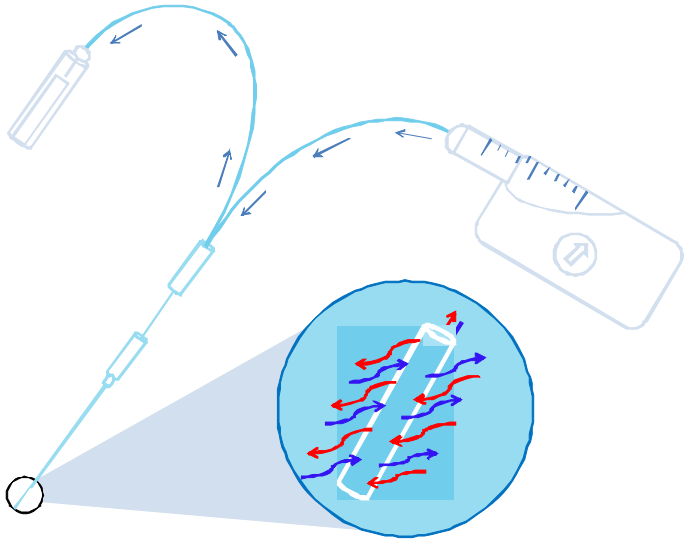


Figure 4.1 Diagram showing the microdialysis catheter with two ends (inlet and outlet) and in the circle the permeable membrane.

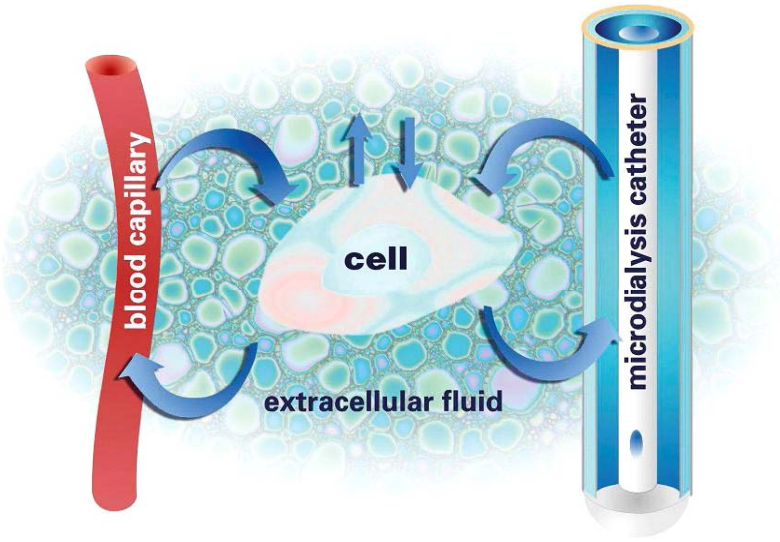


Figure 4.2 Diagram to show the perfusion of fluid through the microdialysis catheter into extracellular fluid compared to the mechanism occurring during blood elements exchange (CMA).

The sample (the dialysate) is collected over time, and stored at –20°C to analyze later for endogenous or exogenous substances. The dialysate contains only unbound molecules. Thus highly lipophilic and protein bound molecules are not detected.

4.3 Tape stripping

Effective drug therapy requires that the active agent should be delivered to the site of action in adequate and sufficient concentrations, with few side effects. Bioavailability (BA) is defined in the FDA CFR 320.1 (a), as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action (FDA, 2008). Thus measuring drug concentration including the metabolites can help to monitor a drug's therapeutic level. Many *in vivo* methods for measuring dermal absorption of drugs are invasive or not feasible (Herkenne et al., 2008), such as a venous cannula inserted into a vein to withdraw blood, where complications may arise in the vein as a result of the cannulation procedure. Complications include haematoma, infiltration (drug not reaching the vein), embolism (occurs when air enters the blood vessel), phlebitis (results from an inflammation of the vein). Other methods need ethical consideration, and are costly. These methods include microdialysis and the skin blister. Some methods are slow, such as collection of urine samples over long periods of time. Thus, there is a significant need for the development of a method to determine drug concentrations locally.

Tape stripping (TS) of the outermost skin layer, the stratum corneum (SC), is a fast and relatively non-invasive technique to measure a drug absorbed into the skin (Tokumura et al., 1999, Reddy et al., 2002, Herkenne et al., 2007). TS is usually performed by placing an adhesive tape strip onto the skin surface. Gentle pressure is applied to ensure a good contact and subsequently the tape is removed by a quick upward movement. The procedure was proposed as dermatopharmacokinetics (DPK) in 1998 in the FDA draft guidance, although, it was withdrawn in 2001 for drugs not targeting the SC. Later, the FDA carried out an investigation to develop a new technique and reinstated DPK in bioequivalence and bioavailability studies of transdermal drugs (FDA, 2002) for a disrupted SC. The SC may be disrupted by dermal disease, physical and mechanical

problems. DPK is relatively painless, given that only dead cells (corneocytes) in the outermost layers in the skin are removed.

The technique supports the development of transdermal drug therapy. However, it is required that the drug must be delivered into the rate-limiting barrier of skin, the SC. In skin stripping studies, an area of skin is exposed to a substance for a period of time and then cleaned of drug residue. The FDA guidance suggests that 12 strips be applied to the area exposed, and they be removed in a sequenced manner to maintain the substance concentration versus time profile. The FDA realized that discarding the first two strips, to clean the residue of the drug, may cause a variable estimate. However, 90% of the drug is found in the first 10 tape strips (Benfeldt et al., 2007), and the quantity steadily decreases with deeper layers (Caron et al., 1990). Thus, using a cotton pad to clean the residue, and then applying 11 strips and discarding the first TS may give a good result.

The determination of the concentration of a chemical in TS is performed in conjunction with a validated bioanalytical assay for the measurement of substances, such as HPLC (Shah et al., 1991), and CE. Tape stripping has been verified as producing a superior and reproducible result in SC harvesting in various studies such as, anti acne (Pershing et al., 2003), dermatologic corticosteroid products (Pershing et al., 2002a), antifungal (Pershing et al., 2002b), and antipsoriasis drugs (Umemura et al., 2008), using the human volar forearm. The results obtained from these studies, can be used to approximate permeability coefficients and partition coefficients.

The current study was performed to quantify the amount of tetracaine delivered through the SC into healthy human skin by tape stripping.

4.4 Ametop

Ametop gel is a tetracaine based formulation. It consists of 4% tetracaine base in a white/yellow semitransparent gel. Tetracaine has a short onset of action and long duration of action, and has become a superior alternative to other local anaesthetics. Similar to EMLA cream, Ametop is a topical drug used for dermal anaesthesia especially in children undergoing invasive operations. Several clinical studies were performed to

compare EMLA and Ametop, and they have concluded that Ametop has a rapid onset of action of 30–45 minutes, and has a longer duration of local anaesthesia, with less methemoglobinemia (Bishai et al., 1999, Shah et al., 2008). The application of Ametop for 30 minutes was clinically equivalent to a 1 hour application of EMLA cream (Bishai et al., 1999, Choy et al., 1999). The short onset of action of Ametop is an advantage especially in busy clinics and wards, by offering time and cost benefits.

The use of topical anaesthetics has increased in recent decades. Most biochemical and pharmacological effects of drugs take place in tissues or cells, especially locally acting drugs which are most closely applied to the site of action. Thus assessing tissue concentration is meaningful and useful for drug pharmacology studies.

Ametop is a local anaesthetic applied to skin, and its concentration is high in local tissue compared to plasma. At this time, MD and TS are available to provide information from the extracellular space and outermost layer of the skin, respectively. Thus, using skin samples is more meaningful than using blood samples, especially when the systemic concentration of tetracaine is low or nil. Many studies did not detect tetracaine, and only detected the metabolite 4-butyaminobenzoic acid (BABA) even in analyzed plasma directly after taking blood (Kudo et al., 2001). Tetracaine is mainly hydrolyzed either in plasma by pseudocholinesterase and/or by esterase in the liver resulting in breakdown of tetracaine into its metabolite (BABA).

The mechanism of action of Ametop is dependent on a phase change in the formulation. Tetracaine forms a meta stable hydrate in the presence of water, resulting in a reduction in the melting point from 42 to 29°C (Dennis et al., 2004). Also tetracaine has higher lipid solubility compared to lidocaine and prilocaine. When tetracaine is applied locally, it melts at skin temperature and forms an oily solution, which facilitates its penetration through the lipid layer. Tetracaine exerts its effect by reversibly blocking the nerve ends close to the site of application, preventing impulses for both conduction and generation. This results in temporary loss of sensation, ‘‘numbness’’, in the local tissue due to the reduction in sodium ion permeability through the channel in the cell membrane, from the intracellular storage of the cell, by competing with calcium, causing an inhibition of

depolarisation. The dermis has nociceptive input which is blocked by the local anaesthetics (Shah et al., 2008).

4.5 Objective

The main objective of this study was to determine the best approach to the pharmacokinetic measurement of topical products. Skin stripping with adhesive tape (TS), microdialysis (MD) fluid, and plasma samples were compared, using the PK analysis of tetracaine and its metabolite (BABA).

4.6 Study approval

The study protocol was approved by the East London and The City Health Authority Research Ethics Committee, reference number 3 of 05/Q0605/98, dated 15th February 2008. Written informed consent was obtained from all healthy volunteers enrolled in the study.

4.7 Materials and methods

4.7.1 Treatment

Ametop gel (containing 4% w/w tetracaine) was supplied in a 1.5 g tube and stored in a refrigerator until the study day for each volunteer (one mL of Ametop gel). This is required by the manufacturer and the characteristics of storage stability.

4.7.2 Apparatus

CMA 60 MD catheters were used together with CMA 107 MD pumps supplied by CMA microdialysis Ltd. (Sweden) for the MD study. Adhesive tape for the TS study was TESA 4204 PV5 supplied by TESA UK Ltd.

4.7.3 Perfusion fluid

Perfusion fluid was an isotonic sterile perfusion fluid with osmolality of 290 mosm/kg, and pH 6. The perfusion fluid contents were Na^+ 147mmol/L , K^+ 4 mmol/L, Ca^{2+} 2.3 mmol/L, and Cl^- 156 mmol/L.

4.7.4 Subjects

Twelve healthy men and women were recruited by advertisement to staff and students of Queen Mary, University of London, Barts and the London NHS Trust, and to the general community, as approved by a Central Office for Research Ethics Committee (COREC).

4.7.5 Study design

The study involved a single dose with two visits, pharmacokinetic study for MD and systemic measurement in one visit, and skin stripping in the second visit in healthy volunteers with a minimum of 1-week gap in between. The study lasted for a maximum of 6 hours for each visit.

4.7.6 Screening evaluation

Prospective subjects attending the study site had a screening visit within 4 weeks of study commencement. In this visit the nature of the study, the procedures, and the risks were fully explained. Before any screening procedures occurred they signed an informed consent form in which they acknowledged that they were willing to be enrolled and would follow all the study protocols. Subjects participating in this study were screened for the inclusion and exclusion criteria referred to appendix 6. This included a medical history, health questionnaires, physical examination (age, blood pressure, heart rate, height, sex, and weight), concomitant medication, and urine tests, a urine specimen was obtained for drug screening, and a pregnancy test for females. The volunteers were fully informed of the nature of the pharmacokinetic study at this time. The subjects were permitted to join the study if they passed the screening.

4.7.7 Study procedure and protocol

Subjects entering the study unit for initiation of the study, had blood pressure and heart rate checked. All hairs were removed from the volar forearm on both hands using a razor. The sites were thoroughly cleaned with water and wiped dry with a towel. The arms were checked for cuts or abrasions which would invalidate the study. The subject layed supine on a bed and one arm was cannulated with a 20G cannula for collection of serial blood samples. The first blood sample (5mL) was drawn immediately after canulation for a baseline reading. On the contralateral arm from the venous sampling, a circular area of 10 cm^2 was marked with a pen in the centre of the volar forearm using a template for the treatment application.

4.7.8 DMD probe implantation

The MD guide cannula was inserted in the dermis (subcutaneously) at about 1 cm below the marked area. Probe implantation was performed without anaesthesia under sterile conditions by a physician using a puncture needle before inserting the introducer. The MD probe was inserted through the tip of cannula and the needle then pull back, leaving the probe implant, about 3 cm long in the dermis (subcutaneous) below the marked area. The inlet and outlet tubing was attached to the skin using self adhesive fabric tape. The inlet tubing was connected to the MD pump and the outlet tubing was connected to the microvial.

Upon successful MD probe implantation, the subject was allowed to recover for 60 minutes to diminish skin reactions (i.e. increase in skin blood flow and histamine release) (Anderson et al., 1994) before the onset of the experiment. The perfusion fluid was perfused for 20 min at a flow rate of 1.0 µL/min and a 5 mL venous blood was collected for a baseline sample. Twenty minutes after collection of the MD sample, Ametop gel was applied to the skin at the marked area and occluded with a dressing for the period of 1 hour. Then, the residual drug was removed and the skin cleared with tissue. Dialysate sampling was commenced for 4 h replacing the micro vial every 20 min. Venous samples were collected at 0, 20, 40 minutes, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h post dose. Both venous and MD samples were stored at –20°C until further analysis by a validated liquid

chromatography-mass spectrometry (LC-MS/MS) method with adequate sensitivity and accuracy to measure a low concentration of drugs in plasma, and the low volume of sample in MD.

After completion of all of the above procedures, both the MD and intravenous catheter were removed and the subject was discharged. The subject had a minimum of a 1-week washout period for the TS study.

4.7.8.1 Implantation of the catheter

A 30 mm length specific catheter with a dialysing membrane was used for MD in the subcutaneous tissue. The probe had diffusion characteristics that permitted exchange of substances in the ECF. The inlet tubing was connected to the pump and the outlet tubing ended in a needle fixed into a microvial holder and the microvial to collect the sample. The microvial was changed every 20 minutes during the study, and then stored at -20° C until the analysis. The catheter was pre-loaded into a unique parallel slit cannula introducer that allowed easy insertion into the tissue.

Prior to catheter implantation into the subcutaneous tissue, the membrane was pre-wetted (without removing the protection tube surrounding the catheter), and checked for its validity using a MD pump to flush the catheter with sterile perfusion fluid. This was to prevent any damage and/or tissue blockage to the probe membrane during the time of insertion into the subcutaneous tissue.

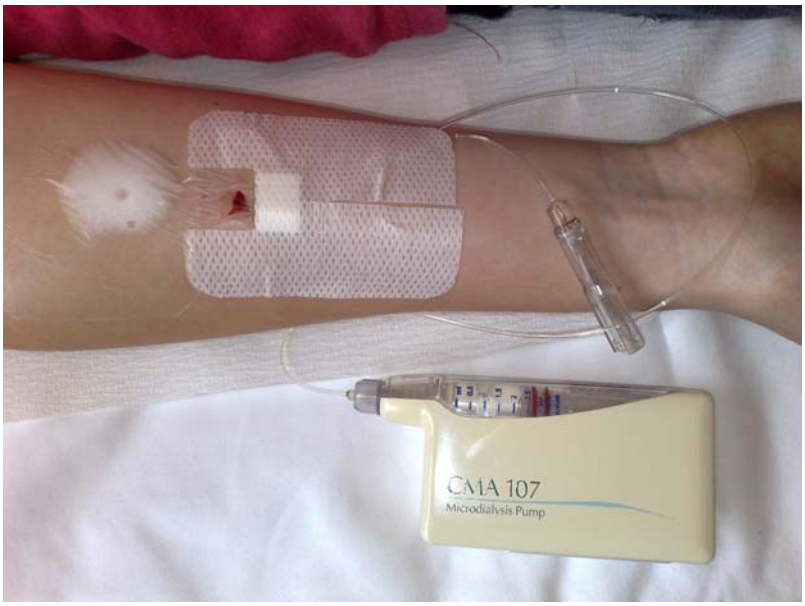


Figure 4.3 Volar forearm picture of a healthy volunteer from the microdialysis clinical study, showing an implanted microdialysis catheter, connected at the outlet with microdialysis pump and at the inlet with a microvial holder and microvial.

4.7.9 Tape stripping procedure

A total of nine rectangular sites (sites 1–9), each 6 cm^2 (2 cm x 3 cm) of skin surface area were demarcated on the ventral forearm using a template. The treated area (sites 2–8) was at the central volar forearm which was 3cm above the wrist and 3cm below the antecubital fossa. Site 1, a minimum of 3cm above the antecubital fossa, served as a control (0 h sampling). The treatment was 1mL Ametop, applied on all of the 8 application sites by using a 2.5 mL syringe followed by an occlusive dressing. Ametop was distributed across the treated area using a metal spatula, and to maintain treatment in its place, subjects were asked to keep their hand in a horizontal position. All the 9 sites represented the uptake and the elimination phase. TS was performed at 0, 15, 30, 45 minutes and 1 hour for the uptake phase (sites 1, 2, 3, 4, and 5) and 1.5, 2, 3, and 4 hours for the elimination phase (sites 6, 7, 8, and 9).

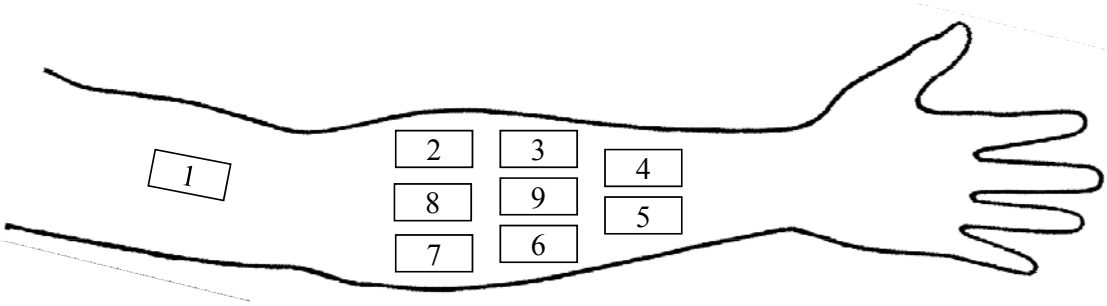


Figure 4.4 Treatment and tape stripping application area on the volar forearm (modified from Barts medical image library at Queen Mary).

4.7.9.1 Removal of the residual products and application of tape to the skin

The SC harvesting was performed directly on site 1 before Ametop gel application. Before SC harvesting from the treated skin sites, the residual product was removed from the skin surface using 3 independent dry cotton pads. The residual product was removed at 15, 30, 45 min from sites 2, 3, and 4. For sites 5 to 9, the residual product was removed at 1 hour. After product removal from the skin, the first adhesive tape was applied and briskly rubbed with blunt ended forceps to harvest the SC. The tape was removed using the forceps. The first adhesive tape was discarded due to the potential contamination from the residual product not removed with dry cotton wool. The remaining 10 adhesive tapes were applied sequentially using the same procedure described above. All the tapes were placed in polypropylene tubes and kept frozen at –20° C until further analysis for the tetracaine content. The product application and SC harvesting were performed by a single person to minimize variability. The forceps used for the application and removal of the tape were wiped with 70% isopropyl alcohol between skin sites to avoid contamination. A pin prick test was performed at the strip site to determine the degree of numbness.

4.7.9.2 Pin prick procedure

In the skin stripping study, the degree of anaesthesia was assessed using a pinprick technique prior to the stratum corneum harvesting at 15 minutes, 30 minutes, 45 minutes, 1h, 1.5h, 2h, 3h, and 4h. The pin prick test was repeated on subjects prior to skin stripping procedures on each site of the study.

Pinprick testing was performed by using a safety pin, which was inserted through a rubber stopper with a mass of 30g. The apparatus was lowered down perpendicularly on the skin and rested gently on the skin surface. The 30g stimulus was to ensure the pressure was always the same for every pinprick and did not penetrate the skin (Bucalo et al., 1998). Pain scores after the pinprick test were recorded using a verbal rating score (VRS) and visual analogue score (VAS). In the VRS assessment, the volunteers were asked the following questions: How strong was the pain of the procedure?, and were provided with a choice of four categories: 1) no pain; 2) minimal sensation; 3) moderate pain; and 4) sharp pain. The volunteer selected one answer for each time point by circling the number. In the VAS assessment, a 100 mm horizontal line with endpoints that were anchored by descriptors ‘no pain’ and ‘sharp pain’ was shown. For each time point, the volunteer was asked ‘What did the procedure feel like?’ and then was requested to make a vertical line across the tramline which represented the intensity or unpleasantness of their pain from the procedure. Values were measured in millimetres from the left of the tramline.

4.8 Analytical methods

Plasma and MD samples were analyzed for tetracaine and BABA concentrations by a LC-MS-MS method. This highly sensitive method was used so that the expected low concentrations of tetracaine and BABA in plasma and the concentrations in the small volume of MD samples (20 µL) could be measured.

In contrast, tape samples contained an expected high amount of tetracaine, so that analysis could be performed by different separation techniques with UV detection. Thus

two different chromatography techniques with UV detection have been used and validated for the analysis of tetracaine. The first was rapid and simple CE (Al-Otaibi et al., 2009), and the second was HPLC. In addition a simple and rapid CE method was developed to detect the BABA concentration in the tape samples. These methods were discussed in detail in Chapter 3.

4.8.1 LC-MS-MS method for the analysis of plasma and MD samples

4.8.1.1 Chemicals

Chemicals are referred to in chapter 3, except clobazam, and bupivacaine hydrochloride were supplied by Sigma Aldrich Company, Poole, UK.

4.8.1.2 Instruments

Solvent delivery was achieved using an Agilent 1100 pump. Sample injection was performed using an Agilent 1100 autosampler, and an Agilent 1100 column oven was used to control the column temperature.

Detection was by Applied Biosystems Sciex API 4000 mass spectrometer.

4.8.1.3 Chromatographic system

A Sciex API 4000 triple quadrupole mass spectrometer equipped with a heated-ion spray (heated electro-spray ionisation, ESI) was used to introduce the sample into the mass spectrometer.

Plasma sample

A 50 mm x 4.6 mm Hypersil 3µ C18-BDS column (Hichrom, Reading, UK) was used, and maintained at 50°C. The mobile phase was 35% acetonitrile: 65% aqueous 0.1% formic acid, pumped at 800 µL/minute. The sample was injected in a volume of 25 µL to the mass spectrometer.

Dialysate

A 100 mm x 4.6 mm Supelcosil 5 µ Silica LC-SI column was used. The mobile phase was 25% acetonitrile: 75% 0.1% aqueous formic acid, and pumped at 1000 µL/minute. The sample was injected in a volume of 25 µL to the mass spectrometer.

4.8.2 Assay procedures

4.8.2.1 Calibrators and quality control samples for MD

4.8.2.1.1 Tetracaine and BABA calibrator

A combined stock solution containing tetracaine, BABA was prepared by pipetting 1 mL of combined solution (5mg/mL) into a 100 mL volumetric flask and making up to the mark with 50% methanol in de-ionized water to produce a sub-stock.

4.8.2.1.2 Tetracaine and BABA quality

A combined stock solution containing tetracaine, BABA was prepared by pipetting 1 mL of combined solution (5mg/mL) into a 100 mL volumetric flask and making up to the mark with 50% methanol in de-ionized water to produce a sub-stock.

4.8.2.1.3 Internal standard

Bupivacaine with final concentration of 100 µg/L was used as I.S. for tetracaine, and clobazam with final concentration of 100 µg/L was used as I.S. for BABA.

4.8.2.1.4 Ringer solution aliquots

Calibrator

1 mL of the stock solution was pipetted into a 50 mL volumetric flask and made up to the mark with Ringer solution to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with Ringer solution as tabulated in Table 4.1.

Table 4.1 Calibration solution preparations from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
8	10	12.50	0.125
7	10	25.00	0.250
6	10	50.00	0.500
5	10	100.0	1.000
4	10	200.0	2.000
3	10	400.0	4.000
2	10	600.0	6.000
Cal 1 = Sub stock		1000.0	

The calibration solutions were stored at approximately 4ºC.

Calibrator 1 and calibrator 8 were used as upper limit of quantitation (ULOQ), and lower limit of quantitation (LLOQ).

4.8.2.1.5 Quality control (QC) samples

0.5 mL of the stock solution was pipetted into a 50 mL volumetric flask and made up to the mark with Ringer solution to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with Ringer solution as tabulated in Table 4.2.

Table 4.2 Quality control preparations from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
1 (low)	10	50.0	1
2 (medium)	10	500.0	10
Cal 1 = Sub stock		500.0	

4.8.2.2 Calibrators and quality control samples for plasma

4.8.2.2.1 Tetracaine and BABA calibrator

A combined stock solution containing tetracaine, BABA was prepared by pipetting 1 mL of combined solution (0.5mg/mL) into a 100 mL volumetric flask and making up to the mark with 50% methanol in de-ionized water to produce a sub-stock.

4.8.2.2.2 Tetracaine and BABA quality control

A combined stock solution containing tetracaine, BABA was prepared by pipetting 1 mL of combined solution (0.5mg/mL) into a 100 mL volumetric flask and making up to the mark with 50% methanol in de-ionized water to produce a sub-stock.

4.8.2.2.3 Internal standard

Bupivacaine with final concentration of 100 µg/L was used as I.S. for tetracaine, and clobazam with final concentration of 100 µg/L was used as I.S. for BABA.

4.8.2.2.4 Calibrator samples

1 mL of the stock solution was pipetted into a 50 mL volumetric flask and made up to the mark with 50% acetonitril to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with 50% acetonitril as tabulated in Table 4.3.

Table 4.3 Calibration solution preparations from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
7	10	0.50	0.05
6	10	1.00	0.10
5	10	5.0	0.50
4	10	10.0	1.00
3	10	25.0	2.50
2	10	50.0	5.00
Cal 1 = Sub stock		100.0	

The calibration solutions were stored at approximately 4ºC.

Calibrator 1 and calibrator 8 were used as upper limit of quantitation (ULOQ), and lower limit of quantitation (LLOQ).

4.8.2.2.5 Quality control (QC) samples

0.5 mL of the stock solution was pipetted into a 50 mL volumetric flask and made up to the mark with 50% acetonitril to produce a sub-stock (cal1). Then working calibration solutions were prepared by diluting the sub stock (cal 1) with 50% acetonitril as tabulated in Table 4.4.

Table 4.4 Quality control preparations from Sub stock (Cal1).

Cal No.	Total Volume (mL)	Assay conc (µg/L)	Volume of sub stock (mL)
1 (low)	10	12.5	2.5
2 (medium)	10	40.0	8.0
Cal 1 = Sub stock		50.0	

4.8.2.3 Extraction

A simple liquid-liquid extraction was used for plasma samples.

Dialysate mixture

10 µL dialysate were transferred to a tube containing 100 µg/L clobazam, and 100 µg/L bupivacaine in 1mL of 50% methanol, and then mixed before injection.

Plasma extraction

100 µL plasma samples were transferred to a tube, to which was added 500 µL acetonitrile and 25 µL internal standard. The contents were mixed and centrifuged at 13684 g (Relative Centrifugal Force (RCF)) for 5 minutes at room temperature. The clean sample in the top layer was then taken and injected.

4.9 Results

Twelve healthy volunteers, 6 males and 6 females (Table 4.5) were recruited and successfully completed the study according to the protocol of the tetracaine study, with no side effects/events observed during or after the study. All of the samples obtained from the TS study were successfully analyzed by two validated techniques CE-UV, and HPLC-UV. The two methods have been compared and tested by Bland Altman to assess the percentage error of the difference. Both tests were shown to have an acceptable difference. In addition, tape samples have been analyzed for BABA in another CE-UV method. The results showed no BABA was detected in the samples. All of these methods have been described in detail in Chapter 3. In contrast, MD and plasma samples were analyzed by a LC-MS-MS method to detect tetracaine and BABA. The LLOQ was set at 5.0, and 12.5µg/L for plasma analysis, and microdialysis sample, respectively. The separation was 1.0, and 1.9 minutes for BABA and tetracaine, respectively. The data analysis shows a quantified BABA concentration in both MD and plasma, although tetracaine was detected but not quantified in MD samples.

Table 4.5 Demographic data for 12 subjects in tetracaine pharmacokinetic study.

Subject	Age (years)	Sex	Height (cm)	Weight (kg)	Body mass (kg/m^2)
S01	26	F	166	56	20
S02	22	M	171	57	19
S03	34	M	174	78	26
S04	40	M	168	65	23
S05	29	F	157	55	22
S06	28	M	170	59	20
S07	28	F	160	47	18
S08	23	F	169	56	20
S09	24	F	165	58	21
S10	24	M	174	71	23
S11	31	M	171	76	26
S12	21	F	158	74	30

The analyzed data obtained from TS, MD, and plasma have been plotted against time profiles, tetracaine concentration versus time shown in Figure 4.5 for TS, and BABA concentration versus time in Figure 4.6 and Figure 4.7, for MD and plasma, respectively. In MD two subjects were not included in the results due to failed canulations. Figure 4.8 shows the plots of TS, MD, and plasma profiles on a log scale. The plots and data obtained demonstrated that the C_{max} concentration of tetracaine was 3 times higher in the SC compared to BABA concentrations in the extracellular dialysate and 10 times higher in plasma. Tetracaine was detected in the SC at 15 minutes after the application of Ametop, but was not detected in either MD or plasma samples. However, BABA was detected at 15 minutes for the dialysate, and 40 minutes for plasma samples.

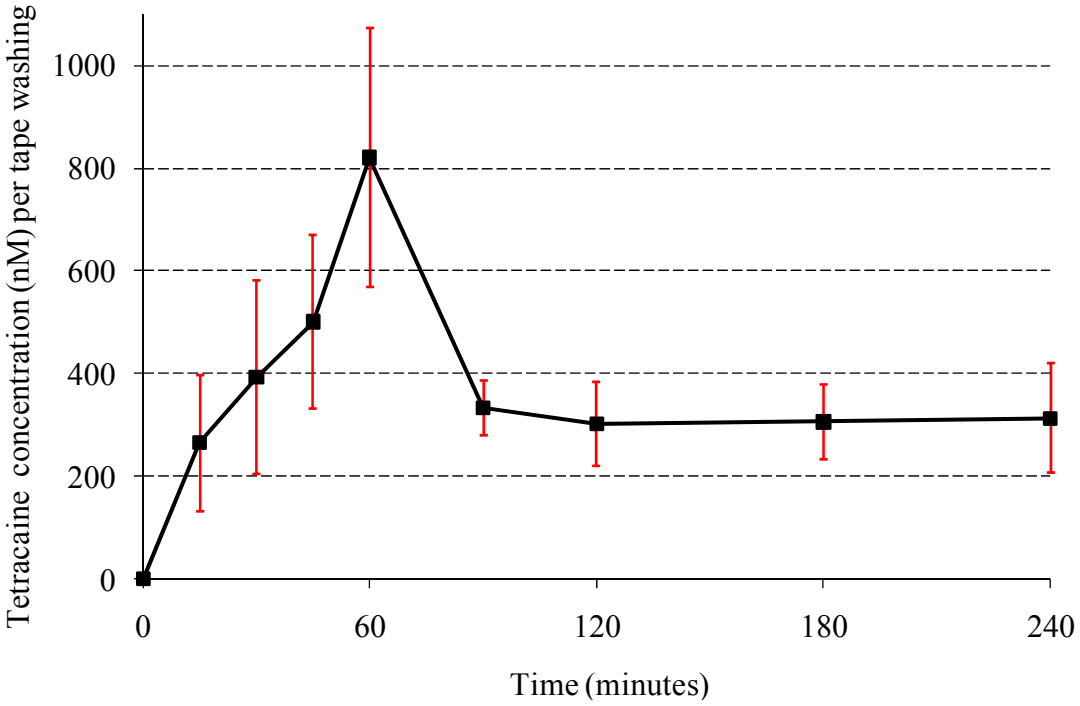


Figure 4.5 Mean tetracaine (nM) versus time (minutes) in tape samples following a 1mL dose of Ametop gel (4% w/w tetracaine), linear concentration axis (n =12, Error Bars= 95% CI).

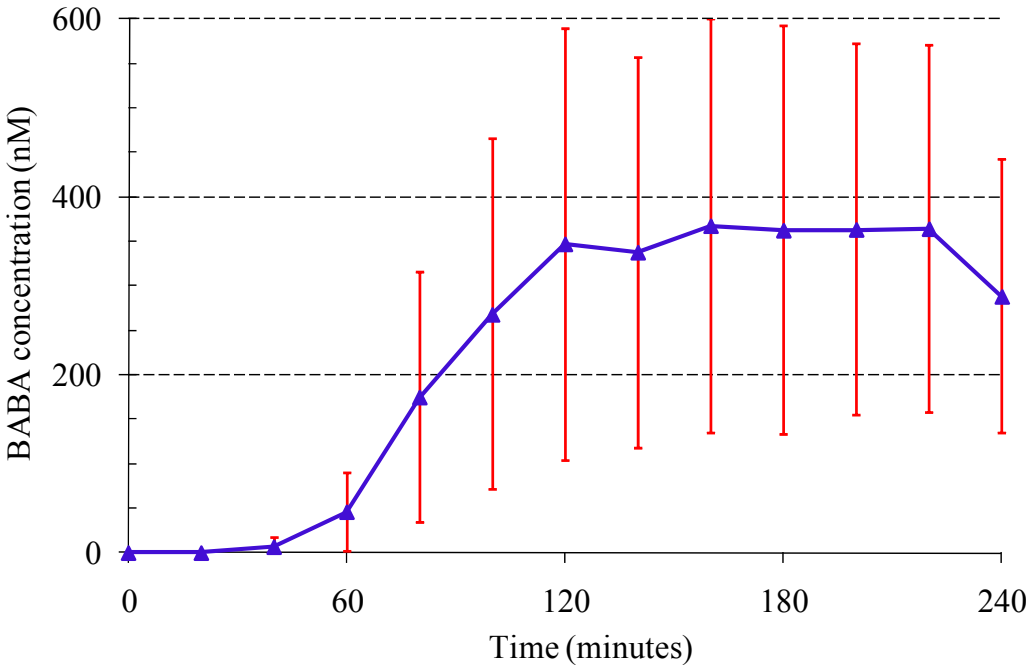


Figure 4.6 Mean BABA concentration (nM) versus time (minutes) in MD samples following a 1mL dose of Ametop gel (4% w/w tetracaine), linear concentration axis (n =10, Error Bars= 95% CI).

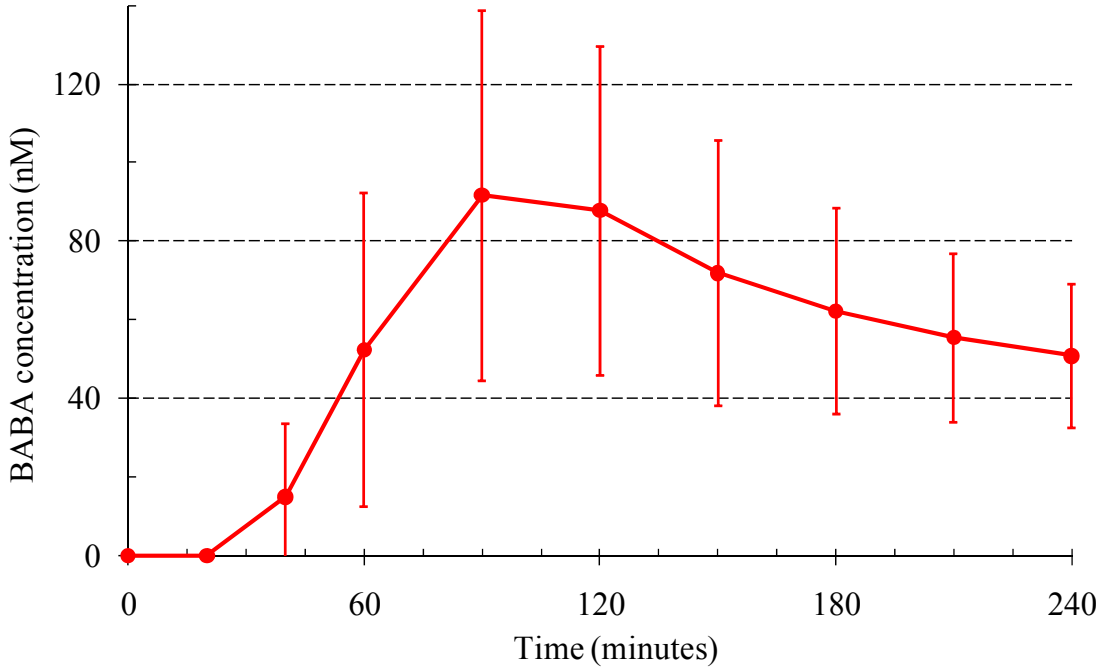


Figure 4.7 Mean BABA concentration (nM) versus time (minutes) in plasma samples following a 1mL dose of Ametop gel (4% w/w tetracaine), linear concentration axis (n =12, Error Bars= 95% CI).

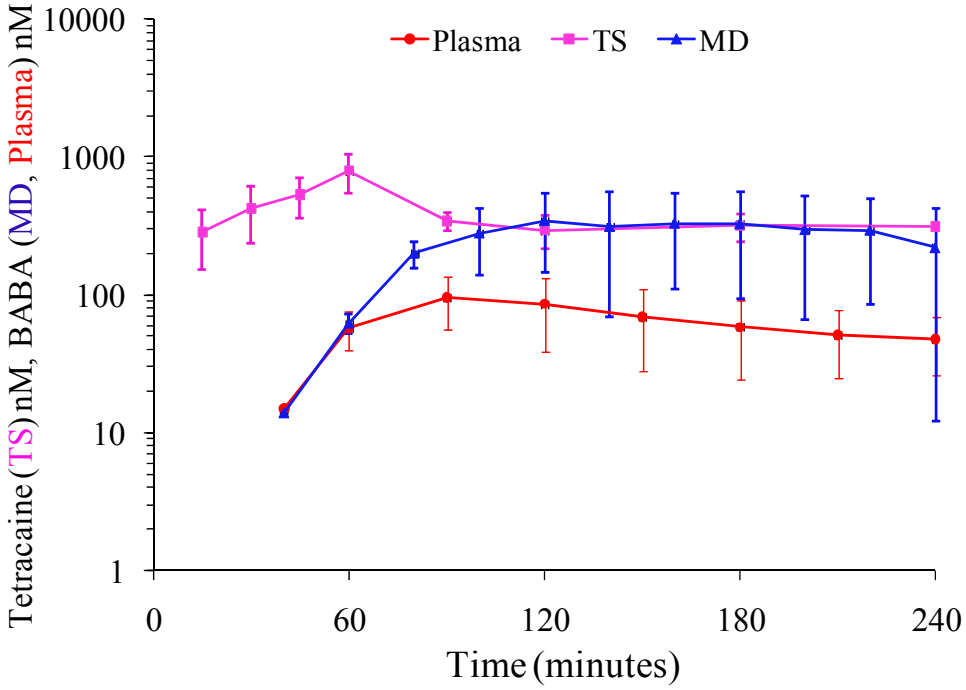


Figure 4.8 Mean tetracaine concentration (nM) in tape samples, and BABA concentration (nM) in MD and plasma, versus time (minutes) following a 1mL dose of Ametop gel 4%, logarithmic axis (n =10, Error Bars= 95% CI).

In addition, the data were analyzed for pharmacokinetic parameters of tetracaine and BABA. Table 4.6 and Table 4.7 show the higher AUC and C_{max} for tetracaine in TS compared to BABA in MD and plasma (Mean $AUC_{0\text{-}4h}$: 88582 nM.min, 55594 and 13208 nM.min: Mean C_{max} (0–4h), 850 nM, 459, 110 nM, respectively).

Tetracaine was detected in TS samples reaching the maximum concentration faster than BABA in dialysate and plasma samples. Wilcoxon's Signed Rank Test, showed a significant statistical difference (p= 0.002) between TS t_{max} and plasma t_{max}, and the median t_{max} was higher in plasma (IQR -52.5 min, CI -105, and -30) compared with tape samples. However, the t_{max} for MD cannot be predicted as the concentration of BABA was endlessly increasing to the last dose time points of sampling (240 minutes).

Table 4.8 show the t_{max} of plasma and TS samples. The mean $t_{1/2}$ of BABA in plasma was 106 minutes with the CV% 45% (Table 4.9).

Table 4.6 Tetracaine $AUC_{0\text{-}4h}$, data obtained from TS, and BABA from MD, and plasma.

Subject	TS	MD	Plasma
	(nM.min)	(nM.min)	(nM.min)
1	60412	25232	14300
2	108703	12378	27576
3	77323	16285	10331
4	104382	124963	17250
5	75094	92512	10536
6	123283	41682	22794
7	58969	12481	1614
8	81534	NA	16005
9	101624	14789	885
10	80046	124331	13402
11	95990	91296	13395
12	79730	41615	6427
	n = 12	n = 11	n = 12
Mean	87258	54324	12876
SD	19639	45171	7805
CV %	23%	83%	61%
	n = 10	n = 10	n = 10
Mean	88582	55594	13208
SD	21436	47406	8284
CV%	24%	85%	63%

NA: not available, n: sample number, TS: tape stripping, MD: microdialysis, nM: nano molar, SD: standard deviation, CV: coefficient of variation.

Table 4.7 Tetracaine C_{max} (0–4h), data obtained from TS, and BABA from MD, and plasma.

Subjects	TS	MD	Plasma
	(nM)	(nM)	(nM)
1	427	255	111
2	1030	88	205
3	761	109	90
4	868	894	194
5	803	914	85
6	840	458	183
7	355	107	22
8	618	NA	114
9	1075	112	15
10	724	869	106
11	1622	792	93
12	1223	288	83
	n = 12	n = 11	n = 12
Mean	862	444	108
SD	346	353	61
CV%	40%	80%	56%
	n = 10	n = 10	n = 10
Mean	850	459	110
SD	354	368	66
CV%	42%	80%	60%

NA: not available, n: sample number, TS: tape stripping, MD: microdialysis, nM: nano molar, SD: standard deviation, CV: coefficient of variation.

Table 4.8 Tetracaine t_{max} (0–4 h), data obtained from TS, and BABA from plasma.

Subjects	TS	Plasma
	(minutes)	(minutes)
1	60	60
2	30	120
3	60	90
4	45	90
5	60	90
6	60	90
7	45	240
8	60	120
9	60	240
10	45	120
11	60	120
12	60	90
Mean	54	122.4
SD	10.2	57.6
CV%	19%	47%

TS: tape stripping, SD: standard deviation, CV: coefficient of variation.

Table 4.9 BABA $t_{1/2}$, data obtained from plasma.

Subjects	Plasma $t_{1/2}$ (minutes)
1	115
2	65
3	81
4	53
5	133
6	76
7	NA
8	208
9	NA
10	87
11	132
12	NA
Mean	106
SD	48
CV%	45%

NA: not available, SD: standard deviation, CV: coefficient of variation.

4.9.1 Least square regression

The $t_{1/2}$ of tetracaine and BABA could not be analyzed from TS and microdialysis samples, respectively (Figure 2.9and Figure 2.10).

Equation 4.1 was fitted to the data using non linear least square regression. The sum of squares between the measured values (yi) and the fitted values (ŷi) (Equation 4.2) was minimised using the Solver add-in of the Microsoft Excel 2007 program.

$$C_t = C_0(e^{-\lambda t} - e^{-k_a t})$$

Equation 4.1 Non linear least square regression.

C_0 = hypothetical concentration at time node (intercept).

C_t = concentration at t time.

λ = elimination (slop).

Ka = absorption (slop).

$$\sum (y_i - \hat{y}_i)^2$$

Equation 4.2 The sum of squares between the measured values (yi) and the fitted values (ŷi).

y_i = measured concentration value.

$\hat{y}_i$ = fitted concentration value.

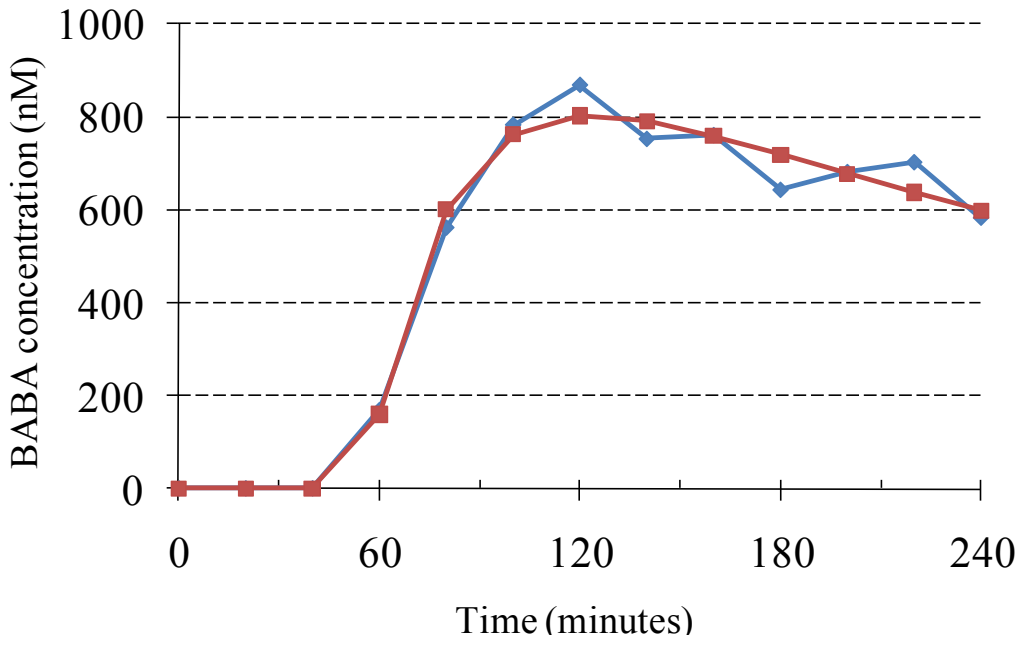


Figure 4.9 BABA concentration (obtained from one subject) with time profile (minutes) in MD samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

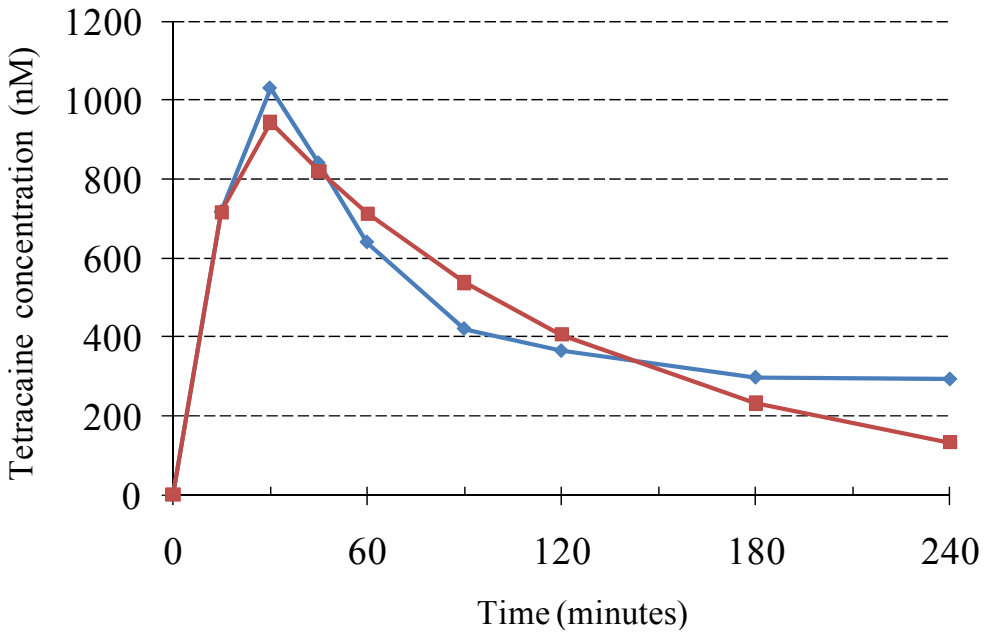


Figure 4.10 Tetracaine concentration (obtained from one subject) with time profile (minutes) in tape samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

In addition, the AUC and C_{max} values demonstrated that the TS study showed low variability compared to plasma and most variability for MD (CV%; $AUC_{0\text{-}4h}$, 24, 63, and 85%: C_{max} (0–4h), 42, 60, 80%, respectively). Figure 4.11 and Figure 4.12 show the plot of Test for Equal Variances (Bartlett's and Levene's test) for AUC and C_{max} from TS, plasma and MD studies. The wider CI can be seen clearly in MD compared to plasma, and the smaller CI in TS for both pharmacokinetic parameters, AUC, and C_{max} (Bartlett's test, p= 0.004 for AUC; and Levene's test, p=0.042, and 0.028, respectively).

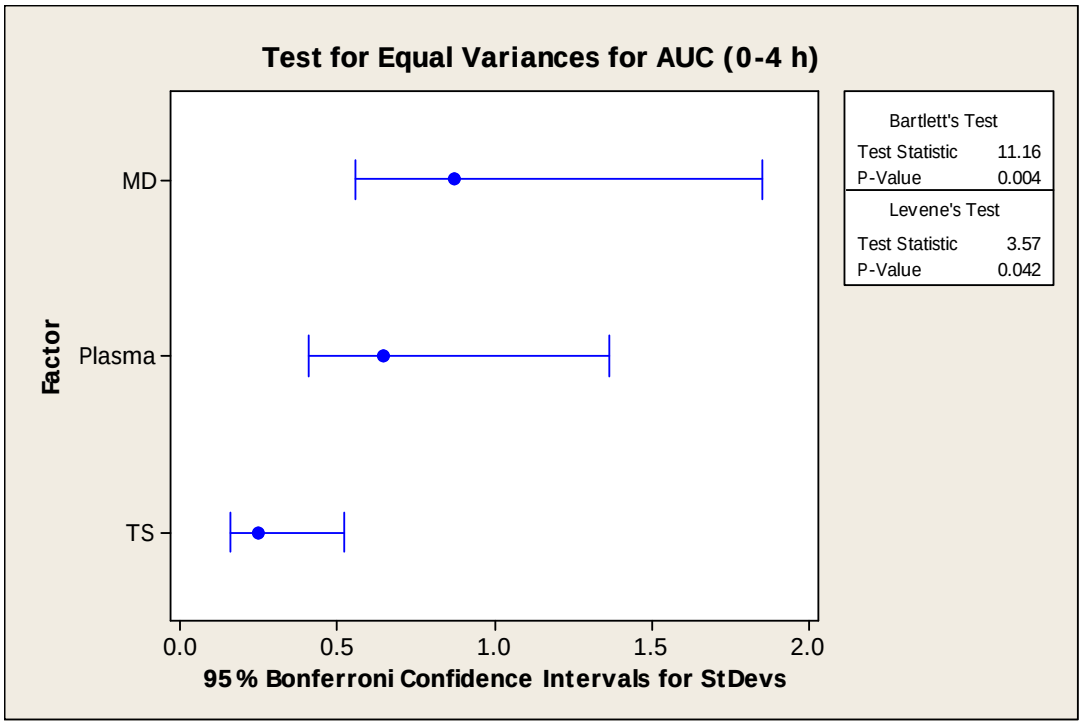


Figure 4.11 Plot of Test for Equal Variance for AUC, with 95% Confidence Interval for tape stripping (TS), plasma and microdialysis (MD).

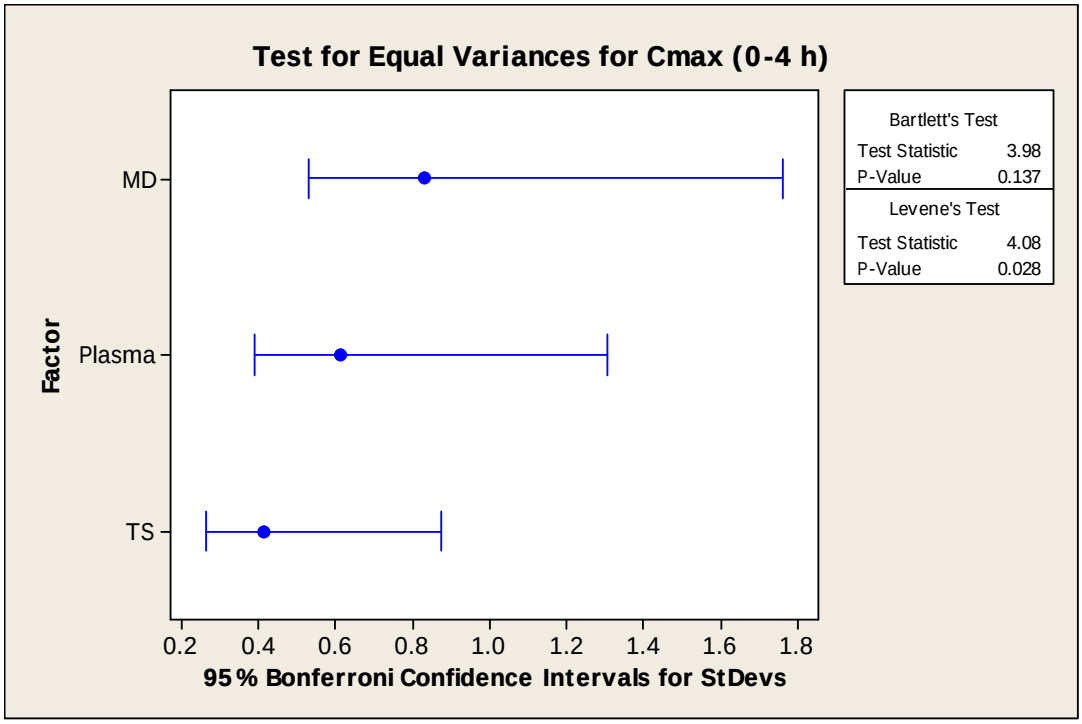


Figure 4.12 Plot of Test for Equal Variance for C_{max}, with 95% Confidence Interval for tape stripping (TS), plasma and microdialysis (MD).

4.9.2 Correlation between TS, plasma and MD

Tetracaine was only quantified in samples obtained by TS with a C_{max} concentration 3 times higher and 10 times higher compared to the metabolite (BABA) concentrations in extracellular dialysate and plasma, respectively. The three measurements differed significantly in the AUC, and also the t_{max} and C_{max} values. A matrix plot for the AUC and C_{max} (Figure 4.13 and Figure 4.14) showed no correlation between the three measurements. An additional test, Pearson Correlation Coefficient was used for further investigation to determine if any correlation existed. However, the results showed there was no correlation between the three methods for all three PK parameters (Table 4.10).

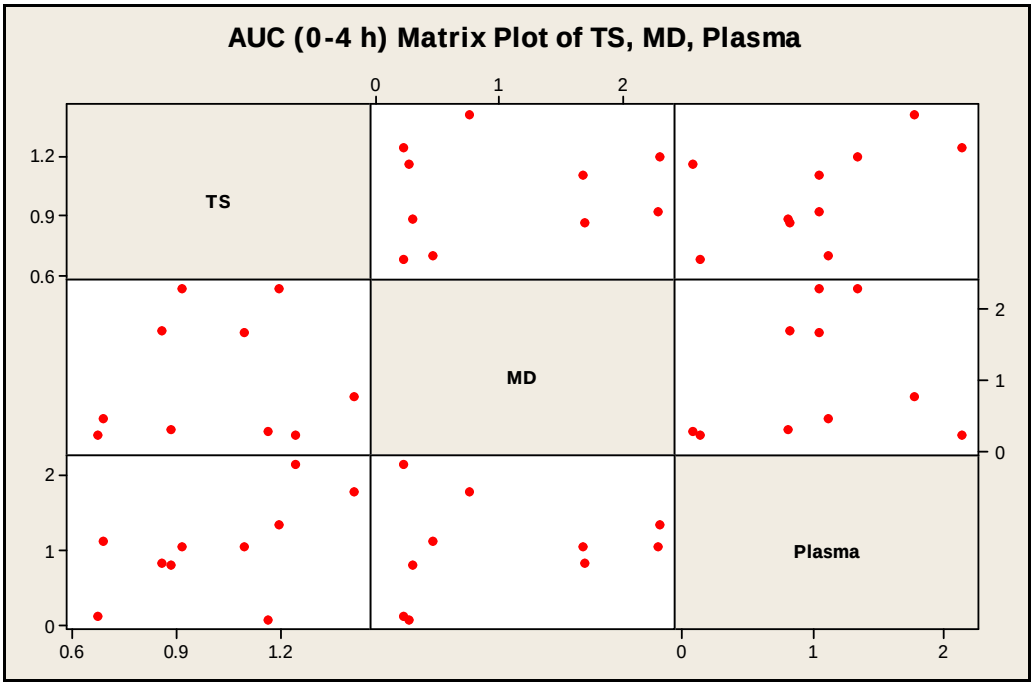


Figure 4.13 Matrix plot of AUC for tape stripping (TS), microdialysis (MD) and plasma.

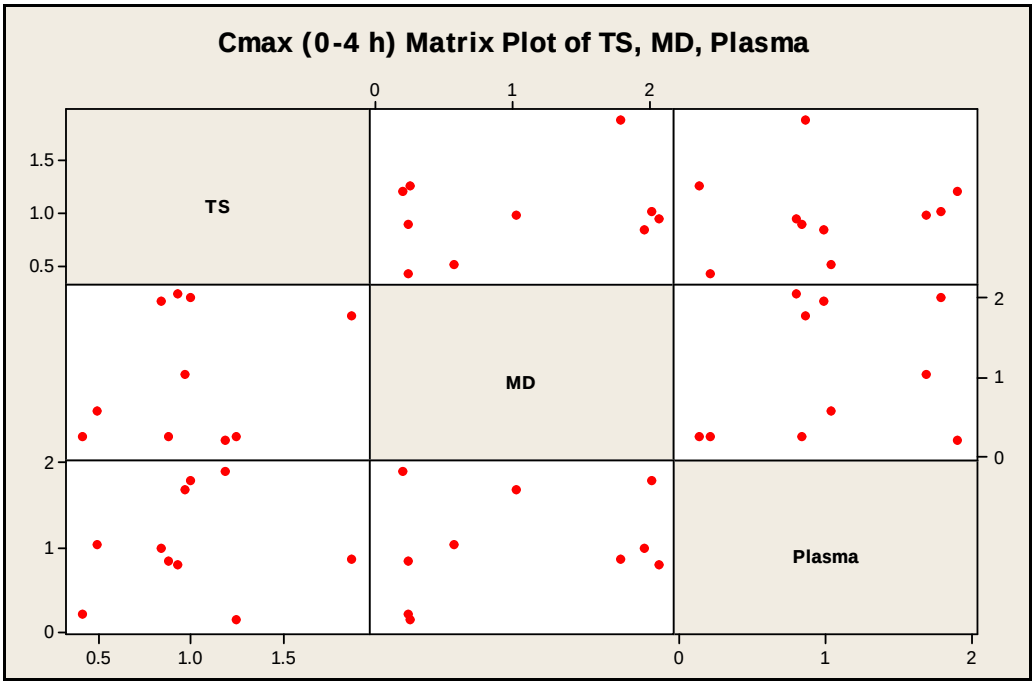


Figure 4.14 Matrix plot of C_{max} for tape stripping (TS), microdialysis (MD) and plasma.

Table 4.10 Pearson correlation coefficient of AUC, C_{max} and t_{max}, between TS, MD and plasma methods of skin study.

		TS			MD		
		AUC	C_{max}	t_{max}	AUC	C_{max}	t_{max}
MD	r	0.106	0.289				
	P	0.772	0.418				
Plasma	r	0.567	0.140	-0.182	0.150	0.243	
	P	0.088	0.700	0.571	0.679	0.498	

r =Pearson correlation coefficient

P value > 0.05 = no significant correlation

4.9.3 Pin prick test

VRS and VAS scores were tested by pin prick test, and plotted in Figure 4.15 and Figure 4.16, respectively. The results showed that lowest VRS was reached at 90 minutes and lasted until 180 minutes post dose (mean, VRS; 1 unit), while for VAS the least pain was felt at 120 minutes post-dose (VAS; 19 mm). The assessments demonstrated a good relationship with the SC tetracaine level in TS (mean t_{max}; 52 minutes) and local tissue

BABA concentrations in plasma (mean t_{max}; 122 minutes). The average BABA level in MD also correlated well with VRS and VAS. Figure 4.17 and Figure 4.18 show a decline in BABA level at point 240 minutes at the same time as there is an increase in both VRS and VAS. The same result was noted in plasma and TS.

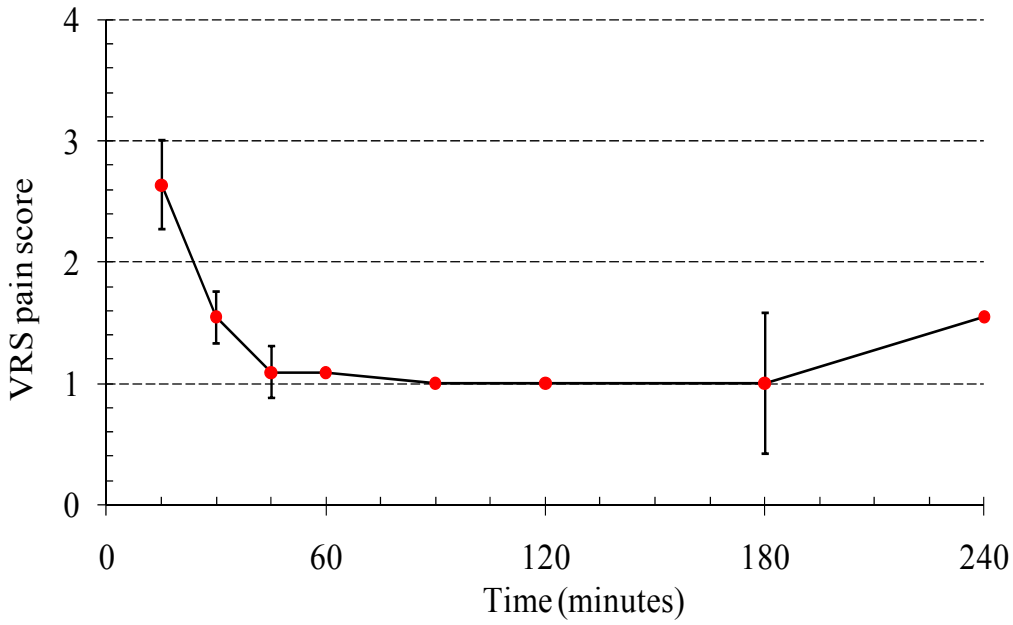


Figure 4.15 Pin prick test showing the mean VRS pain score with time profile (minutes) (n=12, Error Bars= 95% CI).

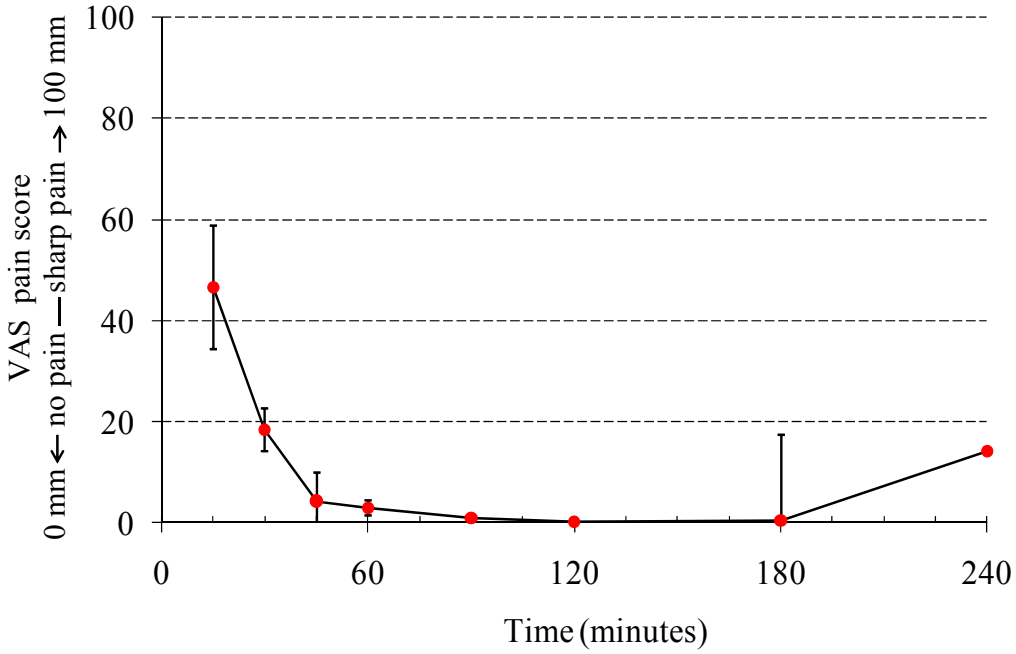


Figure 4.16 Pin prick test show the mean VAS pain score with time profile (minutes) (n=12, Error Bars= 95% CI).

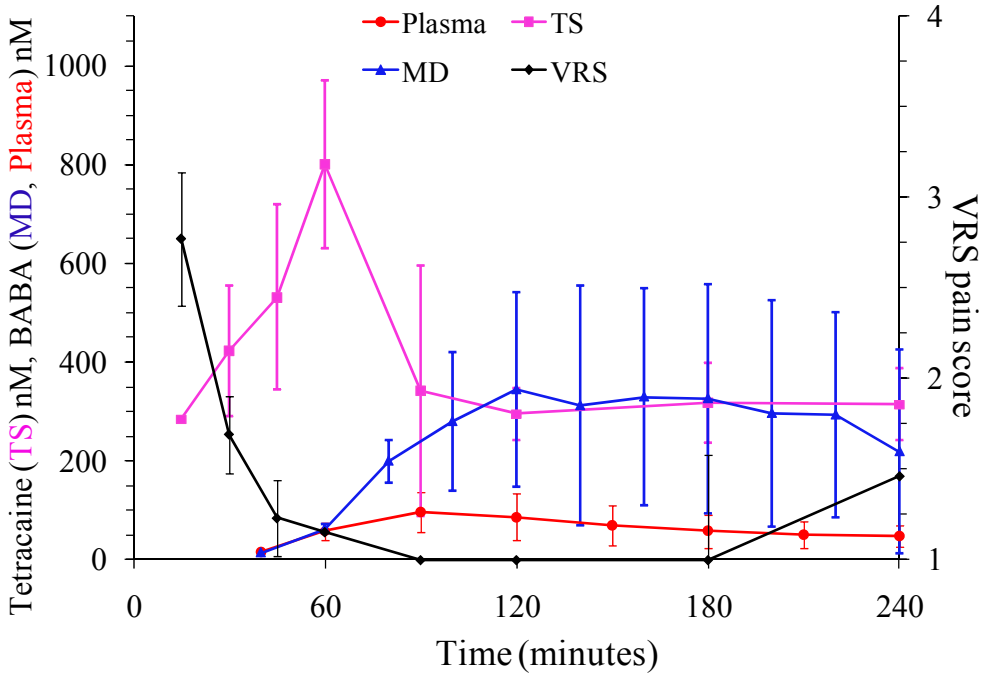


Figure 4.17 Pain visual rating score (VRS), with TS tetracaine, plasma and MD BABA profiles, (n=10, Error Bars= 95% CI).

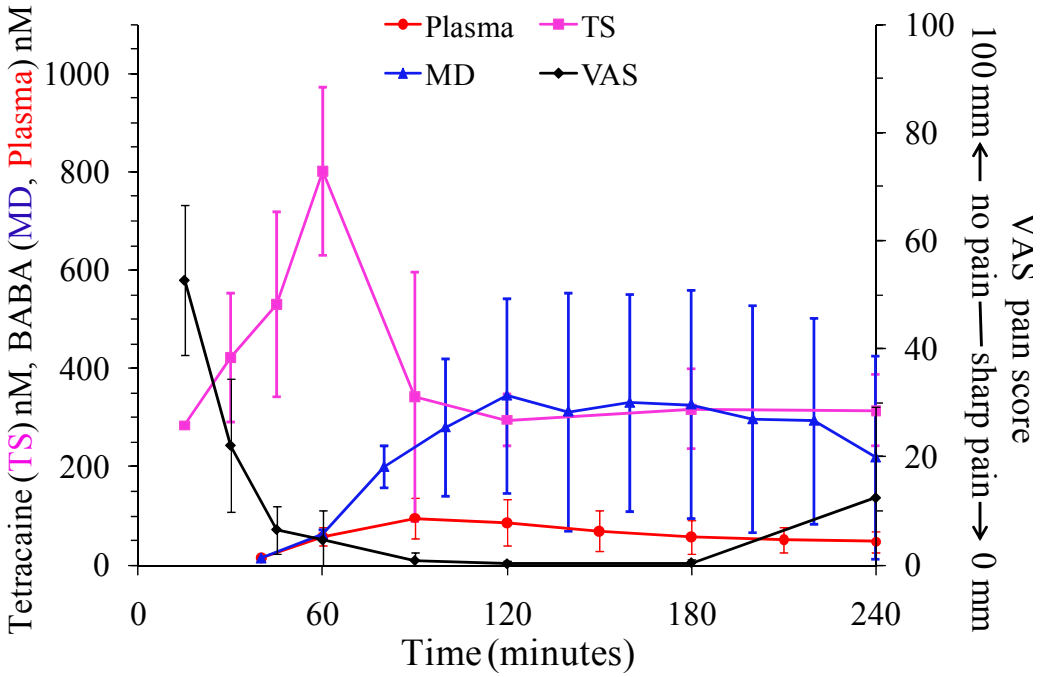


Figure 4.18 Pain visual analogue score (VAS), with TS tetracaine, plasma and MD BABA profiles, (n=10, Error Bars= 95% CI).

4.10 Discussion

Topical application of drugs has an advantage over other routes in its ability to manage specific problems. However, appropriate pharmacokinetic measurement of topical administered drugs is still under investigation. Local anaesthetics of an ester type, such as tetracaine and procaine, are mainly hydrolyzed in plasma by pseudocholinesterase and esterase in the liver, resulting in possible toxicity. Thus, in the measurement of drugs their metabolites must also be analyzed in plasma (FDA, 2001a), and in the dermis for successfully conducted clinical studies.

The most popular methods used to assess drug pharmacokinetics in human skin are tape stripping and microdialysis (Herkenne et al., 2008). The reasons for not using other methods such as skin biopsies, and skin blister fluid sampling are due to the ethical considerations, costs and the lack of reproducibility (Muller et al., 1995), which have limited the application and practice of these methods in clinical drug development. However more practice and comparison between these methods may helpful in providing useful information and critical ideas for the application of these methods in the local tissue or compartment, and to assess the pharmacokinetic profile of the drugs.

In this study, three different types of sample have been collected for the measurement of tetracaine and its metabolite by developed novel methods. The samples were conventional plasma, stratum corneum tape stripping, and dialysate extracellular fluid, after the application of 1 mL of Ametop gel onto the patient's volar surface of the forearm. Nevertheless, the use of MD is limited for several reasons. MD requires very sensitive bioanalytical assays or appropriate assays (Muller et al., 1996, Benfeldt and Groth, 1998), to detect each soluble molecule in the extracellular fluid (ECF) (Muller, 2002). MD needs a skilled person for the insertion of the probe into local tissue to minimize variability between individuals. The recovery of large molecules, highly protein bound or a lipophilic drug in the skin is another challenge in MD (Benfeldt and Groth, 1998). In this study, tetracaine, used as a local anaesthetic was the drug investigated. The topically applied tetracaine is highly lipid soluble and protein bound.

The plasma protein binding is about 75.6% (EMEA, 1997). The LC-MS-MS was a highly sensitive method suitable for the analysis of tetracaine and its metabolite from plasma and microdialysis samples. The separation mode used was referred to as a HILIC system (Hydrophilic Interaction Chromatography or Hydrophilic Interaction Liquid Chromatography). Both plasma and dialysate contained quantified tetracaine metabolite (BABA), although tetracaine was detected in some dialysate samples, but not quantified. The high concentration of tetracaine in tape samples was expected. Hence, an appropriate method has been employed in the development and validation of a rapid and simple CE-UV method (Al-Otaibi et al., 2009). The CE-UV has been compared with a developed and validated HPLC-UV. Both methods were equally valid for the measurement of tetracaine accurately and reproducibly from patients tape samples. In addition, a simple and rapid CE-UV method was developed and used to analyze BABA in patient tape samples, but none was detected. Refer to chapter 3 for more detail of CE-UV and HPLC-UV methods.

The analysis of dialysate appears to be simple and quicker than plasma, and did not require a complex extraction from an endogenous compound, such as lipid and protein which may interfere with the analyte peak. Thus, the dialysate sample can be introduced to the analytical system directly (Davies et al., 2000, Bagger and Bechgaard, 2004, Huang et al., 2008). Nevertheless, the presence of salts in the dialysate is a challenge due to the high ionic strength which may clog the ionization source causing background noise (Davies et al., 2000), and may cause deterioration of the analyte peak. Several methods can be used to resolve the problem by use of off line solid phase extraction prior to injection (Prokai et al., 1998), and desalting the sample with de-ionized water. In this study, samples were analysed without any problem.

All sample matrixes obtained from 12 healthy volunteers have been successfully measured by the analytical methods. The study proved that tetracaine, as a local anaesthetic agent, was absorbed, distributed, and its action occurs in the local tissue, particularly the epidermis including the stratum corneum, the dermis, and subcutaneous tissue. All pharmacokinetic parameters including AUC, C_{max}, t_{max}, and $t_{1/2}$ were measured for BABA. It was not possible to calculate the $t_{1/2}$ in TS samples, due to the presence of a

two compartment model which can be seen clearly in most patient samples (Appendix 22 and Appendix 23), where the concentration declined after reaching t_{max} and then increased again. The same also occurred in the dialysate sample, the AUC and C_{max} were the only pharmacokinetic parameters measured, due to a continuous increase in the concentration of the metabolite BABA. The continuous increase in the BABA concentration in the dialysate sample correlated with the lowest scores from 90 to 180 minutes for VAS and VRS pain score measurements in this study. The elevated drug concentrations observed between 60 and 240 minutes correlated with the local anaesthetic effect of Ametop. Microdialysis is considered to provide information on drug kinetics in the receptor sites beyond the stratum corneum (Muller et al., 1997).

The absorption of tetracaine into the systemic circulation was low and required a longer time to achieve the maximal concentration of its metabolite. In plasma BABA C_{max} was 110 nM and t_{max} was 122 minutes compared with the TS concentration of tetracaine C_{max} 850 nM and t_{max} 54 minutes. BABA concentrations started to decline in the systemic circulation 100 minutes after the application of the dose. This was due to the elimination process of the tetracaine metabolite. The lag time and $t_{1/2}$ observed for BABA from patient plasma samples were 54 minutes and 105 minutes, respectively. The low C_{max} and AUC values, with the longer t_{max} obtained in plasma showed that dermatological drug products, such as local anaesthetics, have limited systemic absorption.

The high variability of C_{max} and AUC in MD compared to plasma and TS may be attributed to the implantation depth, as the same subjects were used for all methods. In addition, the amount of lipid tissue is likely to cause interpatient variability (Lorentzen et al., 1996).

Although tetracaine was stable in tape stripping samples, it was hydrolyzed into BABA quickly and completely in all patients' plasma samples, and almost completely in the dialysate samples. The hydrolysis of tetracaine to its major metabolite was due to the presence of pseudocholinesterase and esterase in the plasma and liver, respectively. The stability of tetracaine and procaine were studied in chapter 3, and it was shown that

tetracaine was hydrolysed/broken down into its major metabolite BABA at 37 °C body temperature and at a pH of 7.8, close to blood pH of 7.4.

TS and MD have been proven to be effective with an excellent rank-order in bioequivalence of topical products (Benfeldt et al., 2007). The tissue concentration of topical drugs obtained by MD is much more precise than plasma estimation (Muller et al., 1995). MD provides a reliable pharmacokinetic model to estimate cutaneous *in vivo* penetration on a routine basis (Kreilgaard, 2001). In this study, TS and MD have been more relevant for assessing the pharmacokinetics of topical anaesthetics products.

In comparison to TS, MD is more invasive. A few challenges are associated with the MD technique, such as when it is used for sampling very lipophilic or very highly protein bound molecules (Benfeldt and Groth, 1998), and it requires a trained person to minimize variability in the study results.

4.11 Conclusions

Topical tetracaine was safe and decreased pain in the healthy subjects. The study results demonstrated that skin stripping with adhesive tape and MD were useful methods of pharmacokinetic assessment of topical drugs. However, in comparison with plasma, skin stripping with adhesive tape was less and microdialysis was more variable. Nevertheless both methods measured local drug concentrations and thus may be more relevant methods of measurement for assessing the pharmacokinetics of drugs such as local anaesthetics. A few challenges need to be resolved with the MD technique, such as its high variability, and low reproducibility.

Thus, more studies concerning the consistency of TS and MD are needed to consider the ideal pharmacokinetic measurement for transdermal drug delivery.

Chapter 5 General discussion

This thesis mainly describes pharmacokinetic studies performed in healthy human volunteers. The projects included the study of diazepam delivery through the skin with a liquid formulated to promote absorption, developing and validating methods to anlayze drugs obtained from skin samples, and appropriate methods to measure and assess the transdermal delivery of drugs. The aims of this thesis as listed in the objectives (1.10, page 66) in Chapter 1 were achieved with varying degrees of success and there is a need for further studies.

1. The TDS® system has been combined with diazepam, in a phase I pharmacokinetic study to compare TDS® diazepam with Diastat® in 12 healthy adult subjects.

The stability and quality control of Diastat® and TDS® diazepam were analyzed by a new HPLC-UV assay. The results showed that for Diastat® the mean weight of the rectal diazepam gel was 2.13 g and it contained an average dose of 9.84 mg diazepam with a CV of 2%. In the TDS® diazepam, five sprays, 1 mL, gave a mean weight of diazepam of 10.17 mg with a CV of 1%. Diazepam in the plasma samples was assayed by a newly developed HPLC-MS method to obtain the necessary high sensitivity. The developed method successfully analyzed all samples in this study without any problems.

The transdermal delivery of diazepam was studied *in vivo* by Schwarz (Schwarz et al., 1995). They showed that by using submicron emulsions, diazepam was effective and the concentrations achieved were equivalent to the parental delivery. In the present study, diazepam was successfully delivered by the TDS® system. It had good safety and tolerability. However, the TDS® diazepam was not bioequivalent to the existing rectal diazepam (Diastat®) and the mean ratios of TDS® diazepam/Diastat® for diazepam and desmethyldiazepam were 11.6 and 47.4%, respectively. The low concentration of diazepam was mainly due to the barrier layer of the stratum corneum. Diazepam is a lipophilic drug and accumulates in the lipid tissue. Although, desmethyldiazepam was present in all subjects in the TDS® group, diazepam was not detected in subject number

S05, and was delayed in subject number S08. This is may have been due to the metabolism of diazepam in the skin. Additionally, the presence of desmethyldiazepam concentration in the first time point of the TDS® system suggests that the washout period between treatments was insufficient to allow for the long half life of desmethyldiazepam. Any future study needs to allow a longer time to clear desmethyldiazepam.

Benzodiazepines are the treatment of choice for the management of acute seizures. These types of drugs are active against many types of seizure, have a rapid onset of action, and have been well studied (Martindale, 1996a). Although, the rectal route of administration is not liked by the patients, their family, and the clinicians, it is difficult and hazardous to administer the drug by IV and IM injections, or orally when the patient is actively convulsing. In this study, diazepam was successfully delivered through the skin into the systemic circulation by the TDS® system. Although not bioequivalent to rectal administration, the route was safe and tolerated by all the volunteers, and devoid of any risk that may affect safety and compliance by the other routes of administration such as IV, IM and oral.

Drug therapy, linked with pharmacokinetics provides useful information to select the optimum dosage regimen that gives an optimum therapeutic effect. Therapeutic range is the concentration of drug that exerts maximum effect with minimum side effects. The presence of the active metabolites and/or stereoisomers or toxicity is not defined. Thus, it is possible to identify the correlation between drug concentrations and the drug effect, by the measurement of plasma drug concentrations.

The plasma concentration of diazepam needed to stop convulsions is not well established, although a range of 150–350 µg/L has been suggested (Agurell et al., 1975), which was supported by Knudsen with a range 200–300 µg/L (Knudsen, 1977). In our study, the plasma diazepam concentrations were within or slightly above the suggested diazepam concentration range (199–379 µg/L) in 11 out of 12 subjects in the rectal diazepam study, but not following the TDS® diazepam (7–67 µg/L). The anticonvulsive range was maintained for 1–5 hours in rectal diazepam group. The wide plasma concentration range observed in this study was due to the high variability in the metabolism of diazepam, and

the differences in the body lipid contents and protein binding affinity between subjects. The pharmacokinetic variability between subjects has been observed in previous studies (Greenblatt et al., 1989, Ogutu et al., 2002). The pharmacokinetics and skin metabolism of diazepam obtained from this study should be used for further development of the TDS® system and diazepam skin delivery, to make this route viable to the patients.

2. Different bioanalytical methods have been explored in this thesis for the analysis of diazepam in the two dosage forms and in plasma, and tetracaine in different biological matrixes, including their metabolites. The methods have been chosen to be sensitive, selective, accurate, precise, reproducible, and stable.

Two new short end (reversed) capillary electrophoresis (CE) with ultra violet (UV) detection methods have been developed, and used to analyze tetracaine and BABA in the tape samples obtained from the TS study. Since, the tape samples contained only tetracaine, the first method was optimized and validated for this purpose (Al-Otaibi et al., 2009). In addition, a high performance liquid chromatography (HPLC) with UV detection method was also developed and validated to investigate the quantitative performance compared to the developed CE, in analyzing tetracaine in skin using tape samples obtained from healthy volunteers given tetracaine as part of pharmacokinetic drug studies. In the comparison between the two methods, the accuracy and selectivity of both methods allowed the measurement of tetracaine in all samples obtained from a skin tape stripping study in healthy subjects, referred to in Chapter 4. The separation was fast for both methods, especially for short end direction (reversed) CE. Although the imprecision was higher, the lower cost and volume of solvent used and faster running time made CE preferable over HPLC for this kind of study. This finding agreed with previous results (Clohs and McErlane, 2003, Aurora Prado et al., 2005, Muscarella et al., 2005, Carlucci et al., 2007, Kowalski and Plenis, 2007, Kocevar et al., 2008). The benefit of a fast analytical method satisfied the high demand for analysis of samples in busy pharmaceutical companies, and laboratories. Although, BABA was not assayed in tape samples, the stability of tetracaine and procaine has been studied by the second CE-UV method in a pH media of 7.8 close to blood pH of 7.4.

3. The correlation of tetracaine measurements have been explored between methods applied for the pharmacokinetic profiles of transdermal drugs by skin tape stripping, microdialysis, and conventional systemic measurement in blood. The results obtained from this study would be appropriate for pharmacokinetic, toxicokinetic, and forensic measurements of topically applied drugs.

Ametop gel was available to target local tissue for an anaesthetic effect. The conventional pharmacokinetic assessment of a drug in plasma may not be an appropriate method for a topical drug. The drug concentration locally may be higher than the drug concentration in plasma due to many factors including drug metabolism in plasma. Thus, tetracaine and BABA were assayed by three different pharmacokinetic measurements. The study was conducted in 12 healthy volunteers and performed for the measurement of tetracaine and its metabolite (BABA) in plasma, MD, and TS samples. Ametop gel was used as a model local anaesthetic, applied on the forearm. The analyzed samples were successfully assayed without any problem. For TS both methods of CE-UV (Al-Otaibi et al., 2009) and HPLC were used; dialysate and plasma samples were analyzed by a developed LC-MS-MS method. The results showed that tape harvesting of the stratum corneum in TS and dialysate samples in MD methods were more reliable than the systemic plasma measurements, in the assessment of the pharmacokinetics of transdermally delivered drugs such as a local anaesthetics. Although tetracaine was not found in plasma or the dialysate samples, the concentration of BABA was log linearly related to the anaesthetic effect of Ametop gel in the dialysate. Microdialysis is considered to provide information on drug kinetics in the receptor phases beyond the stratum corneum (Muller et al., 1997). This finding demonstrated that BABA is an important metabolite/breakdown compound of tetracaine that needs to be assayed if tetracaine is given to subjects.

Drugs can interact with protein (s) in blood plasma and elsewhere resulting in variation of drug-protein binding. High affinity binding of molecules to protein results in low levels of free drug that can reach the target sites, and therefore there is less therapeutic effect. The most abundant human blood proteins are albumin, lipoprotein, and glycoprotein. In drugs that are highly plasma protein bound (> 95%) such as diazepam and desmethyldiazepam, the free unbound drugs are less than 1.5 and 3% (average),

respectively (Allen and Greenblatt, 1981). Thus a small change in protein binding can increase the free unbound drug, that is responsible for the pharmacological action, which affects drug elimination and pharmacokinetics, and toxicity is likely to occur.

In the TDS® diazepam study, the elimination half life of diazepam was 85 hours after rectal administration, but could not be measured for TDS® diazepam. This is may have been due to the accumulation of diazepam in the lipid tissue of the skin. In addition, the half life of desmethyldiazepam could not be measured in both dosage forms. The presence of desmethyldiazepam in 5 subjects in the TDS® diazepam group at the zero time point confirmed that desmethyldiazepam has a half life of more than 14 days.

Tetracaine is a highly protein bound and lipophilic drug. The plasma protein binding of tetracaine is about 75.6% (EMEA, 1997). The measurement of highly protein bound and lipophilic drugs are difficult in MD (Benfeldt and Groth, 1998). Tetracaine was not measured in MD samples due to its protein binding, accumulation in the lipid tissue, and possibly metabolism in the skin.

Protein binding may also be saturable, resulting in a rise of unbound drug proportional to an increase in drug concentration. Other factors that may affect protein binding include drug-drug interactions, the drug has a high binding affinity to plasma protein, which may displace the bound drug and increase its unbound fraction. Drug protein binding is usually reversible, and only unbound drug undergoes metabolism. The drug may dissociate from the protein, thus free drug is released and metabolized. The metabolism of drug facilitates drug elimination, and tends to keep plasma drug concentrations in equilibrium.

5.1 Clinical trials and their regulations

Clinical trials (CTs) are research studies conducted for drugs or appliances to observe their effects on numbers of subjects to attain useful information on safety and efficacy. In Britain, it was recognized that there was a need for a Clinical Trials Committee after the development of insulin in 1920s, which led to the creation of the Medical Research Council in 1931 (Valier and Timmermann, 2008). The Medical Research Council

together with medical manufacturers designed guidelines on the appropriate conduct of clinical trials. Consequent needs and the success of clinical trials created the Tuberculosis Chemotherapy Trials Committee, which led to the organization of the world's first randomized controlled clinical trial in 1946 by a British research team using streptomycin for the treatment of tuberculosis (Yoshioka, 1998, Valier and Timmermann, 2008). The subsequent medical interventions led to the establishment of the Nuremberg Code in 1947, and ethics in medical research adapted that in the Declaration of Helsinki in 1964. The Declaration of Helsinki has been implemented for use in medical ethics to afford directives for the medical researcher in the conduct of clinical trials involving human subjects. The amendment and changes to the Declaration of Helsinki has forced many countries to adopt further regulations in their medical research, including good clinical practice (GCP), and good laboratory practice (GLP).

The European Clinical Trials Directive (2001/20/EC) came into effect in the United Kingdom in May 2004 via the Medicines for Human Use (Clinical Trials) Regulations 2004. The clinical trial regulations control research conduct in Clinical Trials of Investigational Medicinal Products (CTIMPs). Health authorities and organisations such as the NHS support and maintain systems that are suitable so that CTIMPs can be directed and performed in accordance with clinical trials regulations and the research governance framework. The outcome is to facilitate and simplity the application process for clinical drug research and to improve research studies.

In addition, there is considerable importance placed on the regulations including the protection of the research subject, by improving the previous standard regulatory and ethics inspections of all drug research that is required for commercial drug research.

The Medicines for Human Use Act authorized the Amendment of the Regulations of 2004 and were passed in Parliament on July 2006, and came into effect on August 2006. This regulation implemented the EU Directive (2005/28/EC) on GCP, the arrangement of fees/expenses, informing the licensing authority of any breach in the system including GCP, protocols, and any warning notices.

In order to apply for clinical drug research, the CTIMPs require;

1) A registration with an available regulatory agency, such as the Medicines and Healthcare Products Regulatory Agency (MHRA), in the UK. MHRA is the authorized national agency responsible for this.

2) Ethical consideration and approval is provided by a research ethics committee (REC), on behalf of the Central Office for Research Ethics Committees (COREC).

3) The research study requires funding and support usually by a sponsor with legal responsibility such as the institute, or the pharmaceutical company, to ensure that the trial is conducted in accordance with The Medicines for Human Use legislation.

Additional to the above requirements:

4) Maintenance of documents and archiving of the study is also important.

Further amendments and additional changes in the regulations have led to the delay of many clinical trials. The number of drug research papers in anaesthetic drugs mainly in post marketing (phase IV) drug developments declined by 15 and 29% in Europe and in the United Kingdom, respectively, within 3 years following the introduction of the European Clinical Trials Directive (Walker et al., 2009). The decline observed in European anaesthetic development was due to the high financial demand and complex paper work required by ethics committees, which forced local hospitals and other medical institutions to conduct fewer clinical trials. However, large organizations, such as pharmaceutical companies, may prefer to conduct their clinical trials in less regulated countries.

However, there was a delay for a least 1 year in the diazepam study so that it would comply with these regulations. The Medicines for Human Use (Clinical Trials) Regulations 2004 and the Medicines for Human Use (Clinical Trials) Amendment Regulations 2006 were referred to as the Clinical Trials Regulations in this thesis.

5.2 Appropriate transdermal monitoring and drug analysis

Drugs have different receptor sites that may interact and/or bind with a specific target protein. Thus, once a drug is absorbed from the site of the application it may be affected by many factors such as first pass metabolism as in oral dosage form, or the thickness of the stratum corneum such as in transdermal drug delivery, before the drug is distributed to the site of action. A drug must reach its therapeutic concentration to exert its effect by interacting with the receptor. Thus a drug should be measured near its receptor or target site. The benefit of this is to evaluate the drug concentration that can give the optimum therapeutic effect, with the least side effects.

Most drug measurements are usually determined in blood plasma or serum as the total unbound protein concentration. Other biological matrixes can be useful in the measurement of a drug such as saliva, urine, and extracellular fluid samples. The plasma drug concentration is well understood, and the drug concentration is in equilibrium within the body. A drug may metabolise or bind to a protein in plasma, thus the measurement of a drug must include as many metabolites as possible.

Topically applied drugs, such as local anaesthetics, target receptors which are located in skin tissue especially at the site of application. Thus, the measurement of drug concentration in local tissue is vital for locally applied medication rather than assessing the drug concentration in plasma. The first method of evaluation of topically applied drugs was the use of Franz cell apparatus for transdermal drug delivery. The method has been used for the study of cutaneous drug diffusion (Koch et al., 1987), by using animal skin, human skin, or artificial membranes. Although, the method has been used for many years, this *in vitro* model has several limitations. These include the absence of drug elimination, skin metabolising enzymes, blood vessels, and the integrity of the skin. Therefore, this method determines percutaneous absorption instead of cutaneous bioavailability of the topically applied drug.

Recently, researchers in the field recognized that to obtain clinically relevant information about the measurement of a local drug in the local tissue *in vivo* pharmacokinetics must

be applied. Therefore, many techniques/methods have been established and evaluated including tape stripping (TS), microdialysis (MD), and skin biopsies. From these methods, MD and TS are commonly used in transdermal drug delivery due to their consistency.

The tape stripping (TS) method is relatively simple and showed good reproducibility when applied and used as a pharmacokinetic method for transdermal drugs. In this thesis, TS was reproducible with less variability compared to plasma and microdialysis (MD). It has been confirmed in previous studies that TS is the method of choice for the measurement of bioequivalence (Shah et al., 1998), and bioavailability (Weigmann et al., 1999b) of a topically applied drug. The TS method is useful to quantify metabolic enzymes in the skin such as the esterase enzyme in the stratum corneum (Beisson et al., 2001).

In plasma, only BABA was found because tetracaine was broken down. This breakdown could have been due to esterases in blood or may simply have been due to the pH of blood. The latter is more likely, since tetracaine is stable when in contact with the stratum corneum, although it known that the stratum corneum contains esterases (Beisson et al., 2001). In disodium tetraborate buffer at pH 7.8, tetracaine is rapidly broken down to BABA, and a similar breakdown has been noted for procaine (Iglesias-Martinez et al., 2006). Further studies are needed to confirm this finding.

Tape stripping of the outer skin layer, the stratum corneum, and assessment of the extracellular fluid are among the most prevalent and reliable methods to use for the measurement of drug concentrations in transdermal drug delivery. Although, the methods have been extended, a few challenges still need to be addressed before they can be regarded as generally applicable routine techniques for cutaneous drug delivery assessments.

The tape stripping (TS) method is applied with the standard practice of repeated application and removal of adhesive tape on the epidermis. The tape sample contains stratum corneum which contains an amount of applied drug. Although, TS is not difficult to perform, many intrinsic and extrinsic factors need to be investigated. It has even been

suggested that the amount of drug recovered by the tape is influenced by seasons of the year (Tokumura et al., 1999), and the site of application (Tokumura et al., 1999, Loffler et al., 2004). To minimize the seasonal time and the site of application in TS method, the study was performed in the autumn, and at one site of application (e.g. the forearm). Additionally, the inter-individual variability was difficult to minimize in this study, such as the amount of stratum corneum (SC) in the tape sample, which is affected by many factors such as SC thickness, amount of cells, the composition of the lipid layer, age, gender and race of the patient (Lademann et al., 2009). Further investigations are needed to address some question about these factors.

Extrinsic factors that may affect the amount of SC include the type of the tape, the pressure and time the tape was applied for prior to tape stripping, the applied force for removal from the skin surface, and the nature of the topically applied drug and its formulation (Lademann et al., 2009). The high surface area of the tape sample compared to the amount of drug dispersed may result in the loss of compound by evaporation, if a volatile drug is being studied. In such cases, the analysis of tape samples should be performed immediately after the removal of the adhesive tape from the skin. Alternatively, the tape sample can be transferred to dry ice instantaneously after removal from the skin surface.

Normally, TS studies require consistent practice in order to minimize any within and between variability between individuals. The type of the tape used is considered important for good stripping and results. In the TS study, the composition of the adhesive part was uniformly distributed and did not contain any empty spaces. Prior to the study, the tape was tested to prevent any reaction with the subject's skin that might occur, such as hypersensitivity. A constant tape size was used for all subjects and application areas. Additionally, the pressure applied was another challenge in the TS procedure. Some workers have used a rollers, or constant weight, to apply uniform pressure to the skin (Lademann et al., 2009). Although it has not been described in previous studies, in this study, a spatula was used by just one person to give a constant pressure and minimize individual inter-variability.

Another factor that may affect results is the speed of removal of the tape. It has not been standardized, but high speed may decrease the amount of stratum corneum harvested and vice versa for low speed (Loffler et al., 2004). Usually constant and rapid speeds are needed, which have been performed in this TS study. Weighing the tape prior to and after tape stripping was often used to measure the amount of SC removed (Weigmann et al., 1999a). It is not without error, as it is based on the assumption that sebum, the drug, and interstitial fluid may increase the weight. Other methods, such as measurement of protein content in the corneocytes (the major component of the SC) to determine the amount of SC, and using microscopy to determine the amount of SC, are among the more time consuming processes (Lademann et al., 2009). Additionally, transepidermal water loss (TEWL) may be used but this can also be affected by environmental factors, the topically applied drug, and interstitial fluid (Lademann et al., 2009).

MD is well established for the assessment of topically applied drugs. However, many limiting factors can affect drug concentrations in MD samples. The most important factor is the low recovery of molecules, particularly those with a large molecular weight (Groth, 1996, Clough, 2005), such as insulin (Jansson et al., 1993, Rosdahl et al., 2000), highly lipid soluble drugs (Groth, 1996, Clough, 2005), highly protein bound drugs (Clough, 2005), a low concentration gradient, and molecular charged drugs. Tetracaine is a highly lipophilic and protein bound drug. In this study, tetracaine was applied for 1 hour to the same site for all volunteers to minimize variability. Tetracaine was not measured in the dialysate because of its lipid solubility and protein binding. However, the water soluble metabolite of tetracaine (BABA) was measured. The flow rate and duration (Clough, 2005) which may affect drug concentration in the dialysate, were minimized by using a constant flow rate of 1 µL per minute, and for a fixed duration of time for all subjects. Additionally, the length of the dialysis membrane was kept constant using the same type of probe catheter. The implantation depth can affect drug concentration. Although, it was done for all subjects by the same physician, it was difficult to eliminate inter-individual variability. The dialysis membrane is affected by factors including; the probe location (Clough, 2005), probe membrane permeability, probe area, and perfusion fluid properties (Chaurasia, 1999, Ettinger et al., 2001). These factors were minimized in this study by implanting the catheter in the same site on the forearm, and as mentioned before with

same catheter type, and the use of Ringer solution similar to the physiological fluid. The small volume and low concentrations of drug in the dialysate sample may limit MD usage, which requires highly sensitive techniques for analysis (Benfeldt and Groth, 1998). In this study, LC-MS-MS was needed to analyze the plasma and dialysate samples.

These problems with MD have contributed to a high variability in the pharmacokinetic parameters. The large variability can be seen between individuals, compared to within individuals. The high intra-individual variability may be contributed to the implantation depth of the probe, and/or probe to probe variability (Ettinger et al., 2001). Since, tetracaine is a highly lipid soluble and highly protein bound drug. The variability in this study may have come from the inter-individual variability in the lipid layer of the skin, protein binding, and the probe implantation depth. Standard procedures for the use of MD should be available to overcome some errors, such as the insertion of the probe, by using ultrasonography to evaluate the depth of probe (Klimowicz et al., 2007), which must be performed by trained physician.

5.3 Transdermal drug delivery: progress and problems

Transdermal drug delivery is an alternative drug administration choice. The human skin is a readily accessible surface for drug delivery. Michaels and colleagues determined that the ideal diffusion coefficient of drugs in the stratum corneum results in significant permeability of some drugs (Michaels et al., 1975). However, to consider the cutaneous route as viable, a sufficient amount of drug delivery is required. Poor absorption is due to the protective barrier of the SC in the skin, limiting local drug application. Many methods have been tested to facilitate the delivery of molecules through the skin and the only limitation was the success to which a drug can reach its site of action in sufficient quantity. This was implemented in the development of the first patch, and was approved in 1979 to deliver scopolamine for the prevention of motion of sickness (Shaw and Chandrasekaran, 1978).

Currently, transdermal drug delivery methods are available as topically applied creams, ointments, patches, and gels. The drug targets local tissue or other organs through the systemic circulation, avoiding factors that may affect drug bioavailability by the oral route, such as hepatic first-past effect, dose missing, and food-drug interaction. Another advantage for the transdermal delivery of drugs is that treatment can be interrupted or eliminated when necessary (Godwin and Michniak, 1999). Patient compliance can be improved, which is especially notable in patches that require only once weekly application e.g. the once a week transdermal estradiol adhesive patch appears to be an acceptable means of hormone replacement therapy compared to the twice a week patch (Harrison et al., 1997).

Since some drugs have poor penetration through the SC, a chemical enhancer may be used. However, drug absorption in the skin may be limited, and there is need for a potent transdermal delivery system. There appear to be promising transdermal delivery systems available, but they are still under investigation such as liposomal, iontophoresis, magnetophoresis, sonophoresis (phonophoresis).

Liposomes, are small vesicles composed of phospholipids, which when dispersed in an aqueous phase (hydration) form bilayer membranes in the shape of a sphere (Yarosh, 2001). Liposome structures consist of amphiphiles characterized by a hydrophilic (head), and hydrophobic (tail) (Yarosh, 2001). The hydrophilic groups align toward the aqueous, and the hydrophobic tails point to the inner lamellae. Liposomes were introduced in 1980, and used for topical drug delivery (Mezei and Gulasekharam, 1980, Mezei and Gulasekharam, 1982, Moghimi and Patel, 1993). Drug delivery into and through the skin depends upon the type and composition of liposomes. The liposome encapsulation of a drug can increase drug permeation across the skin, and prolong the local anaesthetic effect of tetracaine compared with EMLA cream (Fisher et al., 1998). The benefits are to control drug concentration in the systemic circulation, to avoid a drug's adverse effects and toxicity, or to target local tissue with drugs such as local anaesthetics. Several methods with liposomes have been tested to determine outcomes. For example, the penetration of carboxyfluorescein through the skin was inversely related to the particle size of the liposomes used (Verma et al., 2003). The delivery of clindamycin phosphate

through the skin was increased when an electrical charge was applied to the liposomes (Shanmugam et al., 2009). In addition, the presence of a chemical enhancer such as ethanol containing vesicles called ethosomes has been investigated. The ethosomal system considerably enhanced the skin permeation of minoxidil *in vitro*, testosterone from an ethosomal patch *in vivo* and *in vitro* (Touitou et al., 2000), and *in vitro* and *in vivo* studies have demonstrated the penetration of finasteride through the skin by liposomes and niosomes (Tabbakhian et al., 2006).

Nevertheless, the main progress in transdermal delivery systems can be demonstrated by the application of a small electric potential to the skin so that charged drug molecules can penetrate into the skin more readily. The penetration is due to electrophoresis, electro-osmosis, and the amount of transported molecules is dependent on the quantity of charge penetrated (Kaliaa et al., 2004). The method of iontophoresis uses a small amount of electric current to drive polar and neutral molecules through the skin (Banga et al., 1999, Denet et al., 2003). It has been used to deliver drugs through the skin such as diclofenac (Fang et al., 1999), piroxicam (Curdya et al., 2001) and sumatriptan (Patel et al., 2007). In addition, iontophoresis can be combined with a chemical penetration enhancer, resulting in a synergistic effect. For example, iontophoresis with geraniol was effective in transporting diclofenac *in vivo* (Kigasawa et al., 2009). Electroporation, similar to iontophoresis, uses high voltage treatment for a short time to provide short pulses of current to induce transient pores in the skin. The pores are responsible for ion and molecular transport, and each pore can transport many ions and molecules (Weaver et al., 1999). Methotrexate was successfully delivered through isolated porcine skin by the combination of electroporation, iontophoresis, lipid enhancers, and local hyperthermia (Wong et al., 2005). Magnetophoresis is a method of drug delivery, using a magnetic field to transport a drug through the skin. The influence of magnetic field strength was determined, and it was shown that by increasing the magnetic field, the diffusion flux of the drug was increased (Murthy, 1999). Additionally, sonophoresis uses ultrasound energy to transport a drug through the skin. Although, the mechanism is not clear, mechanical cavitations, and thermal effects may be the method of drug transportation (James et al., 1993, Escobar-Chávez et al., 2009). Also, the use of low level ultrasound

waves can be useful for increasing the permeability of human skin, including the high molecular weight drugs such as insulin (Mitragotri et al., 1995).

5.4 Conclusions

The development of new/existing drugs is fundamental for the management/treatment of disease and can increase the quality of life (well being) of individuals. The process includes discovery of a new entity, modification of an existing product such as improvement of drug structure, dosage form, and formulation. Progress in transdermal drug delivery offers advantages over other routes of drug delivery. Nevertheless, the limiting barrier of the stratum corneum requires an effective transdermal drug delivery system and an appropriate method of drug measurement in the skin.

The TDS® delivery system has been developed to offer a new method of transporting a drug molecule into and through the skin, providing advantages over other routes or delivery systems. The TDS® preparation was shown to deliver diazepam systemically through human skin in this project. This study demonstrated that the TDS® delivery system could be combined with a drug to form a powerful transdermal drug delivery system. Further development of the TDS® preparation is required to deliver drugs through the skin in sufficient amounts for a therapeutic effect.

The development of an analytical method for pharmacokinetic and pharmacodynamic studies requires sensitive, selective, accurate, precise, reproducible and stable methods. Rapid and simple methods should be available in clinical studies because of increasing numbers of samples to assay. The development of capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) methods using UV detection were successfully used in the analysis of tetracaine. Although, the imprecision was higher, the lower cost and volume of solvent used and a faster running time made CE preferable over HPLC for this kind of study.

For most drugs, the pharmacokinetics are characterized by systemic measurement of plasma concentrations. However, to be effective, local anaesthetics need to maintain high concentrations in the local tissues. *In vivo* methods to determine the best approach to

study the pharmacokinetics of tetracaine following topical administration and to compare the drug concentration measurements using skin stripping with adhesive tape (TS), in microdialysis (MD) fluid, and in plasma were investigated. The study results demonstrated that skin stripping with adhesive tape and MD were useful methods for the pharmacokinetic assessment of topical drugs. Both methods measured local drug concentrations and these may be more relevant measurements for assessing the pharmacokinetics of drugs such as local anaesthetics. However, a standard protocol for TS and MD is required for their validity in the application and the assessment of topically applied drugs, which can fully satisfy the criteria of the regulatory authorities.

Chapter 6 References

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Appendices

Appendix 1 Ethics approval letter for TDS® diazepam.

St Thomas' Hospital Research Ethics Committee
Ethics Committee Office
Block 5 South Wing (Gr Fl)
St Thomas' Hospital
London
SE1 7EH

Telephone: 0207 188 2257
Facsimile: 0207 188 2258

29 August 2006

Professor Richard Langford
Consultant in Anaesthesia and Pain Medicine
Pain Research Group Anaesthetics Laboratory
St Bartholomew's Hospital
West Smithfield
London
EC1A 7BE

Dear Professor Langford

Full title of study:	**A Randomised, Single-Dose, Two-Period, Cross-Over Phase I Pharmacokinetic Study to Compare TDSÂ®-Diazepam with Rectal Diazepam in Healthy Adult Subjects**
REC reference number:	**06/Q0702/102**
Protocol number:	**1**
EudraCT number:	**2006-002609-29**

Thank you for your letter of 17 August 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

An advisory committee to South East London Strategic Health Authority

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		30 June 2006
Investigator CV		20 June 2006
Protocol	1	25 June 2006
Covering Letter		30 June 2006
Summary/Synopsis	1	25 June 2006
Letter from Sponsor		30 June 2006
Peer Review		
Compensation Arrangements		
Advertisement	1	25 June 2006
GP/Consultant Information Sheets	1	25 June 2006
Participant Information Sheet: Appendix 2	1.0	17 August 2006
Participant Consent Form: Screening	1.0	17 August 2006
Participant Consent Form: Study	1	17 August 2006
Response to Request for Further Information		17 August 2006
Copy of Request Form to MHRA		
Letter from Funder		22 June 2006
Statement of Indemnity		30 June 2006

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0702/102 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Adrian Hopper
Chair

Enclosures: *Standard approval conditions*
Site approval form

Copy to: Queen Mary, University of London (QMUL)
Joint Research and Development Office
3rd Floor Rutland House; 42-46 New Road; Whitechapel
London
Guy's & St Thomas' R & D Department
Clinical Trials Unit, MHRA

St Thomas' Hospital Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	06/Q0702/102	**Issue number:**	1	**Date of issue:**	29 August 2006
Chief Investigator:	Professor Richard Langford				
Full title of study:	A Randomised, Single-Dose, Two-Period, Cross-Over Phase I Pharmacokinetic Study to Compare TDSÂ®-Diazepam with Rectal Diazepam in Healthy Adult Subjects				

This study was given a favourable ethical opinion by St Thomas' Hospital Research Ethics Committee on 29 August 2006. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes (1)*
		Pain Research Group	East London and the City Research Ethics Committee 1	29/08/2006	

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/~~Administrator~~)

(delete as applicable)

..... (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

SF1 List of approved sites

Appendix 2 Ethics approval letter for pharmacokinetic study of dermatopharmacokinetics.

East London & The City Research Ethics Committee 3
Clinical Leadership
Floor 2
Room 24 Burdett House
Mile End Hospital
Bancroft Road
E1 4DG

Tel: 020 8223 8602

Professor Atholl Johnston
Professor of Clinical Pharmacology
Clinical Pharmacology
Willliam Harvey Research Institute
Charterhouse Square
London EC1M 6BQ

15 February 2008

Dear Professor Johnston

Study title: **Comparative pharmacokinetic study of a combined percutaneous local anaesthetic/Nitric Oxide generating system and EMLA cream: Evaluation by systemic measurement, skin stripping and microdialysis**
REC reference: **05/Q0605/98**
Amendment number:
Amendment date: **19 October 2007**

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 18 December 2007 and subsequently following discussion with Dr Ingram.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMPs)		19 October 2007
Protocol	3.1	13 April 2007
Participant Information Sheet	5.1 AMETOP Tracked	13 April 2007
Participant Information Sheet	5.1 AMETOP Clean	13 April 2007
Participant Information Sheet: NO Lidocaine Clean	5.1	13 April 2007
Participant Information Sheet	5.1 NO Lidocaine Tracked	13 April 2007
Advertisement	2	13 April 2007
Participant Consent Form: NO Lidocaine Clean	3.1	13 April 2007
Participant Consent Form: AMETOP	3.1	13 April 2007
Drug data sheet		14 May 2007

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0605/98:	**Please quote this number on all correspondence**

Yours sincerely

Miss Sandra Burke
Acting Committee Co-ordinator

E-mail: sandra.burke@thpct.nhs.uk

Enclosures	List of names and professions of members who were present at the meeting and those who submitted written comments
Copy to:	Barts & The London School of Medicine and Dentistry

East London & The City Research Ethics Committee 3

Attendance at Sub-Committee of the REC meeting on 18 December 2007

Name	*Profession*	*Capacity*
Dr David A Ingram	Consultant Clinical Neurophysiologist (Chairman)	Expert
Dr Ralph White	Non-Medical Lay Member	Lay

Appendix 3 Informed consent form (sample).

WRITTEN CONSENT FORM: **REC Number: 05/Q0605/98 Ver. 3.1**

Title of research proposal: ***Study of a combined percutaneous local anaesthetics: Evaluation by systemic measurement, skin stripping and microdialysis.***

Name of Patient / Volunteer (Block Capitals)**:**

Address:

The study organisers have invited me to take part in this research.	☐
I understand what is in the leaflet about the research.	☐
I have a copy of the Patient's Information leaflet version 5.1 to keep.	☐
I have had the chance to talk and ask questions about the study. know what my part will be in the study and I know how long it will take.	☐
I have been told about any special drugs, operations, tests or other checks I might be given. know how the study may affect me. I have been told if there are possible risks.	☐
I understand that I should not take part in more than one study at a time. I know that the local North East London and City Health Authority Research Ethics Committee Has seen and agreed to this study.	☐
I understand that personal information is strictly confidential: I know the only people who may see information about my part in the study are the research team or an official representative of the organisation which funded the research.	☐
I freely consent to be a subject in the study. No-one has put pressure on me.	☐
I know that I can stop taking part in the study at any time.	☐
I know that if there are any problems, I can contact:	☐

Prof. Atholl Johnston

Department of Clinical Pharmacology,
William Harvey Research Institute,
Charterhouse Square,
London. EC1M 6BQ.

Tel. No: **(020) 78823414**

Dr Arthur T. Tucker

The Ernest Cooke Clinical Microvascular Unit,
4th Floor Dominion House,
St. Bartholomew's Hospital,
London. EC1A 7BE.

(020) 76018498

Patient's / Volunteer's Signature: ..... Date: .....

The following should be signed by the Clinician/Investigator responsible for obtaining consent
As the Clinician / Investigator responsible for this research or a designated deputy, I confirm that I have explained to the patient / volunteer named above the nature and purpose of the research to be undertaken.

Investigator's Name seeking consent: .....

Investigator's Signature: ..... Date: .....

Patient Information Sheet, Ametop Version 5.1 (13/04/07)

Appendix 4 Patient information sheet (sample).

Study of a combined percutaneous local anaesthetics: Evaluation by systemic measurement, skin stripping and microdialysis.

Lay title: "Study of skin anaesthetic drug delivery-Ametop"

Study no. :05/LA 003/01

Invitation to participate in a Research Project

We invite you to take part in a research project, which we think may be important. The information, which follows, tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part in the study is entirely your choice. Please ask any questions you want to know about the research and we will try our best to answer them.

Why have we approached you?
Procedures which involve insertion of needles through the skin are commonly perceived as painful and may lead to apprehension and anxiety, especially in children. Bad experiences with needles can often lead to problems with future procedures. The introductions of anaesthetic creams, which are applied on the skin have been a great help. However, current anaesthetic creams are slow to have an effect (30 minutes to 1½ hours) and this may lead to difficulties in organising clinics, wards and operating theatre routines around their application time. A local anaesthetic cream with a more rapid effect would be an important development both for the patient and for the smooth running of hospital procedures.

Lidocaine has been used for many years as a local anaesthetic. Normally it takes a very long time to penetrate skin and hence it is mainly given by injection. In this study, we will mix the nitric oxide system with lidocaine, apply it onto your skin, and carry out a series of studies called "pharmacokinetics" in order to measure the effectiveness of the system. We believe that the nitric oxide produced by this system may increase the speed of penetration of lidocaine and produce a more rapid anaesthesia. We will compare this new drug mixture with EMLA cream, and Ametop gel. EMLA cream, and Ametop gel are a local anaesthetic skin cream currently used for skin anaesthesia in the venepuncture and minor procedure. We have been completed this study with EMLA, now want to continue with Ametop which is a local anaesthetic gel.

Most drug content studies in the body are normally done by the measurement of blood. However, measurement of a drug in the blood is not always useful for evaluating skin-based medications (especially local skin anaesthetics). Anaesthetics designed to target the local skin to which they are applied do not readily diffuse into the rest of the body and the blood system. For this reason normal methods of drug measurement using blood samples have proved unhelpful.

In this study, we will therefore measure the amount of drug in the surface layers of the skin using adhesive tape and a needle procedure, called microdialysis This will allow us to carefully compare the drug levels in the blood with the drug levels in the skin at any moment in time. We will then be able to decide which of the methods is most effective.

Microdialysis is a technique which allows measurement of the amount of the test drug in the skin itself and involves placing a special needle (or cannula) under the skin for the duration of the study. This is a safe procedure, but you may feel some transient discomfort when the needle is inserted. Skin samples are taken with normal everyday sticky tape by applying and then removing the tape. This results in removal of those outermost layers of the surface of skin which are normally shed into clothing during the course of the day. Both methods will allow measurement of the varying amount of drug in the skin during the study period.

The main purpose of this study is to find the best technique for measuring the dose and effectiveness of skin based drug delivery. The results from this study will, however, also be of indirect benefit as they may lead to improvements in anaesthetic delivery systems for clinical use.

What would I do in the study, if I took part?
If you agree to take part, we will take a detailed medical and a physical examination, including blood pressure, pulse rate and rhythm. Signs of drug and alcohol abuse will also be checked. **We will treat all the drug screening results as confidential and the results will not be used for any purpose other than inclusion or exclusion from this study.**

The study involves two visits with a minimum of one-week gap in between. The study will last for the maximum of six hours for every visit.

Visit 1: Microdialysis and systemic measurement

Upon entry to the study ward for the initiation of the study, we will check your blood pressure and heart rate. The hairs from forearm on both of your arms will be removed by using a shaver and thoroughly cleaned with water and wiped dry with a towel. Your arms will be checked for cuts or scrapes which could complicate the study. You will be asked to lay down on a bed and we will start by inserting a small cannula (a small tube, same size as a blood test needle) into a vein in your arm. By using the cannula we will be able to take regular small blood samples during the study without having to insert a blood test needle each time.

On your other arm, a circular area of 10 cm^2 will be marked with a pen in the centre of the forearm. The microdialysis guide needle will be inserted in the just under the skin about 1 cm from the marked area. The microdialysis probe will be inserted through the tip of needle, which is then removed, leaving the probe (about 3 cm long) below the marked area. Inlet and outlet tubing will be attached to the sampling probe and secured to the skin using adhesive tape. The inlet tube will be connected to the microdialysis pump and the outlet tubing connected to a small collection pot. You will have to carry the pump (about the size of a mobile phone) with you during the study. This pump moves a special liquid through the probe in order to measure the drug concentration in the skin. Please take care of it. These are safe procedures, but you may feel some discomfort when the needles are inserted, which should quickly fade

After the microdialysis probe implantation, you will be allowed to rest for 60 minutes to allow the skin to recover before the start of the measurements. For 20 min a special fluid will be pumped through the microdialysis probe and collected for later testing. This fluid is very safe, used frequently in research and, in fact, you will not even notice this pumping through implant. A five-millilitre blood sample (about a teaspoon full) will also be collected from the needle in your other arm. After these measurements, the test drug (Ametop gel) will be applied to the skin at the marked area and covered with a dressing for 1 hour. After one hour, the remaining drug will be removed and your skin gently cleaned. The special fluid will be passed through the microdialysis probe and collected for 4 hours after the drug is applied. Five millilitre blood samples (about a teaspoon full) will be collected at 20 and 40 minutes and at 1, 1.5, 2, 2.5, 3, 3.5, and 4 hours after applying the drug.

At the end of the study day, we will remove the small cannula and the microdialysis probe from your arms and put on a dressing before you leave to prevent bleeding.

One week later you will come again for tape stripping study.

Visit 2: Tape stripping study
In this study, we will measure the amount of the drugs, which enter the outermost layers of skin (known as the “stratum corneum”). We will mark nine 2 x 3 cm rectangular sites (sites 1-9), on your forearms which is used for microdialysis study, with a pen. Site 1, positioned 3 cm above the elbow will be served as a control (no drug). One gram of Ametop gel will be applied on all the sites 2 to 9. We will remove the cream from the application site at 15, 30, 45 min., and 1h. We will perform the tape strip at 0, 15, 30, 45 min., 1, 1.5, 2, 3, and 4 h. Before performing the tape strip, we will measure the effectiveness of the anaesthetic by assessing your abilty to perceive a small pinprick in the areas of the drug application on your skin. We will ask you about any sensation you feel by touching your skin with the needle (without puncturing it) and ask you a few questions about what you felt.

- **If you have any medical problems or have to take any medicines, e.g. a course of antibiotics, during the two weeks before the study or during the time immediately afterwards it is important that you let us know as soon as possible.**

If you have any problems after the study you should contact **Dr Art Tucker** (020) 7601 8498 or **Professor Atholl Johnston** in the Clinical Pharmacology Department (020) 7882 3413. Outside working hours you can contact us via the hospital switchboard.

The study has been seen and approved by the local East London and City Authority Research Ethics committee. Any personal information will remain strictly confidential.

If you agree to join the study we will notify your GP that you are taking part.

If you feel any discomfort during the investigations, you must say so and we will stop the tests at any time.

Will this study help me?
This study may not benefit to you directly. It may, however, lead to the development of more effective skin anaesthesia.

Will I be paid?
For attending the Screening session we will compensate you with £10-00. At the end of the study we will be pleased to pay you £85-00for your time, commitment to the study and expenses (a total maximum payment of £95-00).

Could I come to any harm if I take part in the study?
The doses of the drugs have been chosen so that it should cause an effect only on the skin of your hands and not the rest of your body.
Ametop gel is a local anesthetic(tetracaine), it is safe and widly used for many years and is used at a very low concentration to reduce the pain prior to venepuncture or venous cannulation

There are very few risks involved in inserting a needle into a vein in the hand for venous sampling and subcutaneous tissue for microdialysis probe implantation and sampling. You may feel transient pain when the needle is placed under the skin which is similar to having a blood test. You may also experience discomfort and there may be a small bruise around the area which may last for a couple of days.

If you feel unacceptable discomfort or for any reason you do not wish to continue, than we will stop the test immediately.

Are there any factors which would exclude me from taking part in the research? (and which are not known by the investigators) e.g. Pregnancy or other medications.

We need to know whether you are taking any medication or if you have had any reactions to drugs in the past as this may also exclude you from the study.

There may be a risk that taking part in this trial might harm an unborn child and therefore pregnant women will not be included in this study. If you think that there is any chance that you might be pregnant, please inform the investigator and, with your consent, a pregnancy test will be performed.

You should not take part in this study if you are already involved in any other study.

You will also not be able to take part in the study if you are taking drugs of abuse, either short or long term.

How will confidentiality be protected?

All the information obtained about you in the course of the study is confidential and will be kept in a locked room. Only the investigators will have access to the data.

The investigators performing the study will have access to the data collected in this study. Official representatives of the Drug Regulatory Authorities may at some stage in the future request access to the data collected in this study. You will not be identifiable in any publication arising from the study.

If you would like more information about the study or are worried about any aspect of it please feel free to contact:

Dr Arthur Tucker
Ernest Cooke Clinical Microvascular Unit,
4th floor, Dominion House,
St Bartholomew's Hospital,
London EC1A 7BE.
Tel No. (020) 7601 8498

Mr Faisal Al-Otaibi
Clinical Pharmacology,
William Harvey Research Institute,
Charterhouse Square,
London EC1M 6BQ.
Tel No. (020) 7882 3413

You don't have to join the study. You are free to decide not to be in this trial or to drop out at any time.

What happens if you are worried or if there is an emergency?
You will always be able to contact an investigator to discuss your concerns and/or to get help

Name: **Prof. Atholl Johnston**
Post: **Professor of Clinical Pharmacology**
Address: **Department of Clinical Pharmacology, Charterhouse Square, London.**
Telephone/Fax: **(020) 7882 3413**

What happens if something goes wrong?
We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, we carry insurance to make sure that if your health does suffer as a result of your being in the study, then you will be compensated. In such a situation, you will not have to prove that the harm or injury, which affects you, is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

Appendix 5 inclusion and exclusion criteria for the volunteers (Chapter 2).

Inclusion criteria

1. Male and female Caucasian subjects are between 18 and 50 years of age, inclusive.

2. The subject is willing and able to read and understand the Subject Information Sheet and provide written informed consent.

3. The subject has a body mass index (BMI) within 18–35 kg/m^2.

4. The subject is in good health as determined by medical history and physical examination.

5. Female subjects must be practicing an acceptable method of birth control. Acceptable methods of birth control include hormonal contraceptives or double-barrier methods (condom or diaphragm with a spermicidal agent or IUD). If practising an acceptable method of birth control, a negative urine pregnancy test must be obtained at screening and on each Treatment Day.

6. The subject is a non-smoker.

7. The subject must agree to comply with the placement of an indwelling catheter on two occasions and the drawing of blood samples for the pharmacokinetic assessments during the study.

8. The subject is willing and able to comply with all testing and requirements defined in the protocol.

9. The subject is willing and able to return to the study site for all visits.

Exclusion criteria

Subjects meeting any of the following criteria will be excluded from entry into the study:

1. The subject has any relevant deviations from normal in physical examination (including any skin condition that in the investigator's judgment may affect the transdermal absorption of diazepam), electrocardiogram (ECG), or clinical laboratory tests, as evaluated by the investigator.

2. The subject has had a clinically significant illness within 30 days preceding entry into this study.

3. The subject has a history of significant neurological, hepatic, renal, endocrine, cardiovascular, gastrointestinal, pulmonary, or metabolic disease.

4. The subject has a known allergy or history of hypersensitivity to diazepam or similar compounds.

5. The subject has used any prescription medication within 14 days or over-the-counter (OTC) medication or alcohol within 48 hours of dosing or intends to use any prescription or OTC medication during the study that may interfere with the evaluation of study medication (excluding oral contraceptives).

6. Subject is pregnant or breast-feeding.

7. The subject has donated or lost a significant volume of blood (>450 mL) within four (4) weeks of the study, and their haemoglobin concentration and haematocrit have not returned to within 5% of normal.

8. The subject has a history of substance abuse or a current positive urine drug screen or urine alcohol test.

9. Alcohol consumption greater than community norms (i.e. more than 21 standard drinks per week for males, or more than 14 standard drinks per week for females).

10. Subjects who have received an investigational drug or have used an investigational device in the 30 days prior to study entry.

Appendix 6 inclusion and exclusion criteria for the volunteers (Chapter 4).

Inclusion criteria

In order to be eligible to enter the study, volunteers must meet the following criteria:

1 Male and female aged between 18 and 45 years of age.

2 Free of significant abnormal findings as determined by medical history, urinalysis (including specific gravity), and vital signs (sitting blood pressure, sitting pulse rate, within 1 week of commencement of the study.

3. BMI between 18 and 30

4 No history or signs of drug abuse (including alcohol), licit or illicit.

5 Agrees not to use any medications (prescribed or over-the-counter including herbal remedies, but excluding oral contraceptives) judged to be clinically significant by the Principal Investigator during the seven (7) days preceding the study, and during the course of the study.

6 Not pregnant and is using an acceptable form of contraception, or judged unable to become pregnant (for females), and willing to take precautions to prevent pregnancy with their partner (for males) until completion of the study.

7 Able to understand and sign the written Informed Consent Form.

8 Able and willing to follow the Protocol requirements.

Exclusion criteria

Volunteers will not be admitted to the study if they meet any of the following exclusion criteria:

1 Any significant history of allergy and/or sensitivity to any of the contents of study drugs.

2 A pulse rate of less than 50 beats/minute, a sitting systolic blood pressure >160 or <80 mmHg and/or a sitting diastolic pressure of >100 or <60 mmHg.

3 Any significant illness during the four (4) weeks preceding the screening period of the study.

4 Any contraindication to blood sampling.

5 Any contraindication to local anaesthetics administration.

6 Positive urine drug screen or indication

7 Is currently breast feeding.

8 Positive screening Pregnancy test (females only).

9 Participation in any clinical study during the 8 weeks proceeding the dosing period of the study

10 Donation of blood during the eight (8) weeks proceeding the screening period of the study or during the investigation.

Appendix 7 Certificate of analysis of diazepam-D_5, and metabolites-D_5 (Chapter 2)

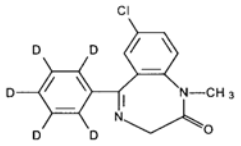
Cerilliant
Science. Down to a science.

D-902
23696-09O
Revision 0

Certificate of Analysis

Diazepam-D_5

7-Chloro-1,3-dihydro-1-methyl-5-pentadeuterophenyl-2H-1,4-benzodiazepin-2-one



Catalog Number:	D-902
Solution Lot:	23696-09O
Expiration Date:	September 2010
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Diazepam-D_5	99%	100.0 ± 3.1 µg/mL	99 ± 0.5 µg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (µg/mL)*	*% Difference from Target*
New Lot	23696-09O	99.0	-1.0
Previous Lot	23696-09N	98.8	-1.2

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (µg/mL)*	*Mean*	*% RSD*
Early	99.1		
Middle	98.6		
Late	99.2	99.0	0.3

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Cerilliant certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation using balances calibrated with NIST traceable weights. Precision is guaranteed by triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature: Lara Sparks

Lara Sparks, Quality Assurance Director

September 19, 2005

Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78664 800-848-7837 / 512-238-9974

Cerilliant
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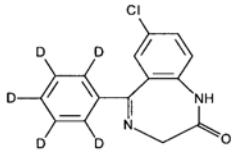
N-903
35268-34B
Revision 1

Certificate of Analysis

Nordiazepam-D_5

7-Chloro-1,3-dihydro-5-pentadeuterophenyl-2H-1,4-benzodiazepin-2-one



Catalog Number: N-903
Solution Lot: 35268-34B
Expiration Date: August 2010
Solvent: Methanol
Amount per Ampule: 1 mL
Storage: Protect from light, refrigerate.
Handling: We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use: For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Nordiazepam-D_5	99%	100.0 ± 3.1 µg/mL	100.7 ± 0.4 µg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (µg/mL)*	*% Difference from Target*
New Lot	35268-34B	100.7	0.7
Previous Lot	35012-57E	94.9	-5.1

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (µg/mL)*	*Mean*	*% RSD*
Early	100.0		
Middle	101.2		
Late	101.0	100.7	0.6

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Cerilliant certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation using balances calibrated with NIST traceable weights. Precision is guaranteed by triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature: Lara Sparks

Lara Sparks, Quality Assurance Director

January 12, 2006

Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78664 800-848-7837 / 512-238-9974

Cerilliant
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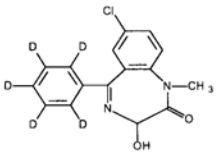
T-902
35233-35C
Revision 0

Certificate of Analysis

Temazepam-D_5

7-Chloro-1,3-dihydro-3-hydroxy-1-methyl-5-pentadeuterophenyl-2H-1,4-benzodiazepine-2-one



Catalog Number: T-902
Solution Lot: 35233-35C
Expiration Date: April 2010
Solvent: Methanol
Amount per Ampule: 1 mL
Storage: Protect from light, refrigerate.
Handling: We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use: For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Temazepam-D_5	99%	100.0 ± 3.1 µg/mL	98.4 ± 0.9 µg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (µg/mL)*	*% Difference from Target*
New Lot	35233-35C	98.4	-1.6
Previous Lot	35233-35A	99.5	-0.5

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (µg/mL)*	*Mean*	*% RSD*
Early	97.6		
Middle	98.3		
Late	99.4	98.4	0.9

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Cerilliant certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation using balances calibrated with NIST traceable weights. Precision is guaranteed by triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature: Lara Sparks

Lara Sparks, Quality Assurance Director

April 28, 2005

Date

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Cerilliant
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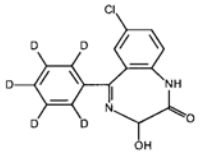
O-901
35268-77B
Revision 0

Certificate of Analysis

Oxazepam-D_5

7-Chloro-1,3-dihydro-3-hydroxy-5-pentadeuterophenyl-2H-1,4-benzodiazepin-2-one



Catalog Number: O-901
Solution Lot: 35268-77B
Expiration Date: April 2010
Solvent: Acetonitrile
Amount per Ampule: 1 mL
Storage: Protect from light, refrigerate.
Handling: We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use: For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Oxazepam-D_5	99%	100.0 ± 3.1 µg/mL	101.0 ± 0.3 µg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (µg/mL)*	*% Difference from Target*
New Lot	35268-77B	101.0	1.0
Previous Lot	35070-85B	100.4	0.4

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (µg/mL)*	*Mean*	*% RSD*
Early	101.3		
Middle	100.6		
Late	101.3	101.0	0.4

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Cerilliant certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation using balances calibrated with NIST traceable weights. Precision is guaranteed by triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature: Lara Sparks

Lara Sparks, Quality Assurance Director

April 27, 2005
Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78664 800-848-7837 / 512-238-9974

Appendix 8 Delegation Log (sample).

Study title:

All those involved in the above study must read the protocol (and the amendment if applicable) and understand their role as outlined in protocol

Name (print)	Job title	Signature	Sign initial	List duty categories	PI signature and date	Date of leaving the team (if appl.)

Key for list of duty categories:

1. **Obtaining informed**
2. **Physical Exam/ Clinical Evaluations**
3. **Source document entry (ie. Medical notes)**
4. **CRF completion / data entry**
5. **Resolving data queries**
6. **Review & reporting adverse event & SAE**
7. **Medical prescriptions**
8. **Drug accountability**
9. **Maintaining investigator file**
10. **Archiving**

Other duties specific to above study please specify below

11. **Principle Investigator**
12. **Study Support**

Appendix 9 Screening Log (sample).

Study title:

Date	Patient Initials	Date of birth	Hospital Number	Sex	Enrolled (yes or no)	Reason for exclusion

Appendix 10 Enrolment Log (sample).

Study title:

Subjects ID code	Subject initials	Date of birth	Date of consent	Date of randomization	Date of withdrawal	Date completed

Appendix 11 Plasma diazepam concentration following rectal administration for 12 subjects (Chapter 2).

Time	Subject / Concentration (μg/L)												
(h)	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	Average
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	266.9	367.4	120.8	215.4	174.1	201.0	250.7	118.1	169.1	60.4	210.3	162.4	193.1
0.5	308.7	234.2	230.6	309.7	198.7	349.2	254.3	207.9	359.2	132.7	268.8	294.3	262.4
0.75	246.4	242.8	286.8	252.6	156.1	333.0	250.1	323.4	303.1	138.3	297.8	346.8	264.8
1	240.1	375.4	173.5	192.3	161.5	297.9	233.3	343.3	301.4	129.2	272.5	378.7	258.3
1.5	210.9	190.9	133.3	126.3	133.1	215.6	151.1	294.3	217.7	108.2	223.8	324.7	194.2
2	177.8	235.2	86.7	109.5	123.2	178.9	130.5	222.1	152.8	82.0	178.3	253.2	160.9
2.5	138.6	126.5	92.8	89.2	111.8	150.2	132.2	222.4	132.7	63.4	138.9	230.6	135.8
3	125.5	102.8	78.4	87.9	109.9	141.5	115.0	183.6	121.9	66.2	118.6	200.9	121.0
4	109.0	87.7	97.8	79.1	102.2	126.5	121.9	179.6	120.2	56.3	88.1	176.2	112.1
5	102.1	82.4	96.9	75.8	100.5	133.1	113.0	154.6	109.6	44.8	95.4	139.4	104.0
6	108.2	79.0	85.2	81.6	99.7	141.1	102.7	124.8	108.2	38.0	83.8	124.7	98.1
8	67.4	59.7	73.2	73.7	93.0	129.2	104.7	83.7	89.9	48.0	83.6	109.4	84.6
10	78.6	83.9	75.9	69.5	84.0	114.7	72.1	84.0	93.0	34.0	65.9	96.2	79.3
12	61.2	49.1	73.3	67.5	73.4	110.2	79.9	93.8	75.2	36.6	61.4	66.3	70.7
24	52.0	75.2	94.3	63.0	69.9	83.6	64.1	49.1	57.8	23.2	60.5	72.1	63.7
32	61.4	49.4	70.2	48.0	57.7	72.0	45.7	41.9	30.0	15.3	45.5	47.5	48.7
48	48.4	45.8	68.9	0.0	44.5	50.0	51.7	32.3	33.9	7.8	38.0	26.4	40.7
72	35.8	36.1	48.7	0.0	36.9	30.2	33.8	25.2	43.3	5.1	24.6	22.0	31.1

Appendix 12 Plasma diazepam concentration following TDS® administration for 12 subjects (Chapter 2).

Time	Subject / Concentration (μg/L)												
(h)	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	Average
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
0.25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
0.75	7.3	0.0	11.8	0.0	0.0	14.1	0.0	0.0	0.0	5.1	5.2	0.0	3.6
1	8.9	0.0	12.9	0.0	0.0	19.4	0.0	9.5	5.6	12.5	9.3	11.9	7.5
1.5	21.3	7.5	29.7	0.0	6.0	26.5	0.0	35.6	14.2	13.0	31.3	36.8	18.5
2	29.9	10.2	33.8	0.0	14.3	22.7	0.0	58.9	21.8	17.0	59.2	47.7	26.3
2.5	36.3	15.2	30.4	0.0	17.5	14.4	0.0	63.2	23.6	14.4	66.8	45.5	27.3
3	37.0	15.8	29.2	0.0	20.9	16.0	0.0	63.4	20.8	12.6	57.4	53.1	27.2
4	25.9	21.0	30.5	0.0	19.2	16.4	0.0	47.3	16.9	9.5	46.0	37.5	22.5
5	27.0	17.4	27.5	0.0	15.5	18.0	0.0	36.7	18.0	8.6	32.7	35.4	19.7
6	21.9	16.2	24.2	0.0	16.9	19.5	0.0	30.8	15.6	9.5	26.1	31.6	17.7
8	20.3	18.0	24.0	0.0	14.1	13.8	0.0	29.1	17.7	11.6	25.9	34.0	17.4
10	14.2	19.0	26.7	0.0	14.2	19.1	0.0	28.5	17.3	12.4	26.4	30.6	17.4
12	12.4	17.3	27.3	0.0	27.6	16.9	0.0	30.8	20.6	10.9	21.7	31.7	18.1
24	18.6	19.4	27.8	0.0	16.3	12.7	7.3	34.0	22.8	14.0	29.9	32.4	19.6
32	16.4	15.7	23.8	0.0	26.9	5.5	7.2	34.0	20.9	11.6	19.7	33.1	17.9
48	18.1	14.5	21.8	0.0	26.4	0.0	6.8	19.7	16.0	8.9	18.7	20.7	14.3
72	12.9	12.1	19.4	0.0	20.8	0.0	7.1	9.0	10.0	0.0	14.6	10.1	9.7
Final	5.2	5.3	5.2	0.0	12.9	0.0	0.0	0.0	6.8	0.0	0.0	0.0	3.0

Appendix 13 Plasma desmethyldiazepam concentration following rectal administration for 12 subjects (Chapter 2).

Time	Subject / Concentration (μg/L)												
(h)	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	Average
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
0.25	0.0	5.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.1
0.5	0.0	5.0	0.0	6.8	0.0	11.1	0.0	0.0	6.0	0.0	6.9	5.2	6.8
0.75	0.0	5.7	0.0	8.7	0.0	14.8	6.6	5.5	6.9	0.0	8.5	8.7	8.2
1	0.0	10.4	0.0	8.2	0.0	19.4	7.7	7.1	7.9	5.8	8.9	9.6	9.4
1.5	5.9	8.5	0.0	8.8	5.1	19.6	8.4	10.0	9.6	9.3	10.9	12.8	10.3
2	7.1	12.3	0.0	8.9	6.0	20.4	9.8	13.3	9.8	8.6	13.1	13.5	11.6
2.5	6.1	7.8	6.0	8.5	6.4	19.8	10.6	15.7	10.3	9.3	12.4	13.8	11.0
3	7.9	7.8	5.1	9.9	6.7	22.2	11.1	15.2	10.5	10.7	13.4	14.5	11.6
4	8.4	8.5	7.2	9.8	8.1	22.6	13.0	18.0	12.8	11.6	13.5	18.9	13.1
5	8.9	9.4	8.4	9.8	8.7	25.2	14.8	19.0	12.1	10.7	15.9	19.3	13.9
6	11.9	10.1	9.0	11.9	11.2	31.2	15.5	17.9	13.4	10.7	16.0	19.2	15.1
8	9	9.4	8.8	12.9	12.8	34.4	19.5	15.5	13.5	15.2	19.8	21.9	16.7
10	12.4	14.0	11.0	13.9	14.1	36.6	18.7	17.9	17.0	14.6	18.8	24.5	18.3
12	10.6	9.9	11.7	17.1	14.2	38.5	19.3	21.2	14.8	16.6	19.3	18.3	18.3
24	14.5	20.3	28.3	23.7	23.0	52.1	29.9	24.4	24.2	20.7	30.4	32.5	28.1
32	24.4	17.3	25.8	23.3	25.9	54.0	30.0	27.3	18.4	19.1	29.5	32.2	27.5
48	25.2	22.0	35.9	0.0	30.7	57.6	37.7	30.4	26.7	20.0	36.4	28.8	32.6
72	27.6	22.9	38.7	0.0	41.1	54.2	41.0	31.8	37.6	16.8	34.7	27.2	34.6

Appendix 14 Plasma diazepam concentration following TDS® administration for 12 subjects (Chapter 2).

Time	Subject / Concentration (μg/L)												
(h)	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	Average
0	0.0	7.7	0.0	0.0	0.0	0.0	9.3	13.9	7.9	0.0	5.0	0.0	10.5
0.25	9.9	8.2	19.9	0.0	0.0	0.0	10.8	12.4	8.2	0.0	5.5	0.0	10.6
0.5	9.4	7.6	19.7	0.0	5.5	0.0	9.5	11.9	8.6	0.0	5.5	0.0	11.3
0.75	11.6	10.2	18.5	0.0	5.4	0.0	9.5	11.3	10.0	0.0	5.2	0.0	11.4
1	10.3	8.1	19.7	0.0	5.0	0.0	10.1	12.5	10.3	0.0	0.0	0.0	12.4
1.5	11.4	9.3	19.0	0.0	6.1	0.0	11.6	12.6	10.7	0.0	6.0	0.0	11.9
2	11.1	7.3	19.6	0.0	5.1	0.0	10.6	11.5	10.8	0.0	6.6	0.0	11.9
2.5	11.1	8.2	18.2	0.0	5.7	0.0	10.8	12.4	11.6	0.0	7.6	0.0	12.2
3	11.6	9.5	18.7	0.0	5.7	5.0	10.8	13.7	10.6	0.0	7.1	0.0	11.8
4	11.2	11.0	19.6	0.0	6.3	5.4	10.2	16.6	10.4	0.0	8.2	0.0	12.2
5	12.0	8.8	19.0	0.0	5.5	6.8	10.4	15.5	11.2	0.0	7.9	0.0	13.0
6	10.8	9.4	18.2	0.0	6.0	6.3	9.1	14.5	10.5	0.0	7.5	5.3	11.9
8	11.3	9.5	18.1	0.0	5.7	6.1	10.5	14.2	11.6	0.0	9.8	6.9	12.5
10	7.3	11.3	19.9	0.0	5.9	10.3	8.8	16.3	11.1	0.0	10.5	6.7	12.6
12	8.0	9.0	19.4	0.0	11.6	13.1	9.5	18.2	11.6	0.0	9.9	7.3	13.1
24	11.4	10.2	21.7	5.1	11.8	15.2	9.4	20.9	14.3	6.5	13.8	10.3	13.6
32	12.3	10.7	22.5	6.8	15.7	13.4	10.6	25.4	15.6	8.1	12.9	12.6	15.2
48	14.4	12.3	21.6	0.0	17.3	5.8	10.4	23.2	14.6	8.7	16.5	15.1	15.5
72	15.0	13.5	25.5	0.0	19.7	0.0	10.1	18.5	15.0	7.8	17.7	14.0	13.3
Final	9.6	9.2	13.4	0.0	13.0	0.0	8.2	8.2	13.4	0.0	12.1	8.0	11.3

Appendix 15 Plasma tetracaine metabolite (p-butyl amino-benzoic acid) concentration for 12 subjects (Chapter 4).

Time	Subject / Concentration (μg/L)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	NA	0
40	6	2.2	0.6	NA	0	14.3	0	NA	NA	0	0	NA	2.9
60	21.5	15.5	13.5	16.7	1.4	33	0	0	0	1.6	8.2	NA	10.1
90	19.7	29.6	17.3	37.5	16.5	35.4	0	11.6	0	12.3	16.8	16	17.7
120	14.6	39.7	11.5	21.1	15.7	24.4	1	22.1	0	20.5	17.9	15	16.9
150	11.8	33.6	9.6	13.9	11.7	17.7	1.6	22	0.7	18	15.7	10.4	13.9
180	10.4	26.6	7.6	11.1	9.4	15.1	2.4	20.1	1.3	15.4	13	NA	12.0
210	9.4	23.7	6.4	9.8	9.2	11.8	3.3	19.1	2.3	12.2	10.6	NA	10.7
240	8.6	20.1	5	7.6	8.4	10.8	4.2	16.4	2.8	13.2	10.9	NA	9.8

NA: not available.

Appendix 16 Tetracaine metabolite (p-butyl amino-benzoic acid) concentration from dialysate fluid for 12 subjects (Chapter 4).

Time	Subject / Concentration (μg/L)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0	0	0	0	0	0	0	0	NA	0	0	0	47.6	4.3
20	0	0	0	0	0	0	0	NA	0	0	0	17.7	1.6
40	0	6.1	6.6	0	0	0	0	NA	0	0	0	17.7	2.8
60	4.4	9.1	6	28.6	0	0	8	NA	0	32.6	0	25.9	10.4
80	15.6	16.6	11.5	103.0	7.6	9.9	19.9	NA	13.6	108.6	31.3	30.2	33.4
100	24.3	17.0	18.1	146.3	41.6	12.3	18.5	NA	16.8	151.4	71.0	28.6	49.6
120	21.1	16.3	14.9	172.8	90.3	25.3	20.6	NA	13.2	168.0	127.9	34.4	64.1
140	23.3	11.8	12.7	150.1	118.5	47.6	7.3	NA	17.0	145.5	118.6	39.2	62.9
160	36.7	6.3	16.3	148.0	151.2	49.1	12.2	NA	16.4	147.1	126.5	33.9	67.6
180	28.5	8.9	21.0	150.3	176.6	66.8	9.3	NA	17.7	124.3	96.7	35.7	66.9
200	28.45	10.5	20.6	116.9	120.6	88.5	9.6	NA	21.7	131.7	153.1	40.7	67.5
220	37	12.7	19.8	139.2	132.6	74.7	12.1	NA	16.9	135.8	122.5	46.5	68.2
240	49.3	8.7	19.7	104.5	109.8	57.1	6.2	NA	19.2	112.7	69.1	55.6	55.6

NA: not available.

Appendix 17 Tetracaine amount from tape stripping harvesting sample analyzed by CE method for 12 subjects (Chapter 4).

Time	Subject / Concentration (μg/L)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15.0	36.3	215.4	61.3	95.2	61.3	104.2	50.6	70.4	28.9	62.7	139.0	32.9	95.9
30.0	43.6	309.7	115.6	135.5	88.5	93.4	40.4	73.2	117.5	115.0	210.3	76.1	124.6
45.0	48.9	252.6	122.3	261.0	80.7	141.3	106.9	106.3	205.8	217.9	160.8	104.3	145.9
60.0	128.5	192.3	228.7	258.0	241.6	252.6	101.2	186.0	323.3	194.6	487.9	367.7	178.6
90.0	88.6	126.3	87.1	93.8	112.9	118.2	62.1	108.1	139.0	113.8	89.0	67.3	131.2
120.0	78.3	109.5	60.5	118.2	79.3	131.8	63.2	104.6	144.2	58.3	44.4	99.0	131.6
180.0	88.6	89.2	85.1	114.8	86.3	173.9	84.2	93.6	79.1	95.2	57.3	58.5	107.4
240.0	61.7	87.9	106.0	106.5	52.0	213.5	91.2	100.9	86.9	67.2	71.3	84.2	114.5

Appendix 18 Tetracaine amount from tape stripping harvesting sample analyzed by HPLC method for 12 subjects (Chapter 3).

Time	Subject / Concentration (μg/L)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15.0	33.5	27.5	57.5	94.0	65.7	94.2	47.2	75.6	48.9	70.4	146.6	31.1	91.5
30.0	43.7	119.5	130.6	155.0	93.4	103.7	38.9	81.0	96.6	119.4	211.5	87.0	141.2
45.0	46.4	221.1	135.1	278.2	89.3	143.1	100.2	118.3	114.6	226.9	185.8	93.8	158.9
60.0	127.9	297.8	240.6	284.0	254.9	214.4	93.7	200.0	143.0	190.2	448.3	343.1	191.2
90.0	97.4	130.5	99.8	88.3	NA	114.7	60.6	114.4	93.7	119.4	92.3	62.8	148.5
120.0	76.2	133.4	59.0	138.2	88.8	129.0	57.7	109.5	94.9	63.9	42.1	109.4	144.9
180.0	83.1	74.7	100.9	111.9	94.4	160.4	78.4	99.0	72.2	102.0	53.1	67.8	119.5
240.0	57.0	83.1	120.8	123.5	58.4	186.2	84.6	NA	100.2	73.7	74.2	94.2	109.2

NA: not available.

Appendix 19 VRS scores by pin prick test following the application of 1mL tetracaine gel to 12 subjects (Chapter 4).

Time	Subject / VRS Score (Category 1-4)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0.0	3.0	2.0	2.0	2.0	3.0	3.0	3.0	3.0	3.0	2.0	3.0	4.0	2.8
15.0	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	1.0	3.0	1.7
30.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	1.0	1.0	2.0	1.3
45.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	1.0	1.0	1.2
60.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
90.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
120.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
180.0	3.0	1.0	2.0	2.0	1.0	3.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5
240.0	3.0	2.0	2.0	2.0	3.0	3.0	3.0	3.0	3.0	2.0	3.0	4.0	2.8

Appendix 20 VAS scores by pin prick test following the application of 1mL tetracaine gel to 12 subjects (Chapter 4).

Time	Subject / VAS Score (0-100 mm)												
(min)	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	Average
0.0	60.0	20.0	35.0	24.5	49.5	77.0	28.5	83.0	63.0	28.5	63.5	90.5	51.9
15.0	5.0	4.5	0.0	0.0	36.5	35.5	23.0	35.0	22.0	28.5	0.0	50.0	20.0
30.0	1.5	2.0	0.0	0.0	10.0	0.0	19.0	24.0	0.0	7.5	0.0	13.5	6.5
45.0	3.0	2.5	0.0	0.0	0.0	0.0	0.0	27.0	27.0	0.0	0.0	0.0	5.0
60.0	4.0	0.0	0.0	0.0	6.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9
90.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
120.0	2.5	1.5	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.4
180.0	62.5	2.5	10.0	14.0	5.5	61.5	0.0	0.0	1.0	0.0	0.0	4.5	13.5
240.0	60.0	20.0	35.0	24.5	49.5	77.0	28.5	83.0	63.0	28.5	63.5	90.5	51.9

Appendix 21 Patients plasma diazepam and desmethyldiazepam concentrations (Chapter 2).

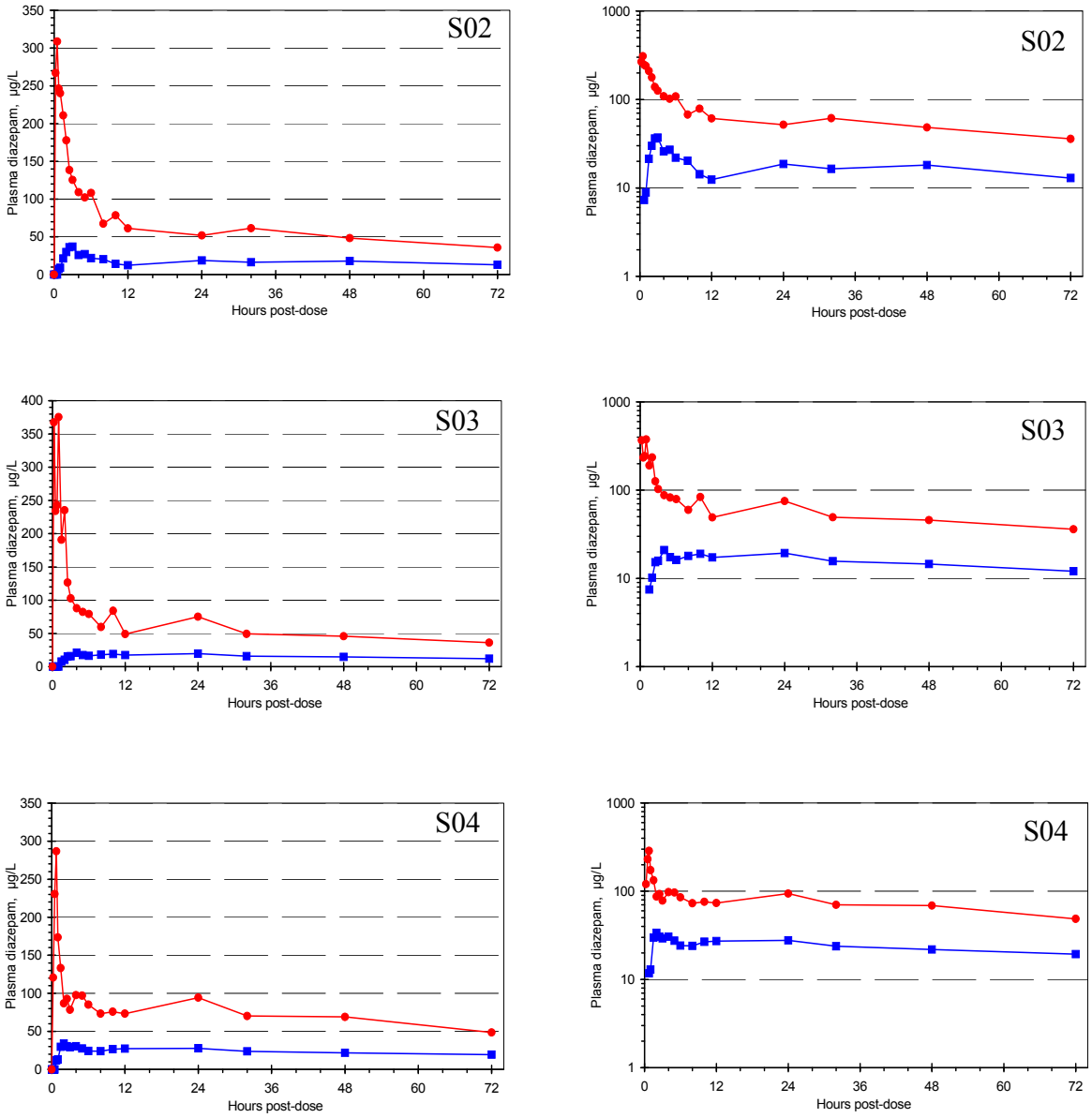


Figure 20.A Plasma diazepam (subjects S02–S04) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

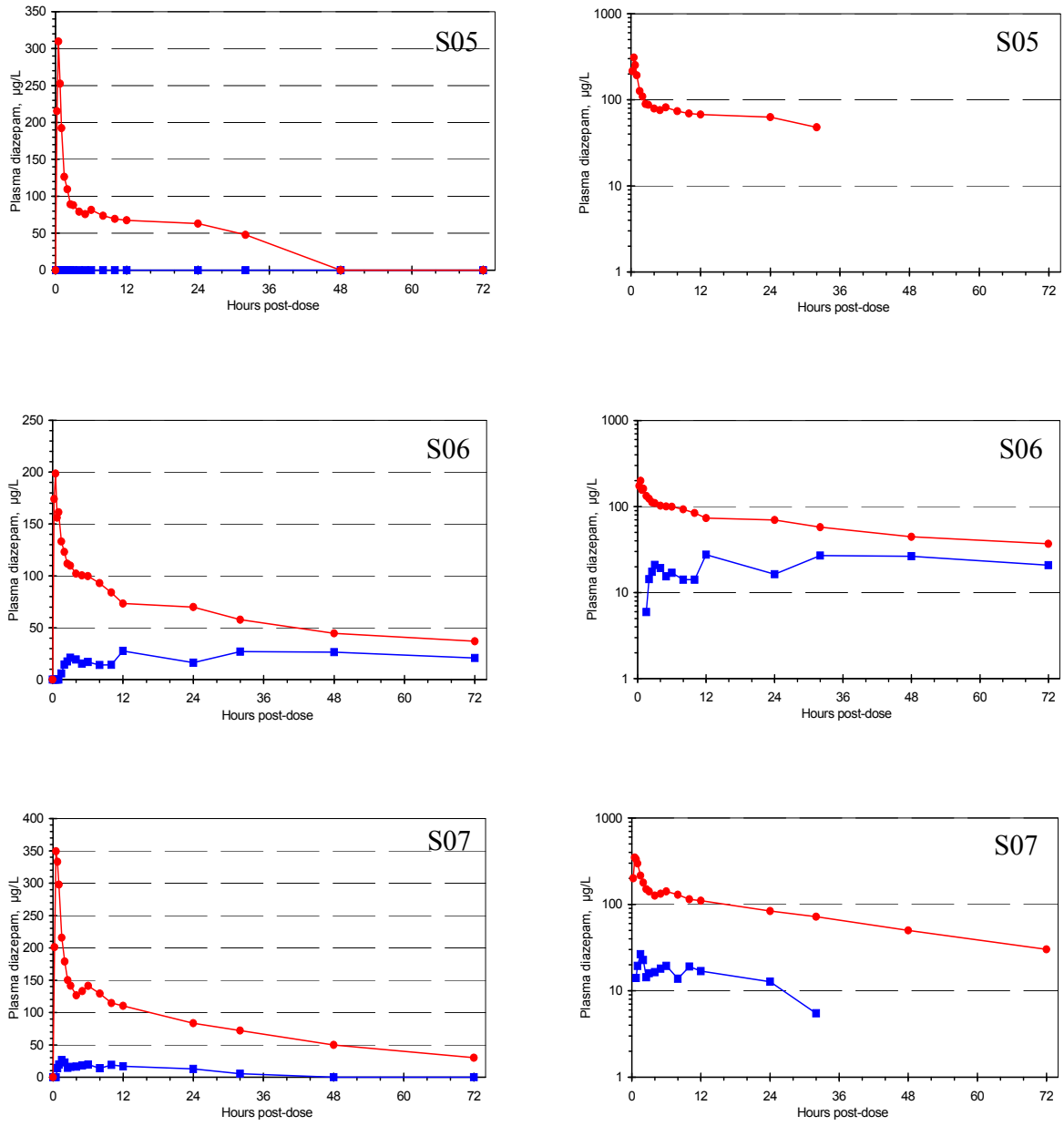


Figure 20.B Plasma diazepam (subjects S05–S07) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

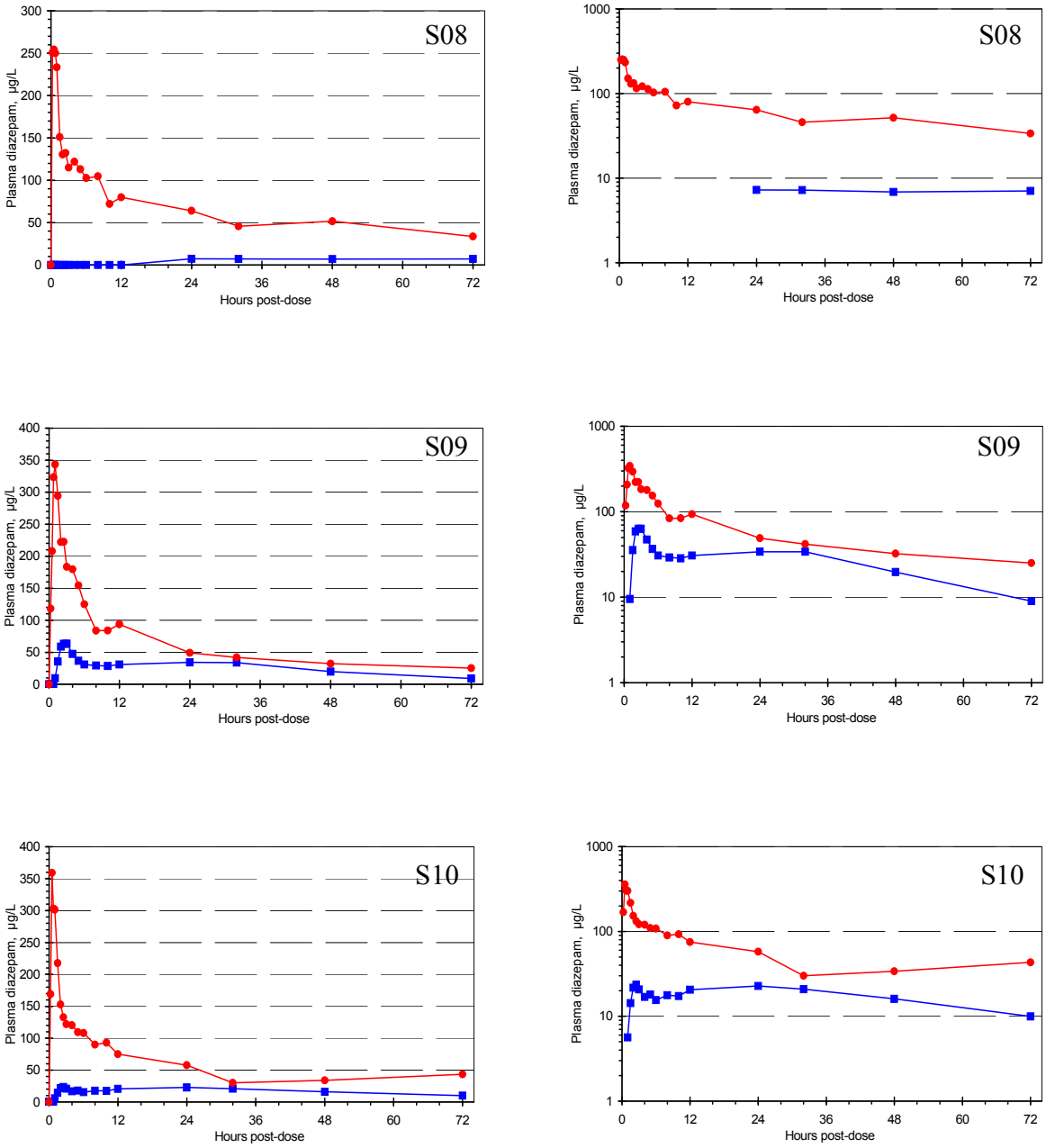


Figure 20.C Plasma diazepam (subjects S08–S10) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

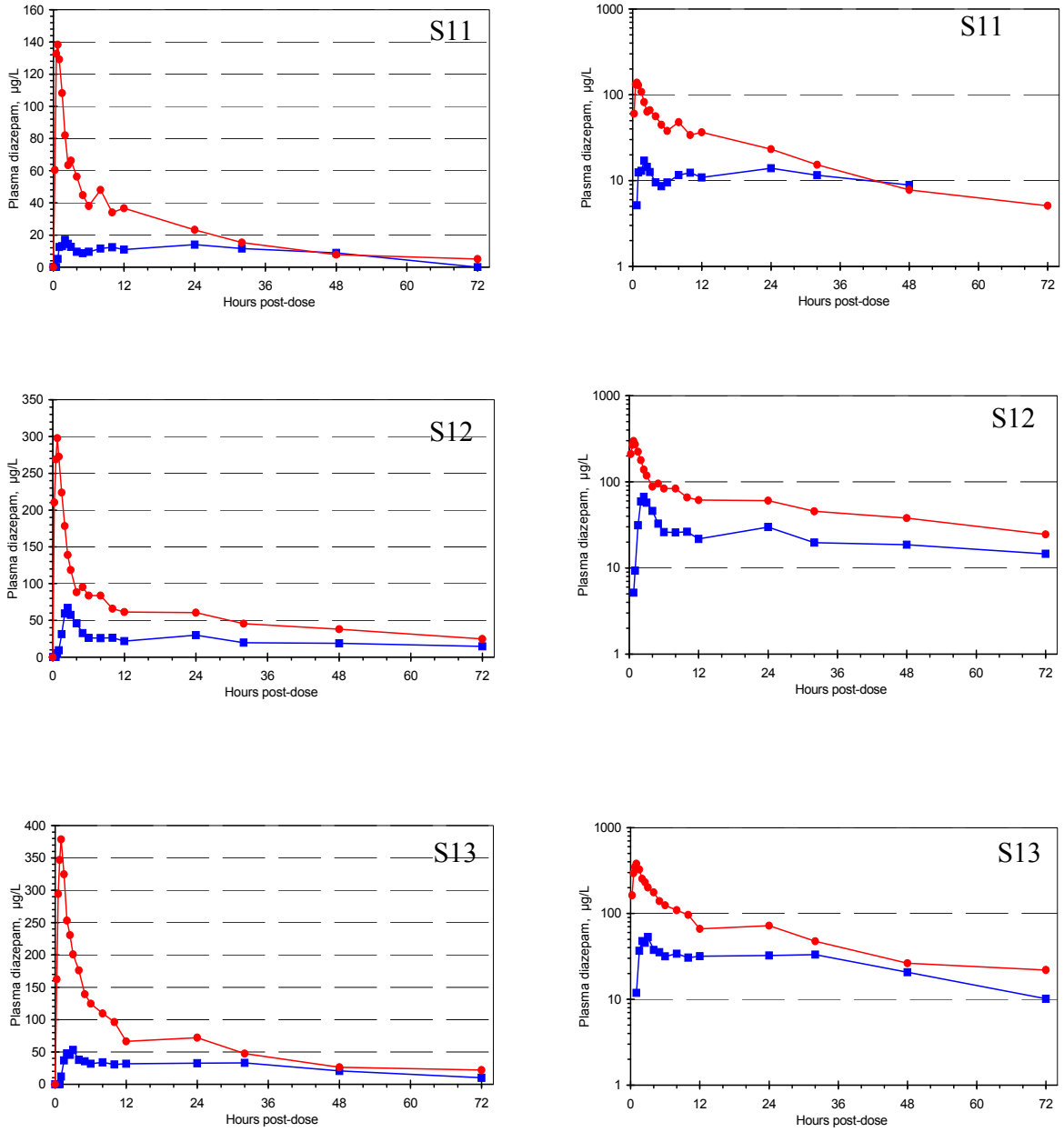


Figure 20.D Plasma diazepam (subjects S11–S13) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

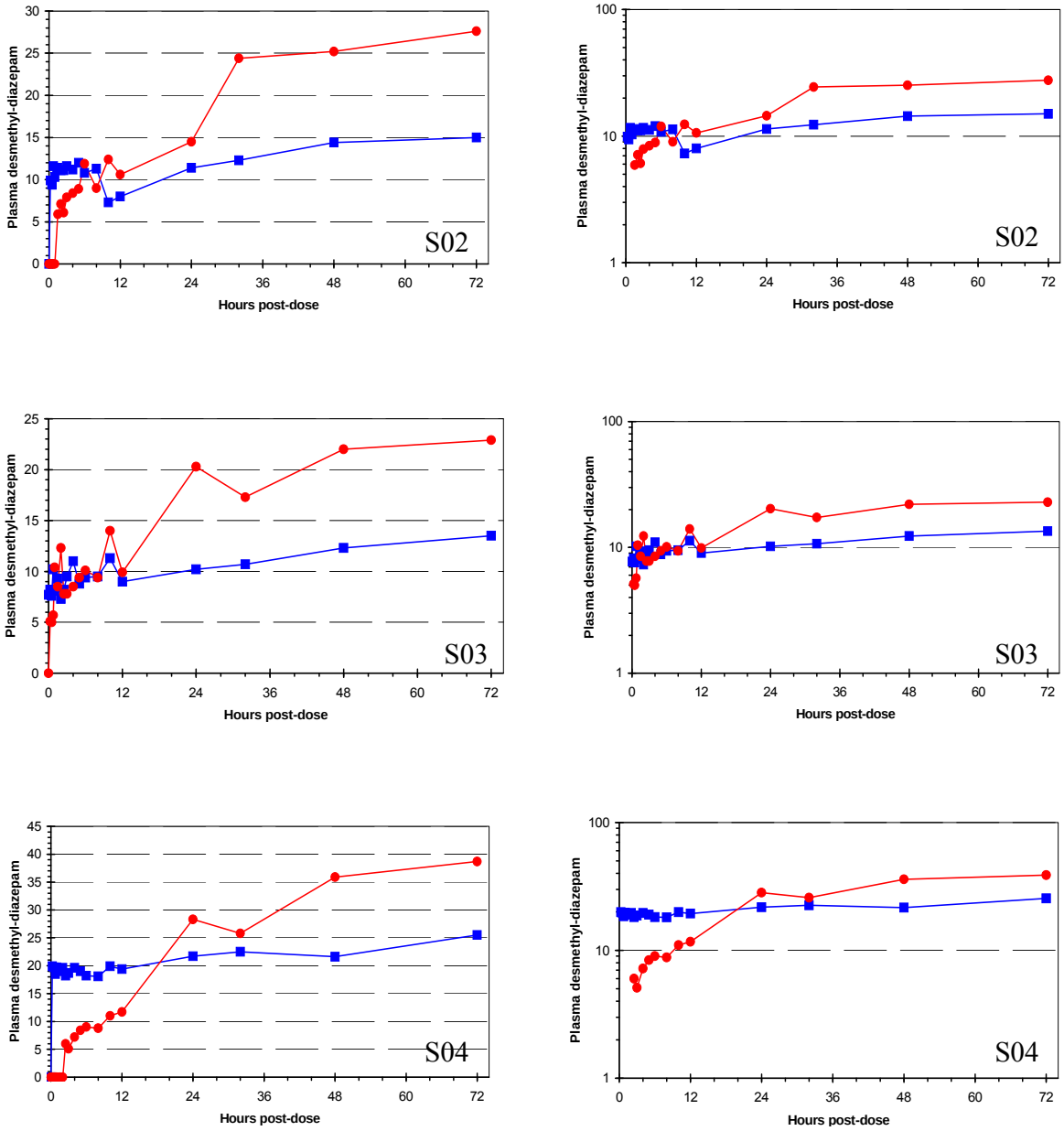


Figure 20.E Plasma desmethyldiazepam (subjects S02–S04) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

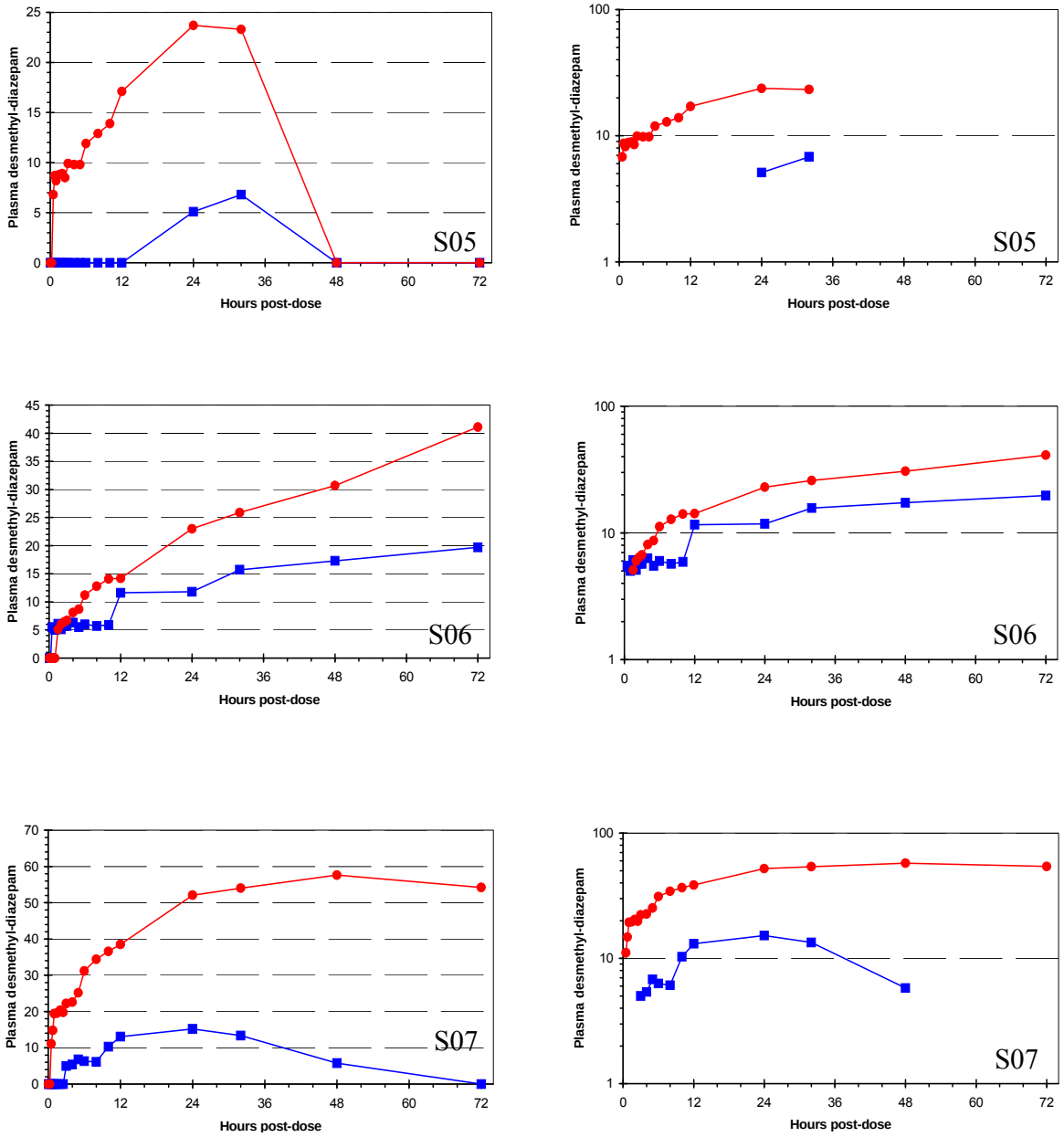


Figure 20.F Plasma desmethyldiazepam (subjects S05–S07) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

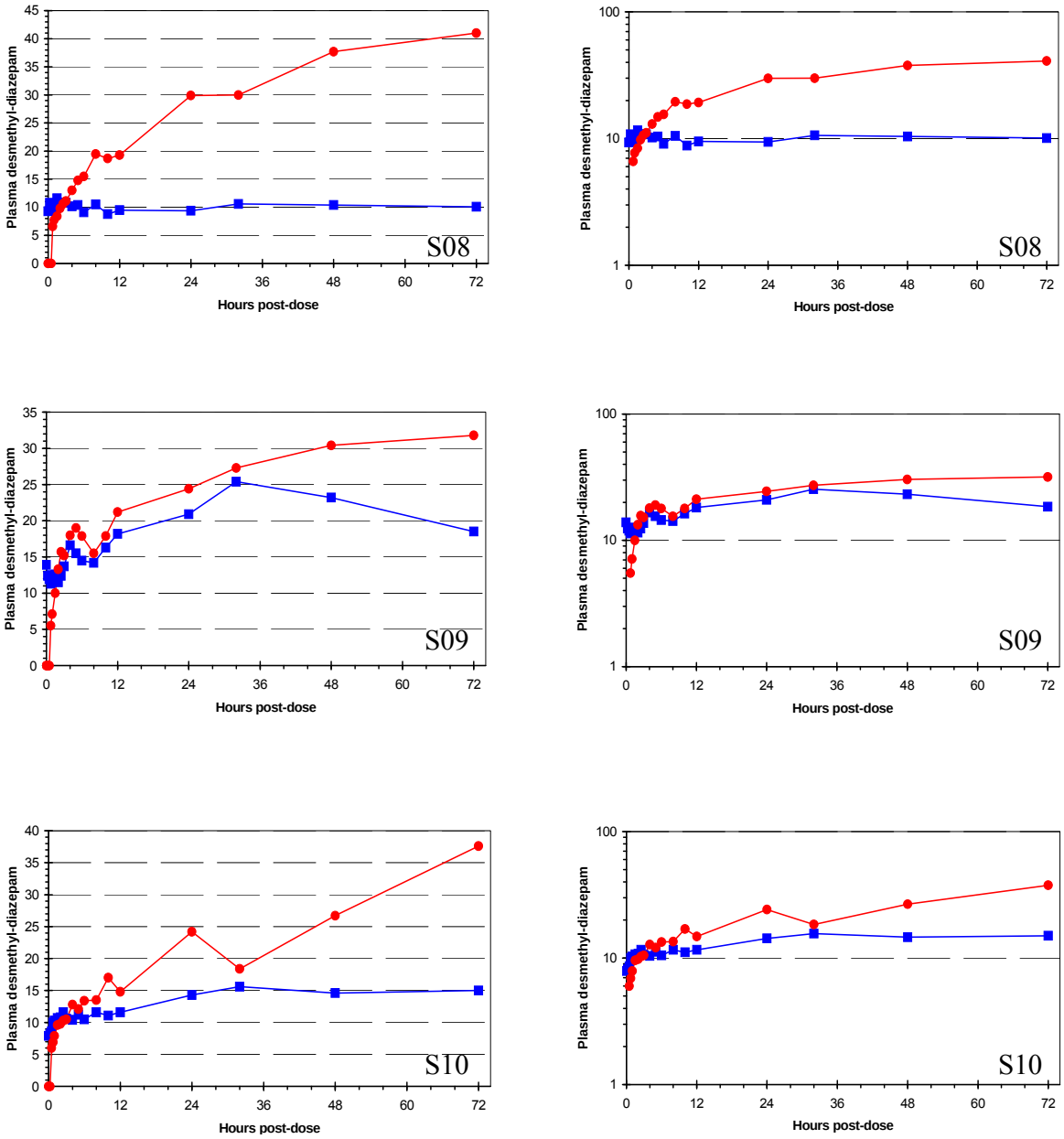


Figure 20.G Plasma desmethyldiazepam (subjects S08–S10) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

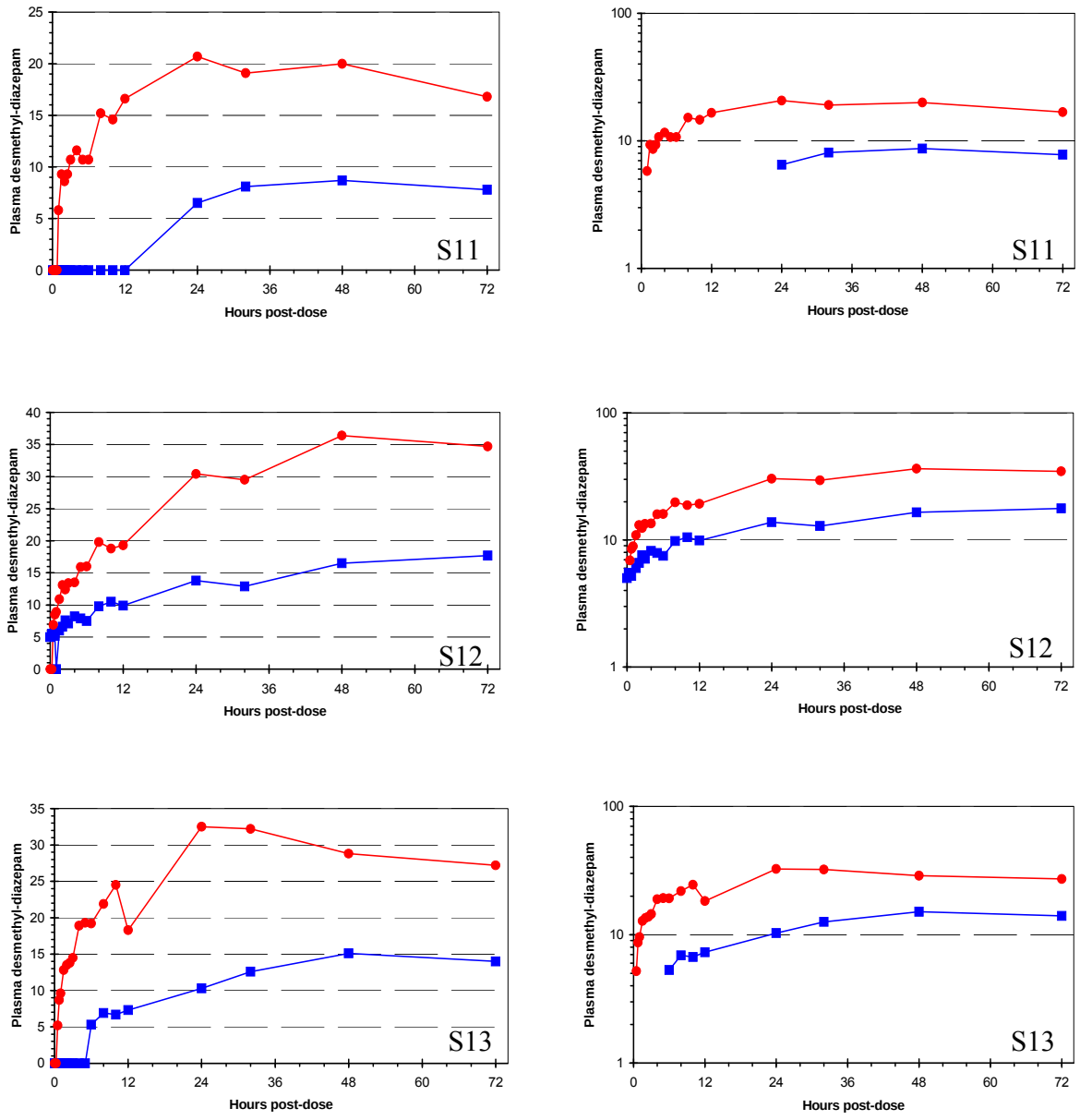


Figure 20.H Plasma desmethyldiazepam (subjects S11–S13) versus time following 10 mg rectal (filled red circles) and TDS® diazepam (filled blue squares), linear concentration axis, and logarithmic concentration axis.

Appendix 22 Volunteers tetracaine concentration with time profile in tape samples (Chapter 4).

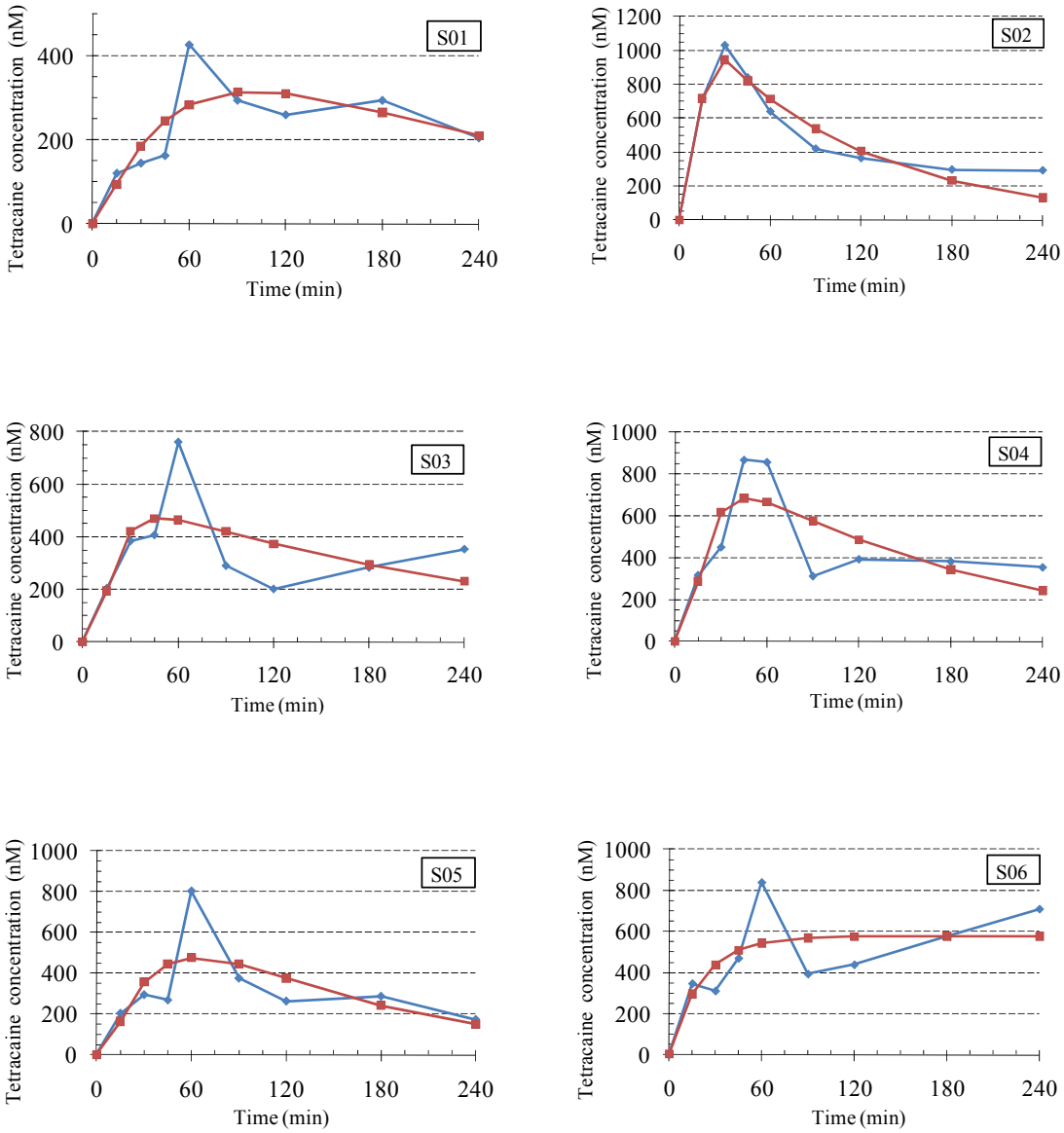


Figure 21.A Tetracaine concentration (obtained from subjects S01–S06) with time profiles in tape samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

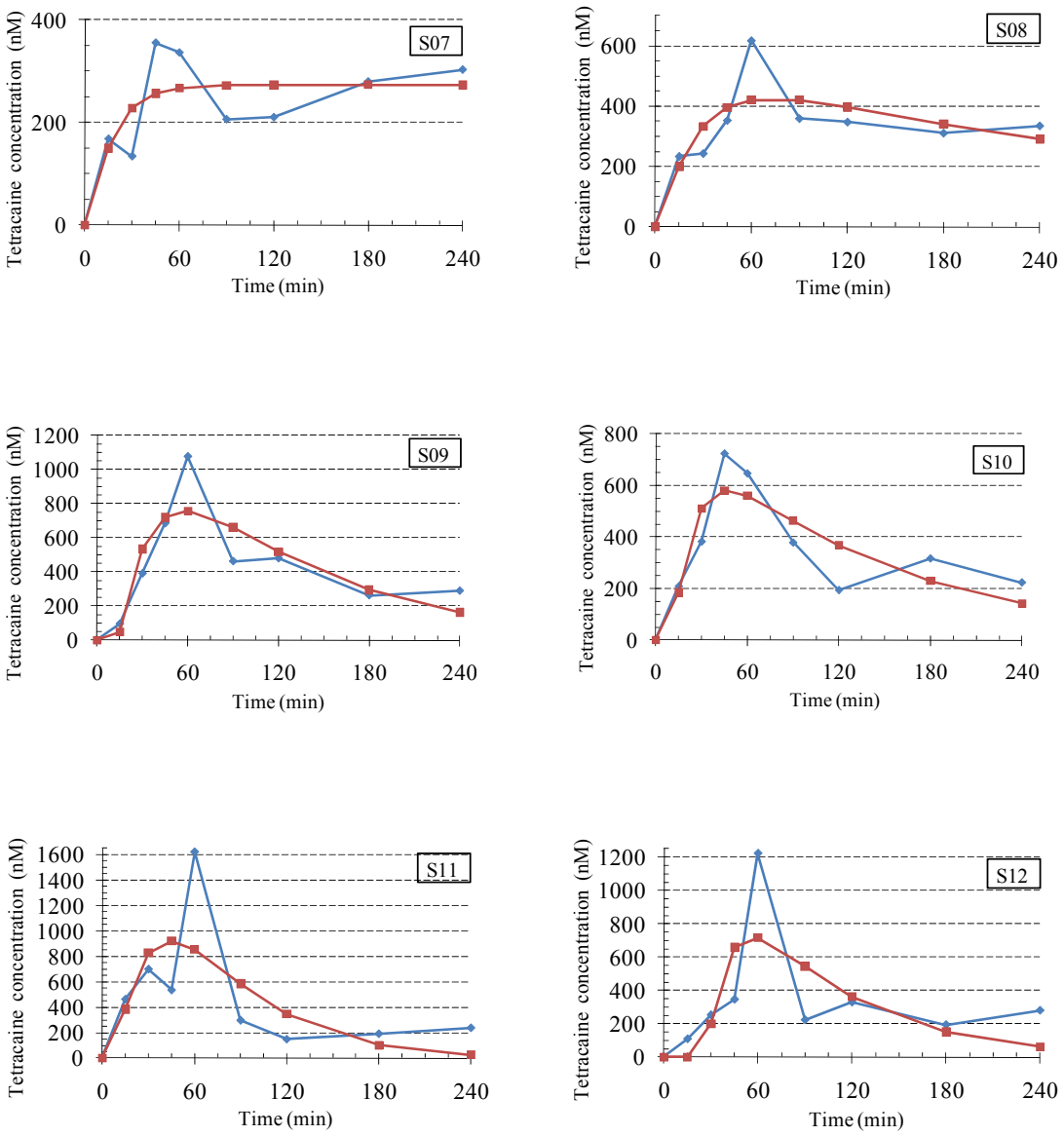


Figure 21.B Tetracaine concentration (obtained from subjects S07–S12) with time profiles in tape samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

Appendix 23 Volunteers BABA concentration with time profile in microdialysis sample (Chapter 4).

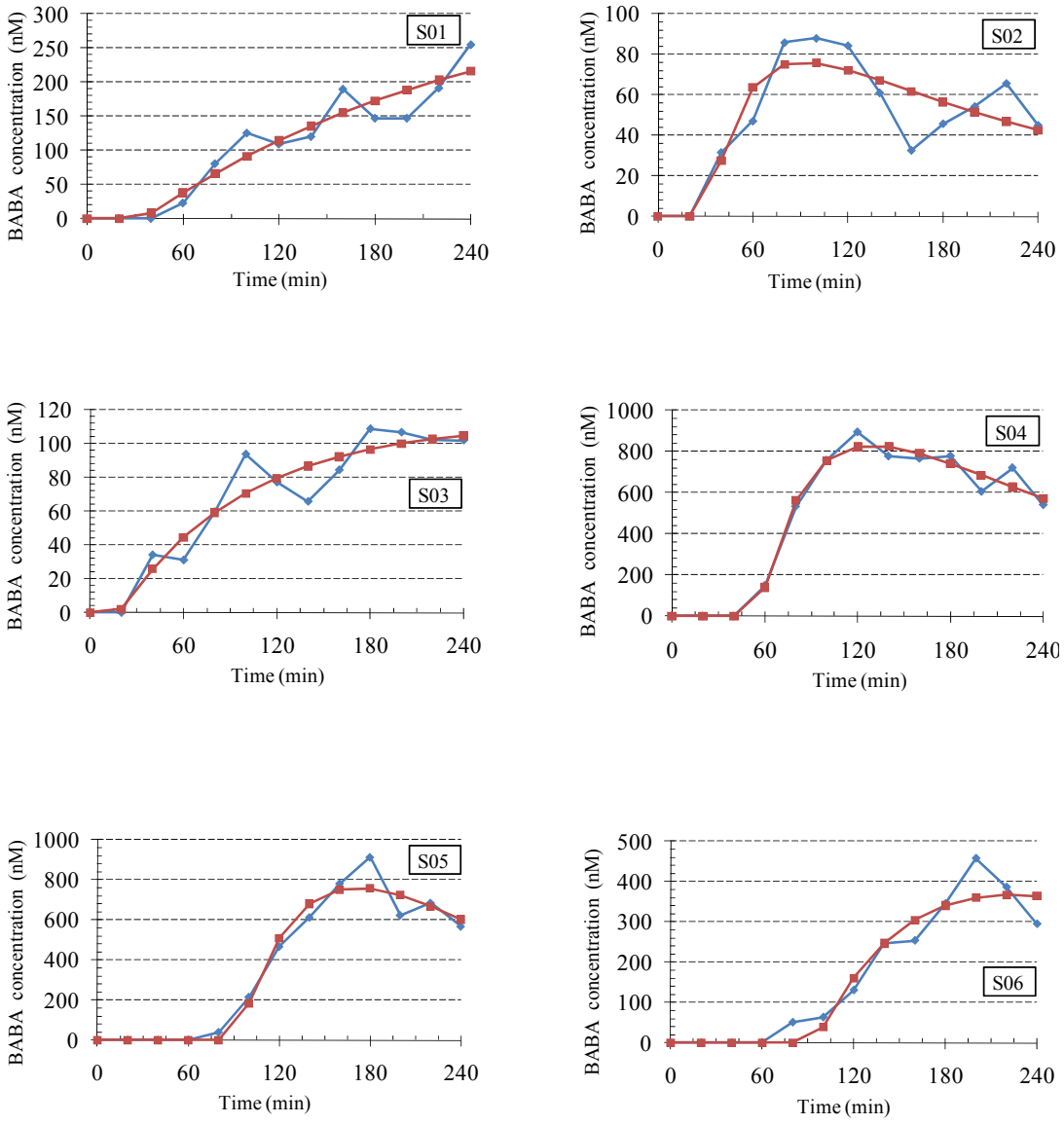


Figure 22.A BABA concentration (obtained from subjects S01–S06) with time profiles in microdilaysis samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

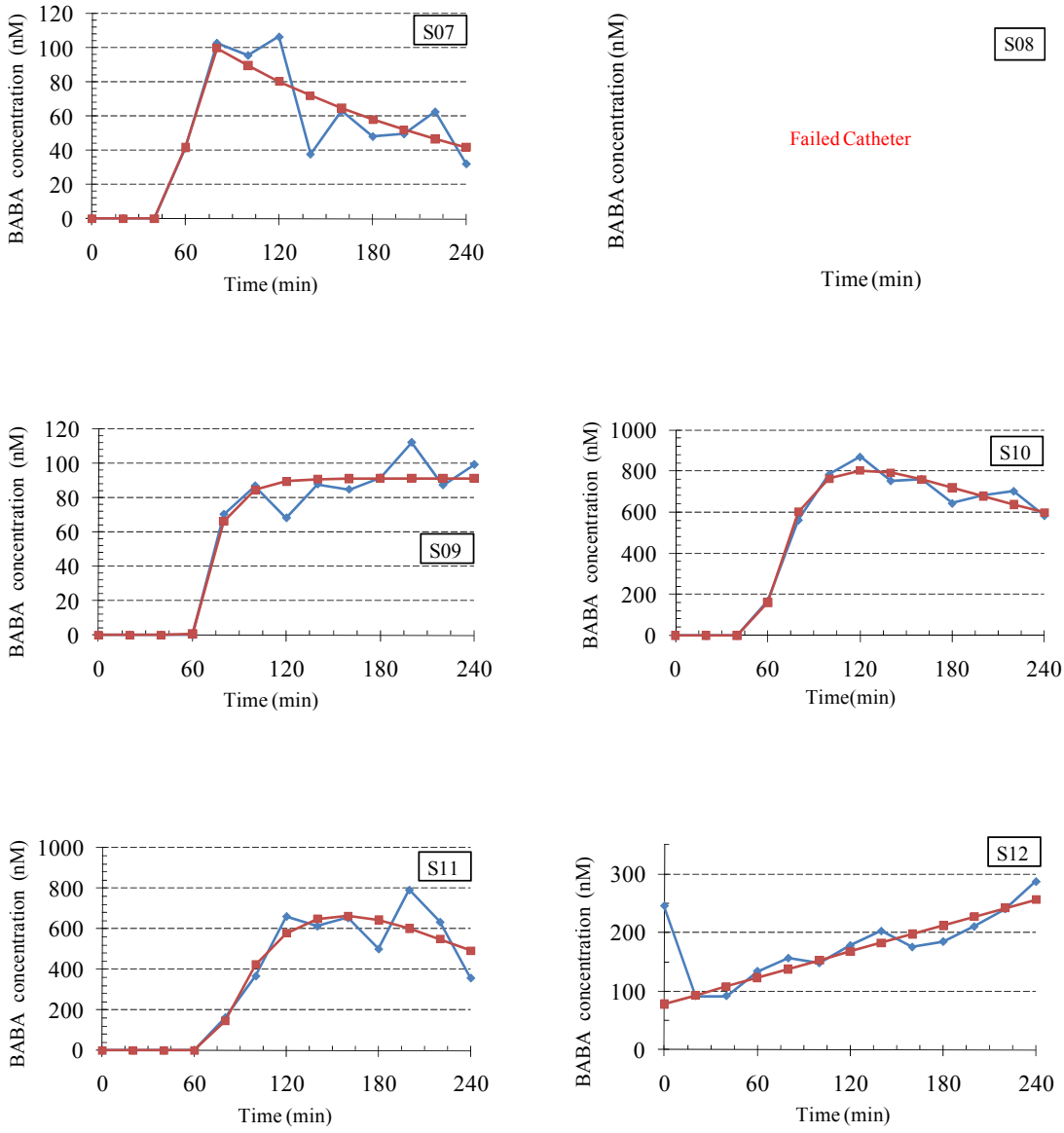


Figure 22.B BABA concentration (obtained from subjects S07–S12) with time profiles in microdilaysis samples after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

Appendix 24 Volunteers plasma BABA concentration (Chapter 4).

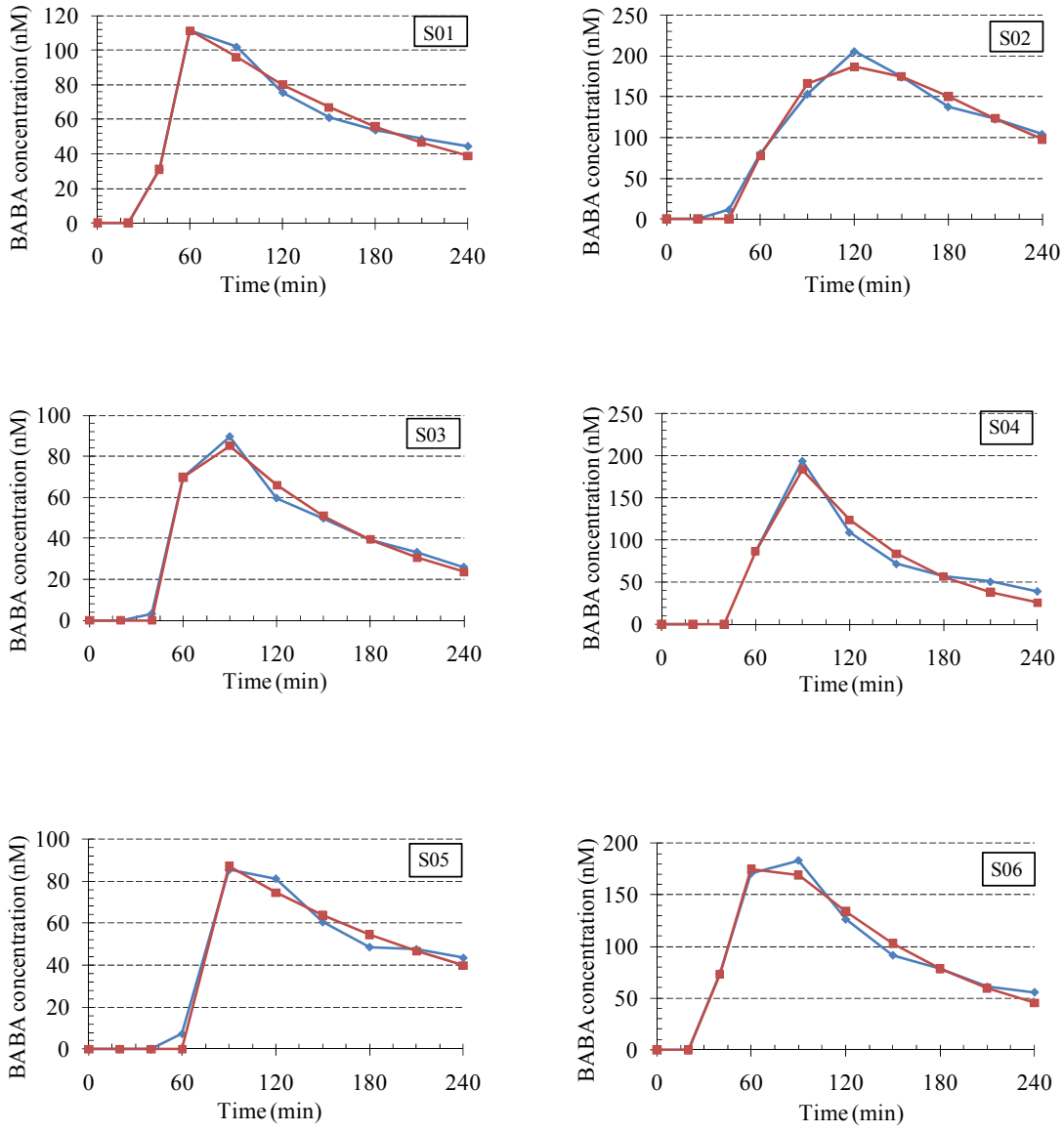


Figure 23.A Plasma BABA concentration (obtained from subject S01–S06) with time profile after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

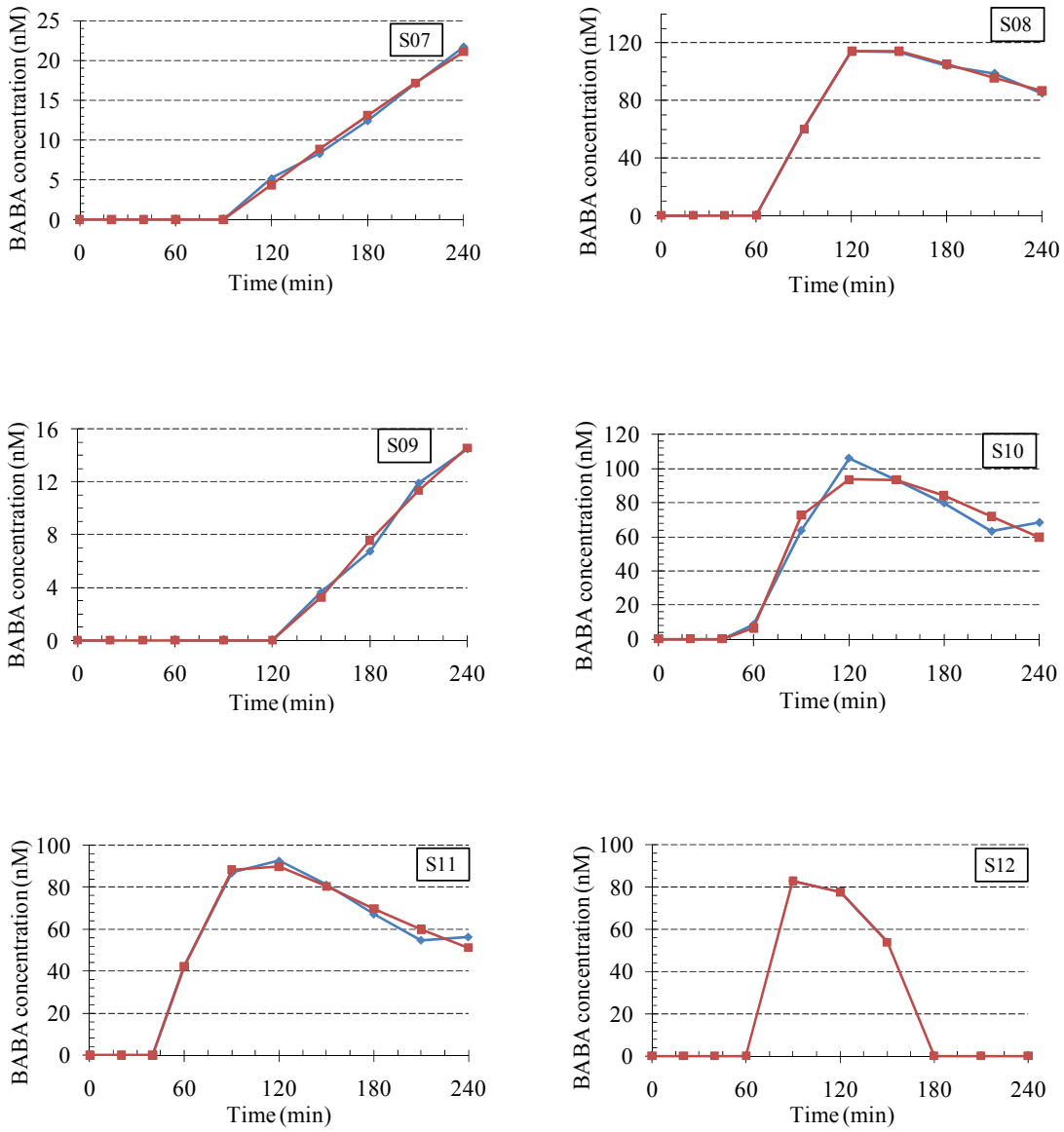


Figure 23.B Plasma BABA concentration (obtained from subjects S07–S12) with time profiles after application of 1 mL Ametop gel (4% w/w tetracaine) blue line (raw data), red line (fitted data) unresolved.

Research Article

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Rapid analysis of tetracaine for a tape stripping pharmacokinetic study using short-end capillary electrophoresis

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ABSTRACT: A rapid and simple short-end (reverse) capillary zone electrophoresis method was developed and validated for the separation and quantification of tetracaine in skin using tape samples. The separation was performed in a 485 mm (400 mm to window) × 50 μm internal diameter fused silica capillary using a background electrolyte of phosphoric acid–Tris pH2.5 at −25 kV. The extraction of tetracaine from tape samples was achieved using methanol diluted to 50% with water before injection. Procaine was the internal standard. The migration times for procaine and tetracaine were 1.25 and 1.36 min, respectively. The limit of quantification for tetracaine was 50 μg, with a signal-to-noise ratio greater than 10. The calibration curve was linear from 50 to 1200 μg with r^2 greater than 0.99. The CV for both within- and between-assay imprecision and the percentage inaccuracy for the quality control samples including lower and upper limits of quantitation were <12.1% and <11%, respectively. The absolute mean recovery of tetracaine was >97%. The accuracy and selectivity of this method allowed the rapid measurement of tetracaine in tape samples obtained from a skin tape stripping study of local anaesthetics in healthy subjects.

Keywords: capillary electrophoresis; short-end injection; tape stripping; local anaesthetics

Introduction

Local anaesthetics can be classified according to their chemical structure into amide or ester groups. Tetracaine (amethocaine, 2-dimethylaminoethyl 4-butylamino-benzoate) was developed in the early 1990s and is a potent local anaesthetic belonging to the amino ester class (Fig. 1). It is used for topical anaesthesia, ophthalmology, antiprurit, spinal anaesthesia and as a nerve block. Also tetracaine has been incorporated into a mucosa adhesive polymer film to relieve the pain of oral lesions resulting from radiation and antineoplastic therapy.

Tetracaine hydrochloride can be found in solutions, creams, gels and as the base in ointments. The topical anaesthetic action of tetracaine is more prolonged than the mixture of lidocaine and prilocaine known as EMLA cream (Martindale, 1996). It also appears to have a more rapid onset of action, i.e. 30–45 min, than EMLA cream, without any risk of methemoglobinaemia. In addition, it causes local vasodilatation, which may be an advantage when used prior to central venous catheter placement, and where obtaining i.v. access or blood is difficult, especially in the newborn.

Most biochemical and pharmacological effects take place in the local tissue and most drugs exert their effect in target tissue or cells, especially local drugs, which are closer to the site of action. Thus assessing tissue concentration is both meaningful and useful for drug pharmacology studies. Ametop is a local anaesthetic applied to skin; its concentration is high in local tissue compared with plasma. At present, microdialysis and tape stripping are available to provide analytical data concerning drug levels in the extracellular space and outermost layer of the skin, respectively.

Tape stripping of the outermost skin layer, the stratum corneum (SC), is a fast and relatively noninvasive technique to measure drug absorbed into the skin (Stinchcomb *et al*., 1999, Christophe *et al*., 2007), usually performed by placing an adhesive tape strip onto the skin surface, after removing the residue of the drugs followed by gentle pressure to ensure a good contact and subsequent removal by a sharp upward movement. The procedure is relatively painless, given that only dead cells (corneocytes) in the outer most layers in the skin are removed.

The determination of tetracaine in biological samples has been performed mainly in plasma. Several methods are available, such as high-performance liquid chromatography (HPLC), (Menon and Norris, 1981; Yang *et al*., 1984; Mazumdar *et al*., 1991), nevertheless human plasma contains proteins and endogenous compounds that may interfere with the chromatographic system. There are several reported ways to extract tetracaine from plasma, e.g. organic extraction or solid-phase extraction, but these are time-consuming. Thus the use of coupled column (RAM-columns) or micellar liquid chromatography (MLC) as

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Abbreviations used: BGE, background electrolyte; MLC, micellar liquid chromatography.

Contract/grant sponsor: Saudi Arabia Government.

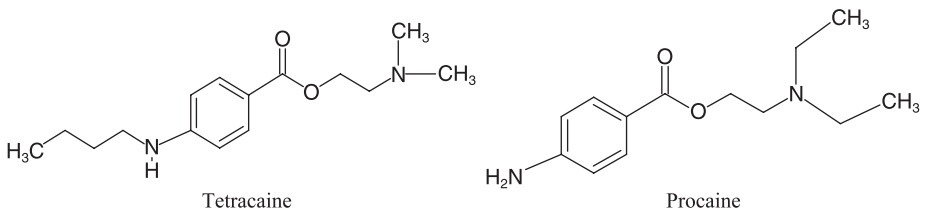

Figure 1. Chemical structures of tetracaine and procaine.

alternative methods has been proposed (Gilabert *et al*., 2001). In addition gas chromatography–mass spectrometry (GC-MS) (Hino *et al*., 2000; Yukiko *et al*., 2002), direct ultraviolet spectrophotometry and colourimetry (Robert and Albert, 2006) can also be used.

Capillary electrophoresis (CE) is an alternative to the chromatographic techniques in drug analysis. It was developed in the 1980s and expanded in the 1990s, and it has become a complementary separation technique that can separate a variety of compounds with separations being based on differences in electrophoretic mobility. CE with UV detection is a simple and fast way to determine local anaesthetics in tape-stripping pharmacokinetic studies since high sensitivity is not usually required to detect the drug levels involved, and it has a cost benefit compared with MS detection.

The purpose of this study was to develop and validate a fast, high-throughput but simple CE method using a short-end injection approach. This method was used to determine tetracaine in skin using tape samples from volunteers given Ametop gel as a part of pharmacokinetic drug delivery study.

Experimental

Chemicals

Tetracaine hydrochloride (HCl; lot no. 065k1501; 99.9% purity) for calibrator and control sample preparation and procaine hydrochloride (HCl; lot no. 114k0569; 100% purity) for use as internal standard were obtained from Sigma Aldrich (Poole, UK).

HPLC-grade solvents were obtained from Rathburn Chemicals Ltd (Walkerburn, UK). All other AnalaR grade reagents were obtained from Merck (BDH) Ltd (Poole, UK).

CE Instrumentation

The analysis and separations were carried out on an Agilent 3DCE G1600AX capillary electropherograph controlled by 3D-CE Chemstation Rev. B.02.01[244] (Agilent, West Lothian, UK). Fused silica capillaries, 485 mm total length with a window at 85 mm from one end × 50 μm i.d. (Composite Metal Services, Ilkley, UK) were conditioned on first use by flushing with 1 M NaOH (BDH, Poole, UK) at >950 mbar, 40°C, for 20 min. Pre-conditioning on injection was a 3 min flush with 0.1 M NaOH (BDH, Poole, UK), then a 3 min flush with background electrolyte (BGE). In the final separation a potential of –25 kV was employed. The capillary was thermostated at 25.0°C. The injections of the samples into the system were carried out hydrodynamically for 10 s at –50 mbar. Detection was by photodiode array over 195–360 nm, but 315 nm with bandwidth 10 nm was used for quantitation since 315 nm is the λ_{max} for tetracaine. The run time was 2 min. Both BGE vials were replenished every 12 injections. All samples and standards in the auto-sampler were kept at ambient temperature.

Experimental Design

Background electrolyte. As the aqueous background electrolyte 0.1 M Tris–phosphate at pH 2.5 was used. The buffer was prepared by diluting 20 mL phosphoric acid (0.5 M) to 80 mL with deionized water followed by adding 15 mL of 0.5 M Tris to obtain a solution at pH 2.5. This solution was made up to 100 mL with deionized water. Then the pH was checked and adjusted if necessary.

Calibrators and quality control samples. Stock solutions for tetracaine (5 mg/mL) were prepared in 50% methanol–water and for procaine 300 μg/mL in water (internal standard, IS). All stock solutions were stored at –20°C. All calibrators and quality control samples were prepared by appropriate dilution of the stock. Calibrations were nominally 50, 200, 400, 600, 800, 1000 and 1200 μg tetracaine; 50 and 1200 μg were the lower limit of quantity (LLOQ) and the upper limit of quantity (ULOQ), respectively, plus 1500 μg IS, and quality controls were nominally 100, 250, 1000 μg tetracaine plus 1500 μg IS.

Preparation of tape sample and extraction. For the purpose of validation and assay calibration, a length of self-adhesive polypropylene tape (Tesa 404 PV5, Beiersdorf, Hamburg Germany) was cut into 10 samples, each approximately 3 × 2 cm. A 100 μL volume of standard or quality control solution was placed onto the adhesive part of the tape. The solution was distributed approximately evenly over the tape. The sample was left to dry at room temperature, which required approximately 30 min. The samples then were transferred into a 10 mL polypropylene tube and stored frozen at –20°C until analysis.

Extraction procedure. A 5 mL aliquot of methanol was dispensed into each tube containing standard/QC tape or samples. The contents were mixed for 45 min with a shaker. A 5 mL aliquot of solution of 300 μg/mL procaine (InternalStandard.) in water was added to the tube to make 50% methanol–water solution. The contents was mixed again using a shaker for further 15 min. A 200 μL aliquot of the solution was transferred into a 250 μL auto-injector vial for CE analysis.

Method Development

Optimization of buffer pH. The role of the pH of 0.1 M Tris-phosphate BGE was studied over the range 2–3.5. pH 2.5 was the optimum with respect to the resolution of tetracaine and procaine.

Optimization of temperature. Various temperatures were studied, 15, 20, 25 and 30°C, and 25°C was found to be optimum.

Optimization of buffer concentration. Different buffer concentrations at constant pH in the range 0.05–0.2 were investigated

and 0.1 M was the optimum buffer concentration with respect to peak resolution and current.

Optimization of voltage. A voltage of −25 kV gave the best separation with respect to analysis time and gave only a moderate current, typically 50–70 μA.

Optimization of injection time. Injections time were studied from 1 to 65 s, and 10 s was optimum for both resolution and sensitivity.

Calculating inaccuracy and imprecision. Inaccuracy was tested by the determination of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. Each control sample contained tetracaine. The nominal values for the low, medium and high control samples were 100, 250 and 1000 μg, respectively. The nominal values for the ULOQ and LLOQ were the same nominal concentration as the highest and the lowest calibration standards, respectively.

Assay imprecision was measured both within-batch and between-batch by the analysis of three control samples, the LLOQ and the ULOQ. Within-batch and between-batch imprecision were calculated by the nested analysis of variance (ANOVA) using internationally agreed methods (ISO 5725:1994).

Validation Procedures and Results

Specificity. Six samples of blank tape and six samples of tape place with tetracaine were prepared and carried through the extraction. The concentration of tetracaine used was 5 μg/mL LLOQ and the internal standard was 150 μg/mL. No significant interfering peaks were found at the migration time of tetracaine or procaine. The signal-to-noise ratios at the LLOQ for both drugs were greater than 10. Figure 2 shows the electropherogram obtained from blank tape spiked with 50 μg tetracaine with added intenal standard of 1500 μg procaine while Figures 3 and 4 show the electropherograms of Ametop gel solution and one of the tape samples from the study, respectively.

Calibration curve/linearity. Calibrations were nominally 50, 200, 400, 600, 800, 1000 and 1200 μg tetracaine plus 1500 μg procaine (internal standard). Seven batches of calibration curve were plotted using the area ratio of tetracaine to internal standard vs known concentration of tetracaine. All the results were calculated using a $y = ax + b$ linear regression (Table 1). The regression coefficients for all the calibration curves obtained were greater than 0.99.

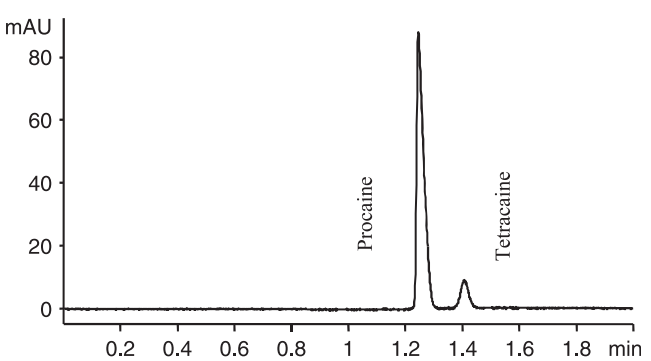

Figure 2. Electropherogram obtained from extracted tape sample spiked with 50 μg tetracaine and added internal standard of 1500 μg procaine. Displayed at 315 nm.

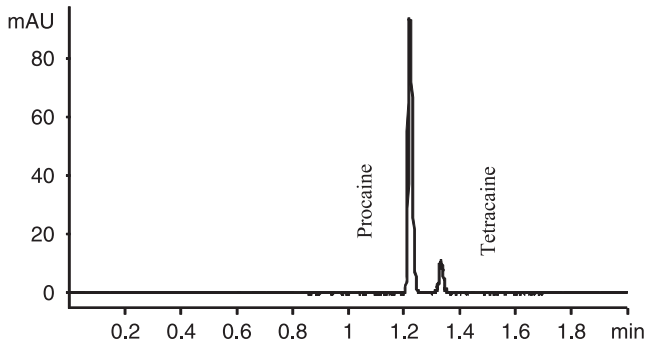

Figure 3. Electropherogram obtained from Ametop gel solution containing 50 μg tetracaine and added internal standard of 1500 μg procaine. Displayed at 315 nm.

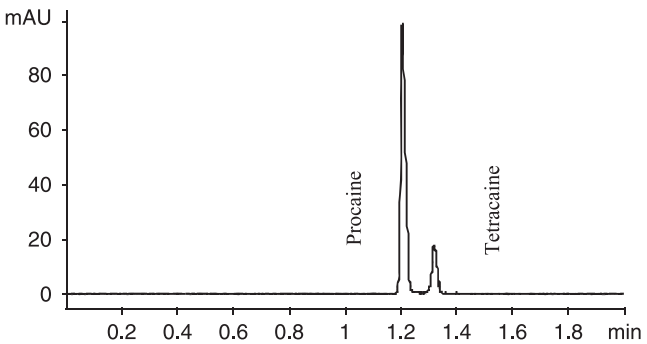

Figure 4. Electropherogram obtained from tape sample at 4 h post dose with added internal standard of 1500 μg procaine. Displayed at 315 nm.

Table 1. Calibration line parameters for 5 separate runs

Batch	Slope (*A*)	Intercept (*B*)	r^2
1	0.0194	−0.0192	0.9992
2	0.0195	0.0099	0.9993
3	0.0194	0.0155	0.9986
4	0.0191	0.0070	0.9998
5	0.0198	0.0041	0.9997

Inaccuracy. The within- and between-assay and the total variability obtained from the ANOVA are summarized in Table 2. The percentage inaccuracy for all the quality control samples including LLOQ and ULOQ was below 11%.

Within-assay reproducibility. For within-batch and between-batch imprecision the LLOQ and ULOQ and the three control samples were each assayed six times in three separate assays. Each assay had an individual calibration curve. The coefficient of variation (CV) for imprecision for all the quality control samples including LLOQ and ULOQ was below 2.5%.

Between assay repeatability. For each of the three assays mentioned above, the mean concentration for each assay was used to calculate the between-assay reproducibility. The CV for imprecision for all the quality control samples including LLOQ and ULOQ was below 12.1%.

Stability. Samples were stable at room temperature for 24 h and for at least 3 weeks when stored at –20°C.

Table 2. The within- and between-batch and the total variability obtained from the nested analysis of variance (ANOVA)

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal concentration (μg/mL)	50	10	25	100	120
Mean (μg/mL), $n = 18$	55.29	10.27	27.35	106.36	121.00
Inaccuracy (%)	10.58	2.77	9.42	6.36	0.83
SDw	0.052	0.254	0.309	2.063	1.862
SDb	0.560	1.239	1.611	8.996	3.270
SDt	0.563	1.265	1.640	9.230	3.763
CVw (%)	0.94	2.47	1.13	1.94	1.54
CVb (%)	10.13	12.06	5.89	8.46	2.70
CVt (%)	10.18	12.31	6.00	8.68	3.10

W = within batch; b = between batch; t = total.

Recovery. Absolute recovery of tetracaine was determined using tape samples doped with tetracaine at the same nominal concentration as the quality control samples. Peak area measurements from extracted samples were compared with the peak areas from direct solvent injection of the test compounds. Mean and standard deviation were calculated from at least six measurements at each level. Two samples of solution and six samples of tapes loaded with 50, 100, 200, 400, 600, 800, 1000 and 1200 μg of tetracaine were used, and 1500 μg procaine as internal standard. The absolute mean recovery of tetracaine ranged from 97 to 104%.

Discussion

Bioanalysis of biological fluids for the quantitative determination of drugs and their metabolites plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetics and toxicokinetics studies. The huge number of samples and the quality of these studies, which is directly reflected by the results obtained, require the development of rapid and simple analytical methods. Thus the validation of the present methods employed the latest guidance and principles of validation approved (FDA, 2001).

In this study we have developed a very short end direction (reverse) CE method. Applying a short end direction (reverse) by negative voltage resulted in tetracaine and procaine being fully separated. The short migration times obtained for procaine (1.25 min) and tetracaine (1.36 min) are an advantage of CE compared with the 5 min retention time for tetracaine using HPLC (Mazumdar *et al*., 1991). All the validation results meet the international requirements as outlined by the FDA's 2001 bioanalytical method validation guidelines (FDA, 2001). In an attempt to reduce the migration time even further, the capillary length was shortened to 35 cm from the original 48.5 cm, but this resulted in tetracaine and procaine not being fully separated and the run time was only reduced by about 30 s. The purpose of this work was to develop a fast and simple CE method that was fully validated. This method is currently being used to determine tetracaine in skin using tape samples from healthy volunteers given tetracaine as a part of a pharmacokinetic drug delivery study.

Conclusions

A simple and rapid short end direction (reverse) method to determine tetracaine in skin using tape samples has been developed and validated for the separation and quantification using capillary zone electrophoresis with UV detection. The method was successfully used to analyse hundreds of tape samples from a tape stripping study.

Acknowledgements

We would like to acknowledge the Saudi Arabia Government for funding this study. Also we would like to acknowledge Tesa UK Ltd for providing us with the adhesive tape used in this study.

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UNIVERSITY OF LONDON

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(St Bartholomew's and the Royal London School of Medicine and Dentistry)

Faisal Al Otaibi

having completed the approved course of study and passed the examinations has this day been admitted by Queen Mary and Westfield College to the University of London Degree of

MASTER OF SCIENCE

with Merit
in Clinical Drug Development

Principal, Queen Mary and Westfield College

Vice-Chancellor

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المكتب الثقافي السعودي
في بريطانيا

SAUDI ARABIA CULTURAL ATTACHE UK
الملحق الثقافي السعودي في بريطانيا

9 November 2006

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Joint Research & Development Office

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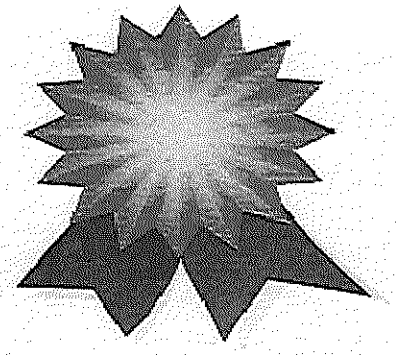

Research Governance

Granted: 25/5/05

Johanna Piper

Johanna Piper
Research Governance & GCP Manager