Pharmacokinetic studies for the development of transdermal drug delivery systems

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by

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This thesis is submitted for the degree of Doctor of Philosophy of the University of London

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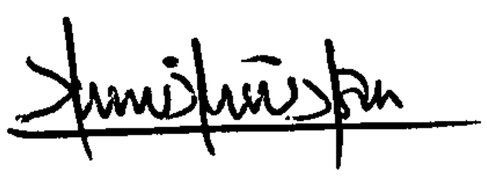
To my parents, wife and all my kids

Statement of originality

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



Zamri Chik, March 2007

Abstract

This thesis mainly describes a series of Phase I pharmacokinetic studies conducted on the TDS® delivery system which combined with lidocaine, testosterone, and a new drug, Melanotan-I for the transdermal drug delivery. Pharmacodynamic studies have also been carried out in certain areas to support the pharmacokinetics.

The initial challenge was the development and validation of a method to analyse lidocaine in human plasma by LCMS-MS. The sensitivity and reliability of the developed method has enabled the analysis of lidocaine plasma levels from the TDS®-Lidocaine study. The results from the study have shown that the TDS® system has been able to deliver the drug effectively through the skin. This finding had a positive impact on the future development of the TDS® system in combination with other drugs.

The combination of the TDS® system with testosterone had been successfully tested in 12 healthy male subjects. TDS®-Testosterone was found to be bioequivalent to Androgel®. This result gave an insight into further development of this preparation if it is to be regarded as an alternative treatment for hypogonadism. Various methods of correcting for endogenous testosterone were performed on the data and the influence on bioequivalence was studied. Testosterone was used as a model drug and used to explore potential guidelines for the bioequivalence assessment of endogenous compounds.

Finally, the TDS® system has been combined with a new, peptide derived drug, Melanotan-I (MT-I). This drug is currently under development for the cosmetic purposes and the treatment of various skin conditions related to sun allergies. A dose escalation study of TDS®-Melanotan for the protective tanning of skin was carried out and the result was presented. In addition, *in vivo* techniques, such as microdialysis and tape stripping, have also been explored to investigate the feasibility of measuring pharmacokinetic of a transdermal drug instead of using the conventional systemic measurements.

Publications

Articles

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2. Z.Chik, T.D. Lee, D.W. Holt, A. Johnston, A.T.Tucker. Validation of high-performance liquid chromatographic-mass spectrometric (LC-MS-MS) method for the analysis of lidocaine in human plasma. Journal of Chromatographic Sciences.Volume 44;5, 2006:262-265.
3. Z.Chik, A.Johnston, A.T. Tucker, S.L. Chew, L.Michaels, C.A.S. Alam Pharmacokinetics of a new testosterone transdermal delivery system, TDS®-testosterone in healthy males. British Journal of Clinical Pharmacology, Volume 61;3, 2006: 275-279.
4. A.T.Tucker, Z.Chik, L.Michaels, K.Kirby, MP Seed, A.Johnston, and C.A.S. Alam. Study of a combined percutaneous local anaesthetic and the TDS® system for venepuncture. Anaesthesia, Volume 61; 2, 2006: 123-126.

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1. Z.Chik, A.T.Tucker, J.I.Shiel, D.J.Collier, D.Perrett, T.D.Lee and A.Johnston. Comparative pharmacokinetic assessments of topically applied drugs: Evaluation by dermatopharmacokinetics, microdialysis and systemic measurement. *Proceedings of the British Pharmacological Society (BPS) Winter Meeting, Oxford, UK.* December 2006.
2. Z.Chik, A. Johnston, A. T. Tucker, S. L. Chew, L. Michaels, C. A. S. Alam, K.B. Kirby. A New Testosterone Transdermal Delivery System, TDS®-testosterone, Has Superior Pharmacokinetics Compared to an Existing Transdermal Preparation in Healthy Males. *Proceedings of the ENDO 2006, The Endocrine Society's 88th Annual Meeting, Boston, Massachusetts, USA.* 24 – 27 June 2006.
3. Z.Chik, A.Johnston, A.T.Tucker, C.A.Alam. The influence of correcting endogenous concentrations in the bioequivalence assessment of testosterone.

Proceedings of the British Pharmacological Society (BPS) Winter Meeting, London, UK. December 2005.

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5. Z.Chik, A. Johnston, A.T. Tucker, L. Michaels, S.L. Chew, C.A.S Alam. Pharmacokinetic of the new testosterone transdermal delivery system, TDS®-Testosterone in healthy subjects. *Proceedings of the British Pharmacological Society (BPS) Winter Meeting, London, UK.* December 2004.

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Chapter 1 Introduction

1.1 The skin

In the study of transdermal drug delivery, understanding the nature and the structure of the skin is vital. Skin is man's largest organ providing around 10% of the body mass of an average individual. Skin has many essential functions, including protection, thermoregulation, immune responsiveness, biochemical synthesis, sensory detection, and social and sexual communication. Although the skin is a large and easily accessible area for the administration of therapeutic agents, it also forms a highly efficient barrier between internal and external parts of the body. Skin is highly resistant to the penetration by any exogenous compound including chemicals and also organisms such as bacteria and viruses. The structure of human skin can be divided into three distinct layers, which are epidermis, dermis and hypodermis (subcutaneous adipose tissue) (Figure 1.1)

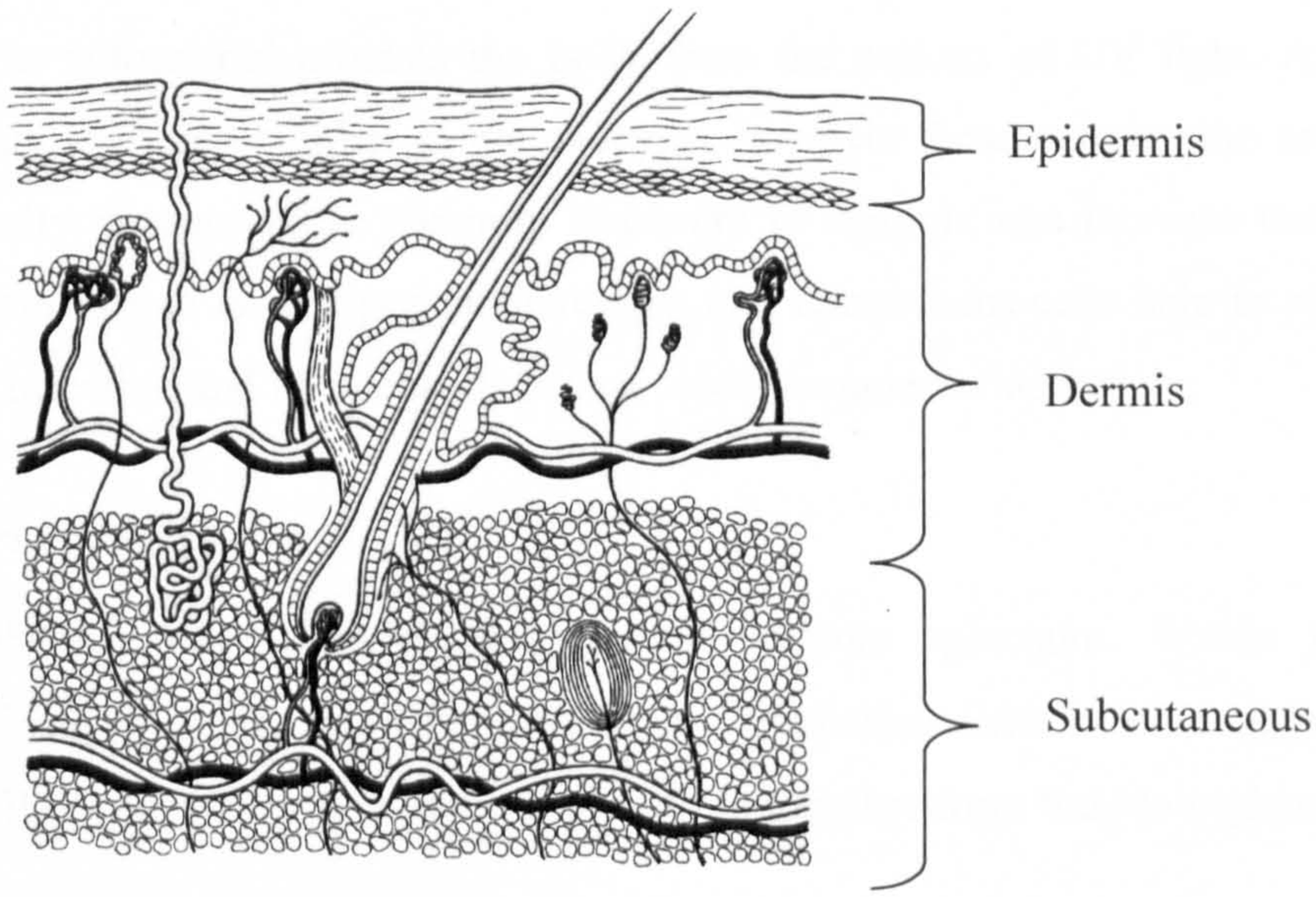


Figure 1.1 A diagram of skin structure showing the three main layers of the skin

1.1.1 The epidermis

The epidermis originally from the Greek word, ('epi', on top and 'derma', the skin) is the outermost layer of the skin. The epidermis is a multiple layered membrane and its thickness varies depending upon the part of the body, from around 0.06mm on the eyelids to around 0.8mm on the palms of the hand and soles of the feet. It contains no blood vessels and is built up by several cells which can be further divided into: 1) Stratum germinativum or stratum basale, followed by 2) Stratum spinosum, 3) Stratum granulosum and, 4) Stratum corneum, from the inside to the outside of the skin.

1) Stratum germinativum

The stratum germinativum is also referred to as the stratum basale is the basal layer which contains cells similar to other tissues in the body. It also contains keratinocytes, melanocytes, Langerhans' cells, and Merkel cells (sensory). The main skin cells are keratinocytes, thus named because they produce keratin, also a basic component of hair and nails. Melanocytes are responsible for production of melanin, the pigment that gives skin its colour and protects the body from the actions of UV light. All people have roughly the same number of melanocytes, however those people who are dark-skinned normally produce more melanin. Exposure to sunlight can increase the production of melanin, which is why people develop a tan. Langerhans cells help to protect the body from infection and Merkel cells have a role in cutaneous sensation.

2) Stratum spinosum

On the top of the basale layer is the stratum spinosum. Within this layer, the keratinocytes begin to differentiate and synthesise keratins that aggregate to form tonofilaments. Stratum basale and spinosum together form the Malpighian layer.

3) Stratum granulosum

From the stratum spinosum to the stratum granulosum, the keratinocytes continue to differentiate, synthesise keratin and start to flatten. It also contains keratohyalin granules which are composed of profillagrin, loricrin, and cystein-rich protein. The fillaggrin subunits of profillagrin play the important role of matrix molecule to aggregate and align the keratin filaments.

4) Stratum corneum

The stratum corneum is the outermost part containing a dry keratinized layer of dead cells and are represented as a 'brick and mortar' model (Elias, 1981;Williams and Elias, 1987). The layers of flat keratinized dead cells are vertically stacked to form a highly organised tissue structure. Its thickness varies between different parts of the body between approximately 10 to 50μm thick. It is thickest on the palms and soles and thinnest on the lips. The stratum corneum provides a pricipal barrier to the permeation of drugs through the skin (Harry E.Bodde, 1989), and it also assists in regulation of water loss from the body.

1.1.2 The dermis

The dermis is typically 3-5mm thick, and is made up of blood vessels, nerve endings, and connective tissue. Collagen and elastin, which combine in fibres are the compounds in the dermis which provide support and also elastic tissue to give man flexibility in the movement. Collagen is strong and hard to stretch whilst elastin, is elastic. In older people, some of the elastin-containing fibres disappear, which is one reason why the skin looks wrinkled.

The dermis also contains a person's sebaceous glands and sweat producing glands. Sebaceous glands, which surround and empty their secretions into our hair follicles and pores, produce oil called sebum that lubricates the skin and hair. Sebaceous glands are found mostly in the skin on the face, upper back, shoulders, and chest. Sweat glands (eccrine and apocrine) are helpful in regulating the body temperature by producing sweat. Eccrine glands can be found almost everywhere in our bodies, although they are mostly located in the forehead, palms, and soles of the feet. Apocrine glands become active at puberty and are concentrated in the armpits and pubic region. The sweat from the apocrine glands is more copious than that produced by the eccrine glands.

1.1.3 The subcutaneous tissue

The subcutaneous tissue can be considered as the bottom layer of the skin. This layer is made up of connective tissue, and cells that store fat. This layer also sometimes calls as hypodermis. Due to the existence of fat, this layer helps to retain the body's heat. The subcutaneous fatty layer can also provide a readily available supply of high-energy

molecules, whilst the principal blood vessels and nerves are carried to the skin in this layer.

1.2 Drug Delivery

Drug delivery is one of the most important subjects in pharmacology. The discussion of drug delivery normally involves the nature of a drug's formulation and how drugs can be delivered into the body to exert their action. Drugs can be delivered into the body by several mechanisms, depending upon the nature of drugs and the route of administration. The routes of administration are the pathways by which a drug or any other substances are brought into the body to exert their pharmacologic effects. Normally a substance needs to be transported from the site of administration to the site of action.

The route of administration can be divided into three classifications:

Enteral – oral, rectal, gastric feeding tube etc.

Parenteral – intravenous, intramuscular injection or infusion, subcutaneous implant, etc.

Topical/transdermal – epicutaneous (application on the skin), inhalation, intranasal, buccal mucosa, etc.

From the three major routes listed above, the enteral route, especially oral delivery of drug is the most commonly used and least invasive. The villi of the upper intestine provide an extremely large surface area (~ 200m^2) (Snyder, 2001) for the absorptions of drug into circulation. Most of the drugs need to be absorbed into the systemic circulation for drugs to reach the site of action.

However, there are certain cases where a drug cannot be taken orally due to the physicochemical properties of the drugs. Some drugs such as insulin are partly destroyed or neutralised in the stomach or intestine, and some of them such as propranolol, lidocaine, propafenone, verapamil, and nitroglycerin has been extensively metabolised by the liver before reaching systemic circulations (Lalka et al., 1993). The bioavailability of a drug may be low, which means that a higher dose is needed to ensure efficacy. The higher dose of a drug could increase the risk of side effects or adverse events. A very common example of the unwanted side effects of an orally administered drug is hepatic toxicity. The liver is the first major organ to be exposed to

the ingested drug due to its portal blood supply. A drug is normally metabolised and excreted by the liver in the process known as first pass metabolism. However this process sometimes can cause liver injury such as necrosis, lipidosis, cholestasis, chirrhosis, etc. For example, many of the diverse group of pharmacologic agents available for the treatment of rheumatic diseases such as most of the NSAIDS (*Non steroidal anti inflammatory drugs*) have the potential to cause serious hepatotoxicity (Brass, 1993;Tolman, 1998).

A drug may be administered via the rectal mucosa for the systemic effects if the oral or another parenteral route is not available. Rectal administration is not a common route for drug delivery in the United Kingdom, and only used for certain types of drugs for the treatment of sedation, seizures control, analgesia, etc. (Beebe et al., 1992;Malinovsky et al., 1995;Pannuti et al., 1982;Uthman and Wilder, 1989). The rectum has a rich of blood supply and a thin wall which facilitates drug absorption. In some cases drug uptake can be almost as rapid as intravenous administration. This may cause adverse events in the cardiovascular or central nervous systems (American Academy of Pediatric, 1997). A consequence of this can be seen in the death reported after rectal administration of multiple doses of morphine (Gourlay and Boas, 1992). Although the rectal route can achieve rapid drug absorption, the irregular uptake, patient-to-patient variability and patient acceptability limits its usefulness (American Academy of Pediatrics, 1997).

To avoid the first pass metabolism by the liver, other routes of drug delivery can be considered, such as parenteral and topical administration. However, parenteral administration such as intravenous injection or infusion and subcutaneous implant, are invasive and inconvenient for some patients especially children. Needle phobia, which affects many children and some adults, can limit the usefulness of this method. Intravenous injection also has the possibility of introducing infection. There are a few systems that have been developed recently to overcome needle phobia, such as a needleless injection and microneedles. The companies marketing the devices claim the systems are successful in delivering a therapeutic dosage through the skin. However, there are still some problems to be resolved when using these techniques. These include proper handling of the device which is necessary to avoid loss of the drug dosage entering the body which could result in high variability. Also the systems have a higher cost of manufacture, which in turn passes on extra costs to the users. Therefore, another

alternative such as topical or transdermal delivery has became increasingly popular for certain drugs.

1.3 Transdermal Delivery

Many terms have been used to describe transdermal delivery of drugs, i.e., penetration of a substance from outer skin through the skin layers and into the blood stream. Some people have used other terms such as percutaneous absorption, sorption, and permeation. However, transdermal delivery can be defined as a passive movement of the substance such as a drug through the outer and middle structure of the skin until the systemic circulation is attained (Scheuplein and Blank, 1971) or produces the local effect. Transdermal drug delivery can be used as alternative for systemic delivery of very potent drugs with low oral bioavailability such as protein and peptide (Lambkin and Pinilla, 2002), as this route avoids hepatic first-pass metabolism. Also potential for long-term controlled release such as patches system, which is unoffered by the usual gastrointestinal transit of oral dosage forms. Furthermore it offers a few other advantages over the oral route. These are listed by Berner and John (Berner and John, 1994), as:

- The ability to discontinue administration by removal of the system
- The ability to modify the properties of the biological barrier to absorption (i.e stratum corneum) without any serious damage to the organ.
- The ability to avoid a changing physiological environment, avoiding chemical or metabolic degradation (e.g. changing pH, luminal microflora involvement, etc., in the gut)
- The ability to change the site of drug delivery, thereby reducing the risks of adverse reaction or toxicity due to repeated exposure at a single site.
- A relatively large area of application and absorption compared to oral transit system.

Various types of transdermal drug delivery are widely used currently including liquid formulation, ointment, gel and cream or lotion. Liquid formulation can be a simple single phase solution - either aqueous, solvent or miscible co-solvents. It can also be two phase systems, such as oil in water or water in oil emulsions. However emulsions

are more widely used in creams. An ointment can be explained as a greasy or semi-solid preparation. Ointment may contain other excipients such as silicone to improve the spreadibility of the formulations. Gels are normally formed from a liquid that has been thickened with other component(s). The liquid essentially forms a continous phase, with thickening agent providing a porous scaffold to maintain the semi-solid consistency. Also proving to be quite popular recently are transdermal patches which can be applied to the skin via adhesive action. The drug content in the patches can be locally or systemically delivered for a certain period of time. The delivery of drug also can be terminated by the removal of the system.

The releases of drug from transdermal formulations and the penetration of drug through the skin is usually driven by using certain mechanism such as penetration enhancers, particles, laser ablation, ultrasound, electricals (iontophoresis and electroporation), etc. However, penetration enhancers are known to be widely used in the transdermal formulations.

1.3.1 Transdermal delivery pathways

When the topical drug products are applied on the skin, three processes involve;

i) The drug releases from the formulation

ii) Penetration / diffusion of drug through viable epidermis

iii) Movements of drug to the site of action or reaching systemic circulations

There are a multiple steps between the first drug apply on the skin until it appears in the systemic circulations or in the local tissues. The drug applied on the skin in the vehicle such as liquid formulation, emulsion, gel, etc. The molecules adjacent to the stratum corneum will partition into the membrane based on their physicochemical properties. Lipophilic molecules have a better partition in the stratum corneum due to the lipid properties of the stratum corneum. However the drug must possess both lipoidal and aqueous solubilities. Too hydrophilic molecule is unable to permeat through stratum corneum and too lipophilic molecule tends to retain in the stratum corneum (Naik et al., 2000). There are two potential pathways of drug to penetrate the skin (Barry, 2002), (Figure 1.2);

i) Passive movement across the stratum corneum

ii) Through the shunt route, hair follicles or via the sweat duct.

Passive movement through the stratum corneum is often regarded as the main route of drug across the skin

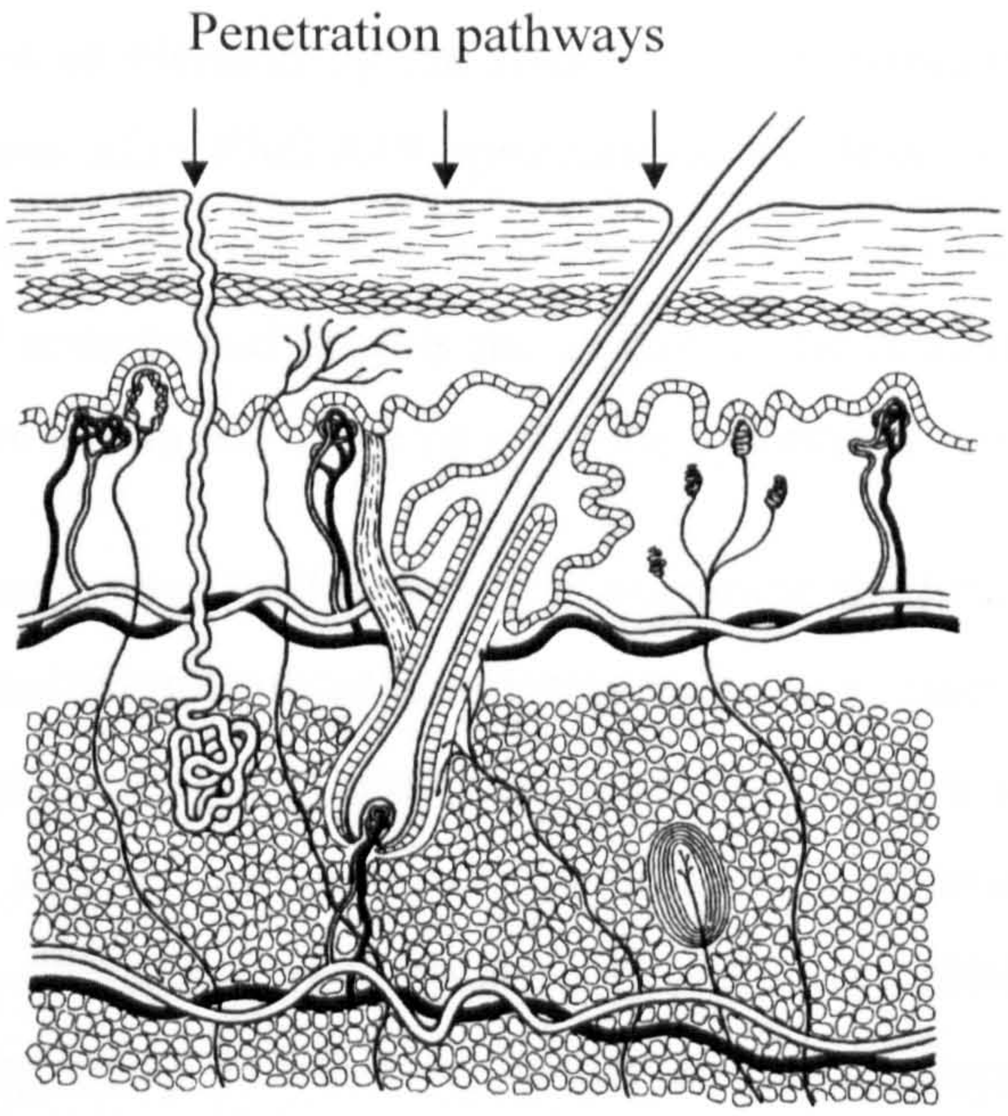


Figure 1.2 Skin structure shows the penetration routes through the skin

The shunt route, hair follicles and sweat ducts normally offer pores for the drug to bypass the barrier function of the stratum corneum. However, shunt pathways only account for approximately 0.1 % of the total skin surface (Scheuplein, 1967). Therefore their contributions to the permeation of drug through the skin are small (Scheuplein and Blank, 1971). Large molecules such as peptides and proteins may require this transappendageal route as they permeate poorly through the stratum corneum. For the molecules cross passively through stratum corneum, they can either diffuse through corneocytes (highly hydrated keratin) in a transcellular route, or by intercellular route, between lipid bilayers. However, intercellular lipid route is the most proposed mechanism for most of the drugs (Albery and Hadgraft, 1979).

1.3.2 Stratum corneum barrier properties

Healthy human skin presents a natural barrier to exogenous chemical agents, including many pharmaceuticals, presenting a clinical problem in the delivery of drugs via this route. In general, the epidermis (specifically, the stratum corneum) provides the major control element of percutaneous absorption. A few studies have been conducted to

monitor the role of stratum corneum as an effective barrier for any exogenous compound to penetrate the skin. A study by Morgan et al. (Morgan et al., 2003), showed that the absorption of penciclovir and aciclovir increased by 1300 and 440-fold, respectively, when the stratum corneum removed by tape stripping prior application. In another study by Singer et al. (Singer et al., 1998), they have shown that the absorption of EMLA™ cream was accelerated by the removal of the stratum corneum. The pain assessment at 15 minutes after EMLA™ applications was less for tape strips (stratum corneum removal) patients compared to patients without tape strips. Therefore, the successful indicator of transdermal drug is the ability of the compound to penetrate the stratum corneum, act locally in the tissue or enter the systemic circulation.

Many systems have been currently developed to overcome the limitation of the stratum corneum as a barrier including drug/vehicle interactions (e.g. eutectic system), vesicles and particles (e.g. liposomes and analogues), stratum corneum modification (e.g. chemical enhancers), stratum corneum bypass (e.g. laser ablation), and electrically assisted methods (e.g. ultrasound, iontophoresis etc.) (Barry, 2001). Stratum corneum modifications, by using the penetration enhancer are widely used at present in the development of a transdermal delivery system. The penetration enhancers use substances that can temporarily diminish the barrier of the skin such as water, sulphoxides and their analogues, pyrrolidones, esters and alcohols, azone and its derivatives, surfactant (anionic, cationic and non-ionic), etc. (Barry, 2001). It therefore can enhance the drug flux. Hadgraft (Hadgraft J., 1984) defined the term penetration enhancer as a substance that increases the permeability of skin without severe irritation or damage to its structure. The ideal properties of a penetration enhancer as described by Hadgraft, are as follows:

- Elicit no pharmacological response
- Specific in its action
- Immediate acting with a predictable duration; its action should be reversible
- Chemically and physically stable and compatible with formulation
- Odourless, colourless, and tasteless
- Non-toxic, non-allergic, and non-irritant, both acute and chronically

- Systemically non-toxic

1.4 Another transdermal routes

Another transdermal route that avoided the stratum corneum barrier properties is the delivery of drug through mucosal membrane. The respiratory tract including nasal mucosa, hypopharynx, etc. provides a large area for drug absorption. The drug can be absorbed systemically through inhalation process. Drugs for asthma treatment such as salbutamol are normally given through inhalation. Drug also can be applied directly on the mucosal surface in the nasal mucosa administration. Applications via the liquid spray on the mucosa surface has shown rapid systemic response after the application of intranasal midazolam (Karl et al., 1992). However, this method of application is not favourable for children due to the discomfort and unpleasant taste of the formulations. Another mucosa surface is the oral transmucosal route. The drug administers through this route also known as sublingual or buccal. There are several drugs have been used for the delivery through this route such as midazolam (Karl et al., 1993), opioid (Weinberg et al., 1988), etomidate (Streisand et al., 1998), testosterone etc. (Stuenkel et al., 1991).

1.5 TDS® delivery system

In this project, we have studied a proprietary liquid formulation, TDS® (Transdermal Delivery System), which can be applied to the skin via a metered pump dispenser, to deliver a drug locally and systemically. The TDS® (Transdermal Technologies Inc, Florida, USA) is a drug delivery system using a novel technology, which has been developed for use in pharmaceutical, cosmetic and over-the-counter products. The system is composed of substances which are nutritional and / or neutral and harmless at their respective concentrations. The TDS® is typically composed of a compatible solvent such as ethanol and propylene glycol, supplemented by other excipients such as cationic surfactants (quaternary ammonium compounds) and non-ionic surfactants (long chain fatty acids) that enable a sufficient dose of the drug to be put into a relatively small volume of liquid. TDS® also contains other excipients, designed to support the skin and maintain the integrity of the barrier and the health of the skin. The application of quaternary ammonium compound, later developed as TDS® system, was claimed effective in the temporary relief of pain associated with arthritis, tendonitis, and bursitis

(Hadley et al., 1998a). Penetran Plus®, which contains this quaternary ammonium compound, has recently been approved as over-the-counter self-administered analgesic (http://www.penetran.com/).

The TDS® enables skin penetration by altering the fluidity of the lipid layers in the stratum corneum. The way lipids surround the skin cells of the stratum corneum is the way mortar surrounds bricks in a wall. The TDS® temporarily and reversibly alters the alignment of those lipids allowing drug molecules to slip through. It camouflages the drug protecting it from the skin and the skin from it. This means that the TDS® should be safe and deliver drugs without causing any damage to the skin (Transdermal Technologies Inc., 2006). The safety of TDS® system has been evaluated and confirmed by the Institute for *In Vitro* Sciences in Gaithersburg, Maryland USA for primary dermal irritation, skin sensitisation and toxicity (Raabe and Nathan, 2005).

The above formulation of the TDS® has been shown to enable rapid delivery and is capable of delivering large (greater than 400 Daltons) drug molecules, proteins, as well as lipophobic and non-polar molecules in doses sufficient to reach therapeutic levels (Alam and Willoughby, 2002). The system uses a liquid vehicle to deliver drug across skin, measured by unit dose or metered pump spray. The dose is routinely compounded into approximately 1mL of very stable fluid. There is no patch or application appliance required other than unit dose packaging or metered pump sprayer.

1.6 Lidocaine

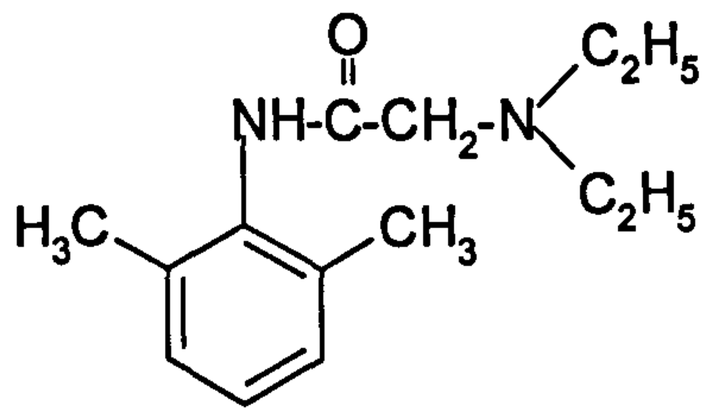


Figure 1.3 Lidocaine structure

Lidocaine (2-diethylaminoaceto-2',6'-xylidide), (Figure 1.3), an amino ethyl amide, is a commonly administered drug widely used as a local anaesthetic and antiarrhythmic for the treatment of ventricular arrythmias (Ellenhorn MJ, 1998). It has also been used as an anticonvulsant, an analgesic and in the management of neuropathic pain. Local anesthetics are drugs used clinically to reversibly block the conduction of impulses in peripheral nerves, leaving a loss of feeling (numbness) at the application area especially

the skin. The chronological discovery of local anesthetics began the centuries ago, when the Peruvian Indians had discovered that chewing "khoka" leaves produced exhilaration and relief from fatigue and hunger (Liljestrand, 1967). In 1884, Carl Koller demonstrated the extract from these leaves (cocaine) had a benefit as a local anesthetic for the eyes, and earned him the distinction of the "Father of Local Anesthesia". Few compounds had been synthesised until the discovery of procaine in 1904, which was accepted to replace cocaine. Procaine, an ester type was used as local anesthetic until the discovery of lidocaine. Lidocaine was discovered in 1943 by the Swedish chemist Nils Lofgren and since then remains one of the most popular local anesthetic agents.

Lidocaine is the alternative choice for individuals sensitive to ester-type local anaesthetics such as procaine and amethocaine or tetracaine. The amides are preferable to the esters because their metabolites are less likely to cause an allergic reaction. This is the first amide anesthetic derived from xylidine. It has more intense, longer lasting and more extensive anaesthesia than does an equal concentration of procaine but has shorter action than those of bupivacaine and prilocaine (Martindale, 1982). It is moderately lipid soluble with pKa of 7.9. It's duration of action of about 1-2 hours (Covino, 1986), indicate it is desirable when used for treatment requiring a rapid recovery (e.g. ambulatory surgery) but in less desirable for prolonged anesthesia.

Pharmacokinetics of lidocaine in human can be described from the application EMLA™ cream on the human skin. Absorption of lidocaine through the skin from EMLA™ application is directly related to both the duration and the area of application. When 60g of EMLA™ cream (1.5g lidocaine and 1.5g prilocaine) applied to 400cm2 of the skin (lateral thigh) for 3 hours, the C_{max} achieved was 120ng/mL and for 24 hours application the C_{max} was 280ng/mL (AstraZeneca, 2005). Similarly, when 10g of EMLA™ cream applied onto facial skin for 2 hours, the C_{max} achieved was 150ng/mL, but for the same dosage and application time onto the forearm, the C_{max} was only 18ng/mL (Juhlin et al., 1989).

The pharmacological and toxic effects of lidocaine depend on the concentrations of drug in plasma. At therapeutic plasma concentration about 64% is bound to plasma protein (Covino, 1986). High concentration of lidocaine in plasma (~ 5.3μg/mL) can cause toxicity such as methemoglobinemia (Bangha et al., 1996). Lidocaine is metabolised in the liver through dealkylation by mixed-function oxidases to monoethylglycine xylidide (MEGX) and glycine xylidide (GX). Both monoethylglycine xylidide and glycine

xylidide retain local anesthetic activity as potent as lidocaine (Catterall W and Mackie K, 2001) but its relative toxicity is approximately that of lidocaine (Kenkel et al., 2004). These metabolites are also responsible for the occurrence of toxicity which can occur during lidocaine therapy (Boyes et al., 1971). When administered intravenously, the steady state volume of distribution is 1.1 to 2.1L/kg. The elimination half – life from the plasma following IV administration is approximately 65 to 150 minutes.

1.7 Testosterone

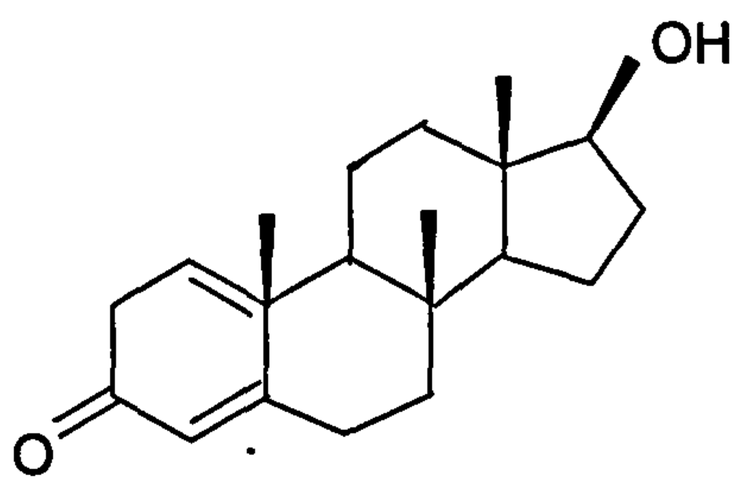


Figure 1.4 Testosterone structure

Testosterone (17 α-hydroxyandrost-4-ene-3-one) (Figure 1.4), an endogenous hormone is the most important androgen secreted into the blood by Leydig cells of the testes in man and corpus luteum and the adrenal cortex of ovaries in woman (Snyder, 2001). The magnitude of testosterone secretion is greater in men than in women at all stages of life. This is the differences between men and women. Testosterone is the principle male sex hormone which plays an important role in male development. In healthy young men, testes produce between 3 and 10mg (average of 6mg) of testosterone per day (Mazer, 2000) and the normal range is between 3 – 10ng/mL. Testosterone is responsible for the three major functions in animals;

- The development of secondary male sex characteristics such as, increased growth of body hair, beard growth, deep voice, penis development, aggressiveness, sexual behaviour, libido and the maturation of the sperm. Testosterone measurements are helpful in evaluating the hypogonadal states

- Promotion of the protein biosynthesis that is responsible for the highly anabolic functions of testosterone such as, accelerates muscle build-up, increases the formation of red blood cells, speeds up recovery time after injuries or illness etc.

- Inhibition of the gonad regulating cycle, including the hypothalamohypophysial testicular axis. This function is to control the production of testosterone. If the body produce enough testosterone, the testes will signal the hypothalamus to release less LH (luteinizing hormone) and FSH (follicle stimulating hormone) result in the reduction of testosterone productions.

LH is a major stimulus of testosterone production in men. Testosterone concentrations are higher during puberty, but decline with age (Bremner et al., 1983). Testosterone secretion is also pulsatile and diurnal, with the highest plasma concentrations attained in early morning and minimal levels in the evening. Testosterone is metabolised to two other active metabolites, dihydrotestosterone and estradiol. Both metabolites are as active as testosterone. Therefore some effects of testosterone are by itself, but some effects are from these two metabolites. Testosterone is also metabolised in the liver to another two inactive metabolite, androsterone and etiocholanolone (Snyder, 2001).

Male hypogonadism is the state where the production of testosterone is below the normal level (testosterone deficiency). At this stage, testosterone replacement therapy may be indicated. Testosterone replacement therapy can increase and maintain the testosterone at the normal level in hypogonadal man. A number of testosterone preparations have been studied and are currently available for replacement therapy.

1.8 Melanotan

α-MSH (melanocyte stimulating hormone), a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) is the pigmentary hormone that is released from the pars intermedia of the pituitary gland in some animals and keratinocytes and melanocytes in human skin. A major role of this hormone is to regulate integumental melanin pigmentation of the skin and hair in many species including human and some animals (Bolognia et al., 1989;Hadley M.E., 1996).

The substitution of the amino acid methionine (Met) with norleucine (Nle) at position 4 and L-phenylalanine (L-Phe) with D-phenylalanine (D-Phe) at position 7, give an analogue of α-MSH called Melanotan or more specifically as Melanotan-I (MT-I), (Figure 1.5). These substitutions make this analogue 26 times as potent as α-MSH in the adenylate cyclase assay (Sawyer et al., 1980). Melanotan was previously synthesized in the mid 1980's by a group of scientists at the University of Arizona, USA for the

treatment of skin cancer. After synthesizing hundreds of molecules the compound which is now known as MT-I which is commonly identified as [Nle^4-D-Phe^7] - α-MSH was found which enhanced the potency and prolonged the biological activity of α-MSH (Abdel Malek et al., 1985;Hadley et al., 1985). In addition, these analogues also exhibited prolonged (residual) melanotropic activity, as evidenced by sustained maximal tyrosinase stimulation in cultured mouse melanoma cells (Abdel Malek et al., 1985). Unlike α-MSH, MT-I is resistant to inactivation by serum enzymes or by purified proteolytic enzymes (eg., α-chymotrypsin) (Castrucci et al., 1984).

The above results have very important impacts for the use of MT-I in the therapeutic darkening of the skin to protect it from harmful ultraviolet (UV) light. In response to the sun exposure, UV radiation induces α-MSH to stimulate the melanocyte in the skin to produce melanin, the pigment that gives the skin its colour (Bolognia et al., 1989). The fact that melanocytes can induce melanin pigmentation of the human skin can be described in Addison's disease where the direct action of ACTH (a melanotropic peptide) results in hyperpigmentation of the patient's skin especially in the mouth, hand, face and all flexural regions (Kumar and Clark, 1998). Melanin acts as a shield to protect the body from dangerous level of UV light. When exposed to the sunlight, the body is protected. It can be considered as an internal sunscreen. Since melanin pigmentation is considered to be the most effective mechanism the body has to protect against radiation-induced damage, it is important to stimulate the skin's own 'protective mechanism' of tanning without exposure to the sunlight. MT-I, like sunlight (or UV lights), induces the production of melanin in the skin, resulting in a tan. By producing melanin without exposing the skin to dangerous levels of UV light, MT-I has the potential to reduce skin damage and thus to reduce the incidence of associated skin cancer.

Fitzpatrick (Fitzpatrick, 1988) has classified the individual skin type in relation to their susceptibility to sunburn by the following criteria:

Type 1: The skin that never tans, but always burns

Type 2: The skin that sometimes tans, but mostly burns

Type 3: The skin that mostly tans, but sometimes burns

Type 4: The skin that always tans, and never burns

Several studies have suggested that individuals whose skin tends to burn easily on exposure to the sun and who do not tan readily are at higher risk of both non-melanoma skin tumours and of cutaneous melanoma (Gilchrest and Eller, 1999). UV radiation has many harmful effects and is now identified as a major risk factor in the etiology of skin cancers, including malignant melanoma (Farmer and Naylor, 1996;Holman et al., 1983). Skin cancer is a global health issue affecting millions of lives. Non-melanoma skin cancer is the most common cancer in the UK. Official figures have over 62,000 cases diagnosed in the UK in 2001. But this figure is an underestimate as registration is generally incomplete (Cancer Research, 2005). There are also a few more common types of sun allergies that are caused by or aggravated by exposure of the skin to sunlight, such as Polymorphous Light Eruption (PMLE), Actinic Prurigo (hereditary PMLE), Photoallergic Eruption, Solar Urticaria, etc. Beside to induce the skin tanning, the development of MT-I is hoped to reduce the number of diseases associated with the sun exposure.

The pharmacokinetic and safety of MT-I have been reported in a few studies. The MT-I doses of 0.16mg/kg were administered intravenously (IV) and orally (PO), and doses from 0.08 to 0.21mg/kg subcutaneously in three healthy volunteers for ten days (Ugwu et al., 1997). The results showed that the bioavailability of SC doses was comparable to IV doses, but no drug levels were observed in plasma following oral doses. The pharmacokinetic parameters obtained from SC and IV doses were summarised in Table 1.1 and Table 1.2, respectively. The doses given were well tolerated with minimal side effects such as mild dermatologic and gastrointestinal effects. Two subjects became visibly flushed in the face and neck area within minutes after SC dosing and one of them experienced mild nausea after oral and IV dose.

Table 1.1 Pharmacokinetic parameters for Melanotan-I following SC administration to humans (n=3) (Ugwu et al., 1997).

Parameter	Subject 1	Subject 2	Subject 3
Dose (mg/kg)	0.08	0.21	0.20
Half-lives (h)			
absorption (α)	0.07	0.79	0.16
beta (β)	0.78	1.47	1.66
$AUC_{0-\infty}$ (ng/mL/h)	427.2	1802.3	1226.1
MRT (h)	1.21	2.74	2.59
CL_s (L/kg/h)	0.19	0.12	0.16
$V_d\beta$ (L/kg)	0.21	0.25	0.39

Table 1.2 Pharmacokinetic parameters for Melanotan-I following IV administration to humans (n=3) (Ugwu et al., 1997).

Parameter	Subject 1	Subject 2	Subject 3
Dose (mg/kg)	0.16	0.16	0.15
Half-lives (h)			
alpha (α)	0.25	0.14	0.52
beta (β)	0.48	0.66	2.08
$AUC_{0-\infty}$ (ng/mL/h)	296.7	405.1	518.9
CL_s (L/kg/h)	0.54	0.39	0.29
$V_d\beta$ (L/kg)	0.38	0.38	0.87

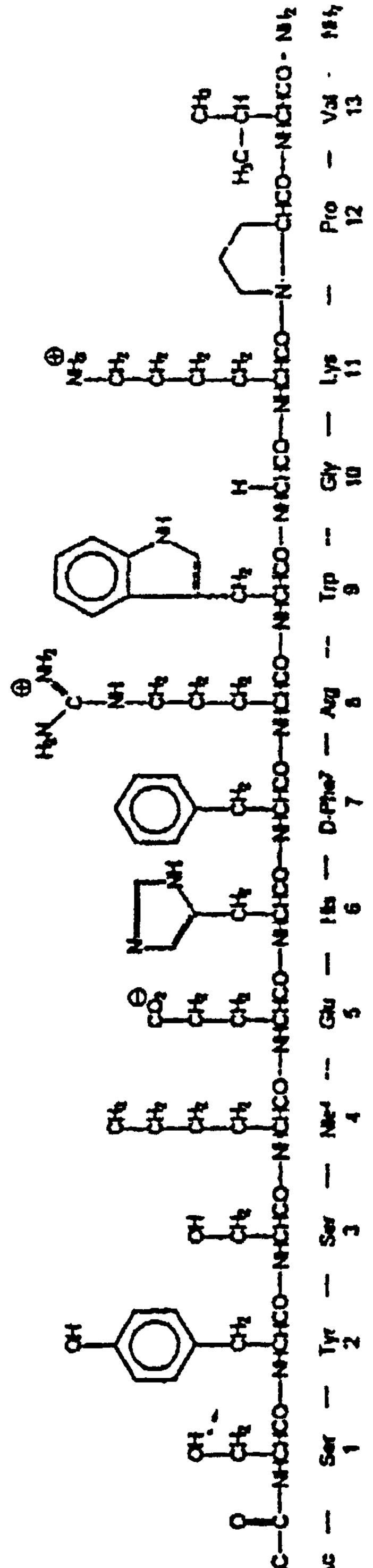


Figure 1.5 Structure of Melanotan-I (EpiTan Limited, 2004)

1.9 Pharmacokinetic studies

Pharmacokinetics involves the kinetic study of drug absorption, distribution, metabolism, and elimination (ADME). The study of pharmacokinetics must involves both the theory and experimental. The theoretical aspects involve the design of the study and the prediction of the model to use in the experimental phase. Experimental aspects of the study involve the conduct of the clinical trial, sample collection, analytical methods development and validation, and statistical analysis of pharmacokinetic data. Pharmacokinetic studies or clinical trials are normally conducted in several phases - Phase I, II, III, and IV.

Phase I is an early phase trial normally conducted in a small number of healthy subjects. These trials investigate how the human body handles the new drug or new formulations. Where possible the studies include an assessment the pharmacologic activity, side effects and the effectiveness of a drug in the human body. Most of the pharmacologic activity studies in phase I are to obtain the pharmacokinetic and pharmacodynamic properties of a drug. The information obtained from these studies can be used to design a Phase II study, normally conducted in a bigger number of patients. Phase II trials aim to give an idea of efficacy and safety of the drug in patients. During the early stages of Phase II, a limited number of patients may be given a low dose of drug and closely monitored for their tolerability with the treatment. During this phase, the suitable dosage and adverse effects shoud be identified and observed.

If the study is successful and adequate degree of efficacy exists with the acceptable adverse effects, the bigger trials involving hundreds to thousands of patients will be conducted in Phase III in order to obtain the necessary marketing approval. Phase III is the main safety and efficacy study to optimise the drug effectively and may involve a multi centre studies. Phase IV trials are normally performed after the drug has been approved for marketing. They may be used to clarify some questions that may remain unanswered after finish the Phase III, such as effectiveness and safety on certain groups of people. These groups of people include children, the elderly, etc.

1.10 Good Clinical Practice (GCP)

Good Clinical Practice is an international ethical and scientific quality standard for design, conduct, performance, monitoring, auditing, recording, analyses, and reporting

of clinical trials. It provides assurance that the data and reported results are credible and accurate and the rights, integrity and confidentiality of trial subjects are protected [EMEA, ICH GCP, 1.24] (EMEA, 2002). GCP compliance is now a legal requirement in the EU as of May 2004 which means that everyone involved in the investigation of medicinal products has the legal duty to ensure that all of the research processes are carried out in compliance with these regulations (HMSO, 2004).

1.11 Objectives of the project

This PhD project involved a series of pharmacokinetic studies conducted in healthy volunteers. The studies conducted were involved the transdermal delivery of a number of drugs currently used as a topical administration such as testosterone, lidocaine and new drug formulation, melanotan-I in order to improve the current problem or limitation associated with these products.

The aim of this project is to:

1) Conduct pharmacokinetic studies in healthy human subjects for the development of a novel transdermal drug delivery system, TDS® which are combined with a few drugs currently use for transdermal delivery. Pharmacodynamic aspects of the treatments also have been investigated in certain areas to support the pharmacokinetic findings. The pharmacokinetic and other information obtained will be used for future development of the TDS® system.

2) Explore, develope, improve and validate the analytical tools available for the analysis of drugs in different matrixes obtained from all the clinical studies conducted such as analysis of lidocaine in plasma from TDS®-Lidocaine and EMLA™ cream studies, lidocaine in dialysate and tape from microdialysis and tape stripping studies, respectively.

3) Explore and compare the various methods available to measure pharmacokinetic profiles of transdermal or topical drugs such as conventional systemic measurement in blood, microdialysis and the tape stripping techniques. The outcome from this study may contribute the finding towards the establishment of the pharmacokinetic methods for topical drug administration.

Chapter 2 Lidocaine

2.1 Introduction

In percutaneous local anaesthetic, the drugs targets are the pain receptors (nociceptors), located at the dermo-epidermal junction. Local anaesthetic agents stabilize neuronal membranes by inhibiting the ionic fluxes required for the initiation and conduction of impulses for the local anaesthetic action. For this purposes, local anaesthetic is normally given by subcutaneous injection. When administered via subcutaneous injection, it produces a rapid onset of action and rapidly spreads through surrounding tissue for the anaesthetic effect. However, procedures involving the insertion of a needle through the skin are painful and may induce great fear and anxiety, especially in children. For the above reasons, many researchers have searched for a local anaesthetic method which is needleless and pain free via topical application. Many researches have been conducted and published on lidocaine and other agents for development of a topical local anaesthetic.

The investigations into the efficacy of topically applied local anaesthetics were first carried out by Monash in 1957 (MONASH, 1957). He showed that application of a 2% alcoholic solution of several local anaesthetic bases, produced percutaneous local anaesthesia within 45 – 60 minutes application. Since then, many formulations have been tested including the used of dimethyl sulfoxide, dimethyl acetamide and other solvents to increase the permeability of the solution into the skin (Brechner et al., 1967). The introduction of Eutectic Mixture of Local Anaesthetics (EMLA™) cream (Astra Pharmaceutical Ltd.) represented a major advance in the investigation of transdermal local anesthetics. EMLA™ cream (5% lidocaine:prilocaine) was developed in the 1980s (Evers et al., 1985), and is now in clinical use and has contributed to reducing the needle phobia in many European countries. The most common use of EMLA™ cream is for the management of pain associated with venepuncture or intravenous cannulation, especially in children. Several studies have been conducted to assess the effectiveness of EMLA™ as a local anaesthetic. Hopkins et al. (Hopkins et al., 1988) have evaluated the efficacy of EMLA™ in 111 young children, ages 1-5 years in the alleviation of venipuncture pain at intravenous induction using 27-gauge needles. 75 children received EMLA™ cream and 36, placebo. Pain was assessed by VAS and VRS and the result

showed that the EMLA™ treated group had significantly lower rating score (no pain) than who received placebo.

Although EMLA™ is effectively used as local anaesthetic, long application times (at least 1 hour) for it to achieve acceptable analgesia, limit its usefulness. Another local anaesthetic agent, amethocaine or tetracaine (U.S.P) has been extensively studied (McCafferty D.F., 1995;McCafferty et al., 2000) to overcome the slow onset time of EMLA™. Amethocaine is a potent ester type local anaesthetic. Its local anaesthetic action is greater than that of lidocaine and normally used in lower concentrations (Martindale, 1982). The development of Amethocaine gel in Belfast in 1980's, has led to the introduction of Ametop Gel and is now also in clinical use as a topical local anesthetic (Smith & Nephew Healthcare Ltd.). Ametop, contained 4% amethocaine has been claimed that more effective than EMLA™ in providing skin anaesthesia. In the double blinded study involving 20 healthy volunteers, the various concentrations of amethocaine gel to provide anaesthesia were assessed. The study concluded that the 3% and above of gel concentrations are effective in providing skin anaesthesia after 30 minutes application time (Woolfson and McCafferty, 1996) In another comparison study between EMLA™ and Ametop gel in 110 children , the 40 minutes application of EMLA™ and Ametop on the skin resulted in Ametop the most effective than EMLA. In the pain assessment, 62% of the subject treated by Ametop scored no pain compared to only 32% in the EMLA™ treated group (Morton NS, 1996).

The delivery of lidocaine through the skin by iontophoresis has also been investigated recently by a few researchers (Galinkin et al., 2002;Rose et al., 2002;Zempsky and Parkinson, 2003). In two studies conducted by Rose et al. (2002) and Galinkin et al. (2002), iontophoresis local anesthetic system has shown to be more effective than placebo and comparable to EMLA. However it still requires at least 15 minutes of onset time (Zempsky et al., 1998). Nitric Oxide generating system (sodium nitrite + ascorbic acid + KY jelly™) when combined with lidocaine also shown to be able to produce skin analgesia when applied on the skin (Tucker et al., 2002). The 10 minutes application of this system on the dorsal hand can significantly reduced the pain caused by venous cannulation, compared to placebo. Giving thorough consideration, this system is potential to be a topical local anaesthetic with a fast onset time. There are other systems currently under investigation such as lidocaine patches (Padula et al., 2003) and liposomal system (Bucalo et al., 1998;Eichenfield et al., 2002).

2.2 Objectives

This study had two main objectives. The first objective was to develop and validate the sensitive and accurate liquid chromatography-mass spectrometry (LC-MS-MS) method to measure low levels of lidocaine in human plasma. The second objective was to determine the ability and the effectiveness of the TDS® local anaesthetic system to anaesthetise the skin. Also to determine which anaesthetic system, the TDS®α Anaesthetic System (alcohol based) or the TDS®β Anaesthetic System (water based) is effective in producing skin anaesthesia.

2.3 Validation of Liquid Chromatography - Mass Spectrometry (LC-MS-MS) method for the analysis of Lidocaine in human plasma.

2.3.1 Introduction

The absorption of lidocaine from intact skin into the blood circulation for transdermal delivery is very poor compared to intravenous injection. Therefore the systemic level of lidocaine is considerably lower. To analyse the plasma samples obtained from the clinical study of transdermal delivery of lidocaine, the sensitive and accurate method should be applied. Various analytical methods based on High Performance Liquid Chromatography (HPLC) with UV detection (Abraham et al., 1997;Adams et al., 1989;Chen et al., 1992;Kang et al., 1999;Klein et al., 1994;Lotfi et al., 1997), Liquid Chromatography-Mass Spectrometry (LC-MS-MS) (Abdel-Rehim et al., 2000;Bo et al., 1999), and Gas Chromatography (GC) (Watanabe et al., 1998) have been used in the quantification of lidocaine. Regardless the LC-MS-MS and GC-MS method, the HPLC with UV detection was not sensitive enough to detect low concentration of lidocaine which all the papers above claimed the LOQ ranging from 4 to 100ng/mL. In this study, the simple and rapid LC-MS-MS method for the analysis of lidocaine in human plasma using bupivacaine as an internal standard has been developed and validated.

2.3.2 The principle of LC-MS-MS

LC-MS-MS is a combination of two techniques to identify and analyse chemical compounds. Liquid chromatography can separate a component of a mixture into separate compounds and then characterise them by mass spectrometry by their molecular weight. The separation of a mixture by liquid chromatography can be achieved by using a column. A column is normally packed with certain materials form different types of phases, such as normal phase, reverse phase, ion exchange, etc. After separation in the column, the samples are then introduced into the mass spectrometer's ion source, where the molecular components are ionised. The mass spectrometer will separate these ions based on their mass to charge ratio. For the purpose of simplicity, assume that all ion forms are singly charged, therefore the denominator of the mass to charge ratio (m/z) is always 1. Consequently, any ion observed at a particular mass is that mass. Therefore a compound can be identified either to confirm the presence of the

compound, quantitation by using standard curve or determine the elements within the sample, if unknown.

2.3.3 Good Laboratory Practice (GLP)

This validation was conducted at the Analytical Unit, St. George's Hospital Medical School in accordance with United Kingdom Statutory Instrument 1999, No. 3106, 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.

2.3.4 Experimentals

2.3.4.1 Chemicals

Lidocaine hydrochloride and bupivacaine hydrochloride (internal standard) was obtained as a white powder from the Sigma-Aldrich Company, Poole, UK. 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. The high purity air and nitrogen was generated using generators obtained from Peak Scientific Instruments Ltd., Renfrew, Scotland.

2.3.4.2 Apparatus

Solvent delivery was achieved using a Perkin Elmer series 200 pump set at 1mL/min. Sample injection was performed by using a Perkin Elmer series 200 auto injector. A Shimadzu CTO-10A was used as a column oven. Detection was by PE SCIEX API2000 Mass Spectrometer. All PE SCIEX software was supplied by Perkin Elmer, England. The NM20ZA high purity nitrogen and air generators were supplied by Peak Scientific Instruments Ltd., Renfrew, Scotland.

2.3.4.3 Chromatographic system

The analytical column, a Supelcosil LC-Si (4.6mm ID × 100mm), obtained from Merck (BDH) Limited, Dorset, England, was maintained at 50° C. The mobile phase was 2% formic acid in acetonitrile:water (50:50). The flow rate was fixed at 1mL/min. A PE SCIEX API2000 triple quadrupole mass spectrometer equipped with a turbo-ion spray (heated electro-spray, ESI) was used to introduce the sample into the mass spectrometer

through off-axis at an angle of 45°. The sample was introduced through a 10:1 splitter, which meant only 10µL of the sample entered the mass spectrometer. Nitrogen was used as the collision gas. PE SCIEX Analyst software (1.3 Version) was used to control the HPLC/MS, record the output from the detector, perform integration of peak areas and calculate the lidocaine concentrations.

Bupivacaine was used as an internal standard. All calculations were based on peak area ratios of lidocaine and internal standard. The precursor ion for lidocaine and bupivacaine were m/z 234.99 and 289.09, respectively and after collisional dissociation the product ions were 85.98 and 140.09, respectively. Molecular structure and the molecular weight of precursor and product ions are shown in Figure 2.2. The retention time for lidocaine and bupivacaine during the assay were 4.9 and 5.1 minutes, respectively.

2.3.5 Assay Procedures

2.3.5.1 Preparation of stock solutions

2.3.5.1.1 Lidocaine Calibrator

A stock solution of lidocaine was prepared by dissolving 5.8mg lidocaine hydrochloride in 100mL 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming a purity 100 %) 50µg/mL.

2.3.5.1.2 Lidocaine Quality Control (QC)

A stock solution of lidocaine was prepared by dissolving 5.7mg lidocaine hydrochloride in 100mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming a purity 100.0%) 49.1µg/mL.

2.3.5.1.3 Bupivacaine (Internal Standard)

A stock solution of bupivacaine was prepared by dissolving 5.8mg bupivacaine hydrochloride in 100mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming a purity of 99.9%) 50µg/mL. A working strength solution was prepared by diluting 1mL of the stock to 100mL with de-ionised water giving a final concentration of 500ng/mL.

All the above stock solutions were stored at approximately -20°C.

2.3.5.2 Plasma Aliquots

2.3.5.2.1 Calibrators

250µL of the lidocaine stock solution was pipetted into a 50mL volumetric flask and made up to the mark with analyte free plasma to produce a sub-stock (Cal 1). Working calibration solutions were prepared by diluting the sub stock with plasma as tabulated in Table 2.1.

Table 2.1 Working calibration solution (cal.) with related dilution in plasma.

Volume of sub stock (plasma)	Volume of plasma added	Total volume (plasma)	Nominal concentration	Measured concentration	Inaccuracy	Cal. No.
(mL)	(mL)	(mL)	(ng/mL)	(ng/mL)	%	
0.00	20	20.00	0.0	0.00		9
0.05	25	25.05	0.5	0.50	0.0	8
0.10	25	25.10	1.0	1.00	0.0	7
0.20	20	20.20	2.5	2.48	-0.8	6
0.80	20	20.80	10.0	9.62	-3.8	5
2.25	20	22.25	25.0	25.28	1.1	4
4.00	16	20.00	50.0	50.00	0.0	3
10.00	15	25.00	100.0	100.00	0.0	2
250µL stock solution	49.750	50.00	250.0	250.00	0.0	1 (Sub stock)

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

2.3.5.2.2 Quality Control (QC) samples

500µL of the lidocaine stock solution was pipetted into a 25mL volumetric flask and made up to the mark with analyte free plasma to produce a sub-stock (QC 4, 982ng/mL).

Working controls were prepared by diluting the sub stock with plasma as tabulated in Table 2.2.

Table 2.2 Working control solution (ctrl.) with related dilution in plasma.

Volume of sub stock (plasma)	Volume of plasma added	Total volume (plasma)	Nominal concentration	Measured concentration	Inaccuracy	Ctrl. No.
(mL)	(mL)	(mL)	(ng/mL)	(ng/mL)	%	
0.075	50.0	50.075	1.5	1.47	-2.0	1
1.20	25.0	26.2	45	44.98	0.0	2
4.50	25.0	29.5	150	149.8	-0.1	3
500µL stock solution	24.5	25.0	1000	982	-1.8	4 (Sub stock)

2.3.5.3 Extraction

A simple liquid-liquid extraction was performed for this assay. A 0.5mL volume of plasma, 0.1mL internal standard (I.S) solution (500ng/mL), 0.1mL 1M NaOH and 3mL of methyl-tert-butyl ether (MTBE) were placed in a 4.5mL propylene tube. The content was mixed for the minimum of 5 minutes by the shaker and then centrifuged at 3000-3500rpm for 5 minutes. The top layer was then transferred to a 4.5mL propylene tube containing 0.25mL of 0.1% Formic acid. The tube was mixed again for the minimum of 5 minutes by the shaker and then centrifuged at 3000-3500rpm for 5 minutes. The top solvent layer was discarded by vacuum and the remaining solution was transferred into an autosampler vial. A 100µL volume was injected into the analytical column. Figure 2.1 shows the schematic diagram of the extraction procedure.

Lidocaine LC/MS/MS

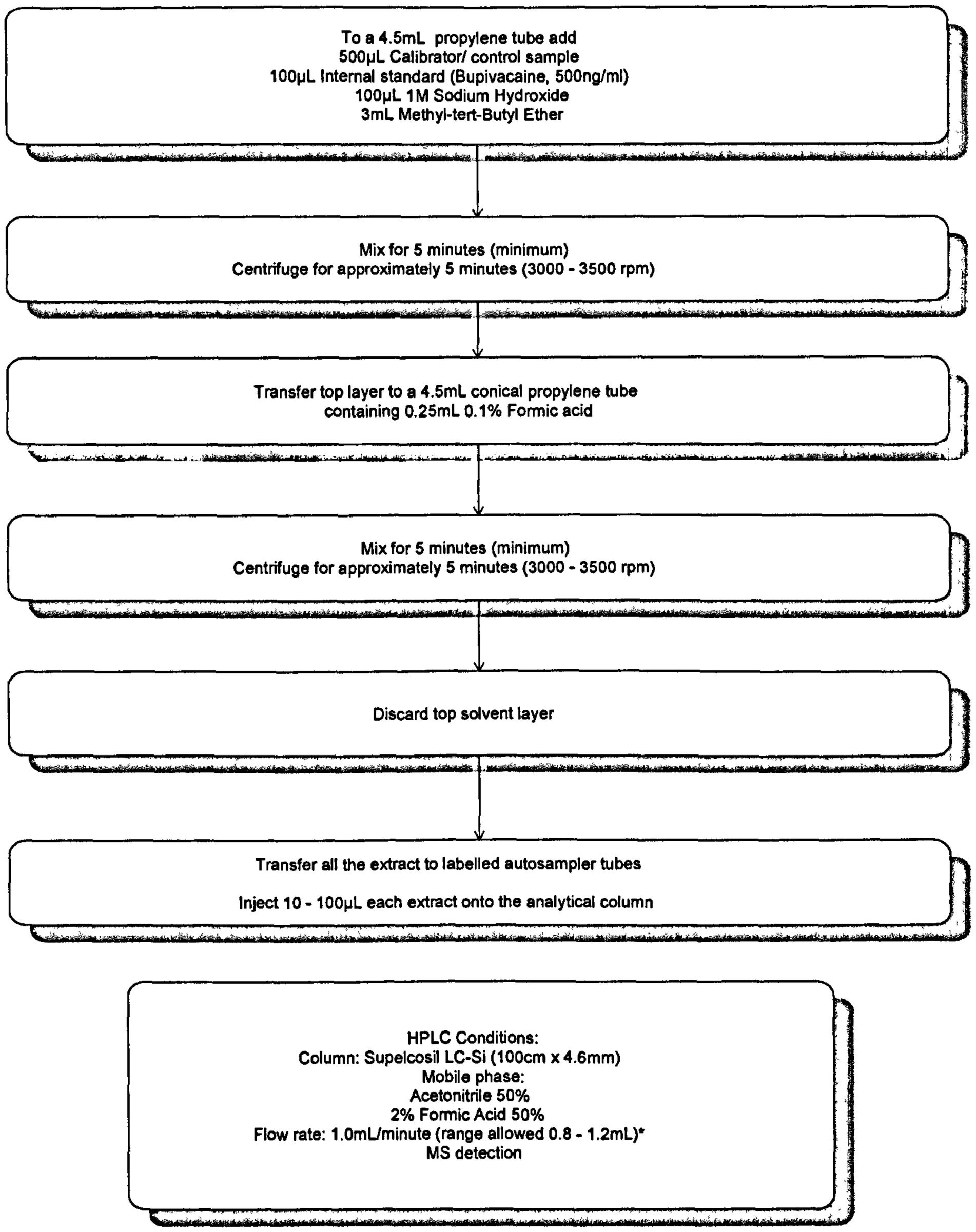


Figure 2.1 Schematic diagram of the lidocaine extraction procedures

2.3.6 Method Validation

All the validation procedures and the acceptance criteria used in this study were adapted from the Food and Drug Administration (FDA) and The European Agency for the Evaluation of Medicinal Products (EMEA) guideline for method validation (EMEA, 1995;FDA, 2001b).

2.3.6.1 Specificity

Six samples of blank plasma and six samples of plasma spiked with lidocaine and the internal standard were prepared and the extraction was carried out. The nominal concentration of lidocaine used was 0.5ng/ml (LLOQ) and the internal standard was 500ng/mL. The signal to noise ratio for lidocaine and internal standard should be greater than 5.

2.3.6.2 Calibration / linearity

The calibration consists of eight non zero, calibrators assayed in duplicate (nominal values 0.5, 1.0, 2.5, 10, 25, 50, 100 and 250ng/mL). Two lidocaine free samples were analysed, one with the internal standard and one without the internal standard; neither being included when fitting the calibration line. The correlation coefficient (r) between concentration and peak area ratio should be equivalent to, or better than, 0.98. The simplest mathematical model that adequately describes the concentration-response relationship was used.

The following conditions should be 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. Excluding any calibrators should not change the model used.

2.3.6.3 Inaccuracy and Imprecision

Inaccuracy was tested by determinations of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. The nominal values for low, medium and high control samples were 1.5, 45 and 150ng/mL, 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 were measured both within-batch and between-batch by the analysis of the three control samples, the LLOQ and the ULOQ. For within-batch and between-batch imprecision the LLOQ, ULOQ and the three control samples was each assayed six times in three separate assays. Each assay has an individual calibration curve.

The within- and between-batch mean inaccuracy for the high and medium control sample concentration must be within ±15% of the expected or nominal concentration and within ±20% of the expected or nominal concentration for the lowest control sample. The within-batch and between-batch imprecision for the high and medium control sample concentration must be within ±15% and within ±20% for the lowest control sample.

At the LLOQ, the mean inaccuracy and imprecision must be within ±20% of the expected or nominal concentration for at least five of the six control samples. At the ULOQ, the mean inaccuracy and imprecision must be within ±15% of the expected or nominal concentration for at least five of the six control samples. At least 66% of the controls must meet the above criteria.

2.3.6.4 Recovery

Absolute recovery of lidocaine was tested using human plasma spiked with lidocaine at the same nominal concentrations as the quality control samples. Absolute recovery of bupivacaine, (internal standard) was tested at a nominal concentration of 100ng/mL. Peak area measurements obtained from the extracted samples were compared to the peak area measurements obtained from direct solvent injection of the test compounds. Mean and standard deviation were calculated from at least three measurements at each level.

2.3.6.4.1 Extracted control sample:

100µL of each aquoues control sample, 500µL blank plasma, 100µL internal standard solution were processed as the extraction method.

2.3.6.4.2 Non-extracted control sample:

100µL of each control sample (aqueous) was mixed with 100µL internal standard solution and 50µL 0.1% formic acid.

2.3.6.5 Sample stability

2.3.6.5.1 Freeze and thaw stability

Stability of lidocaine was measured in a sample of analyte-free human plasma spiked with lidocaine at the same nominal concentrations as the quality control samples. These samples were subjected to repeated freezing and thawing. A minimum of three freeze/thaw cycles were tested. The storage conditions were representative of normal conditions (domestic freezer, nominally -20°C). The maximum and minimum temperatures during this period were recorded. The mean and the standard deviation were calculated from at least three measurements of each sample, for each freeze/thaw cycle.

2.3.6.5.2 Stability at ambient and 4°C

The stability of these control samples were examined at ambient temperature, approximately 20°C, and at approximately 4°C, for a period of at least 24 hours. The mean and the standard deviation were calculated from at least three measurements of each sample. The maximum and minimum temperatures at which both sets of samples were held during this period were recorded.

2.3.6.5.3 Stability of the sample extracts

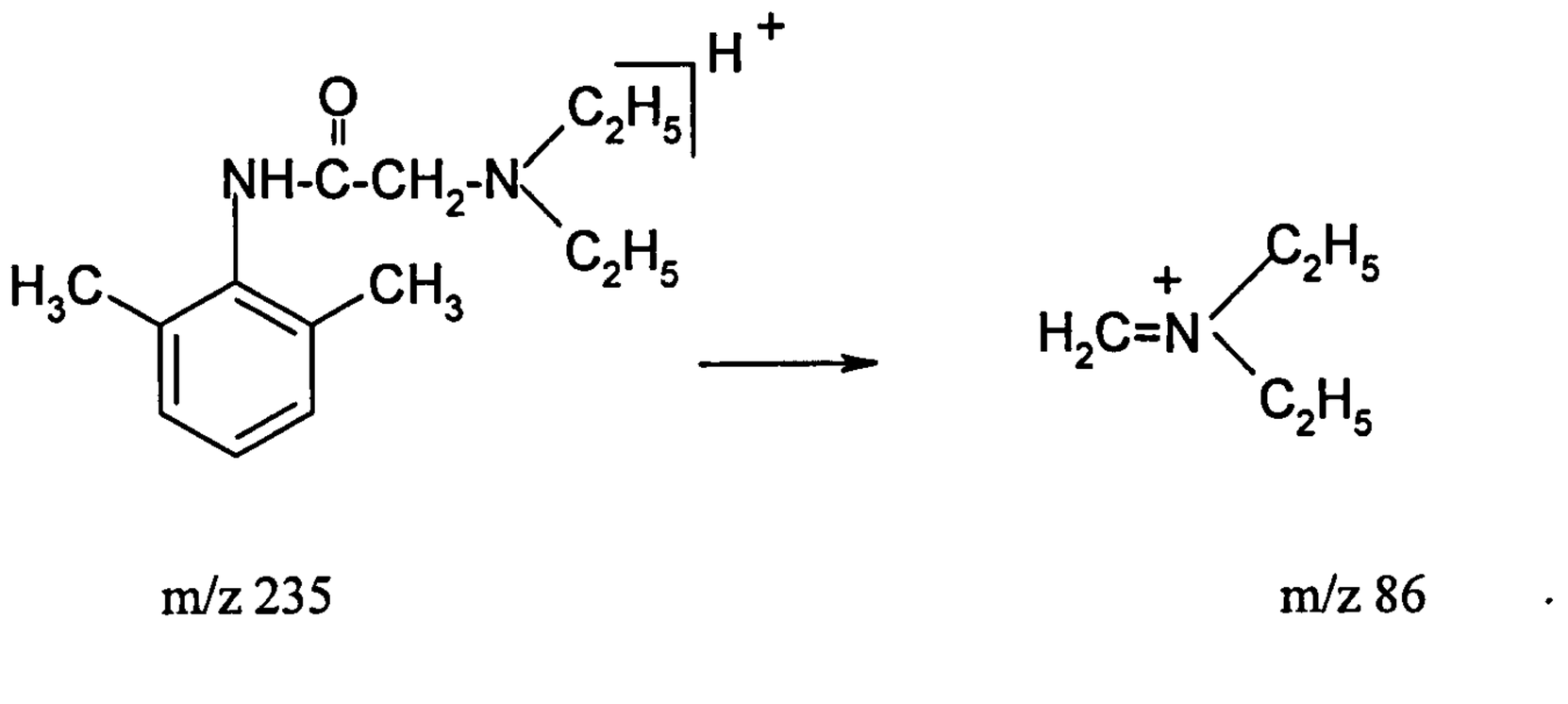
The stability of the sample extracts were tested at the same nominal concentrations as the quality control samples. The three control samples, prepared in human plasma, was each extracted in such a way as to yield a total volume of extract sufficient to allow aliquots to be placed in the auto-sampler at room temperature and injected over a period of not less than 24 hours. A minimum of six measurements were made at each

concentration. The maximum and minimum temperatures during this period were recorded.

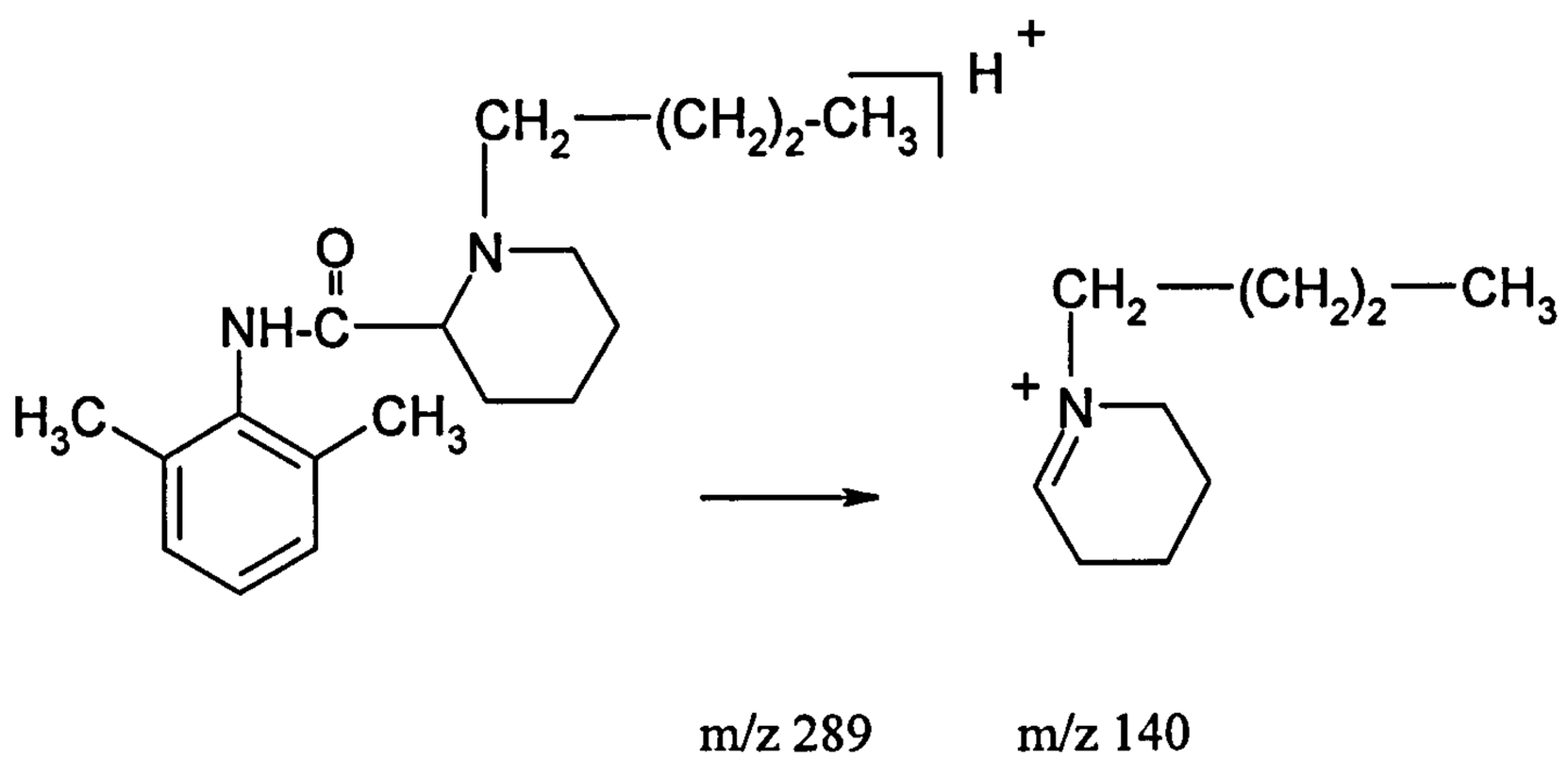
2.3.7 Results and discussions

2.3.7.1 Selection of operating protonated ions

Figure 2.2 shows the chemical structure and the protonated ions of lidocaine and bupivacaine used in this study related to their mass. The fragment ions at 86 m/z for lidocaine and 139.7 m/z for bupivacaine were selected for the assay. The mass spectrum scans for the above two analytes are shown in Figure 2.3.

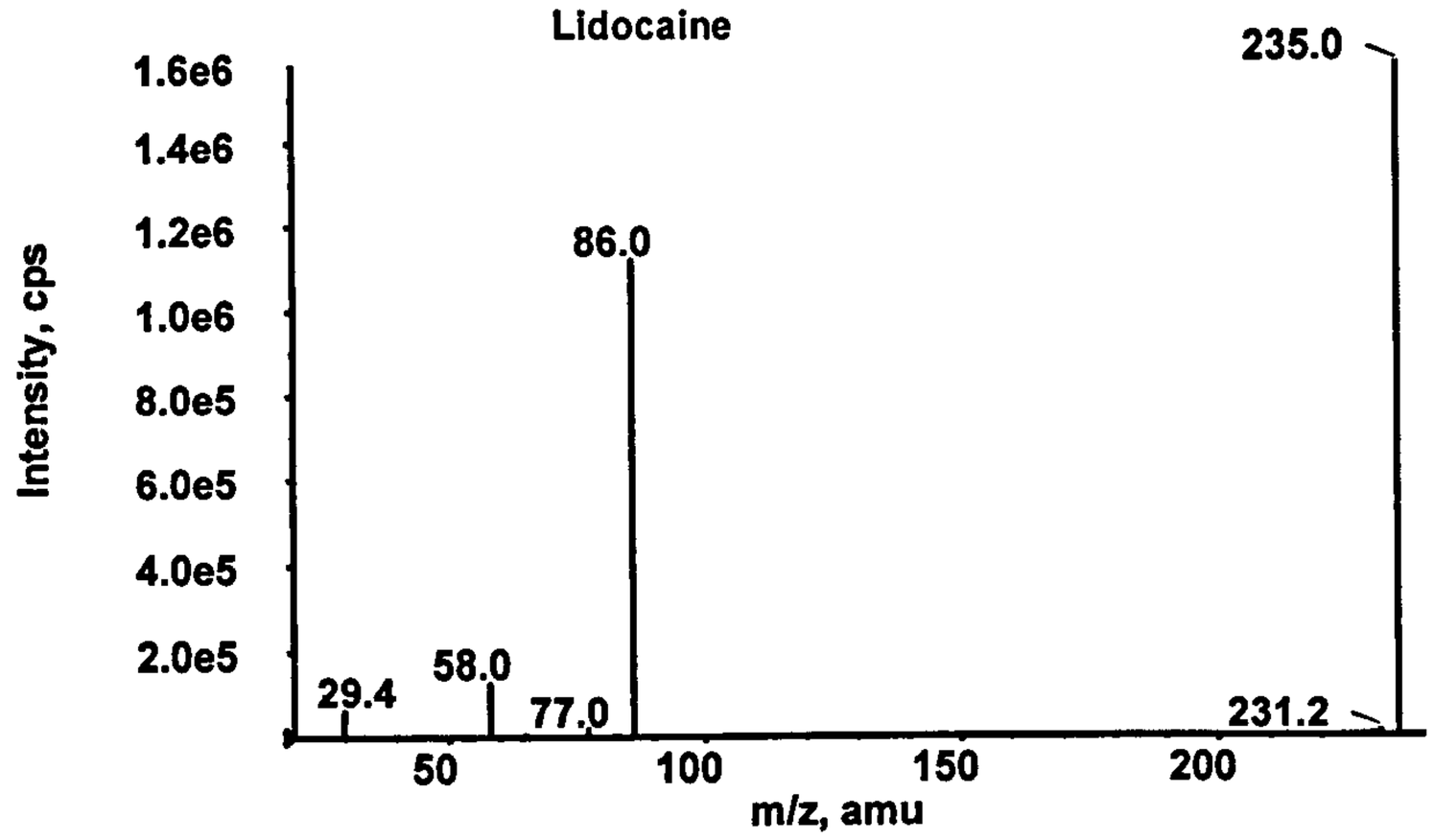


LIDOCAINE



BUPIVACAINE

Figure 2.2 Chemical structures of precursor and product ions of protonated lidocaine and bupivacaine



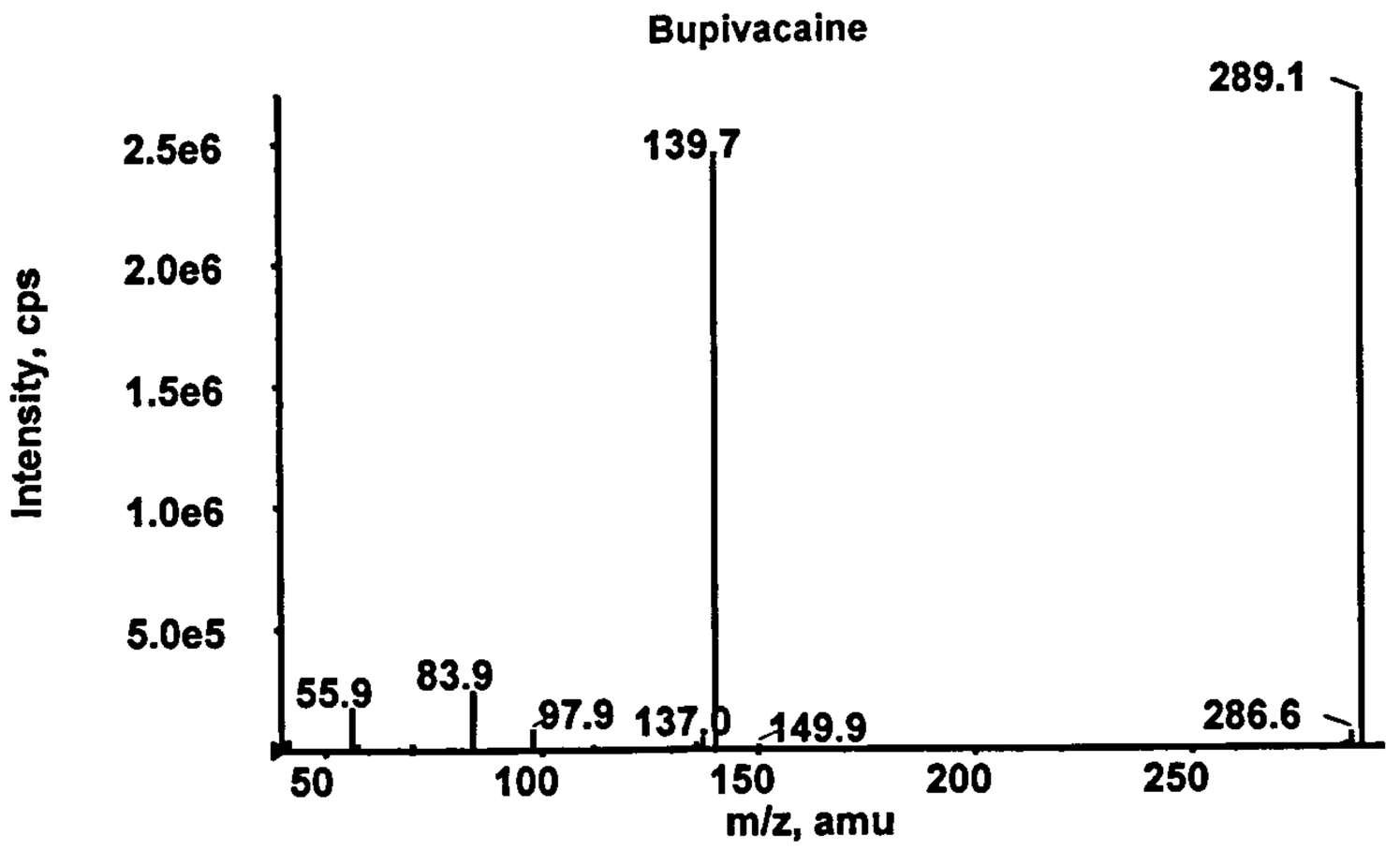


Figure 2.3 Masss spectra of precursor and products ion scan for lidocaine and bupivacaine

2.3.7.2 Specificity

No significant interfering peaks were found at the retention time of lidocaine or the internal standard. The signal to noise ratio for both lidocaine and the internal standard were both greater than 5. Figure 2.4 shows the chromatogram obtained from the blank plasma, blank plasma spiked with 0.5ng/mL lidocaine and blank plasma spiked with 500ng/mL bupivacaine.

2.3.7.3 Calibration

The concentration range of lidocaine measurement was 0.5 to 250ng/mL. To correctly weight the quadratic regression line for this 500 fold range the residuals were weighted by the reciprocal of the nominal concentration value squared. This achieves an allocation of equal importance to each standard value. That is, a constant coefficient of variation is assumed across the calibration range. 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. The regression coefficient for all the calibration curves were greater than 0.99. Mean results obtained from five curves are summarized in Table 2.3.

Table 2.3 Regression parameters for five calibration curves during validation

Batch	(C)	(A)	Intercept(B)	r^2
1	6.12 E-6	0.00743	0.000394	0.9995
2	7.75 E-6	0.00772	0.000682	0.9995
3	7.51 E-6	0.00783	0.000210	0.9987
4	7.79 E-6	0.00816	0.000282	0.9994
5	8.52 E-6	0.00830	0.000434	0.9997

$y = Cx^2 + Ax + B$

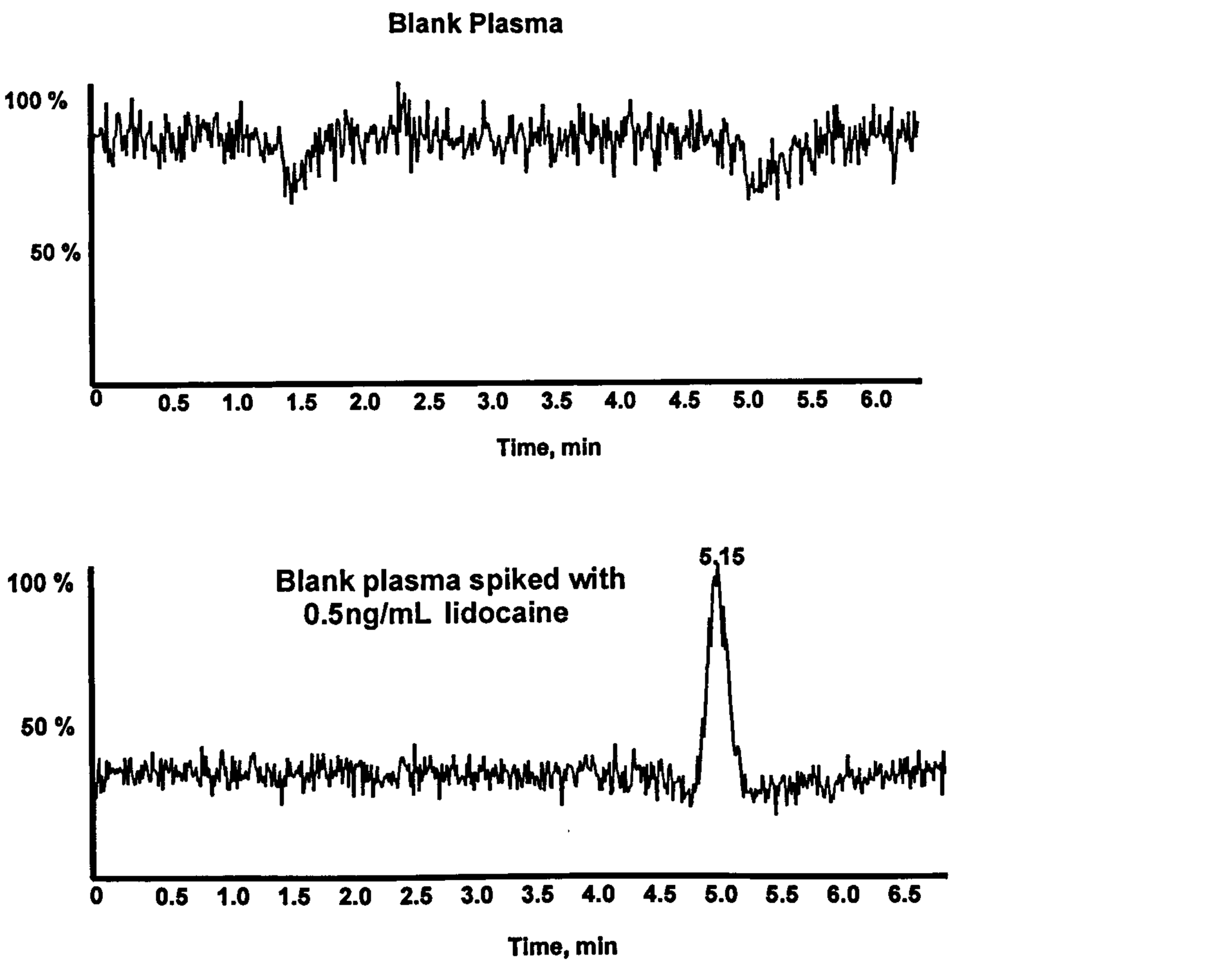


Figure 2.4 Chromatograms obtained from extracted blank plasma, blank plasma spiked with 0.5ng/mL lidocaine, and blank plasma spiked with 500 ng/mL bupivacaine.

2.3.7.4 Imprecision and Inaccuracy

2.3.7.4.1 Within-assay reproducibility

The CV% and the percentage for within assay imprecision and inaccuracy including LLOQ and ULOQ were all within the accepted range with ranging from 1.7 to 13% and 0 to 10%, respectively.

2.3.7.4.2 Between assay repeatability

The CV% and the percentage for between assay imprecision and inaccuracy including LLOQ and ULOQ were all within the accepted ranges of between 1.9 to 8.3% and 1 to 5%, respectively. From the nested Analysis of Variance, the within and between-batch and the total variability for all the QC samples including ULOQ and LLOQ were all below 11%. Table 2.4 and Table 2.5 summarises the within and between assay imprecision and inaccuracy achieved during the validation study, whilst Table 2.6 summarises the within and between-batch, and the total variability obtained from the nested analysis of variance. All the results obtained were below than the limit accepted for validation.

Table 2.4 Within assay imprecision and inaccuracy in plasma

Batch No	Nominal concentrations	Mean n=6	SD	CV	Mean inaccuracy
	(ng/mL)	(ng/mL)		(%)	(%)
1	0.50 (S/N* ratio > 5)	0.54	0.03	6.44	7
	1.47	1.34	0.09	6.77	-10
	44.98	45.58	0.95	2.08	1
	149.8	151.25	4.04	2.67	1
	250.00	250.01	6.91	2.76	0
2	0.50 (S/N ratio > 5)	0.46	0.06	13.33	-10
	1.47	1.52	0.09	5.69	3
	44.98	47.48	1.71	3.60	5
	149.8	156.01	8.17	5.24	4
	250.00	267.10	9.73	3.64	6
3	0.50 (S/N ratio > 5)	0.52	0.03	5.28	4
	1.47	1.46	0.09	5.88	0
	44.98	44.09	1.09	2.47	-2
	149.8	156.37	2.83	1.81	4
	250.00	272.00	4.49	1.65	8

* S/N : Signal to noise ratio

Table 2.5 Between assay imprecision and inaccuracy in plasma

Nominal concentration	Mean (n=18)	SD	CV	Mean inaccuracy
(ng/mL)	(ng/mL)		(%)	(%)
0.50	0.50	0.04	8.34	1
1.47	1.44	0.09	6.40	-2
44.98	45.72	1.70	3.72	2
149.8	154.54	2.86	1.85	3
250.00	263.04	11.55	4.39	5

Table 2.6 Within and between batches variability from the nested analysis of variance (ANOVA).

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal concentration (ng/mL)	0.5	1.47	44.98	149.8	250
Mean (ng/mL); n = 18	0.50	1.43	45.72	154.54	263.04
SDw	0.03	0.09	1.34	5.34	6.99
SDb	0.04	0.08	1.55	2.38	11.18
SDt	0.06	0.12	2.05	5.88	13.18
CVw (%)	6.6	6.1	2.9	3.5	2.7
CVb (%)	8.7	5.9	3.4	1.5	4.2
CVt (%)	10.9	8.5	4.5	3.8	5.0

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

2.3.7.5 Recovery

The absolute recovery of lidocaine and bupivacaine ranged from 84 to 89% and 82 to 86%, respectively. All the values are summarised in Table 2.7.

2.3.7.6 Stability

Table 2.8 lists the stability data for lidocaine in plasma after three freeze and thaw cycles, after 48 hours at room temperature, and after 48 hours at 4°C. The stability of the sample extract (in autosampler) was summarized in Table 2.9. Figure 2.5 shows the graph of log concentration (ng/mL) versus time (h) for autosampler stability test. Stability data shows that lidocaine was stable for all of the parameters mentioned above.

Table 2.7 Percentage of lidocaine and bupivacaine recovery from plasma

Lidocaine				Bupivacaine			
Nominal concentration	Mean area		Recovery	Nominal concentration	Mean area		Recovery
	Extracted samples	Non extracted samples			Extracted samples	Non extracted samples	
(ng/mL)	Peak area	Peak area	(%)	(ng/mL)	Peak area	Peak area	(%)
1.47	45178	53922	83.8				
44.98	1334951	1599427	83.5	500	4228486	5059871	83.6
149.8	3866634	4367950	88.5				

Table 2.8 Results of stability tests carried out on lidocaine: values expressed in percentage of the concentration difference between, before and after the test.

	Concentration (ng/mL)		
	1.47	44.98	149.80
Time 0 stability data. Mean (n=4)	1.34	45.58	151.25
In plasma, after three freeze-thaw cycles. Mean (n=4)	1.52	44.58	141.70
Difference (%)	13.40	-2.20	-6.31
In plasma after 48 hours room temperature. Mean (n=4)	1.47	40.19	134.29
Difference (%)	10.40	-11.84	-11.21
In plasma after 48 hours at 4°C. Mean (n=4)	1.54	44.94	149.24
Difference (%)	15.46	-1.41	-1.33

Table 2.9 Autosampler stability data

Sample type	Expected concentration	Measured concentration	Elapsed time
	(ng/mL)	(ng/mL)	(h)
QC1	1.47	1.33	0
		1.68	4.3
		1.36	8.7
		1.59	17.0
		1.28	22.1
		1.37	24.5
QC 2	44.98	40.24	0
		41.53	4.3
		41.33	8.7
		39.7	17.0
		40.07	22.2
		40.3	24.6
QC 3	149.8	148.95	0
		138.49	4.3
		138.47	8.7
		139.71	17.0
		142.79	22.1
		142.31	24.5

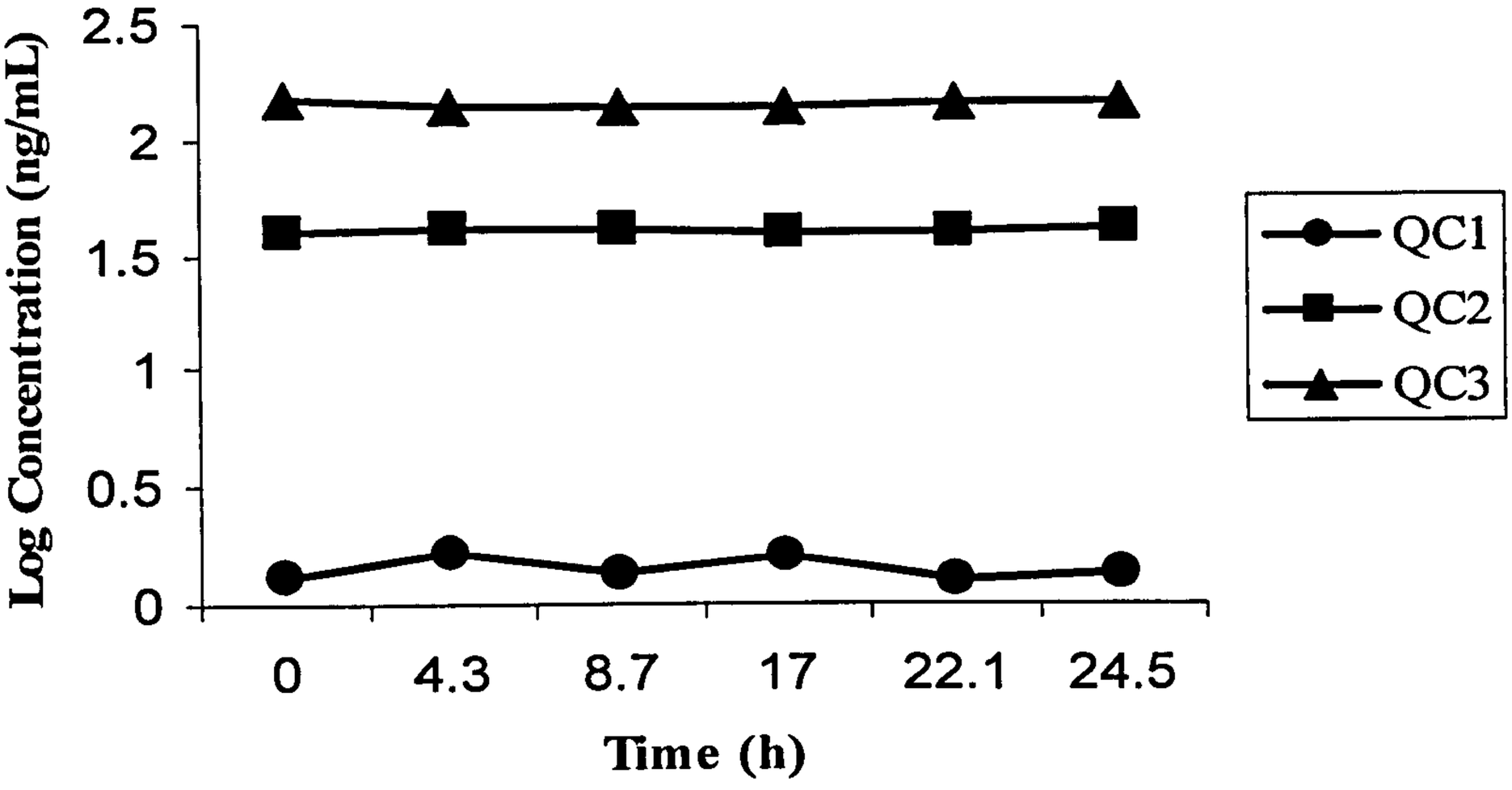


Figure 2.5 Graph of log concentration (ng/mL) versus time (h) for autosampler stability test

2.3.8 Discussion and conclusion

All of the validation results met the international requirements as outlined in the Guideline for Analytical method validation (EMEA, 1995;FDA, 2001b). In this study, a simple liquid-liquid extraction which only needs 0.5mL of plasma has been developed and validated. The small amount of plasma used in the extraction procedure is suitable for analysis of the small volume of blood samples, especially from pediatric patients. Back extraction methods used in this study offered advantages in getting the clean final effluent to be injected into the chromatographic system, therefore reducing impurity peaks. The sensitivity, precision and accuracy achieved by this method enabled then analysis of very low serum lidocaine concentrations obtained from the pilot study of two TDS®-Lidocaine local anaesthetic system, which was conducted in 100 healthy volunteers.

2.4 Study of a combined percutaneous local anaesthetic and the TDS® System for venepuncture.

2.4.1 Introduction

The pain caused by common minor procedures such as venepuncture and minor surgery, is often ignored by clinicians. While it may be a trivial problem in adults, for young people, especially children, the pain is significant and can lead to the development of "needle phobia", an intense fear of needles that triggers immediate anxiety. This also affects some adults. Local anaesthetics are normally given by injection and are used to reduce the pain by inducing a loss of feeling (numbness) of skin and mucous membranes. To avoid the pain and anxiety of venipuncture, topical anaesthetics have been in use to provide needleless induction of local anaesthesia.

Current topical local anaesthetics such as EMLA®, Astra Pharmaceuticals Ltd. (Evers et al., 1985) and AMETOP® gel, Smith & Nephew Healthcare Ltd. (Browne et al., 1999) are effective but require long onset time. The one hour prior application of EMLA® (AstraZeneca, 2005) and the 30 to 45 minutes for AMETOP® (Smith & Nephew Healthcare Ltd., 2005), limit clinical and patient acceptance. Thus topical anaesthetics are excluded from the procedures requiring acute anaesthesia, as well as those outside the institutional environment. The development of a topical delivery system with faster time of anaesthetic onset would be helpful in emergency cases and for increasing the number of surgical day cases seen, especially in paediatrics. Shortening the period of anticipatory anxiety while achieving the maximal desensitising of the skin would be advantageous.

As the TDS® system had shown in the earlier study was able to deliver drug molecules through the skin (Hadley et al., 1998a), we therefore combined this system with lidocaine as local anaesthetic in order to overcome the slow onset time of some of the currently available systems as described above. This was a pilot study to evaluate the ability of our new local anaesthetic system to give the anaesthetic effect and also to deliver the lidocaine across the skin.

2.4.2 Study Approval

The study was approved by the North East London Research Ethics Committee. Reference no. RS/SA/P/02/087, dated 8th.August 2002.

2.4.3 Materials and Methods.

2.4.3.1 Study materials

Study Materials were supplied by Transdermal Technologies Inc. Florida USA.

1) TDS®α Anaesthetic System (alcohol based) containing 4% w/v lidocaine and 2% w/v tetracaine.
2) TDS®β Anaesthetic System (water based) containing 4% w/v lidocaine and 2% w/v tetracaine.
3) TDS®α and TDS®β placebo.

2.4.3.2 Study design

This was a double blinded and placebo controlled study, with a one week washout period, involving 100 healthy volunteers. Based on the previous study on the combination of lidocaine with a nitric oxide generating system (Tucker et al., 2002), 100 subjects recruited in this study would be sufficient to produce an 80% power to detect a difference of 25% in the primary outcome measures at $p < 0.05$.

2.4.3.3 Subject recruitment / screening

Each subject was screened for standard blood biochemistry, drugs of abuse, and answered a health questionnaire including demographic. Skin was assessed for erythema, oedema, itching, broken skin, or other signs of pathology. Body mass, height, body mass index, and blood pressure were recorded. Only subjects age range of 20-40, were included in the study. Subjects were not permitted any form of analgesia within 1 week of the trial.

2.4.3.4 Admission and procedures

2.4.3.4.1 Study Protocol

Subjects were admitted to the Study Unit having fulfilled all the inclusion criteria assessed during screening. Blood pressure and heart rate were measured after the subjects had rested for 10 minutes. A blood sample was taken from an antecubital vein to establish a baseline measurement of plasma lidocaine concentration. TDS®β (active) was applied to the dorsal surface of a right hand and the TDS® (placebo) was applied on the left hand. The treatments were blinded from the volunteers. Administrations of the

formulations were achieved by metered pump spray of 1mL to the area of $4cm^2$. Five minutes after application the hands were routinely cleaned using Alcowipes prior to venepuncture.

A vein on each hand within the treatment area was then cannulated using a 20G butterfly needle. The success of cannulation was confirmed by the ability to withdraw 1-2mL of blood. Two methods of pain assessment, a Likert Verbal Rating Score (VRS) and a Visual Analogue Scale (VAS) were used to assess the pain of the procedure. Both systems have been fully validated in the literature (McCafferty et al., 1989;Woolfson et al., 1990). Following successful bilateral cannulation, a VRS and VAS pain classifications were used with respect to each hand.

Two hours after the treatment application, another blood sample was taken to assess the systemic concentration of lidocaine. The plasma was transferred to cryo-vials and stored at -20°C until analysis. All the procedures, including treatment applications and bilateral cannulation were each performed blindly by separate investigators. One week later, the volunteers repeated the above procedure using the second formulation (TDS®β). For TDS®β application, the order of the treatment was changed to the active treatment on the left hand and the placebo on the right hand.

2.4.3.4.2 Verbal Rating Score (VRS)

The volunteers were asked the following question: "How strong was the pain of the procedure?" and provided with a choice of five categories: 1, no pain; 2, minimal sensation; 3, mild pain; 4, moderate pain; 5, severe pain. The volunteer selected one answer for each hand by circling the number.

2.4.3.4.3 Visual Analogue Scale (VAS)

In the VAS assessment, a 100mm horizontal line with endpoints that are anchored by descriptors 'no pain' and 'severe pain' was used. For each hand, the volunteer was asked "What did the procedure feel like?" and then requested to make a vertical line on the horizontal line which represented the intensity or unpleasantness of their pain experienced during the procedure. Values were measured in millimeters from the left hand edge of the horizontal line that is 0mm represented no pain and 100mm represented severe pain.

2.4.3.5 Analytical Method

Plasma concentration of lidocaine was analysed by using the liquid chromatography-mass spectrometry (LC – MS - MS) method as described previously.

2.4.3.6 Statistical analysis

All of the data was analysed using Microsoft Excel worksheet, 2003 and Minitab 14 statistical software (www.minitab.com/). The VRS and VAS score of active treatments were compared to the placebo control using Wilcoxon's Signed Rank test. The lidocaine concentrations at 2 hour post dose for TDS®α and TDS®β were also compared by using Wilcoxon's Signed Rank test.

2.4.4 Results

One hundred healthy volunteers (41 males and 59 females) were successfully recruited, and the demographic data is presented in Table 2.10. Of the 100 subjects, 65 were Caucasian, 22 Asian, 4 African/Caribbean, and 9 from other ethnic groups. The cannulation procedures were successfully completed at the first attempt for all 100 volunteers. All of the subjects tolerated the procedure well and complied with the study protocol.

In the VRS pain classification, the active treatment of TDS®α was not significantly different to that of the placebo (estimated median = 0.0; p= 0.64 (NS);Figure 2.6). However the active treatment of TDS®β resulted in a significant reduction in pain response to cannulation compared to the placebo treatment (estimated median = -0.5; p = 0.018;Figure 2.7).

Similarly, the VAS also showed no significant different between placebo and active for TDS®α (estimated median = -0.5; p = 0.827 (NS);Figure 2.8) but significantly different for TDS®β (estimated median = -6.0; p = 0.016;Figure 2.8). The distribution of scores for the active treatment group in Phase II (TDS®β) was generally shifted from the two highest scores (moderate and severe) to the lower level of score (mild), with no subjects on the active treatment rating the pain as severe and reducing the numbers of those who scored the pain as moderate by 36.4% (Figure 2.7).

However, the analysis of plasma samples for TDS®α and TDS®β at 2 hour post dose were found no significant difference between TDS®α and TDS®β (Wilcoxon's Sign

Rank test; p = 0.574). The median plasma level for TDS®α was slightly higher than TDS®β with the median [IQR] were 0.930 [1.930] ng/mL and 0.560 [2.185] ng/mL, respectively.

Table 2.10 Demographic data of study volunteers

Parameters	Mean (SD)	Median [range]
Sex;M:F	41:59	
Age;years	26.45 (5.2)	25.0 [20-40]
Body mass index(BMI)	23.3 (3.9)	22.6[15.2-37.5]

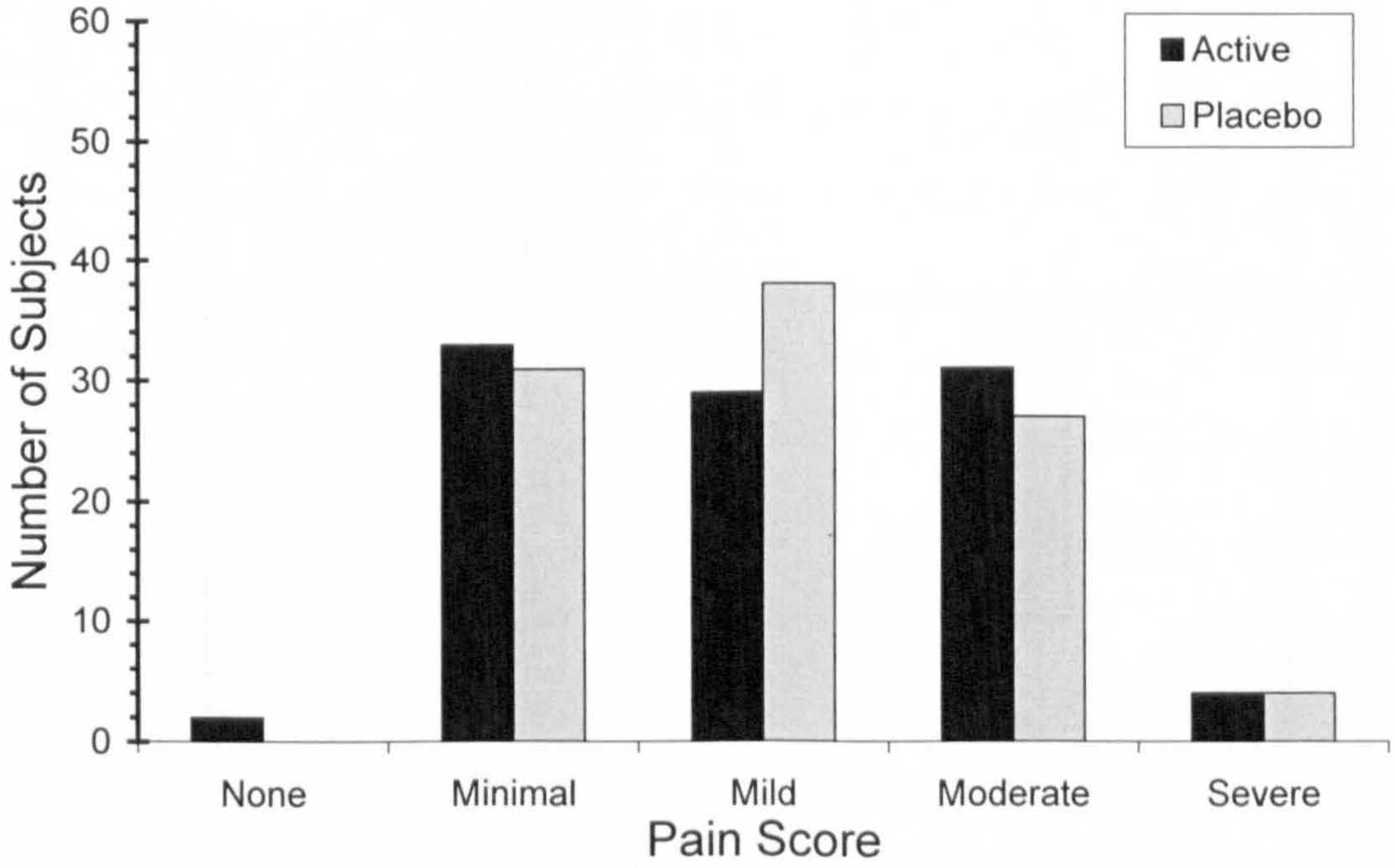


Figure 2.6 Verbal Rating Score (VRS) for TDSα. Values are subjects percentage vs. categories; n = 100; p=NS, Wilcoxon's Signed Rank test

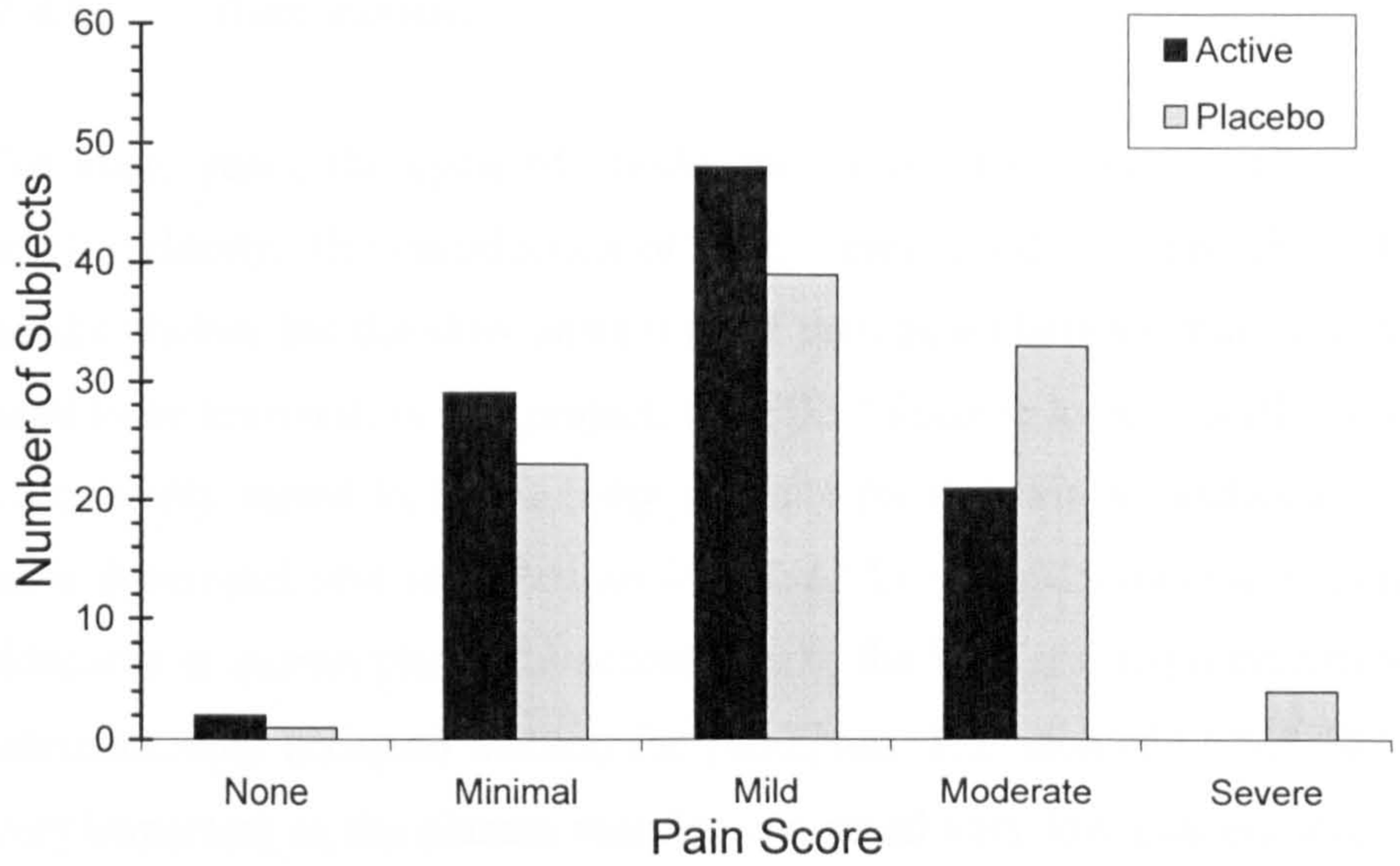


Figure 2.7 Verbal Rating Score (VRS) for TDSβ. Values are subjects percentage vs. categories; n = 100; P=0.018, Wilcoxon's Signed Rank test.

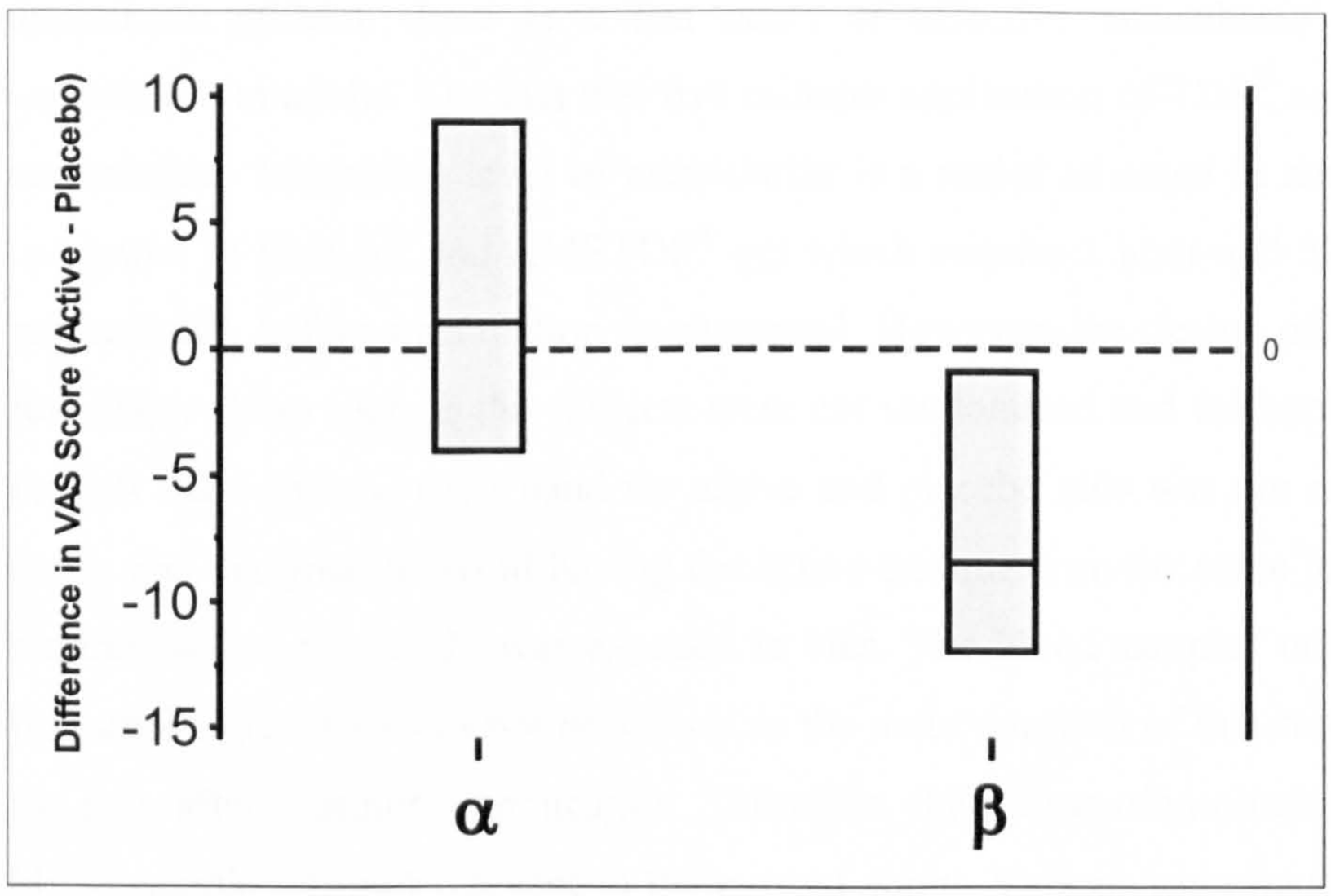


Figure 2.8 The median differences in VAS score of active and placebo (α = TDSα and β = TDSβ). Values are median ± 95% CI; n=100; p= NS for TDSα and p< 0.016 for TDSβ, Wilcoxon's Signed Rank test.

2.4.5 Discussion

For many years, the cycle of needle phobia affected many people especially children and the elderly. The introduction of EMLA cream and Ametop gel has finally broke the needle phobia, but the slow onset time of both anaesthetics remained a critical point that need to be resolved. In this project, the TDS lidocaine local anaesthetic system has been successfully tested in 100 healthy subjects for the skin anaesthesia. In this study, we have developed and validated an improved LC-MS-MS method validation to analyse lidocaine in human plasma in accordance to the UK GLP requirements which meets the internationally accepted method for validation. The used of LCMS-MS in this study is very important as the plasma samples contained very low concentration of lidocaine as many other analytical methods are not capable in performing this task. This method has been successfully applied in the analysis of plasma samples from TDS®-Lidocaine study.

The result from this study suggests that immediately following application of a TDS® anaesthetic system, there is a fast onset of effective anaesthesia for the venous cannulation in adults. The fact that five minutes application of TDS® anaesthetic system can produce acceptable level of anaesthesia is a major advance in anaesthetic system compared to EMLA® and AMETOP® gel which require 1 hour and 30 to 45 minutes, respectively, before cannulation is attempted. However, the design of this study has a few major flaws such as the subjects were not randomised and the application between the left hand and the right hand for active and placebo also was not randomised. This study was designed to avoid having the active treatment on the same hand, but without randomisation, this study was exposed to bias. The blood samples taken at two hours post application also was not necessary as the main question of this study was to assess the pain after 5 minutes application. Therefore, the information obtained from 2 hours blood sample was not relevant to the current study. Various sampling points for blood was necessary to investigate the amount of lidocaine absorb from both formulations. The used of 5 minutes application time was also arguable. This was an exploratory study to compare the two TDS® systems (water and alcohol base). A future study should be designed to assess various application times and using the pin prick test to assess the pain.

Between the two TDS® systems tested, the water based anaesthetic system (TDS®β) was found to be effectives in providing dermal anaesthesia, whereas the alcohol based

(TDS®α) was ineffectives and no different from placebo. However, from the plasma lidocaine analysis, the median concentration of TDS®α was slightly higher than TDS®β at 2 hours post dose suggesting that TDS®α may be able to deliver lidocaine through the skin but needs longer onset time for the anaesthesia. Therefore TDS® anaesthetic system can be manipulated to adjust the onset and degree of topical anaesthesia, and will be used as a basis for investigations into application periods and increased levels of anaesthesia. Other TDS® anaesthetic systems, such as using alternative combinations of local anaesthetic agents rather than the lidocaine/tetracaine used in this study are currently under investigation. The development of a rapid onset topical local anaesthetic would enable the replacement of invasive method of local anaesthesia, consequently increases the effectiveness of clinic or day surgery centre in the management of pain associated with local anaesthetic.

2.4.6 Conclusions

The result from TDS®-Lidocaine study, which has shown that the water based (TDS®β) local anaesthetic system has been able to give skin anaesthesia after 5 minutes application time, was a major achievement in the development of local anaesthetic system. This result has a significant contribution on the future development of more effective local anaesthetic system compared to currently available treatments. The bigger and proper phase 1 pharmacokinetic study in healthy subjects is needed in the development of this system.

Chapter 3 Testosterone

3.1 Introduction

Testosterone deficiency in men or hypogonadism is a syndrome that can be defined by low level of testosterone (i.e. serum total testosterone < 10nmol/L or < ~2.88ng/mL). This condition may result from a primary defect of the testes, or from a disorder of the hypothalamus or anterior pituitary resulting in inadequate gonadotrophic stimulation of the testes (Arver et al., 1997). Klinefelter syndrome also can caused hypogonadism (Nieschlag et al., 2004). Primary hypogonadism, always associated with raised level of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) due to diminish testosterone productions and secondary hypogonadism with low testosterone and low or normal LH and/or FSH (Nieschlag et al., 2004). There are also a number of conditions that can cause decreasing amount of testosterone in men, such as diabetes mellitus, atheroschlerosis, myocardial infarction, chronic heart failure, chronic renal failure, AIDS, alcohol abuse, etc. (Kaufman and Vermeulen, 1997). At this stage, testosterone replacement therapy may be indicated.

There are a few treatments currently available to increase the amount of testosterone in the systemic circulation such as oral and sublingual preparations (Johnsen et al., 1974;Stuenkel et al., 1991), transdermal patches (Dobs et al., 1999;Korenman et al., 1987), subcutaneous implants (Handelsman et al., 1990), testosterone esters, enanthate and cypionate (Matsumoto, 1994) and also the gel applications (Jockenhovel, 2003;Swerdloff and Wang, 2003). Free, unesterified testosterone was absorbed well, but undergone hepatic catabolism in the liver rapidly following oral ingestion (Gluud et al., 1983;Snyder, 2001). Behre (Behre H.M., 1998) reported that oral administration of testosterone gives rise to wide fluctuations with high within and between individual variability in serum testosterone concentrations. It has been reported that the methyl - testosterone can cause hepatic toxicity and adversely affects cholesterol concentrations following long-term usage (Bird and Vowles, 1977;Lowdell and Murray-Lyon, 1985;Westaby et al., 1977).

To avoid first-pass metabolism, testosterone rectal preparations was developed. The systemic absorption was high, but lasting for only 4 hours. Therefore needs to administer frequently to maintain the level. This is not ideal for testosterone replacemement therapy (Nieschlag et al., 1976). Intra nasal application also has been

studied, but the same result as rectal has been observed (Danner and Frick, 1980). Sublingual preparations of testosterone also resulted in rapid increases in serum concentrations, unfortunately decline to below the normal range after 2 hours (Stuenkel et al., 1991). Since free testosterone undergone rapid metabolism, testosterone esters have been evaluated for intramuscular injection, such as testosterone enanthate, propionate. However, the testosterone level declines to very low level before the next injection. This meant that the substance needs frequent injections, and do not suitable for long term therapy (Behre H.M., 1998). Another testosterone ester cypionate , and undecanoate, however can maintain the testosterone level for up to two weeks, and 12 weeks within injection interval, respectively (Nankin, 1987;Nieschlag et al., 1999).

Subdermal testosterone implants also have been evaluated for their efficacy (Handelsman et al., 1990;Kelleher et al., 2004). Implants are using stainless steel and administered under sterile conditions under routine minor surgery. The procedure is usually completed within 10-15 minutes (Handelsman et al., 1997). In a pharmacokinetic study on three different regiments of pellets (6 × 100mg, 6 × 200mg, 3 × 200mg), 43 androgen-deficient men were implanted for the period of 6 months. Testosterone levels peaked at the first month and were maintained at physiological levels for 4 to 5 (600mg doses) or 6 (1200mg dose) months after a single implantation (Handelsman et al., 1990). In another study comparison study between, intramuscular injection of testosterone ester, oral testosterone undecanoate, and subcutaneous pellet, the results showed that the testosterone was elevated for up to 4 months. The patients treated with intramuscular injection and oral only showed the testosterone peaked at the first treatment week and fell down with some fluctuations and most variables plasma level in orally treated group (Conway et al., 1988). Since the procedure to apply and removal of the implant is inconvenient, some patients may not prefer this method for testosterone replacement therapy.

Transdermal delivery of testosterone has been found to be an effective and more convenient for testosterone replacement therapy. The advantages of transdermal delivery are the avoidance of hepatic first pass effect and the testosterone level obtained from the treatments closely mimic the circadian rhythm of testosterone secretion in healthy males. The first ever transdermal application is the scrotal patches which can be applied on the scrotal skin. This system is available as Testoderm®, and consists of a film containing 10 or 15mg testosterone (Zitzmann and Nieschlag, 2000). The result from the study conducted on healthy and hypogonadal males subject found testosterone

levels maintained in the normal range during a 12-week treatment period. No side-effects or adverse events have been observed. This type of application offers a new approach to androgen substitution therapy (Bals-Pratsch et al., 1986). In a long term study of this system, scrotal patches were been applied everyday for 14 months in seven hypogonadal men. The result showed that all patients were clinically well substituted and responded with good compliance (Bals-Pratsch et al., 1988).

Another form of transdermal patch, Androderm® which can be applied on the non-scrotal skin has been developed recently. To optimise testosterone delivery and hormone normalizations, the preferred sites for applying the system are the back, thigh, upper arm, and abdomen (Meikle et al., 1996). The long term study which applies the treatment for up to 12 months had shown that the morning serum testosterone levels has been normalized in 93% of the patients treated. Majority of the patients treated also showed improvement in the hypogonadism symptoms such as decreased libido and fatigued, after 2-4 weeks treatment (Arver et al., 1997).However the further studies on Androderm® had found that some patients experienced local skin irritation or local contact dermatitis (Arver et al., 1997;Rolf et al., 1999). Occasional mild redness or itching of the skin is also common with transdermal patches. Although scrotal patches causes less skin irritation than conventional transdermal preparations, usage of the forma leads to an increase in dihydro-testosterone (DHT) concentrations after 3 months of treatment (Behre et al., 1999).

Testosterone in a hydroalcoholic gel, AndroGel® (Unimed Pharmaceutical Inc.) has been evaluated for the replacement therapy. The delivery of testosterone is generally good with increases in testosterone level up to 5 fold after application of 100 mg testosterone gel. Serum testosterone level reached steady state and remained at the same level for the duration of gel application and returned to baseline after stopping application (Wang et al., 2000a). Another version of testosterone gel, TestimTM (Auxilium Pharmaceuticals, Inc.) was recently approved for used in the USA (Tenover, 2003). The pharmacokinetic study to compare the bioavailability of Testim and AndroGel® has been conducted in 29 hypogonadal men. The results showed that TestimTM was not bioequivalent to AndroGel® where TestimTM providing higher serum levels and greater bioavailability than AndroGel® (Marbury et al., 2003).

Although testosterone gels are effective for replacement therapy, care must be taken to prevent the transfer of testosterone to another person. Patients must wash their hands

after applying the gel to a substantial surface area of the body and they must cover the application site with clothing once the gel has dried (Unimed Pharmaceutical Products, 2002). Furthermore, AndroGel® required large surface area of application as the study conducted by Wang et al. (Wang et al., 2000a) showed that the application of the same gel amount at 4 sites of the body contained approximately 4 times higher testosterone than 1 site. Rolf et al. (Rolf et al., 2002) recently showed that newly developed hydroalcoholic gel was effective for replacement therapy. The advantage is the gel can be washed after 10 minutes application to avoid transfer to another person.

The delivery of testosterone through buccal mucosa has also been evaluated recently. Baisley et al. (Baisley et al., 2002) has evaluated the buccal adhesive testosterone tablet (BATT) which is under development by Abbott Laboratories, USA. BATT was applied to the gingival, in the region of infranasal fossa. The results have showed that the three dosages of BATT were effective in maintaining the testosterone level within the normal range in healthy males at least 24 hours after application. The steady state of testosterone was reached at day 5 from the ten application days.

None of the currently available testosterone replacement therapy has achieved the goal due to some disadvantages as discussed above. Ideal testosterone replacement therapy should in theory, produce and maintain physiologic serum concentrations of the hormone and its active metabolites without significant side effects or safety concerns (Hellstrom, 2004). An *in vivo* pilot study has been conducted earlier, to investigate the effectiveness of a novel TDS® - Testosterone to deliver testosterone systemically in rodents (Alam and Willoughby, 1999). The application of TDS®- Testosterone to the dorsal surface of rodents facilitates transport of quantifiable levels of testosterone in the systemic circulation. An increase 3 to 6 fold in the level of serum testosterone was seen in all animals treated with TDS®- Testosterone. In this study, TDS®- Testosterone has been evaluated in healthy males through two parts of the study.

A preliminary study to look at the ability of TDS®-Testosterone to deliver a therapeutic amount of testosterone systemically has been conducted in five healthy males receiving five different dosages. The suitable dosage concluded in this study was used in the pharmacokinetic study of the TDS®-Testosterone conducted in twelve healthy males in comparison with a TDS®- Placebo and AndroGel®.

3.2 Objectives

The main objective of preliminary pharmacokinetic study in 5 healthy males was to assess the ability of TDS® system to deliver a therapeutically acceptable serum testosterone concentration and to identify a therapeutically acceptable dose of testosterone.

The main objective of pharmacokinetic and bioequivalence study in 12 healthy males was to measure the pharmacokinetic profiles and bioavailability of a new TDS®-Testosterone compared to Androgel® 1% and TDS®-placebo in healthy males.

The second objective of the above two studies was to assess a safety and tolerability of TDS®-Testosterone in healthy males subject.

3.3 An open label, single dose study of 5 different doses of TDS®- Testosterone (Preliminary study)

3.3.1 Study Approval

The study was approved by the East London and The City Health Authority Research Ethics Committee. Reference no. RS/SB/P03180, dated 9th. October 2003.

3.3.2 Materials and methods

3.3.2.1 Study treatment

The study treatment was a topical application of the TDS®-Testosterone system (batch number MBR-BFLIQ84) containing 10, 20, 30, 40, or 50mg of testosterone. The treatment application was via a metered-pump dispenser, each spray delivering 0.2mL containing 10mg of testosterone.

3.3.2.2 Study Design

This study was an open label and single dose of five different doses of TDS®-Testosterone involving five healthy males.

3.3.2.3 Subject recruitment

Five healthy male volunteers were recruited in response to an advertisement. Written informed consent was obtained from all volunteers prior to participation. A medical history was taken, including past illness, allergies, tobacco and alcohol consumption, and current use of other medically active substances. Blood samples were collected for clinical biochemistry, haematology and hormone levels such as testosterone, LH, and FSH. In addition, routine urinalysis and a screen for drugs of abuse were performed. Entry to the study was gained following review of pathology reports, medical history and the hormone level within the normal range.

3.3.2.4 Admission and procedures

The subjects were admitted to the Clinical Microvascular Unit, St Bartholomew's Hospital on the morning of each study day. Blood pressure and heart rate were measured after subjects had rested for 10 minutes. A 20G cannula was placed in a large antecubital vein for blood collection. Subjects were randomised (Table 3.1) to one of

five TDS®-Testosterone doses (10, 20, 30, 40 or 50mg), and the dose applied to the left arm via metered dose spray and gently rubbed onto the skin. Regular meals and beverages were provided throughout the study day. After dosing subjects were permitted to engage in normal daily activities, but excluding significant physical exertion or activities likely to stimulate endogenous testosterone production.

Table 3.1 Randomisation table for the five TDS®-Testosterone doses

Subject no.	TDS®-Testosterone doses (mg)
1	20
2	50
3	30
4	40
5	10

3.3.2.5 Blood samplings

On the study day, intravenous in-dwelling cannulae were maintained for 8 hours following the TDS®-Testosterone application. A 4mL blood sample was collected at 0 hours (prior to dosing) to establish a baseline measurement of serum testosterone concentration. Subsequently, serial 4mL blood samples were collected at 0.5, 1, 2, 3, 4, 6, and 8 hours post dose. The integrity of the cannulae was maintained using a 2mL saline flush after each collection.

After the collection of a blood sample at each time point, the sample was allowed to clot for approximately 20 minutes and then centrifuged at 1800g for 10 minutes. The serum was transferred to labelled tubes (cryo-vials) and stored at 4°C. At the end of the study day the serum samples were transported and stored at –20°C until analysis.

3.3.2.6 Analytical Method

Testosterone concentrations were measured in serum using an Enzyme Link Immunosorbent Assay (ELISA) method. The ELISA came as a kit, complete with all of the reagents to perform the analysis on a 96 well plate. The testosterone Elisa kit was obtained from DRG Instruments GmBH, Germany, lot number 29K064. The kit has already been validated to demonstrate adequate sensitivity, specificity, inaccuracy and imprecision (within and between assays). For the accuracy of measurements and the

GCP requirements, all the samples were analysed together with quality control samples containing three different concentrations. The three level testosterone control standards were obtained from Bio-Rad Laboratories, USA (lot no. 40631, 40632, and 40633 for level 1, 2, and 3, respectively). The plate reader used was a GENios, computer controlled spectrophotometer from TECAN Company, Austria.). The limit of quantification was 0.2ng/mL. All of the standards, samples and controls were analysed in duplicate.

3.3.2.6.1 ELISA Principles

ELISA is an Enzyme immunoassay (EIA) used to measure an antigen or antibody. It is a heterogeneous, solid phase assay that requires the separation of reagents. ELISA is based on the fact that an antigen or antibody can be attached to a solid phase support yet retains immunological activity, and either antigen or antibody can be linked to an enzyme while the complex retains immunological and enzymatic activity. It has two available techniques, the sandwich and the competitive techniques. The ELISA used in this study was a sandwich technique. In this technique, the polystyrene plate comes with an antibody bound in the well. When adding the antigen to be measured in the well, it will form an antigen-antibody immune complex. An enzyme conjugate is then added to the well and binds with the immune complex. The substrate is added to the enzyme conjugate which is bound onto the complex and if any enzyme conjugate complex exists, a colour change will occur. The intensity of the colour will depend on the amount of complex inexistance. Normally the colour will change to blue. The more complexes that exist, the more intense the colour. Stop solution is added to stop the reaction between substrate and enzyme conjugate and the blue colour will turn to yellow. The darker blue colour will change to the darker yellow, and the colour intensity can be observed by using spectrophotometer.

3.3.2.6.2 Experimental procedures

All the reagents and sample specimens were allowed to come to room temperature before starting the assay. The desired number of coated wells on the plate was labelled for blank, standards, controls, and samples. 25µL of testosterone standards, controls and samples were dispensed into the appropriate wells, and 200µL of enzyme-conjugate was added to each well. The contents of the wells were thoroughly mixed for 10 seconds by shaking the plate by hand. The plate was incubated for 60 minutes at room temperature

without covering the plate. After 60 minutes incubation, the contents of the wells were removed and each well was thoroughly rinsed three times with diluted wash solution (≈ 400µL per well). The wells were struck on absorbent paper to remove residual droplets. 200µL of substrate solution was added into each well and then the plate was incubated again for 15 minutes at room temperature. 100µL of stop solution was added to each well to stop the enzymatic reaction. The intensity of the colour change was measured by the determination of absorbance at a wavelength of 450±10nm by spectrophotometer.

3.3.2.6.3 Data analysis

From the raw absorbance data obtained from the plate reader at 450 nm, the four parameters logistic calibration curves absorbance versus concentration (ng/mL) was plotted using Microsoft Excel Worksheet 2003. The four parameters logistic curve was plotted based on the equation 1, below:

$$A = NSB + \frac{(Top - NSB)}{1 + \left(\frac{EC_{50}}{[Testosterone]}\right)^{slope}} \longrightarrow \text{Equation 1}$$

A = Absorption value

NSB = The lower value of the curve – non specific binding

Top = The upper value of the curve – maximal binding

EC_{50} = The midpoint value of the curve

$Slope$ = The slope of the curve

The serum concentration of samples and controls were obtained from the calibration curve and calculated using the above formula. The mean serum concentration of testosterone was plotted against the sampling time for every subject to obtained the concentrations profile of testosterone from 0 to 8 hours. Mean serum concentration of testosterone change from baseline (0 hour) against the sampling time has also been plotted to compare the different profiles of drug concentration between the five doses given.

3.3.3 Results and discussion

Five healthy subjects were successfully recruited in this study. All the serum samples were analaysed for testosterone concentration by using ELISA technique as described above. Figure 3.1 shows the calibration curve plotted for the mean absorbance versus testosterone concentration (ng/mL). The testosterone serum concentration in all the study samples and QC samples were obtained by extrapolating the absorbance value from the calibration curve.

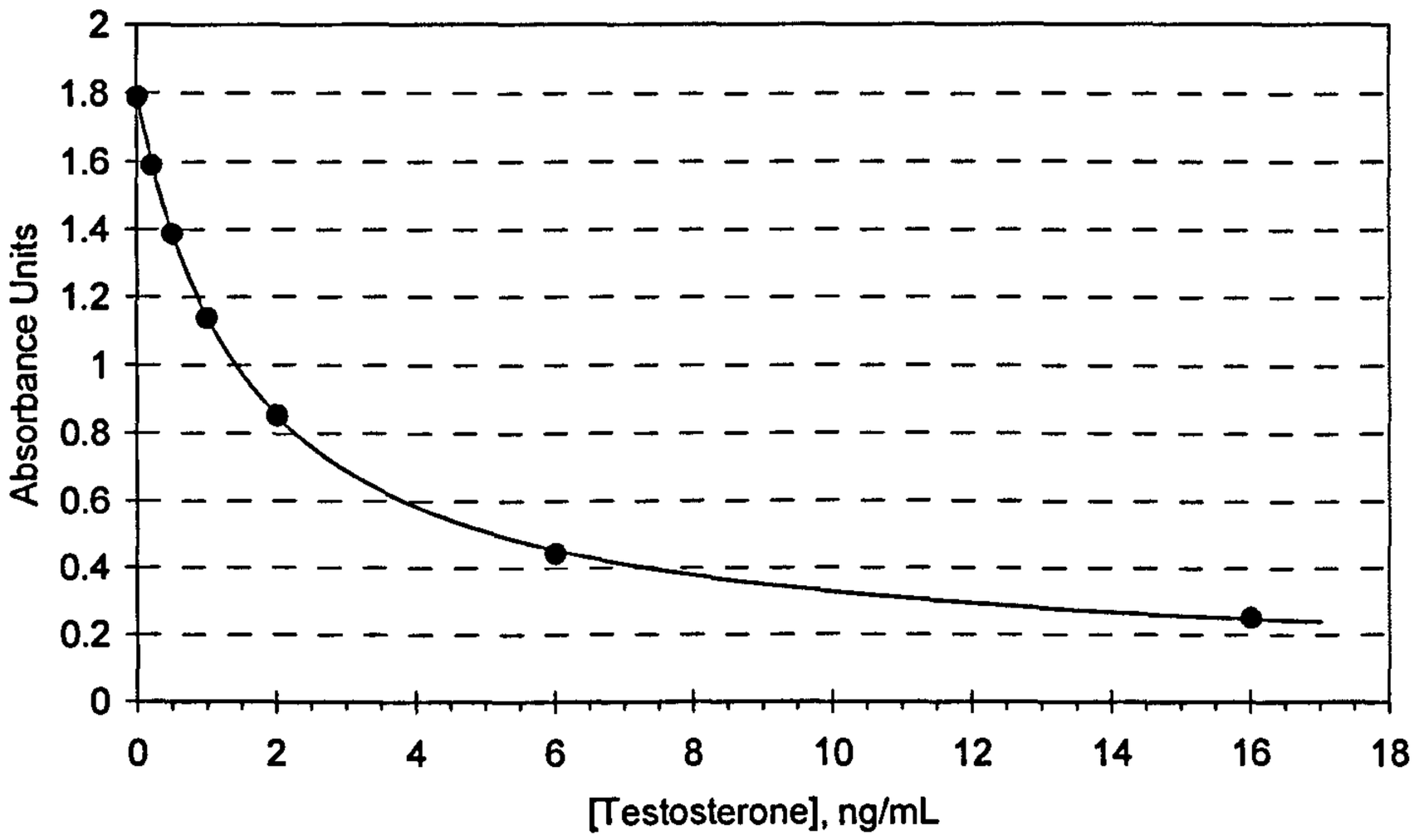


Figure 3.1 Calibration curve for the Absorbance versus concentration (ng/mL)

Table 3.2 summarises the QC samples result obtained during the analysis of testosterone by ELISA. From the concentrations obtained, the mean, standard deviation and the coefficient of variation (CV%) were calculated. The CV for imprecision were all below 15%.

Table 3.2 Coefficient of Variation (CV) for all the Quality Control (QC) samples achieved during the assay

Control no.	QC1	QC2 (ng/mL)	QC3
1	0.79	3.98	9.25
2	0.88	3.88	11.12
3	0.82	4.21	8.22
4	0.92	3.65	9.35
5	0.85	3.77	9.78
6	0.9	3.55	9.21
7	0.98	3.46	8.1
8	0.72	4.1	11.21
9	0.73	3.98	9.36
10	0.94	3.25	7.21
Mean	0.85	3.78	9.28
SD	0.09	0.30	1.26
CV (%)	10.27	8.03	13.52

Figure 3.2 and Figure 3.3, show the plot of mean serum concentrations of testosterone (ng/mL) versus time (h) and mean serum concentration of testosterone changed from baseline (0 hour) from 0 to 8 hours for five subjects and five different dosages, respectively.

The plot of plasma concentration changed from baseline for five different doses showed that 40 and 50mg doses have a reasonable higher absorption profile compared to 10, 20, and 30mg with 50mg is slightly higher than 40mg. The testosterone profiles also show a slight fluctuation in all the subjects within the sampling period, obviously at three hours post dose. All the subjects were well tolerated the dosages well. No serious or unexpected adverse events were reported or observed during the study day.

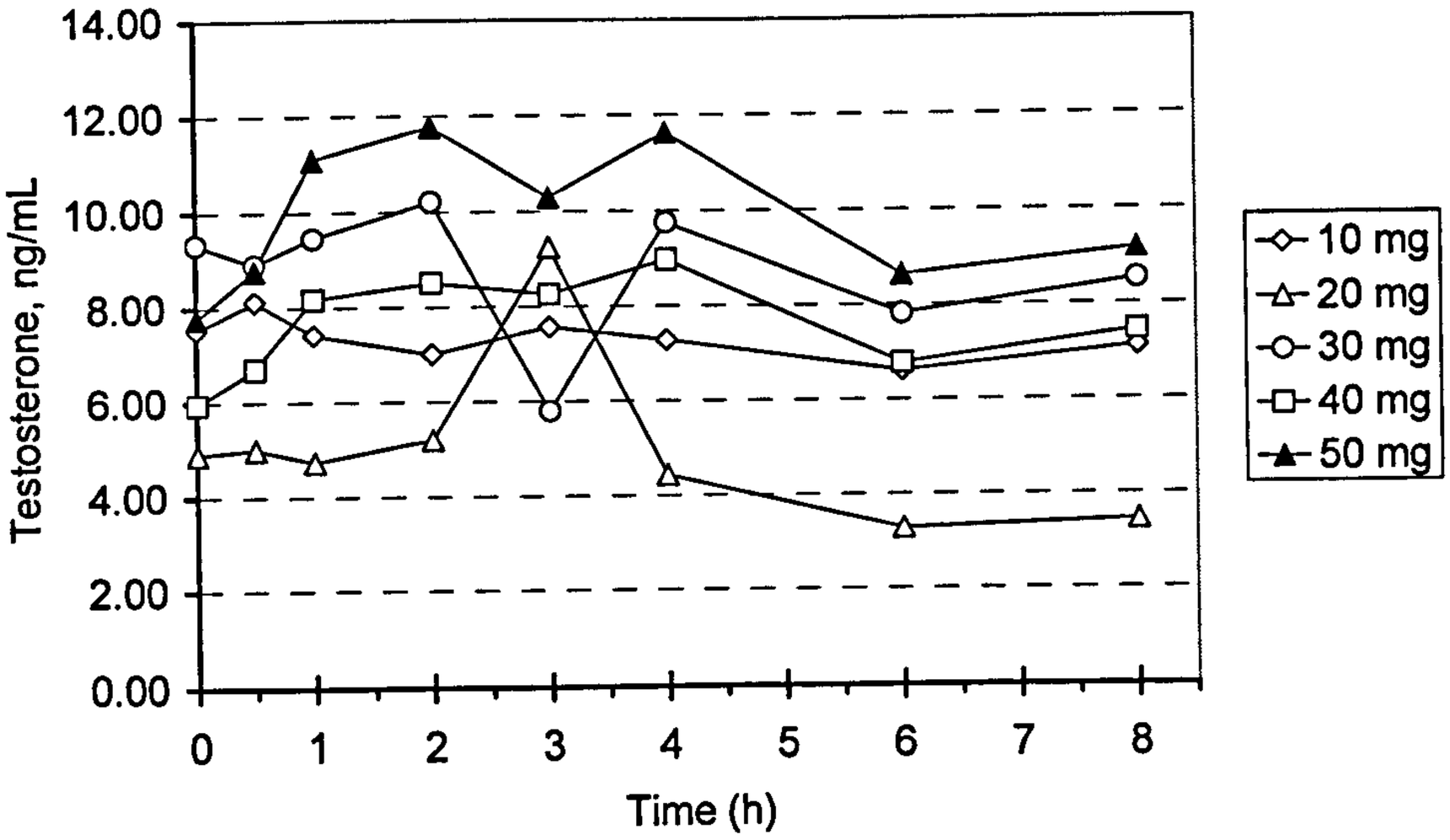


Figure 3.2 Serum concentration versus time for five different doses (10 -50mg)

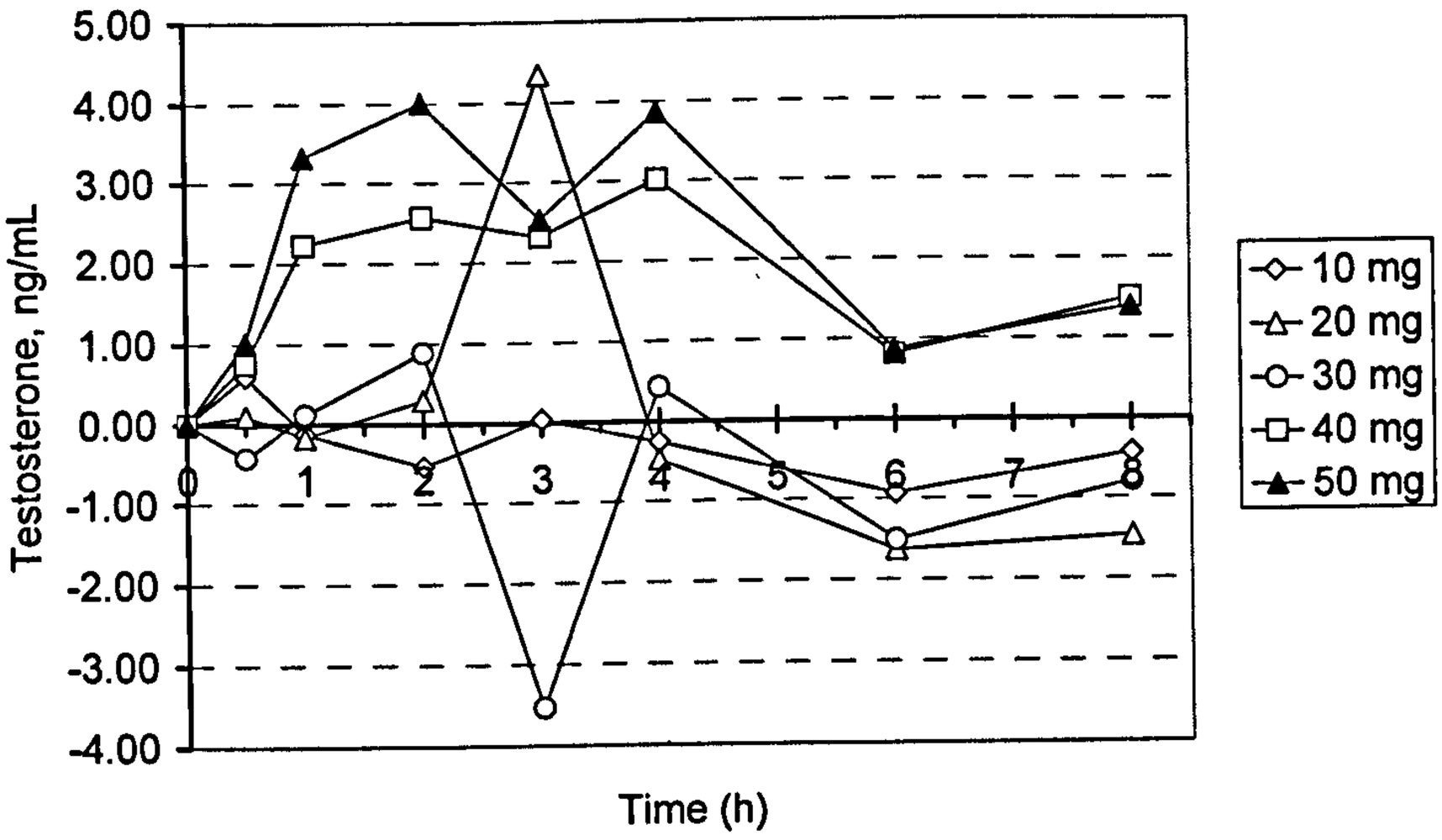


Figure 3.3 Serum concentration (change from baseline) versus time for five different doses (10 - 50mg)

3.3.4 Conclusion

This was a pilot study for dose ranging. The dose that has been chosen for the main pharmacokinetic study of testosterone was the minimum that could be shown different from the lowest dose. The lowest dose was effectively a "placebo" dose. The result from this study showed that TDS®-Testosterone was effective in delivering testosterone systemically through the skin and a 50mg dose was found to be the most effective dose with a maximum of a 4 fold increases in the concentration of testosterone without any adverse event to the subject. Therefore, 50mg dose of TDS®-Testosterone was an

effective dosage that can be used in the three way comparative bioequivalence study of TDS®-Testosterone, AndroGel® and the TDS®-Placebo.

3.4 Pharmacokinetic and bioequivalence study of 50mg TDS®-Testosterone in 12 healthy males

3.4.1 Study approval

The study was approved by the East London and The City Health Authority Research Ethics Committee. Reference no. DAI/SB/P03180, dated 6th. April 2004.

3.4.2 Materials and methods

3.4.2.1 Study Materials

The TDS®-Testosterone system, 50mg (batch number MBR-BFLIQ84), TDS®-Placebo and AndroGel® 1% (50mg) (batch number 20293 LC) were supplied by TransDermal Technologies, Inc., Florida, USA. The TDS®-Testosterone and TDS®-Placebo were supplied as a liquid formulation, delivered by metered pump, with each spray containing 10mg of testosterone. AndroGel® was supplied as a gel in unit-dose aluminium foil packets of 5g each containing 50mg testosterone.

3.4.2.2 Study design

This was a single dose, randomised (Table 3.3), three-way crossover study (with 3 treatments, 3 periods, and 6 sequences) with a minimum of one week washout period between each treatment.

Table 3.3 Randomisation table for the three treatments given in 12 healthy males

Subject	1	2	3	4	5	6	7	8	9	10	11	12
Period 1	A	B	C	B	C	A	A	B	C	B	C	A
Period 2	B	C	A	A	B	C	B	C	A	A	B	C
Period 3	C	A	B	C	A	B	C	A	B	C	A	B

A = TDS®-Testosterone; B = TDS®-Placebo; C = AndroGel®

3.4.2.3 Subject recruitment

Recruitment procedures are the same as mentioned in 3.3.2.3, but involving 12 healthy males.

3.4.2.4 Admission and procedures

On the morning of the study day, blood pressure and heart rate were measured after subjects had rested for 10 minutes. A 20G cannula was placed in a large antecubital vein for blood collection. The drug formulation was then applied to the left arm and gently rubbed into the skin. Regular meals and beverages were provided throughout the study day. After dosing, subjects were permitted to engage in normal daily activities, but excluded from significant physical exertion or activities likely to stimulate endogenous testosterone production.

3.4.2.4.1 Blood sampling

Intravenous in-dwelling cannulae were maintained for 12h following the treatment application. Approximately 4mL of blood was collected at -0.5 (30 minutes before dosing) and 0 hour (immediately prior to dosing), to establish a baseline measurement of serum testosterone concentration. Subsequently, serial blood samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, and 24 hours post-dose. Samples were allowed to clot for approximately 20 minutes at room temperature (26°C) and were then centrifuged at 1800g for 10 minutes. The serum was transferred to labelled tubes and stored at –20°C until analysis.

3.4.2.5 Testosterone analysis

Testosterone concentrations were measured in the serum using an Enzyme Link Immunosorbent Assay (ELISA) method as described previously.

3.4.2.6 Data analysis

Pharmacokinetic parameters and the statistical analysis were performed using Kinetica Version 4.2 software. C_{max} and t_{max} were determined directly from the individual serum concentration-time curves. The AUC was calculated using the linear trapezoidal method. The difference between treatments for AUC_{0-12} and C_{max} were analysed after

logarithmic transformation using the analysis of variance (ANOVA) for cross over studies that accounts for variation due to sequence, subject, formulation, and period.

Bioequivalence testing was based upon the 90% confidence interval (CI) for the ratio of population mean between two treatments. This method is equivalent to the corresponding two one-sided test procedure, with the null hypothesis of bioequivalence at the 5% significance level. Formulations were considered bioequivalent if the 90% CI of the ratio, test to reference, was contained within 80 to 125% (EMEA, 2001;FDA, 2003;Pabst and Jaeger, 1990).

3.4.3 Results

Twelve healthy males successfully completed the protocol. Figure 3.4 and Figure 3.5 show the plots of mean serum concentration of testosterone (ng/mL) versus time (h), and mean serum concentration changes from baseline (0 hour) (ng/mL). Testosterone concentrations fluctuated slightly in most of the subjects during all of the treatments. Most of the subjects treated with AndroGel® and TDS®-Testosterone showed the higher value compared to placebo at 24 hours post dose.

The pharmacokinetic parameters, AUC and C_{max} are summarised in Table 3.4, Table 3.5, and Table 3.6 for TDS®-Testosterone, Androgel® and TDS®-Placebo, respectively. The AUC and C_{max} values were calculated for both 0-12 and 0-24 hour. The mean AUC_{0-12} was higher following application of TDS®-Testosterone compared to AndroGel® and TDS®-Placebo. However, the mean AUC0-24 for AndroGel® was higher than TDS®-Testosterone and TDS®-Placebo. The mean C_{max} (0-12) was similar for TDS®-Testosterone and AndroGel® and these values were higher than that obtained for TDS®-Placebo. Owing to the higher concentrations of testosterone at 24 hours in some subjects, the mean C_{max} (0 – 24) value for AndroGel® was higher than those for TDS®-Testosterone and TDS®-Placebo. Due to no samples taken between 12 to 24 hours and the higher testosterone concentration at 24 hours for most of the subjects in the active treatments, only the 0-12 hour data was used for the determination of bioequivalence. The t_{max} values including the median and the interquartile range (IQR) for all the treatments are summarise in Table 3.7.

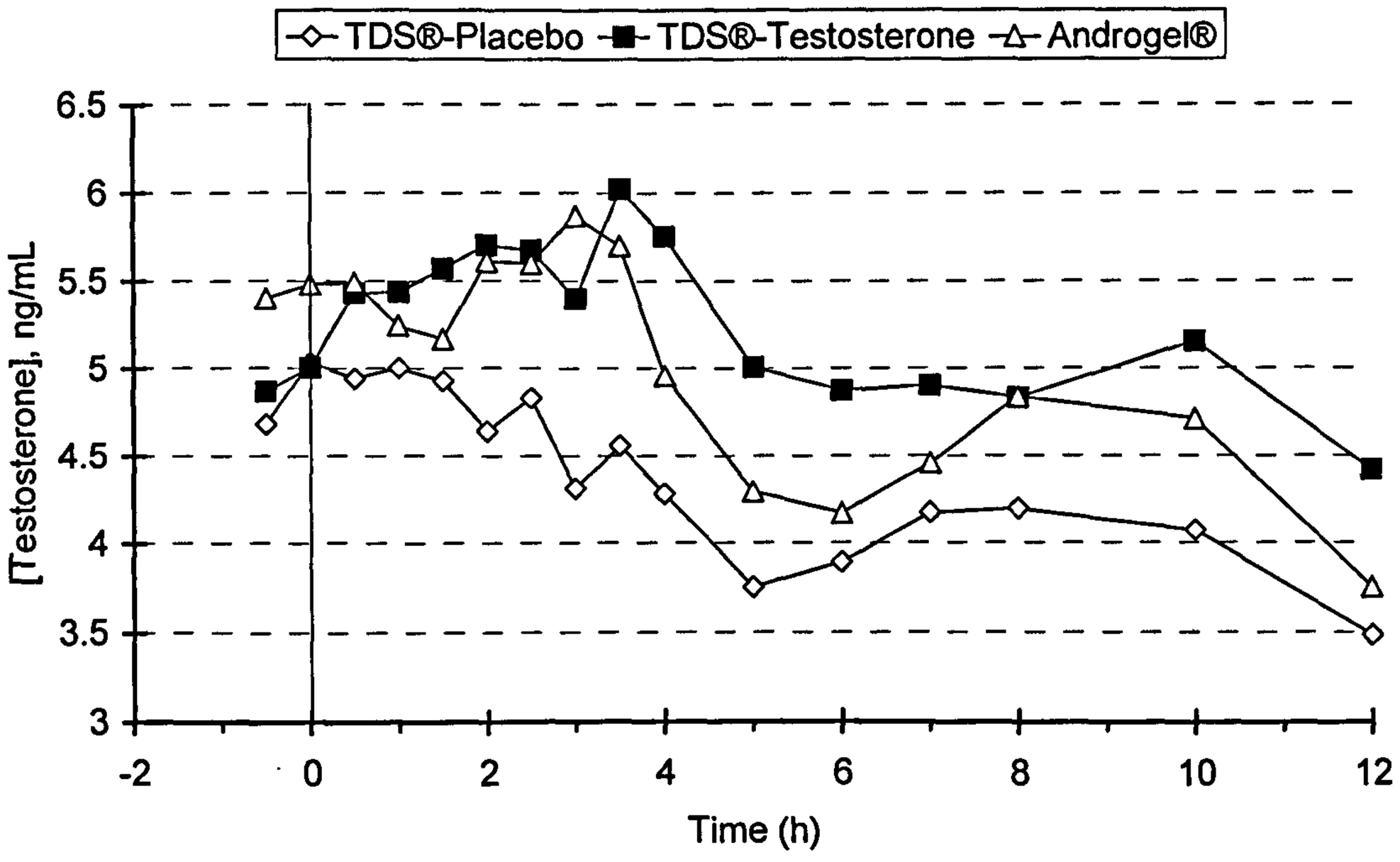


Figure 3.4 Plots of mean serum testosterone concentration (ng/mL) versus time (h) for each treatment from 0-12 hours

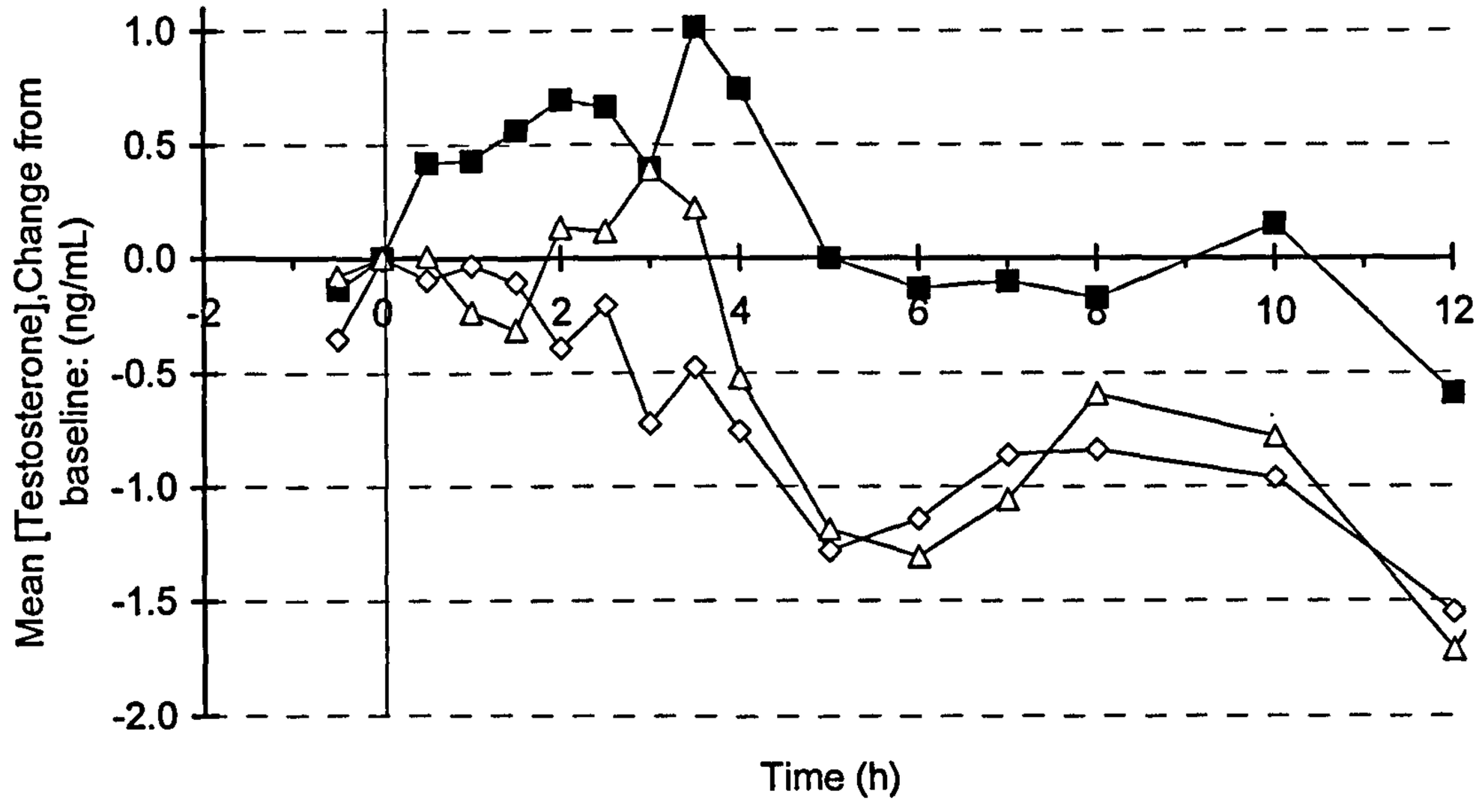


Figure 3.5 Plots of mean serum testosterone concentration change from baseline (ng/mL) versus time (h) for each treatment from 0-12 hours.

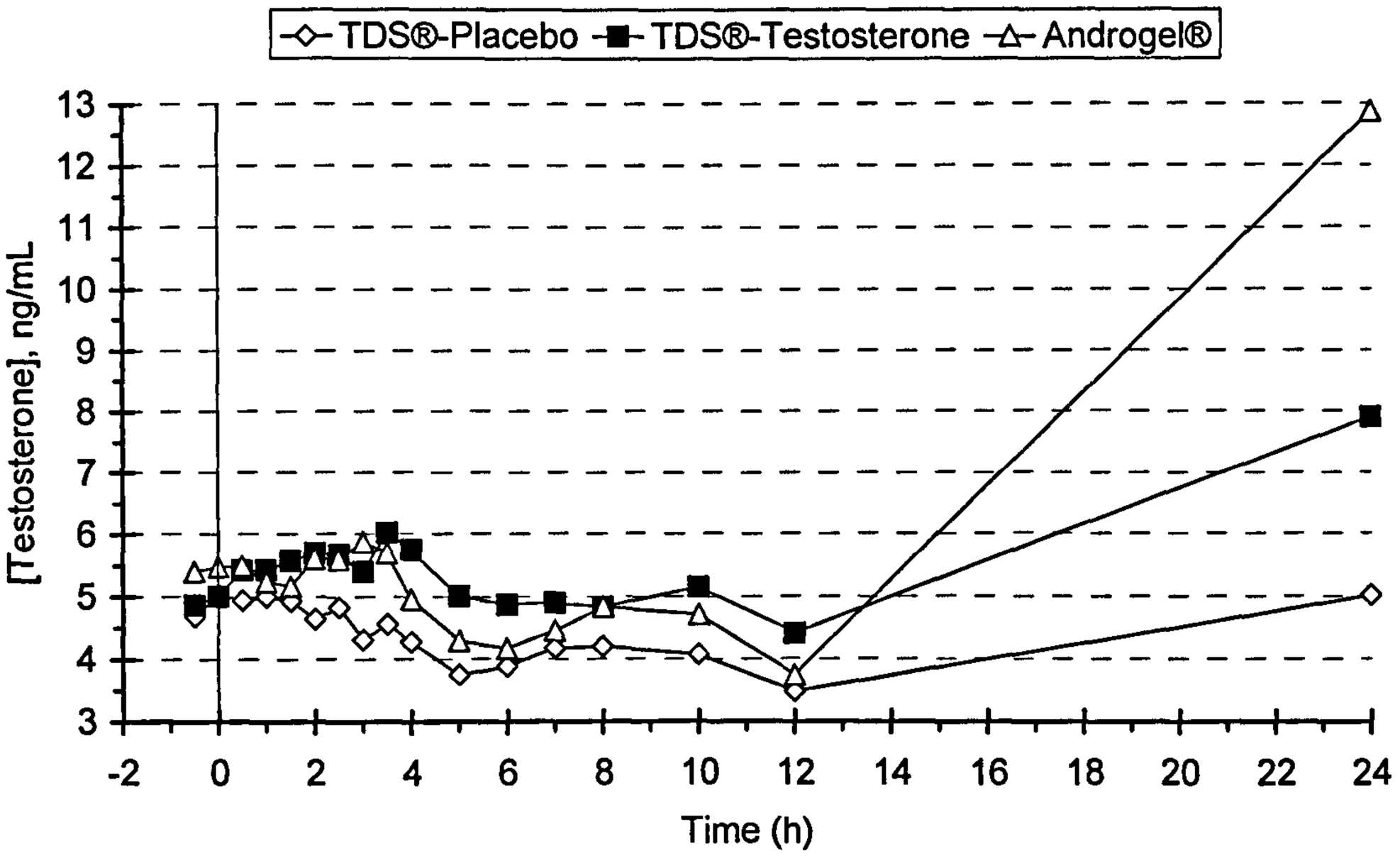


Figure 3.6 Plots of mean serum testosterone concentration (ng/mL) versus time (h) for each treatment from 0-24 hours

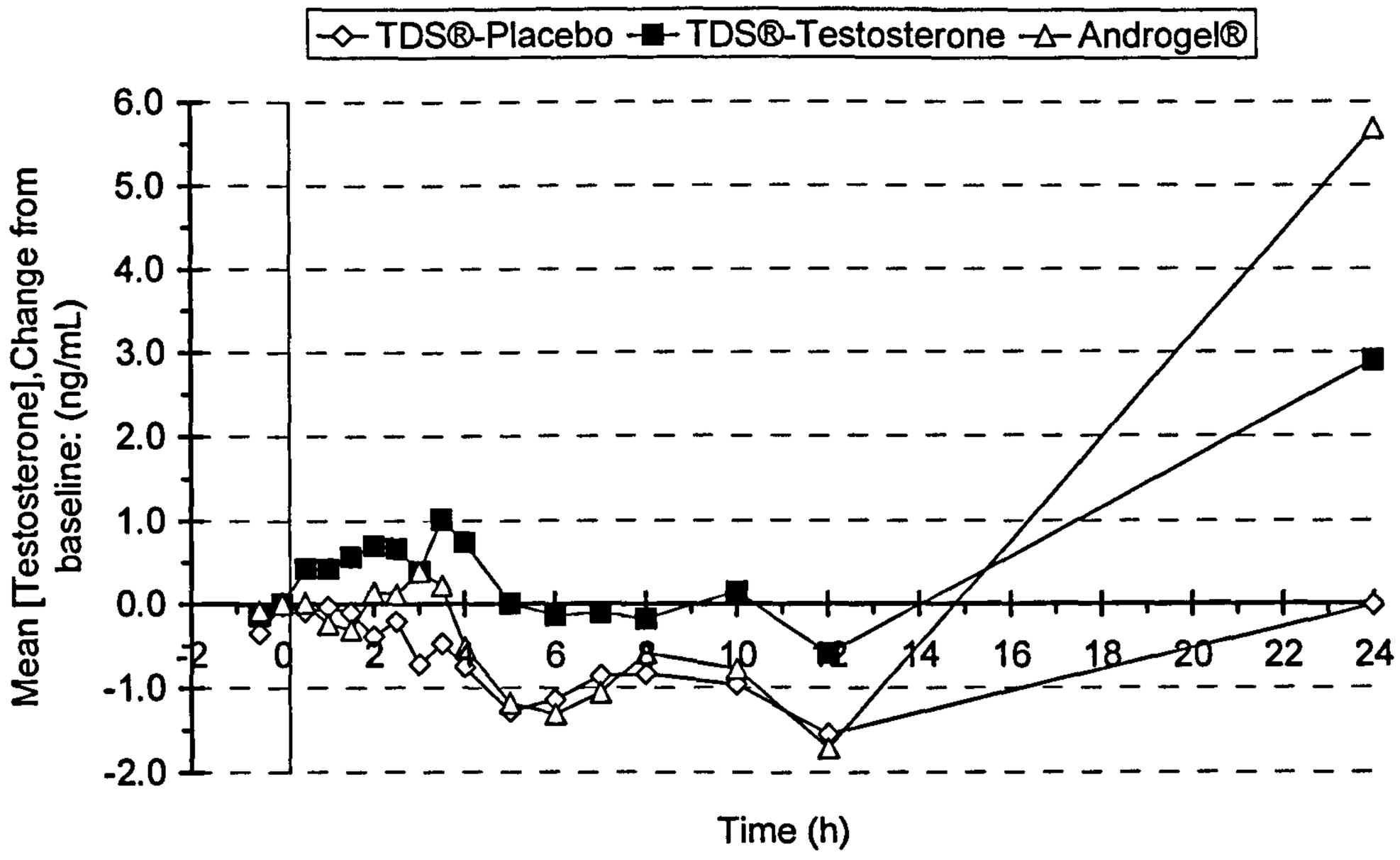


Figure 3.7 Plots of mean serum testosterone concentration change from baseline (ng/mL) versus time (h) for each treatment from 0-24 hours.

Table 3.4 C_{max} and AUC values with mean, SD and CV% for TDS®-Testosterone

Subject	C_{max} (0-12)	AUC_{0-12}	C_{max} (0-24)	AUC_{0-24}
	(ng/mL)	(h.ng/mL)	(ng/mL)	(h.ng/mL)
1	6.45	65.21	8.46	142.43
2	4.02	38.57	4.02	76.25
3	8.79	77.42	8.79	162.09
4	5.60	46.56	5.60	88.68
5	9.23	96.81	19.23	259.71
6	7.59	72.18	7.59	141.66
7	6.28	57.73	7.04	123.85
8	5.16	53.31	5.16	104.19
9	6.91	59.32	6.97	127.06
10	6.65	58.26	6.65	120.84
11	5.59	52.20	13.59	150.96
12	7.45	64.58	7.45	131.48
Mean	6.64	61.85	8.38	135.77
SD	1.49	15.28	4.16	46.30
CV %	22.40	24.70	49.65	34.10

Table 3.5 C_{max} and AUC values with mean, SD and CV% for Androgel®

Subject	C_{max} (0-12)	AUC_{0-12}	C_{max} (0-24)	AUC_{0-24}
	(ng/mL)	(ng/mL.h)	(ng/mL)	(ng/mL.h)
1	5.78	54.06	5.90	110.04
2	5.63	44.30	5.63	87.26
3	8.25	64.39	12.77	166.21
4	6.49	56.41	6.49	114.55
5	8.27	74.66	49.35	399.62
6	9.16	76.89	25.67	261.45
7	5.60	55.30	9.32	130.78
8	4.67	49.90	5.12	100.48
9	4.71	45.85	4.91	94.93
10	8.51	69.83	8.51	143.93
11	5.81	51.66	18.82	181.68
12	5.55	48.76	5.55	98.02
Mean	6.54	57.67	13.17	157.41
SD	1.58	11.14	13.07	90.82
CV %	24.11	19.32	99.25	57.69

Table 3.6 C_{max} and AUC values with mean, SD and CV% for TDS®-Placebo.

Subject	C_{max} (0-12)	AUC_{0-12}	C_{max} (0-24)	AUC_{0-24}
	(ng/mL)	(ng/mL.h)	(ng/mL)	(ng/mL.h)
1	5.95	50.94	5.95	104.88
2	4.13	32.48	4.13	60.44
3	5.22	42.03	6.85	105.03
4	6.33	51.95	6.33	110.81
5	8.32	75.81	8.66	160.59
6	5.45	45.38	5.45	97.34
7	5.90	60.40	5.90	110.80
8	3.98	36.97	3.98	67.83
9	4.44	42.54	4.44	83.39
10	5.83	56.37	6.61	120.33
11	6.60	61.64	6.60	108.62
12	6.45	51.45	6.45	89.97
Mean	5.72	50.66	5.95	101.67
SD	1.21	11.94	1.32	25.92
CV %	21.13	23.57	22.18	25.50

Table 3.7 t_{max} values with median and interquartile range (IQR) for all the treatments

Subject	TDS®-Testosterone		AndroGel®		TDS®-Placebo	
	t_{max} (0-12) (h)	t_{max} (0-24) (h)	t_{max} (0-12) (h)	t_{max} (0-24) (h)	t_{max} (0-12) (h)	t_{max} (0-24) (h)
1	4.0	24.0	2.0	24.0	1.0	1.0
2	3.5	3.5	1.5	1.5	3.5	3.5
3	3.5	3.5	0.5	24.0	0.0	24.0
4	3.5	3.5	2.0	2.0	10.0	10.0
5	1.0	24.0	3.0	24.0	2.5	24.0
6	3.5	3.5	0.5	24.0	0.0	0.0
7	1.5	24.0	2.5	24.0	7.0	7.0
8	0.5	0.5	2.0	24.0	0.5	0.5
9	1.5	24.0	1.0	24.0	10.0	10.0
10	3.0	3.0	2.5	2.5	1.0	24.0
11	0.0	24.0	1.0	24.0	1.5	1.5
12	3.5	3.5	3.5	3.5	2.5	2.5
Median	3.3	3.5	2.0	24.0	2.0	5.3
IQR	2.4	20.5	1.5	21.3	5.5	19.4

The 90% confidence intervals (CI) on the relative difference of the ratio for the AUC_{0-12} and the C_{max} (0-12) between TDS®-Testosterone and AndroGel® were contained within the bioequivalence limit (80 – 125%), (C_{max} (0-12):87.0 to 119.1% and AUC_{0-12}:91.0 to 123.8%). Serum testosterone concentrations were lower following TDS®-Placebo and were not bioequivalent either to the gel or the spray. The CI values obtained for all the treatments' comparison are summarised in Table 3.8. Table 3.9 summarises the QC sample results obtained during the analysis of testosterone by ELISA. From the concentrations obtained, the mean, standard deviation and the coefficient of variation (CV%) were calculated. The CV for imprecision and inaccuracy were all below 15%. No serious or unexpected adverse events were reported or observed during the study day. The applied dosages and protocol requirements were well tolerated by all subjects.

Table 3.8 Confidence interval (CI) for all the treatments' comparison

Comparison	90 % CI	
	AUC_{0-12}	C_{max} (0-12)
TDS®-Testosterone vs. Androgel®	91.0 – 123.8	87.0 – 119.1
TDS®-Testosterone vs. TDS®-Placebo	105.0 – 142.1	99.0 – 135.4
Androgel® vs. TDS®-Placebo	98.5 – 134.0	97.2 – 133.0

Table 3.9 Coefficient of Variation (CV) for all the Quality Control (QC) samples achieved during the assay

No of control	C1	C2	C3
	ng/mL		
1	0.83	4.28	8.63
2	0.96	3.91	10.25
3	0.84	3.26	8.99
4	0.93	3.66	9.45
5	0.80	3.14	8.87
6	0.99	3.47	9.64
7	0.70	3.23	11.89
8	0.63	3.49	10.01
9	0.94	3.15	6.82
10	0.99	2.86	8.50
11	0.70	3.18	8.88
12	0.60	2.99	8.34
13	0.77	3.12	8.24
14	0.76	2.68	10.31
15	0.92	3.69	10.47
16	0.73	3.47	7.56
17	0.78	3.28	7.75
18	0.92	2.64	9.88
19	0.71	2.60	6.44
20	0.85	3.24	8.89
21	0.81	3.82	7.81
22	0.91	3.34	10.50
23	0.96	3.49	9.32
24	0.92	3.40	10.37
25	0.82	3.82	8.38
26	0.82	3.15	7.96
27	0.70	3.56	10.10
28	0.85	2.78	8.97
29	0.89	3.28	8.56
Mean	0.83	3.31	9.03
SD	0.11	0.39	1.21
CV	12.96	11.92	13.42

3.4.4 Discussion and conclusion

The systemic delivery of testosterone has been successfully achieved by using the TDS® system in this study. The 50 mg dose applied was well tolerated in all the twelve subjects tested. However, the real amount of TDS®-Testosterone and AndroGel® applied on the skin surface cannot be confirmed as the glove has been used to rub both of the treatments on the skin. Some of the testosterone from the solution or the gel may stick to the glove. This would contribute to the inter subjects variability. This factor of variability has been minimised by using the same application method into all the subjects. Although we have assumed that the amount stuck to the glove is very little and has only a small contribution to the exact dosage amount, it would be ideal if the drug content in the glove could be analysed so that the exact amount applied can be confirmed. Therefore, the future study on the development of TDS®-Testosterone needs to include this analysis.

In conclusion, the TDS®-Testosterone preparation was shown to be able to deliver testosterone systemically in humans. The concentrations of hormone in the first 12 hours following TDS® administration were found to be bioequivalent to an existing topical delivery gel. However higher pharmacokinetic profiles such as AUC and C_{max} were observed for TDS®-Testosterone compared to AndroGel®. Both TDS-Testosterone and AndroGel® were not bioequivalent to TDS® - Placebo.

This conclusion is based on the direct analysis of serum testosterone profile from 0 to 12 hours for the three treatments given. However, circulating endogenous testosterone may influence the bioequivalence assessment of exogenously administered testosterone. The following section will explain the detail of the endogenous correction method that can be applied for more accurate bioequivalence assessment of testosterone.

3.5 Methods of correction for endogenous concentration for bioequivalence assessments of testosterone.

3.5.1 Introduction

In the evaluation of new drug preparations, a bioequivalence (BE) study in healthy volunteers normally has to be conducted in order to show that the new preparation is as effective as current preparations. Guidelines for the determination of bioequivalence of pharmaceutically active formulations are fully documented by Food and Drug Administration (FDA) in the USA (FDA, 2003) and by the Committee for Proprietary Medicinal Products (CPMP) for the European Union (EMEA, 2001). Bioequivalence studies compare the rate and extent of absorption for a new treatment against that of the reference product using the parameters of maximum serum concentration (C_{max}) and area under the curve (AUC). For exogenously administered endogenous compounds, like testosterone, the comparison needs to allow for the normal circulating concentrations of the compound. However, the circulating concentrations of testosterone, complicate the analysis of pharmacokinetic parameters when these compound is administered exogenously. Therefore, correction of the data to remove the influence of endogenous testosterone is necessary to obtain the concentration of testosterone that is attributable to the exogenous source.

Except levothyroxine sodium (FDA, 2001c) and potassium chloride (FDA, 1994), the current BE guidelines offer no guidance on the correction for the endogenous concentrations. The four endogenous correction methods have been evaluated in this study for the proper determination of bioequivalence of testosterone.

3.5.2 Correction Methods

Three methods of data correction were used to subtract the influence of endogenous concentrations of testosterone from the total concentration measurements. The AUC and the C_{max} were then calculated from the data generated in corrections 1, 2 and 3. Correction 4 was carried out on the AUC and C_{max} values obtained from uncorrected data.

- **Correction Method 1**

The mean pre-dose testosterone concentration (-0.5 and 0 hour) was subtracted from each testosterone concentration after dosing for each subject and treatment.

- **Correction Method 2**

The endogenous data was modelled from the placebo data using a polynomial equation and then subtracted from the measured treatment values. The endogenous concentration obtained for each time point was then subtracted from the analogous time point for active treatments.

- **Correction Method 3**

The concentrations on the placebo day were subtracted from the active treatment concentrations at the analogous time point for each subject.

- **Correction Method 4**

The testosterone C_{max} and AUC values calculated without data correction on the placebo day were subtracted from the parameters calculated from the two active treatment days.

Some of the negative values were generated after subtractions. These values are due to the variability in the endogenous testosterone level without any increased from exogenously administered testosterone. Therefore, all the negative values can be considered zero for more reliable statistical analysis. The corrected serum testosterone concentrations (ng/mL) were then plotted versus times (h) along with corrections 1, 2, and 3.

3.5.3 Pharmacokinetic and statistical analysis

C_{max} was determined by observation and AUC was calculated using the linear trapezoidal method. The AUC and C_{max} for the uncorrected and corrected data were determined from 0-12 hour data for each treatment. Analysis of variance (ANOVA) were carried out on the log transformed AUC and C_{max} to determine the bioequivalence between TDS®-Testosterone and AndroGel®. All the statistical analyses were carried out using Kinetica version 4.2 Software (Kinetica, 2006).

3.5.4 Results

3.5.4.1 Serum testosterone profiles

3.5.4.1.1 Uncorrected serum testosterone data

The plot of mean testosterone serum concentration for TDS®-Testosterone, TDS®-Placebo and AndroGel® was shown in Figure 3.4. Higher testosterone concentration profiles were observed for both of the active treatments compared to placebo. Serum testosterone profiles for TDS®-Testosterone and AndroGel® were lower at the pre dose, and increased after the treatments application. Both the active treatments achieved the highest concentration after 3 to 4 hours post dose and declined following a period of 12 hours. However, the TDS®-Placebo showed a decreased in serum testosterone profile from pre dose until the 12 hours post dose.

3.5.4.1.2 Correction Method 1

Following the 12 hours sampling period, the TDS®-Testosterone showed a higher profile of testosterone concentration compared to AndroGel® and the TDS®-Placebo (Figure 3.8). AndroGel® also showed the small increase from 0 up to 2.5 hours and decrease through out the day to the same level as the placebo. The TDS®-Placebo treatment remains constant and slightly fluctuated for the whole sampling period.

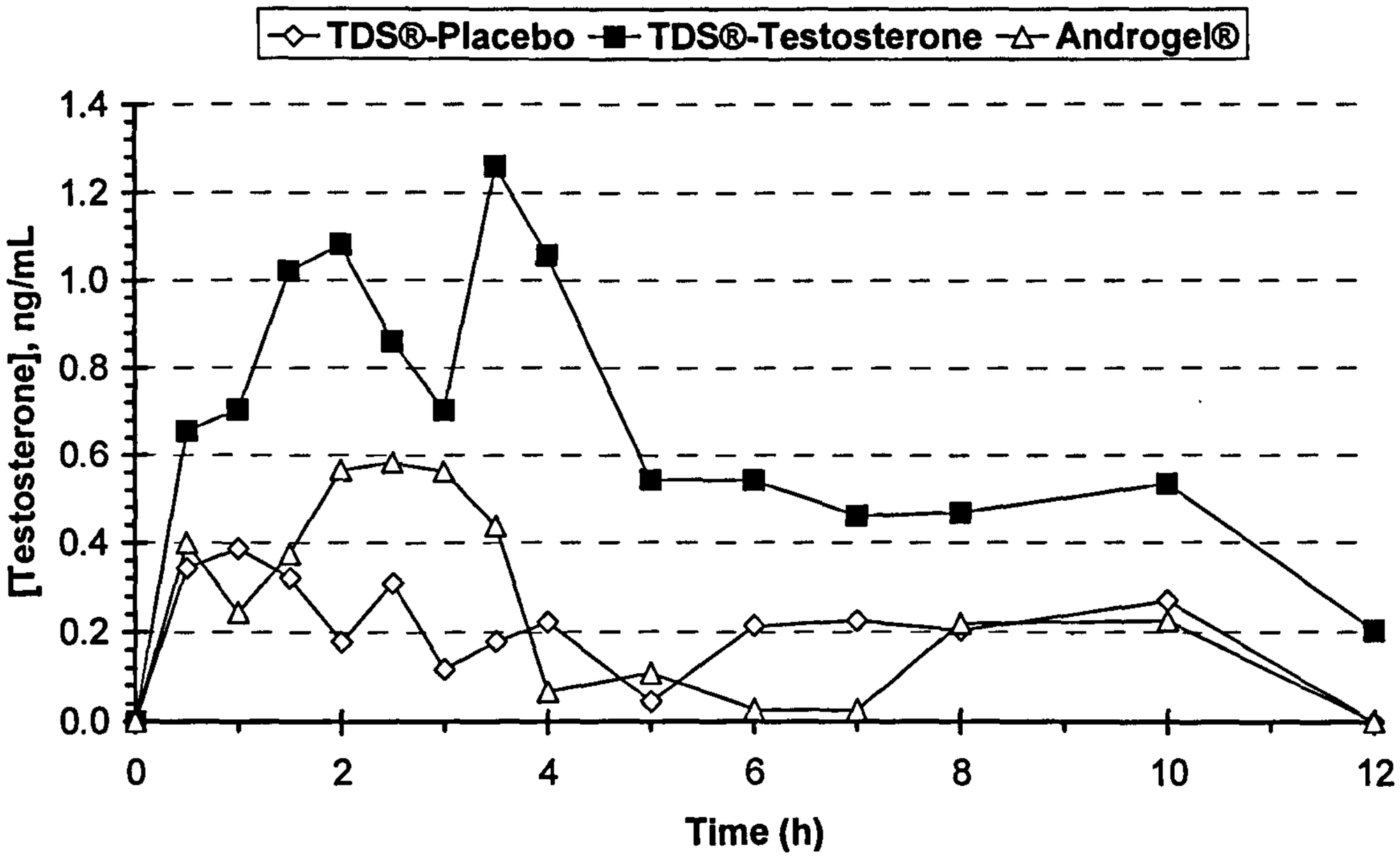


Figure 3.8 Plot of mean serum testosterone concentration (ng/mL) versus time (h) for each treatment based on Correction Method 1.

3.5.4.1.3 Correction Method 2

Figure 3.9 shows the testosterone profile for the placebo treatments for 12 subjects with the examples of polynomial regression and equation on selected subjects. Figure 3.10 shows the plot of testosterone concentration versus time for TDS®-Testosterone and AndroGel® based on Correction Method 2. The plots for TDS®-Testosterone and AndroGel® were quite a similar with the concentration peaks at 3.5 hours and 3 hours, respectively. However, the testosterone concentrations were higher for the TDS®-Testosterone compared to AndroGel® for most other sampling times.

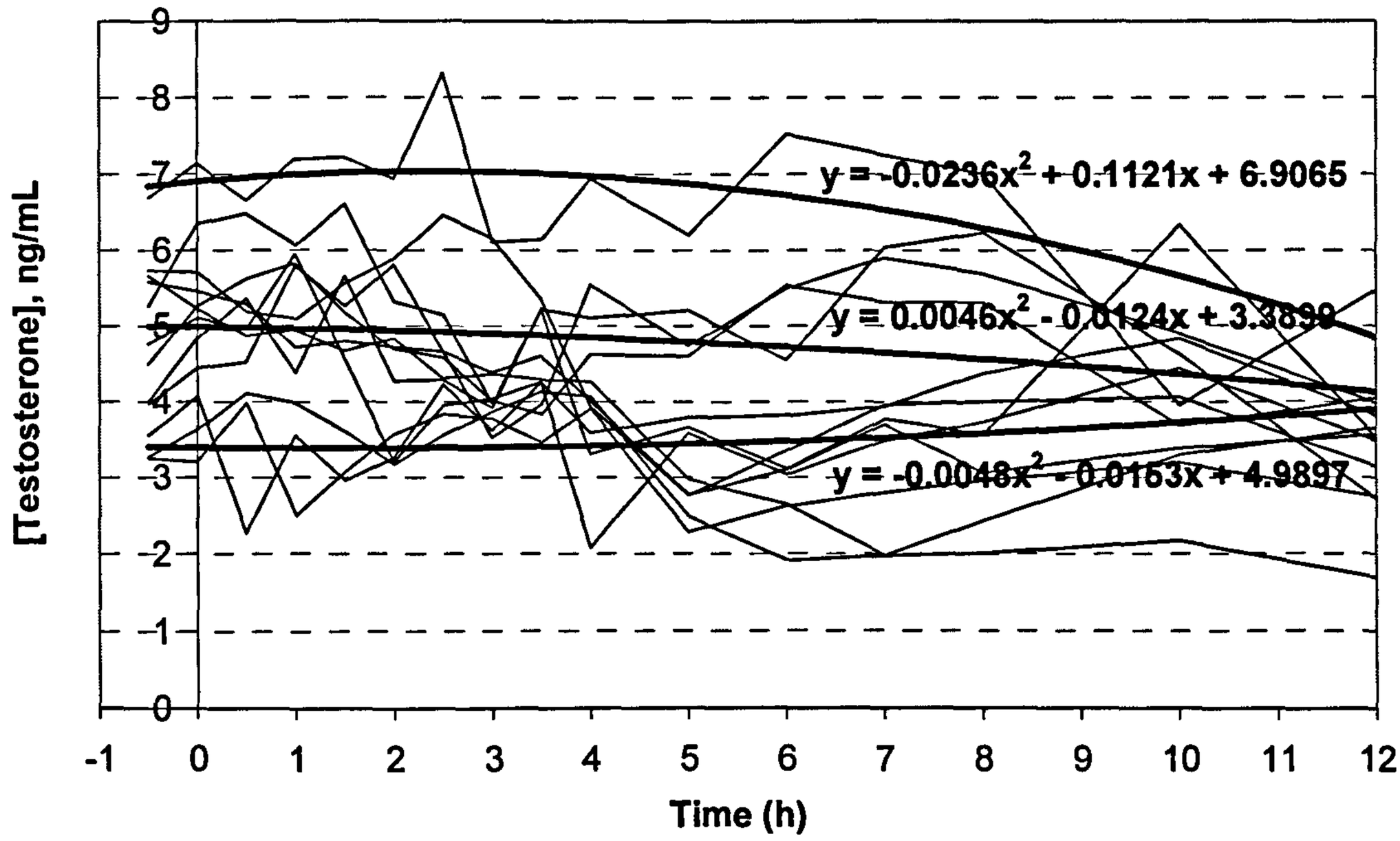


Figure 3.9 Plots of concentration vs. time for TDS®-Placebo in 12 subjects with examples of polynomial regression on selected lines.

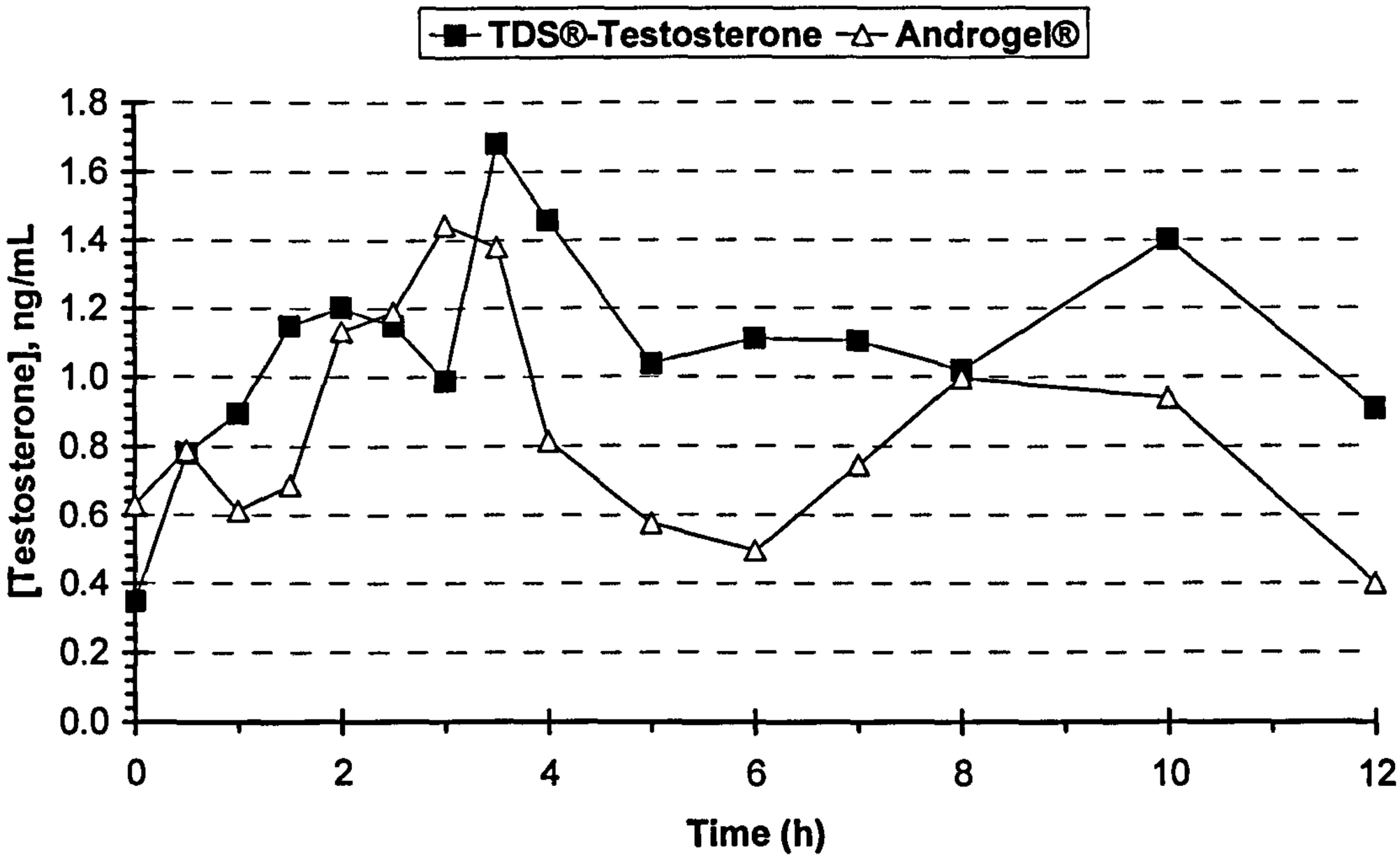


Figure 3.10 Plots of mean serum testosterone concentration (ng/mL) versus time (h) for TDS®-Testosterone and AndroGel® based on Correction Method 2.

3.5.4.1.4 Correction Method 3

The mean serum testosterone concentrations for AndroGel® were higher than for the TDS®-Testosterone after application up to 3 hours, but decreased dramatically to lower than the pre dose value, especially at 12 hours post dose. The serum testosterone concentration profile for the TDS®-Testosterone were increased after application and peaked at 4 hours, before decreased constantly through out the day (Figure 3.11)

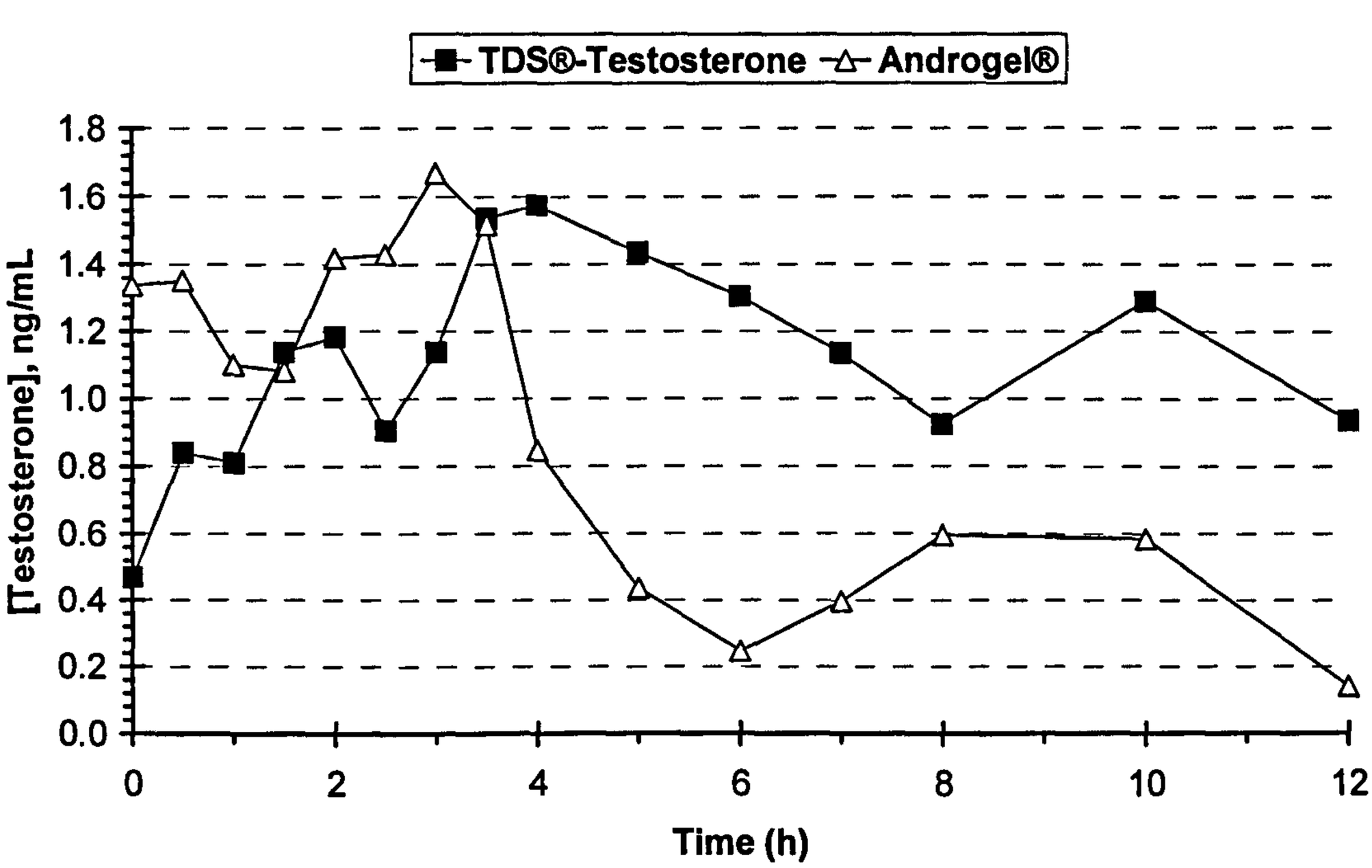


Figure 3.11 Plots of mean serum testosterone concentration (ng/mL) versus time (h) for TDS®-Testosterone and Androgel® based on Correction Method 3.

All the plots for serum testosterone concentration (ng/mL) versus time (h) based on uncorrected and Correction Methods 1, 2, and 3 showed the higher testosterone profile for the TDS®-Testosterone than AndroGel®.

3.5.4.2 Bioequivalence and statistics

The relative bioavailability comparison of the AUC and C_{max} for the TDS®-Testosterone and AndroGel® are summarises in Table 3.10 for all uncorrected, Correction Methods 1, 2, and 3 data. The AUC and C_{max} for TDS®-Testosterone and AndroGel® were not bioequivalent with the TDS®-Placebo using the uncorrected and Correction Method 1 data. However, the 90% CI of the AUC and C_{max} for the comparison between the TDS®-Testosterone and AndroGel® gave a different results between uncorrected and Correction Methods 1, 2, and 3. The TDS®-Testosterone and

AndroGel® were found to be bioequivalent based on uncorrected data, but they were not bioequivalent with Correction Methods 1, 2, and 3.

Table 3.10 Relative bioavailability (90% CI) for uncorrected and all corrections data.

Correction Methods	Bioequivalence (90% CI)	
	AUC_{0-12}	C_{max} (0-12)
Uncorrected	91-124	87-119
Correction 1	52-106	50-258
Correction 2	71-655	87-286
Correction 3	67-315	88-157

Reference: AndroGel®

Table 3.11 summarises the percentage differences of AUC and C_{max} between TDS®-Testosterone and AndroGel®. The differences were lower for uncorrected data compared to corrected data. Within the corrected data, the largest difference was seen in Correction Method 1. Similarly, the other correction methods showed higher AUC and C_{max} values for the TDS®-Testosterone compared to AndroGel®. Thus, data corrected for endogenous serum testosterone concentrations showed increased testosterone profiles for the TDS®-Testosterone compared to AndroGel®, with correction AUC by 30 to 190% and correction C_{max} by 12 to 55%, depending on the method of corrections. ANOVA could not be performed on Correction Method 4 due to the negative values generated. Therefore, only the average of AUC and C_{max} were reported.

Table 3.11 Percentage differences (%) in mean AUC and C_{max} between TDS®-Testosterone and AndroGel®.

	Mean Values		Percentage differences (TDS®-AndroGel®)	
	AUC_{0-12} (ng/mL.h)	C_{max} (0-12) (ng/mL)	AUC_{0-12} (%)	C_{max} (0-12) (%)
Uncorrected				
TDS®-Testosterone	61.92	6.63	7.18	1.47
AndroGel®	57.77	6.54		
Correction Method 1				
TDS®-Testosterone	7.47	1.69	190.15	54.90
AndroGel®	2.57	1.09		
Correction Method 2				
TDS®-Testosterone	13.61	2.42	36.37	29.41
AndroGel®	9.98	1.87		
Correction Method 3				
TDS®-Testosterone	13.86	2.88	30.07	16.45
AndroGel®	10.65	2.48		
Correction Method 4				
TDS®-Testosterone	11.15	0.95	59.29	11.76
AndroGel®	7.00	0.85		

3.5.5 Discussion

Currently licensed transdermal delivery systems for testosterone in UK are available as patches, gels and buccal tablets. However they do not fully achieved the goal for testosterone replacement therapy as described in Section 3.1 (Introduction). TDS®-Testosterone preparation appears to provide a more convenient transdermal delivery of testosterone with rapid drying and no skin irritation compared to some of the other transdermal systems. TDS®-Testosterone has proved to be more effective than AndroGel® in delivering testosterone systemically, in the same dosage (50mg) given. AndroGel® was used for the comparison due to the same mode of application, availability and widely used currently in UK for the treatment of hypogonadism.

In the bioequivalence evaluation of exogenously administered endogenous compound such as testosterone, the circulating endogenous compound should be taken into account. Without proper consideration, the judgement of bioequivalent may be inaccurate. There are a few methods that have been published in consideration of the endogenous compound by using correction methods.

Blakesley (Blakesley, 2005) has proposed several correction models in the bioequivalence assessment of Levotyroxine sodium (T_4). Correction for endogenous T_4 has been done by three different approaches. In Method 1, the mean of three pre-dose values were subtracted from each concentration at post dose. In correction method 2, the post dose concentrations were corrected based on hypothetical decay of endogenous T_4 with a seven day half life. Corrrection Method 3 used the concentration T_4 measured on the day prior to dosing to subtract the post dose concentrations at the analogous time point. This study concluded that, without endogenous correction, the bioequivalence conclusion showed a failure to identify differences between T_4 products varying as much as 25%-33% in dosage strength.

Other studies conducted on testosterone used another approach for correcting endogenous concentration. The pharmacokinetic study to compare the three dosage regimens of buccal adhesive testosterone tablets has been conducted (Baisley et al., 2002). In this study three injections of leuprorelin acetate to compress the testosterone productions were administered in healthy males, followed by androgen replacement therapy (oral testosterone undecanoate) within the three weeks of screening phase. At the end of replacement therapy, whose morning serum testosterone ≤ 5.38nmol/L were

entered the baseline and treatment phase of the study. The endogenous concentration was corrected by deducting the baseline AUC_{0-24} from the treatment AUC_{0-24}. This approach has an advantage of minimizing the fluctuation of testosterone in healthy males, hence contributed to less interference from endogenous testosterone.

The similar approach in testosterone suppression method has been carried out by Rolf et al. (Rolf et al., 2002). In this study the testosterone production has been suppressed by a single intramuscular injection of 400mg norethisterone enanthate 5 days before the start of the study. The testosterone gel was applied daily for 10 days and the serum testosterone concentration was studied at pre-dose (basal level), day 1, day 5, and day 10 of treatment's application. The plot of testosterone basal level after injection of norethisterone had really shown a complete suppression without any diurnal variation in the testosterone level within the 24 hours observed. The testosterone plot showed a higher level at day 1, 5 and 10 compared to the basal level.

Based on the above described correction methods, four correction methods on endogenous testosterone have been carried out in this study. The outcomes from the correction methods that have been carried out can be considered as an interesting finding in which the results showed the different outcomes between corrected and uncorrected data. Although all the correction methods gave the same conclusion in the bioequivalent of TDS®-Testosterone and AndroGel®, there are potential advantages and disadvantages existed, which may influence the accuracy of each correction method used.

The first correction method (Correction Method 1) was based on the assumption that the endogenous testosterone level remained constant at all times. Therefore, the mean pre dose (-0.5 and 0 hour) concentration value can be used to subtract the post dose values to obtain the correct amount attributable exogenous administration. The advantage of this method is that the pre dose values were obtained exactly before the administration of testosterone, which minimised the period error. However, this method also offers some disadvantages, as it is known that testosterone secretion is likewise pulsatile and diurnal. In healthy males, recorded plasma testosterone concentration is normally higher in the morning, at about 8 am and lowest in the evening (~ 8 pm) (Snyder, 2001).This condition can be seen in the pattern of mean testosterone concentration from the placebo treatment in Figure 3.6, which were higher at the beginning and lower throughout the day (up to 12 hours) and increased again to the same level as 08:00 at 24 hours post

dose. Our placebo data also correlated well with the literature values of an observed and predicted testosterone level in healthy young males as published by Gupta et al. (Gupta et al., 2000)

The second correction (Correction Method 2) was based on the inhibition of the gonad regulating cycle, which can cause the body to reduce production of testosterone when its level is too high. During exogenous administration of testosterone, endogenous testosterone release may be inhibited through feedback inhibition of pituitary luteinizing hormone (LH), resulting in declining levels of testosterone. Correction Method 2 may offer a slight advantage over Correction Method 1, as the average level of testosterone production can be estimated through the best fit of a polynomial regression for the placebo modelled data. This data can therefore be accepted as an average endogenous level of testosterone on that individual subject. However, the inhibition of endogenous testosterone productions may only happen with large doses of exogenously administered testosterone. No data was available to date to suggest the exact amount of testosterone that may inhibit the production of testosterone. Assuming the diurnal variation of testosterone as explained previously, Correction Methods 1 and 2 have similar disadvantages.

Correction Method 3 and 4 were based on the analogous placebo data can be assumed as the endogenous level of testosterone. Therefore by subtracting the placebo's testosterone concentration values at the analogous time points and AUC and C_{max} for each subject and treatment in Method 3 and 4, respectively, the amount from exogenous administration can be obtained. These methods may offer further advantages over Correction Methods 1 and 2, as these methods can overcome the diurnal variation of testosterone in healthy males. However, this method may have a disadvantage as the analogous placebo values, which were obtained at different periods may not represent the endogenous testosterone level on the day the active treatments were administered. This can be explained by the high variability in pre-dose testosterone concentration values (0 hour). As manifest by the within subject coefficient of variation (CV) of the mean C0 values which ranged from 1.4 to 28.2%. The study by Andersson and co-workers (Andersson et al., 2003) also suggested that there was variability in the testosterone levels from month to month. To minimise this error, a placebo treatment may need to be conducted in the days immediately before the active treatment is given.

The conclusions about bioequivalence in the uncorrected and corrected data in this study are based on the 90% CI values from the ANOVA. It can be seen that the 90% CI for all the correction methods performed were not only outside the bioequivalence limit (80-125%), but contained a wider range of intervals. This condition resulted from the variability in the results obtained after endogenous subtractions. Most of the plasma levels obtained after subtractions are fluctuated closed to zero, especially for AndroGel®. Twelve subjects used in this study were not enough to overcome the variability arose from the testosterone level after endogenous corrections. Therefore, to obtain a more accurate bioequivalence assessment with endogenous corrections, the sample size needs to be increased, such as using 24 subjects instead of using 12 subjects in this study.

3.5.6 Conclusions

TDS®-Testosterone is found to be more effective in delivering testosterone systemically in much smaller volume and more convenient to use than AndroGel®. Hence, TDS®-Testosterone has the potential to be an alternative treatment for hypogonadism. The phase II study in a bigger number of hypogonadal patients has to be carried out for this purpose.

Different results obtained in the relative bioavailability between the TDS®-Testosterone and AndroGel® for uncorrected and corrected data, suggest that correcting endogenous concentrations is important for the proper determination of bioequivalence for endogenous compound such as testosterone. Without endogenous data correction, an incorrect conclusion about bioequivalence may result with products being declared bioequivalent when they are actually not bioequivalent or *vice versa.*

Chapter 4 Comparative pharmacokinetic assessments of topical drugs: Evaluation by dermatopharmacokinetics, microdialysis, and systemic measurement.

4.1 Introduction

The conventional pharmacokinetic profiles of drugs are mainly described through the plasma concentration versus time profiles. This is vital for most orally administered drugs which undergo systemic absorption before the drug can be distributed to the site of action. However some drugs such as local anaesthetics, antifungal agents, topical corticosteroids, etc. are designed to target the local tissue where the drugs are applied and, as such, have limited systemic absorption. The distribution and the action of these drugs are more appropriately measured in the local tissue rather than systemically. Thus, conventional drug measurement using systemic blood samples is inappropriate. The concentrations of a drug in systemic circulation may be too low to detect, hence hampering the pharmacokinetic profiling of the drug. Although the concentration of the drug can be detected, it may not directly relate to the amount of drug in the local tissue in which they are producing the therapeutic effects. Some of the topically administered drugs such as local anaesthetics are retained in the local tissue rather than absorbed into the the systemic circulation. Hence the direct measurement of these drugs in the local tissue tends to be more appropriate for a pharmacokinetic assessment. To date, a number of in vitro and in vivo techniques are available for direct measurement of pharmacokinetic or clinical relevance information about the drug contents in the target tissue and skin.

In vitro experimentation tends to be a simple method to study drug permeation through the skin, especially in the early development of drugs. Animal skin or excised human skin obtained from a skin bank or by donations from a patient undergoing a surgical procedure are always used in *in vitro* experiments. These excised human or animal skin samples are used as a membrane and normally fixed to a diffusion cell where a membrane is clipped between two compartments, termed as donor and receptor. Drug applied on the membrane on the donor side will diffuse through the membrane to the receptor side where the concentration can be analysed. There are a few types of

diffusion cell with different designs but similar in their functioning. The well known and commonly used diffusion cell is the Franz type diffusion cell (Franz, 1975). However, it should always be kept in mind that the data obtained from excised skin may not translate directly to the *in vivo* situation. The barrier properties of the skin may be disrupted when the skin undergoes the excision process and the most important properties, the integrity of the skin in terms of water retention, blood supply and skin metabolism may be lost. Furthermore, it is impossible to obtain a large quantity of human skin therefore animal skin and artificial membranes are frequently used in *in vitro* experimentation. Although it is possible to get a large amount of animal skin, the structure and the function of animal skin is different to that of human skin and different between species.

In order to obtain clinically relevant information about a drug's action in the skin, especially for pharmacokinetic study, *in vivo* techniques must be applied. There are a few techniques currently available to obtain drug information in the skin and also a pharmacokinetic profile, techniques include dermal microdialysis (DMD) (Anderson et al., 1992;Benfeldt, 1999;Hegemann et al., 1995), tape stripping - dermatopharmacokinetic (DPK) (Pershing et al., 2003), skin blister fluid method (Nowak and Klimowicz, 1990), magnetic resonance imaging (Jynge et al., 1990), and biopsy followed by tissue homogenisation (Roos and Brorson, 1990). Among the various techniques mentioned above, microdialysis and tape stripping are widely used in the skin pharmacokinetics compared to the other methods due to their reliability and reproducibility. Due to higher costs, ethical problems, invasive procedures, and lack of reproducibility, some of the other methods mentioned above are not practicable to use in drug development.

Since both DMD and DPK are still under investigation by many researchers, they have not yet been accepted as the valid techniques for the pharmacokinetic study, especially for bioequivalence. But these techniques are useful for determining the pharmacokinetic profile of drugs especially for transdermal application. The future challenge for assessment of these techniques lies in the sampling of lipophilic compounds, which constitute the majority of novel drugs targeted to the skin. Lidocaine is a good lipophilic model drug for this purpose as it possesses local activity and is widely used as a topical local anaesthetic.

4.2 Microdialysis

Microdialysis has been shown in the previous publications (Benfeldt et al., 1999;Benfeldt, 1999;Benfeldt and Groth, 1998;Hegemann et al., 1995;Murakami et al., 1998;Okahara et al., 1995) to provide reliable estimates of the drug content in the skin after topical administration. It enables the measurement of the quantity of the drug compound directly in the target organ, i.e., the dermis or subcutaneous layers of the skin after topical administration. Microdialysis is a sampling technique where a semipermeable membrane probe is placed in an extracellular compartment and perfused by perfusion fluid with a similar ionic strength and pH as the tissue fluid. It can be used for measuring endogenous and exogenous compounds in extracellular spaces. The technique was originally developed in neuroscience to monitor the relationship between neurochemistry and behaviour (Groth, 1996). Bito et al. (Bito et al., 1966) first described the idea to sample extracellular by dialysis and was followed by Delgado et al. (Delgado et al., 1972). The technique was evolved and after further refinements, microdialysis has become the major bioanalytical sampling tool in brain research (Groth, 1996). Nowadays, microdialysis has been widely used in clinical drug studies in several compartments such as adipose tissue. Microdialysis offers several advantages over traditional methods of measuring tissue drug concentrations such as skin biopsy and suction blister. Although biopsy and blister samples can give information about drug concentrations, the number of samples is limited and frequent sampling is not possible due to the invasive nature of the procedure and ethical considerations. Time courses of drug concentrations are therefore difficult to obtain. More sophisticated and non-invasive techniques, such as magnetic resonance imaging, are expensive and require specialised equipment and expertise.

In contrast, microdialysis technique allows simultaneous sampling at the same site or multiple sites. By placing the microdilaysis probe in the dermis or any other tissues, continuous sampling can be done by continuously perfusing the sampling site with buffer solution. It is also be able to monitor topical drug penetration continuously for a certain period of time with detailed real-time chronology. In one animal experiment, a microdialysis probe was implanted in rat tissue with the other end connected to liquid chromatography (LC) or capillary electrophoresis (CE), (Wang et al., 2005;Zhang et al., 2006) for real-time analysis. The technique also allowed multiple site drug administration with each site of application having no effect on the other sites, therefore

allowing different dosages to be applied to one subject for a topical drug study. This method can significantly reduce the number of subjects required. Furthermore, microdialysis causes only moderate pain, when compared to intramuscular and subcutaneous injection and is also easy to perform.

4.2.1 Principles of microdialysis

Microdialysis involves the insertion of a microdialysis probe into a tissue or (body) fluid to monitor the chemistry of the extracellular space in the living tissue. The probe consists of a semipermeable fibre membrane forming a thin hollow 'tube' (typically 0.2–0.5 mm diameter). The probe also connects to the impermeable tube which further connects to a micropump at one side and sampling vial at another side. The probe is continuously perfused with Ringer solution which has the same ionic strength as the extravascular fluid. When this physiological salt solution is slowly pumped through the microdialysis probe, the solution equilibrates with the surrounding extracellular tissue fluid. Any molecule which smaller than the membrane pore size may pass through the membrane and permeate into the perfusion medium across the concentration gradient and will be collected in the sampling vial for further analysis. The movements of the molecules follow the law of simple diffusion (Benveniste and Huttemeier, 1990). However bigger molecules, larger than the membrane cut-off (e.g. proteins and enzymes), will be excluded (Benfeldt et al., 1999). There are a few types of probe that are available for these purposes such as; linear, concentric, flexible, and shunt (Davies et al., 2000). Concentric, linear, and flexible probes are the most commonly used and are commercially available. A concentric probe is commonly used in animal studies such as rat and rabbit and is normally inserted into the brain. A linear probe and flexible probe are widely used in human studies and can be inserted intravenously or into the dermis for transdermal delivery studies. Figure 4.1 shows a diagram of a flexible probe and how it can be connected to the microdialysis pump and microvial.

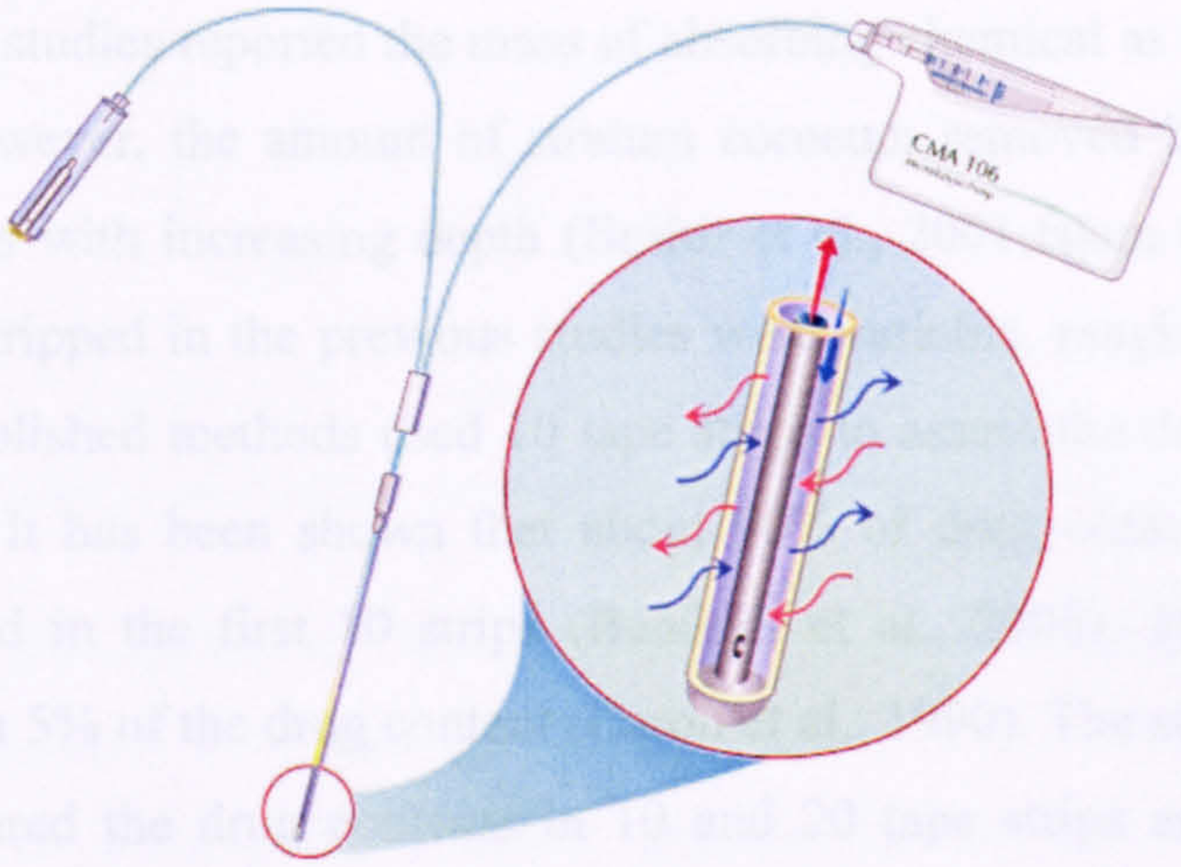


Figure 4.1 A diagram of flexible probe (in circle) with connections to the pump and microvial (Courtesy of CMA Microdialysis AB)

For cutaneous microdialysis, the probe is implanted in the dermis of the skin via a guide cannula. The probe is slowly perfused (typically 0.1–5 μL/min) with a Ringer solution, which equilibrates with the extracellular fluid of the surrounding tissue. Any drug concentrations occuring in surrounding tissue will enter the probe and diffuse into the perfusion fluid. Perfusion fluid entering the probe is known as perfusate and that exiting the probe is known as dialysate.

4.3 Tape stripping – Dermatopharmacokinetic (DPK)

Many *in vivo* methods for measuring dermal absorption of chemicals are invasive such as the need to cannulate a vein for blood sampling, skin blister, and probe insertion in microdialysis studies, etc. Tape stripping of the outermost skin layer, the stratum corneum (SC), is a simple and relatively noninvasive technique used to measure the drug content in the skin (Pershing et al., 2003;Stinchcomb et al., 1999). This approach is based upon the theory that the stratum corneum is a rate limiting barrier for any substance entering the body through the skin. In order for a drug to enter the site of action in the skin, it must first penetrate the stratum corneum. A drug is normally retained in the stratum corneum before slowly diffusing to a viable epidermis and the dermis. By sampling the stratum corneum it is possible to measure the drug quantities that penetrate the skin per unit time.

In DPK experiments, a certain area is marked on the skin and exposed to a drug for a set period of time and then cleaned. A number of adhesive tapes are applied to and removed from the exposed area in sequence so that sufficient quantities of stratum corneum can

be sampled. Many studies reported the mass of absorbing chemical as a function of tape strip numbers. However, the amount of stratum corneum removed by each tape strip generally decreases with increasing depth (Bashir et al., 2001;Islam et al., 1999). The numbers of tape stripped in the previous studies were variable, ranging from 5 to 100. However most published methods used 10 tape strips to assess the drug content in the stratum corneum. It has been shown that about 90% of drug content in the stratum corneum are found in the first 10 strips (Benfeldt et al., 2006). The next 10 strips contained less than 5% of the drug content (Caron et al., 1990). The study by Franz and co-workers compared the drug contents in 10 and 20 tape strips and also found no difference between 10 and 20 strips (Shah, 2005).

The drug contents in the tape can be analysed by a validated bioanalytical assay such as liquid chromatography and capillary electrophoresis methods (Shah et al., 1991). The DPK method has been shown to demonstrate reproducibility in its application to antifungal drugs (Pershing et al., 2002b), and dermatologic corticosteroid products (Pershing et al., 2002a), using the human volar forearm.

4.4 EMLA (Eutectic Mixture Local Anaesthetic)

The term eutectic mixture refers to the phenomenon where the melting point of a mixture of two local anaesthetics is lower than the melting points of each, alone (Buckley and Benfield, 1993). At room temperature, both lidocaine and prilocaine exist as liquid oil rather than as crystal in a ratio of 1:1. Each gram of 5 % EMLA cream contains 25 mg lidocaine and 25 mg prilocaine in the mixture with other compounds as an excipient and purified water as a medium. These mixtures form an oil-in water emulsion with the mixture of local anaesthetics as the disperse phase and water as the continuous phase (Brodin et al., 1984;Nyqvist-Mayer et al., 1985). Lidocaine, an amide type is widely used as local anaesthetic since it was discovered in 1948. Besides local anaesthetic effects, lidocaine is also used as an antiarrythmic, anti thrombic drug, etc. Prilocaine (Figure 4.2) is also an amide type local anaesthetic which has an action and potency similar to lidocaine. Hence, by combining these two local anaesthetics for topical application, and using it prior to cannulation or injection, it was hoped that needle phobia, which affects many people especially children would be reduced.

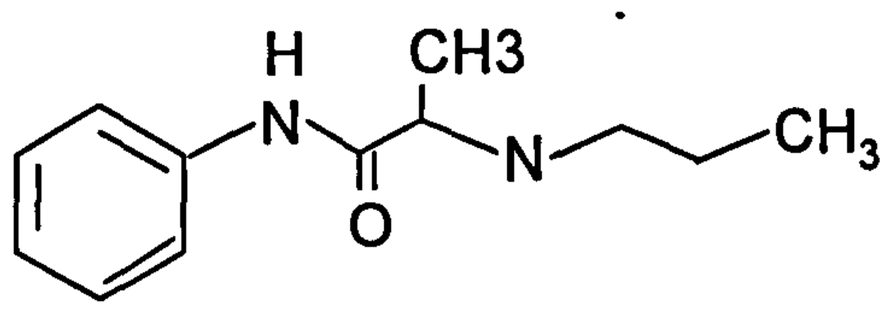


Figure 4.2 Prilocaine chemical structure

4.4.1 Mechanism of action and pharmacokinetics of EMLA™

EMLA™ cream when applied on the skin under occlusive dressing provides dermal analgesia approximately 1 hour after application. Low level of lidocaine and prilocaine can be detected in plasma after topical applications of EMLA™. The plasma concentrations of lidocaine and prilocaine after EMLA™ applications have been studied by a few researchers. Plasma concentrations of prilocaine tend to be lower than those of lidocaine (Buckley and Benfield, 1993). In a healthy volunteer study by Evers et al. (Evers et al., 1985), 20g of EMLA™ cream was applied to a 300cm^2 area of the thigh for 60 minutes. The maximum plasma concentration of lidocaine and prilocaine were 74.5 and 24.7ng/mL, respectively. The maximum concentration was achieved approximately 3 hours after application. In another study in infants (Engberg et al., 1987), 2mL of EMLA™ cream was applied to a 16cm^2 are of skin for 4 hours. The maximum concentrations achieved were 410ng/mL for lidocaine and 130ng/mL for prilocaine. The amount of lidocaine and prilocaine absorbed systemically are related to the duration of application and the application area. Buccal mucosa showed higher absorption compared to normal skin. The maximum concentration measured was 418ng/mL for lidocaine and 223ng/mL for prilocaine when 8g of EMLA™ was occluded to 18cm^2 of buccal mucosa for 30 minutes (Vickers et al., 1997).

4.5 Objective

The objective of this study was to investigate the outcome of the three techniques, DMD, DPK, and systemic blood measurements in the pharmacokinetic evaluation of topically applied drugs. The results obtained from these three methods have been compared to show the reliability and integrity of each method in pharmacokinetic evaluation. For the above purposes, the analyses of lidocaine content in all of the parameters of evaluations are the main outcome measures.

4.6 Study Approval

The study was approved by the East London and The City Health Authority Research Ethics Committee. Reference no. 05/Q0605/98, dated 9th. September 2005.

4.7 Study Material

4.7.1 Treatment

EMLA™ cream (Astra Zenecca, UK) containing 2.5% lidocaine and 2.5% prilocaine

4.7.2 Apparatus

CMA 60 microdialysis catheter was used together with CMA 106 microdialysis pump supplied by CMA Microdialysis Ltd. Sweden for DMD study. Adhesive tape for DPK study was a TESA 4204 PV5 supplied by TESA UK Ltd.

4.8 Subjects

The study involved twelve healthy subjects.

4.9 Study Methods

4.9.1 Study Design

The study was an open label, involving two visits with a minimum of a one-week washout period in between. DMD and systemic drug measurements were performed on one occasion and DPK alone on the other.

4.9.2 Study Protocol

Prospective subjects attending the study had the nature of the study fully explained to them, the procedures and the risks. Before any screening procedures occured they had signed an Informed Consent Form in which they acknowledged that they were willing to take part and follow all of the study protocols. In the screening evaluations, subjects were checked for the inclusion/exclusion criteria, physical examinations (blood pressure and heart rate), health questionnaires, concomittant medications, drugs of abuse and they also provided urine samples for urinalysis and a pregnancy test for females. Subjects were permitted to participate in the study soon after they had passed the

screening. The arms were checked for cuts or abrasions which would invalidate the study. The subject was asked to lay supine on a bed and one arm was cannulated with a 20G cannula for serial blood samples collection. A 5mL blood sample was drawn immediately after cannulation for baseline sampling. On the contralateral arm of the venous sampling, a circular area of $10cm^2$ was marked with a pen in the centre of the left volar forearm using a template for the treatment applications.

DMD probe implantation

The DMD guide cannula was inserted subcutaneously at about 1cm below the marked (treatment) area so that the probe lies horizontally below the treatment area. Probe implantation was performed without anaesthesia under sterile conditions. The DMD probe was inserted through the tip of the cannula and the needle then retracted, leaving the 3cm long probe implated in subcutaneous tissue below the marked area. The inlet and outlet tubing were attached to the skin using the adhesive mefix. The inlet tubing was then connected to the DMD pump and the outlet tubing connected to the microvial. Upon successful DMD probe implantation, the subject was allowed to recover for 60 minutes to diminish skin reactions (i.e. increased skin blood flow and histamine release) (Anderson et al., 1994;Rougier et al., 1985) before onset of the experiment. The perfusion fluid (Ringer solution) was perfused for 20 minutes at the flow rate of 1.0μL/min before the treatment applications for baseline sampling.

The treatment, 1g of EMLA™ cream (2.5% lidocaine / 2.5% prilocaine, AstraZeneca, UK) was applied to the skin at the marked area and occluded with a dressing. The DMD microvial was replaced for the 20 minute sampling and the vial was continuously replaced every 20 minutes for up to 4 hours. The residual drug was removed from the treated area after 1 hour of application. Venous samples were further collected at 20, 40 minutes, 1 hour, 1.5, 2, 2.5, 3, 3.5, and 4 hours post dose. Both venous and DMD samples were stored at -20˚C until further analysis. After completing all of the above procedures, both the DMD and intravenous catheter were removed and the subjects discharged. After a minimum of a one week washout period, subjects returned for the DPK study.

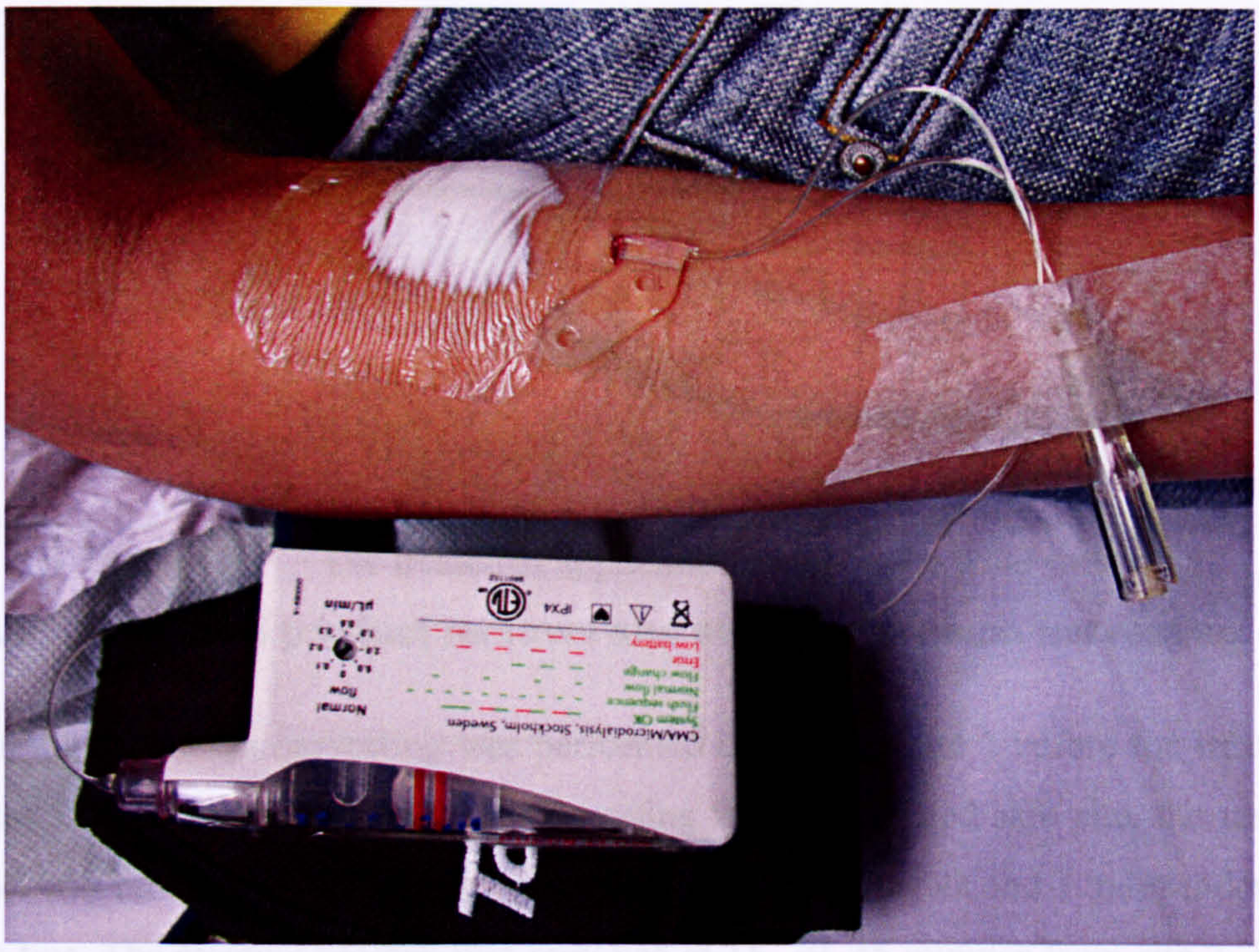


Figure 4.3 A photo taken of a study subject showing a DMD procedure

4.9.3 Tape stripping procedure

A total of 9 rectangular sites (site 1-9), each 6cm^2 (2 cm ×3 cm) of skin surface area were demarcated on the ventral forearm using a template (Figure 4.4). The treated areas (sites 2-8) were on 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 hour sampling). The treatment, 1g of EMLA™ cream was applied on each of the 8 application sites by using a syringe. The cream was distributed to cover the whole treatment area by a metal spatula. The subject was asked to maintain their hand in a horizontal position during the treatment period. All 9 of the sites were represented in the uptake and the elimination phase. DPK was performed at 0, 15, 30, 45 minutes and 1 hour for the uptake phase (site 1, 2, 3, 4, and 5) and 1.5, 2, 3, and 4 hours for the elimination phase (site 6, 7, 8, and 9).

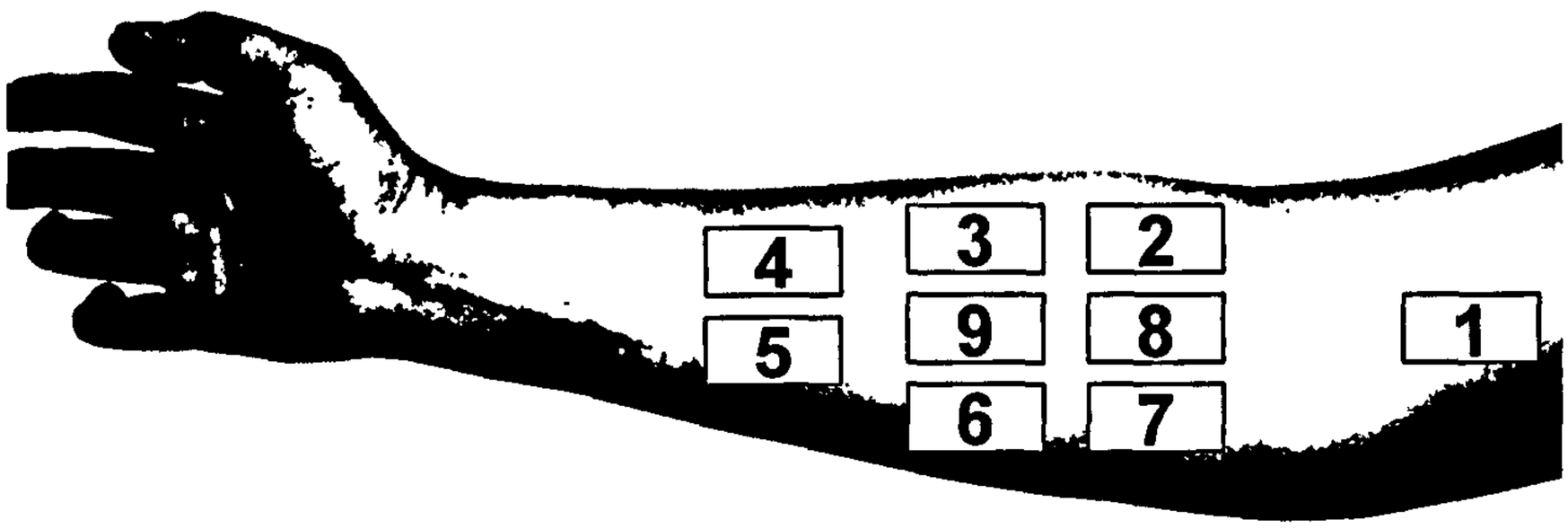


Figure 4.4 The treatment application areas on the volar forearm

4.9.3.1 Residual product removal and stratum corneum harvesting

The stratum corneum harvesting was performed directly on site 1 before the treatment application. Before stratum corneum harvesting from the treated skin site, the residual product was removed from the skin surface using a metal spatula and 3 independent dry cotton tips. The residual product was removed at 15, 30, 45 minutes from site 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 the blunt ended forceps to harvest the stratum corneum. The tape was removed using the forceps. The first adhesive tape was discarded as a result of the potential contamination from the residual product not removed with dry cotton wool. The remaining 9 adhesive tapes were applied sequentially using the same procedure described above. All the tapes were placed in a polypropylene tube and kept frozen at -20°C until further analysis for the lidocaine content. The product application and stratum corneum harvesting were performed by a single person. The forcep used for the application and removal of the tape was washed with water and wiped with 70% isopropyl alcohol between skin sites to avoid contamination. Pin prick testing was carried out at the stripping site to measure the degree of anaesthesia.

4.9.4 Pin prick procedure

Pin prick 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 onto the skin and rested gently on the skin surface. The 30g stimuli was to ensure the pressure was always the same for every pin-prick and the needle did not penetrate the skin (Dreher et al., 1998). The pin prick was performed at an adjacent untreated site, first followed by the treatment site. Pain scores after the pin prick test were recorded

using a Likert verbal rating score (VRS) and visual analogue score (VAS) (Tucker et al., 2006). In the VRS assessment, the subject was asked the following question: What did the pin prick feel like?, compared to an adjacent untreated site. The prick at the untreated site was considered sharp pain (category 4 and 100mm). The subject was provided with a choice of four categories: 1, no pain; 2, minimal sensation; 3, moderate pain; 4, sharp pain. The subject selected one answer for each time point by circling the relevant number. In the VAS assessment, a 100mm horizontal line with endpoints that are anchored by descriptors 'no pain' for 0mm and 'sharp pain' for 100mm was made. For each time point, the subject was asked the same question as above and then requested to make a vertical line across the tramline which represented the intensity or unpleasantness of their pain caused by the procedure. Values were measured in millimeters from the left of the tramline.

4.10 Analytical

Lidocaine concentration from plasma and DMD samples were analysed by the validated LC-MS-MS method due to expected low concentration of lidocaine in plasma and the small volume of DMD samples (20μL). Therefore a highly sensitive analytical method such as LC-MS-MS is required for the above purposes. As the LCMS-MS method for analysis of lidocaine in plasma has been validated previously (Chapter 2), the re-validation procedures were performed on the assay by using Ringer solution as a matrix for the DMD samples.

On the other hand, tape samples contained an expectedly high concentration of lidocaine, for which analysis can be performed by various chromatography techniques with UV detection. For analysing the tape samples, we have developed a fast and simple capillary electrophoresis (CE) with UV detection method.

4.11 LC-MS-MS validation for analysis of DMD samples.

4.11.1 Experimentals

All of the chemicals, and the chromatographic systems used are similar as explained in the validation of LC-MS-MS method for the analysis of lidocaine in Chapter 2.

4.11.2 Assay Procedures

4.11.2.1 Preparation of stock solutions

4.11.2.1.1 Lidocaine Calibrator

A stock solution of lidocaine was prepared by dissolving 5.8mg of lidocaine hydrochloride in 100mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming purity 100%) 50µg/mL.

4.11.2.1.2 Lidocaine Quality Control (QC)

A stock solution of lidocaine was prepared by dissolving 5.8mg lidocaine hydrochloride in 100mL 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming purity 100.0%) 50µg/mL.

4.11.2.1.3 Bupivacaine (Internal Standard)

A stock solution of bupivacaine was prepared by dissolving 5.8mg of bupivacaine hydrochloride in 100mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming purity 99.9%) 50µg/mL. A working strength solution was prepared by diluting 0.2mL of the stock to 100mL with de-ionised water giving a final concentration of 100ng/mL.

All the above stock solutions were stored at approximately -20°C.

4.11.2.2 Ringer solution aliquots

4.11.2.2.1 Calibrator

0.5mL of lidocaine stock solution was pipetted into a 50mL volumetric flask and made up to the mark with ringer solution to produce a sub-stock (Cal. 1). Working calibration

solutions were prepared by diluting the sub stock with Ringer solution as tabulated in Table 4.1

Table 4.1 Working calibration concentrations with related dilution in Ringer solution

Volume of sub stock (Ringer)	Volume of ringer added	Total volume (Ringer)	Nominal concentration	Measured concentration	Inaccuracy	Cal. No.
(mL)	(mL)	(mL)	(ng/mL)	(ng/mL)	%	
0.02	19.98	20.0	0.5	0.5	0.0	8
0.04	19.96	20.0	1	1	0.0	7
0.4	19.6	20.0	10	10	0.0	6
1.0	19.0	20.0	25	25	0.0	5
2.0	18.0	20.0	50	50	0.0	4
4.0	16.0	20.0	250	250	0.0	3
10.0	10.0	20.0	500	500	0.0	2
0.5mL stock solution	49.5	50.0	500	500	0.0	1 (Sub stock)

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

4.11.2.2.2 Quality Control (QC) samples

0.5mL of the lidocaine stock for control was pipetted into a 25mL volumetric flask and made up to the mark with ringer solution to produce a sub-stock (QC 4, 1000ng/mL).Working controls were prepared by diluting the sub stock with Ringer solution as tabulated in Table 4.2.

Table 4.2 Working control concentrations with related dilution in ringer solution

Volume of sub stock (Ringer)	Volume of ringer solution	Total volume (Ringer)	Nominal concen-tration	Measured concen-tration	Inaccuracy	Cal No.
(mL)	(mL)	(mL)	(ng/mL)	(ng/mL)	%	
0.015	9.985	10.0	1.5	1.5	0.0	1 (low)
2.00	8.0	10.0	200.0	200.0	0.0	2 (medium)
4.50	5.5	10.0	450.0	450.0	0.0	3 (high)
0.5mL stock solution	24.5	25	1000	1000	0.0	4 (Sub stock)

4.11.2.3 Extraction

The same liquid-liquid extraction procedure as described in Chapter 2 was used to extract lidocaine from dialysate. However, the amount of sample used was only 10µL and the concentration of the internal standard used was 100ng/mL.

4.11.3 Validation procedures and results

4.11.3.1 Specificity

Six samples of blank ringer solution and six samples of ringer solution spiked with lidocaine and the internal standard were prepared and the extraction carried out. The concentration of lidocaine used was 0.5ng/mL (LLOQ) and the internal standard was 100ng/mL. No significant interfering peaks were found at the retention time of lidocaine and the internal standard. The signal to noise ratio for both lidocaine and the internal standard were both greater than 5. Figure 4.5 shows the chromatogram obtained from blank ringer solution spiked with 0.5ng/mL lidocaine and 100ng/mL bupivacaine.

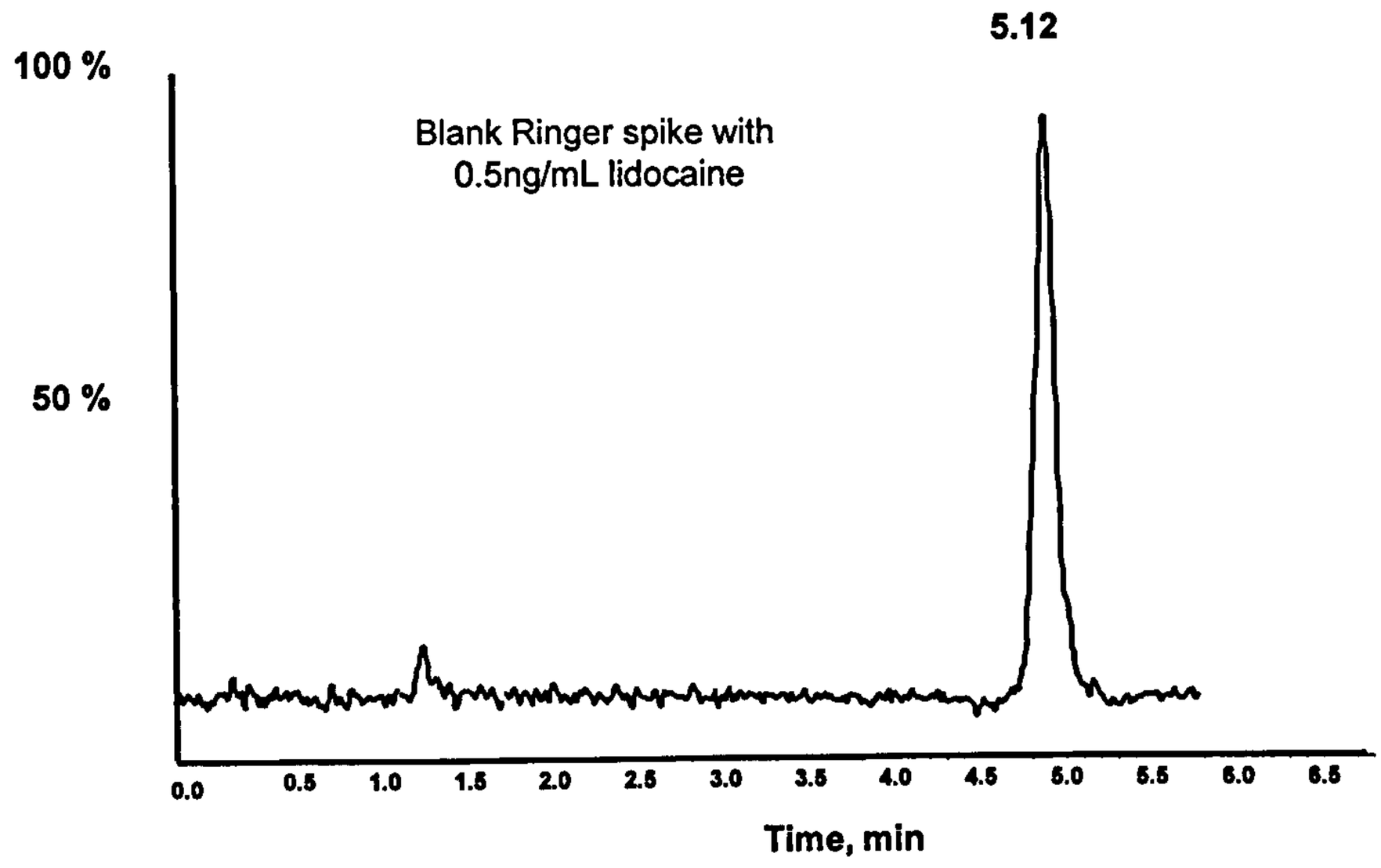


Figure 4.5 Chromatograms obtained from extracted blank ringer solution spiked with 0.5ng/mL lidocaine, and blank ringer spiked with 100ng/mL bupivacaine.

4.11.3.2 Calibrations

A calibration curve consists of eight non zero, calibrators assayed in duplicate (nominal values 0.5, 1.0, 10, 25, 50, 100, 250 and 500ng/mL). All of the results were calculated using a $1/x^2$ weighted quadratic 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. The regression coefficient for all the calibration curves were greater than 0.99. Mean results obtained from five curves are summarised in Table 4.3.

Table 4.3 Regression parameters for five calibration curves during validation

Batch	Curvature (C)	Slope (A)	Intercept (B)	r^2
1	7.18 E-7	0.00200	0.00160	0.9912
2	7.81 E-7	0.00152	0.00164	0.9910
3	9.00 E-7	0.00210	0.00330	0.9988
4	2.79 E-6	0.00234	0.00237	0.9991
5	6.63 E-7	0.00179	0.00139	0.9948

Second-order equation: $y = Cx^2 + Ax + B$

4.11.3.3 Inaccuracy and Imprecision,

Inaccuracy and imprecision were carried out as mentioned in Chapter 2. The concentrations of the quality control used were 1.5, 200, and 450ng/mL for QC1, QC2, and QC3, respectively. The concentrations for LLOQ and ULOQ were 0.5ng/mL and 500ng/mL, respectively.

4.11.3.3.1 Within-assay reproducibility

The CV % and the percentage for within assay imprecision and inaccuracy including LLOQ and ULOQ were all within the accepted range with ranging from 1.5 to 13.5% and 0 to 10 %, respectively (Table 4.4).

4.11.3.3.2 Between assay repeatability

The CV% and the percentage for between assay imprecision and inaccuracy including LLOQ and ULOQ were all within the accepted range with ranging from 1.6 to 10.7% and 0 to 3%, respectively (Table 4.5).

4.11.3.3.3 Nested ANOVA

From the nested Analysis of Variance, the within and between-batch and the total variability for all the QC samples including ULOQ and LLOQ were all below than 12 % (Table 4.6).

Table 4.4 Within assay imprecision and inaccuracy in ringer solution

No. of batches	Nominal conc.	Mean	SD	CV	Mean inaccuracy
	(ng/mL)	N=6		(%)	(%)
	0.5	0.50	0.06	12.00	0
	1.5	1.55	0.10	6.45	3
	200	186.54	10.56	5.66	-7
	450	460.35	26.49	5.75	2
	500	519.85	9.04	1.74	4
	0.5	0.52	0.05	9.62	4
	1.5	1.35	0.12	8.89	-10
	200	197.75	7.41	3.75	-1
	450	449.07	12.09	2.69	0
	500	529.35	7.08	1.34	6
	0.5	0.52	0.07	13.50	4
	1.5	1.45	0.10	6.90	-3
	200	199.58	5.56	2.79	0
	450	478.63	10.74	2.24	6
	500	515.50	7.83	1.52	3

Table 4.5 Between assay imprecision and inaccuracy in ringer solution

Nominal concentrations	Mean	SD	CV%	Mean accuracy
(ng/mL)	n=18		(%)	(%)
0.5	0.51	0.05	10.66	3
1.5	1.45	0.13	8.97	-3
200	194.62	9.66	4.96	-3
450	462.68	20.99	4.54	3
500	500.66	7.86	1.57	0

Table 4.6 Within and between batch variability from the nested analysis of variance (ANOVA).

Nominal concentration (ng/mL)	LLOQ 0.5	QC1 1.5	QC2 200	QC3 450	ULOQ 500
Mean (ng/mL); n = 15	0.51	1.49	193.98	464.12	502.16
SDw	-	0.05	-	7.66	1.29
SDb	0.06	0.09	10.40	19.04	7.68
SDt	0.06	0.10	10.40	20.52	7.78
CVw (%)	-	3.1	-	1.7	0.3
CVb (%)	11.3	5.9	5.4	4.1	1.5
CVt (%)	11.3	6.6	5.4	4.4	1.5

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

4.11.3.4 Extraction recovery

Absolute recovery of lidocaine was tested using Ringer solutions spiked with lidocaine at the same nominal concentration as the quality control samples. The absolute recovery of lidocaine and bupivacaine ranged from 81 to 91% and 94%, respectively. All of the values are summarised in Table 4.7.

4.11.3.5 Microdialysis probe recovery

Three DMD probes (probe 1, 2, and 3) were placed in three eppendorf tubes containing 1.5ng/mL lidocaine in ringer solution. Using the DMD pump, the blank ringer solution was perfused through the probe and the dialysate was collected every 20 minutes for 80 minutes and labelled sample 0, 1, 2, and 3. The first 20 minute sample (sample 0) was not used in the analysis. The probes were then transferred into another eppendorf tube containing 200ng/mL lidocaine and the above procedures were repeated and finally with 450ng/mL lidocaine. The samples were analysed for their lidocaine concentration. Relative Recovery (RR) was calculated by the following formula:

$$RR = \left(\frac{Cout}{Cm}\right) \times 100\%$$

Cout = Drug concentration in the dialysate

Cm = Drug concentration in the medium

The mean percentage probe recovery for low concentration (1.5ng/mL) is higher compared to medium (200ng/mL), and high concentrations (450ng/mL) with the values of 94, 83, and 74%, respectively. The percentage recoveries were found to decrease when the concentration increased. All of the values are summarised in Table 4.8. The analysis of variance for the within, between and total probe variabilities found there to be higher variability for low concentrations compared to medium and high. Table 4.9 summarises the within, between and the total probe variability obtained from the nested analysis of variance (ANOVA).

Table 4.7 Percentages of lidocaine and bupivacaine recovery from ringer solution

Lidocaine				Bupivacaine			
Nominal concentrations	Mean area		Recovery	Nominal concentrations	Mean area		Recovery
	Extracted samples	Non extracted samples			Extracted samples	Non extracted samples	
(ng/mL)	Peak area	Peak area	(%)	(ng/mL)	Peak area	Peak area	(%)
1.5	34416	37803	91				
200	2938722	3623634	81	100	9111822	9700436	94
450	6435345	7161796	90				

Table 4.8 Percentages of in vitro DMD probe recovery

Probe	Nominal concentration					
	1.5 ng/mL		200 ng/mL		450 ng/mL	
	Mean dialysate concentration	Recovery	Mean dialysate concentration	Recovery	Mean dialysate concentration	Recovery
	(ng/mL)	(%)	(ng/mL),	(%)	(ng/mL)	(%)
Probe 1	1.5	100.0	189.3	94.7	336.5	74.8
Probe 2	1.3	84.4	156.2	78.1	338.3	75.2
Probe 3	1.5	98.0	154.6	77.3	323.9	72.0
Mean	1.41	94.1	166.7	83.4	332.9	74.0
SD	0.13	8.5	19.6	9.8	7.6	1.7

Table 4.9 Within and between probe variability from the nested analysis of variance (ANOVA)

	Low concentration	Medium concentration	High concentration
Nominal concentration (ng/mL)	1.5	200	450
Mean observed concentration (ng/mL)	1.41	166.72	332.91
SDw	-	18.34	-
SDb	0.30	11.98	29.20
SDt	0.30	21.90	29.20
CVw (%)	-	11.0	-
CVb (%)	21.5	7.2	8.8
CVt (%)	21.5	13.1	8.8

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

4.12 Validation and application of capillary electrophoresis for the analysis of lidocaine in DPK study.

4.12.1 Introduction

Various techniques are available to measure lidocaine in plasma such as HPLC with UV detection, LC-MS-MS, and GCMS, as explained in Chapter 2. Most of the analysis of lidocaine (Padula et al., 2003) and other compounds (Pershing et al., 2003;Weigmann et al., 1999) from tape samples has been done by using HPLC with UV detection. The drug contents in the tape obtained from a DPK study is normally higher than in plasma especially for local anaesthetic studies. Therefore, the high sensitivity techniques such as LC-MS-MS or GCMS are not required since UV detection is sufficiently sensitive to detect such levels of a drug. Moreover, the cost of running analysis by UV detection is less compared to MS detection.

On the other hand, capillary electrophoresis (CE) is an alternative to the chromatography techniques used in drug analysis which can separate a variety of compounds by using an electric field. The separation of the compounds by electrophoresis in CE is based on the differences in electrophoretic mobility and the voltage applied. Like HPLC, CE also can be coupled with a UV detector (CE-UV) and MS (CE-MS) forming a powerful analytical method for drug analysis. Capillary electrophoresis with UV and MS detection for the analysis of lidocaine in pharmaceutical formulations have been reported previously (Geiser et al., 2003;Geiser et al., 2005;Wang et al., 2001). In this study, we have performed a method validation and CE analysis of lidocaine in tape samples obtained from a DPK study in healthy subjects.

4.12.2 Experimentals

4.12.2.1 Chemicals

Lidocaine Hydrochloride (99.9% purity) and Procaine Hydrochloride (99.9% purity) (internal standard) were obtained from Sigma-Aldrich Company, Poole, UK. The chemical structure of lidocaine and procaine are shown in Figure 4.6. 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.

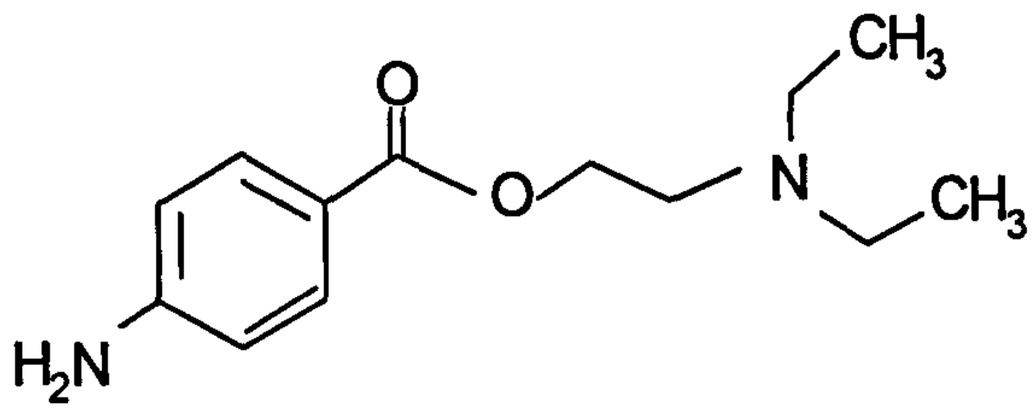


Figure 4.6 Chemical structure of procaine

4.12.2.2 Adhesive tape

Tesa 4204 PV5, Beiersdorf, Hamburg Germany supplied by TESA UK ltd. Tape dimension was 19mm widths, and the backing material is polypropylene.

4.12.2.3 Background electrolyte

The aqueous background electrolyte used in this analysis was 100mM Tris-phosphate buffer at pH 2.5. To prepare this solution, 20mL phosphoric acid (0.5M) was diluted with 80mL water and 15mL Tris (0.5M) was added to obtain a solution at pH 2.5. The solution was topped up to 100mL with deionised water.

4.12.2.4 CE instrumentation and capillaries

Separations were carried out on an Agilent ^{3D}CE G1600AX capillary electropherograph (Agilent, West Lothian, UK) controlled by Chemstation B.02.01 (Agilent, West Lothian, UK). 350mm (265mm to window) x 50µm i.d., fused silica capillaries (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 minutes. Pre-conditioning on injection was 3 minutes flush with 0.1M HCl (BDH, Poole, UK), then a 2 minute flush with background electrolyte (BGE). Separation was at a potential difference of +25.0kV. The capillary was thermostated at 25.0°C. Detection was by photodiode-array over 195 - 300nm, but 200nm with bandwidth 6nm, was used for quantitation. Run time was 4 min. Both BGE vials were replenished every 12 injections. All samples and standards in the autosampler were kept at ambient temperature. The injections of the samples into the system were carried out hydrodynamically for 12 seconds at 50mBar.

4.12.2.5 Assay Procedures

4.12.2.5.1 Preparation of stock solutions

A stock solution of lidocaine and procaine was prepared in 50% methanol. All of the sub-stock solutions were stored at -20°C. All the calibrators and QC sample concentrations were prepared by appropriate dilution of the sub-stock.

Lidocaine Calibrator

A stock solution of lidocaine was prepared by dissolving 2900mg of lidocaine hydrochloride in 50mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming a purity of 100%) 50mg/mL.

Lidocaine Quality Control (QC)

A stock solution of lidocaine was prepared by dissolving 1450mg of lidocaine hydrochloride in 25mL of 50% methanol. The concentration of the stock solution was (after correcting for the salt and assuming a purity of 100.0%) 50.0mg/mL.

Procaine (Internal Standard)

A stock solution of procaine was prepared by dissolving 139.2mg procaine hydrochloride in 1 L deionised water. The concentration of the stock solution was (after correcting for the salt and assuming purity 99.9%) 120µg/mL.

The stock solutions for lidocaine calibrator and quality control were stored at approximately -20°C.

Working standard solutions

Calibrator

20mL of the lidocaine stock solution was pipetted into a 100mL volumetric flask and made up to the mark with 50% methanol to produce a sub-stock (Cal 1). Working calibration solutions were prepared by diluting the sub stock with 50% methanol as tabulated in Table 4.10.

Table 4.10 Working calibration concentrations with related dilutions in 50% methanol

Volume of sub stock	Volume of 50% methanol	Total volume	Nominal concentration	Measured concentration	Inaccuracy	Cal No.
(mL)	(mL)	(mL)	(mg/mL)	(mg/mL)	%	
1.25	23.75	25.0	0.5	0.5	0.0	6
2.50	22.50	25.0	1.0	1.0	0.0	5
6.25	18.75	25.0	2.50	2.5	0.0	4
12.50	12.50	25.0	5.0	5.0	0.0	3
18.75	6.25	25.0	7.5	7.5	0.0	2
20mL stock solution	80.00	100	10	10.0	0.0	1 (Sub stock)

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

Quality Control (QC) samples

10mL of the lidocaine 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, 20mg/mL).Working controls were prepared by diluting the sub stock with 50% methanol as tabulated in Table 4.11.

Table 4.11 Working control concentrations with related dilutions in 50% methanol

Volume of sub stock	Volume of 50% methanol	Total volume	Nominal concentration	Measured concentration	Inaccuracy	Cal No.
(mL)	(mL)	(mL)	(mg/mL)	(mg/mL)	%	
0.8	19.2	20	0.8	0.8	0.0	1 (Low)
4.0	16.0	20	4.0	4.0	0.0	2 (Medium)
8.0	12.0	20	8.0	8.0	0.0	3 (high)
10mL stock solution	15	25	20.0	20.0	0.0	4 (sub stock)

4.12.2.5.2 Preparation of tape samples

For the purpose of validation and assay calibration, a length of self adhesive tape (Tesa 4204 PV5) was cut into 10 samples so that each tape was approximately 3 x 2cm. 100µL volume of standard or quality control solution were spiked on the adhesive part of the tape. The solution was distributed approximately evenly on all of the tapes. The samples were left to dry naturally at room temperature. This required, approximately 30 minutes. The samples were then transferred into 10mL polypropylene tubes and stored frozen at -20°C until analysis. Samples were obtained from patients by applying the tape (3 x 2cm) on the skin and stripped off by using forceps. The procedure was repeated 10 times to obtain 10 samples at each skin site. The samples were placed in 10mL polypropylene tubes and stored frozen at -20°C until analysis.

4.12.2.5.3 Extraction procedure

5mL of methanol was dispensed into the tube containing standard/QC tape or samples. The contents were mixed for 45 minutes by using a vortex mixer. 5mL of internal standard solution containing 120µg/mL procaine in water was added to the tube to make a 50% methanol solution. The contents were mixed again by a vortex mixer for another

15 minutes. A 200µL quantity of the solution was transferred into 250µL auto injector vial for CE analysis.

4.12.3 Validation procedures and results

4.12.3.1 Specificity

Six samples of blank tape and six samples of tape spiked with lidocaine were prepared and the extraction was carried out. The amount of lidocaine spike was 50µg (LLOQ) and the internal standard added was 600µg. No significant interfering peaks were found at the retention time of lidocaine or procaine. The signal to noise ratio for both drugs were greater than 10.

Figure 4.7 shows the electropherogram obtained from blank tape spiked with 50µg lidocaine and added IS of 600µg procaine. Whilst

Figure 4.8 and Figure 4.9 shows the electropherograms of EMLA cream solution and one of the tape samples, respectively.

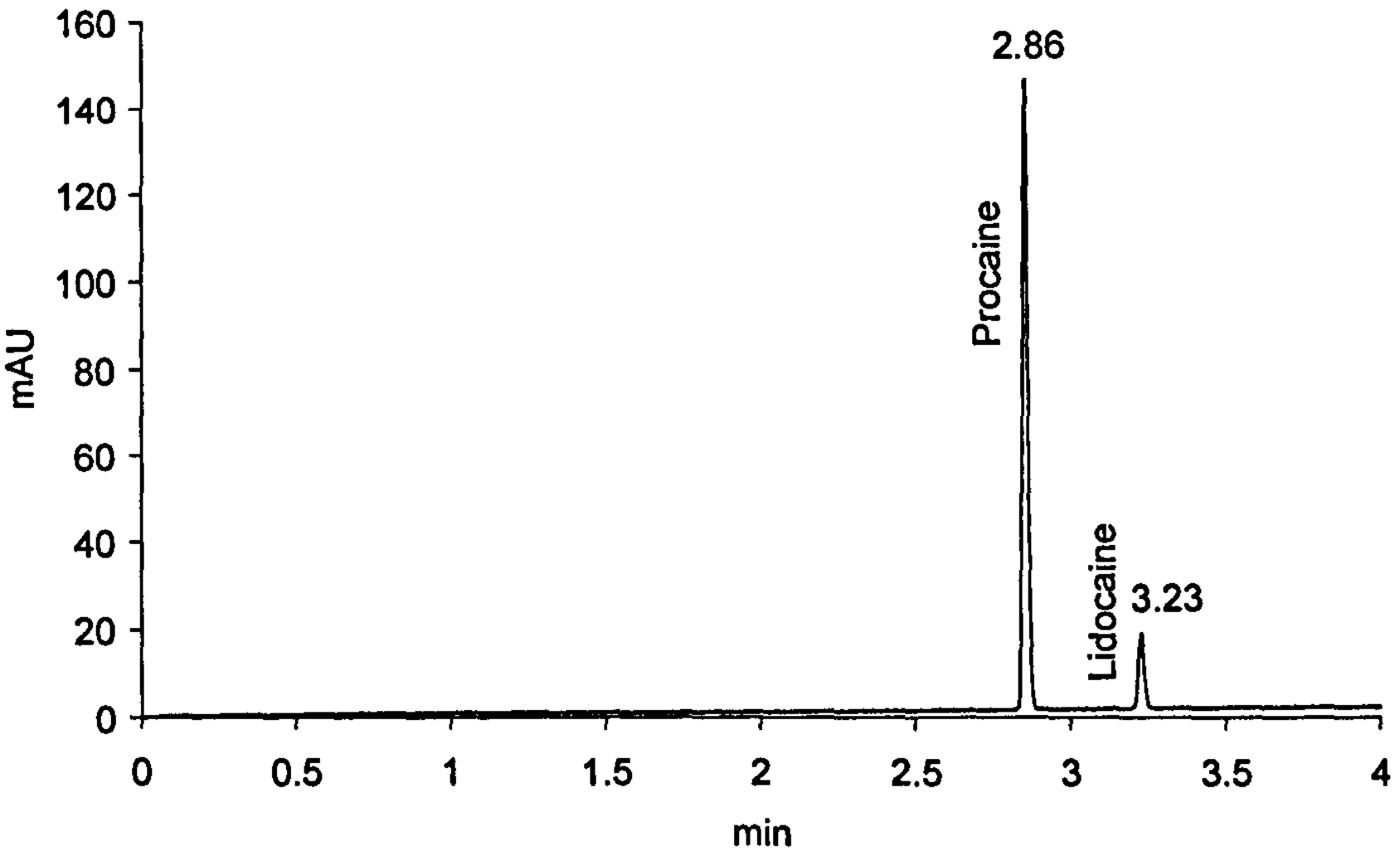


Figure 4.7 Electropherogram obtained from extracted tape sample spiked with 50µg lidocaine and added IS of 600µg procaine.

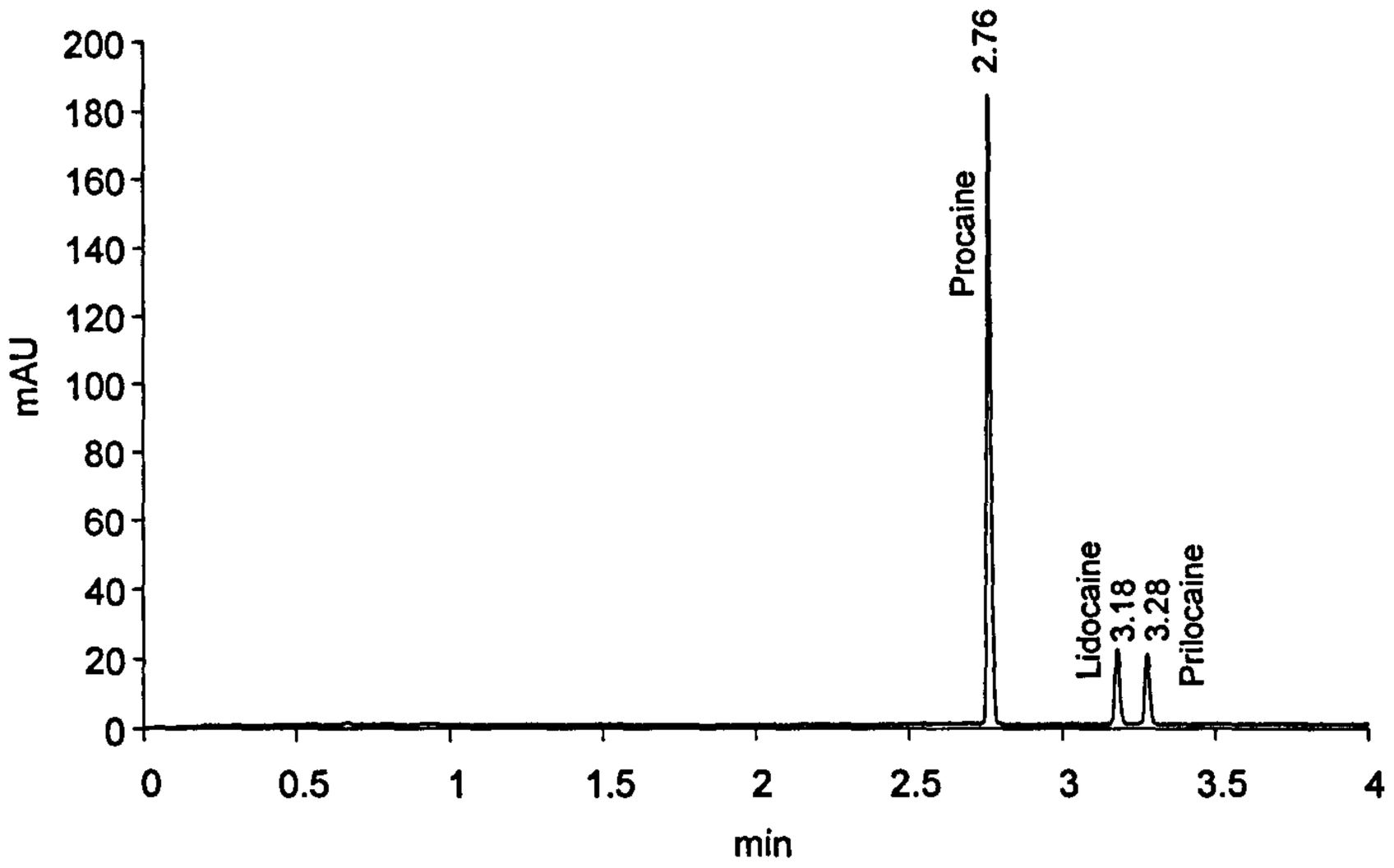


Figure 4.8 Electropherogram obtained from EMLA cream solution containing 50µg lidocaine and prilocaine and added IS of 600µg procaine.

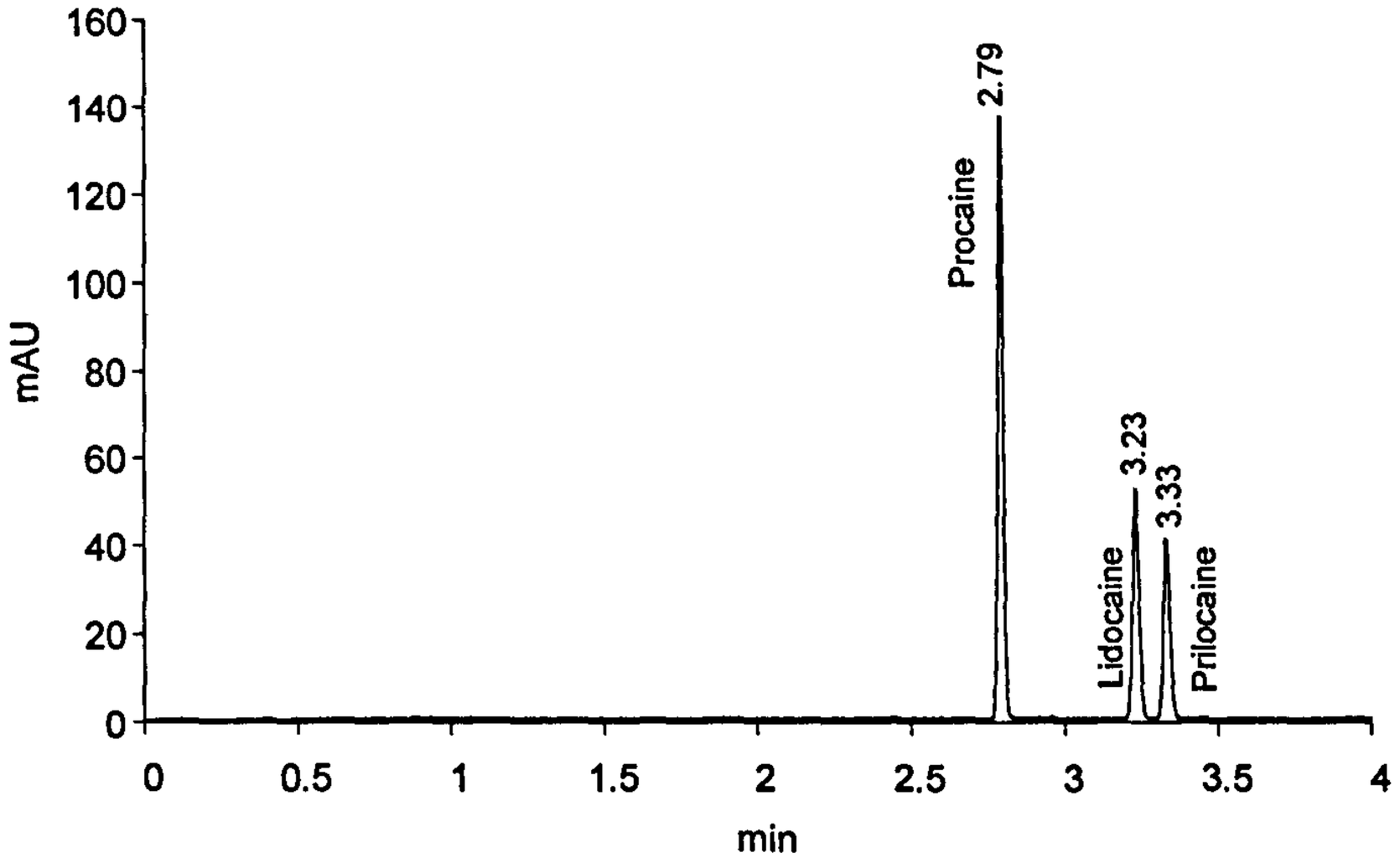


Figure 4.9 Electropherogram obtained from tape sample at 4 hours post dose with added IS of 600µg procaine.

4.12.3.2 Calibration curve/ linearity

The calibration curve consists of six non-zero calibrators with nominal values of 50, 100, 250, 500, 750, and 1000µg and 600µg procaine as internal standard (IS). Five batches of calibration curves were prepared for validation purposes. The calibration curve was plotted using the area ratio of lidocaine to IS versus known concentrations of lidocaine. All of the results were calculated using a $y = Ax + B$ linear regression. The regression coefficient for all of the calibration curves obtained were greater than 0.99. Regression parameters obtained from five curves are summarised in Table 4.12

Table 4.12 Regression parameters for five calibration curves during validation.

Batch	Slope (A)	Intercept (B)	r^2
1	0.0257	0.0867	0.9986
2	0.0262	0.0816	0.9974
3	0.0255	0.0995	0.9967
4	0.0257	0.0661	0.9997
5	0.0240	0.1232	0.9914

Linear equation: $y = Ax + B$

4.12.3.3 Inaccuracy and Imprecision

The method for inaccuracy and imprecision were the same as mentioned in Chapter 2. The nominal values for low, medium, and high control samples were 80, 400 and 800µg, respectively. The concentration for LLOQ and ULOQ were 50 and 1000µg, respectively.

4.12.3.3.1 Within-assay reproducibility

The CV for imprecision and the percentage of inaccuracy for all the quality control samples including LLOQ and ULOQ were below than 2% and 14%, respectively (Table 4.13).

4.12.3.3.2 Between-assay repeatability

The CV for imprecision and the percentage of inaccuracy for all of the quality control samples including LLOQ and ULOQ were below than 2% and 11%, respectively (Table 4.14).

4.12.3.3.3 Nested ANOVA

From the nested Analysis of Variance, the within and between-batch and the total variability for all of the QC samples including ULOQ and LLOQ were all below than 7% (Table 4.15).

Table 4.13 Within assay imprecision and inaccuracy in tape samples.

No. of batches	Nominal concentration	Mean n=6	SD	CV	Mean inaccuracy
	(µg)	(µg)		(%)	(%)
1	50	44.93	0.65	1.45	-10
	80	85.82	0.45	0.52	7
	400	428.04	2.46	0.58	7
	800	830.69	1.88	0.23	4
	1000	1025.33	6.44	0.63	3
2	50	47.42	0.47	0.99	-5
	80	78.83	0.35	0.45	-1
	400	410.58	2.07	0.50	3
	800	807.11	1.95	0.24	1
	1000	970.58	2.10	0.22	-3
3	50	42.85	0.74	1.74	-14
	80	81.76	0.38	0.46	2
	400	401.67	0.82	0.21	0
	800	827.38	1.26	0.15	3
	1000	1043.12	7.25	0.69	4

Table 4.14 Between assay imprecision and inaccuracy in tape samples

Nominal concentration	Mean n=18	SD	CV	Mean inaccuracy
(µg)	(µg)		(%)	(%)
50	44.41	0.58	1.31	-11
80	82.01	0.49	0.59	3
400	413.43	2.12	0.51	3
800	821.73	1.94	0.24	3
1000	1005.27	5.62	0.56	1

Table 4.15 Within and between batch variability from the nested analysis of variance (ANOVA).

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal concentration (µg)	50.0	80.0	400.0	800.0	1000.0
Mean ; n =18 (µg)	45.1	81.9	415.0	822.4	1013.0
SDw	-	0.31	0.11	-	2.78
SDb	0.06	0.39	2.28	2.13	5.73
SDt	0.06	0.50	2.28	2.13	6.37
CVw (%)	-	3.8	0.3	-	2.7
CVb (%)	1.4	4.7	5.5	2.6	5.7
CVt (%)	1.4	6.1	5.5	2.6	6.3

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

4.12.3.4 Recovery

Absolute recovery of lidocaine was tested using tape samples spiked with lidocaine at the same nominal concentrations as the quality control samples. Peak area measurements obtained from the extracted samples were compared to the peak area measurements obtained from direct solvent injection of the test compounds. Mean and standard deviation were calculated from at least three measurements at each level. The absolute recovery of lidocaine ranged from 97 to 103%.

4.13 Results

Twelve healthy subjects successfully completed the study according to the protocol. All of the samples obtained from DPK study were successfully analysed by the validated CE-UV method whilst plasma and DMD samples were analysed by the validated LC-MS-MS method.

4.13.1 Lidocaine concentration profile and pharmacokinetic parameters

The plots of mean lidocaine concentration versus time obtained from the analyses of DPK, DMD and plasma, are shown in Figure 4.10, and Figure 4.12, respectively. Figure 4.13 shows all the plots for DPK, DMD and plasma plotted on the log scale. From all of the plots, we can see clearly that the lidocaine profile is higher in stratum corneum by 1000 fold compared to dialysate and 100 000 fold compared to plasma. Lidocaine was detected in the stratum corneum at 15 minutes after dosing but was not detected until 1.5 hours in the plasma and at 1.3 hours post-dose in the dialysate.

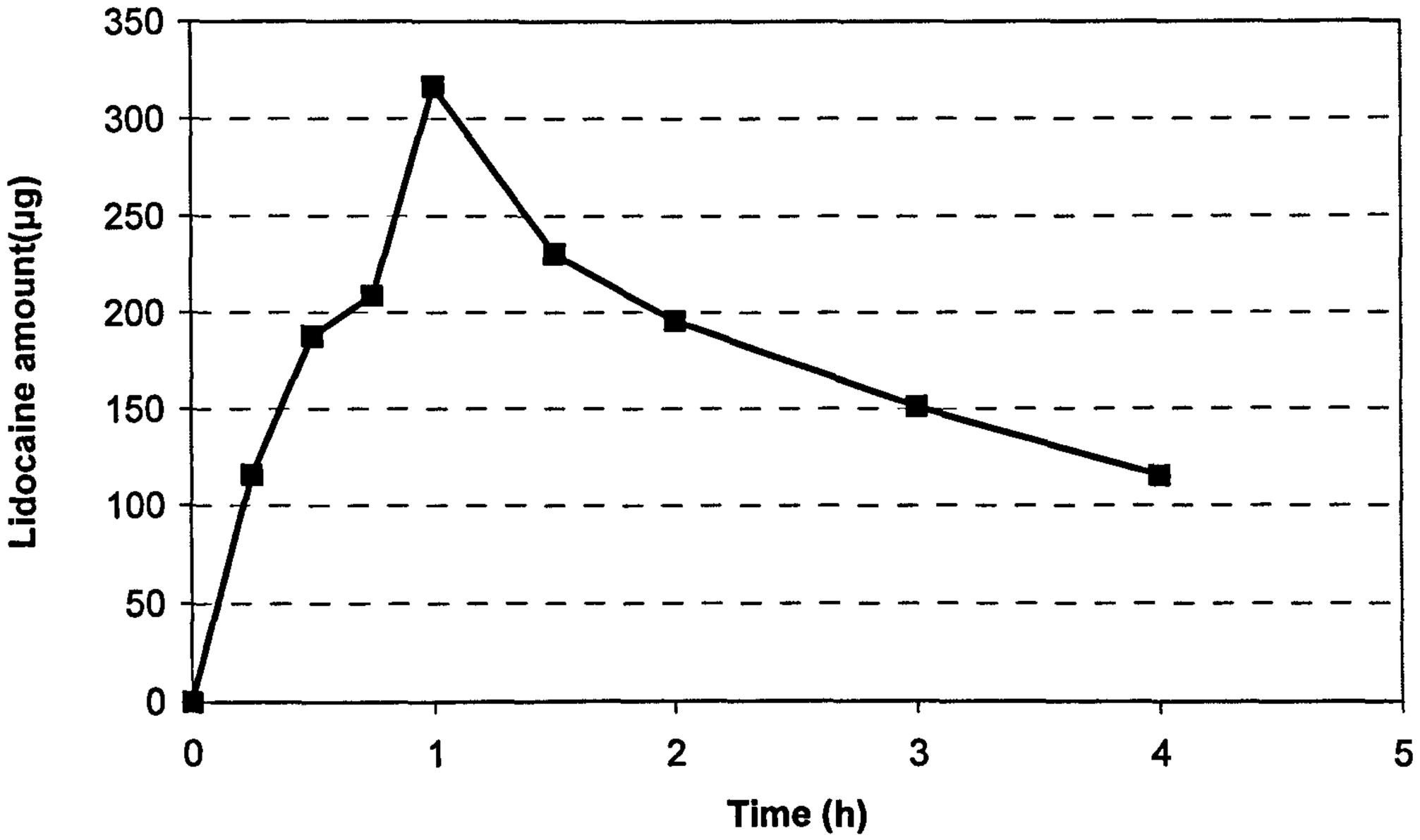


Figure 4.10 Plot of mean lidocaine concentration (µg) versus time (h) in tape samples obtained from DPK study in 12 healthy subjects.

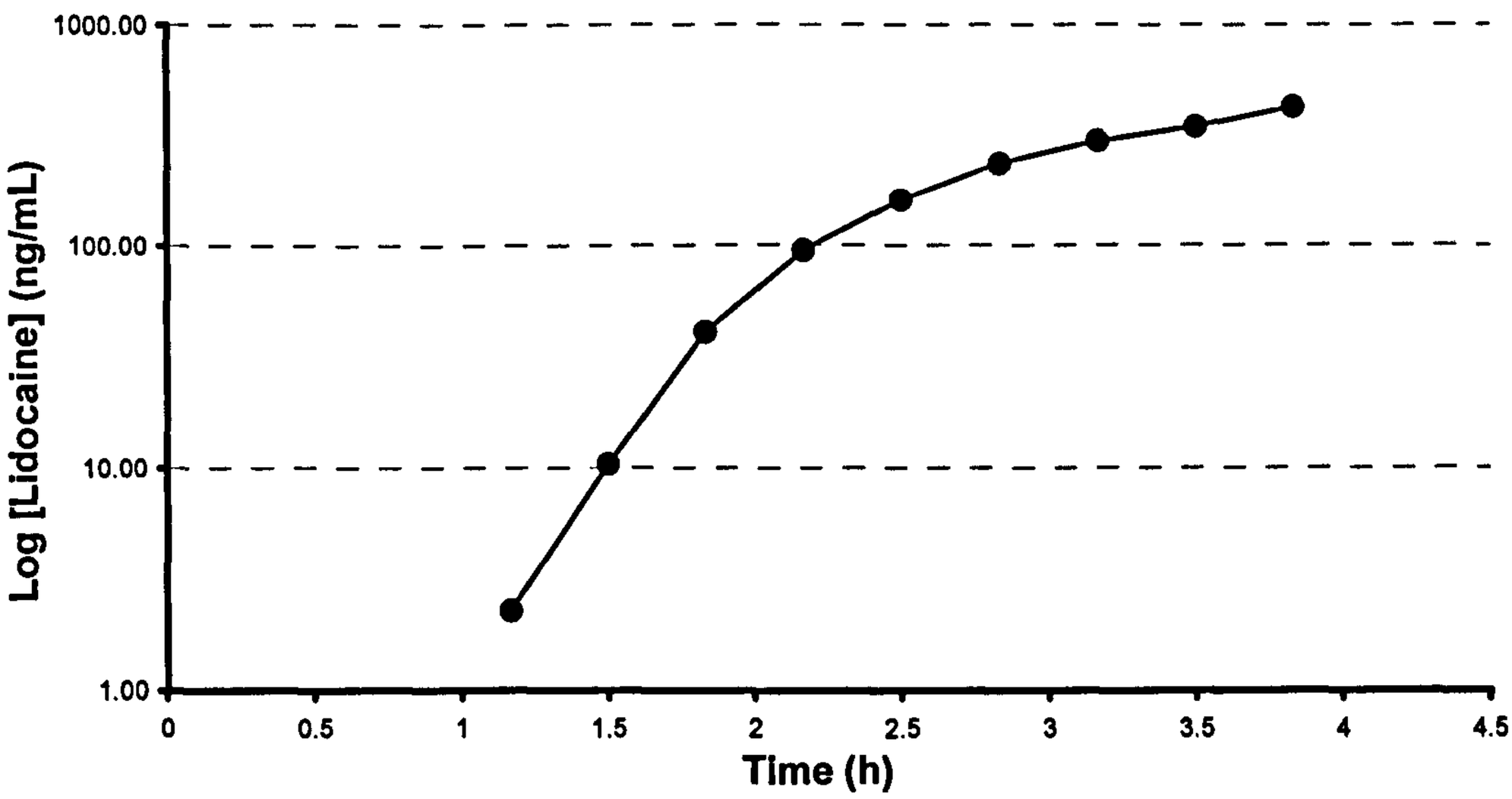


Figure 4.11 Plot of mean lidocaine concentration (ng/mL) versus time (h) in DMD samples obtained from DPK study in 12 healthy subjects.

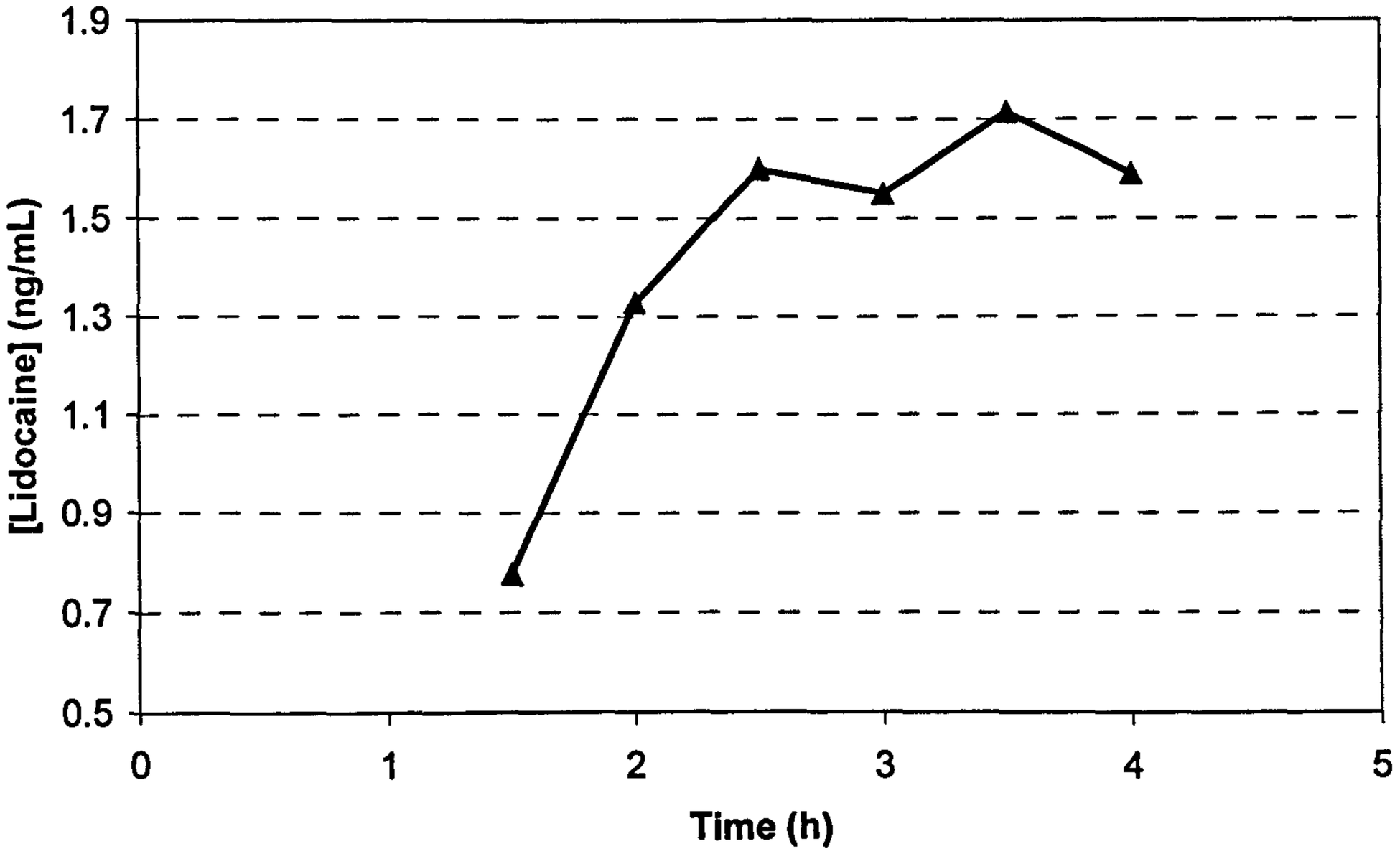


Figure 4.12 Plot of mean lidocaine concentration (ng/mL) versus time (h) in plasma samples obtained from DPK study in 12 healthy subjects.

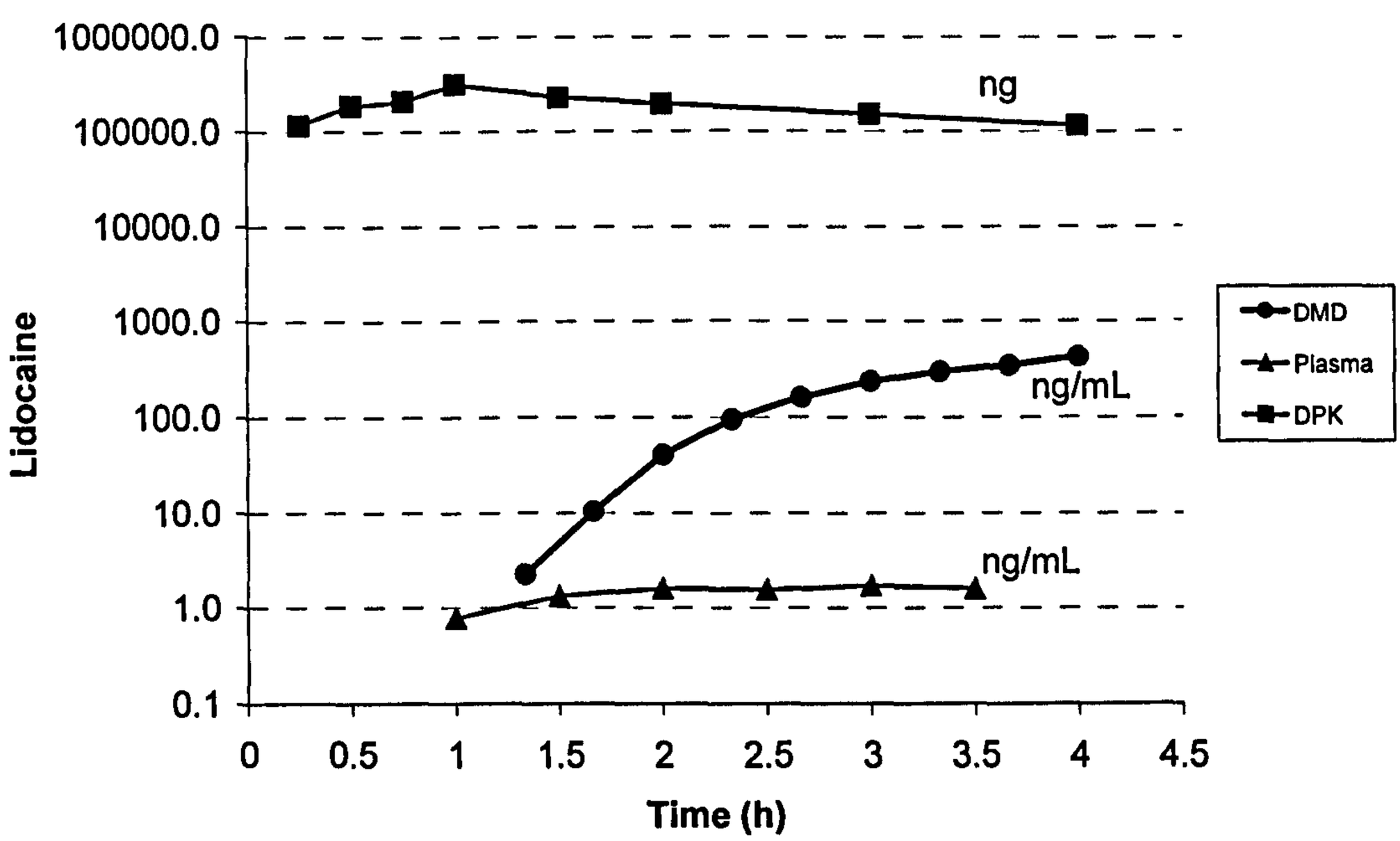


Figure 4.13 Plot of mean lidocaine concentration versus time (h) in tape, dialysate, and plasma samples on log scales

The analysis of the pharmacokinetic parameters of lidocaine showed the higher AUC and C_{max} in DPK compared to DMD and plasma (Mean AUC_{0-4}: 712.3µg.h, 458 and 4.1ng/mL.h: Mean C_{max} (0-4), 320.8µg, 426 and 2.5ng/mL, respectively). Table 4.16 and Table 4.17 show the AUC and C_{max} values of lidocaine in 12 healthy subjects obtained from DPK, DMD and plasma studies.

The lidocaine amount in stratum corneum soon achieving the maximum concentration compared to dialysate and plasma. The median (IQR) t_{max} was shorter in the stratum corneum, 1 (0.0) hour, compared to plasma 3.5 (0.5) hours. The different was statistically significant (Wilcoxon's Signed Rank test; $p = 0.003$). The t_{max} for dialysate cannot be predicted as the concentration of lidocaine was still increasing in value at the last sampling point (4 hours post dose) in almost all of the subjects. Therefore, the terminal half life ($t_{1/2}$) cannot be calculated for DMD. The $t_{1/2}$ was calculated using the following formula: $t_{1/2} = 0.693/K_{el}$, where K_{el} is the elimination rate constant that can be calculated from the terminal phase slope. K_{el} for DPK was calculated from at least 3 terminal phase concentration points and from 2 to 4 points in plasma. The mean $t_{1/2}$ obtained from DPK were not significantly different to the mean value calculated from plasma with 2.5 hours and 1.9 hours, respectively (paired 't' test test; $p = 0.337$). Table 4.18 and Table 4.19 show the t_{max} and $t_{1/2}$ values of lidocaine in 12 healthy subjects obtained from DPK and plasma studies.

Table 4.16 AUC_{0-4} of lidocaine with mean, SD and CV% obtained from DPK, DMD and Plasma in 12 healthy subjects

Subject	DPK	DMD	Plasma
	(μg.h)	(ng/mL.h)	(ng/mL.h)
1	1155.67	123.40	4.12
2	688.24	431.48	7.03
3	650.77	101.68	0.72
4	431.20	2284.60	2.35
5	575.55	174.18	4.09
6	700.65	535.50	3.31
7	1180.61	1298.47	7.22
8	730.69	57.70	2.41
9	647.93	264.02	3.25
10	399.65	21.45	3.77
11	911.48	62.83	3.73
12	475.13	136.69	6.72
Mean	712.30	457.67	4.06
SD	255.18	675.34	2.00
CV %	36%	148%	49%

Table 4.17 C_{max} (0-4) of lidocaine with mean, SD and CV% obtained from DPK, DMD and Plasma in 12 healthy subjects

Subject	DPK (μg)	DMD (ng/mL)	Plasma (ng/mL)
1	486.88	272.60	2.69
2	296.60	450.50	3.22
3	281.51	123.30	0.57
4	194.53	2006.40	1.33
5	311.22	249.70	7.95
6	344.92	436.80	1.72
7	424.77	1044.80	2.99
8	334.66	88.40	1.61
9	359.47	239.10	1.64
10	222.20	10.00	1.53
11	347.43	105.80	1.78
12	244.98	85.10	3.42
Mean	320.76	426.04	2.54
SD	82.58	569.55	1.91
CV %	26%	134%	75%

Table 4.18 t_{max} of lidocaine with mean, SD and CV% obtained from DPK and Plasma in 12 healthy subjects

Subject	DPK (h)	Plasma (h)
1	1.0	2.5
2	1.0	3.0
3	1.5	3.5
4	1.0	3.5
5	1.0	3.0
6	1.0	3.5
7	1.0	3.0
8	1.0	4.0
9	1.0	3.5
10	1.0	3.5
11	1.0	3.5
12	1.0	4.0
Median	1.0	3.5
IQR	0.0	0.5

Table 4.19 $t_{1/2}$ of lidocaine with mean, SD and CV% obtained from DPK and Plasma in 12 healthy subjects

Subject	DPK (h)	Plasma (h)
1	1.5	2.0
2	4.6	1.0
3	3.1	0.8
4	1.4	1.4
5	1.5	4.1
6	3.5	1.6
7	3.6	1.7
8	3.1	NA
9	1.6	2.8
10	1.8	1.2
11	2.6	NA
12	2.4	NA
Mean	2.6	1.9
SD	1.0	1.0
CV %	40%	56%

The AUC and C_{max} values obtained from the DPK study also showed low variability compared to plasma and very high variability for DMD (CV%; AUC_{0-4}, 36, 49, and 148%: C_{max} (0-4), 26, 75 and 134%, respectively).

Figure 4.14 and Figure 4.15 show the plot of Test for Equal Variances (Bartlett's and Levene's test) for the AUC and the C_{max} from all the studies. The plots clearly showed the wider confidence interval for the DMD compared to plasma and DPK for the AUC and C_{max}, meaning the variability is higher in the DMD results compared to plasma and DPK (Bartlett's test, $p = 0.000$).

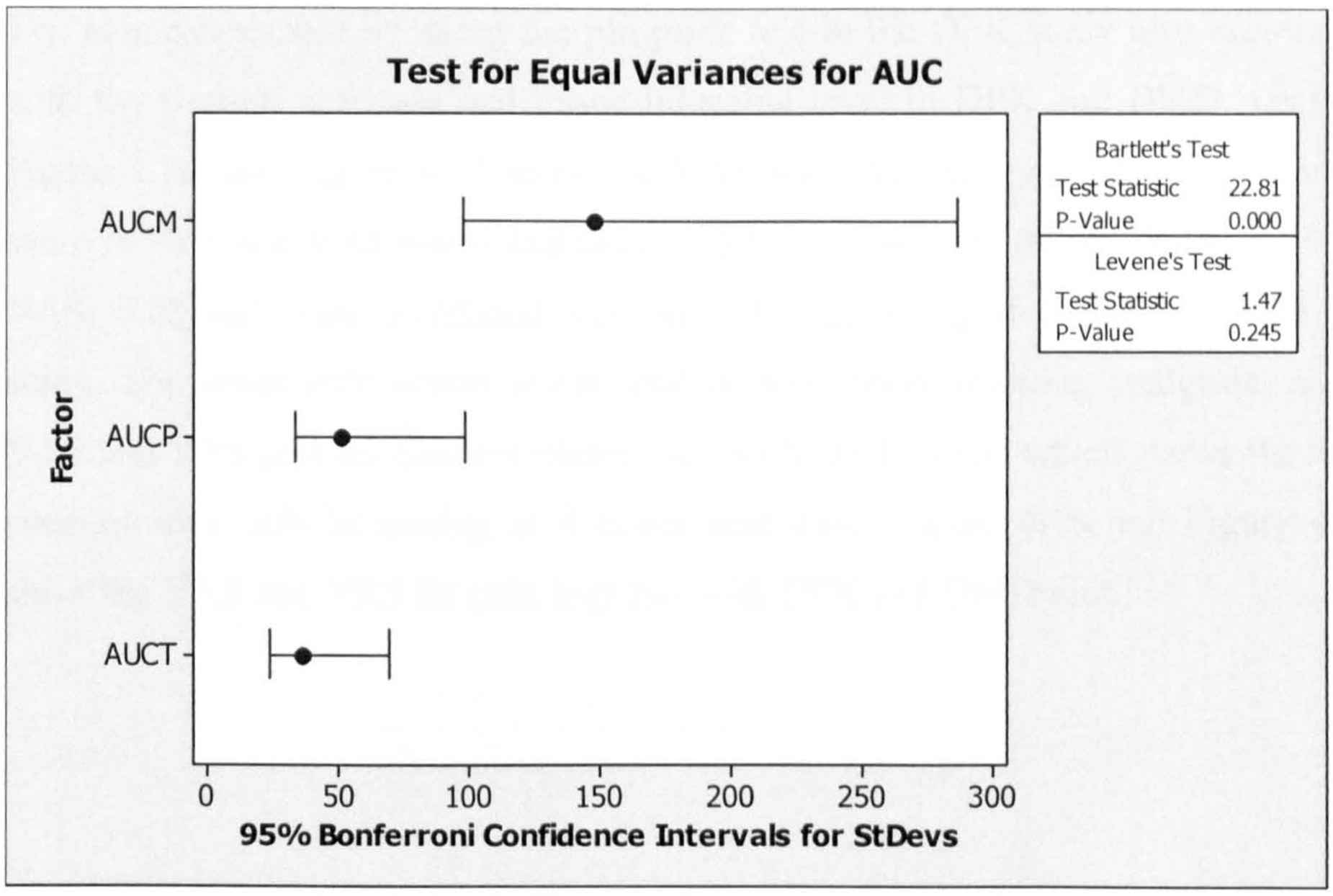


Figure 4.14 Plot of Test for Equal Variance with 95% confidence interval (AUCT= AUC DPK, AUCP = AUC plasma, and AUCM = AUC microdialysis

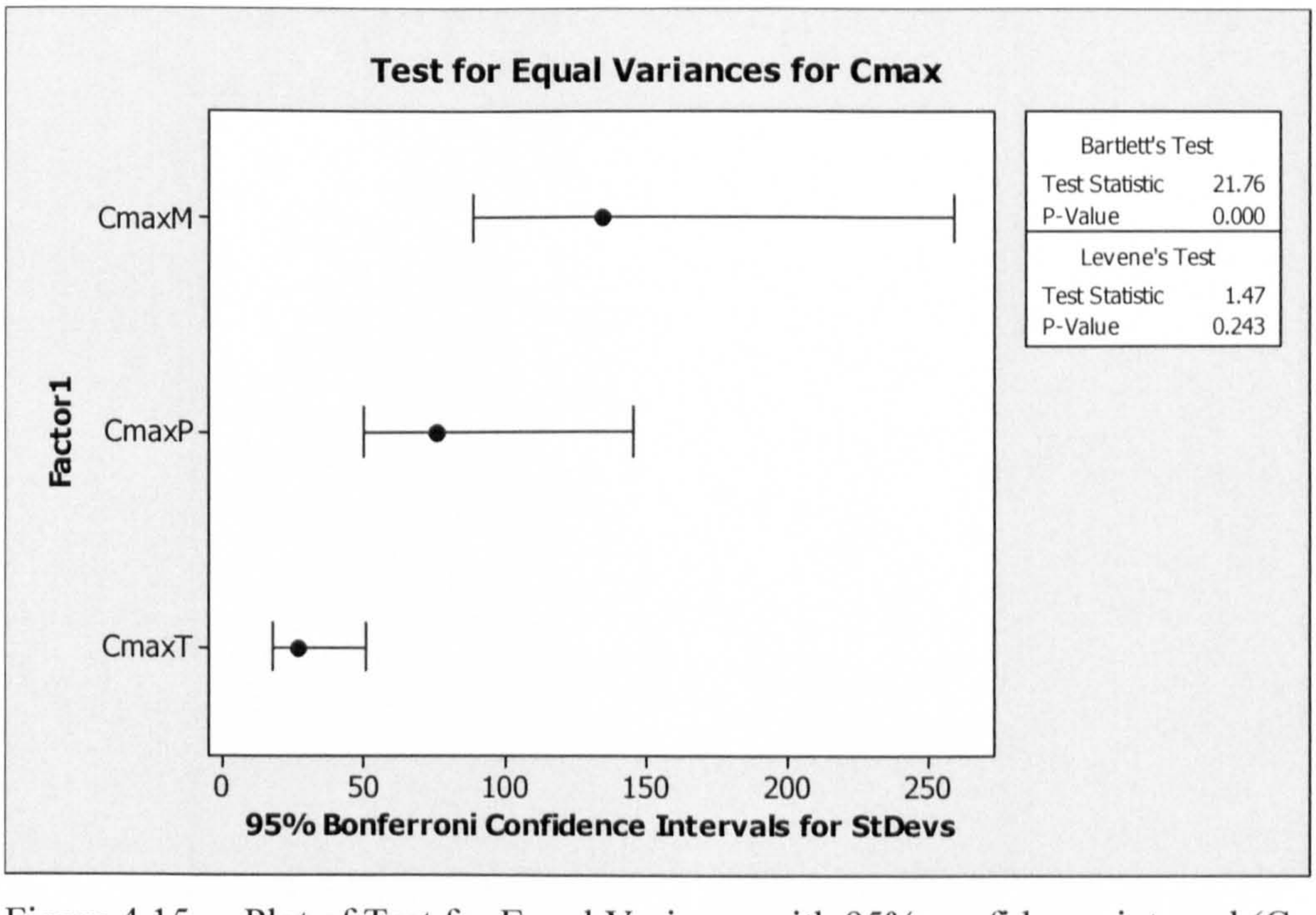


Figure 4.15 Plot of Test for Equal Variance with 95% confidence interval ($C_{max}T$= C_{max} DPK, $C_{max}P$ = C_{max} plasma, and $C_{max}M$ = C_{max} microdialysis)

The pain assessment by using the pin prick test in the DPK study also correlated well with the stratum corneum and tissue lidocaine level in DPK and DMD, respectively. Figure 4.16 and Figure 4.17 show the VAS and VRS for pain plots. The lower pain score in VRS and VAS were obtained at 1.5 hours post dose (Mean, VRS; 1.17 unit and VAS; 9.08mm) were correlated well with the mean t_{max} at 1 hour obtained in DPK study. The lower pain scores maintained up to 4 hours (prolong analgesia) as seen in VAS and VRS plot are also correlated well with DMD result which shows the lidocaine concentration still increasing at 4 hours post dose. Figure 4.18 and Figure 4.19 and show the VAS and VRS for pain together with DPK and DMD plot.

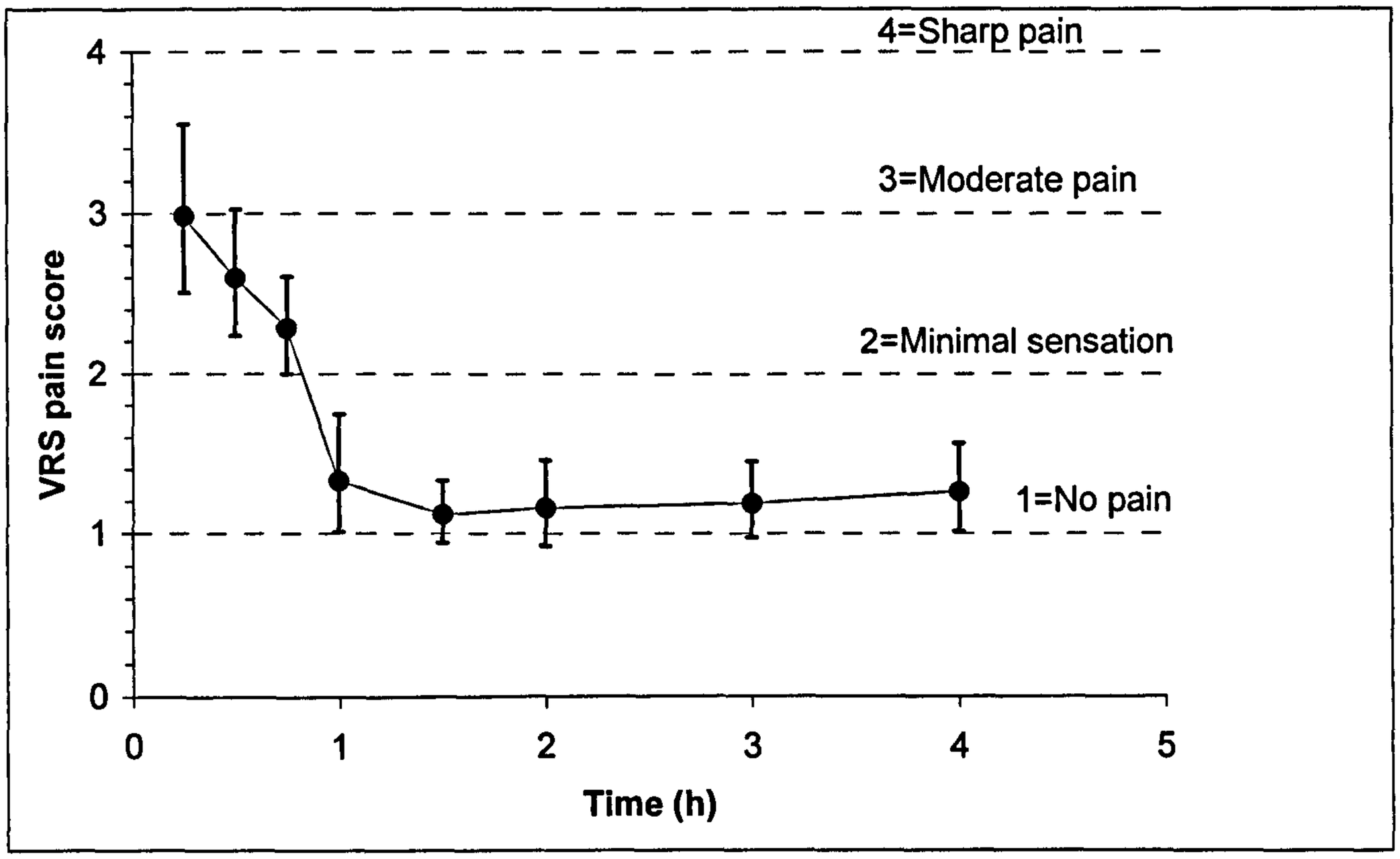


Figure 4.16 Mean VRS pain score corresponding to the pin prick time (n=12. Error bars = CI)

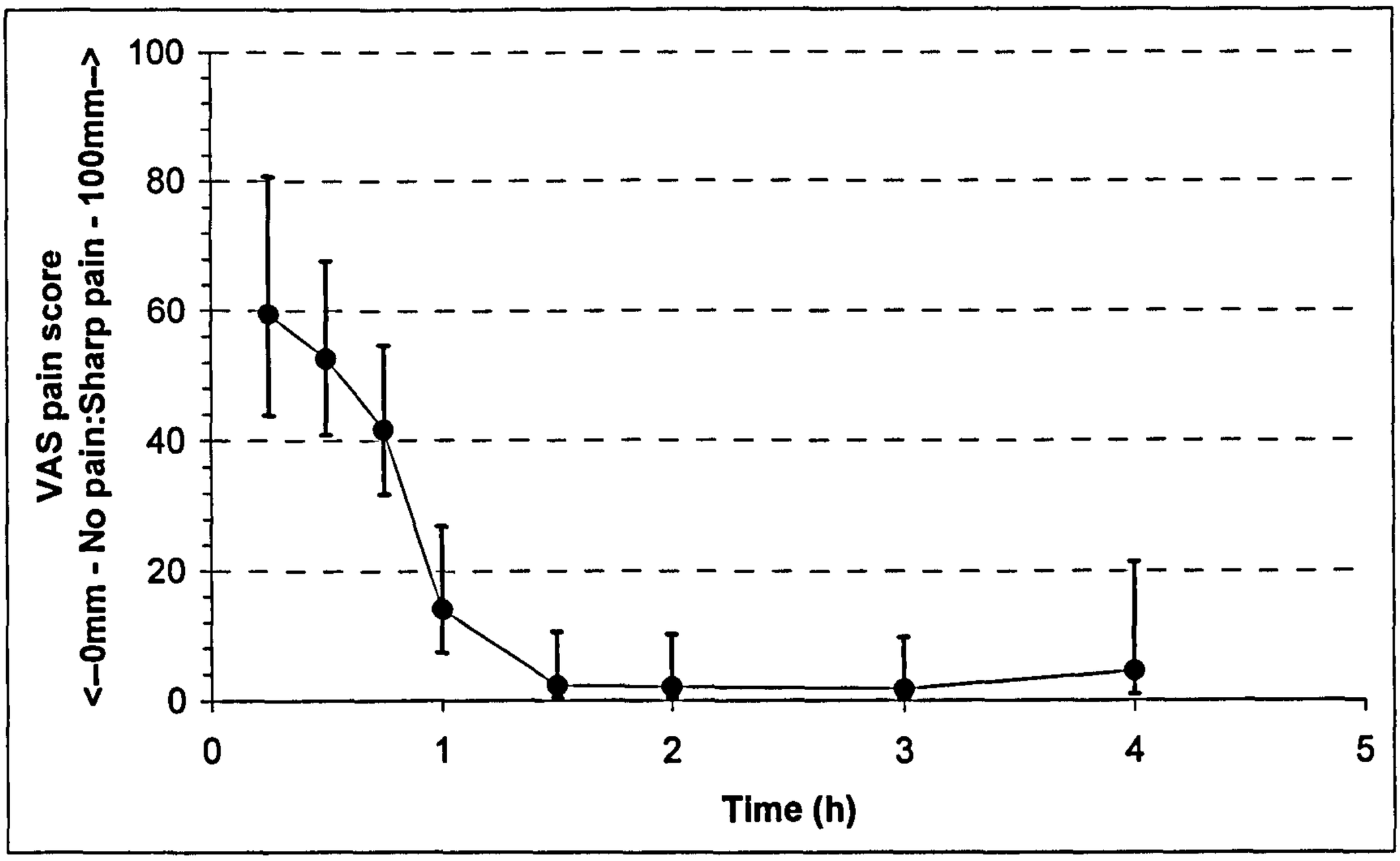


Figure 4.17 Mean VAS pain score corresponding to the pin prick time (n=12. Error bars = CI)

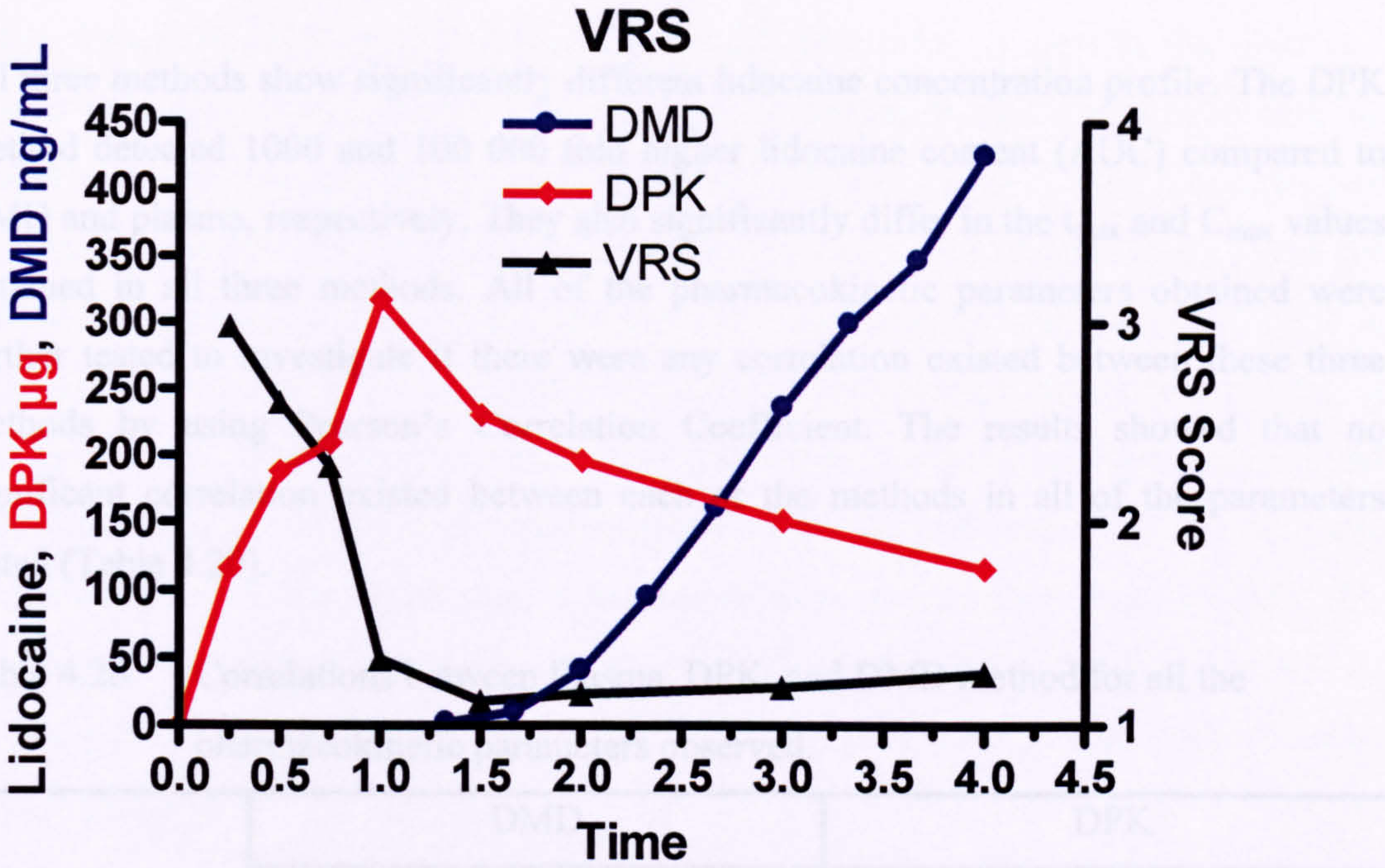


Figure 4.18 Plot of Verbal rating score (VRS) for pain with DPK and DMD lidocaine profile

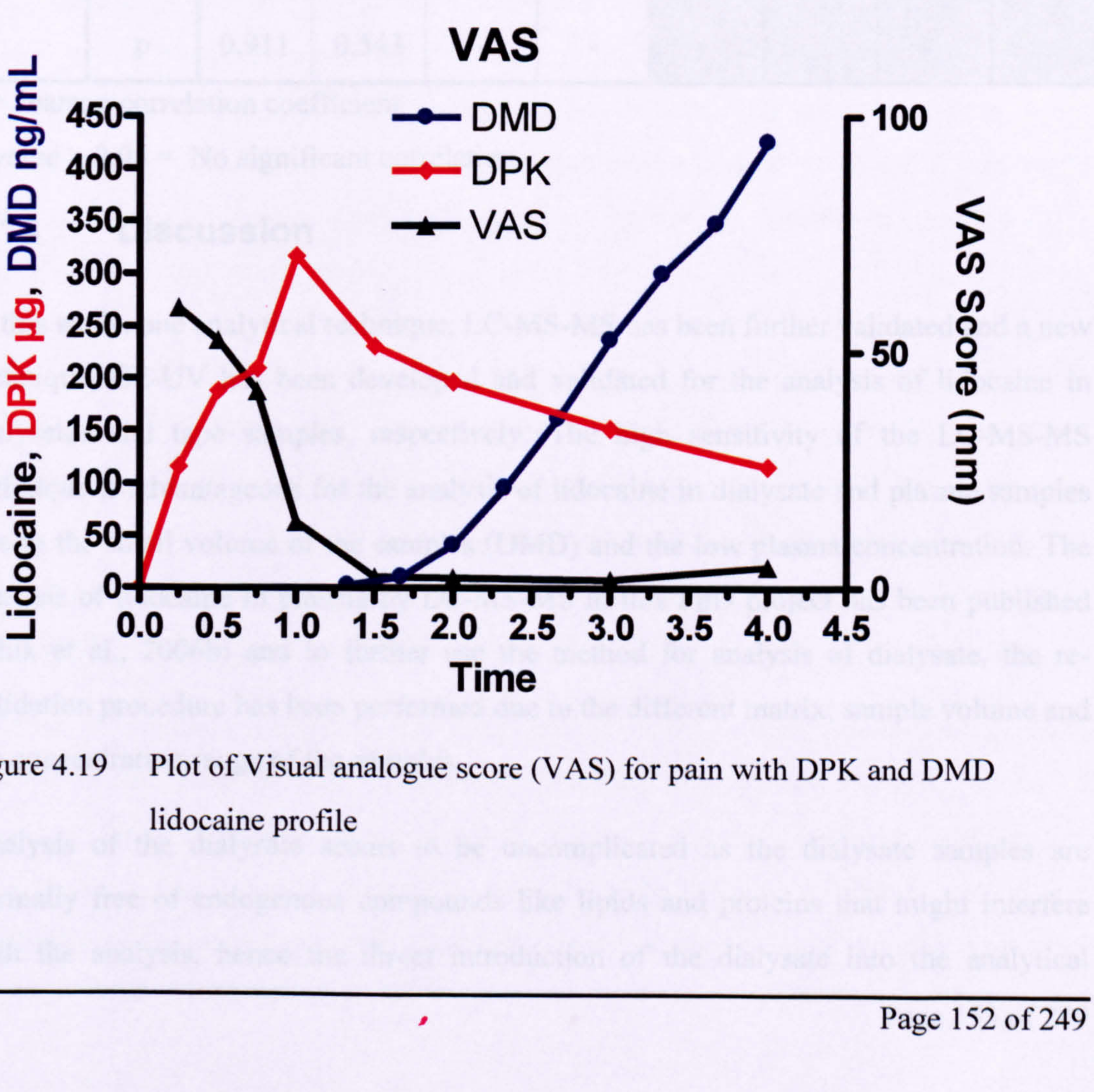


Figure 4.19 Plot of Visual analogue score (VAS) for pain with DPK and DMD lidocaine profile

4.13.2 Correlation between methods

All three methods show significantly different lidocaine concentration profile. The DPK method detected 1000 and 100 000 fold higher lidocaine content (AUC) compared to DMD and plasma, respectively. They also significantly differ in the t_{max} and C_{max} values obtained in all three methods. All of the pharmacokinetic parameters obtained were further tested to investigate if there were any correlation existed between these three methods by using Pearson's Correlation Coefficient. The results showed that no significant correlation existed between each of the methods in all of the parameters tested (Table 4.20).

Table 4.20 Correlations between Plasma, DPK, and DMD method for all the pharmacokinetic parameters observed.

		DMD				DPK			
		AUC	C_{max}	t_{max}	$t_{1/2}$	AUC	C_{max}	t_{max}	$t_{1/2}$
Plasma	r	0.053	-0.097	-	-	0.274	0.103	0.248	-0.521
	p	0.871	0.764	-	-	0.388	0.750	0.489	0.150
DPK	r	-0.036	-0.195	-	-				
	p	0.911	0.543	-	-				

r = Pearson correlation coefficient

p value > 0.05 = No significant correlation

4.14 Discussion

In this study, one analytical technique, LC-MS-MS has been further validated and a new technique, CE-UV has been developed and validated for the analysis of lidocaine in dialysates and tape samples, respectively. The high sensitivity of the LC-MS-MS technique is advantageous for the analysis of lidocaine in dialysate and plasma samples due to the small volume of the samples (DMD) and the low plasma concentration. The analysis of lidocaine in plasma by LC-MS-MS in this PhD project has been published (Chik et al., 2006b) and to further use the method for analysis of dialysate, the re-validation procedure has been performed due to the different matrix, sample volume and the concentration range of the samples.

Analysis of the dialysate seems to be uncomplicated as the dialysate samples are normally free of endogenous compounds like lipids and proteins that might interfere with the analysis, hence the direct introduction of the dialysate into the analytical

system is possible (Bagger and Bechgaard, 2004;Davies et al., 2000;de Lange et al., 2000). However, high ionic strengths due to the proportion of salts in the dialysate, present a challenge when analysed by MS. The salt content in the dialysate can clog the ionization source and create high background noise (Davies et al., 2000). The existence of salts also deteriorated the shape of the analyte peak in our observations. There are several methods that can be used to alleviate this problem, such as the use of off line solid phase extraction prior to introduction into the chromatographic system (Prokai et al., 1998), and desalting the sample with deionised water. Since our liquid-liquid extraction in our previous study was easy and simple to perform, all of the dialysate samples have been treated the same as plasma samples prior to analysis by LC-MS-MS. Further work needs to be carried out for the analysis of lidocaine in the dialysate, so that the sample can be directly introduced into the chromatographic system without off or on-line treatments.

On the other hand, tape samples obtained from the DPK study contained a huge amount of lidocaine. Therefore LC-MS-MS was not suitable to analyse all of the DPK samples as the detector was almost saturated with a high concentration of lidocaine. CE with UV detection was found to be a suitable method for this task due to its simplicity and reliability. In the development of this CE method we have attempted to reduce the migration time by a short – end injection (Geiser et al., 2005) and this resulted in the migration time of procaine at about 1 minute and lidocaine at about 1.2 minutes. However, prilocaine from EMLA™ cream in all of the samples cannot be fully separated from lidocaine by using this method.

Reducing the capillary length to 35cm from the original, 48.5cm, resulted in lidocaine and prilocaine being fully separated and took only about 2 minutes more than by short-end injection. Short migration times obtained for procaine (2.7 minutes) and lidocaine (3.2 minutes) is an advantage of CE compared to the 5 minute retention time for lidocaine using LC-MS-MS. This method has been used for the analysis of lidocaine in tape samples obtained from the DPK study in 12 healthy subjects.

Analysis of lidocaine content in all of the sample matrixes have shown that the DPK method is the most reliable method of measuring the pharmacokinetic profile of lidocaine. This can be seen through all of the pharmacokinetic parameters obtained such as AUC, C_{max}, t_{max} and $t_{1/2}$. The results showed that the amounts of lidocaine are higher in stratum corneum compared to the dermis layer of the skin and blood 1 hour after

application of EMLA™ cream. From the DPK, DMD and plasma lidocaine results, we can prove that the local anaesthetic agents mainly absorbed, distributed and active within the local tissue, especially the outermost layer of the skin, epidermis and underneath layer, dermis and subcutaneous tissue. Within the local tissue, it shows that local anaesthetic is higher in the stratum corneum compared to underneath tissue, the dermis and the subcutaneous. The DPK result showed that the lidocaine was detected in stratum corneum soon after applications and higher concentration at 1 hour post applications. The decrease in the amount of lidocaine after 1 hour in DPK samples can be explained by the increased lidocaine concentration in the dialysate. This remained elevated even at the 4 hours sampling time. Numerous studies have demonstrated that EMLA™ cream requires at least 45 to 60 minutes to achieve effective analgesia (Ehrenstrom-Reiz et al., 1983;Hallen et al., 1984) which continues to increase after removal of the cream (Arendt-Nielsen and Bjerring, 1988;Evers et al., 1985). The lowest score at 1.5 hours for VAS and VRS for pain in this study is correlated to the onset time of EMLA™ and the continued increased of lidocaine concentration in dialysate is the answer of the prolong analgesia as observed in this study and as reported in the above literatures.

The absorption of lidocaine into the systemic circulation is very low and took a longer time to achieve maximal concentration. This can be explained through the mean C_{max} and t_{max} result from plasma analysis are 2.5ng/mL and 3.25 hours compared to 320.8µg and 1 hour by the DPK methods, respectively. Although the concentration of lidocaine in dialysate was still increasing at 4 hours post dose, the lidocaine concentration in blood, however started to decreased after 3.5 hours. This condition may be due to elimination or metabolism of lidocaine into its major metabolites monoethylglycinexylidide (MEGX) and glycine xylidide (GX) by the cytochrome P-450 (Bargetzi et al., 1989). The lag time for the lidocaine as observed from the plasma result was 1.5 hours, and the $t_{1/2}$ obtained was 1.9 hours, therefore by 3.5 hours, half of lidocaine may already be eliminated from the body. The mean t_{max} value obtained from plasma result (3.25 hours) is very closed to the result obtained by Evers et al. (Evers et al., 1985) who reported the maximal concentration of lidocaine and prilocaine occcured 180 minutes after EMLA application. The lower C_{max} and AUC and the longer t_{max} obtained in plasma showed that dermatological drug products such as local anaesthetic have a limited systemic absorption.

Higher variability in DMD data compared to plasma and DPK may be largely attributed to the probe implantation depth as the same subjects were used for all three techniques. Also the validation of within and between probe recovery showed a low variability in the three probes studied. Therefore between and within probe variability was minimal and did not contribute significantly to the variability seen.

4.15 Conclusion

The results from this study show that, DPK and DMD have been proven more reliable methods for the pharmacokinetic assessment of topical drugs than systemic drug measurements. Good reproducibility and the higher lidocaine concentration - time profile shown by DPK and also by the DMD method are advantageous when compared to the low lidocaine profiles derived from systemic measurements. Between the DPK and the DMD methods, good reproducibility and less variability was shown by the DPK method.

However, more research and data regarding the reliability of DMD and DPK are necessary for the methods to be considered ideal for the pharmacokinetic assessment of transdermal drug delivery. A study such as to monitor the relationship of implantation depth of the microdialysis probe to the drug content is necessary to answer the variability observed in the DMD study. Once the results are confirmed that there is variability in the drug content related to the implantation depth, another study would need to be conducted investigating how to minimise the variation in the probe depth such as using ultrasound methodology.

On the other hand, another study needs to be carried out to monitor the relationship between the pressure of the adhesive tape applied related to the tape's drug content. This also can help to improve and standardise the DPK method for the pharmacokinetic assessment of transdermal drug.

Chapter 5 Melanotan

5.1 Introduction

Over 1000 analogues of α-MSH have been synthesized and biologically evaluated by a group of researchers in Arizona (Hadley et al., 1998b). Several of these α-MSH analogues have exhibited superpotency and prolonged activity with respect to their melanogenic (skin tanning) properties. Among them only two α-MSH analogues, Melanotan I (MT-I) (Hadley et al., 1993;Levine et al., 1991) and Melanotan II (MT-II) (Dorr et al., 1996) have been extensively studied. Both Melanotan I and II have tanning capabilities, but because MT-II caused spontaneous penile erection as a side effect, it is now also being developed as an erectile dysfunction drug.. The tanning and penile erection capabilities of melanotan II have been shown in the study conducted by Dorr and co-workers (Dorr et al., 1996), and other studies on melanotan II have shown that the drug can initiate penile erection and increased sexual desire in some of the subjects (Hadley et al., 1998b;Wessells et al., 1998;Wessells et al., 2000). On the other hand MT-I has been further developed as a tanning agent (Dorr et al., 2000;Hadley and Dorr, 2006). The delivery of MT-I is problematic as it is a peptide drug with a short half-life, metabolic instability, relatively high polarity, and larger molecular size which limits its transport via gastrointestinal absorption (Hadley et al., 1998b). Therefore to date, the delivery of melanotan has been studied using intravenous injection, subcutaneous implant, and transdermal delivery.

Transdermal delivery of MT-I has been conducted and tested on mice (Levine et al., 1987). MT-I was topically applied to an area of the back of the mice and within 24-48 hours, eumelanin production was visible microscopically within hair bulb melanocytes in both treated and untreated areas. Thus, these results demonstrated that MT-I was successfully delivered through the skin and had the action of melanin production. However similar studies using rat skin showed poor penetration of MT-I (Dawson et al., 1988). In another in vitro study on human skin (Dawson et al., 1990), MT-I has successfully delivered across human skin and was measurable using radioimmunoassay (RIA) to test for quantity and biologic activity, respectively.

Preliminary clinial trials on MT-I to demonstrate tanning of skin were carried out in Arizona, USA by Norman Levine (Levine et al., 1991). Twenty eight healthy white men with history of either poor or good tanning skin were administered with 10

subcutaneous injection of MT-I. The results showed that a tan induced by Melanotan in the head and neck region, were produced in the same way as a natural tan and persisted for a similar time. This result has been considered the first demonstration of a stable drug candidate that could induce a natural tan in human beings. A study by Ugwu and co - workers (Ugwu et al., 1997) also showed a significant tanning of the forehead, arms, and neck following IV or SC dosing of MT 1 in three healthy voluteers.

In a pharmacokinetic study (Evans AM, 2002) conducted in 12 volunteers in Australia of a daily subcutaneous injection of 0.16mg/kg of Melanotan for ten days demonstrated that Melanotan has a half-life after subcutaneous administration of about 30 minutes with little or no activity in the plasma after 4 hours. There appeared to be negligible accumulation of Melanotan in subjects (Evans AM, 2002). A Phase II study conducted at two sites in Australia (Royal Prince Alfred Hospital, Sydney and Royal Adelaide Hospital, Adelaide) during 2003 recruited 81 subjects (61 Melanotan and 20 placebo). The subjects enrolled into the active arm received 30 subcutaneous injections of 0.16mg/kg/day of MT 1 for thirty days, given for every 10 days at the beginning of 3 consecutive months. Significant increases in skin melanin were seen in the active group compared to placebo ($p < 0.0001$) at the end of the 90 day study period (Datapharm Australia, 2004).

The safety and tolerability of a slow-release (depot) formulation of Melanotan is being tested in a human trial in Australia. This trial involves the subcutaneous insertion into the abdominal wall of biodegradable polylactide rods containing increasing doses of Melanotan. Subjects are being monitored for safety, pharmacokinetic levels of the drug in the blood and skin tanning effects over a 60 day period. Preliminary results with a 20mg and 40mg implant have shown the average skin melanin density increases from 2.81 + 0.33% to 4.03 + 0.29% within 10 days of starting treatment and persisted over the 60 day observation period. Adverse events were principally limited to the cosmetic effects (rapid tanning, freckling) induced by the rapid onset of melanogenesis. Other mild adverse events which have been reported less commonly include headache, satiety, rhinitis, chemical taste in the mouth, yawning, muscle twitching, diarrhoea, light-headedness and nervousness. Adverse events associated with sustained-release implants have been mainly restricted to irregular skin tanning, darkening of nevi (moles) and freckling. The darkening of moles and freckling are not strictly adverse events as they represent the natural effects of the drug, however the rapidity of the change was such as may be recorded as an unexpected event.

Recently, Clinuvel Pharmacetical Limited (previously known as Epitan Limited) has announced that it has successfully completed a phase II trial on injectable implants of Melanotan-I for the treatment of Polymorphous Light Eruptions (PMLE). The results show that Melanotan I can be an efficacious drug for the treatment of PMLE. They now plan to start the Phase III trial in nine centres across the European continent in early 2007 (Clinuvel Pharmaceutical Limited, 2006).

All of the above forms of administration are expensive to administer, time-consuming and inconvenient to the subject. If this is to become an over the counter (OTC) product, there is a need to establish the pharmacokinetics of a more user-friendly dosage regimen. In this study, the TDS® system has been combined with MT-I to assess the capabilities of the system to deliver MT-I for the assessment of tanning and it's pharmacokinetic properties. This is a more convenient mode of application compared to a subcutaneous implant and IV injection. The expected difference in bioavailability in a subcutaneous versus a transdermal dose has resulted in the selected dose escalation from 1 mg up to 40mg administered for 10 consecutive days in this study. The total dose of up to 400mg was received by subjects based on the tanning and toxicity criteria. This criteria and full dosage regime will be explained in the methods section.

5.2 Study Objectives

The main objective of this study was to assess the ability of the TDS® system to deliver MT-I systemically to induce the production of skin's melanin and give the tanning effect in healthy Caucasian subjects.

The second objective of this study was to assess the pharmacokinetic properties and therapeutic dosage of MT-I in conjunction with the safety and tolerability of MT-I in healthy subjects.

5.3 Study Approval

The study was approved by the St. Thomas' Hospital Research Ethics Committee. Reference no. 04/Q0702/120, dated 11th. November 2004.

5.4 Subjects

This study involves 30 healthy Caucasian subjects enrolled over a period of 9 months.

5.5 Study Methodology

5.5.1 Study treatment

The TDS® system as prepared under GMP manufacturing and FDA guidelines by Natural Vitamin Co., Las Vegas, Nevada, USA and dispensed by Transdermal Technologies Inc. (TTI) Lake Park, Florida, USA. TTI was sent TDS® and Melanotan to be combined and formulated by Controlled Therapeutics, Glasgow, United Kingdom. TDS® - Melanotan 1, 5, 10, 20, and 40mg/mL solutions were supplied in metered pump dispenser with one entire spray containing 0.2mL solution.

5.5.2 Design

This study was a dose escalation study starting with a 1mg dose for Cohort 1 and ending with a 40mg dose for Cohort 5. Each cohort contained 6 subjects who received a dose for 10 continous days. The detail of the cohorts and doses are shown in the Table 5.1. The lowest dose (1mg/day) is approximately $1/10^{th}$ of the effective dose established in previous studies of ten daily IV doses of 0.16mg/kg/day [100mg delivered over 10 days to a 62kg person] (Ugwu et al., 1997).

Table 5.1 Doses and corresponding cohorts for TDS-Melanotan administration

Cohort (Treatment) Number	No. of subjects	Dose (mg/day)	Total Dose (mg)
1	6	1	10
2	6	5	50
3	6	10	100
4	6	20	200
5	6	40	400

5.5.3 Screening Evaluation

Prospective subjects attended the study site for a screening visit within 4 weeks of study commencement. The nature of the study, the procedures and the risks were fully explained. Before any screening procedures occured they had signed an Informed

Consent Form for Screening and an Informed Consent Form for tissue storage. At the screening sessions, blood samples were taken for routine biochemical, haematological, endocrinological and serological screenings. A urine specimen was also obtained for urinalysis and drug screening and pregnancy testing for females. A further 10mL of blood was taken for the assessment of MC1R genotype. Each subject was then provided with Uritainer and requested to collect all of their urine over the following 24-hour period for the measurement of 24-hour urinary cortisol levels. Routine physical examinations, inclusion/ exclusion criteria and assessment of skin type were also conducted during the screening. Skin type has been recorded based on Fitzpatrick scale (Fitzpatrick, 1988) as list down below.

i) Type 1: Never tans, always burns

ii) Type 2: Sometimes tans, mostly burns

iii) Type 3: Mostly tans, sometimes burns

iv) Type 4: Always tans, never burns

In addition to the subject's skin type assessed according to Fitzpatrick classification, a more definitive assessment of skin type will be assessed according to the "melanocortin-1 receptor" (MC1R) genotype (Palmer et al., 2000). A 10mL blood sample was collected into a standard EDTA blood tube for this purpose. After mixing well, the tube was labelled and stored refrigerated (not frozen) until further analysis.

5.5.4 Study Procedures

5.5.4.1 Dose escalation strategy

Upon acceptance into the study, volunteers were allocated subject numbers and hence to a Cohort number for administration of Melanotan at one of the pre-assigned doses. Cohorts 1, 2, and 3 were run in parallel. Cohort 4 for the 20mg dose was started approximately 1 month after completion of day 30 of the first three cohorts, after review of all of the haematology and clinical biochemistry results at Day 30. Upon completion of a review of all of the results, (and found) no related grade 3 or 4 toxicity as defined by the National Cancer Institute Common Toxicity Criteria (National Cancer Institute, 1999), no abnormal pathology results and no serious adverse events, another six

subjects were enrolled in Cohort 4 to receive the 20mg/day dose. The above procedures were also carried out for the enrolment of Cohort 5 (40mg/day dose).

5.5.4.2 Treatment procedures (per cohort)

5.5.4.2.1 Day 0

Subjects reported to the Study Centre at approximately 0830 hours on Study Day 0. The volunteers underwent a check-in procedure during which Informed Consent for Study Participation was obtained. A urinary pregnancy test was conducted on females to make sure that they were not pregnant. Subjects then had skin reflectance measurements performed according to the procedures outlined in Section 5.5.8. Prior to discharge from the Study Centre, subjects were given Diary Cards which they were asked to fill in on a daily basis to record daily exposure to sun, applications of sunscreen to the skin, concurrent medications and any adverse events. Subjects were requested to bring these diary cards to the Study Centre at each study visit for review.

5.5.4.2.2 Days 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10

On Day 1, subjects attended the Study Centre at about 0730 hours. Subjects were checked for any concomitant medications and had a blood pressure (BP) check before insertion of a cannulae into an antecubital vein of the left arm. A 10 mL blood sample was obtained before the treatment application. The treatment dose was sprayed topically to the inner aspect of the right inner upper arm and gently rubbed into the skin. A surgical glove was worn when applying the treatment to prevent self-dosing. The applied drug was allowed to dry for approximately 5 minutes prior to the subject covering the arm with clothing. Following dosing, subjects remained under observation at the Study Centre. Serial 10mL blood samples were collected at 20min, 40min, 1, 1½, 2, 2½, 3, 4, 5, 6, 8, 10 and 12 hours post dose. On the remaining treatment days (2 – 10), a 10mL blood sample was drawn just prior to the treatment being applied. The treatment was applied at the same application sites for 10 continuous days. On day 5 and 10, skin reflectance measurement was carried out by using a spectrophotometer. On day 10, a blood sample was taken for pathology assessment and subjects were provided with a Uritainer and requested to collect their urine over the following 24 hour period. Subjects were asked to return the Uritainer on the following day for 24-hour urinary cortisol measurement.

5.5.4.2.3 Day 20 (+/- 1 day)

On this day, subjects attended Study Centre at about 0830 hours for Skin reflectance measurements. Subjects also were also assessed for concomitant medication, vital signs and adverse events.

5.5.4.2.4 Day 30 (+/- 1 day)

On this day subjects attended the Study Centre at about 0830 hours for skin reflectance measurements (Section 5.5.8) prior to blood collection for safety measurements (haematology, biochemistry and endocrinology), and a urine sample was obtained from female subjects for pregnancy testing. Subjects were then provided with a Uritainer and repeat the urine collection procedure as mentioned above.

On each of the above visits, subjects were assessed for concomitant medication, vital signs and adverse events. Subjects were also reminded to record their daily activity related to sun exposure, any adverse events and medication taken, in the diary book supplied.

5.5.4.3 Restrictions

5.5.4.3.1 UV Exposure

Subjects were required to refrain from excessive exposure to UV light from 1 week prior to the study start until after the Day 30 visit. Subjects were not permitted to use UV beds and had to apply sunscreen to all exposed body regions during prolonged ($\geq$ 30 minutes full sun) sun exposure.

5.5.4.3.2 Concurrent Medications

Subjects were instructed to abstain from medications (prescribed or over-the-counter, including herbal remedies, but excluding oral contraceptives) deemed to be significant by the Principal Investigator for the 7 days preceding the dosage phase (Days 1-10), and throughout the study (until Day 30). Subjects were asked to inform Study Personnel if any additional medication was required. All the details of concomitant medications were recorded in the subject's Diary and Case Report Form (CRF).

5.5.5 Parameters for Evaluation

5.5.5.1 Skin Reflectance Measurements

Skin reflectance measurements were performed on Days 0, 5, 10, 20 and 30. Reflectance by the skin, of wavebands of light that are 15 nm wide were measured at 20 nm intervals in the wavelength range of 400 to 700nm with a Minolta 2500d spectrophotometer. Refer to Appendix 22 for the whole procedures of the skin reflectance measurements. Nine different anatomical sites were assessed as follows:

Site 1 : Forehead

Site 2 : Left cheek

Site 3 : Right side of neck

Site 4 : Left shoulder (scapula)

Site 5 : Left inside upper arm

Site 6 : Right inside upper arm

Site 7 : Left medial forearm

Site 8 : Right side of lower back

Site 9 : Left calf

Four skin reflectance parameters were measured, in triplicate, on an area of 8mm x 8mm at each anatomical site, these are: L-value, b-value, R_{420} and R_{400} (the latter two readings were used to calculate Melanin Density (MD)). Reflectance measurements were recorded as L-values, corresponding to luminescence or brightness from black to white, and b-values, representing colour hues from blue to yellow (Porges et al., 1988;Seitz and Whitmore, 1988). Visually apparent tans are typically associated with $\geq$ 1 unit decrease in the L-value and $\geq$ 1 unit increase in b-value, compared to baseline (Ugwu et al., 1997). In addition, the density of cutaneous melanin was estimated from reflectance at that site of wavebands of light centred at 400 nm and 420 nm according to the results of Dwyer et al. (Dwyer et al., 1998;Dwyer et al., 2000). The equation used is:

$$MD = 100 \times (0.035307 + 0.009974(R_{420} - R_{400}))$$

Where;

MD = Melanin density

R_{400} and R_{420} = Reflectance at 400 nm and 420 nm, respectively.

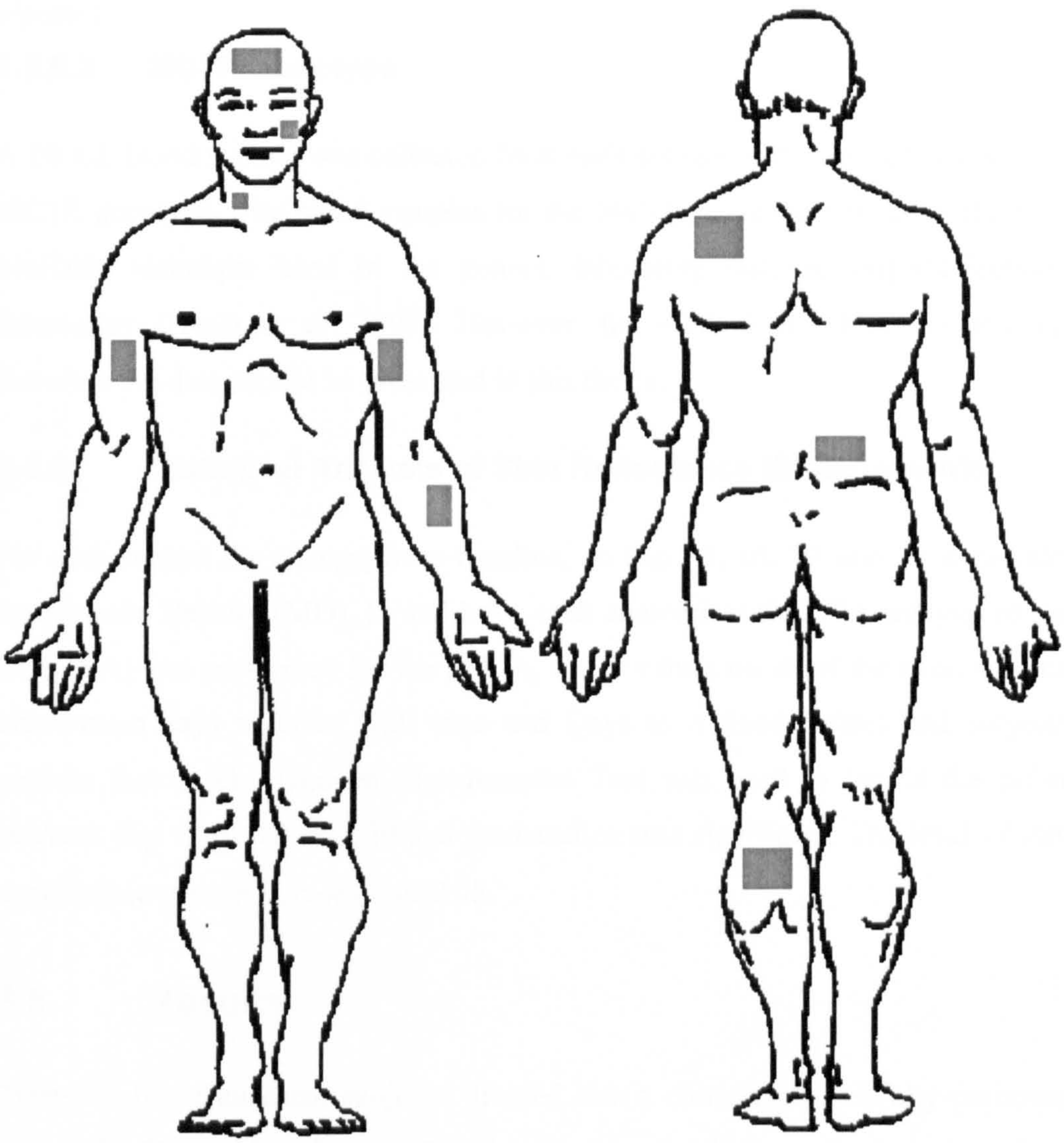


Figure 5.1 A body diagram shows the 9 anatomical sites (grey shaded area) for the skin reflectance measurement.

5.5.5.2 Melanotan Assay

Melanotan concentrations will be measured in plasma using a validated LC-MS-MS method. At the time this thesis was submitted, the method is still under development. Therefore, the melanotan plasma concentration and pharmacokinetic analysis cannot be reported.

5.5.5.3 MC1R Genotype

A 10 mL blood sample was collected from each subject at the time of screening for the MC1R genotype. The blood samples for the MC1R genotype will be analysed by any available technique used in the genetic laboratory such as oligonucleotide DNA microarray (Hacia et al., 1999). However, the method is still under development, therefore the data cannot be presented in this thesis.

5.5.6 Statistical Analysis of Skin Reflectance Measurements

For each subject the change, from baseline, on Days 5, 10, 20, and 30 were calculated for Melanin Density (MD), L, and b for each anatomical site. The analysis of variance (ANOVA) was performed for the MD, b, and L values on all of the sites, subjects, and observation days together with sites and Days as a fixed factors and subjects as a random factor. The Dunnett Simultaneous Test was used to test if the differences between day 5, 10, 20, and 30 and the baseline was significant. The level of statistical significance was set at α-level of 0.05.

5.6 Results

Thirty healthy Caucasian subjects, divided into 5 cohorts, successfully participated in this study. One subject from Cohort 3 refused to finish the study for personal reasons, but was replaced by another subject. All of the subjects followed the protocol well and restricted their sun esposure. The average length of sun exposure in all of the subjects studied for the whole 30 days study period was, mean [range] 25 [0-82] hours. After completing the 30 day study on each Cohort, no significant skin tanning was observed in any of the subjects. The results of MD, b and L at day 5, 10, 20, and 30 also showed no difference compared to baseline for all anatomical sites and all of the cohorts accepted the final cohort, the 40 mg dose was where site 7 (left medial forearm) showed a significant increase and a decrease in the MD and 'b' values, respectively. Therefore, only the results from cohort 5 will be presented and discussed.

The analysis of skin reflectance parameters by each subject had found the MD, b and L values were inconsistence throughout the 30 day observation period for all the subjects and the sites studied. Only site 7 (left medial forearm) appeared to have a significant increase in MD and b values, and a significant decreased in L values for most of the subjects. For simplicity, the analysis for MD, b, and L were performed at each site. The plot of mean MD and b change from baseline showed no increase for all of the anatomical sites except site 7 (left medial forearm) (Figure 5.2 and Figure 5.3). Similarly, the plot of mean L change from baseline also showed no decreased for all the anatomical sites except for site 7 (Figure 5.4). Further detail on site 7 also showed most of the subjects had an increase in MD and b, and this correlated with the decrease in L values from day 0 to day 30. Figure 5.5, Figure 5.6, and Figure 5.7 show the plots of MD, b, and L change from baseline for site 7 for all of the subjects in cohort 5. Whilst Figure 5.8 shows clearly the plot of mean MD, b, and L values change from baseline for site 7.

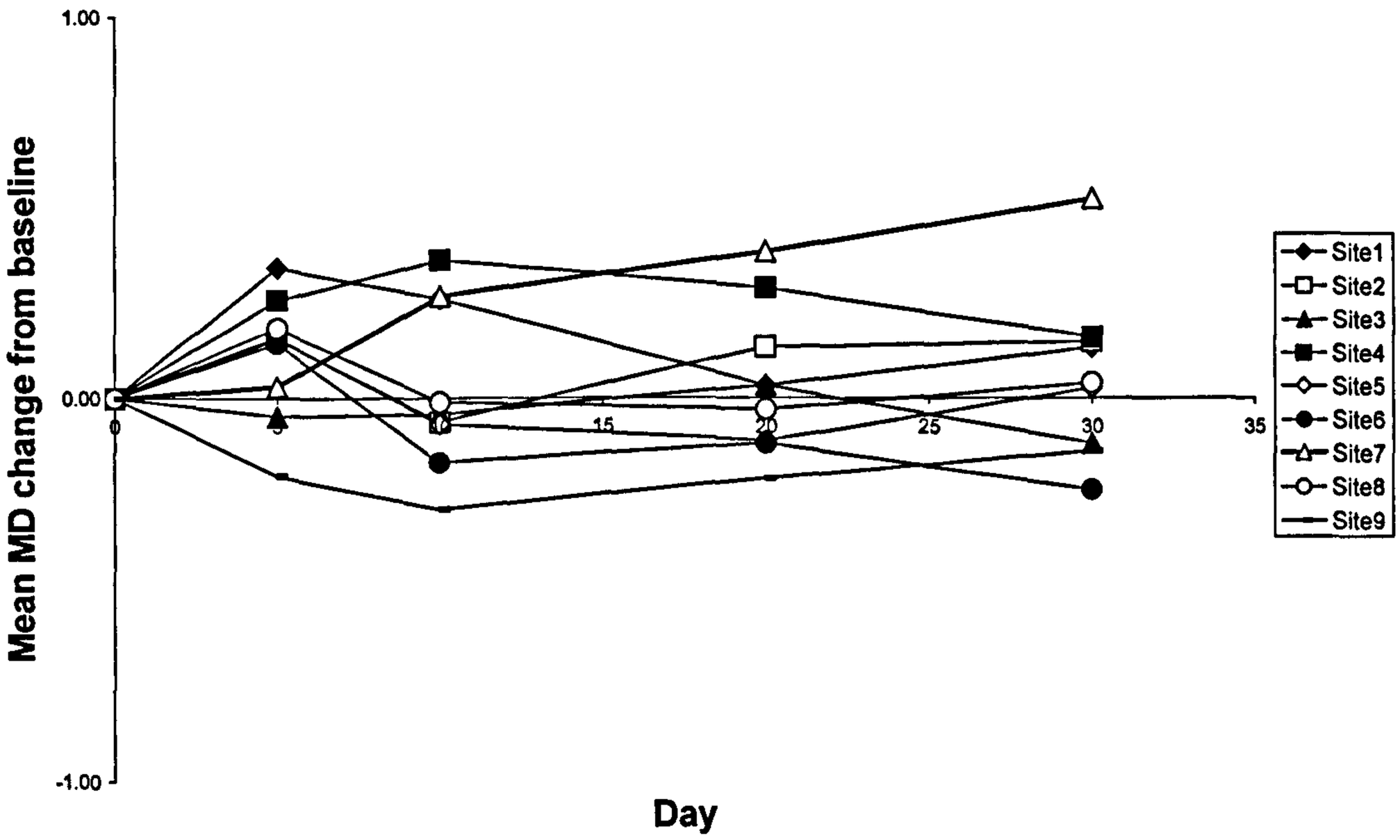


Figure 5.2 Plot of mean MD change from baseline for all the anatomical sites in Cohort 5

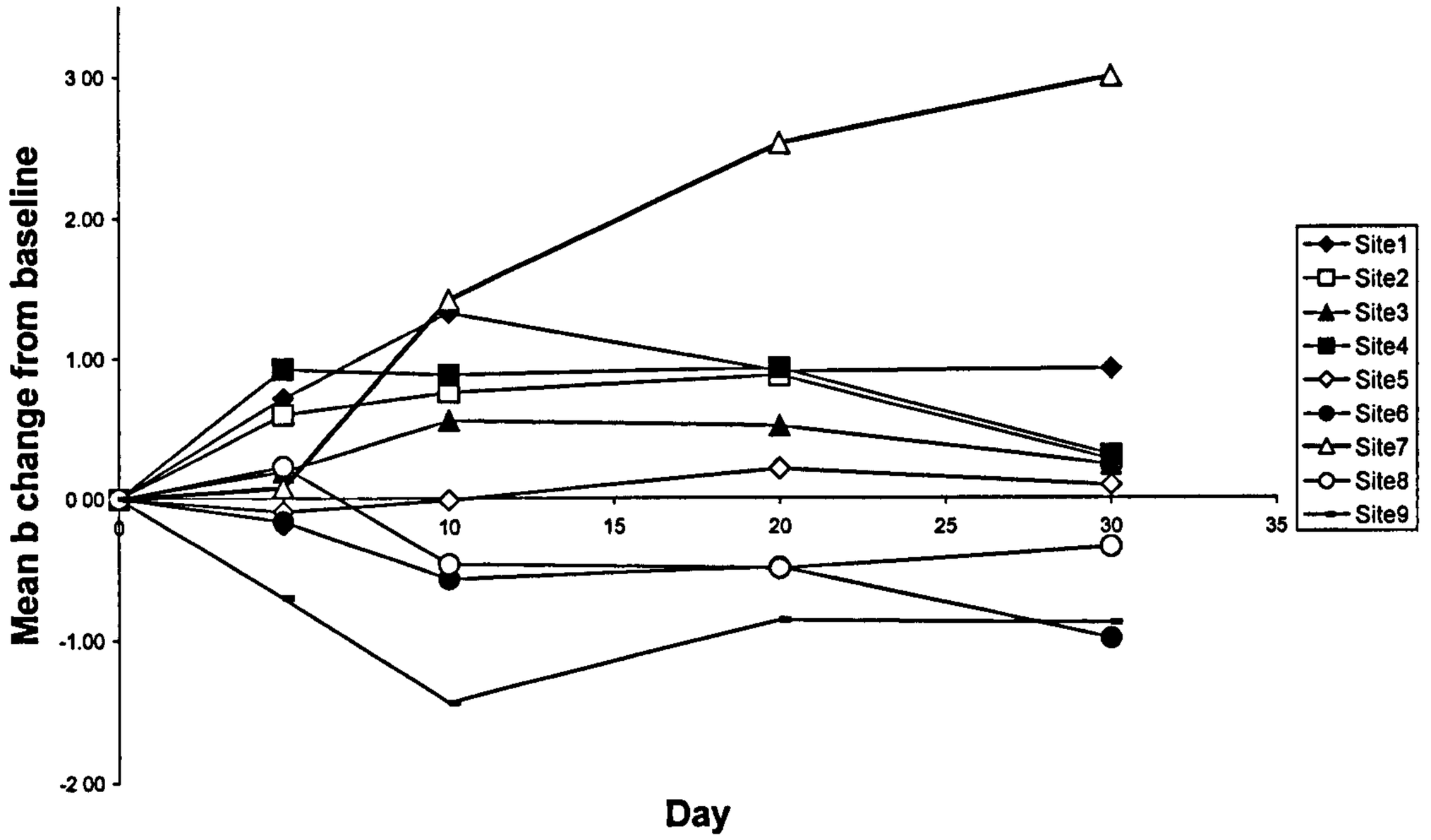


Figure 5.3 Plot of mean b change from baseline for all the anatomical sites in Cohort 5

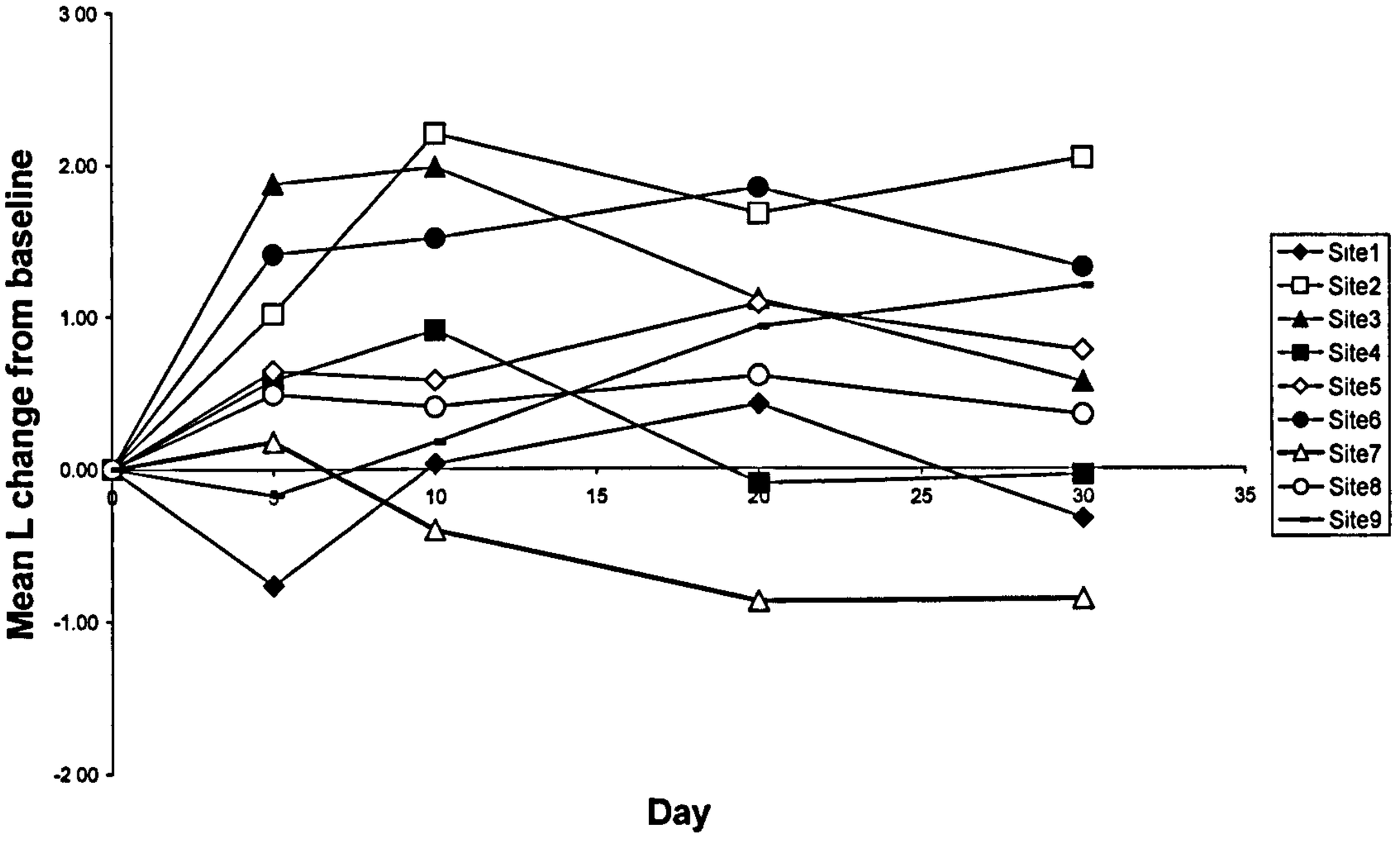


Figure 5.4 Plot of mean L change from baseline for all the anatomical sites in Cohort 5

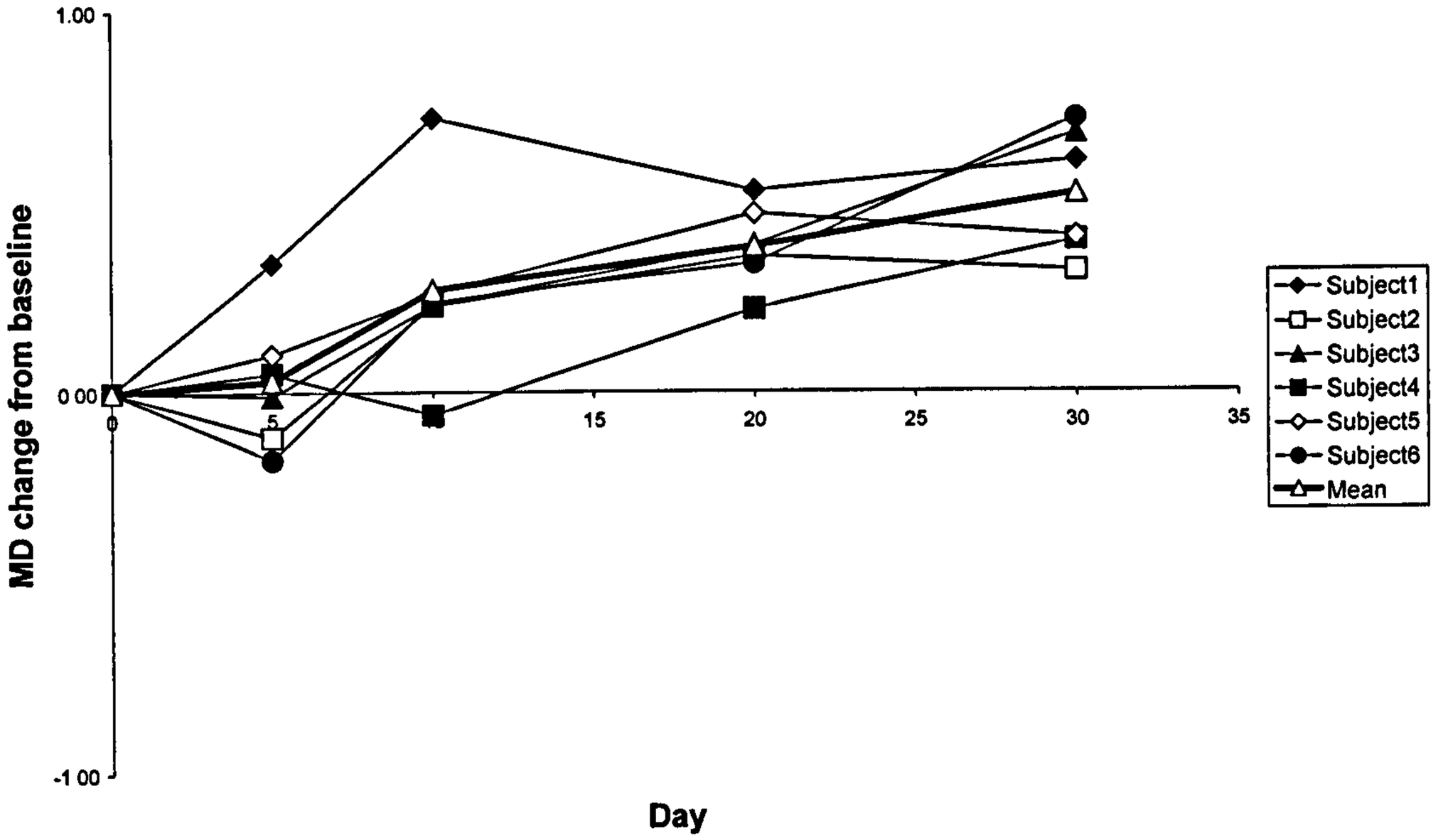


Figure 5.5 Plot of MD values change from baseline for site 7 for all the subjects in Cohort 5.

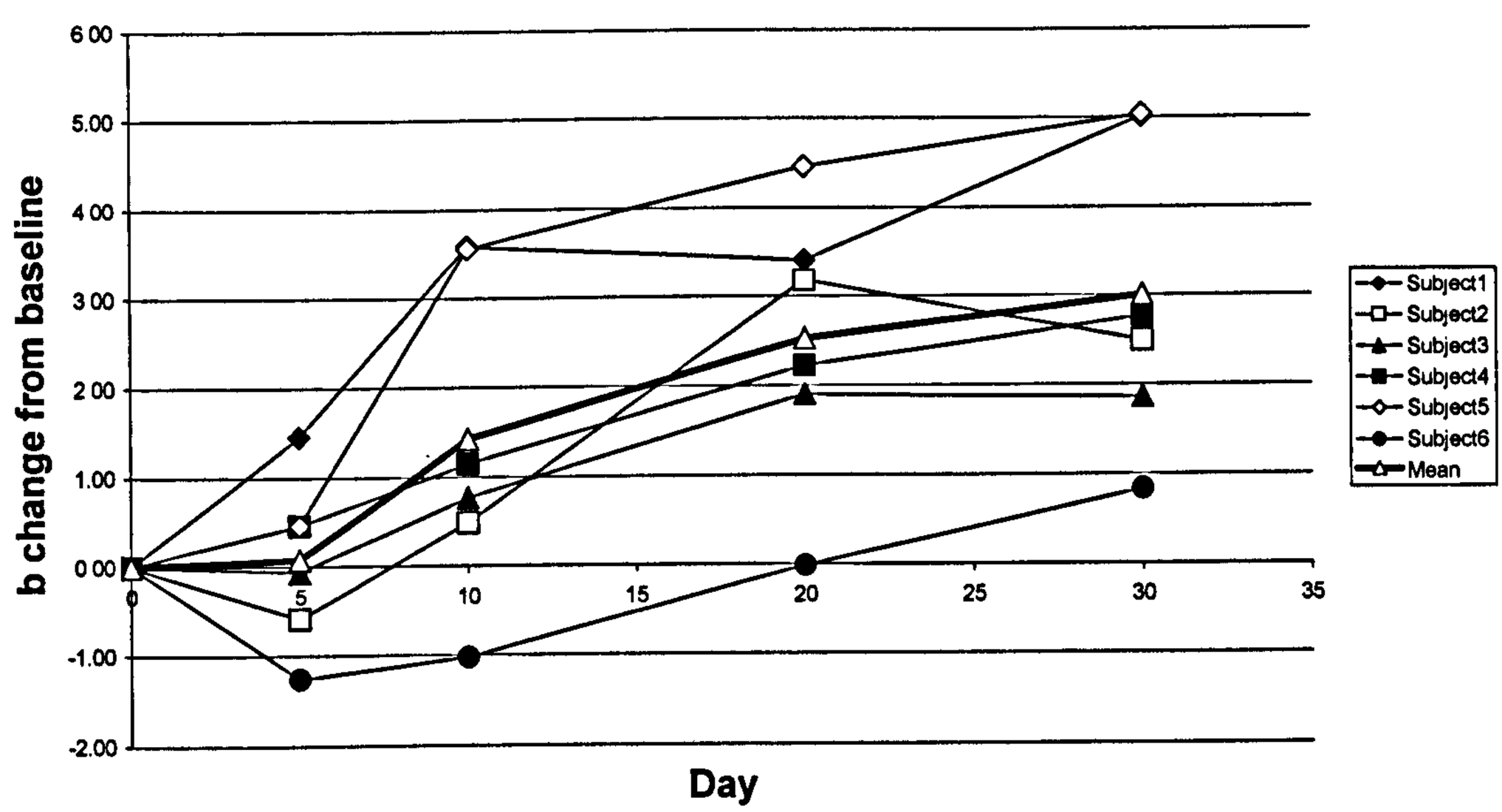


Figure 5.6 Plot of b values change from baseline for site 7 for all the subjects in Cohort 5.

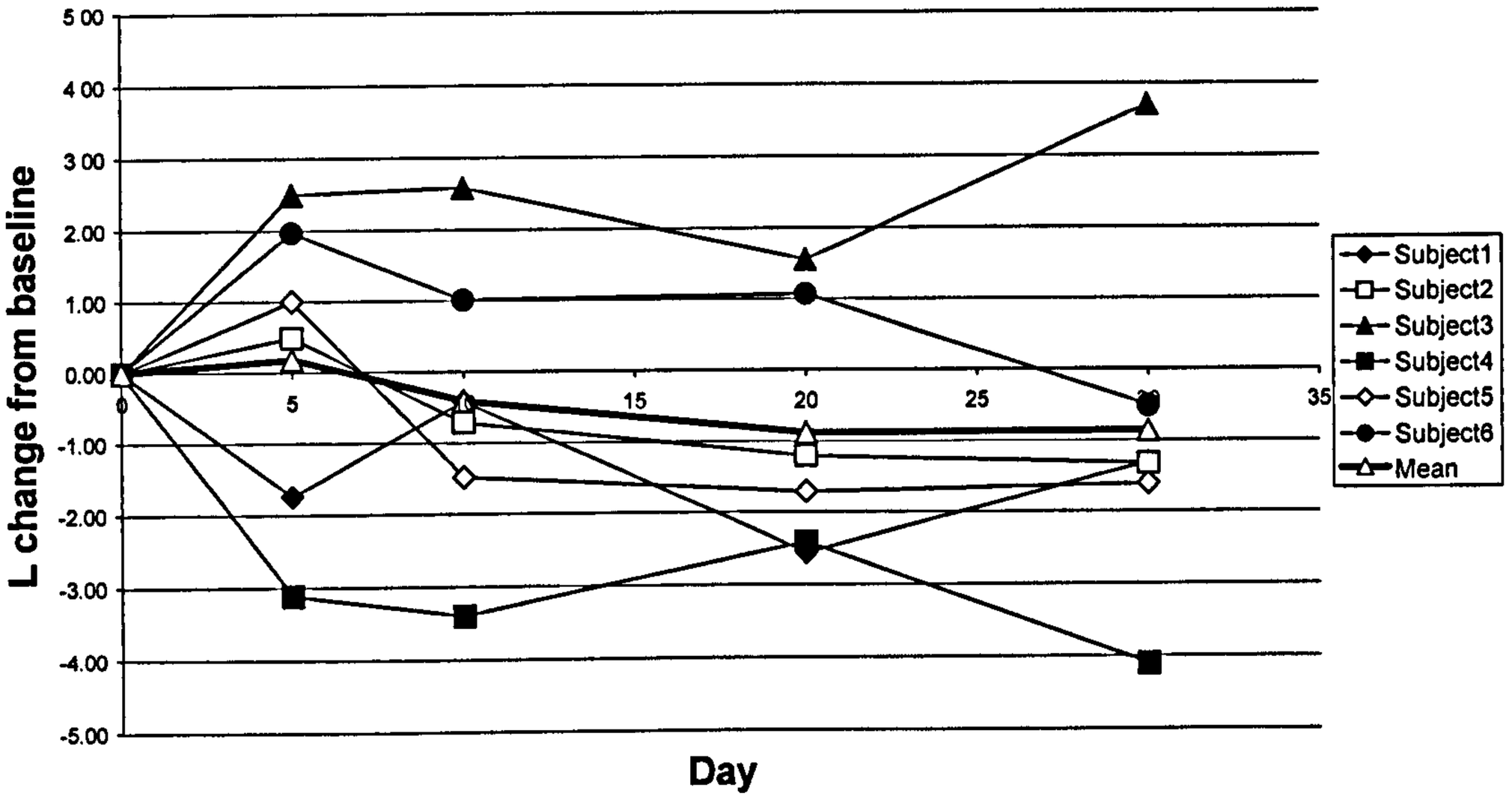


Figure 5.7 Plot of L values changed from baseline for site 7 for all the subjects in Cohort 5.

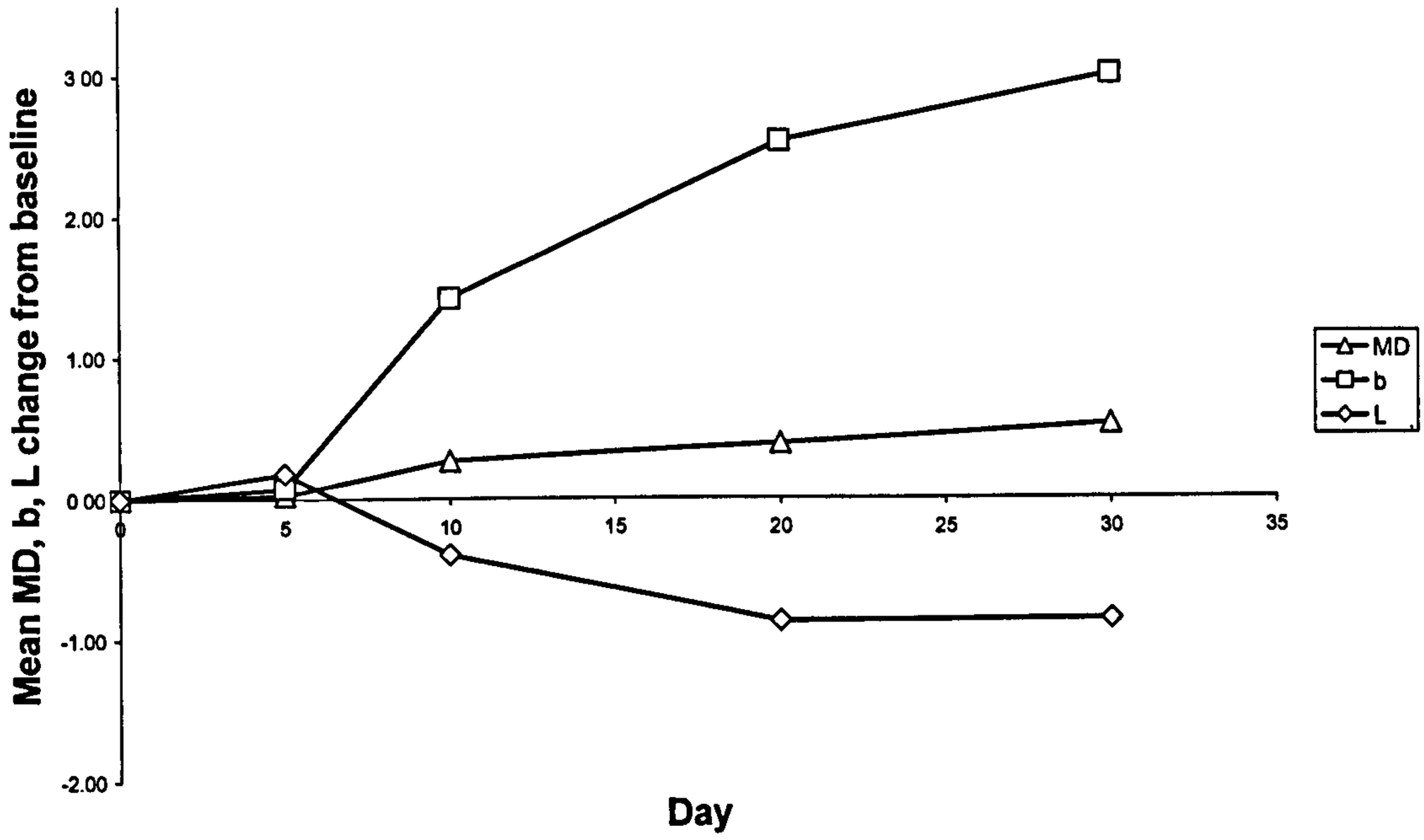


Figure 5.8 Plot of mean MD, b, and L change from baseline for site 7

The analysis of variance (ANOVA) was performed for the MD, b, and L values on all sites and subjects together for the difference between Day 5, 10, 20, and 30 from the baseline (n = 270 observations). No significant differences were observed between day 5, 10, 20, and 30. The 95 % confidence interval (CI) and the p values are summarised in Table 5.2. The ANOVA performed for MD, b, and L on each site also showed no difference between day 5, 10, 20, and 30 for all sites except site 7. The MD and b values for site 7 were statistically different at day 10, 20 and 30. Although the mean L values were decreased at day 10, 20, and 30, the differences were not statistically significant. The confidence interval and the p values of MD, b, and L for site 7 are summarised in the Table 5.3.

The analysis of all the blood samples for dose 40 mg also showed that MT I were not detected in plasma in any of the sampling point in all the subjects. This result showed that the TDS system may not be able to deliver MT I systemically and that is the likely answer for the lack of tanning in all the subjects studied.

No serious adverse events were reported by all the subjects studied. From 30 subjects, only 6 subjects reported headache at some point during the 30 day study period, but were only mild and lasted for a short period of time. Two subjects caught a cold and one subject experienced stomach pain, but this was not related to the study drug. All of the subjects tolerated the treatment applications and there was no local reaction observed at the application site.

Table 5.2 The confidence interval (CI) and p values for MD, L, and b obtained from ANOVA for the differences between Day 5, 10, 20, 30 from baseline for all the observation sites and subjects

Day	MD		L		b	
	CI	p	CI	p	CI	p
Day 5	-0.1466 - 0.3736	0.6591	-0.7446 - 1.920	0.6556	-0.6167 - 1.014	0.9366
Day 10	-0.2312 - 0.2890	0.9962	-0.5072 - 2.158	0.3604	-0.5414 - 1.089	0.8283
Day 20	-0.2149 - 0.3053	0.9799	-0.5901 - 2.075	0.4560	-0.3591 - 1.271	0.4523
Day 30	-0.1999 - 0.3203	0.9450	-0.7757 - 1.889	0.6963	-0.5247 - 1.106	0.7977

Table 5.3 The confidence interval (CI) and p values for MD, L, and b obtained from ANOVA for the differences between Day 5, 10, 20, 30 from baseline for all the subjects in site 7.

Day	MD		L		b	
	CI	p	CI	p	CI	p
Day 5	-0.1802 - 0.2402	0.9863	-1.6360 - 2.0022	0.9963	-1.2250 - 1.3820	0.9995
Day 10	0.0598 - 0.4802	0.0098	-2.2210 - 1.4172	0.9374	0.1180 - 2.7250	0.0302
Day 20	0.1748 - 0.5952	0.0004	-2.6960 - 0.9422	0.5286	1.2230 - 3.8300	0.0002
Day 30	0.3132 - 0.7335	0.0000	-2.6790 - 0.9588	0.5445	1.7000 - 4.3070	0.0000

5.7 Discussion and Conclusion

Although the primary endpoint, the skin tanning, was not achieved in this study, the analysis of the skin reflectance data have showed a significant increase in the MD and b and the decrease in L values at one site of the body. However, the difference is only significant for MD and b, but not for L. Therefore we cannot conclude that the TDS®-Melanotan worked.

A few suggestions can be made from the above observations. At the doses used the TDS® system may not capable of delivering the necessary therapeutic dosage of MT 1 through the skin to induce enough production of melanin to give a tanning effect. This is may be because of MT-I is a peptide which is by its nature has a large molecular weight, is hydrophilic, and is a polar compound (Bodde et al., 1989) presenting difficulties for effective transdermal drug delivery. Oral administration for peptides is not feasible as peptides are rapidly metabolised in the intestinal tract by pancreatic and intestinal proteolytic enzymes to form a smaller structure (Steffansen et al., 2005) Also the large molecular size of peptides almost restricts the compounds from entering enterocytes in the intestine (Steffansen et al., 2005). Therefore the delivery of a peptide through the skin presents a challenge for the future development of the TDS® system. These can be confirmed with the result from plasma analysis of all the subjects in 40mg dose group which were found no MT I detected at any time points.

Another parameters that can be considered to influence the tanning capabilities is the MC1R (Melanocortin 1 receptor) polymorphism. MC1R is a G-protein–coupled receptor existing in skin melanocytes, that can activates adenylate cyclase to elevate cyclic adenosine monophosphate levels upon stimulation by the proopiomelanocortin-derived peptides α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (Thody and Graham, 1998). Stimulation of MC1R by α-MSH and other pro-opiomelanocortin (POMC) peptides leads to eumelanogenesis and is central to the tanning response of human skin under UV exposure (Rouzaud et al., 2005;Suzuki et al., 1999). MC1R also regulates the balance of two melanin types, the red/yellow pheomelanin and black/brown eumelanin.

The MC1R gene has found to be highly polymorphic in Caucasian populations (Rouzaud et al., 2005). More than 30 allelic variants of the human MC1R gene have been identified, mostly in European and Australian populations (115,116,120,153).

Among the variants reported, R142H, R151C, R160W, and D294H are known to be associated with a red hair phenotype (116, 118, 120, 154). It can be seen in a study on a series of individuals from a general Irish population. 75 % contained a variant in the MC1R gene, with 30% containing two variants. The Arg151Cys, Arg160Trp, and Asp294His variants were significantly associated with red hair and fair skin (poor tanning capabilities) ($p = 0.0015$, $p < 0.001$, and $p < 0.005$, respectively) (Smith et al., 1998). UV induced tanning maybe ineffective in numerous 'fair-skinned' individuals, which contain functional disruption of MC1-R (D'Orazio et al., 2006).

Although the MC1R genotype may contribute to the poor tanning capabilities as discuss above, the significant of this factor to the result obtained in this study is highly unlikely, especially since the blood results of the highest dose, 40mg, were unable to confirm the absorption of MT I.

Chapter 6 General Discussion

The aim of this thesis was to:-

1) Conduct pilot pharmacokinetic and pharmacodynamic studies in healthy human subjects for the development of a novel transdermal drug delivery system, TDS® which are combined with a few drugs currently used for transdermal delivery.

TDS® is a state of the art technique for transdermal drug delivery. TDS® is a liquid formulation that can be combined with any drug entity to form a novel and more convenient transdermal delivery system. The system would provide a method of delivering drugs that is simple, easy, fast, and effective. An earlier studies has shown that the TDS® system is capable of transporting drug molecules across the human skin (Hadley et al., 1998a). The clinical studies outlined in this thesis are among the early works ever conducted for the TDS® system on humans. The studies reported in this thesis have successfully achieved the aim as outlined in the studies' objectives.

The TDS® system has been combined with two well known drugs, lidocaine and testosterone, and a new drug under investigation the α-melanocyte stimulating hormone, melanotan I. For TDS®-Lidocaine, a pilot study for the efficacy and tolerability of the system has been conducted in 100 healthy subjects and the results have been published in the journal of Anaesthesia (Tucker et al., 2006). Showing that five minutes post application of TDS®-Lidocaine can give acceptable anaesthesia is the major achievement for the local anaesthetic system.

The Phase 1 pharmacokinetic study comparing TDS®-Testosterone, AndroGel® and TDS® - Placebo has been conducted in 12 healthy males. The analysis of the results from the study not only showed that the TDS®-Testosterone was bioequivalent to Androgel, but also attained a higher systemic profile of testosterone than Androgel. The results and the detail of this study have been published in the British Journal of Clinical Pharmacology (Chik et al., 2006a). Due to the existence of endogenous levels of testosterone, a few models for correction and removing the influence of endogenous testosterone have also been studied.

The dose escalation for the investigation of the pharmacokinetic and the skin tanning properties of TDS®-Melanotan has been conducted in 30 healthy Caucasians. The highest dosage, 40mg, given to the subjects did not produce significant tanning on the

skin, but an increases in the melanin density in some regions of the body were observed. However, these result may not being contributed from the administration of MT I as the analysis of melanotan in all the blood samples for the 40 mg dosage have found no MT I detected.

2) Explore, develop, improve and validate the analytical tools available for the analysis of lidocaine in plasma, dialysate and tape samples, and testosterone in plasma.

A few analytical methods have been explored for the analysis of lidocaine in different matrixes and testosterone in serum. For lidocaine in plasma obtained from TDS®-Lidocaine study, the LC-MS-MS method has been developed and validated to show sensitivity, selectivity, accuracy, and precision. This method has improved upon the previously published methods in term of the amount of plasma used and the extraction strategy. The same method has been further validated and applied for the analysis of lidocaine in the dialysate. The method has been shown to be very sensitive as it is capable of analysing the small amount of dialysate from the DMD study (~ 10 μL).

A new method to analyse lidocaine in the tape samples from the DPK study has been developed and validated by using CE with UV detection. Available methods that have been published for the analysis of lidocaine by CE – UV are from the pharmaceutical formulation. The CE method developed for the analysis of lidocaine has been modified in terms of the capillary length to decrease the analysis time. Also the new extraction procedure for the tape samples has been introduced. Whilst for the analysis of testosterone in serum, the ELISA method that has been validated, and came in a kit which was used for it's the ease and simplicity without sacrificing accuracy and precision. To show the necessary accuracy and precision, the controls from a commercial company were used when performing the sample analysis.

3) Explore and compare the various methods available to measure pharmacokinetic profiles of transdermal or topical drugs.

The conventional pharmacokinetic drug measurements in plasma may not be suitable for the pharmacokinetic assessment of transdermal or topical drugs delivery as the drug is designed to target the local tissue. Hence, the comparison study performing systemic measurement, microdialysis and tape stripping has been conducted in 12 healthy subjects by using a local anaesthetic system, EMLA™ cream as a model drug. In this project, the DPK and DMD methods have shown to be far more reliable than systemic

measurement for the pharmacokinetic assessment of a transdermal drug delivery system, especially a local anaesthetic.

6.1 Clinical Trials of a new medicine

Clinical trials as defined by Meinert (Meinert, 1986) are a research activity that involves the administration of a test treatment to some experimental unit in order to evaluate the treatment. Clinical trials of drugs can be defined as a research activity conducted via administration of a drug into healthy subjects or a particular type of patient in order to assess the drug's activity. Piantadosi (Piantadosi, 1997) simply defined a clinical trial is an experimental test of a medical treatment on human subjects. Refering to the above definitions, the investigation of a drug on animals is not considered to be clinical trials. Clinical trials involving healthy volunteers or patients have played an important role in the understanding, prevention, or treatment of the diseases. It is also a key aspect in the development of new medicines or modifications of the existing formulations. Three key aspects involved in the clinical trials are the subjects, the treatments, and the assessments.

Subject refers to the study participants or targeted population, such as healthy volunteers in specific age groups, or a certain ethnic group of ethnicity. Subjects could also be patients with a certain type of disease, etc. The treatment refers to the study drug intended for evaluation. It can be an active ingredient or a placebo. Assessment refers to the evaluation of efficacy of the treatment. It can be a drug or medical device. Clinical trials also, sometimes assess the quality of patient life resulting from the treatment, pharmacogenomics, or pharmacoeconomic. In performing a clinical trial, clinical researchers need to develop the protocol. A protocol is a study plan that contains the details about the study to be carried out and how the samples or data are to be collected and analysed. The protocol is used as the basis of all clinical trials (ABPI, 2003). A proper plan including the drug handling, storage, randomisation, time frame of the study, etc. need to be included in the protocol. The protocol has to be reviewed by the Medicine and Healthcare products Regulatory Agency (MHRA), and approved by an Independent Ethics Committee.

6.2 History and regulations surrounding the clinical trials

The Committee on Clinical Trials was formed by the Medical Research Council of the Great Britain in 1931 (Chow and Liu, 2004) to provide guidelines on the proper conduct of clinical trials. However the legal guidance on the conduct of trials only came into effect in the late twentieth century. Different laws and regulations have been enacted to ensure that clinical trials and their discoveries are effective and safe. A series of suspect medical interventions led to the establishment of the Nuremberg code in 1947 and the first Declaration of Helsinki in 1964. The Declaration of Helsinki has become an important document of ethical code to provide direction for physicians and other participants in medical research involving human subjects. Since then, the Declaration has been subsequently amended in the years 1975, 1983, 1989, 1996, 2000, 2002, and 2004 (World Medical Association, 2004). Many countries including the UK have adopted the Declaration of Helsinki into their clinical trial regulations and it forms part of Good Clinical Practice (GCP).

Europen Union Directive 2001/20/EC of 4 April 2001 (Directive 2001/20/EC, 2001) for the implementation of good clinical practice (GCP) in the conduct of clinical trials on medicinal products for human use was published in May 2001 by the European Parliament and the Council of The European Union. It took about three years to incorporate into national legislation and compliance by May 2004. This EU Directive was incorporated into UK Law by means of 'The Medicines for Human Use (Clinical Trials) Regulations 2004' (HMSO, 2004). UK Law now requires that all the trials must adhere to the principles of GCP. The Directive requires the "competent authorities" of the Member States to perform certain functions in relation to clinical trials. In the UK, the competent authority responsible for those functions is the (MHRA). In relation to the above directive, the UK's current practice in the clinical trial has had major changes as detailed below (MHRA, 2004)

- Pharmacology studies in healthy human volunteers (Phase I studies) require authorisation from the MHRA where previously they only needed a favourable opinion of an ethics committee;
- Investigational medicinal products (IMPs) must be manufactured to good manufacturing practice (GMP) standards and the manufacturer must have a manufacturing licence; and

- Each trial must have an identified sponsor who takes responsibility for its initiation, management and conduct. The Regulations allow a group to collaborate to take on these responsibilities.

The above changes have placed all the clinical trials in UK under a further layer of bureaucratic scrutiny. The Directive imposes a heavy burden of extra costs, complexity and paperwork upon universities and hospitals carrying out low-budget studies on medicinal products. With a large number of clinical trials conducted in the UK each year, it means that the process to obtain approval for one trial seems to be longer than ever. The UK is among the leading countries in the world in the development of medicinal products. Nearly a quarter of the world's top 100 medicines were developed in the UK (ABPI, 2003). With new legislation, it has caused a major delay to every clinical trial to be conducted. The two clinical studies in this PhD project (TDS®-Testosterone and TDS®-Melanotan) fell under the remit of the clinical trial regulations, therefore needed to comply with new legislation. To comply with all of the above requirements, the TDS®-Melanotan study was delayed for nearly one year beyond the original schedule.

6.3 Pharmacokinetics and Pharmacodynamics

Pharmacokinetic, often abbreviated to "PK" is a concept of describing and predicting the time course of a drug's action in the body. The word pharmacokinetics was originally derived from the Greek root *pharmacon*, a drug or poison, and *kinetikos*, meaning movement. Hence pharmacokinetics is a very important area in a drug delivery study as it provides the important information regarding the drug's behaviour in the body. This can be obtained through the four principles of PK, which are absorption, distribution, metabolism, and excretion or usually abbreviated to ADME. In other words pharmacokinetics explore how the body handles a foreign substance following it's administration into the body.

Following drug administration, through oral, intravenous injection, trasdermal methods, etc., the goal is to achieve the desired therapeutic outcome. The desired therapeutic outcome only can be achieved if the amount of drug administered is sufficient enough to interact with the specific receptor site, which will then provide the response or outcome. The evaluation of the responses or outcomes from the results of a drug's reaction with the receptor sites with time is called pharmacodynamics. This means that any changes in pharmacokinetics will effects the pharmacodynamic outcomes. It is often assumes that

drug doses and the therapeutic effects or the outcome are linearly or log linearly related. This can be seen in the application of local anaesthetic, where the degree of anaesthesia is log linearly related to the lidocaine content in the skin as observed in microdialysis study of EMLA cream in this project. In addition, pharmacodynamic outcome is useful to support the pharmacokinetic data.

6.4 Which pharmacokinetic methods for the study of transdermal drug delivery?

The body can be represented as a series of compartments such as blood and tissue that have a close relationship between each other. In the compartmental analysis, the pharmacokinetics of drugs entering the body has always been evaluated through it's plasma/serum or sometime urine profiles. Theoretically, the pharmacokinetics of drugs should be evaluated at the receptor site, at which the drug binds for its action. However, this evaluation is not possible as some of the receptor sites for drugsare not clearly understood. Therefore the easiest way to assess a drug concentration related to its effect is via the blood. Blood is easily accessible and the concentration of drug in the blood is always in equilibrium with the receptor.

For topical drugs that are designed to target the local tissue, it is quite clear that the receptor sites are located within the skin and probably at the application site. Therefore it is possible to monitor the drug profile directly at the receptor site rather than in blood. In recent years, various methods to monitor the pharmacokinetics of drugs within the local tissue have been established and evaluated including DMD, DPK, skin blister fluids methods, etc. Among them, DMD and DPK are widely used due to their reliability and convenience. Despite being widely and successfully used in drug delivery studies, the question arises as to why DMD and DPK are not yet accepted as the standard methods for the pharmacokinetic assessment of a transdermal drug. A few issues still need to be addressed before they can be regarded as generally applicable routine techniques for cutaneous drug delivery assessments.

A major limitation of the DMD technique is the low recovery for molecules with large molecular weights such as insulin (Jansson et al., 1993) or those with a high lipophilicity (Groth, 1996). Sensitive analytical techniques such as LC-MS-MS are also required due to the small sample volumes and low drug concentrations obtained in the DMD experiments. High variabilities in the results obtained are also a subject of

argument against DMD. The implantation depth of the probe may contribute to the large variability of the pharmacokinetic parameters, as variability appears to be larger between individuals, compared to within individuals when multiple probes are used in a cutaneous drug penetration studies (Benfeldt and Serup, 1999;Kreilgaard, 2001;Kreilgaard et al., 2001). (Kreilgaard, 2002) reported that large variability is often found in cutaneous drug delivery determined by the DMD technique (CV typically 50-100%). In a human microdialysis study, pharmacokinetic profiles of lidocaine were similar for paired DMD probes under the same application site (CV 4% of lag time). However, the inter-individual, CV 38% was obtained in the lag time result (Kreilgaard et al., 2001). The good reproducibility of the DMD technique for sampling in the skin and subcutaneous tissues was shown by lower variability of DMD following oral administration of acetaminophen in healthy subjects (Muller et al., 1995). In this study, the CV, 30% obtained for DMD was not substantially different from the variability of parameters determined directly from plasma samples.

On the other hand, DPK is a method with great potential and requires thorough consideration as a pharmacokinetic method in the skin. Although DPK showed a good reproducibility and relatively is simple to perform, many factors remain to be investigated. There are many opportunities for experimental artifacts to develop. For example, tape strip samples have high surface area to volume ratios, and losses by evaporation can be significant especially for a volatile compound. The analysis of the samples should be completed soon after tape strip removal from the skin (Islam et al., 1999) or to minimise this condition, the stripping tape should be kept in dry ice immediately after the stripping process for labile drugs

Other factors that may contribute to the source of variation include the pressure applied to the tape prior to stripping, and the peeling force applied for removal. There are no standard methods to describe how the tape can be applied to the skin. This fact may increase inter-subject and inter-investigator variations if not given proper consideration. In the study by Saqib et al. (Bashir et al., 2001) they attempted to improve standardisation, quantifying the mass and thickness of stratum corneum removed by the tapes utilized. The tapes were pressed onto the skin with a standardised 10 kPa pressure for 2 seconds. This study provides clear guidance on how to standardise the amount of SC removed. The study also quantified the mass of SC removed for different types of tapes and at different sites. For the DPK performed on the dorsal and volar forearm of each subject, with three types of tape (D-Squame®, Transpore®, and Micropore®) there

was no significant difference in the amount of SC removed by any of the tapes utilised, although there was a difference in the degree of barrier disruption to water. Also there was no significant difference in the amount of SC removed between the dorsal and the ventral site for each tape. The conclusion made following this study is for the tapes and sites tested, neither the site nor the type of tape significantly influenced the mass of SC removed by the tapes.

Another factor that can influence the amount of stratum corneum that can be removed by a piece of tape is the existence of furrows in the human epidermis. Van der Molen (van der Molen et al., 1997) validated the tape-stripping method with X-ray microanalysis in the mapping mode by scanning electron microscopy using TiO_2 as a marker. They have found that TiO_2 applied on the skin still present even after ten tapes were stripped and specifically located on the rim of the furrows. A histological section that was tape stripped 20 times also showed non-stripped skin in the furrows. This finding gave some impact to the question concerning concerns regarding consistency of the tape stripping method. If the materials applied to the skin accumulated in the furrows, the interpretation of the tape stripping result will have a slight variation. This consequence is very important, especially when performing the bioequivalence study.

Recent publication by Shah (Shah, 2005) on the *in vitro-in vivo* correlation for topically applied preparations contained a contradiction results of DPK study. The results of a bioequivalence tretinoin + isotretinoin (Ortho and Bertek products) study conducted by Franz (FDA, 2001a) and Pershing (Pershing et al., 2003) on the same brand were found them to be different. Although the two studies obtained the same conclusion of bioinequivalence, the order of DPK profiles of the two products was reversed in the two studies. Pershing's study showed that the Ortho product had a higher DPK profile than Bertek, but Franz's study showed the opposite. These contradictory results have had an adverse impact on the perceived reliability and accuracy of the DPK method.

6.5 Progress in transdermal delivery system

The development of alternative routes of drug administration, such as a transdermal route compared to the conventional oral route has gained considerable importance during the recent decade. Transdermal delivery systems have been evaluated for more than 20 years, since the first scopolamine patches for the prevention of motion sickness, approved in 1979 (Langer, 2004). It is now regarded as one of the important routes for

drug administration especially for orally problematic drugs. The transdermal delivery systems such as patches, cream, ointment, liquid formulations, or gel have been widely discussed as the most popular mode of application in transdermal drug technology. However, there are a few more systems currently under investigation that have potential to be a powerful drug delivery system such as a liposomal drug delivery sytem, iontophoresis, magnetophoresis, sonophoresis, etc.

Liposomes, also known as lipid vesicles, which fully surround by an aqueous volume. Liposomes can trap hydrophilic molecules within their aqueous region. Liposome structures are made of amphiphilies which can be characterised by a hydrophilic (head), and hydrophobic (tale) (Yarosh, 2001). When created, the hydrophilic heads align along the vesicle surface with hydrophobic tales pointed inward. Multilamellar vesicles can be created by repeating this pattern. Liposomes were first used in transdermal delivery by (Mezei and Gulasekharam, 1980). Encapsulation of a drug into a lipid vesicle can increase the drug permeation across the skin. Various liposomes have been evaluated for transdermal delivery such as topical anaesthesia (Fisher et al., 1998), liposomal tacrolimus (Dutta et al., 1998), liposomal triamcinolone acetonide (Abraham et al., 1983), inhaled liposome-encapsulated fentanyl (Hung et al., 1995), etc. also showed a good result. There are many other 'somes' that have been evaluated such as ethosomes (Dayan and Touitou, 2000;Touitou et al., 2000), niosomes (Uchegbu et al., 1994), etc. With all of the positive results obtained, liposomal delivery system might be useful in the treatment of various skin diseases (Schmid and Korting, 1994).

The most significant progressions in transdermal delivery have been seen in the application of electrical potential gradient to move charged of drug molecules across the skin. Iontophoresis has been shown recently, to provide enhanced transportation for topically applied therapeutic agents (Fang et al., 2000;Huang et al., 2005;Jaw et al., 1995). The combination of iontophoresis with chemical penetration enhancers such as sodium dodecyl sulphate also increased the drug flux as studied by (Wang et al., 2000b) on the delivery of hydrocortisone . Electroporation like iontophoresis, involves the application of electric pulses to the skin which has been suggested to induce the formation of transient pores. The pores are responsible for an increase in skin permeability (Weaver et al., 1999). Magnetophoresis, by using magnetic fields to deliver drug through the skin has also has been evaluated. It has been shown that increasing the magnetic field can increase the diffusion flux of a drug (Murthy, 1999). Low-frequency ultrasound was also shown to increase the permeability of human skin

to many drugs, including high molecular weight proteins such as insulin (Mitragotri et al., 1995).

6.6 Hypogonadism: The treatments and the consequences

Male hypogonadism is a disturbing factor which significantly reduces quality of life. Hypogonadism can affect men at any stage of life, but is mostly seen through ageing. Ageing can be a major cause of testosterone deficiency. (Vermeulen and Kaufman, 1995) estimate that 7% of men aged 40-60 years have serum total testosterone concentrations of < 12 nmol/L, and this percentage increases to 21% and 35% of 60-80 and > 80 year olds, respectively. Many hypogonadism patients are untreated due to lack of diagnosis or possibly due to the taboo surrounding 'maleness' and sexuality (Nieschlag et al., 2004). Testosterone replacement therapy is the only option for the treatment of hypogonadism.

Although hypogonadism can be treated by using testosterone replacement therapy, most of the therapies that have been developed were not really effective or were inconvenient to the patients as widely discussed in Chapter 3. Among the many treatments options for hypogonadism, only the transdermal preparation is widely used due to its effectiveness and convenience of use. Testosterone gel formulations provide stable levels of serum testosterone within the normal range, and little skin irritation compared to patches. The only deficiency of testosterone gel treatment is the possibility of transfer of testosterone from the patient to another person if there is skin contact at the application site, and the application site cannot be washed for several hours after application. According to the World Health Organisation Guidelines for use of Androgens in Men (1992) (Nieschlag et al., 2004), the ideal testosterone replacement therapy should offer: safety, efficacy, value for money, convenience, a good release profile, dosing, flexibility, and effective normalisation of testosterone levels. Refering to this definition, none of the currently available replacement therapies can be considered as an ideal therapy. TDS® - Testosterone is hoped to be an ideal testosterone replacement therapy as the results obtained in this PhD project are considerably promising.

Although testosterone replacement therapy can successfully restore the physiological level of testosterone, there are some adverse events reported following the treatments. Some of the testosterone replacement therapies as mentioned above have been reported to cause some adverse side effects including gynaecomastia, polycythemia, weight gain,

and increased prostate size (Arver et al., 1996;Dobs et al., 1999;Meikle et al., 1997). A change in lipid profile has also been noted, such as decreased HDL-cholesterol and increased LDL-cholesterol under testosterone replacement therapy (Jockenhovel et al., 1999). In the meta analysis of 19 studies associated with testosterone replacement and males ≥ 45 years old, a significantly higher cases of adverse events were recorded for the replacement therapy (Calof et al., 2005). The combined rates of all prostate events were found to be higher in testosterone-treated men than in placebo-treated men. Rates of prostate cancer, prostate-specific antigen, and prostate biopsies were also higher in the testosterone group rather than in the placebo group. Testosterone treated men are also nearly four times as likely to have a hematocrit level of > 50 % higher than placebo treated men.

6.7 Conclusion

The end of the 20th Century had seen an explosion in the discovery of medicinal products. These included the development of new drugs or modifications of existing drugs. The development of new drugs is essential due to the increasing number of untreated diseases and for the enhancement of the quality if life. Modifications of an existing drug include improvement in the formulations or the creation of new delivery systems to enable the drug to be more effective. Progress in the drug delivery has seen transdermal delivery method became increasingly popular especially for the various skin treatments and for systemic delivery of orally problematic drugs as discussed in the earlier chapters.

The TDS® delivery system has been created to offer this advantage and act as alternative to the oral and other delivery systems. The TDS® system has been found to be effective in transporting lidocaine and testosterone through the skin in this project. However, the delivery of the peptide drug, MT I cannot be confirmed until the plasma samples obtained from the study are assayed. The phase II pharmacokinetic study of TDS®-Testosterone in hypogonadal patients is essential for further development of this system as a hormone replacement therapy. A proper pharmacokinetic study in healthy subjects is needed for the future development of TDS®-Lidocaine as a local anaesthetic system. The outcomes from this project give an early indication of the future acceptance of the TDS® system which can be combined with any drugs to form a powerful tool in drug delivery.

The requirement to develop analytical techniques that are sensitive, accurate and have short analysis time is important. Simple and effective analytical techniques should be available to cope with the increasing number of drugs being developed. This has led to a growing number of immunoassay techniques which are as reliable and comparable in costs to chromatographic techniques.

The increasing number of developing transdermal drug delivery systems has also contributed to the establishment of a few methods to study the behaviours of the drugs in the body. *In vivo* methods for the pharmacokinetic assessment of topical drug products such as DMD and DPK have been studied extensively for transdermal delivery drugs. These methods have been shown to offer clinically relevant advantages over conventional systemic measurements in blood. From the overall perspective, DMD and DPK have the potential to be standardised as pharmacokinetic methods for transdermal drugs, but thorough consideration of certain aspects of the methods are necessary for their validity.

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Appendices

Appendix 1 Ethics approval letter for TDS®-Lidocaine study

North East London NHS
Health Authority

Aneurin Bevan House
81 Commercial Road
London E1 1RD

Tel: 020 7655 6600
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Arthur T. Tucker PhD SRCS
Research Scientist
The Ernest Cooke Clinical Microvascular Unit
4th Floor, Dominion House
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London EC1A 7BE

Our Ref: RS/SA/P/02//087

8th August 2002

Dear Mr Tucker

Re: P/02/087 – Study of a combined percutaneous local anaesthetic and the TDS system for venepuncture

Thank you for your letter dated 30th July 2002 regarding the above protocol. I acknowledge that the following have been received and added to our files:

- Details of the Medicines Control Agency Exemption Certificate (DDX)
- Indemnity cover for both the BLT and the Medical School.

As written confirmation of indemnity has now been received, you may proceed with the study.

I can also confirm approval under Chairman's Action on behalf of the North East London Research Ethics Committee to your proposal of using internal e-mail to recruit volunteers to the study.

Yours sincerely

Mr Richard Smith
Chairman
North East London Research Ethics Committee

Appendix 2 Ethics approval letter for TDS®-Testosterone study

NHS

East London and The City Local Research Ethics Committee
Aneurin Bevan House, 81 Commercial Road, London E1 1RD
Telephone Number: 020 7 655 6622
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Arthur Tucker PhD SRCS
Research Scientist
The Ernest Cooke Clinical Microvascular Unit
4th Floor, Dominion House
Department of Clinical Physics
Clinical Support Services
St Bartholomew's Hospital
London EC1A 7BE

Our Ref: RS/SB/P03180 9th October 2003

Dear Dr Tucker

Re: P/03/180 – Study of a combined percutaneous testosterone and the TDS System for hormone replacement

Thank you for your recent letter addressing the points of the Committee's earlier letter. I am happy to tell you that I am now able to approve this study on Chairman's action to be noted at future meeting of the Committee.

Please note the following conditions to the approval:

1. The Committee's approval is for the length of time specified in your application. If you expect your project to take longer to complete (I.e. collection of data), a letter from the principal investigator to the Chairman will be required to further extend the research. This will help the Committee to maintain comprehensive records.

2. Any changes to the protocol must be notified to the Committee. Such changes may not be implemented without the Committee or Chairman's approval.

3. The Committee should be notified immediately of any serious adverse events or if the study is terminated prematurely.

4. You are responsible for consulting with colleagues and/or other groups who may be involved or affected by the research, such as extra work for laboratories.

5. You must ensure that, where appropriate, nursing and other staff are made aware that research in progress on patients with whom they are concerned has been approved by the Committee.

6. The Committee should be sent one copy of any publication arising from your study, or a summary if there is to be no publication.

I should be grateful if you would inform all concerned with the study of the above decision.

Your application has been approved on the understanding that you comply with Good Clinical Practice and that all raw data is retained and available for inspection for 15 years.

Please quote the above study number in any future related correspondence.

Yours sincerely

P.p. Senior Administrator
MR RICHARD SMITH
Chairman
East London and The City Research Ethics Committee

East London & The City HA Local Research Ethics Committee 3

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:	05/Q0605/98	**Issue number:**	1	**Date of issue:**	09 September 2005
Chief Investigator:	Professor Atholl Johnston				
Full title of study:	Comparative pharmacokinetic study of a combined percutaneous local anaesthetic/Nitric Oxide generating system and EMLA cream: Evaluation by systemic measurement, skin stripping and microdialysis				

This study was given a favourable ethical opinion by East London & The City HA Local Research Ethics Committee 3 on 31 August 2005. 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)*
Prof Atholl Johnston	Professor of Clinical Pharmacology,	Barts and the London NHS Trust	East London & The City HA Local Research Ethics Committee 3	09/09/2005	

Approved by the Chair on behalf of the REC:

S. Grate ..... (Signature of Chair/Administrator)
(delete as applicable)

Sandra Grate ..... (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.*

Appendix 3 Ethics approval letter for comparative pharmacokinetic study of dermatopharmacokinetic, microdialysis and systemic measurement

East London & The City HA Local Research Ethics Committee 3
3rd Floor, Aneurin Bevan House
81-91 Commercial Road
London
E1 1RD
Telephone: 020 7655 6612
Facsimile:020 7655 6655

09 September 2005

Professor Atholl Johnston
Professor of Clinical Pharmacology
Barts and The London, Queen Maryâ€™s School of Medicine & Dentistry
Clinical Pharmacology
Willliam Harvey Research Institute
Charterhouse Square, London
EC1M 6BQ

Dear Professor Johnston

Full title of study: **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 number: **05/Q0605/98**

Thank you for your letter of 08 September 2005, 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.

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.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	1	09 May 2005
Investigator CV	1	03 May 2005
Protocol	2	04 May 2005
Peer Review	1	03 May 2005

Copies of Advertisements	1	03 May 2005
GP/Consultant Information Sheets	1	25 April 2005
Participant Information Sheet	2	(None Specified)
Participant Consent Form	2	03 May 2005
Response to Request for Further Information		19 August 2005
Response to Request for Further Information		08 September 2005
Patients Information Sheet	2	08 September 2005
e-mail from MHRA		17 August 2005
Covering Letter	1	04 May 2005
Checklist	2	03 May 2005
Letter to Sponsor	1	25 April 2005
Letter to Clinical Trials Unit	1	04 May 2005
Letter to R&D – Indemnity	1	25 April 2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Notification of other bodies

The Committee Administrator will notify the research that the study has a favourable ethical opinion.

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

With the Committee's best wishes for the success of this project,

Yours sincerely

pp **Dr David Ingram**
Chair

Email: sandra.grote@nelondon.nhs.uk

SF1 list of approved sites

East London & The City HA Local Research Ethics Committee 3

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:	05/Q0605/98	**Issue number:**	1	**Date of issue:**	09 September 2005
Chief Investigator:	Professor Atholl Johnston				
Full title of study:	Comparative pharmacokinetic study of a combined percutaneous local anaesthetic/Nitric Oxide generating system and EMLA cream: Evaluation by systemic measurement, skin stripping and microdialysis				

This study was given a favourable ethical opinion by East London & The City HA Local Research Ethics Committee 3 on 31 August 2005. 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]
Prof Atholl Johnston	Professor of Clinical Pharmacology,	Barts and the London NHS Trust	East London & The City HA Local Research Ethics Committee 3	09/09/2005	

Approved by the Chair on behalf of the REC:

S. Grote ..... (Signature of Chair/Administrator)
(delete as applicable)

Sandra Grote ..... (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.*

Appendix 4 Ethics approval letter for TDS®-Melanotan study

Dr A T Tucker
The Ernest Cooke Clinical Microvascular Unit
4th Floor, Dominion House
Department of Clinical Physics
Clinical Support Services
St Bartholomew's Hospital
London EC1A 7BE

ST THOMAS' HOSPITAL
RESEARCH ETHICS COMMITTEE
Ethics Committee Office
Block 5, South Wing,
St Thomas' Hospital
London SE1 7EH

Chairman – Dr A Hopper/Dr AJ Williams
Administrator - Ms S Hirsch

Phone: 020 7188 2257
Fax: 020 7188 2258
Email: Stella.hirsch@gstt.sthames.nhs.uk

11 November 2004

Dear Dr Tucker

REC reference number: 04/Q0702/120
Full title of study: ***A Dose-Escalation study of topical application of TDS-Melanotan to Assess the Pharmocokinetics and skin melanin density in Healthy Adult Subjects***

Thank you for your letter of 10 November 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman on 11 November 2004.

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.

The favourable opinion applies to the following research site:

Site: Barts and the London NHS Trust, Barts and the London School of Medicine and Dentistry
Chief/Principal Investigator: Dr Charlotte Mary Proby Consultant Dermatologist & Senior Lecturer

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.

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document Type: Application
Dated: 24/09/2004
Date Received: 06/10/2004

Document Type: Investigator CV
Dated: 24/09/2004
Date Received: 04/10/2004

Document Type: Protocol
Version: 4.3
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: Covering Letter
Dated: 20/09/2004
Date Received: 04/10/2004

Document Type: Summary/Synopsis
Version: 4.3
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: Letter from Sponsor
Dated: 29/09/2004
Date Received: 04/10/2004

Document Type: Peer Review
Dated: 07/09/2004
Date Received: 04/10/2004

Document Type: Compensation Arrangements
Dated: 06/10/2004
Date Received: 06/10/2004

Document Type: Sample Diary/Patient Card
Version: 1.0
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: Copies of Advertisements
Version: 1.0
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: GP/Consultant Information Sheets
Version: 1.0
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: Participant Information Sheet
Version: 1.1
Dated: 10/11/2004
Date Received: 11/11/2004

Document Type: Participant Consent Form
Version: 1.0
Dated: 16/09/2004
Date Received: 04/10/2004

Document Type: Investigator's Brochure
Version: Edition No: 6
Dated: 14/05/2004
Date Received: 04/10/2004

Document Type: Response to Request for Further Information
Dated: 10/11/2004
Date Received: 11/11/2004

Management approval

The study may not commence until final management approval has been confirmed by the organisation hosting the research.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant host organisation before commencing any research procedures. Where a substantive contract is not held with the host organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Notification of other bodies
We shall notify the research sponsor, Barts and the London School of Medicine and Dentistry that the study has a favourable ethical opinion.

Statement of compliance *(from 1 May 2004)*
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.

REC reference number: 04/Q0702/120 Please quote this number on all correspondence

Yours sincerely,

Dr A J Williams
Co-Chairman

Enclosed: **Standard approval conditions SL-AC2**
Membership list

Copy:R&D

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:	04/Q0702/120	**Issue number:**	1	**Date of issue:**	15 November 2004
Chief Investigator:	Dr Charlotte Mary Proby				
Full title of study:	A Dose-Escalation study of topical application of TDS-Melanotan to Assess the Pharmacokinetics and skin melanin density in Healthy Adult Subjects				

This study was given a favourable ethical opinion by St Thomas' Hospital Research Ethics Committee on 11 November 2004. 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]
Dr Charlotte Mary Proby	Consultant Dermatologist & Senior Lecturer,	Barts & The London NHS Trust and Barts & The London School of Medicine & Dentistry. Clinical Trials Unit, Clinical Pharmacology, 2nd Floor, John Vane Building, Charterhouse Sqaure, LondonandThe Ernest Cooke Clinical Microvascular Unit, 4th Floor Dominion House, St. Bartholomew's Hospital.	East London & The City HA Local Research Ethics Committee 3	15/11/2004	

Approved by the Chair on behalf of the REC:

* ..... (Signature of Chair/~~Administrator~~*)

(**delete as applicable*)

A J WILLIAMS ..... (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.*

Appendix 5 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 anaesthetic/Nitric Oxide generating system and EMLA cream: 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 5.0 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.	☐
I 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
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: .....

Appendix 6 Study Information Sheet (sample)

Study of a combined percutaneous local anaesthetic/Nitric Oxide generating system and EMLA cream: Evaluation by systemic measurement, skin stripping and microdialysis.

Lay title: "Study of skin anaesthetic drug delivery"

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.

Nitric Oxide [*NO*] is a simple gas of huge importance for the circulation system and it was also found to exert a series of other functions. We know today that *NO* also acts as a signal in the nervous system, as a weapon against infections, as a controller of blood pressure and blood flow. *NO* is present in most living creatures and made by many different types of cells. We have found a simple way of making NO by combining two substances together (Sodium nitrite and Vitamin C). We have already found that this mixture increases blood flow when placed on the skin and is very safe.

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. EMLA cream is a local anaesthetic skin cream currently used for skin anaesthesia in the venepuncture and minor procedure.

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.

<u>What would I do in the study, if I took part</u>?

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 three 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 of EMLA

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 (EMLA cream) 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 EMLA cream 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.

Visit 3 and 4: NO Lidocaine study

After a minimum of one week from visit 2, you will come again to repeat the above procedures with NO Lidocaine treatment

- **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. Having completed the first two visits, we will pay you £100-00 and pay you another £100-00 if you completed the visit 3 and 4.

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. The two substances used in the gels have both been used already in patients and volunteers for other reasons. Ascorbic acid (Vitamin C) is used as a vitamin supplement and is known to be safe. Sodium nitrite is used for the treatment of chemical poisoning.

The anaesthetic (lidocaine) used in this study has been used safely for many years and is used at a very low concentration.

EMLA cream is safe and widely used currently as a skin-based local anaesthetic.

There are very few risks involved in inserting a needle into a vein in the hand for venous sampling and subcutaneous tissue for microdialysis 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. Zamri Chik
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 7 Delegations Log (sample)

Study title:

All those involved in the above study must read the protocol (and amendments if applicable) and understand their role as outlined in the protocol

Name (print)	Job title	Signature	Signed Initials	List duty categories	PI signature & date	Date of leaving study team (if appl.)

Key for list of duty categories :

1. Obtaining informed consent
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 events & SAE
7. Medical prescriptions
8. Drug accountability
9. Maintaining investigator file
10. Archiving

Other duties specific to above study Please specify below

11. Principal Investigator
12. Study Support

Appendix 8 Screening Log (sample)

Study Title:

Date	Patient Initials	Date of birth	Hospital Number	Sex	Enrolled (yes or no)	Reason for exclusion

Appendix 9 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 10 Raw data of pain score (VAS and VRS) for TDS®- ß in Chapter 2

Subject	Sex	Age	Treatment/ Result			
			TDS®- ß (Active)		TDS®- ß (Placebo)	
			VRS	VAS	VRS	VAS
		(y)	(Unit)	(mm)	(Unit)	(mm)
1	F	25	4	48	2	11
2	F	34	4	62	3	46
3	M	30	4	71	4	82
4	F	29	3	38	2	13
5	M	23	4	71	3	39
6	F	28	3	35	3	30
7	F	32	3	23	4	41
8	F	30	4	34	4	46
9	M	28	2	22	3	29
10	M	23	2	21	5	76
11	F	36	4	63	3	47
12	F	26	3	45	4	69
13	M	23	2	26	3	48
14	F	32	4	72	4	65
15	M	35	2	30	3	38
16	F	29	2	16	3	70
17	M	25	2	18	3	44
18	M	31	3	39	3	39
19	F	33	4	71	5	88
20	F	28	4	57	4	64
21	F	28	3	46	4	76
22	F	40	3	27	4	41
23	F	32	2	22	3	50
24	F	24	3	24	2	1
25	F	23	3	39	3	53
26	F	23	4	77	3	48
27	F	23	4	74	4	86
28	F	27	4	74	2	22
29	F	23	3	41	4	63
30	F	25	2	30	4	53
31	M	37	3	29	2	7
32	F	30	2	26	4	58
33	M	29	2	38	3	47
34	F	31	3	18	4	51
35	F	42	3	41	3	42
36	M	26	4	52	2	25
37	M	29	2	38	2	14
38	F	34	3	48	2	24
39	M	22	3	33	3	40
40	M	25	2	22	4	60
41	M	38	2	24	2	23
42	M	21	3	52	2	22
43	M	32	3	34	3	31
44	F	30	4	59	2	25
45	F	21	3	30	3	16
46	F	21	2	18	3	27
47	F	27	2	27	4	60
48	F	20	3	37	2	16
49	F	28	4	80	4	78
50	F	27	3	30	3	11

51	F	21	3	27	3	24
52	F	30	4	69	5	88
53	M	22	3	36	2	24
54	F	24	2	55	4	67
55	M	23	3	22	4	34
56	M	22	2	30	2	47
57	F	27	3	48	3	47
58	F	25	2	40	3	48
59	F	22	3	26	4	25
60	F	25	2	10	3	39
61	M	28	2	15	2	24
62	F	30	1	14	2	17
63	F	40	3	48	4	73
64	F	22	3	19	4	43
65	M	20	1	15	2	34
66	M	21	2	22	3	44
67	F	25	2	26	4	70
68	M	21	2	33	4	64
69	F	20	3	56	3	63
70	F	20	2	20	3	39
71	F	22	3	35	4	75
72	M	21	3	32	3	33
73	M	22	3	22	2	34
74	F	20	3	23	2	14
75	M	21	2	18	3	40
76	M	20	3	41	3	42
77	M	21	3	44	2	22
78	M	22	3	71	4	88
79	M	23	3	38	2	16
80	F	21	3	35	3	47
81	M	26	3	48	3	40
82	F	29	2	24	3	37
83	F	25	2	29	3	41
84	M	23	3	37	4	57
85	F	26	3	35	4	61
86	M	32	3	31	3	39
87	F	22	4	67	3	53
88	M	22	3	37	4	69
89	F	31	4	49	4	52
90	M	20	2	35	4	63
91	M	21	3	57	2	35
92	M	24	4	50	3	38
93	F	26	4	73	3	47
94	F	24	3	45	4	65
95	M	39	3	44	4	64
96	M	35	2	28	1	3
97	F	29	3	20	2	11
98	M	24	3	39	3	31
99	F	22	4	63	5	78
100	F	28	3	18	4	62

Appendix 11 Raw data of pain score (VRS and VAS) for TDS®-α in Chapter 2

Subject	Sex	Age	Treatment/ Result			
			TDS®- α (Active)		TDS®- α (Placebo)	
			VRS	VAS	VRS	VAS
		(y)	(Unit)	(mm)	(Unit)	(mm)
1	F	25	3	45	3	37
2	F	34	4	53	3	54
3	M	30	4	76	3	55
4	F	29	3	51	2	33
5	M	23	3	31	4	72
6	F	28	3	36	2	25
7	F	32	2	20	4	52
8	F	30	2	14	3	40
9	M	28	3	61	5	84
10	M	23	4	69	2	21
11	F	36	2	22	4	59
12	F	26	5	94	4	75
13	M	23	4	73	2	32
14	F	32	4	90	4	75
15	M	35	3	33	2	20
16	F	29	4	64	3	44
17	M	25	2	34	4	57
18	M	31	2	33	4	79
19	F	33	3	34	2	10
20	F	28	4	67	3	52
21	F	28	2	29	4	66
22	F	40	4	65	2	18
23	F	32	4	64	4	60
24	F	24	3	39	4	60
25	F	23	3	30	3	59
26	F	23	3	55	4	73
27	F	23	4	85	4	73
28	F	27	5	96	4	67
29	F	23	3	41	3	38
30	F	25	2	16	2	16
31	M	37	2	15	3	41
32	F	30	4	75	2	23
33	M	29	4	62	4	60
34	F	31	4	60	2	22
35	F	42	2	26	3	38
36	M	26	4	42	4	64
37	M	29	2	19	2	12
38	F	34	2	9	3	31
39	M	22	1	36	3	65
40	M	25	4	68	2	28
41	M	38	2	11	2	14
42	M	21	2	21	3	45
43	M	32	3	37	2	15
44	F	30	2	19	2	18
45	F	21	2	11	4	51
46	F	21	2	12	3	41
47	F	27	2	11	4	72
48	F	20	3	34	2	18
49	F	28	3	50	5	93
50	F	27	4	66	2	5

51	F	21	2	8	2	22
52	F	30	4	72	5	91
53	M	22	3	52	4	74
54	F	24	2	28	4	63
55	M	23	2	23	2	10
56	M	22	4	59	3	50
57	F	27	2	20	2	16
58	F	25	3	50	2	37
59	F	22	3	39	3	40
60	F	25	2	14	3	46
61	M	28	1	16	3	26
62	F	30	2	6	2	9
63	F	40	2	32	2	33
64	F	22	5	86	5	85
65	M	20	3	41	2	16
66	M	21	4	67	3	55
67	F	25	2	33	3	57
68	M	21	3	44	4	66
69	F	20	3	44	4	66
70	F	20	2	16	3	23
71	F	22	4	66	3	52
72	M	21	2	17	2	18
73	M	22	4	49	3	24
74	F	20	3	48	2	11
75	M	21	1	3	2	30
76	M	20	4	65	3	49
77	M	21	3	45	2	27
78	M	22	4	62	3	35
79	M	23	2	4	3	33
80	F	21	3	29	3	38
81	M	26	2	32	4	76
82	F	29	3	35	3	26
83	F	25	2	36	3	48
84	M	23	2	37	3	45
85	F	26	4	49	3	32
86	M	32	2	18	2	28
87	F	22	3	45	4	70
88	M	22	2	27	4	74
89	F	31	4	50	3	37
90	M	20	3	42	2	21
91	M	21	4	56	3	48
92	M	24	3	47	3	44
93	F	26	4	74	3	55
94	F	24	5	88	4	61
95	M	39	3	56	2	36
96	M	35	4	74	3	50
97	F	29	3	26	4	25
98	M	24	4	59	4	61
99	F	22	4	66	3	36
100	F	28	4	58	3	26

Appendix 12 Plasma concentrations at 2 hours post dose for TDS®- α and TDS®- ß in Chapter 2

Subject	TDS®- α (ng/mL)	TDS®- ß (ng/mL)
1	0.97	0.53
2	0.00	0.00
3	1.87	5.19
4	1.88	0.00
5	0.00	0.00
6	0.00	0.00
7	55.90	46.51
8	0.77	0.00
9	0.00	0.00
10	0.00	0.00
11	0.52	0.00
12	0.00	0.00
13	0.55	2.02
14	0.00	0.46
15	0.49	0.00
16	0.00	1.10
17	0.60	1.19
18	0.54	16.59
19	0.00	0.00
20	0.00	0.00
21	0.52	25.75
22	0.00	0.00
23	0.00	0.00
24	0.00	0.00
25	0.51	1.00
26	0.00	0.00
27	0.00	0.00
28	0.00	0.00
29	0.00	0.00
30	0.86	0.00
31	0.00	7.18
32	0.43	0.56
33	1.24	0.00
34	0.00	0.00
35	0.00	0.00
36	3.08	0.00
37	0.00	0.00
38	0.65	1.15
39	0.00	0.00
40	0.92	1.09
41	7.24	1.79
42	0.62	0.00
43	1.00	2.22
44	1.00	0.92
45	1.23	0.00
46	7.27	0.00
47	2.45	5.51
48	1.68	0.95
49	0.00	0.00
50	0.00	5.08

51	1.23	0.41
52	0.00	0.41
53	0.00	1.00
54	4.90	2.81
55	2.29	0.00
56	0.00	0.00
57	0.75	0.00
58	0.55	0.00
59	0.00	0.00
60	0.00	0.00
61	0.00	0.00
62	1.91	13.52
63	1.96	1.17
64	0.99	1.45
65	1.11	0.79
66	0.00	3.58
67	0.96	6.22
68	4.43	0.54
69	1.53	10.20
70	0.00	0.00
71	0.00	0.00
72	2.24	4.96
73	21.87	6.88
74	0.55	5.75
75	2.05	0.00
76	0.00	2.08
77	5.04	0.53
78	0.00	0.00
79	1.86	8.88
80	3.13	1.30
81	1.63	0.00
82	1.75	0.00
83	0.93	0.00
84	1.95	0.91
85	3.07	0.44
86	0.00	0.00
87	5.86	2.12
88	1.43	1.53
89	0.64	0.44
90	0.00	0.00
91	0.00	0.00
92	8.51	3.77
93	1.62	28.57
94	6.22	1.83
95	24.39	2.48
96	0.00	0.00
97	2.06	0.76
98	1.89	2.22
99	4.59	1.17
100	1.11	2.15

Appendix 13 Raw data for Figure 3.2 in Chapter 3

Time (h)	Testosterone concentration (ng/mL)				
	10 mg	20 mg	30 mg	40 mg	50 mg
0	7.53	4.90	9.33	5.96	7.77
0.5	8.13	5.00	8.91	6.71	8.78
1	7.41	4.74	9.45	8.18	11.09
2	7.00	5.20	10.22	8.52	11.77
3	7.58	9.24	5.80	8.27	10.29
4	7.28	4.43	9.76	8.97	11.63
6	6.60	3.28	7.82	6.77	8.63
8	7.11	3.44	8.54	7.45	9.16

Appendix 14 Serum concentrations for TDS®- Testosterone in Chapter 3

Time	Subject/ Concentration (ng/mL)												Avg. Conc.
(h)	1	2	3	4	5	6	7	8	9	10	11	12	(ng/mL)
-0.5	3.81	2.61	6.43	5.37	9.30	5.43	5.08	3.51	3.01	4.59	5.27	4.03	4.87
0	5.03	2.50	7.20	4.83	9.08	5.75	4.93	3.80	2.88	4.08	5.59	4.39	5.00
0.5	5.23	3.46	7.91	3.34	9.01	5.56	5.56	5.16	4.20	5.56	5.45	4.71	5.43
1	5.88	3.52	7.61	4.08	9.23	6.64	5.69	4.05	4.82	4.13	4.20	5.40	5.44
1.5	5.37	3.89	7.18	2.98	8.75	6.74	6.28	3.79	6.91	5.26	3.33	6.37	5.57
2	6.36	3.91	7.08	3.95	7.80	7.38	5.86	3.93	6.56	5.38	4.18	6.05	5.70
2.5	5.77	3.47	6.82	5.02	8.31	7.00	6.21	3.93	5.19	5.42	4.91	6.05	5.67
3	3.91	3.43	7.42	4.97	7.60	6.60	4.98	4.01	3.87	6.65	4.81	6.57	5.40
3.5	5.68	4.02	8.79	5.60	8.03	7.59	6.12	4.75	4.95	4.80	4.46	7.45	6.02
4	6.45	3.25	8.11	5.21	6.98	7.38	5.78	4.64	5.28	4.76	4.72	6.47	5.75
5	4.65	3.00	6.89	3.75	7.24	6.05	4.19	4.89	4.93	3.67	4.51	6.31	5.01
6	5.10	3.12	6.10	2.38	8.08	5.67	4.30	4.54	4.87	4.75	3.38	6.18	4.87
7	6.06	2.98	6.40	3.05	7.63	5.67	4.40	4.66	4.67	4.57	4.11	4.64	4.90
8	5.51	2.54	4.63	3.65	7.98	4.97	4.07	4.92	5.05	4.76	4.93	4.96	4.83
10	5.84	3.48	5.70	4.26	8.56	5.83	4.45	4.66	5.04	5.07	4.77	4.18	5.15
12	4.41	2.83	5.48	3.46	7.92	5.00	3.98	3.37	4.32	4.87	2.87	4.47	4.41
24	8.46	3.45	8.63	3.56	19.23	6.58	7.04	5.11	6.97	5.56	13.59	6.68	7.90

Appendix 15 Serum concentrations for TDS®-Placebo in Chapter 3

Time	Subject/ Concentration (ng/mL)												Avg. Conc.
(h)	1	2	3	4	5	6	7	8	9	10	11	12	(ng/mL)
-0.5	4.76	3.27	5.66	4.00	6.69	5.58	3.97	3.25	3.55	4.49	5.26	5.73	4.68
0	5.11	3.63	5.22	4.83	7.14	5.45	4.44	3.20	4.07	5.26	6.35	5.71	5.03
0.5	4.96	4.11	4.87	5.36	6.65	5.29	4.52	3.98	2.27	5.62	6.48	5.19	4.94
1	5.95	3.98	4.95	4.38	7.20	4.72	5.80	2.50	3.55	5.83	6.07	5.09	5.00
1.5	4.56	3.61	4.67	5.66	7.22	4.80	5.26	3.07	2.96	5.17	6.60	5.56	4.93
2	3.20	3.17	4.84	4.27	6.94	4.72	5.81	3.57	3.26	4.70	5.32	5.89	4.64
2.5	3.95	3.55	4.30	4.28	8.32	4.66	4.66	3.83	4.22	4.58	5.14	6.45	4.83
3	4.04	3.87	4.36	3.92	6.11	4.38	3.52	3.77	3.62	4.01	3.97	6.16	4.31
3.5	4.25	4.13	4.29	5.21	6.14	4.60	3.96	3.46	4.25	3.83	5.22	5.34	4.56
4	3.59	4.07	4.26	3.30	6.94	4.01	5.54	3.91	2.08	4.62	5.10	3.93	4.28
5	3.79	2.48	2.97	3.66	6.20	2.77	4.74	2.28	3.58	4.60	5.20	2.76	3.75
6	3.82	1.92	2.64	3.11	7.52	3.10	5.50	2.62	3.04	5.54	4.55	3.35	3.89
7	3.94	1.97	1.98	3.77	7.25	3.70	5.90	2.80	3.48	5.31	6.04	3.96	4.17
8	4.37	2.01	2.42	3.57	7.01	3.06	5.69	2.96	3.73	5.31	6.23	4.00	4.20
10	4.84	2.17	3.29	6.33	3.95	3.38	4.90	3.19	4.44	3.68	4.63	4.06	4.07
12	3.81	1.69	3.65	3.52	5.47	3.56	3.98	2.74	3.48	4.05	2.71	3.14	3.48
24	5.18	2.97	6.85	6.29	8.66	5.10	4.42	2.41	3.33	6.61	5.12	3.28	5.02

Appendix 16 Serum concentrations for Androgel® in Chapter 3

Time	Subject/ Concentration (ng/mL)												Avg. Conc.
(h)	1	2	3	4	5	6	7	8	9	10	11	12	(ng/mL)
-0.5	5.42	3.55	6.83	4.69	8.17	8.75	4.60	3.87	3.92	5.10	4.49	5.46	5.40
0	5.41	4.06	7.18	4.71	7.64	8.85	4.22	4.48	4.27	5.10	4.59	5.25	5.48
0.5	4.16	4.21	8.25	6.10	7.09	9.16	4.82	3.99	3.62	4.28	5.51	4.71	5.49
1	5.36	4.49	6.74	4.70	6.58	7.51	4.77	3.72	4.71	4.07	5.81	4.47	5.24
1.5	4.71	5.63	5.75	5.26	5.62	6.95	4.19	3.58	4.52	5.88	5.45	4.48	5.17
2	5.78	4.98	5.88	6.49	7.00	7.39	5.16	4.67	3.99	7.30	4.56	4.16	5.61
2.5	4.18	4.95	5.46	5.48	6.76	8.53	5.60	4.65	4.07	8.51	4.47	4.54	5.60
3	5.56	5.32	6.08	5.08	8.27	8.54	5.41	4.55	3.68	8.00	4.61	5.34	5.87
3.5	5.12	5.58	6.33	4.74	7.53	8.24	4.96	4.13	4.42	7.47	4.30	5.55	5.70
4	4.29	3.77	5.45	4.23	7.03	7.92	4.42	4.28	4.16	5.72	3.78	4.44	4.96
5	3.68	2.84	4.97	2.75	5.63	6.12	3.90	4.44	3.42	6.14	3.68	3.95	4.29
6	4.36	3.93	3.86	3.68	4.64	5.36	3.95	3.50	4.28	4.87	4.32	3.32	4.17
7	4.47	3.73	5.97	3.99	5.82	5.78	4.50	4.38	2.11	4.47	4.41	3.87	4.46
8	4.99	3.24	5.02	5.55	5.88	5.17	4.68	4.43	4.10	6.25	4.64	4.09	4.84
10	4.37	2.49	4.91	5.37	6.75	5.27	5.53	4.36	4.05	5.83	4.10	3.49	4.71
12	3.43	2.63	4.20	3.82	4.81	5.09	3.26	3.31	3.27	5.07	2.85	3.28	3.75
24	5.90	4.53	12.77	5.87	49.35	25.67	9.32	5.12	4.91	7.28	18.82	4.93	12.87

Appendix 17 Tape lidocaine amount in 12 subjects in Chapter 4

Time	Subject/ Lidocaine amaount (μg)												Average (μg)
	1	2	3	4	5	6	7	8	9	10	11	12	
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	201.37	118.10	82.81	58.33	71.92	118.92	168.90	155.06	93.33	66.20	149.11	105.40	115.79
0.5	350.64	280.85	110.04	164.06	132.66	127.96	299.19	155.03	195.88	67.99	247.60	121.46	187.78
0.75	288.65	213.99	225.43	175.53	178.54	189.90	294.83	258.14	196.32	139.77	220.93	124.42	208.87
1	486.88	296.60	230.49	194.53	311.22	344.92	424.77	334.66	359.46	222.20	347.43	244.98	316.51
1.5	293.19	194.20	281.51	126.22	223.60	173.89	298.69	324.57	271.76	116.06	338.21	125.20	230.59
2	379.08	166.03	146.62	112.82	150.54	181.55	360.57	160.74	169.12	105.44	275.13	141.78	195.78
3	269.39	138.83	144.26	101.44	110.27	167.58	291.24	134.69	99.90	91.81	175.77	91.54	151.39
4	151.41	132.25	136.93	34.04	66.69	152.96	246.55	102.93	71.93	48.50	159.99	80.49	115.39

Appendix 18 Plasma lidocaine concentration in 12 subjects in Chapter 4

Time	Subject/ Lidocaine concentration (ng/mL)												Average
	1	2	3	4	5	6	7	8	9	10	11	12	(ng/mL)
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.67	0.62	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
1.00	0.70	1.14	0.00	0.00	0.50	0.00	0.97	0.00	0.00	0.00	0.00	0.00	0.28
1.50	0.70	1.90	0.00	0.07	0.63	0.57	2.08	0.22	0.83	1.38	0.64	0.31	0.78
2.00	1.06	2.39	0.09	0.57	1.38	0.86	2.56	0.53	0.81	1.50	1.28	2.92	1.33
2.50	2.69	2.46	0.09	1.03	1.28	1.33	2.44	0.81	1.05	1.16	1.56	3.30	1.60
3.00	0.86	3.22	0.38	1.19	1.22	1.20	2.99	1.33	1.13	1.27	1.21	2.61	1.55
3.50	1.46	2.01	0.57	1.33	2.24	1.72	2.57	1.13	1.64	1.53	1.78	2.60	1.72
4.00	1.28	1.57	0.37	1.04	2.06	1.38	2.00	1.61	1.45	1.15	1.76	3.42	1.59

Appendix 19 Dialysate lidocaine concentration in 12 subjects in Chapter 4

Time	Subject/ Lidocaine concentration (ng/mL)												Average
	1	2	3	4	5	6	7	8	9	10	11	12	(ng/mL)
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.33	0.00	4.30	1.60	4.40	1.00	5.30	3.70	0.00	2.60	4.30	0.00	0.00	2.27
1.67	0.60	13.50	7.40	26.20	1.60	29.60	30.30	0.00	7.70	7.60	0.00	0.70	10.43
2.00	5.30	44.20	7.40	91.10	13.90	82.60	176.50	1.20	31.50	10.00	0.60	26.00	40.86
2.33	9.10	108.10	7.70	337.90	22.80	*196.70*	335.80	4.10	66.00	10.00	4.10	42.80	95.43
2.67	21.30	132.60	23.10	713.10	51.80	310.80	486.10	13.60	101.90	7.90	7.30	61.50	160.92
3.00	*42.95*	243.00	32.10	1188.80	116.10	305.60	646.30	25.00	127.70	7.30	22.10	85.10	236.84
3.33	64.60	241.50	46.10	1681.00	*153.30*	351.70	728.80	34.40	168.60	8.10	33.60	82.10	299.48
3.67	133.00	282.00	118.00	1808.10	190.50	303.10	965.50	50.60	166.50	6.70	67.90	71.40	346.94
4.00	272.60	450.50	123.30	2006.40	249.70	436.80	1044.80	88.40	239.10	5.10	105.80	81.20	425.31

Appendix 20 Verbal Rating Score (VRS) in 12 subjects in Chapter 4

Subject	Time (h) / Result (unit)							
	0.25	0.5	0.75	1	1.5	2	3	4
1	4.0	3.0	2.5	1.0	1.0	1.0	2.0	2.0
2	3.0	2.0	2.0	1.0	1.0	1.0	1.0	2.0
3	2.0	2.0	2.0	2.0	2.0	3.0	2.0	1.0
4	2.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0
5	4.0	3.0	3.0	1.0	1.0	1.0	1.0	1.0
6	3.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0
7	3.0	3.0	2.0	1.0	1.0	1.0	1.0	1.0
8	4.0	3.5	2.0	2.0	1.0	1.0	1.0	2.0
9	4.0	3.5	3.5	2.5	2.0	2.0	1.0	2.0
10	2.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0
11	3.0	3.0	2.0	1.0	1.0	1.0	1.0	1.0
12	3.0	3.0	3.0	3.0	1.0	1.0	2.0	1.0
Average	3.08	2.67	2.33	1.46	1.17	1.25	1.25	1.33
SD	0.79	0.62	0.54	0.72	0.39	0.62	0.45	0.49

Appendix 21 Visual Analog Score (VAS) in 12 subjects in Chapter 4

Subject	Time (h) / Result (unit)							
	0.25	0.5	0.75	1	1.5	2	3	4
1	91.0	70.0	50.0	12.0	11.0	3.0	23.0	56.0
2	61.0	47.0	35.0	5.0	5.0	3.0	8.0	32.0
3	34.0	39.0	19.0	20.0	22.0	70.0	44.0	5.0
4	23.0	22.0	22.0	2.0	0.0	0.0	0.0	0.0
5	78.0	66.0	59.0	9.0	0.0	0.0	0.0	0.0
6	68.0	35.0	30.0	9.0	12.0	10.0	13.0	10.0
7	60.0	60.0	49.0	15.0	4.0	2.0	3.0	4.0
8	100.0	85.0	49.0	38.0	0.0	0.0	0.0	32.0
9	100.0	82.0	82.0	52.0	30.0	31.0	0.0	29.0
10	31.0	42.0	39.0	23.0	16.0	17.0	16.0	21.0
11	60.0	57.0	46.0	7.0	0.0	0.0	0.0	0.0
12	77.0	69.0	61.0	68.0	9.0	13.0	27.0	12.0
Average	65.3	56.2	45.1	21.7	9.1	12.4	11.2	16.8
SD	25.9	19.5	17.7	20.6	9.7	20.4	14.1	17.5

Appendix 22 Skin reflectance measurement procedures in Chapter 5

SPECTROPHOTOMETER CM-2500d

1. Switch on the spectrophotometer [power switch at rear of machine]. The display will show the <COND> 1 window. Use wheel at top front of device to move to CALIBRATION mode and press white dot to activate. If the cursor is on ZERO CALIBRATION, use wheel to change to WHITE CALIBRATION. Place the instrument on the white calibration device and press the measuring button located on side of the instrument. Three measurements will be taken and the spectrophotometer will automatically change to measurement mode. Remove the white calibration device. White calibration must be performed before any measurements can be taken and is required every time the machine is switched on.
2. TAKING MEASUREMENTS:
   i) Ensure spectrophotometer is in measurement mode (press BREAK repeatedly until SPECTRAL GRAPH or L* a* b* appears on display).
   ii) Rest the head of the spectrophotometer measuring aperture on the surface of the skin site to be measured (site 1 – forehead). Ensure that there is a complete seal with the skin surface but no depression of the skin (to avoid surface blanching or flushing). Care must be taken to avoid areas of irregular pigmentation such as moles etc or hairy skin. Press the measuring button. The Xenon lamp will make three recordings. Take care not to move the spectrophotometer until the entire measurement cycle has been completed and the results displayed [if movement occurs delete this data – see item 4 below and repeat measurement]. Note the instrument has been set to allow 1 second between the 3 readings and between pressing the button and the first measurement.
   iii) Record exact position of measurement on chart diagram together with the specimen number recorded in upper left corner of display panel. Move to next measurement area and repeat exercise 2.ii until all eight areas of skin are recorded.
   iv) Upon completion of subject, check that nine specimen numbers have been recorded (1-9 for subject 1; 10-18 for subject 2 etc). The sequence of measurements of skin areas should be the same for each subject. If during the course of measurement an erroneous reading was taken either because the spectrophotometer was moved during a reading or an "air" reading was inadvertently taken, this can be deleted as follows;-
3. DELETING DATA FOR SINGLE SPECIMEN MEASUREMENT: Select the desired specimen number to be deleted (move wheel to DEL is highlighted). Press white spot and a confirmation message will come on screen, highlight "OK" and press white spot again. The selected specimen measurement will be deleted and the spectrophotometer will return to measurement mode and the remaining specimen numbers will be renumbered.
4. To switch between subject numbers, move highlight to ◄► position and press white dot, symbol will change to ▼▲. Moving the wheel will now allow screen to move between subjects.
5. Using the "SPEC GRAPH" setting, record all values for 400nm, using the **SCI** values.
6. Change wavelength to 420 using the **SCI** values (or vice versa by firstly resetting ◄ ► and moving back to "nm". Pressing the white spot at this position will allow the wheel to move across the spectral graph.
7. Once all "SPEC GRAPH" 400 and 420nm readings have been recorded, the display screen can be changed to "L* a* b*" mode by selecting "PREV" to return

to <COND> 1 and selecting <FILE> by moving to highlight FILE and pressing the white spot. Move the wheel until "sel" is highlighted and press the white button continuously until DISPLAY is highlighted. Then move wheel to select "DIFF & ABS" and continue pressing white spot to end of this programme. Taking care not to alter any of the other settings. The screen will return to highlight break. Press white spot to return to the measurement mode screen which will now show L* a* b* results (note if ΔLab screen is shown, change by highlighting "DISP" and pressing white button. All L*, a* and b* values for each subject can now be read in the same way as for spectral results by changing to ▼▲ and wheeling between subjects. Use the values listed under **SCI**.
Note that if L* a* b* results are the first to be displayed the procedure outlined above should be reversed. THE SEQUENCE DOES NOT MATTER.

8. When all data has been recorded from all subjects, the spectrophotometer can be switched off. 700 data readings can be stored in the memory. The data must be recorded on the CRFs or downloaded to a printer after each set of results are taken at any one measuring session (i.e. baseline, day 10 etc).

MEASUREMENT SEQUENCE

1. Forehead
2. Left cheek
3. Right side of neck
4. Left scapula
5. Right inside upper arm
6. Left inside upper arm
7. Left medial forearm
8. Right side of lower back
9. Left calf

SPECTROPHOTOMETER STANDARD SETTINGS

Pg1/2	
Mask/gloss	M/I+E
UV setting	UV 100%
Illuminant1	D65
Illuminant2	---
Observer	10°
Display	SPECT. GRAPH or DIFF&ABS - depending on whether you want Lab
or nm.	
Pg2/2	
color space	L*a*b*, ΔE*
Manual Avg times deviation	---
Auto Avg	3
Delay time	0.5s

Appendix 23 Skin reflectance data for all the subjects and all the sites in Cohort 5 in Chapter 5

Subject	Site	Day	L*(D65)	a*(D65)	b*(D65)	400nm	420nm	MD
1	1	1	63.64	10.48	17.79	16.77	16.56	3.32
1	1	5	63.37	11.03	16.71	17.47	17.06	3.12
1	1	10	63.45	11.02	18.1	16.44	16.1	3.19
1	1	20	63.45	11.6	16.99	17.49	17.03	3.07
1	1	30	63.92	10.62	18.64	16.18	16.22	3.57
2	1	1	61.56	13.34	16.52	14.98	14.8	3.35
2	1	5	62.2	12.56	16.8	15.01	15.07	3.59
2	1	10	62.03	12.89	17.19	14.7	14.55	3.38
2	1	20	63.54	12.4	17.32	16.11	15.77	3.19
2	1	30	62.2	13.13	16.91	15.38	15.12	3.27
3	1	1	61.97	13.28	17.02	15.53	15.46	3.46
3	1	5	63.06	11.97	18.04	15.29	15.56	3.80
3	1	10	63.87	12.4	17.6	16.4	16.34	3.47
3	1	20	64.22	12.35	17.65	16.65	16.7	3.58
3	1	30	64.09	12.76	17.62	16.83	16.63	3.33
4	1	1	62.01	13.25	13.78	17.93	17.4	3.00
4	1	5	59.62	14.83	15.6	14.32	14.43	3.64
4	1	10	60.62	13.5	16.06	14.51	14.91	3.93
4	1	20	62.28	12.65	16.3	16.55	16.17	3.15
4	1	30	56.94	17.03	14.67	13.13	13.34	3.74
5	1	1	66.46	10.97	14.29	21.36	20.1	2.27
5	1	5	65.4	11.89	15.88	19.06	18.49	2.96
5	1	10	66.68	11.03	16.53	19.67	19.04	2.90
5	1	20	66.4	11.6	15.64	20.09	19.36	2.80
5	1	30	68.1	9.54	14.91	22.25	20.83	2.11
6	1	1	66.17	10.53	16.37	19.51	19.17	3.19
6	1	5	63.57	11.49	17.05	16.72	16.73	3.54
6	1	10	65.37	11.07	18.27	17.44	17.21	3.30
6	1	20	64.45	12.04	17.3	17.64	17.1	2.99
6	1	30	64.58	11.23	18.57	16.73	16.58	3.38
1	2	1	59.57	11.14	21.64	10.61	11.91	4.83
1	2	5	60.8	11.4	19.78	12.57	13.67	4.63
1	2	10	61.27	10.72	21.49	11.36	12.89	5.06
1	2	20	61.66	10.75	21.63	11.68	13.12	4.97
1	2	30	61.14	11.38	21.19	11.57	13.03	4.99
2	2	1	61.68	12.25	16.52	14.69	14.99	3.83
2	2	5	62.52	12.19	17.11	14.82	15.24	3.95
2	2	10	63.71	11.49	18.2	14.68	15.06	3.91
2	2	20	63.56	12.14	18.05	14.68	15.52	4.37
2	2	30	66.83	9.03	18.77	15.86	17.14	4.81
3	2	1	59.28	13.82	17.84	12.44	13.16	4.25
3	2	5	62.23	12.48	19.01	13.5	14.43	4.46
3	2	10	63.75	11.45	19.17	14.21	15.2	4.52
3	2	20	63.78	10.06	19.78	13.97	15.01	4.57
3	2	30	64.09	11.41	19.15	14.51	15.48	4.50
4	2	1	55.3	10.15	14.56	11.56	12.03	4.00
4	2	5	58.23	11.25	16.85	11.99	12.75	4.29
4	2	10	57.86	9.17	15.01	13.11	13.41	3.83
4	2	20	56.84	9.56	16.19	11.75	12.26	4.04
4	2	30	55.1	11.24	14.13	11.68	12.06	3.91
5	2	1	66.19	10.32	16.74	18.12	18.21	3.62
5	2	5	65.35	11.69	17.49	17.21	17.3	3.62
5	2	10	67.04	9.22	17.98	18.13	18.11	3.51

5	2	20	67.07	9.75	17.9	18.29	18.49	3.73
5	2	30	69.55	9.25	15.8	22.04	21.58	3.07
6	2	1	66.69	9.39	17.06	18.84	18.89	3.58
6	2	5	65.71	10.72	17.72	17.4	17.96	4.09
6	2	10	68.34	8.88	17.08	20.76	20.14	2.91
6	2	20	65.84	11.8	16.08	19.29	19	3.24
6	2	30	64.24	12.53	16.97	17.19	17.39	3.73
1	3	1	60.36	10.6	20.81	11.22	12.03	4.34
1	3	5	61.72	10.27	21.66	11.61	12.44	4.36
1	3	10	61.94	9.82	21.74	11.5	12.49	4.52
1	3	20	61.89	10.08	22.07	11.32	12.32	4.53
1	3	30	59.69	11.6	21.61	10.46	11.31	4.38
2	3	1	65.32	8.35	16.45	16.45	16.87	3.95
2	3	5	65.75	9.15	16.88	17.21	17.12	3.44
2	3	10	66.28	8.28	17.35	17.13	17.22	3.62
2	3	20	66.09	8.47	18.59	16.13	16.48	3.88
2	3	30	65.62	9.11	17.34	16.67	16.63	3.49
3	3	1	57	14.55	19.28	10.49	10.84	3.88
3	3	5	61.66	11.88	21.52	11.76	12.39	4.16
3	3	10	62.54	10.86	20.71	12.43	13.06	4.16
3	3	20	60.79	12.58	18.84	12.78	13.18	3.93
3	3	30	61.45	13.27	18.75	13.35	13.68	3.86
4	3	1	66.2	9.87	17.77	17.13	17.36	3.76
4	3	5	68.2	7.72	17.19	18.81	19.12	3.84
4	3	10	67.31	8.67	17.48	18	18.2	3.73
4	3	20	68.47	7.48	17.91	19.09	19.14	3.58
4	3	30	64.3	11.31	16.44	16.6	16.6	3.53
5	3	1	68.43	6.75	15.61	19.78	19.7	3.45
5	3	5	70.11	5.04	14.64	22	22.08	3.61
5	3	10	70.39	5.56	17.59	20.2	20.41	3.74
5	3	20	68.82	7.42	17.32	19.09	19.13	3.57
5	3	30	70.74	5.67	17.68	20.79	20.98	3.72
6	3	1	68.73	7.8	17.72	20.01	19.47	2.99
6	3	5	69.88	6.86	16.91	21.68	20.82	2.67
6	3	10	69.52	7.39	16.12	22.29	21.11	2.35
6	3	20	66.63	7.07	16.01	19.35	18.9	3.08
6	3	30	67.64	8.86	17.26	19.61	18.76	2.68
1	4	1	58.28	10.89	19.77	10.61	11.48	4.40
1	4	5	60.62	9.79	19.59	12.04	12.91	4.40
1	4	10	65.2	6.34	19.75	14.9	16.15	4.78
1	4	20	64.26	7.33	20.77	13.69	14.94	4.78
1	4	30	62.23	9.35	20	12.77	13.8	4.56
2	4	1	70.61	5.55	17.19	20.71	20.89	3.71
2	4	5	71.43	5.29	18.41	20.56	21.02	3.99
2	4	10	70.55	6.53	17.45	20.1	20.4	3.83
2	4	20	71.18	6.29	17.46	20.93	21.35	3.95
2	4	30	70.61	6.52	16.4	21.52	21.55	3.56
3	4	1	67.89	6.38	14.02	21.53	21.13	3.13
3	4	5	65.99	8.35	20.04	15.28	16.53	4.78
3	4	10	65.32	8.88	20.26	14.46	15.71	4.78
3	4	20	63.59	10.11	20.66	12.82	13.96	4.67
3	4	30	63.86	10.85	19.36	14.11	14.82	4.24
4	4	1	70.63	6.72	14.23	24.52	23.76	2.77
4	4	5	71.47	6.13	15.01	24.32	23.65	2.86
4	4	10	69.16	7.14	15.35	21.96	21.39	2.96
4	4	20	69.13	7.5	15.69	22.48	21.7	2.75
4	4	30	68.32	8.39	14.76	21.81	21.12	2.84
5	4	1	61.78	9.99	23.21	11.25	12.07	4.35

5	4	5	61.48	11.1	22	11.58	12.18	4.13
5	4	10	63.7	8.58	22.93	12.41	13.39	4.51
5	4	20	61.47	11.81	21.43	12.01	12.52	4.04
5	4	30	63.17	10.04	22.19	12.5	13.19	4.22
6	4	1	64.22	9.47	22.55	13.3	13.79	4.02
6	4	5	65.95	8.92	21.48	15.33	15.57	3.77
6	4	10	64.94	10.36	20.54	15.03	15.21	3.71
6	4	20	63.19	10.69	20.54	13.82	14.22	3.93
6	4	30	64.98	9.15	20.11	15.14	15.54	3.93
1	5	1	69.5	5.56	16.46	20.74	20.76	3.55
1	5	5	69.98	4.87	15.99	21.9	21.94	3.57
1	5	10	70.84	4.64	15.64	23.24	23.04	3.33
1	5	20	71.63	4.33	16.27	23.36	23.15	3.32
1	5	30	71.1	4.6	15.98	23.15	23.09	3.47
2	5	1	72.52	4.03	12.93	26.68	25.85	2.70
2	5	5	71.56	4.24	13.58	24.72	24.47	3.28
2	5	10	71.86	3.78	13.74	24.48	24.18	3.23
2	5	20	73.2	4.04	13.19	27.45	27.04	3.12
2	5	30	71.66	4.19	13.89	24.64	24.24	3.13
3	5	1	68.05	6.52	15.26	20.44	20.35	3.44
3	5	5	69.27	5.86	14.35	22.81	22.68	3.40
3	5	10	70.38	5.68	14.06	24.37	23.66	2.82
3	5	20	70.73	5.8	14.7	23.84	23.31	3.00
3	5	30	70.65	5.32	15.09	23.68	23.16	3.01
4	5	1	74.14	3.81	13.3	29.34	27.71	1.90
4	5	5	73.57	3.87	13.85	27.47	25.94	2.00
4	5	10	72.6	3.98	14.17	26.47	24.82	1.88
4	5	20	73.21	3.71	14.77	27.01	25.14	1.67
4	5	30	73.84	3.9	12.73	28.84	27.04	1.74
5	5	1	69.42	5.45	14.37	21.25	21.4	3.68
5	5	5	71.31	3.99	14.38	23.16	23.5	3.87
5	5	10	69.99	4.81	15.49	20.84	21.06	3.75
5	5	20	70.28	5.12	15.99	20.64	21.08	3.97
5	5	30	71.62	4.12	14.71	23.7	23.82	3.65
6	5	1	70.2	5.53	13.49	25.29	24.19	2.43
6	5	5	72.03	4.48	13.1	27.87	26.87	2.53
6	5	10	71.65	4.38	12.64	27.84	26.59	2.28
6	5	20	71.25	4.42	12.16	27.57	26	1.96
6	5	30	69.59	5.28	13.97	23.44	22.78	2.87
1	6	1	69.84	5.04	14.73	22.9	22.91	3.54
1	6	5	69.18	5.19	15.35	21.8	21.99	3.72
1	6	10	69.6	4.73	14.73	22.74	22.82	3.61
1	6	20	69.25	4.87	15.6	21.63	21.71	3.61
1	6	30	68.87	5.41	15.78	21.07	21.04	3.50
2	6	1	72.12	4.22	13.89	25.03	24.47	2.97
2	6	5	72.45	4.28	14.22	25	24.63	3.16
2	6	10	72.93	3.47	12.52	27.57	26.22	2.18
2	6	20	73.21	4.1	13.53	26.93	26.42	3.02
2	6	30	72.62	3.85	12.6	27.4	26.35	2.48
3	6	1	61.65	10.93	18.46	13.28	13.89	4.14
3	6	5	69.19	6.11	14.88	22.07	22.39	3.85
3	6	10	69.9	5.84	15.74	21.69	21.61	3.45
3	6	20	70.12	5.77	15.84	22.05	22.07	3.55
3	6	30	71.14	5.26	15.21	23.99	23.69	3.23
4	6	1	73.52	3.93	12.2	29.69	27.84	1.69
4	6	5	73.5	3.55	13.29	27.9	26.4	2.03
4	6	10	73.19	4.16	12.28	28.87	26.99	1.66
4	6	20	73.59	3.68	12.5	30.19	28.47	1.82

4	6	30	73.78	3.12	10.15	32.25	30.08	1.37
5	6	1	70.72	4.6	14.5	22.8	22.83	3.56
5	6	5	70.62	4.74	15.75	21.12	21.75	4.16
5	6	10	70.9	4.19	15.29	22	22.42	3.95
5	6	20	71.84	4.23	14.8	23.76	23.88	3.65
5	6	30	71.29	4.44	14.77	23.25	23.64	3.92
6	6	1	70.43	5.22	15.06	24.28	23.67	2.92
6	6	5	71.82	4.73	14.37	26.61	25.83	2.75
6	6	10	70.86	4.82	14.84	24.51	23.94	2.96
6	6	20	71.33	4.46	13.6	26.53	25.46	2.46
6	6	30	68.48	5.48	14.36	22.05	21.4	2.88
1	7	1	63.3	8.27	17.88	14.44	14.88	3.97
1	7	5	61.56	8.57	19.34	12.71	13.49	4.31
1	7	10	62.89	7.66	21.46	12.46	13.62	4.69
1	7	20	60.74	8.12	21.29	11.36	12.33	4.50
1	7	30	61.99	8.45	22.89	11.39	12.44	4.58
2	7	1	67.5	5.95	17.63	16.52	17.52	4.53
2	7	5	67.99	5.16	17.04	17.07	17.95	4.41
2	7	10	66.79	6.5	18.12	15.48	16.71	4.76
2	7	20	66.29	7.4	20.81	13.94	15.3	4.89
2	7	30	66.17	7.12	20.13	14.21	15.53	4.85
3	7	1	62.5	9.12	17.64	14.03	14.53	4.03
3	7	5	64.99	8.04	17.58	16	16.49	4.02
3	7	10	65.08	8.11	18.41	15.26	15.99	4.26
3	7	20	64.06	8.47	19.54	13.84	14.73	4.42
3	7	30	66.2	7.44	19.5	15.19	16.37	4.71
4	7	1	69.92	5.38	12.3	23.85	23.88	3.56
4	7	5	66.81	6.66	12.76	20.18	20.26	3.61
4	7	10	66.52	6.3	13.45	19.41	19.38	3.50
4	7	20	67.51	5.85	14.52	19.89	20.14	3.78
4	7	30	65.82	6.77	15.07	17.45	17.88	3.96
5	7	1	68.34	5.98	16.13	18.65	19.18	4.06
5	7	5	69.34	5.22	16.59	19.3	19.93	4.16
5	7	10	66.86	6.75	19.69	15.56	16.35	4.32
5	7	20	66.63	7.31	20.59	14.97	15.97	4.53
5	7	30	66.73	7.39	21.17	14.61	15.55	4.47
6	7	1	67.53	6.82	17.85	17.63	17.87	3.77
6	7	5	69.5	5.6	16.59	20.59	20.65	3.59
6	7	10	68.54	5.87	16.83	19.46	19.94	4.01
6	7	20	68.6	6.61	17.84	18.88	19.46	4.11
6	7	30	67.02	6.68	18.69	16.59	17.55	4.49
1	8	1	69.78	5.77	14.41	23.37	21.93	2.09
1	8	5	70.83	4.86	14.86	24.43	22.74	1.85
1	8	10	69.4	5.53	13.79	23.82	22.16	1.88
1	8	20	70.99	5	13.48	25.92	24.39	2.00
1	8	30	70.07	5.21	13.74	24.37	22.91	2.07
2	8	1	74.75	3.35	15.52	27.34	25.82	2.01
2	8	5	74.47	4.16	16.73	25.63	24.53	2.43
2	8	10	73.96	3.9	17	24.48	23.38	2.43
2	8	20	75.06	4.02	16.59	26.73	25.66	2.46
2	8	30	74.46	4.01	16.49	26.29	24.96	2.20
3	8	1	66.74	7.77	17.77	17.08	16.68	3.13
3	8	5	69.49	6.42	18.76	18.69	18.89	3.73
3	8	10	68.73	7.1	18.54	18.19	17.91	3.25
3	8	20	70.07	6.35	19.23	18.82	18.49	3.20
3	8	30	70.57	5.36	17.91	20.21	20.03	3.35
4	8	1	69.12	6.63	14.81	21.61	20.38	2.30
4	8	5	66.4	6.57	14.06	19.46	18.68	2.75

4	8	10	68.94	6.51	12.86	22.74	21.25	2.04
4	8	20	67.94	6.84	13.62	21.63	20.04	1.94
4	8	30	67.41	7.63	13.37	20.93	19.59	2.19
5	8	1	67.02	6.88	19.8	16	16.47	4.00
5	8	5	68.95	5.89	19.61	17.59	18.08	4.02
5	8	10	66.91	7.05	20.59	15.32	15.81	4.02
5	8	20	66.31	6.91	19.05	15.38	15.82	3.97
5	8	30	66.99	6.97	18.67	16	16.27	3.80
6	8	1	66.3	8.07	20.63	15.79	15.85	3.59
6	8	5	66.54	7.41	20.3	15.83	15.76	3.46
6	8	10	68.22	6.75	17.34	19.26	19.18	3.45
6	8	20	66.98	7.02	17.95	17.56	17.4	3.37
6	8	30	66.32	7.8	20.67	15.26	15.48	3.75
1	9	1	69.82	4.35	13.84	22.52	22.5	3.51
1	9	5	68.6	3.78	11.67	23.18	22.57	2.92
1	9	10	68.03	5.38	13.39	21.67	21.49	3.35
1	9	20	68.94	3.87	13.85	21.56	21.48	3.45
1	9	30	66.99	6.5	13.64	20.77	20.53	3.29
2	9	1	62.26	7.48	17.97	12.25	13.21	4.49
2	9	5	62.22	6.57	18.37	12.16	13.23	4.60
2	9	10	64.14	5.27	17.18	13.66	14.69	4.56
2	9	20	64.13	6.64	19.15	12.85	14.14	4.82
2	9	30	65.31	5.78	17.42	14.6	15.63	4.56
3	9	1	55.68	13	17.53	9.56	10.17	4.14
3	9	5	56.13	10.64	16.79	10.07	10.73	4.19
3	9	10	57.64	10.59	17.07	10.49	11.13	4.17
3	9	20	56.41	11.76	16.53	10.1	10.7	4.13
3	9	30	57.29	11.59	17.73	9.89	10.61	4.25
4	9	1	67.78	5.26	12.12	21.86	21.39	3.06
4	9	5	64.44	7.25	12.51	18.17	17.72	3.08
4	9	10	63.78	7.23	11.68	18.32	17.6	2.81
4	9	20	67.37	4.99	13.59	20.75	20.09	2.87
4	9	30	68.46	6.2	12.89	21.65	21.06	2.94
5	9	1	66.15	7.74	18.23	15.55	16.11	4.09
5	9	5	64.07	7.89	16.88	14.4	14.73	3.86
5	9	10	65.54	6.46	15.8	16.63	16.84	3.74
5	9	20	65.81	7.13	16.71	16.38	16.74	3.89
5	9	30	67.87	5.49	16.46	18.26	18.66	3.93
6	9	1	59.96	11.01	19.23	10.89	11.44	4.08
6	9	5	65.15	7.62	18.48	15.24	15.21	3.50
6	9	10	63.59	7.87	15.18	15.82	15.29	3.00
6	9	20	64.54	8.14	13.9	17.7	17.12	2.95
6	9	30	62.9	8.86	15.46	14.79	14.83	3.57

Validation and application of capillary electrophoresis for the analysis of lidocaine in a skin tape stripping study.

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Abstract

A fast and simple capillary zone electrophoresis (CE) method was developed and validated for the determination of lidocaine in skin using tape samples. Separation was performed in 350mm (265mm to window) x 50μm i.d fused silica capillary using a background electrolyte of phosphoric acid/TRIS pH 2.5. The extraction of lidocaine from tape samples was achieved with methanol, which was diluted to 50% with water before injection. Procaine was the internal standard. The migration times for procaine and lidocaine were 2.9 and 3.2min, respectively. The limit of quantification for lidocaine was 50μg, with S/N ratio was greater than 10. The calibration curve was linear from 50 to 1000μg with r^2 greater than 0.99. The CV for both within and between assayed imprecision and the percentage of inaccuracy for the quality control samples including LLOQ and ULOQ were ≤ 2% and ≤ 14%, respectively. The absolute recovery of lidocaine was >97%. Accuracy and selectivity of this method allowed the measurement lidocaine in tape samples obtained from a skin tape stripping study of local anesthetics in healthy subjects.

Keywords: Capillary electrophoresis, lidocaine; tape stripping: method validation.

1. Introduction

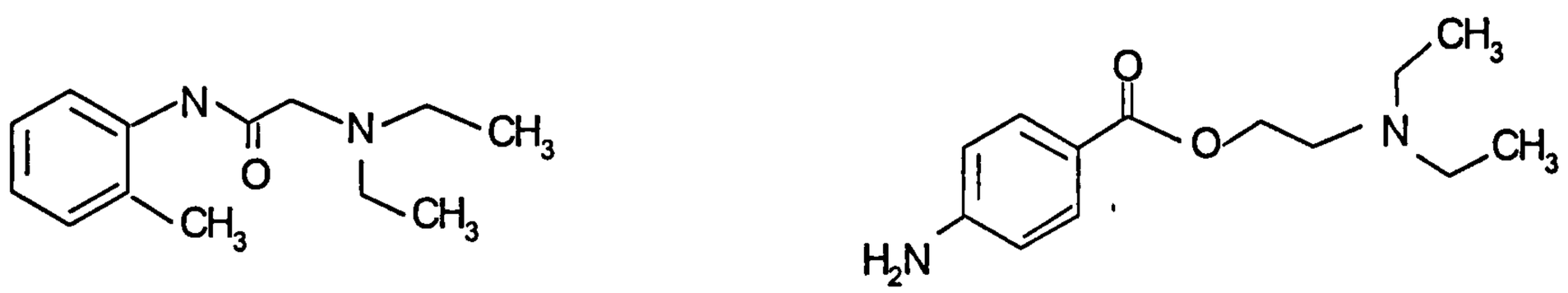
Lidocaine is commonly used as local anaesthetic and anti arrhythmic. Lidocaine is applied onto skin in either cream or patches for local anaesthetic effects. Due to the local activity of lidocaine its concentration is higher in the local tissue compared to systemic circulation. Some in vivo methods such as microdialysis and tape stripping are available to sampling drugs in the local tissue and outermost layer of the skin, respectively (Benfeldt & Groth 1998;Kenkel et al. 2004;Kreilgaard 2001;Kreilgaard et al. 2001;Pershing et al. 2002;Reddy et al. 2002) . Various techniques are available to measure lidocaine in plasma such as HPLC with UV detection (Lotfi et al. 1997), LC-MS-MS (Bo, Mazzucchelli, & Marzo 1999), and GCMS (Watanabe et al. 1998). Most of the analysis of drugs including lidocaine (Pershing et al. 2003;Pershing, Corlett, & Nelson 2002;Weigmann et al. 1999) from tape samples have been by HPLC with UV detection since the drug content in tape samples is normally higher than in plasma, especially for local anaesthetic study. Therefore, the high sensitivity techniques such as LC-MS-MS or GCMS are not required since UV detection is sufficiently sensitive to detect such level of drugs. Moreover, the analysis cost of using UV detection is less compared to MS detection.

On the other hand, capillary electrophoresis (CE) is an alternative to the chromatography techniques in drug analysis which can separate a variety of compounds by using an electric field. The separation of the compounds by electrophoresis in CE is based on the differences in electrophoretic mobility and the voltage applied. Like HPLC, CE also can be coupled with UV detector (CE-UV) and MS (CE-MS) forming a powerful analytical method for drug analysis. Capillary electrophoresis with UV and MS detection for the analysis of lidocaine in pharmaceutical formulations have been reported previously (Geiser, Rudaz, & Veuthey 2003;Geiser, Rudaz, & Veuthey 2005;Wang et al. 2001). In this study, we have performed a method validation for the CE analysis of lidocaine in tape samples obtained from tape stripping study on healthy subjects.

2. Experimental

2.1 Chemicals

Lidocaine Hydrochloride (99.9% purity) and Procaine Hydrochloride (100% purity) (internal standard) were obtained from Sigma-Aldrich Company, Poole, UK. The chemical structure of lidocaine and procaine are shown in **Figure 1.** 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.



Lidocaine Procaine

Figure 1 Chemical structures of lidocaine and procaine

2.2 Background electrolyte

The aqueous background electrolyte used in this analysis was 100mM Tris-phosphate buffer at pH 2.5. To prepare this solution, 20mL phosphoric acid 0.5M was diluted with 80mL water and 15mL Tris 0.5M was added to obtain a solution at pH 2.5. The solution was topped up to 100mL with deionised water.

2.3 CE instrumentation and capillaries

Separations were carried out on an Agilent ^{3D}CE G1600AX capillary electropherograph (Agilent, West Lothian, UK) controlled by Chemstation B.02.01 (Agilent, West Lothian, UK). 350mm (265mm to window) x 50μm i.d., fused silica capillaries (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 minutes. Pre-

conditioning on injection was a 3 min flush with 0.1M HCl (BDH, Poole, UK), then 2 minutes flush with background electrolyte (BGE). Separation was at a potential difference of +25.0kV. The capillary was thermostated at 25.0°C. Detection was by photodiode-array over 195 - 300nm, but 200nm with bandwidth 6nm, was used for quantitation. Run time was 4 min. Both BGE vials were replenished every 12 injections. All samples and standards in the autosampler were kept at ambient temperature. The injections of the samples into the system were carried out hydrodynamically for 12 seconds at 50mBar.

2.4 Assay Procedure

2.4.1 Preparation of stock solution

A stock solution of lidocaine and procaine were prepared in 50% methanol. All the sub-stock solutions were stored at -20°C. All the calibrators and QC sample concentrations were prepared by appropriate dilution of sub-stock dilution.

2.4.2 Preparation of tape samples and extraction

For the purpose of validation and assay calibration, a length of self adhesive polypropylene tape (Tesa 4204 PV5, Beiersdorf, Hamburg Germany) was cut into 10 samples so that each was approximately 3 x 2cm. 100µL volume of standard or quality control solution were spiked on the adhesive part of the tape. The solution was distributed approximately evenly on all the tapes. The samples were left to dry naturally at room temperature. This required, approximately 30 minutes. The samples then transferred into 10mL polypropylene tube and stored frozen at -20°C until analysis. Samples were obtained from patients by applying the tape (3 x 2cm) on the skin and stripped off by using the forceps. The procedure was repeated 10 times to obtain 10 samples at each skin site. The samples were placed in 10mL polypropylene tube and stored frozen at -20°C until analysis.

2.4.3 Extraction procedure

A 5mL methanol was dispensed into the tube containing standard/QC tape or samples. The contents were vigorously mixed for 45 minutes by using a vortex mixer. 5mL of a 120μg/mL procaine (I.S.) in water solution was added to the tube to make a 50% methanol solution. The contents were mixed again using a vortex mixer for a further 15 minutes. A 200μL of the solution was transferred into 250μL auto injector vial for CE analysis.

3. Validation procedures and results

3.1 Specificity

Six samples of blank tape and six samples of tape spike with lidocaine were prepared and carried through the extraction. The concentration of lidocaine used was 50μg (LLOQ) and the internal standard was 600μg. No significant interfering peaks were found at the migration time of lidocaine or procaine. The signal to noise ratio for both drugs were greater than 10. Figure 2 shows the electropherogram obtained from blank tape spiked with 50μg lidocaine and added IS of 600μg procaine. Whilst Figure 3 and Figure 4 show the electropherograms of EMLA cream solution and one of the tape samples from the study, respectively.

3.2 Calibration curve/ linearity

Calibration curve consists of six non - zero calibrators with nominal values of 50, 100, 250, 500, 750, and 1000μg lidocaine and 600μg procaine. Five batches of calibration curves were prepared for validation purposes. Calibration curve was plotted using area ratio of lidocaine to IS versus known concentrations of lidocaine. All the results were calculated using a $y = Ax + B$ linear regression. The regression coefficient for all the calibration curves obtained were greater than 0.99. Regression parameters obtained from five curves are summarised in Table 1.

3.3 Inaccuracy and Imprecision

Inaccuracy and imprecision were measured both within-batch and between-batch by repeated analysis of low, medium and high quality control samples, together with the LLOQ and ULOQ samples. The nominal values for low, medium and high control samples were 80, 400 and 800μg, respectively. The nominal values for the ULOQ and LLOQ were the same nominal concentration as the highest and the lowest calibration standards, respectively. All the control samples, LLOQ, and ULOQ were each assayed six times in three separate assays. Within-batch and between-batches imprecision and inaccuracy were calculated using internationally agreed method (FDA 2001).

3.4.1 Within-assay reproducibility

The three quality control samples, LLOQ and ULOQ were, initially, each extracted six times in one batch. Subsequently, they were extracted six times in two additional batches. On each occasion a separate calibration curve was extracted. The CV for imprecision and the percent inaccuracy for all the quality control samples including LLOQ and ULOQ were below than 2% and 14%, respectively.

3.4.2 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. The CV for imprecision and the percentage inaccuracy for for all the quality control samples including LLOQ and ULOQ were below than 2% and 11%, respectively .

Table 2 summarises the within and between-batch, and the total variability obtained from the nested Analysis of Variance. From the nested ANOVA, the within and between-batch and the total variability for all the QC samples including ULOQ and LLOQ were all <7%.

3.4 Recovery

Absolute recovery of lidocaine was determined using tape samples spiked with lidocaine at the same nominal concentrations as the quality control samples. Peak area measurements obtained from the extracted samples were compared to the peak area measurements obtained from direct solvent injection of the test compounds. Mean and standard deviation were calculated from at least three measurements at each level. The absolute recovery of lidocaine was ranging from 97 to 103%.

Discussion

Clinical trial of drugs, especially pharmacokinetic studies, normally consists of large numbers of samples. Therefore, the development of rapid and fully validated techniques to analyse the samples in a short a period of time as possible is important. In the development of this CE method we attempted to reduce the migration time even further by short – end injection (Geiser, Rudaz, & Veuthey 2005). This resulted in the migration time of procaine at about 1 minute and lidocaine at about 1.2 minutes. Unfortunately, the prilocaine also found in the EMLA cream could not be fully separated from lidocaine so the optimized method employed the standard separation mode.

Reducing the capillary length to 35cm from the original, 48.5cm, resulted in lidocaine and prilocaine being fully separated and only about 2 minutes more than by short- end injection (Figure 3 & 4). The short migration times obtained for procaine (2.7 minutes) and lidocaine (3.2 minutes) is an advantage of CE compared to the 5 minutes retention time for lidocaine using LC-MS-MS in our previous publication (Chik et al. 2006). All the validation results meet the international requirements as outlined by the FDA's 2001 bioanalytical method validation guidelines (FDA 2001). This method has been used for the analysis of lidocaine in tape samples obtained from tape stripping study in 12 healthy subjects. For the purpose of the study, only lidocaine content was analysed from the tape samples.

Conclusions

A fast and reliable method to analyse lidocaine from skin stripping tape samples has been developed and validated by using capillary electrophoresis with UV detection. The method has successfully been used to analyse hundreds of tape samples from tape stripping study.

Acknowledgements

We would like to acknowledge Tesa UK Ltd. for provided us with the adhesive tape used in this study.

Table 1 Regression parameters for five calibration curves during validation.

Batch	Slope (A)	Intercept (B)	r^2
1	0.0257	0.0867	0.9986
2	0.0262	0.0816	0.9974
3	0.0255	0.0995	0.9967
4	0.0257	0.0661	0.9997
5	0.0240	0.1232	0.9914

Linear equation: $y = Ax + B$

Table 2 Within and between batch variability from the nested analysis of variance (ANOVA).

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal concentration (μg/mL)	50.0	80.0	400.0	800.0	1000.0
Mean ; n =18 (μg/mL)	45.1	81.9	415.0	822.4	1013.0
SDw	0.00	0.31	0.11	0.00	2.78
SDb	0.06	0.39	2.28	2.13	5.73
SDt	0.06	0.50	2.28	2.13	6.37
CVw (%)	0.0	3.8	0.3	0.0	2.7
CVb (%)	1.4	4.7	5.5	2.6	5.7
CVt (%)	1.4	6.1	5.5	2.6	6.3

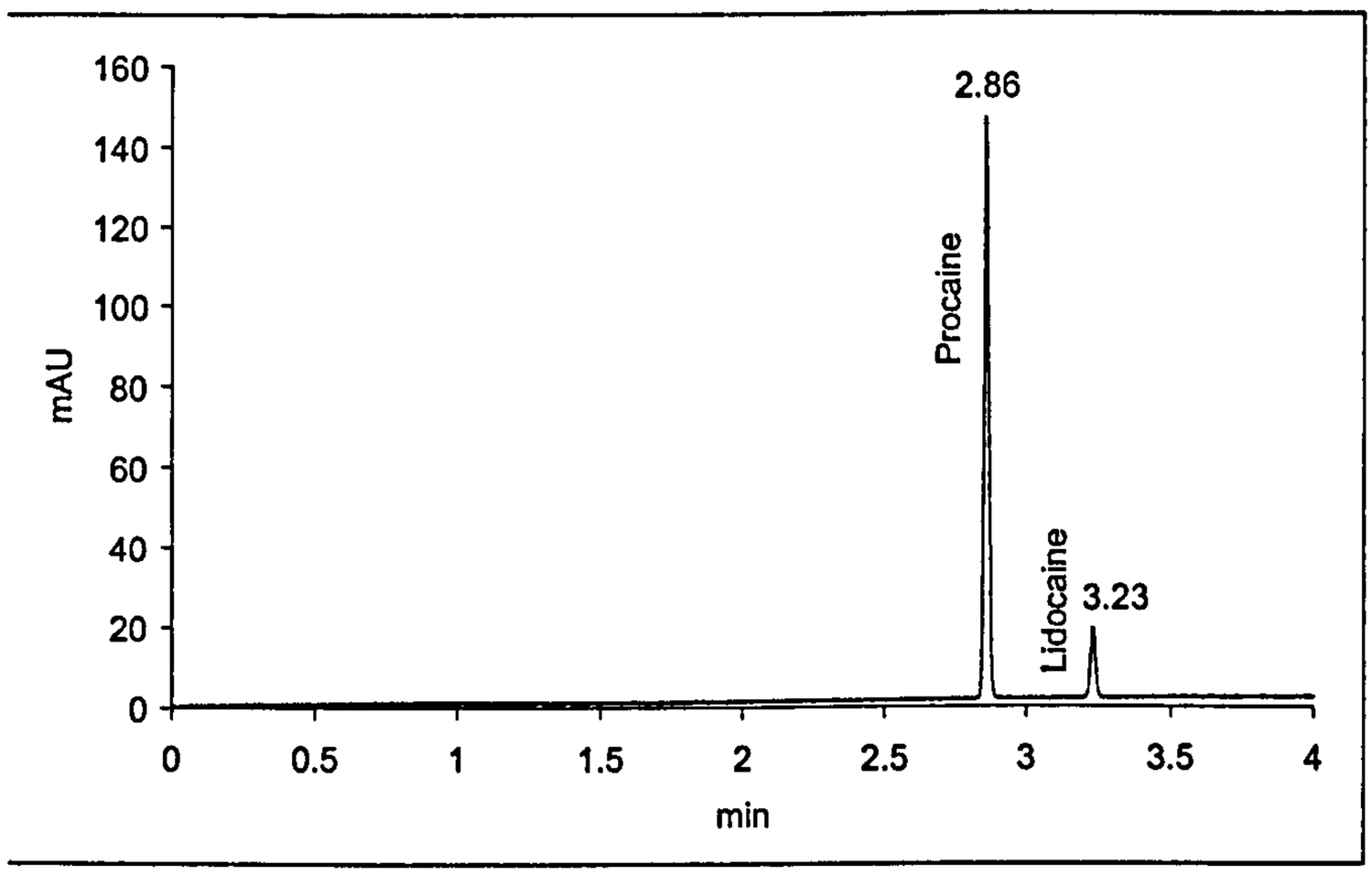


Figure 2 Electropherogram obtained from extracted tape sample spiked with 50μg lidocaine and added IS of 600μg procaine. Displayed at 200nm.

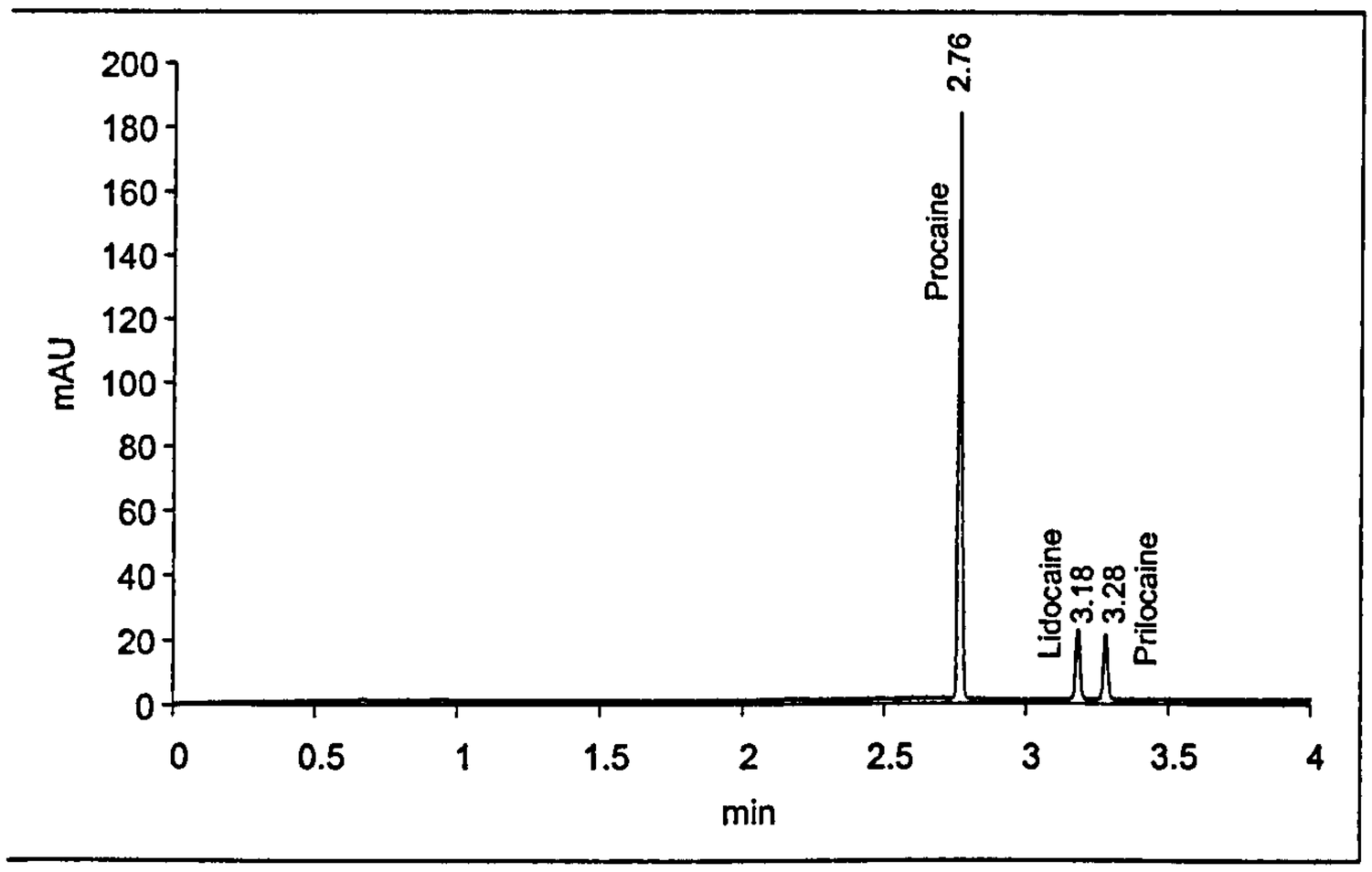


Figure 3 Electropherogram obtained from EMLA cream solution containing 50µg lidocaine and prilocaine and added IS of 600µg procaine. Displayed at 200nm.

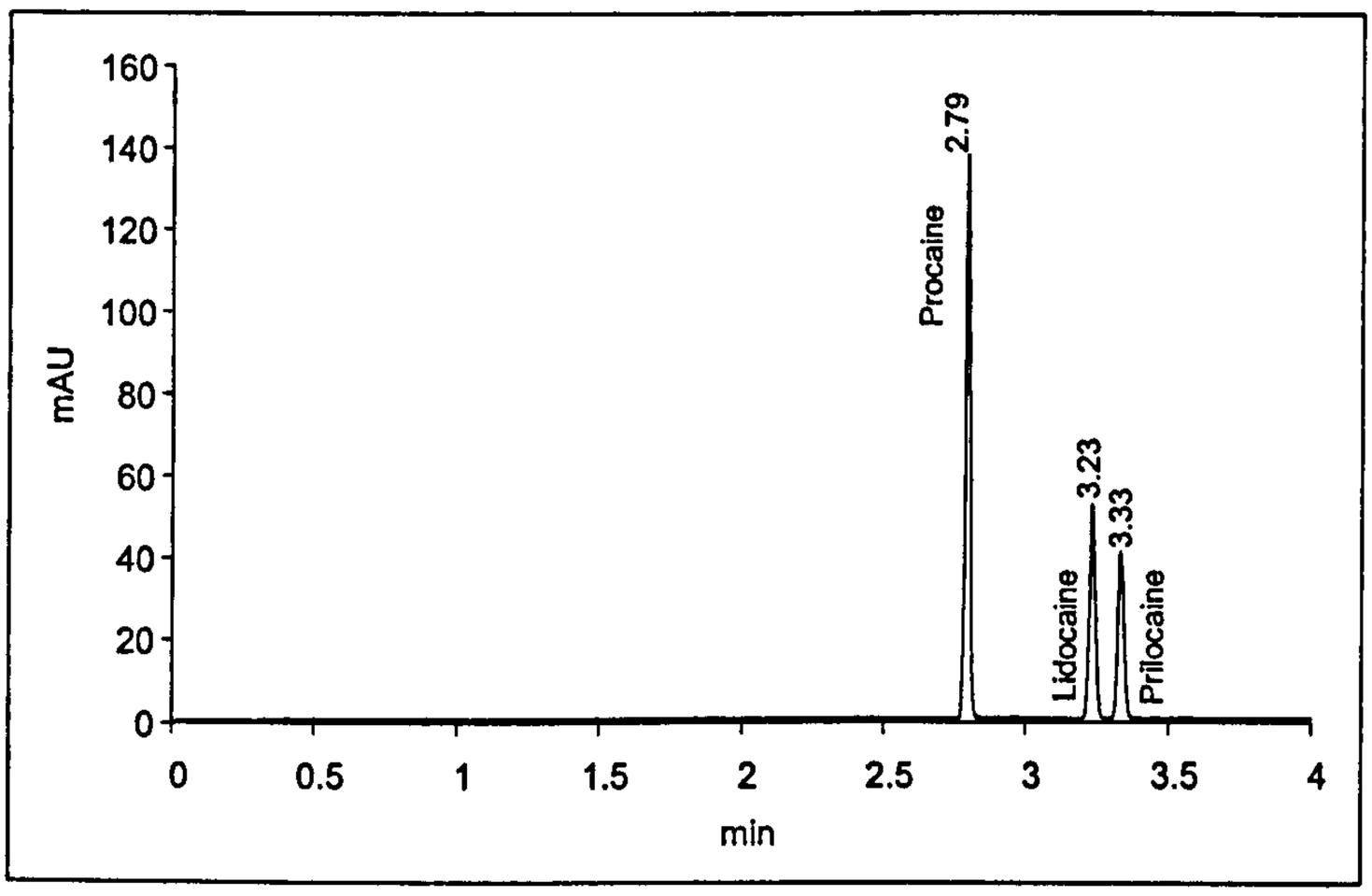


Figure 4 Electropherogram obtained from tape sample at 4 hours post dose with added IS of 600 µg procaine. Displayed at 200nm.

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Validation of High-Performance Liquid Chromatographic–Mass Spectrometric Method for the Analysis of Lidocaine in Human Plasma

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Abstract

A sensitive and simple liquid chromatography–tandem mass spectrometry method is developed and validated for the determination of lidocaine in human plasma. Bupivacaine is used as an internal standard, and the plasma extraction is performed by a simple liquid–liquid extraction. The limit of quantitation (LOQ) is 0.5 ng/mL with a signal-to-noise ratio greater than 5. The calibration curve is linear from 0.5 to 250 ng/mL with an r^2 greater than 0.99. The coefficients of variation for within- and between-assay imprecision, including LOQ, are ≤ 13% and ≤ 8%, respectively. The percentage of inaccuracy for within- and between-assay, including LOQ, are ≤ 9% and ≤ 5%, respectively. The absolute recovery of lidocaine and bupivacaine are greater than 84% and 82%, respectively. The higher sensitivity and accuracy of this method allow the measurement of low concentrations of lidocaine in plasma from a clinical study of topically applied lidocaine in healthy subjects.

Introduction

Lidocaine is a commonly administered drug used both as a local anesthetic and antiarrhythmic. Lidocaine is administered intravenously or parenterally as the oral bioavailability of lidocaine is poor because of the first pass metabolism effect (1,2), and high concentrations of its principal metabolites are neurologically toxic drugs. Procedures involving the insertion of a needle through the skin are painful and may induce great fear and anxiety, especially in children. Bad experiences with a needle may reduce future compliance to the needle procedure. For the described reason, many researchers have searched for a "needle-less" and pain-free local anesthetic method via transdermal delivery.

Absorption from the intact skin for transdermal delivery is poor. Therefore, the systemic absorption is considerably low. To analyze the plasma samples obtained from the clinical study of transdermal delivery of lidocaine, a sensitive and accurate method was needed. In this study, a simple and rapid method for the analysis of lidocaine in human plasma was developed and validated using bupivacaine as an internal standard (IS).

Various analytical methods have been based on high-performance liquid chromatography (HPLC) with UV detection (3,4), liquid chromatography (LC)–tandem mass spectrometry (MS) (5,6), and gas chromatography (GC)–MS (7) for the quantitation of lidocaine. However, the HPLC with UV detection and GC–MS methods are not sensitive enough for dermal absorption studies, as the limit of quantitation (LOQ) of the published methods range from 4 to 100 ng/mL. Only the LC–MS methods are sensitive enough to detect the low concentrations of lidocaine in plasma seen in these studies as the LOQ needs to be of the order of 0.2 ng/mL (5).

Experimental

Chemicals

Lidocaine hydrochloride (99.9% purity) and bupivacaine hydrochloride (100% purity) (IS) were obtained from Sigma-Aldrich (Poole, U.K.). All HPLC-grade solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland). All analytical reagent-grade reagents were obtained from Merck (BDH) (Poole, Dorset, U.K.).

Apparatus

Solvent delivery was achieved using a PerkinElmer series 200 pump (Boston, MA) set at 1 mL/min. Sample injection was carried out using a PerkinElmer series 200 autoinjector. A Shimadzu CTO-10A column oven (Columbia, MD) was used. Detection was by PE SCIEX API2000 MS (Warrington, U.K.). All PE SCIEX software was supplied by Applied Biosystems (Warrington, U.K.). The NM20ZA high-purity nitrogen and air generators were supplied by Peak Scientific Instruments (Reinfrew, Scotland).

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Chromatographic system

The analytical column was a Supelcosil LC-Si (100 × 4.6-mm i.d.), obtained from Merck (BDH) and was maintained at 50°C. The mobile phase was 2% formic acid in acetonitrile–water (50:50). The flow rate was fixed at 1 mL/min. A PE SCIEX API2000 triple quadrupole MS equipped with a turbo-ion spray (heated electrospray) was used to introduce the sample into the MS through off-axis at an angle of 45°. The sample was introduced through 10:1 splitter, which meant only 10 µL of the sample entered the MS. Nitrogen was used as the collision gas. PE SCIEX Analyst software (Version 1.3) was used to control the HPLC–MS, record the output from the detector, perform integration of peak areas, and calculate the lidocaine concentrations.

Bupivacaine was used as an IS. All calculations were based on peak-area ratios of lidocaine and IS. The selection of operating ions is shown in Figure 1. The precursor ions for lidocaine and bupivacaine were *m/z* 234.99 and 289.09, respectively, and after collisional dissociation, the product ions were 85.98 and 140.09, respectively. The retention times for lidocaine and bupivacaine during the assay were 5.1 and 4.9 min.

Assay procedure

Preparation of stock solution

A stock solution of lidocaine and bupivacaine were prepared in 50% methanol. All the sub stock solutions were stored frozen at approximately –20°C. All of the calibrators and quality control (QC) sample concentrations were prepared by appropriate dilution of sub stock dilution.

Extraction procedure

A 0.5-mL volume of plasma, 0.1-mL IS solution (500 ng/mL), 0.1 mL 1M NaOH, and 3 mL of methyl-tert-butyl ether were placed in a 4.5-mL propylene tube. The contents were mix for a minimum of 5 min by the shaker and then centrifuged at 3000–3500 rpm for 5 min. The top layer was then transferred to a 4.5-mL polypropylene tube containing 0.25 mL of 0.1% formic acid. The tube was mixed again for a minimum of 5 min by the shaker and then centrifuged at 3000–3500 rpm for 5 min. The top solvent layer was discarded by vacuum, and the remaining solution was transferred into an autosampler vial. A 100-µL volume was injected into the analytical column.

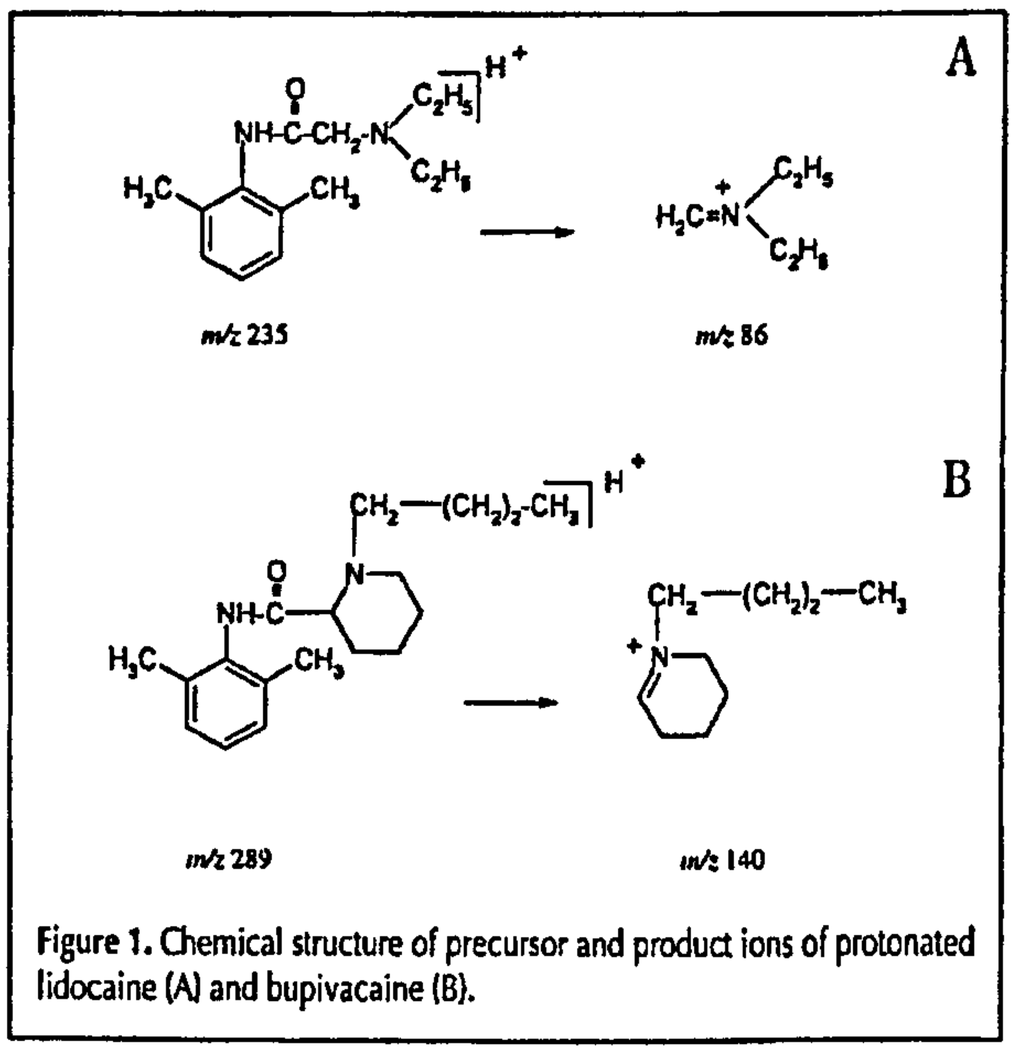

Figure 1. Chemical structure of precursor and product ions of protonated lidocaine (A) and bupivacaine (B).

Results

Selection of operating protonated ions

Figure 1 shows the chemical structure and the protonated ions of lidocaine and bupivacaine used in this study related to their mass. The fragment ions at *m/z* 86 for lidocaine and *m/z* 140 for bupivacaine were selected for the assay. The mass spectrum scans for the two analytes are shown in Figure 2.

Specificity

No significant interfering peaks were found at the retention times of lidocaine or bupivacaine. The signal-to-noise ratios for both drugs were greater than 5. Figure 3 shows the chromatogram obtained from blank plasma spiked with 0.5 ng/mL lidocaine and blank plasma spiked with 500 ng/mL bupivacaine.

Calibration curve

The calibration curve covered the range from 0.5 to 250 ng/mL of plasma with seven calibrators. All of the results were calculated using a $1/x^2$ weighted quadratic regression. The peak-area ratio, regression coefficient, and parameters of the calibration line were calculated from the peak area data by the

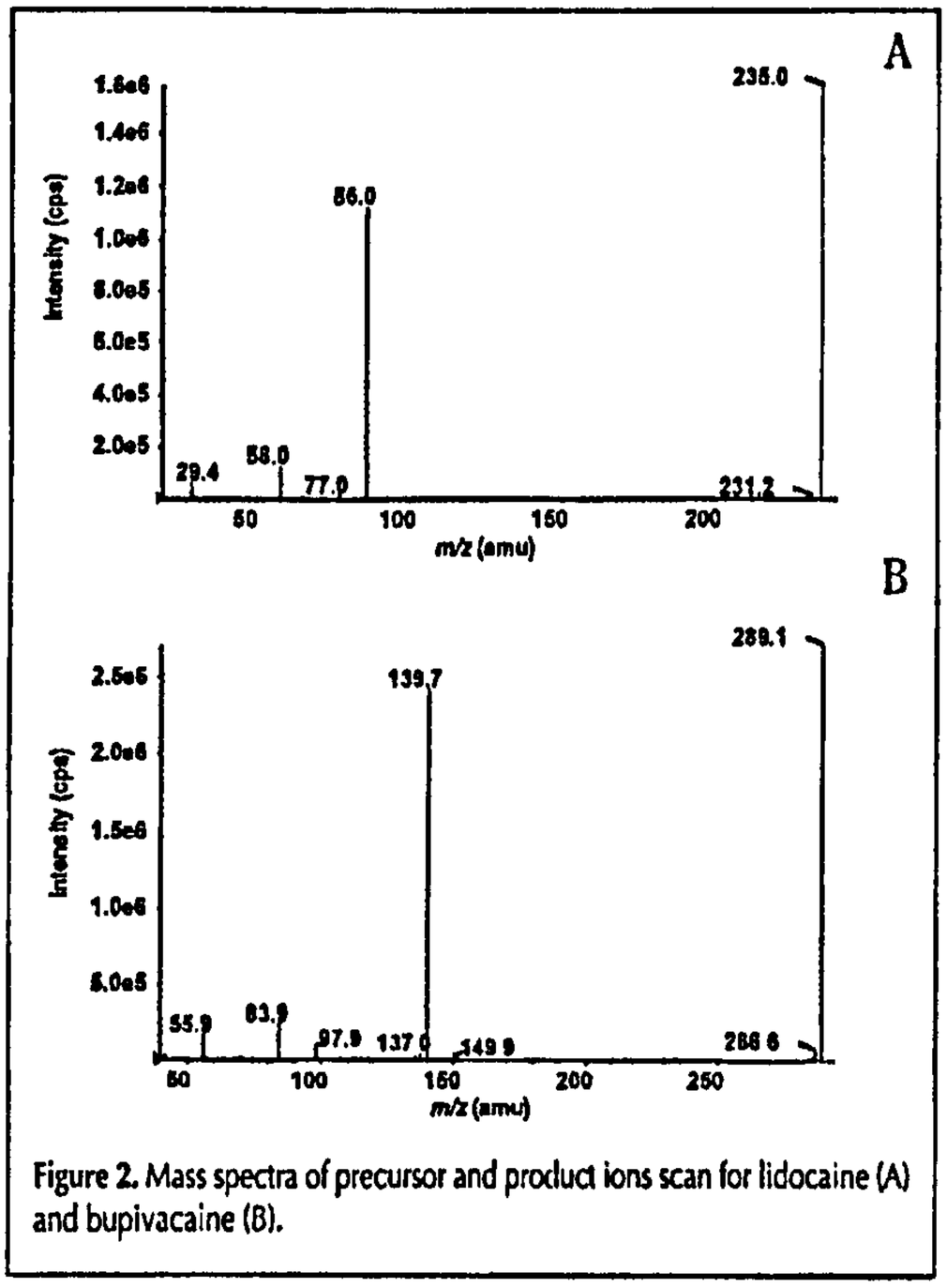

Figure 2. Mass spectra of precursor and product ions scan for lidocaine (A) and bupivacaine (B).

Analyst program. The regression coefficient for all the calibration curves was greater than 0.99. Mean results obtained from five curves are summarized in Table I.

Table I. Regression Parameters for Five Calibration Curves During Validation*

Batch	Curvature (C)	Slope (A)	Intercept (B)	r^2
1	6.12 E-6	0.00743	0.000394	0.9995
2	7.75 E-6	0.00772	0.000682	0.9995
3	7.51 E-6	0.00783	0.000210	0.9987
4	7.79 E-6	0.00816	0.000282	0.9994
5	8.52 E-6	0.00830	0.000434	0.9997

* Second-order equation: $y = C \times 2 + Ax + B$.

Imprecision and inaccuracy

Table II summarizes the within- and between-batch and total variability obtained from the nested analysis of variance (ANOVA). Precision and accuracy were assessed using three QC samples with nominal lidocaine values of 1.5, 45, and 150 ng/mL and a lower limit of quantitation (LLOQ) of 0.5 ng/mL and upper limit of quantitation (ULOQ) of 250 ng/mL.

Within-assay reproducibility

The three QC samples, LLOQ, and ULOQ were, initially, each extracted six times in one batch. Subsequently, they were extracted six times in two additional batches. On each occasion a separate calibration curve was extracted. The coefficient of variation (CV) and the percentage for imprecision and inaccuracy, including the LLOQ and ULOQ, were all within the accepted range, which was 1.7–13% and 0–9%, respectively.

Between-assay repeatability

For each of the three assays previously mentioned, the mean concentration from each assay was used to calculate the between-assay reproducibility. The CV and the percentage for between-assay precision and accuracy, including LLOQ and ULOQ, were all within the accepted range, which was 1.9–8.3% and 1–5%, respectively.

From the nested ANOVA, the within- and between-batch and total variability for all the QC samples, including ULOQ and LLOQ, were all below than 11%.

Table II. Within and Between Batch Variability from the Nested ANOVA*

	LLOQ	QC1	QC2	QC3	ULOQ
Nominal concentration (ng/mL)	0.5	1.47	44.98	149.8	250
Mean (ng/mL); $n = 18$	0.50	1.43	45.72	154.54	263.04
SDw†	0.03	0.09	1.34	5.34	6.99
SDb	0.04	0.08	1.55	2.38	11.18
SDt	0.06	0.12	2.05	5.88	13.18
CVw (%)	6.6	6.1	2.9	3.5	2.7
CVb (%)	8.7	5.9	3.4	1.5	4.2
CVt (%)	10.9	8.5	4.5	3.8	5.0

* Abbreviations: w = within batch; b = between batch; t = total.
† SD = standard deviation

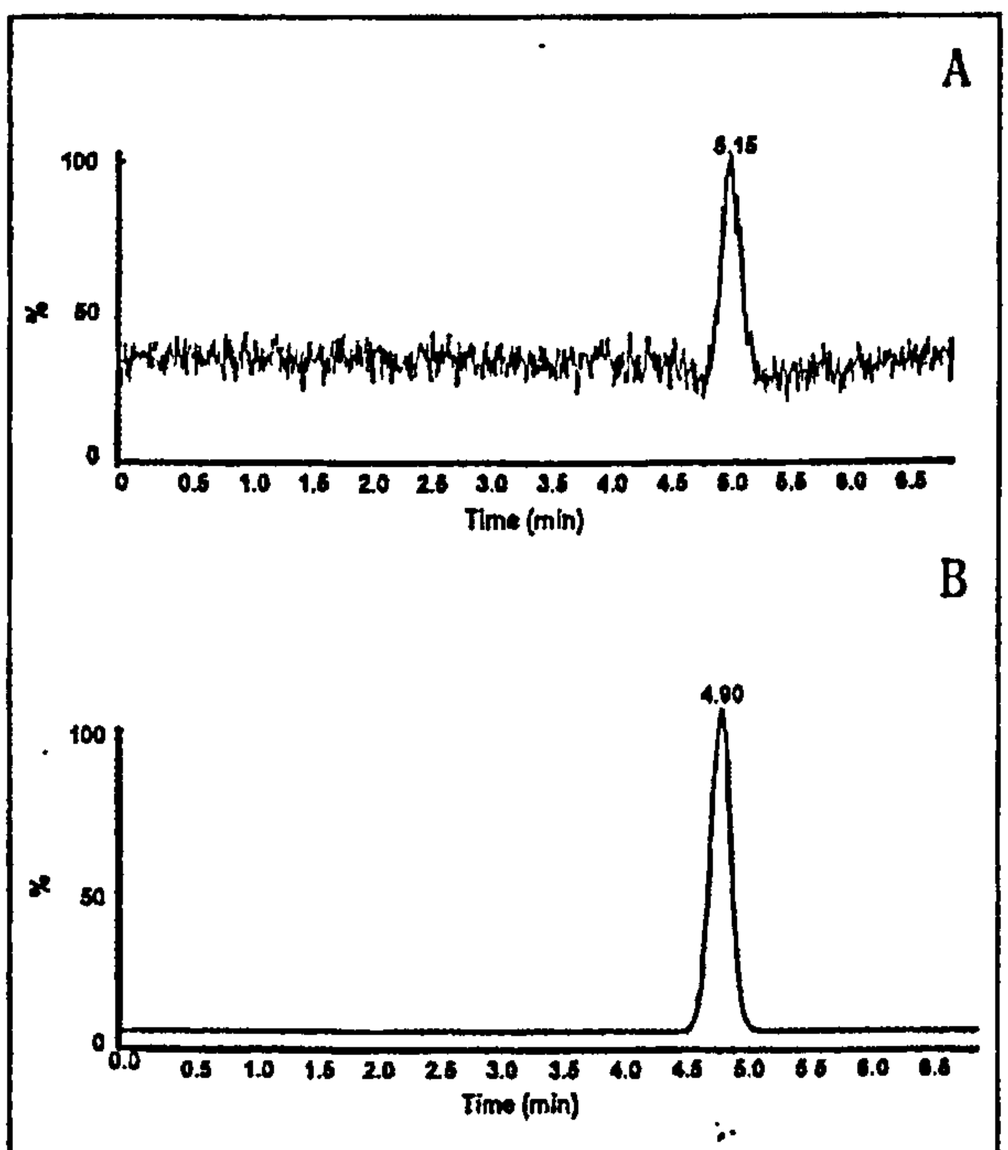

Figure 3. Chromatograms obtained from extracted blank plasma spiked with 0.5 ng/mL lidocaine (A) and 500 ng/mL bupivacaine (B).

Table III. Results of Stability Tests Carried Out on Lidocaine*

		Concentration (ng/mL)		
		1.47	44.98	149.80
Time 0 stability data	Mean ($n = 4$)	1.34	45.58	151.25
In plasma, after three freeze-thaw cycles	n	4.00	4.00	4.00
	Mean	1.52	44.58	141.70
	Difference (%)	14.04	-2.20	-6.31
In plasma after 48 h room temperature	n	4.00	4.00	4.00
	Mean	1.47	40.19	134.29
	Difference (%)	10.40	-11.84	-11.21
In plasma after 48 h at 4°C	n	4.00	4.00	4.00
	Mean	1.54	44.94	149.24
	Difference (%)	15.46	-1.41	-1.33

* Values express in percentage of the concentration difference between before and after test.

Recovery

The absolute recovery of lidocaine was tested using human plasma spiked with lidocaine at the same nominal concentrations as the QC samples. The absolute recovery of bupivacaine was tested at a nominal concentration of 100 ng/mL. Peak area measurements obtained from the extracted samples were compared with the peak area measurements obtained from direct solvent injection of the test compounds. Mean and standard deviations were calculated from at least three measurements at each level. The absolute recoveries of lidocaine and bupivacaine ranged from 84% to 89% and 82% to 86%, respectively.

Stability

Table III lists the stability data for lidocaine in plasma after three freeze-and-thaw cycles, after 48 h at room temperature, and after 48 h at 4°C. Figure 4 shows the graph of log concentration (ng/mL) versus time (h) for the autosampler stability test. The stability of lidocaine was measured in a sample of analyte-free human plasma spiked with lidocaine at the same nominal concentrations as the QC samples. A minimum of three freeze-and-thaw cycles were tested. The stability of these control samples was further examined at ambient temperature, approximately 20°C, and at approximately 4°C for a period of at least 24 h. For the stability of the sample extracts, the three control samples were each extracted such as to yield a total volume of extract sufficient to allow aliquots to be placed in the autosampler at room temperature and injected over a period of not less than 24 h. The autosampler was operated at ambient temperature, approximately 20°C (maximum temperature 20.5°C, minimum temperature 18.0°C). From all the results obtained, lidocaine was found to be stable in all of the parameters tested.

Discussion

This method validation has been conducted in accordance with the Good Laboratory Practice Regulations, Department of Health, London (U.K.), and the Organization for Economic Cooperation and Development (OECD) Principles on Good Laboratory Practice (Paris, France). All the validation results meet the international requirements as outlined in the Bioanalytical method validation, Food and Drug Administration, 2001 (8). This method has been used for the analysis of human plasma obtained from the clinical studies of transdermal delivery of lidocaine on 100 healthy subjects. Although the procedure of liquid–liquid extraction used in this study was similar to the method published by Bo et al. (5), their procedures have been improved by reducing the amount of plasma used in the extraction and the length of the extraction process. The amount of plasma used in this study is suitable to analyze small amount of blood samples, especially from pediatric patients.

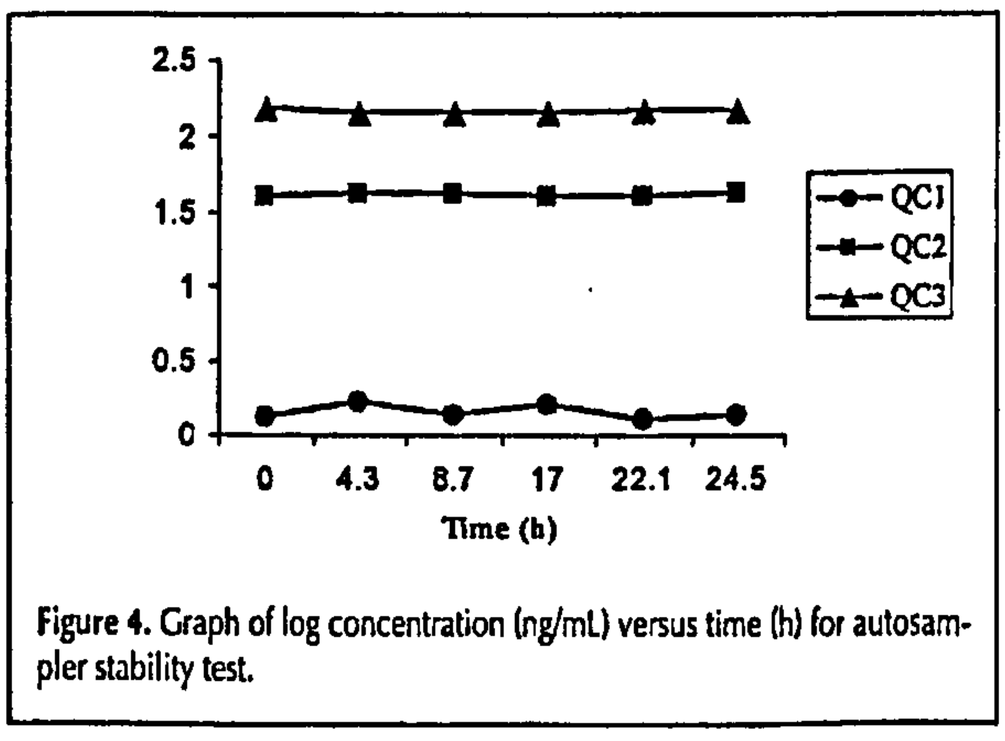

Figure 4. Graph of log concentration (ng/mL) versus time (h) for autosampler stability test.

Conclusion

A simple liquid–liquid extraction and analysis method for lidocaine in plasma has been developed and validated. The sensitivity and accuracy of the assay is suitable for the analysis of low concentrations of lidocaine in plasma for the transdermal delivery study.

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Pharmacokinetics of a new testosterone transdermal delivery system, TDS®-testosterone in healthy males

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Aims
The Transdermal Delivery System (TDS®) is a liquid formulation that can be applied to the skin via a metered pump spray to deliver drug to the systemic circulation. The aims of this study were to assess the ability of the TDS® preparation to deliver testosterone systemically, and to characterize the pharmacokinetic profiles of the hormone in healthy males.

Methods
An open label, comparative, randomized placebo controlled study involving three treatments and three periods with a minimum of a 1 week washout period was conducted. Twelve healthy males received 50 mg TDS®-testosterone, TDS®-placebo, and 50 mg of a commercially available topical testosterone preparation (Androgel®, 1% topical testosterone gel).

Results
The mean AUC(0,12 h) was higher following application of TDS®-testosterone (61.8 ng ml^{-1} h), compared with Androgel® (57.7 ng ml^{-1} h) and TDS®-placebo (50.7 ng ml^{-1} h. The mean C_{max} (0,12 h) was similar for TDS®-testosterone (6.6 ng ml^{-1}) and Androgel® (6.5 ng ml^{-1}) and these values were higher than those for TDS®-placebo (5.7 ng ml^{-1}). Analysis of variance showed that the 90% confidence intervals on the relative difference of the ratio for the AUC(0,12 h) and the C_{max} (0,12 h) between TDS®-testosterone and Androgel®, were contained within the bioequivalence limit (80, 125%) (C_{max} 89.2, 112.3% and AUC 93.5, 120.5%). Serum testosterone concentrations were lower following TDS®-Placebo and were not bioequivalent either to the gel or spray.

Conclusions
The TDS® preparation was shown to deliver testosterone systemically to humans and the concentrations of the hormone in the 12 h following TDS® administration were bioequivalent to an existing topical delivery gel.

Introduction

Testosterone (17α-hydroxyandrost-4-ene-3-one), is the most important androgen secreted into the blood. About 95% of the circulating testosterone present in men is secreted by the testes, which produce between 3 and 10 mg of the hormone per day. Testosterone is responsible for the development of secondary male sex characteristics (e.g. increased growth of body hair, beard, and sexual libido). Hormone concentrations are higher during puberty, but decline with age [1] and

testosterone replacement therapy may be indicated, especially for hypogonadal (testosterone deficiency) men.

A number of testosterone preparations have been tested for replacement therapy. These include subcutaneous implants [2], scrotal transdermal patches [3], non scrotal transdermal patches [4], oral and sublingual preparations [5, 6], testosterone gel [7–9], and the testosterone esters, enanthate and cypionate [10]. With the exception of the transdermal preparations, none is suitable for replacement therapy. Oral administration of testosterone leads to absorption into the hepatic circulation and rapid metabolism by the liver [11]. Behre [12] reported that oral administration of testosterone gives rise to wide fluctuations with high within and between individual variability in serum testosterone concentrations. It has been reported that methyltestosterone can cause hepatic toxicity and adversely affect cholesterol concentrations following long-term usage [13–15].

Sublingual preparations of testosterone result in rapid increases in serum concentrations, which decline to below the normal range after 2 h [6]. Occasional mild redness or itching is common with transdermal patches. Although scrotal patches causes less skin irritation than conventional transdermal preparations, usage of the former leads to an increase in dihydro-testosterone (DHT) concentrations after 3 months of treatment [16]. Testosterone gels have also been developed, but care must be taken to prevent the transfer of testosterone to another person. Patients must wash their hands after applying the gel to a substantial surface area of the body and they must cover the application site with clothing once the gel has dried [17]. We have developed a more convenient method to deliver testosterone through the skin via a metered pump dispenser, using the TDS® delivery system (Transdermal Technologies Inc., Florida, USA). This is a proprietary technology, which has been developed for use in pharmaceutical, cosmetic and over-the-counter products. The system consists of a true solution of ethanol, propylene glycol, monolaurins, vitamins and pro-vitamins and cAMP energy donors. The safety of the TDS® system has been evaluated and confirmed by the Institute for *In Vitro* Sciences in Gaithersburg, Maryland USA, with respect to primary dermal irritation, skin sensitization and toxicity.

The aims of this study were to assess the ability of the TDS® preparation to deliver testosterone systemically and to characterize its pharmacokinetics in healthy males.

Methods

Study materials

TDS®-testosterone (batch number MBR-BFLIQ84), TDS®-placebo and Androgel® (batch number 20293 RC) were supplied by TransDermal Technologies, Inc., Florida, USA. TDS®-testosterone and TDS®-placebo were supplied as a liquid formulation, delivered by metered pump, with each spray containing 10 mg testosterone. Androgel® was supplied as a gel in unit-dose aluminium foil packets of 5 g, each containing 50 mg testosterone.

Study design and treatments

This was a single dose, randomized, three-way crossover study (with three treatments, three periods, and six sequences) with a minimum of 1 week washout period between each treatment. The three treatments were TDS®- testosterone 50 mg, TDS®-placebo, and Androgel® 1% (50 mg).

Subjects

Twelve healthy males successfully completed the protocol. Six subjects were Caucasian, and six were from other racial groups. The mean (SD) age of the subjects was 29.0 (6.2) years, and the mean (SD) BMI was 24.1 (3.2) kg m^{-2}. The study was approved by the East London and The City Health Authority Research Ethics Committee and received a Doctors and Dentists Exemption Certificate (DDX) from MHRA (Medicines and Healthcare Products Regulatory Agency), UK. All the subjects gave written informed consent before taking part in the study, and each fulfilled all entry criteria based on physical examination, medical history, and clinical laboratory tests.

Study protocol

On the morning of each study day, blood pressure and heart rate were measured after subjects had rested for 10 min. A 20G cannula was placed in a large antecubital vein for blood collection. The drug formulation was then applied to the left arm and gently rubbed into the skin. Regular meals and beverages were provided throughout the study day. After dosing, subjects were permitted to engage in normal daily activities, but were excluded from significant physical exertion or activities likely to stimulate endogenous testosterone production.

Approximately 4 ml of blood was collected at –0.5 (30 min before dosing) and 0 h (immediately prior to dosing), to establish a baseline measurement of serum testosterone concentration. Subsequently, serial blood samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, and 24 h post dose. Samples were allowed to clot for approximately 20 min at room tem-

perature (26 °C) and were then centrifuged at 1800 *g* for 10 min. The serum was transferred to labelled tubes and stored at –20 °C until analysis.

Testosterone analysis

Testosterone concentrations were measured in serum using an Enzyme Link Immunosorbent Assay (ELISA) method. The kit was obtained from DRG Instruments GmBH, Germany, lot no. 29K064. The three sets of testosterone control standards were obtained from Bio-Rad Laboratories, USA (lot no. 40631, 40632, and 40633 for set 1, 2, and 3, respectively). The plate reader used was a GENios, computer controlled fluorometer from TECAN Company, Austria. The kit was validated to demonstrate adequate sensitivity, specificity, recovery, accuracy and precision (within and between assays). The limit of quantification was 0.2 ng ml^{-1} for the assay. All the study samples were analyzed together with quality control samples containing three different concentrations. The coefficients of variation for imprecision and inaccuracy were all below 15%.

Data analysis

Pharmacokinetic parameters were determined and the statistical analysis was performed using Kinetica Version 4.2 software. C_{max} and t_{max} were determined directly from the individual serum concentration-time curves, and the AUC was calculated using the linear trapezoidal method. The difference between treatments for AUC(0,12 h) and C_{max} were analyzed after logarithmic transformation using analysis of variance (ANOVA) for crossover studies which accounts for variation due to sequence, subject, formulation, and period.

Bioequivalence testing was based upon the 90% confidence interval (CI) for the ratio of population means between two treatments. This method is equivalent to the corresponding two one-sided test procedure, with the null hypothesis of bioequivalence at the 5% significance level. For formulations to be bioequivalent, the ratio (test : reference) must fall between the 0.8, 1.25 confidence interval [18].

Results

Figures 1 and 2 show the plots of mean serum concentration of testosterone *vs.* time and mean serum concentration changes from baseline. Testosterone concentrations fluctuated slightly in most of the subjects during all the treatments. Most of the subjects treated with Androgel® and TDS®-testosterone achieved higher concentrations compared with placebo at 24 h post dose.

The pharmacokinetic parameters AUC, C_{max}, and t_{max} are listed in Table 1 for each treatment. The AUC and C_{max} values were calculated for both 0–12 and 0–24 h. The mean AUC(0,12 h) was higher following application of TDS®-testosterone compared with Androgel® and TDS®-placebo. However, the mean AUC(0,24 h) for Androgel® was higher than TDS®-testosterone and TDS®-placebo. The mean C_{max} (0–12 h) was similar for TDS®-testosterone and Androgel® and these values were higher than that obtained for TDS®-placebo. Owing to the higher concentrations of testosterone at 24 h in some subjects, the mean C_{max} (0–24 h) value for Androgel® was higher than those for TDS®-testosterone and TDS®-placebo. Only the 0–12 h data were used for the determination of bioequivalence.

The 90% confidence intervals on the relative difference of the ratio for the AUC(0,12 h) and the C_{max} (0–12 h) between TDS®-testosterone and Androgel®, were contained within the bioequivalence limit (80, 125%)

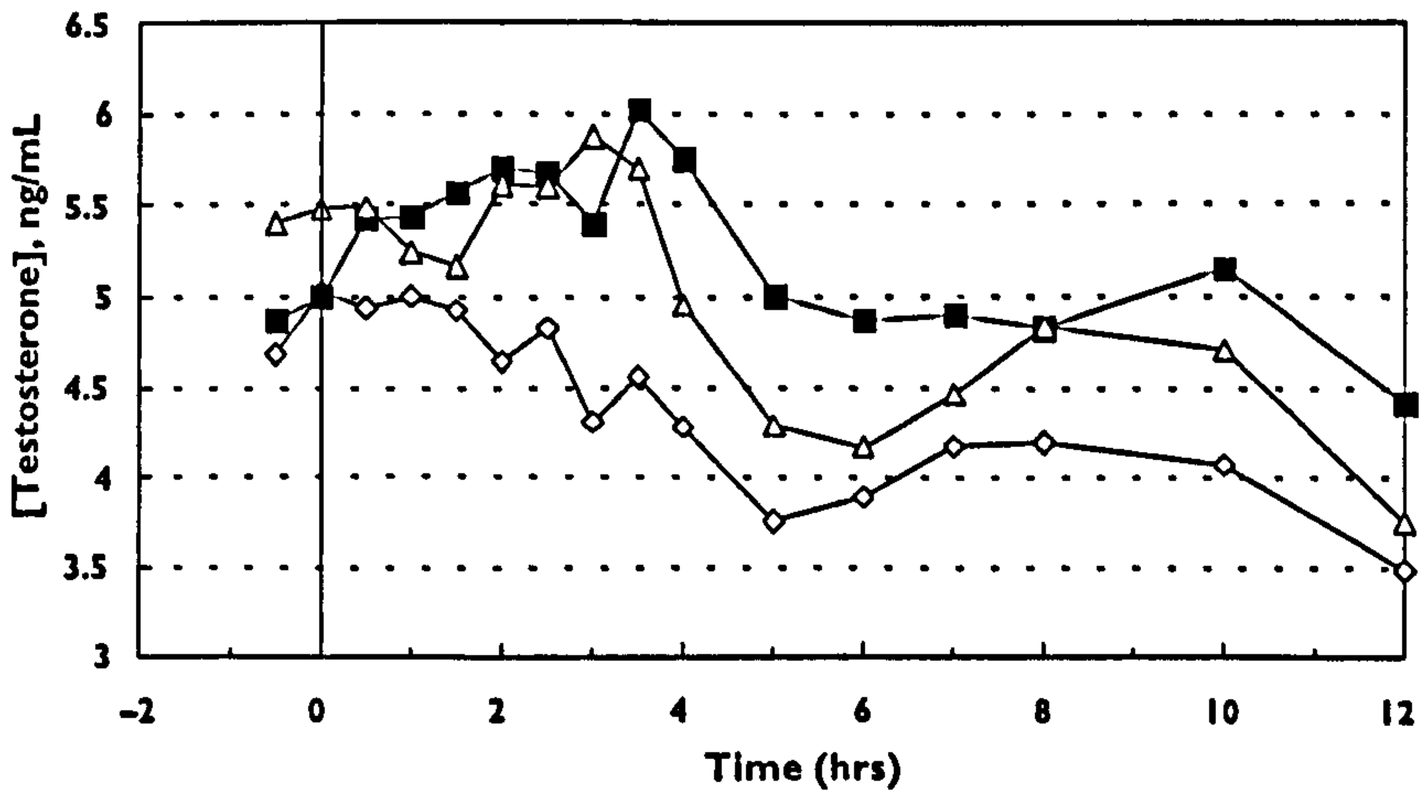

Figure 1
Plots of mean serum testosterone concentration (ng ml^{-1}) *vs.* time (h) for each treatment. TDS®-placebo (◇), TDS®-testosterone (■), Androgel® (△)

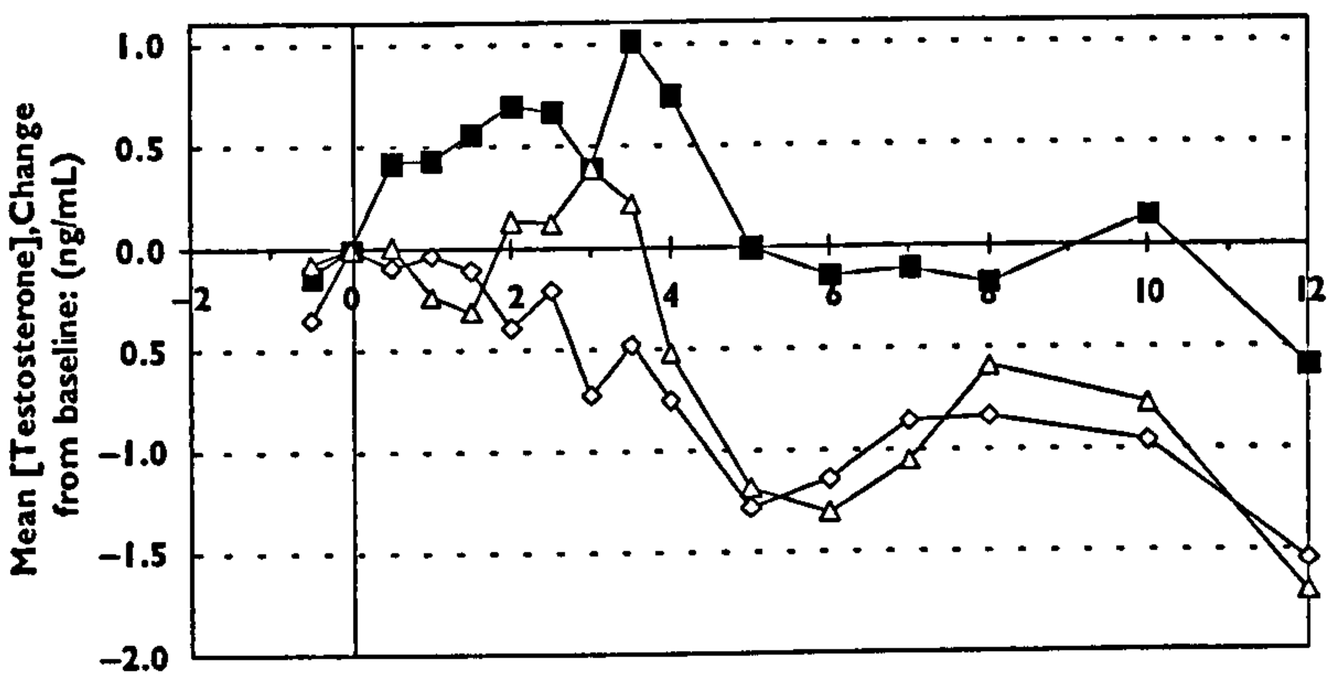

Figure 2
Plots of mean serum testosterone concentration change from baseline (ng ml^{-1}) *vs.* time (h) for each treatment. TDS®-placebo (◇), TDS®-testosterone (■), Androgel® (Δ)

Table 1
Geometric mean (CV percentage) values for C_{max}, t_{max} and AUC for each treatment

	C_{max} (ng ml^{-1})		t_{max} (h)		AUC (ng ml^{-1} h)	
	0–12 h	0–24 h	0–12 h	0–24 h	0,12 h	0,24 h
TDS®-testosterone	6.64	8.38	2.42	11.75	61.85	135.77
	(22.4)	(49.7)	(58.4)	(92.3)	(24.7)	(34.1)
TDS®-placebo	5.72	5.95	3.29	9.00	50.67	101.67
	(21.1)	(22.2)	(111.5)	(107.4)	(23.6)	(25.5)
Androgel®	6.54	13.17	1.83	16.79	57.67	157.41
	(24.1)	(99.2)	(52.4)	(63.5)	(19.3)	(57.7)

(C_{max}: 89.2, 112.3% and AUC(0,12 h): 93.5, 120.5%). Serum testosterone concentrations were lower following TDS®-placebo and were not bioequivalent either to the gel or the spray.

Discussion

No serious or unexpected adverse events were reported or observed during the study day. The drug formulations and protocol requirements were well tolerated by all subjects. Currently licensed transdermal delivery systems are available as patches, gels and buccal tabs. Patients report to us that patches may irritate the skin, gels can take time to dry and leave a residue, while the buccal tabs may be intrusive and have to be applied twice a day. The TDS preparation appears to provide a more convenient transdermal delivery of testosterone with rapid drying, and low to no skin residue or irritation compared with other transdermal systems. Based on these data we now plan a phase II study in hypogonadal males.

The TDS®-testosterone preparation was shown to deliver testosterone systemically to humans. The concentrations of hormone in the first 12 h following TDS® administration were found to be bioequivalent to an existing topical delivery gel.

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Study of a combined percutaneous local anaesthetic and the TDS® system for venepuncture

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Summary

Transdermal Delivery System (TDS®) is a liquid formulation which can be applied to the skin via a metered pump spray to deliver drug across skin. This placebo controlled, double blind trial compared anaesthetic properties of two TDS® systems (TDS®α and TDS®β) with placebo. The active and placebo treatments were applied to the dorsum of the hands, bilaterally and simultaneously for 5 min on 100 healthy volunteers. Following cannulation, pain perception was measured using the verbal rating score (VRS) and visual analogue score (VAS). Lidocaine plasma levels were assessed at 0 and 2 h. The VRS and VAS results show that TDS®β significantly decreased pain score compared to placebo ($p < 0.02$). Blood lidocaine at 2 h post application was also higher for TDS®β than for TDS®α, suggesting that a 5 min application of TDS®β was effective in delivering local anaesthetic and accelerating the onset of skin anaesthesia prior to venous cannulation in adults.

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The pain caused by common minor procedures such as venepuncture and minor surgery is often ignored by clinicians. While it may be a trivial problem in adults, for young people, especially children, the pain is significant and can lead to the development of 'needle phobia', an intense fear of needles that triggers immediate anxiety. This also affects some adults. Local anaesthetics are normally given through injection and used to reduce the pain by inducing a loss of feeling (numbness) of skin and mucous membranes. To avoid the pain and anxiety of venepuncture, topical anaesthetics have been in use to provide needleless induction of local anaesthesia.

Current topical local anaesthetics such as EMLA® (Astra Pharmaceuticals Ltd, Luton, UK) [1] and AMETOP® gel [2] (Smith & Nephew Healthcare Ltd, Hull, UK), whilst effective, require institutional support. One hour prior application of EMLA® [3] and 30–45 min prior application of AMETOP® [4] limit clinical and patient acceptance. Thus, topical anaesthetics are excluded from the procedures requiring acute anaesthesia, as well as those outside the institutional environment. The development of a topical delivery system with faster time of anaesthetic onset would be helpful in emergency cases and to increase the number of surgical day cases seen, especially in paediatrics.

The Transdermal Delivery System (TDS®) is a patented process for creating a formulation to deliver drug across skin using a liquid vehicle, measured by unit dose or metered pump spray. The dose is routinely compounded into approximately 1 ml of very stable fluid. There is no patch or application appliance required other than unit dose packaging or metered pump sprayer. The system is composed of substances that are nutritional and/or neutral and harmless at their respective concentrations. TDS® systems are typically composed of a compatible solvent, supplemented by other excipients that enable a sufficient dose of the drug to be put into a relatively small volume of liquid. TDS® also contains excipients designed to support the skin and maintain the integrity of the barrier and the health of the skin. A TDS® may also contain excipients that can affect the rate of absorption of the drug. The TDS® is usually designed to enable extremely rapid delivery and bioavailability of drugs

equivalent to needle injection, with the added benefit of design, which enables rapid physiological response and blood levels equivalent to oral dosing.

The TDS® has been successfully tested in preclinical models with the following diverse molecules: cystamine, hydroxyzine, acyclovir, morphine sulphate, ibuprofen (three studies), paracetemol (two studies), imidazolinium methyl sulphate, testosterone, progesterone and the peptide alpha melanocyte-stimulating hormone. These drugs vary with molecular weights from the low 100 Da to nearly 2000 Da, both hydro- and lipophilic compounds and doses of 1–500 $mg.ml^{-1}$ of TDS® drug product. The safety of TDS® systems has been evaluated and confirmed by the Institute for In Vitro Sciences in Gaithersburg, Maryland, USA, for primary dermal irritation, skin sensitisation and toxicity. In this study, two TDS® local anaesthetic systems (TDS®α and TDS®β) have been evaluated for their speed of onset of anaesthesia, and drug penetration into the circulation.

Method

Study materials

Study materials were supplied by Transdermal Technologies Inc., Florida.

1 TDS®α Anaesthetic System (alcohol based) containing 4% w/v lidocaine and 2% w/v tetracaine.
2 TDS®β Anaesthetic System (water based) containing 4% w/v lidocaine and 2% w/v tetracaine.
3 TDS®α and TDS®β placebo.

Study design and subjects

This study was prospective, double blinded and placebo controlled, with a 1-week washout period, involving 100 healthy volunteers. Based on our previous study [5], 100 subjects recruited in this study would have an 80% power to detect a difference of 25% in the primary outcome measures at $p < 0.05$. Prior to enrolment, each subject was screened for standard blood biochemistry, drugs of abuse, and answered a questionnaire for demographics. Skin was assessed for erythema, oedema, itching, broken skin, or other signs of pathology. Body mass, height, body mass index, systolic and diastolic blood pressure, and heart rate were recorded. Subjects outside the age range of 20–40 years, with signs of skin pathology, haematology 'out of standard limits' or with positive drug abuse tests were excluded. Subjects were not permitted any form of analgesia within 1 week of the trial. The study was approved by the East London and City Authority Research Ethics Committee and received a Doctors and Dentists Exemption Certificate (DDX) from the MHRA (Medicines and Healthcare Products Regulatory Agency, UK). Subjects were admitted to the investigation having been provided with a verbal and written explanation and signed a consent form.

Admission and procedure

Subjects were admitted to the Study Unit having fulfilled all the inclusion criteria. Blood pressure and heart rate were measured after subjects rested for 10 min. A sample was taken from an antecubital vein to establish a baseline measurement of plasma lidocaine concentration. All subjects were dosed according to the randomisation schedule. In the Phase 1 study, TDS® (active) was applied to the dorsal surface of a randomly selected hand and the TDS® (placebo) was simultaneously applied on the contralateral hand. Administrations of the formulations were achieved by metered pump spray of 1 ml to the area of 4 cm^2. Five minutes after application, the hands were routinely cleaned using alcohol wipes prior to venepuncture.

A vein on each hand within the treatment area was then cannulated using a 20G butterfly needle. The success of cannulation was confirmed by the ability to withdraw 1–2 ml of blood. Two methods of pain assessment, Verbal Rating Score (VRS) and Verbal Analogue Score (VAS), were used to assess the pain of the procedure. Both systems have been fully validated in the literature [6, 7] and the investigators trained in their use [5]. Following successful bilateral cannulation, a VRS pain classification was used for each hand. The volunteers were asked the following question: 'How strong was the pain of the procedure?' and provided with a choice of five categories:

1 no pain
2 minimal sensation
3 mild pain
4 moderate pain
5 severe pain

The volunteer selected one answer for each hand by circling the number.

In the VAS assessment, a 100 mm horizontal line with endpoints that are anchored by descriptors 'no pain' and 'severe pain' was used. For each hand, the volunteer was asked 'What did the procedure feel like?' and then requested to make a vertical line on the horizontal line which represented the intensity or unpleasantness of their pain by the procedure. Values were measured in millimetres from the left hand edge of the horizontal line.

Two hours after the treatment application, another blood sample was taken to assess the systemic level of lidocaine. The plasma was transferred to cryo-vials and stored at −20 °C until analysis. All the procedures, including treatment applications, bilateral cannulation and data recording were each performed blindly by separate investigators. One week later, the volunteers repeated the above procedure using the second formulation (TDS®β).

Analytical method

Plasma concentration of lidocaine was analysed by using the liquid chromatography-mass spectrometry (LC-MS/MS) method. Sample separation and detection was achieved on a Supelcosil LC-Si 10 cm column and PE SCIEX API 2000 mass spectrometer. The method was validated to demonstrate adequate sensitivity, specificity, accuracy and precision. The lower limit of quantification (LOQ) was 0.5 ng.ml^{-1} and bupivacaine was used as an internal standard.

Statistical analysis

All the data were analysed using GraphPad Prism 4.0 (http://www.graphpad.com/prism/Prism.htm) and Minitab 14 statistical software (http://www.minitab.com/). The active treatments were compared to the placebo control using Wilcoxon's Signed Rank test. The lidocaine concentrations at 2 h for TDS®α and TDS®β were compared using Student's paired *t*-test.

Results

One hundred healthy volunteers were successfully recruited, and the demographics data are presented in Table 1. Of the 100 subjects, 65 were Caucasian, 22 Asian, four African/Caribbean, and nine from other ethnic groups. The cannulation procedures were successfully completed at the first attempt for all 100 volunteers. All the subjects tolerated the procedure well and complied with the study protocol.

The median for VRS and VAS scores were different between active and placebo for both TDS®α and TDS®β. In the VRS pain classification, the active treatment of TDS®α was not significantly different from the placebo (p = NS; Fig. 1). However, the active treatment of TDS®β resulted in a significant reduction in pain response to cannulation compared to the placebo treatment ($p < 0.02$; Fig. 2).

Similarly, the VAS also showed no significant difference between placebo and active treatment with TDS®α but was significantly different for TDS®β ($p < 0.02$; Fig. 3). The distribution of scores of the active treatment group in Phase II (TDS®β) was generally shifted from the two highest scores (moderate and severe) to the lower

Table 1 Demographic data of study volunteers.

Parameter	Mean (SD)	Median [range]
Sex; M : F	41 : 59	
Age; years	26.45 (5.2)	25.0 [20–40]
Body mass index (BMI)	23.3 (3.9)	22.6 [15.2–37.5]

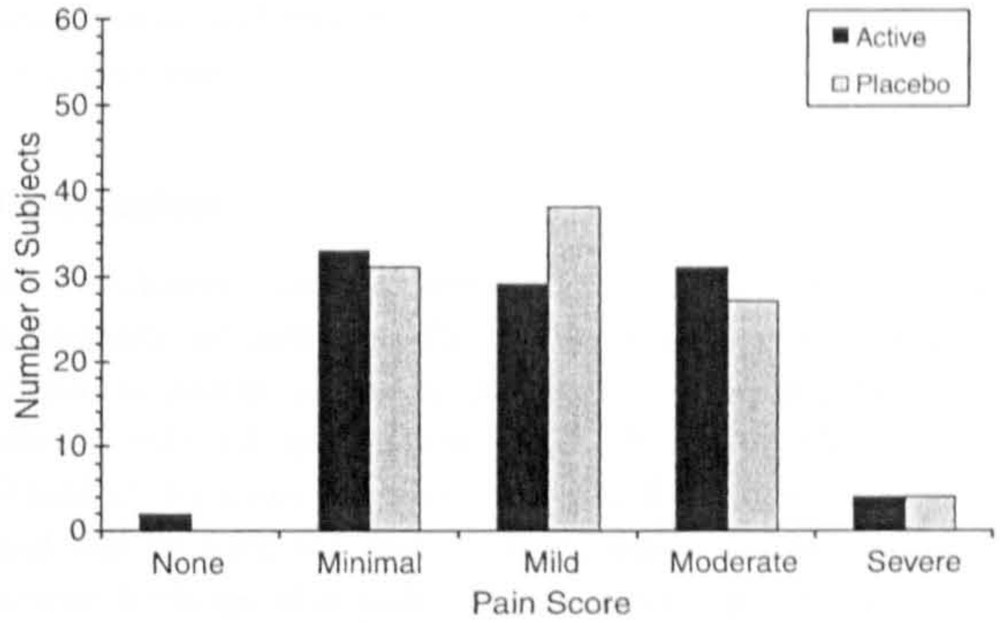

Figure 1 Verbal Rating Score (VRS) for TDS®α. Values are subjects percentage vs. categories; $n = 100$; p = NS, Wilcoxon's Signed Rank test.

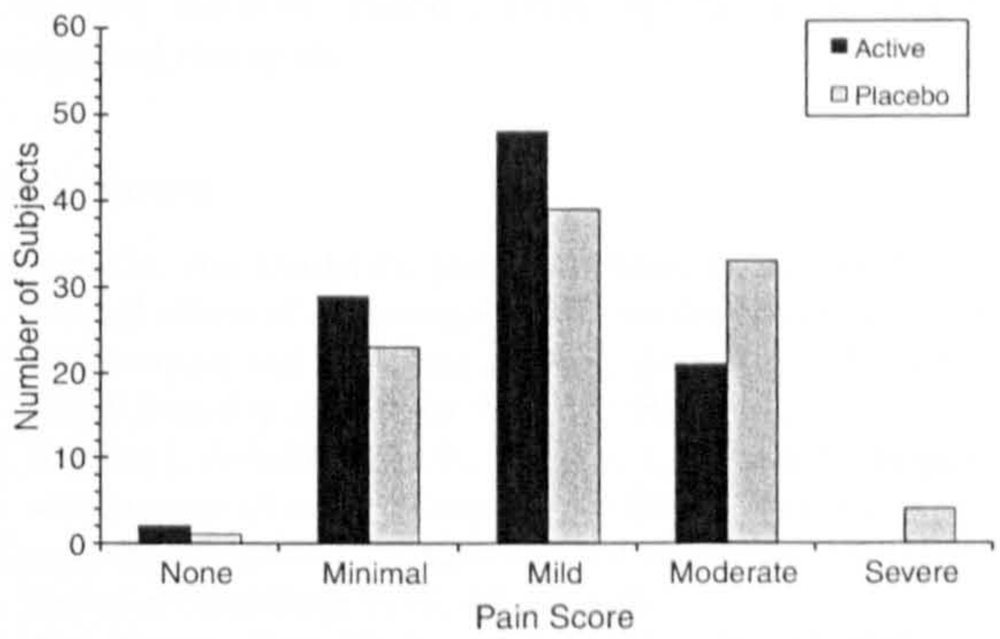

Figure 2 Verbal Rating Score (VRS) for TDS®β. Values are subjects percentage vs. categories; $n = 100$; $p < 0.02$, Wilcoxon's Signed Rank test.

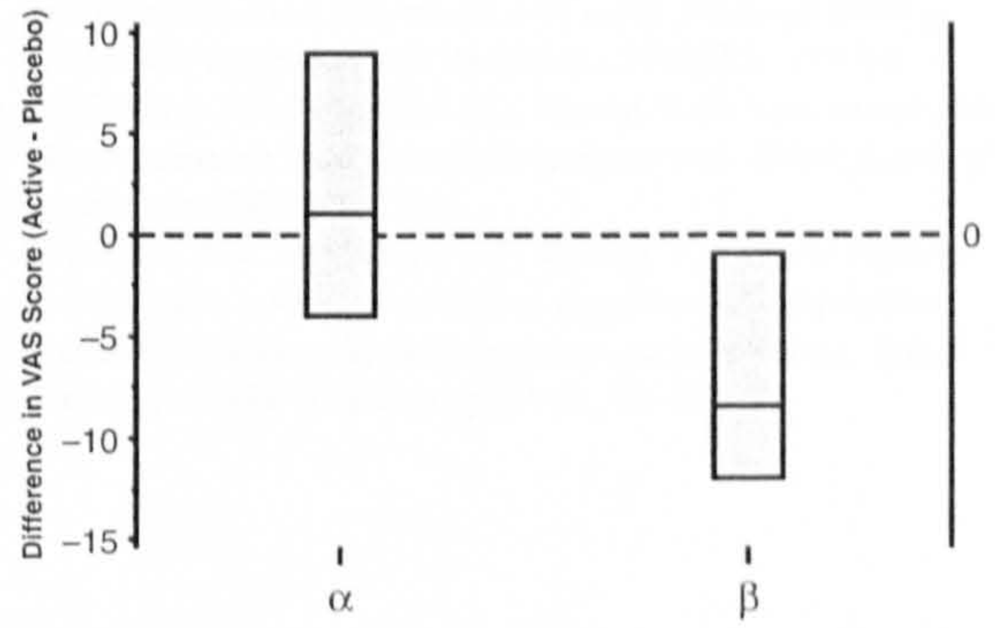

Figure 3 The median differences in VAS score of active and placebo (α = TDS®α and β = TDS®β). Values are median ± 95% CI; $n = 100$; p = NS for TDSα and $p < 0.02$ for TDS®β, Wilcoxon's Signed Rank test.

level of score (mild), with no subjects on active treatment rating the pain as severe. There was a reduction of those who scored the pain as moderate by 36.4% (Fig. 2).

The above result was further supported by the plasma lidocaine concentrations 2 h after the active treatment was applied. Lidocaine was detected in plasma for almost all subjects at 2 h post dose. Although the level of lidocaine was not significantly different between TDS®α and TDS®β ($p = 0.287$, NS; paired *t*-test), the mean plasma level for TDS®β was slightly higher than TDS®α: mean (SD) [range] 3.51 (9.31)[0–64.5] $ng.ml^{-1}$ and 2.51 (6.8)[0–55.9] $ng.ml^{-1}$, respectively.

Discussion

The result from this exploratory study suggests that immediately following application of a TDS® anaesthetic system, there is a fast onset of effective anaesthesia for the venous cannulation in adults. The fact that 5 min application of TDS® anaesthetic system can produce an acceptable level of anaesthesia is a major advance in the anaesthetic system compared to EMLA® and AMETOP® gel, which must be applied 1 h and 30–45 min, respectively, before cannulation is attempted. For operational reasons, randomisation was only performed within the treatment between active and placebo and not between the two systems (TDS®α and TDS®β). However, the first treatment (TDS®α) could not have affected the second treatment (TDS®β) due to the 1 week washout period. This gave more than enough time for the lidocaine to clear from the body and made carry over effects highly unlikely.

Between the two TDS® systems tested, the water based anaesthetic system was more effective than the alcohol based product in providing both transdermal delivery of local anaesthetic and anaesthesia. The TDS® anaesthetic system can thus be manipulated to adjust the onset and degree of topical anaesthesia, and will be used as a basis for investigations into application periods and increased levels of anaesthesia. Other TDS® anaesthetic systems, such as those using alternative combinations of local anaesthetic agents rather than the lidocaine/tetracaine used in this study, are currently under investigation. The development of a rapid onset topical local anaesthetic would enable the replacement of invasive methods of local anaesthesia, and free this procedure from the institutional environment.

Conclusion

In conclusion, topical application of the TDS® local anaesthetic system was effective in providing skin anaesthesia for dorsal hand vein cannulation in healthy subjects, after 5 min of application. TDS®β (water based) was found to be more effective than TDS®α (alcohol based) and can be used for further development of this system. These findings also indicate the rapid transdermal drug delivery by the TDS® system.

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Zamri Bin Chik

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and is competent to carry out phlebotomy

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To whom it may concern:

We hereby confirm that

Zamri CHICK

has participated in the

Course ***"Basic and Advanced Aspects on In Vivo microdialysis"***

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We hereby also confirm receipt of registration fees of

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