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Mechanistic Studies On Skin Permeation Models

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Plagiarism statement

This thesis describes research conducted in the UCL School of Pharmacy between 2013 and 2016 under the supervision Dr Majella E. Lane and Professor Kevin Taylor. I certify that the research described is original and any work conducted by collaboration is clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in any publication.

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Abstract

The assessment of skin permeation is critically important in many fields and a suitable and robust model that may be used to quantify and predict percutaneous penetration is necessary. Currently available models include *in vitro* models using human skin, animal skin, synthetic membranes and cell culture models. Recently, the Skin Parallel Artificial Membrane Permeation Assay (PAMPA) has been proposed as a simple but high throughput screening system that may be suitable to study skin permeation.

In the present study, a lipophilic active, ibuprofen and a hydrophilic active, caffeine were selected to conduct *in vitro* permeation studies in the conventional Franz cell models using silicone membrane, porcine skin and human skin, and the novel Skin PAMPA model. The overarching aim was to determine the utility of Skin PAMPA for routine *in vitro* skin permeation testing with reference to topical formulations.

The *in vitro* permeation studies conducted in Franz cell models using silicone membrane and porcine skin showed that as a lipophilic active, ibuprofen permeated rapidly though silicone membrane and porcine skin. Compared with ibuprofen, caffeine went through the skin more slowly. *In vitro* permeation studies in Franz cell models using human skin indicated that the ibuprofen percentage permeation values for human skin were much lower than corresponding values in porcine skin as expected.

Various *in vitro* permeation studies were conducted in the novel skin PAMPA model for different ibuprofen and caffeine formulations. In general, the Skin PAMPA model did discriminate between different formulation types and different solvent systems compared with other models, with low variability in the permeation data. The more permeable nature of the PAMPA, silicone membrane and porcine tissue models to ibuprofen compared with human skin was also demonstrated, while the permeation of caffeine, a hydrophilic compound, in the PAMPA model was comparable to that in human skin.

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This thesis is the end of my journey to achieve my Ph.D. It was based on research performed in the University College London, School of Pharmacy.

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List of Abbreviations

SC Stratum corneum

VE Viable epidermis

NSAID Non-steroidal anti-inflammatory drug

PDE Phosphodiesterase

cAMP Cyclic adenosine monophosphate

1,2-PENT 1,2-pentanediol

PG Propylene glycol

PEG polyethylene glycol

1,3-BG 1,3-butylene glycol

1,2-BG 1,2-butylene glycol

IPA Isopropyl alcohol

GER Geraniol

LIM d-Limonene

T-BA t-butyl alcohol

Lina Linalool

TPG tri-propylene glycol

IPM Isopropyl myristate

DMI Dimethyl isosorbide

DPPG Propylene glycol dipelargonate

PGMC Propylene glycol monocaprylate

PGML Propylene glycol monolaurate

TRANS Transcutol-P

PEG-6-CCG PEG-6-caprylic/capric glycerides

OSAL Octyl salicylate

PBS Phosphate buffered saline

HPLC High Performance Liquid Chromatography

GC Gas chromatography

LOD Limit of detection

LOQ Limit of quantification

ICH International Conference on Harmonization

R.S.D Relative standard deviation

S.D Standard deviation

ACN Acetonitrile

TFA Trifluoroacetic acid

Log P Partition coefficient

D Diffusion coefficient

h Diffusional path length

ANOVA One-way analysis of variance

V_m Molar volume

E_V Energy of vaporisation

k_p Permeability coefficient

pK_a Acid dissociation constant

$Q_{washing}$ permeation amount from washing

$Q_{extraction}$ permeation amount from extraction

$Q_{permeation}$ permeation amount from permeation

OECD Organisation for Economic Co-operation and Development

TEWL Trans-epidermal Water Loss

CRS Confocal Raman spectroscopy

DS Degree of drug saturation

Chapter 1 General introduction

1.1 The structure of skin

The skin is the largest organ of the body, representing more than 10% of the body mass with an average surface area of 2 m^2 in adults (Washington et al., 1989). It is also a complex living organ which enables the body to interact intimately with its environment. Therefore, the functions of the skin can be classified as protective, maintenance of homeostasis and sensory. The most important role of the skin is to act as a barrier to protect the internal body structure from the relatively hostile external environment, which has a variable temperature and water content, and the presence of environmental dangers, for instance, chemicals, bacteria, allergens, fungi, and radiation. The skin is also a major organ for temperature control and physical protection against mechanical stress and external pressure. Moreover, with the sensory nerves, the skin also acts as an important organ in terms of sensing environmental influences, such as heat, pain, allergens and touch.

In general, the skin consists of three distinct layers: the epidermis, the dermis and the subcutaneous tissue. There are also several appendages: hair follicles, sweat ducts, apocrine glands, and nails. Figure 1.1 shows a schematic representation of human skin.

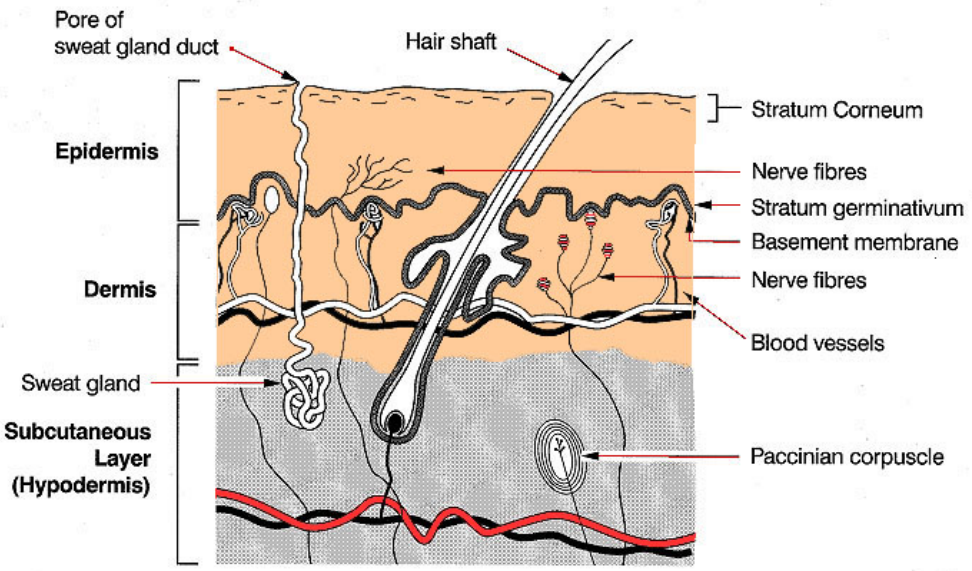

Figure 1.1 A schematic representation of human skin adapted from Simpson and Okubadejo (2001)

1.1.1 The epidermis

The epidermis is a stratified avascular tissue. Its thickness is between 0.8 mm on the palms and the soles, and 0.06 mm on the eyelids. The main cell type in this complex tissue is the keratinocyte which undergoes a maturation process along with the migration of the cell from the proliferative to the outermost layer of epidermis (Barry, 1983). Two distinct layers can be distinguished in the epidermis: the nonviable epidermis, stratum corneum (SC) and the viable epidermis (VE).

The VE is a multilayer tissue, which typically consists of three separate layers on the basis of differing stages of keratinocyte maturation. From the lower layer upwards to the skin surface, the three layers are the stratum germinativum (also known as the basal layer), the stratum spinosum (also

known as the prickle cell layer), and the stratum granulosum (also known as the granular layer).

The stratum germinativum is the innermost and the only active mitotic layer of the epidermis. Immediately below the stratum germinativum is the acellular basement membrane, which provides dermal-epidermal adherence, mechanical support and which controls the passage of cells and proteins (Walters, 2002). Mitosis of the basal cells produces a progressive outward migration of keratinocytes towards the skin surface. This procedure is significant for epidermal turnover and it is balanced by the loss of horny cells from the SC in order to maintain an undifferentiated thickness in healthy skin. The accumulated melanin is held in organelles called melanosomes which are subsequently transferred to adjacent keratinocytes providing pigmentation and protection from UV rays to the skin.

The stratum spinosum is the layer of the epidermis which is next to the stratum germinativum and where the keratinocytes start to produce keratin, to flatten and their nuclei shrink (Walters, 2002). It is also referred to as the prickle cell layer because its keratinocytes are connected by fine prickles.

In the stratum granulosum, the keratinocytes continue their migration to the epidermal surface and there is production and subsequent aggregation of keratin molecules. In addition, the cytoplasm of the cells appears granular at this level because of the accumulation of keratohyalin granules. Finally, at the transition between the stratum granulosum and the SC, the cells secrete

lamellar bodies containing lipids and proteins into the extracellular space and they lose their nuclei and all the other cellular organelles (Alam, 2004).

The final outcome of keratinocyte differentiation is found in the SC. The SC is the outermost layer of the skin and it is the principal barrier to percutaneous absorption of exogenous substances. It has been confirmed that removing the SC by tape stripping increased the permeability of water and other substances by about 1000 times more than through intact skin (Scheuplein, 2011). Several factors contribute to the ability of the SC to control the loss of water and the penetration of exogenous compounds. For instance, it has a high density (approximately 1.4 g/cm^3 dry weight), low hydration (15 to 20% w/w) and a low surface area for diffusion (Walters, 2002). Constant renewal of the cells in the SC by desquamation or regeneration also contributes to maintaining a barrier with a very effective protective function (Egelrud, 2000).

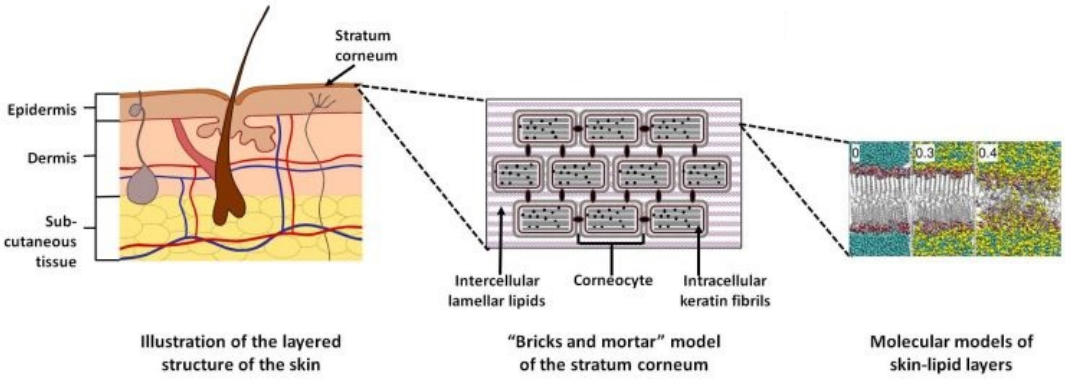

Figure 1.2 A schematic representation of the SC and the potential transport pathways through the SC adapted from Notman (2012)

Figure 1.2 presents a schematic representation of the SC. As can be seen, the SC is a thin (~10-20 μm) heterogeneous layer and is composed of hexagonal, stacked and terminally differentiated keratinocytes, separated by a complex mixture of intercellular lipids (Menon et al., 2012). The structure of the SC has been compared to a brick wall: the keratinised cells are the “bricks” and the intercellular lipids are the “mortar” (Michaels et al., 1975). Each cell is about 34 to 46 μm long, 25 to 36 μm wide and 0.5 to 1 μm thick (Walters, 2002). The dimensions are variable depending on age, anatomical location and external factors such as UV radiation (Walters, 2002).

1.1.2 The dermis

Below the epidermis lies the dermis, which is a connective tissue and which mainly comprises a tough, supportive matrix made up of collagen (75%) and elastin fibres (4%) in an amorphous ground substance of mucopolysaccharides (20%) (Wilkes et al., 1973). The dermis is 20 to 30 times thicker than the overlying epidermis, acting as a highly vascularised tissue which is responsible for the regulation of blood pressure and temperature, the provision of oxygen and nutrients to the skin, the removal of waste products, the immune response and wound healing (Washington et al., 1989). Moreover, a topically applied drug has to reach the vascular network in the dermis to be systemically absorbed (Walters, 2002).

1.1.3 Subcutaneous tissue

The subcutaneous tissue, which is also known as the hypodermis, is the deepest layer of the skin and consists of a network of fat cells linked to the dermis by collagen and elastin fibres (Walters, 2002). The base of the hair follicles and the secretary portion of the sweat glands are also present in this layer (Walters, 2002). However, the subcutaneous tissue spreads all over the body, except the eyelids and male genitalia. The main functions of the tissue include insulation, absorption of mechanical shocks and storage of readily available high-energy molecules in the adipose fat (Burton and Cunliffe, 1992).

1.2 Routes for percutaneous absorption

There are three different purposes for the administration of active substances to the skin: epidermal, topical and transdermal absorption (Trommer and Neubert, 2006). For example, cosmetics, insect repellents and disinfectants aim to maintain the active compound on the surface of the skin. Topical formulations allow the active to penetrate into the deeper layers of the skin, without systemic absorption. Furthermore, transdermal formulations are designed to deliver the active into the systemic circulation.

The routes for percutaneous absorption can be intercellular, transcellular and appendageal (through either eccrine-sweat glands or hair follicles) routes (Figure 1.2). Because the skin appendages only occupy less than 0.1% of the

total human skin surface area, the appendageal route is not considered as a significant route (Illel et al., 1991; Moser et al., 2001).

Using the transcellular route, the drug molecules partition repeatedly between both the lipophilic (lipid matrix) and hydrophilic regions (corneocytes) of this pathway. Although it has been suggested that drugs can traverse the SC by this route, this route has a rate-limiting barrier, which is the multiple bi-layered lipids between the keratinocytes that the drug solutes have to go through(Walters, 2002; Nemanic and Elias, 1980). Albery and Hadgraft (Albery and Hadgraft, 1979) carried out *in vivo* studies on the skin absorption of methyl nicotinate which indicated that the diffusional pathlength was 20 times greater than the SC thickness.

The highly tortuous intercellular route results in a much longer pathway for the active to travel comparing the thickness of the SC. In this route, the active needs to cross the lipid domains between the corneocytes. The idea that the intercellular route is the principle pathway for most drugs through the SC was also confirmed by Potts and Francoeur (1991). They reported a diffusion pathlength for water which was 50 times longer than the thickness of the SC.

1.3 Processes of percutaneous absorption

Drug absorption from a topical drug delivery system into the systemic circulation can be compared to passage through consecutive skin layers (or barriers). It involves the following steps (Figure 1.3):

1. Release from the formulation, for example a gel;

2. Penetration into the SC and permeation/diffusion through it;

3. Diffusion from the SC to the VE before reaching the capillaries located in the dermis.

Once the drug reaches the deepest layer of the VE and dermis, the drug will be rapidly distributed in the systemic circulation through the blood vessels in these regions (Hadgraft, 1991; Walker and Smith; 1996, Scheuplein, 2011).

Drug penetration into the SC is the major limiting factor as for most of the formulations the drug diffuses quickly in the vehicle. In this process, the drug partitioning into the membrane is highly influenced by the drug solubility in the formulation and in the SC (Walker and Smith, 1996). After penetrating into the SC, the drug diffuses at a rate determined by the diffusivity within the SC (Scheuplein, 2011). In the deepest layers of the SC, the drug undergoes a second partitioning step at the SC/VE interface (Hadgraft, 1991). Consequently, for highly hydrophobic drugs the VE constitutes the major

barrier for drug absorption, as the drug has to partition into the more hydrophilic VE (Cross et al., 2003).

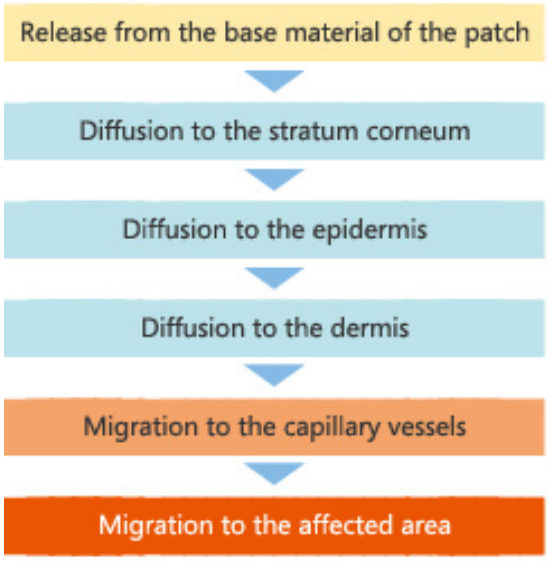

Figure 1.3 Drug permeation through the skin

1.4 Factors affecting active permeation

1.4.1 Physiological factors affecting the skin barrier

1.4.1.1 Age

Many studies have investigated the relationships between the skin age and the skin barrier (Machado et al., 2010a; Gebauer et al., 2012; Luebberding et al., 2013; Boireau-Adamezyk et al., 2014). It has been well-known that skin conditions and skin structure change with age. In new-borns and neonates, the SC is not fully developed (Kalia et al., 1998; Giusti et al., 2001). This has been used as an advantage in the administration of transdermal theophylline

and caffeine (Barrett and Rutter, 1994). From soon after birth until early “old age”, the change in the rate of trans-epidermal water loss across normal, intact skin does not vary greatly. However, the overall lipid and collagen content of human skin decreases with age (Leger et al., 1988; Rogers et al., 1996) in older people, the SC thickens and its hydration is less, which generally improves its barrier function (Boireau-Adamezyk et al., 2014).

1.4.1.2 Gender

Studies have also been conducted to investigate any correlation between gender and the skin barrier function (Hadgraft and Valenta, 2000; Machado et al., 2010a; Darlenski and Fluhr, 2012). Plewig investigated the variation of corneocyte size for four males and three females, all aged 21–31 years (Plewig, 1970). According to this study, corneocyte sizes were larger for women than men for all anatomic sites. Corneocyte surface areas varied from 746 to 1222 μm^2 for males, and from 896 to 1346 μm^2 for females. This contrasts with the research by Rougier et al. (1988) who reported no gender differences in corneocyte surface area for the forehead and for the upper-outer arm for a slightly larger study group (eight males and seven females). Other researchers have also reported that the difference between genders was not significant (Jang et al., 1996; Sandby-Moller et al., 2003; Jacobi et al., 2005).

1.4.1.3 Anatomic site

The skin structures vary to some extent over the human body. For example, the SC is thicker on the palms of the hands and soles of the feet than on the lips or eyelids (Williams, 2003b). However, varying permeability of different anatomic sites does not simply reflect variation in SC thickness. It has been noted that variation in drug permeation could be seen for body sites with similar SC thickness, and some sites with varied thickness of SC showed similar levels of drug permeation (Wester and Maibach, 1989).

Jang et al. measured the basal TEWL from 24 healthy volunteers in order to determine variation in skin barrier function (1996). According to this study, the permeability of the skin barrier could be ranked as follows:

Palm > sole > back = calf = chin, head and neck > forearm

The findings by Machado et al. indicate that the anatomic sites with higher TEWL values were where the corneocyte sizes were smaller (Machado et al., 2010a). According to Machado et al. (2010a), the smallest corneocytes were found in the forehead compared to the forearms and abdomen. This finding is in line with data reported by Plewig and Marples (1970), and Rougier et al. (1988).

1.4.1.4 Race

Many studies that have been reported show no significant difference between African, Asian and European skin (Yosipovitch et al., 2003; Grimes et al. 2004; Machado et al., 2010b). For instance, the study by Machado et al. (2010b) showed that the volar forearm TEWL values of 90 volunteers, males and females, Caucasians and Asians, aged between 20 and 60 years old, were not significantly different. However, there are also many reports showing that there are differences in TEWL values between races (Berardesca et al., 1991; Kompaore et al., 1993). Kompaore et al. reported that the baseline TEWL values obtained from Black and Asian subjects were significantly higher compared with Caucasians. However, the baseline TEWL values between Black and Asian subjects were not significantly different.

1.4.2 Permeant physiochemical factors affecting absorption

1.4.2.1 Partition coefficient

In order to permeate through the stratum corneum, an active permeant has to partition into the membrane first. The partitioning into the membrane acts as the rate-limiting step in the permeation process and the partition coefficient (log P) is the determining factor. Because of the lipophilic region of the SC and the hydrophilic domain of the viable epidermis, permeants with intermediate lipophilicity, namely with a log P of 1 to 3, are ideal molecules for skin penetration (Williams, 2003a). Highly hydrophilic molecules (log P <1) are

unlikely to partition into the lipophilic stratum corneum. Highly lipophilic molecules (lop P >3) will have a greater affinity for the SC and then are unlikely to permeate into the viable epidermis.

1.4.2.2 Molecular size

Drug permeation through the skin is a passive mechanism. Therefore, the size and shape of the molecule is another main factor in determining the flux of a permeant through the skin. For simplicity, the molecular weight is considered as the most appropriate measurement of permeant bulk. As a general rule, a good candidate for topical and transdermal delivery is reported to lie within a relatively narrow range of molecular weight of 100 - 500 Da (Williams, 2003a).

1.4.2.3 Solubility and melting point

The permeation of a permeant is also influenced by its solubility. According to Martin (1993), the solubility of a solid in a vehicle can be expressed as the following equation:

$$-\ln X_2 = \frac{\Delta H_f}{RT}\left(\frac{T_0 - T}{T_0}\right) + \frac{V_2 \emptyset_1^2}{RT}(\delta_1 - \delta_2)^2 \qquad \text{Equation 1.1}$$

Where X_2: molar fraction solubility; Φ1: volume fraction of solvent; V2: molar volume of solute; R: gas law constant; T: temperature in degrees Kelvin; T_0: melting point of the solid; H_f: molar heat of fusion; δ_1: solubility parameter of the solvent; δ_2: solubility parameter of drug.

For a particular temperature and a particular vehicle, the solubility of a certain chemical depends on its melting point to a large extent. In other words, a drug with a high melting point will have low solubility.

1.4.2.4 Ionisation

Considering the nature of the SC barrier to topical and transdermal delivery, the free acid and free base form of a permeant are preferable to be used in skin permeation. This is because the ionised species of a permeant has a lower permeability coefficient compared to its unionised species. However, the ionised species usually has a high aqueous solubility, while the unionised species has a lower aqueous solubility. Although the unionised species may have a high permeability coefficient, due to the low aqueous solubility, it may not show more favourable skin permeation (Hadgraft and Valenta, 2000).

1.5 Models of skin for in vitro permeation studies

1.5.1 Human tissue

Human tissues from cadaver skin, biopsy material or cosmetic surgery are the ”gold standard” models for *in vitro* studies, as the data collected from such studies should better reflect *in vivo* performance (Netzlaff et al., 2005). However, there are a number of legal and ethical issues associated with obtaining and using human tissues. In addition, the high data variability between skin tissues obtained from different donors or different sites of the body increases the difficulties of the studies, and raises the cost of conducting such studies (Batheja et al., 2009).

1.5.2 Animal tissues

Animal skin is an alternative tissue to human skin, which has been widely used in permeability studies of formulations due to its availability and ease of handling and storage. The sources of animal skin include rat, hairless mouse, guinea pig, snake and others (Panchagnula et al., 1997). However, the differences between human skin and animal skin, especially in terms of morphological characteristics and the lipid composition of stratum corneum, are significant (Cilurzo et al., 2007). Therefore, the reliability of conclusions from animal data for human skin has been questioned (Netzlaff et al., 2005). In addition, the ethical problems in relation to using animal skin for testing

purposes have always been controversial. In 2009, EU regulations (76/768/EEC, Feb. 2003) finally began to prohibit the use of animals for gathering toxicological data for cosmetic agents. As a result, animal experiments should be ruled out whenever scientifically feasible. In other words, the application of *in vitro* systems becomes increasingly imperative.

1.5.3 Non-animal membranes

Although after many years of development, many *in vitro*, non-animal models have been introduced, there is no international consensus on specific *in vitro* methods that may be used as alternatives to animal studies. It has been claimed that the replacement of the current animal tests with suitable alternatives will not be achieved for many more years (Adler et al., 2011).

Synthetic membranes are structurally simpler than human skin, and therefore may be applied, where appropriate, as valuable and suitable tools to evaluate partitioning and diffusion phenomena. Artificial membranes are easily available. More importantly, they provide a reproducible alternative to study basic mechanisms governing membrane transport than compared with complex biological tissues for example human skin, because they overcome the variability associated with human skin and variations with age, gender, race and anatomical site (Dias et al., 2007, Ng et al., 2010, Oliveira et al., 2012).

Polydimethylsiloxane (PDMS - silicone) is a commonly used membrane used in *in vitro* diffusion studies (Flynn and Roseman, 1971; Cross et al., 2001; Ng et al., 2010; Oliveira et al., 2011; Oliveira et al., 2012). It is an isotropic, non-porous and relatively inert hydrophobic barrier which is characterised by strong polar Si-O-Si bonds (Colas and Curtis, 2005). Although it has been reported by Sloan et al. (2013) that there is a positive correlation between the maximum flux of permeants across silicone membranes and human skin, it is crucial to bear in mind that the results obtained with model membranes cannot be directly extrapolated to human skin.

A promising alternative may be provided by biotechnologically-manufactured human skin equivalents. Human skin equivalents have been developed and researched during recent years for a variety of applications, for instance skin replacements in burns or wounds (Supp and Boyce, 2005; Shakespeare, 2005), for skin biology research (MacNeil, 2007), for cutaneous irritation and toxicity testing (Ponec and Kempenaar, 1995; Welss et al., 2004) and as models for permeability testing of agents and formulations (Netzlaff et al., 2007; Dreher et al., 2002). These skin substitutes are cultured in a controlled environment in order to mimic the relevant properties of human skin as closely as possible (Batheja et al., 2009). Today, reconstructed skin models, including EpiSkin®, SkinEthic® and EpiDerm®, are commercially available and have a well-defined architecture and lipid composition but are more frequently being used for irritation and toxicity testing and to a lesser extent for evaluation of cutaneous absorption (Perkins et al., 1999).

Moreover, since the introduction of the Biopharmaceutics Classification System (BCS)(Amidon et al., 1995), the development of efficient and effective permeability measurement strategies to classify actives at the earliest stages of drug discovery has become necessary. The Parallel Artificial Membrane Permeability Assay (PAMPA), was developed for this purpose. Its design will be discussed in more detail in a later section.

1.6 Model drugs

A lipophilic drug, ibuprofen and a hydrophilic drug, caffeine were selected for the present work. These two actives were chosen based on their physicochemical properties and wide use in topical formulations.

1.6.1 Ibuprofen

Ibuprofen (Figure 1.4) is classified as a non-steroidal anti-inflammatory drug or NSAID. It works by acting on a group of compounds called prostaglandins and is used to reduce fever and treat pain or inflammation caused by many conditions such as headache, toothache, back pain, arthritis, menstrual cramps, or minor injury.

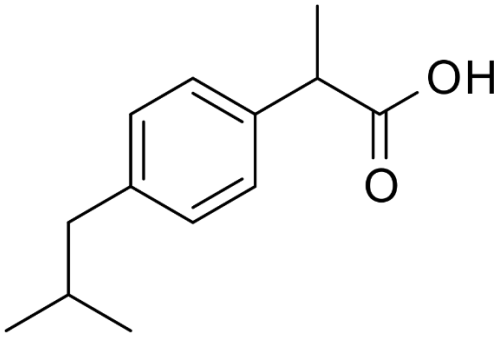

Figure 1.4 Chemical structure of ibuprofen ($C_{13}H_{18}O_2$).

Ibuprofen was synthesised more than 50 years ago and its research has been linked to an understanding of the concepts of the pathogenesis of inflammatory diseases and the actions of therapeutic agents (Rainsford, 2011). The principal initiator of this research leading to the discovery of ibuprofen was Dr Stewart Adams, a pharmacologist in The Boots Pure Drug Company Ltd at Nottingham, United Kingdom (Rainsford, 2015). The goal was to develop a more efficacious treatment for rheumatoid arthritis than aspirin, that was more tolerable and not a steroid. A patent was granted in 1961 (Nicholson and Adam), and subsequently ibuprofen became available commercially in the UK in tablet form in 1969 for the treatment of rheumatoid arthritis (Rainsford, 2015). It became an over the counter medicine in the UK in 1983 and in the same decade the first topical IBU preparation was launched.

Ibuprofen is a relatively lipophilic compound and the physicochemical properties of the molecule are listed in Table 1.1 (Patel et al., 2013).

Table 1.1 Physicochemical properties of ibuprofen

Molecular Mass	**206.30 g/mol**
Solubility (25°C)	21 mg/L
Solubility parameter	10.36 $(cal/cm^3)^{1/2}$
Hansen solubility parameter	17.5 (δ_d) 2.5 (δ_p) 6.5 (δ_h)
Melting range	75-77°C
Log P (octanol/water)	4.0
pK_a	4.4, 5.2

There have been a number of reports which describe the permeability characteristics of ibuprofen with reference to human skin (Akhter and Barry, 1985; Hadgraft and Valenta, 2000; Bock et al., 2004; Watkinson et al., 2009b; Watkinson et al., 2009a). The therapeutic effectiveness of any topically administered drug will be a function of both its penetration through the skin and its potency. The relative topical effectiveness of ibuprofen compared with other NSAIDs has been examined by Hadgraft et al (2000). As ibuprofen is a carboxylic acid the pK_a of the molecule will be an important determinant in its ionisation and hence permeation. Hadgraft and Valenta (2000) have investigated the pH partition behaviour of ibuprofen with reference to the skin. According to their studies, the maximum flux through the skin may occur at a pH where ibuprofen ionisation is high thus optimum topical formulations may not necessarily be for the free acid. The maximum flux is the product of the permeability coefficient and the solubility.

Ibuprofen has been formulated as many different topical preparations to reduce the adverse side effects of the molecule and to avoid hepatic first-pass metabolism. However, it is still challenging to deliver effective amounts of ibuprofen by topical delivery because of its poor skin permeation ability (Yano et al., 1986). In order to enhance the permeation of ibuprofen, nanoemulsions, microemulsions, eutectic systems, niosomes encapsulating ibuprofen–cyclodextrin complexes and ibuprofen-loaded nanostructured lipid carrier-based gels have been explored (Yuan and Capomacchia, 2013; Sharif M et al., 2012; Marianecci et al., 2013; Hu et al., 2014; Suto et al., 2016)

1.6.2 Caffeine

Caffeine (1, 3, 7-trimethylpurine-2, 6- dione) is a methyl xanthine alkaloid which is consumed as a beverage, administered as a medicine or applied for cosmetic purposes (Figure 1.5). Caffeine is also employed as a model hydrophilic compound in skin toxicology; dermal absorption of such marker compounds is used for risk assessment of exposure to hazardous substances in man (OECD, 2004) .

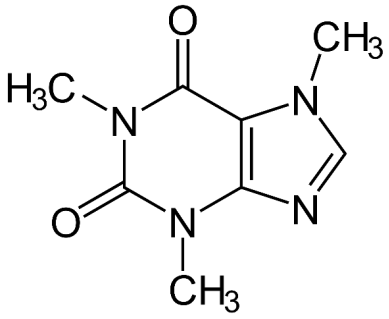

Figure 1.5. Chemical structure of caffeine (C8H10N4O2).

Caffeine is recommended as a test substance by the OECD because it has been studied extensively *in vitro* and *in vivo*. Although coffee and other caffeine containing drinks, such as tea, have been consumed at least since the fifteenth century the molecule itself was not isolated until 1820 (Anft, 1955). The major route for actives to permeate through the skin is via the lipid content of the outermost layer (Menon et al., 2012), the SC. However, caffeine does not possess the properties of an ideal skin penetrant as it is a hydrophilic material with a Log P of -0.07 (Table 1.2). Caffeine also exhibits unusual solubility behaviour in non-aqueous solvents (Bustamante et al., 2002) and forms aggregates in aqueous solutions (Guttman and Higuchi, 1957; Cesaro et al., 1976).

Table 1.2 Physicochemical properties of caffeine.

Molecular weight*	**194.2**
Log P*	-0.07
Solubility*	1 in 46 of water, 1 in 1.5 of boiling water
Solubility parameter**	31 MPa1/2
Melting point*	238 ^{o}C
pK_a*	14 (25 ^{o}C), 10.4 (40 ^{o}C)

*Moffat et al. (2004) and Bustamante et al. (2002)

Caffeine inhibits the phosphodiesterase enzyme and has an antagonistic effect on central adenosine receptors. It is a stimulant of the central nervous system and may produce wakefulness and heightened mental activity. The molecule also increases rate and depth of respiration but it is a weaker bronchodilator than theophylline (Parfitt, 1999). Caffeine has been investigated for its neuroprotective effects in dementia (Ritchie et al., 2007) as well as its potential as an aid to recognise hypoglycaemic symptoms in diabetic patients (Debrah et al., 1996; Watson et al., 2000). The major advantages claimed for use of caffeine in topical cosmetic products are that it (i) prevents excessive fat accumulation in skin, (ii) promotes lymphatic drainage and (iii) protects skin from photodamage. Scientific evidence for many of these proposed benefits is lacking and most studies in the literature are based on cell culture or mouse models (Herman and Herman, 2013). The efficacy of caffeine in the management of gynoid lipodystrophy (cellulite) via proposed adipocyte lipolysis and increased cyclic adenosine monophosphate (cAMP) is controversial. The applications of caffeine in the management of this condition

have been reviewed by Herman and Herman (2013). The authors describe in detail potential roles for caffeine in lipolysis including effects on catecholamine secretion, cAMP levels, lipase activity, PDE inhibition and lymphatic drainage. Although caffeine has been shown *in vitro* to promote follicular proliferation evidence for this effect *in vivo* has not been reported (Fischer et al., 2007).

When administered orally, the bioavailability of caffeine is 100% with values for plasma half-life, volume of distribution and clearance reported, respectively, as 2–10 h, 0.5 L/kg and 1–2 mL/min/kg (Moffat et al., 2004). The extent to which the molecule binds to plasma proteins is about 35% (Blanchard, 1982). For treatment of neonatal apnoea of prematurity the recommended dose is 5–20 mg/kg caffeine (as the citrate) administered orally or intravenously. The recommended oral dose of caffeine for mild stimulant purposes is 50–200 mg daily (Parfitt, 1999). Following oral administration, caffeine may be subject to metabolism by N-demethylation, acetylation and oxidation; approximately 1% of the molecule is excreted unchanged. There is no evidence, to date, that caffeine undergoes appreciable metabolism following application to skin. Poisonings and fatalities have followed ingestion of large oral doses of caffeine but no toxic or skin reactions have been reported following dermal exposure in healthy patients (Moffat et al., 2004).

1.7 Aims

The aims of the present study may be summarized as follows:

- To develop analytical method for the model drugs, ibuprofen and caffeine
- To perform both infinite and finite dose *in vitro* permeation studies and mass balance studies on porcine skin to investigate the permeation behaviour of the model drugs in the Franz cell model
- To perform finite dose *in vitro* permeation studies and mass balance studies on human skin to investigate the permeation behaviour of the model drugs in the Franz cell model
- To verify if the novel Skin PAMPA model may be used to classify and predict passive permeability of model drugs
- To conduct *in vivo* permeation studies with conventional trans-epidermal water loss (TEWL) and tape stripping techniques, and the novel confocal Raman spectroscopy (CRS) technique to investigate *in vivo* permeability of a model drug

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Chapter 2 Analytical Method Development and solubility studies

2.1 Introduction

Because the quality of analytical data plays an important role in the success of a drug development program, the process of method development and validation has a critical impact on the quality of these data. Although conducting a thorough method validation can be tedious, the consequences of performing this incorrectly are wasted time, money as well as resources (Green, 1996).

Solubility is also a critical physicochemical property of a chemical, especially in judging whether a chemical is a suitable candidate in a topical or transdermal delivery system, and in selecting suitable vehicles for a chemical to develop or improve its topical or transdermal delivery.

Therefore, the aims of the work reported in this chapter are as follows:

- To develop analytical methods for the model drugs ibuprofen and caffeine;
- To determine the solubility of the model drugs in a range of solvents in order to select suitable vehicles for *in vitro* and *in vivo* permeation studies.

2.2 Materials and methods

2.2.1. Materials

2.2.1.1 Chemicals and tissues Materials

Ibuprofen was a gift from Wyeth (Haversham, Hants., UK). Analytically pure standards of polyethylene glycol (PEG) 300 and propylene glycol (PG) were obtained from Sigma-Aldrich (UK).

Analytically pure standards of caffeine, 1,2-pentanediol (1,2-pent), 1,3-butylene glycol (1,3-BG)/1,2-butylene glycol (1,2-BG), propylene glycol (PG) and Isopropyl alcohol (IPA) were obtained from Sigma-Aldrich (UK). Geraniol (GER), limonene (LIM), t-butyl alcohol (T-BA), linalool (Lina) and tripropylene glycol (TPG) were purchased from Fisher Scientific (UK). Isopropyl myristate (IPM), and dimethyl isosorbide (DMI) were gifts from Croda Ltd. (UK). 1, 2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), propylene glycol monocaprylate (PGMC), propylene glycol monolaurate (PGML), and Transcutol® (TRANS) were received as gifts from Gattefossé (France). PEG-6-caprylic/capric glycerides (PEG-6-CCG) was a gift from Avon. These solvents are generally regarded as safe (GRAS) chemicals.

HPLC grade water, acetonitrile (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade) and absolute ethanol were purchased from

Fisher Scientific (UK). Phosphate buffered saline (pH 7.4 at 25°C) was prepared using Dulbecco A Tablets (Oxoid, UK).

2.2.1.2 Instruments

A water bath (SUB Aqua 26) was obtained from Grant (UK), an electronic balance (Sartorius 1702) was from Sartorius (Germany) and a sonicator (PDL 356) was obtained from Camlab Serving Science (UK). The HPLC system used consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. The software used to acquire and analyse the data was ChemStation® for LC 3D Rev. A. 09.03 (Agilent Technologies, U.S.A.). A water purification system with a 75 L reservoir (Elga Option 3) was obtained from Veolia Solutions and Technologies (UK). A rotator (SB2) was purchased from Stuart Equipment (U.S.A.). A centrifuge (5415R) was acquired from Eppendorf (U.S.A.).

2.2.2 Methods

2.2.2.1 Analytical method development

2.2.2.1.1 HPLC analysis of ibuprofen

2.2.2.1.1.1 Development of HPLC method for ibuprofen

The chromatographic conditions which were developed for the HPLC method for ibuprofen are listed in Table 2.1.

Table 2.1 HPLC conditions for analysis of ibuprofen

HPLC Conditions	Method
Column	C_{18} 250X4.6 mm
Mobile phase(v/v)	80% methanol
	20% Water with 0.01%TFA
Temperature (°C)	30.0
Flow rate (mL/min)	1.0
Run time (min)	10
Wavelength (nm)	222
Injection volume (µL)	15

2.2.2.1.1.2 Validation of HPLC method for ibuprofen

As mentioned by the Pharmacopeial Convention (1985), “validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application”.

In high-performance liquid chromatography (HPLC) method development, once the chromatographic and the experimental conditions are established, the method needs to be validated by the following parameters: linearity, precision, accuracy, robustness, Limit of detection (LOD), limit of quantification (LOQ) and system suitability.

2.2.2.1.1.2.1 Linearity

Linearity is the ability of an analytical method to obtain results which are either directly, or after mathematical transformation, proportional to the concentration of the analyte within a given range (Hearn, 1992).

Generally, linearity is demonstrated by preparation of standard solutions at five concentration levels, from 50 to 150% of the target analyte concentration (Green, 1996). The five levels are necessary for the detection of curvature in the plotted data and the standards should be prepared and analysed at least three times (Green, 1996). One of the main indicators used to judge the acceptability of linearity data is the correlation coefficient. A correlation coefficient of > 0.999 is generally considered as acceptable (Green, 1996).

2.2.2.1.1.2.2 Accuracy

“Accuracy is a measure of the closeness of test results obtained by a method to the true value” (Pharmacopeial Convention (1985). According to Green (1996), accuracy can be determined by four procedures, with the most widely used being the recovery study. The recovery study is usually performed by spiking the analyte in blank matrices and spiked samples are prepared in triplicate at three levels over a range of 50 to 15% of the target concentration. In addition, for the assay method, accuracy is usually reported as the percent

recovery of a given amount of analyte added to the sample. As noted by (Lam, 2004) the percent accuracy may be expressed by Eq. (2.1)

$$\%\text{Accuracy} = \frac{\text{Mean observed concentration}}{\text{Expected concentration}} \times 100 \qquad \text{Equation 2.1}$$

In this method, the accuracy was assessed from triplicate determinations of three solutions of different concentrations (1, 2 and 20 µg/ml). The results of recovery% and RSD% were subsequently calculated.

2.2.2.1.1.2.3 Precision

"The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample" (Pharmacopeial Convention, 1985). Precision is usually verified by applying the method to test a sample for a sufficient number of times to obtain valid results. To be more precise, the procedure of precision of a method includes determination of repeatability (intra-day) and intermediate precision (inter-day). Repeatability is assessed by carrying out twelve repeated analyses of the same working solution under the same experimental conditions on the same day, and intermediate precision (inter-day) is determined by performing the analysis on three different days by another analyst in the same laboratory (Franz, 1975). The precision is finally expressed as the relative standard deviation (RSD) as expressed in Eq. (2.2):

$$\%R.S.D = \frac{\text{standard deviation} \times 100\%}{\text{mean}} \qquad \text{Equation 2.2}$$

In this equation standard deviation may be expressed by Eq. (2.3)

$$S = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N-1}} \qquad \text{Equation 2.3}$$

Where (X1, X2,..., XN) are the observed values of the sample items and $\bar{x}$ is the mean value of these observations.

The precision of the proposed method was verified through repeatability and intermediate precision by triplicate determinations of three different solutions at low, medium and high concentrations, which were 2, 20 and 80 µg/ml. These experiments were carried out over a period of one week to determine both the intra and inter day variability.

2.2.2.1.1.2.4 Robustness

The robustness of an analytical method is defined as its capability to remain unaffected by small changes in normal test conditions, for instance, different analysts, laboratories, instruments, percent organic content and pH of the mobile phase, buffer concentration, and temperature and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment (Virlichie and Ayache, 1995).

The robustness of the proposed method was determined by analysing the same sample under a variety of experimental conditions; the method essentially involves a mobile phase composition of ACN (85%): water with 0.1% TFA (15%), HPLC column temperature at 40 °C, injection volume of 10 µl and UV detection wavelength of 360 nm.

2.2.2.1.1.2.5 Limit of detection (LOD) and limit of quantification (LOQ)

According to the International Conference on Harmonization (ICH) guidelines, there are three methods for determining the detection and quantification limits for an analytical method, namely visual determination, signal-to-noise determination, and standard deviation and slope determination.

In this validation protocol, the standard deviation and slope determination method was employed. In this method, the limit of detection (LOD) may be expressed as:

$$\mathrm{LOD} = \frac{3.3\ \sigma}{\mathrm{S}}$$

Where σ is the standard deviation of the response, and S is the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out based on the standard deviation of the blank or based on the calibration curve. In the latter method, a specific calibration curve should be studied using samples containing an analyte in the range of LOD. The residual standard deviation of a regression line or the standard deviation of the y-intercepts of regression lines may be utilized as the standard deviation.

Similar to the LOD, the limit of quantification (LOQ) may be expressed as

$$\mathrm{LOQ} = \frac{10\,\sigma}{\mathrm{S}}$$

Where σ is the standard deviation of the response, and S is the slope of the calibration curve.

2.2.2.1.1.2.6 System suitability

The ICH introduced the system suitability test, which is actually used after the method has been validated fully, and when the validated method is being routinely used to analyse real samples (Krull and Swartz, 1999). A system suitability sample is run any day that real samples are being analysed, always before and after the actual batch of samples, often within that run of batches, for the purpose of demonstration that the instrumental system is performing properly (Krull and Swartz, 1999).

For the proposed method, the system suitability was determined by performing six replicate injections from freshly prepared standard solutions at a concentration of 20 µg/ml and a comparison of the chromatogram parameters with a standard trace was performed to allow a comparison of the peak shape, peak width and baseline resolution. The standard trace involves peak area, retention time, symmetry, number of theoretical plates (N) and tailing factors (T).

2.2.2.1.2 HPLC analysis of caffeine

2.2.2.1.2.1 Development of HPLC method for caffeine

The chromatographic conditions for the HPLC method for caffeine are listed in Table 2.2.

Table 2.2 HPLC methods for caffeine

HPLC Conditions	**Method**
Column	C_{18}250X4.6 mm
Mobile phase(v/v)	30% methanol
	70% Water+0.01%TFA
Temperature (°C)	35.0
Flow rate (ml/min)	1.0
Run time(min)	10
Wavelength (nm)	270
Injection volume (µl)	10

2.2.2.1.2.2 Validation of HPLC method for caffeine

As mentioned above, validation is an essential step to make sure the analytical method used in research is reliable, accurate and repeatable six criteria were studied, including linearity, accuracy, precision, robustness, LOD and LOQ, and system suitability. The validation procedure is described in section 2.2.2.1.1.

2.2.2.1.3 Analytical quantification by GC analysis for PG

2.2.2.1.3.1 Development of GC method for PG

A Chromapack Varian model CP9001 (Chrompack, Netherlands) equipped with a flame ionisation detector (FID) was used for PG quantification. The signal was acquired and recorded using a computer connected to the instrument by an analogue-to-digital (A/D) interface (H&A Scientific, Inc., United States). Analyses were performed on a 30 m x 0.35 mm x 1.00 µm open column Zebron ZB-WAX (Phenomenex, United Kingdom). Nitrogen was used as a carrier gas at a flow rate of 6.6 ml/min. The column was operated accordingly to the following gradient method: the initial temperature was at 80^{o}C and then increased to 200^{o}C at a rate of 15^{o}C/min (a total of 8 mins). The temperature of the inlet was 225^{o}C and the temperature of the detector was 300^{o}C. Under these conditions, the retention time for PG was 5.3 min. The

method demonstrated linearity of at least 0.99. Standards were prepared with PG ranging from 0.031 to 1.0 μl/ml.

2.2.2.1.3.2 Validation of GC method for PG

The validation of the GC method was carried out by determining linearity, accuracy, precision, and LOD and LOQ. The validation procedure is described in section 2.2.2.1.1.

2.2.2.2 Solubility studies

2.2.2.2.1 Solubility studies for ibuprofen

For the selection of suitable vehicles and receptor phase for *in vitro* permeation studies, several solvents and solvent systems, which included PBS, PG, IPA and PEG 300, were evaluated.

The solubility studies were performed by adding an excess amount of ibuprofen to a plastic centrifuge tube (n=3) containing 1 ml of the given solvent or solvent systems. The tubes were sealed with Parafilm® and then placed on a rotator at 32 ± 1˚C for 48 hours in order to produce a saturated solution with a visible excess of the drug. A suitable number of pipette tips were also placed into an oven at 32 ± 1˚C to avoid variations of the experimental temperature when they were used. After 48 h, the samples were centrifuged (13,200 rpm,

15 min, 32 ± 1˚C) and a known amount of supernatant was suitably diluted in volumetric flasks in order to achieve a concentration within the range of the HPLC calibration curve. The concentrations of the compounds were then determined by HPLC.

2.2.2.2.2 Solubility studies for caffeine

In order to select suitable vehicles and receptor phases for *in vitro* permeation studies and to develop the optimum formulations, the selected solvents and solvent systems included dimethyl isosorbide (DMI), propylene glycol (PG), Transcutol® (TRANS), propylene glycol monocaprylate (PGMC), 1,2-pentanediol (1,2-pent), geraniol (GER), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), isopropyl myristate (IPM), limonene (LIM), 1,3-butylene glycol (1,3-BG), 1,2-butylene glycol (1,2-BG), t-butyl alcohol (T-BA), linalool (Lina), tripropylene glycol (TPG), PEG-6-caprylic/capric glycerides (PEG-6-CCG), PGMC : TRANS 50:50 (v/v), PEG-6-CCG : TRANS 50:50 (v/v), PEG-6-CCG : PG 50:50 (v/v), DMI : PGMC 50:50 (v/v), DMI : PEG-6-CGG 50:50 (v/v) and 1,2-BG : PGMC (50:50).

Solubility studies of caffeine in each solvent and solvent system were conducted. The method for solubility studies is described in Section 2.2.2.2.2.

2.3 Results and discussion

2.3.1 Validation of HPLC method for ibuprofen

2.3.1.1 Linearity

The peaks areas of ibuprofen at different concentrations are shown in Table 2.3 and Figure 2.1. As shown in Figure 2.1, the representative linear equation was $y=36.74x + 10.39$ ($n=3$, $R^2=0.99$). In this equation, 36.74 represents the slope and 10.39 is the intercept. In principle, if the coefficient of determination (R^2) is ≥ 0.997, it means the method has good linearity (Lam, 2004). Therefore, the value of the determination coefficient of 0.99 confirmed excellent linearity for the calibration curve for the selected method.

Table 2.3 Peak areas of ibuprofen at different concentrations

Concentration(μg/mL)	**Peak area**
0.5	26.1
1	44.6
5	203.7
10	372.2
20	746.7
40	1479.3

As shown in Figure 2.1, the representative linear equation was y=36.74x + 10.39 (n=3, R^2=0.99). In this equation, 36.74 represents the slope and 10.39 is the intercept. In principle, if the coefficient of determination (R^2) is ≥0.997, it means the method has good linearity (Lam, 2004). Therefore, the value of the determination coefficient of 0.99 showed excellent linearity for the calibration curve for the selected method.

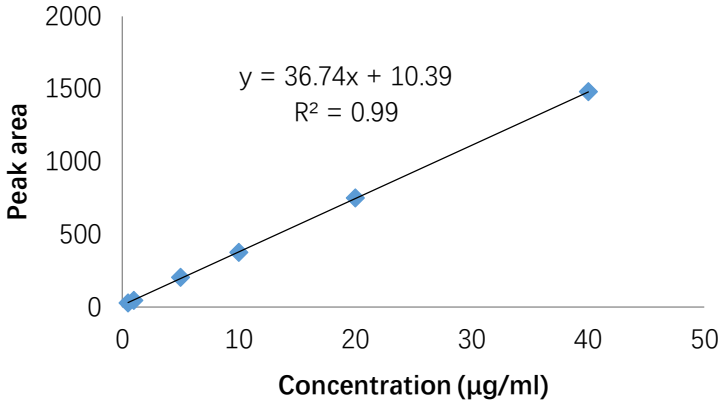

Figure 2.1 Calibration curve of ibuprofen

2.3.1.2 Accuracy

Table 2.4 shows that the recovery values at the three different concentrations were 92.95, 100.26 and 100.01% with R.S.D values of 0.33, 0.12 and 0.36 % respectively. The mean value for recovery was 97.74 % and that of R.S.D was 0.27%. The range for accuracy limit should be within the linear range. According to Lam et al (2004), the expected accuracy of the recovery of drug substances in a mixture is in the range from 95% to 105%. The result shows that the recovery values for the three selected

concentrations, except at 1 μg/ml, and the mean values were in the range between 95% and 105%.

Table 2.4 Accuracy results for ibuprofen

Theoretical concentration (μg/ml)	**Measured concentration (μg/ml)***	**Recovery (%)**	**R.S.D (%)**
1	0.93 ± 0.01	92.95	0.33
20	20.05 ± 0.02	100.26	0.12
40	40.13 ± 0.15	100.23	0.13
		mean 97.81	mean 0.19

*mean ± SD (n=3)

2.3.1.3 Precision

In the assay systems, the variation of all quantitative results should not be beyond the range of ± 2% (Shabir, 2004). As can be seen in Table 2.5, the %R.S.D values of the measurements ranged from 0.10 to 1.09% for intra-day variability studies while the results obtained from intermediate precision studies were 1.19, 0.17 and 0.11% respectively (Table 2.6). Noticeably, the %R.S.D values decreased while the concentration of the solutions increased.

Table 2.5 Summary of intra-day (repeatability) variability data for ibuprofen

Theoretical concentration (µg/mL)	Intra-day measured concentration(µg/mL)*					
	day 1		day 2		day 3	
	Mean ±SD	% R.S.D	Mean±SD	% R.S.D	Mean±SD	% R.S.D
1	0.92 ± 0.01	1.09	2.42 ± 0.04	1.09	0.93 ± 0.01	1.07
20	20.05 ± 0.05	0.25	20.07 ± 0.15	0.15	20.06 ± 0.02	0.10
40	40.00 ± 0.04	0.10	39.99 ± 0.04	0.11	40.19 ± 0.05	0.12

*mean ± SD (n=3)

Table 2.6 Summary of inter-day (intermediate precision) variability data for ibuprofen

Theoretical concentration (µg/mL)	Inter-day measured concentrations(µg/mL)*	
	Mean±SD	%R.S.D
1	2.23 ± 0.01	1.09
20	20.07 ± 0.03	0.17
40	40.06 ± 0.04	0.11

*mean ± SD (n=3); Inter-day precision was determined from three different runs over a 1-week period.

2.3.1.4 Robustness

Table 2.7 presents the results and chromatographic conditions selected as variables in each experiment. There were no obvious differences in the chromatograms when specific modifications were made in the given experiments. The correlation coefficients (R^2) of the calibration curves were 0.99, 0.99, 0.99 and 0.99 respectively and the recovery ranged between 97.36 and 101.79%. All the data mentioned above illustrate that the selected method is robust for ibuprofen.

Table 2.7 The robustness test for HPLC conditions and obtained responses

Method	Wavelength (nm)	Injection volume (µL)	Temperature (°C)	Mobile phase water : ACN (v/v)	R^2	Recovery (%)
1	222	10	30	20:80	0.99	98.22
2	222	15	35	20:80	0.99	97.36
3	225	15	30	20:80	0.99	101.79
4	222	15	30	25:75	0.99	99.43

2.3.1.5 Limit of detection (LOD) and limit of quantification (LOQ)

Based on the standard deviation and slope determination, the LOD and LOQ of this HPLC method were determined to be 0.53 and 1.61 µg/mL separately.

2.3.1.6 Suitability

The results of the system suitability study are listed in Table 2.8. The %R.S.D for peak area and retention time was 0.09 and 0.13 respectively. All these values were within the required limits. In other words, the proposed method met the requirements to produce good resolution between the peaks of interest with high reproducibility.

Table 2.8 System suitability results for ibuprofen

	Mean ± SD*	%R.S.D
peak area	748.02 ± 1.34	0.18
retention time(min)	7.19 ± 0.03	0.42
symmetry	0.84 ± 0.01	1.20

*n=6

2.3.2 Validation of HPLC method for caffeine

2.3.2.1 Linearity

The peak areas of caffeine at different concentrations are illustrated in Table 2.9 and Figure 2.2.

Table 2.9 Peak areas of caffeine at different concentrations

Concentration(μg/mL)	Peak area
0.5	12.2
1	24.7
5	124.9
10	251.7
20	510.3
50	1278.1

The representative linear equation was y=25.54x-1.67 (n=3, R^2=0.99). In this equation, 25.54 represented the slope and -1.67 was the intercept. In principle, if the coefficient of determination (R^2) is $\geq$0.99, it means the method has good linearity (Lam, 2004). Therefore, the value of the determination coefficient of 0.99 showed excellent linearity for the calibration curve for the selected method.

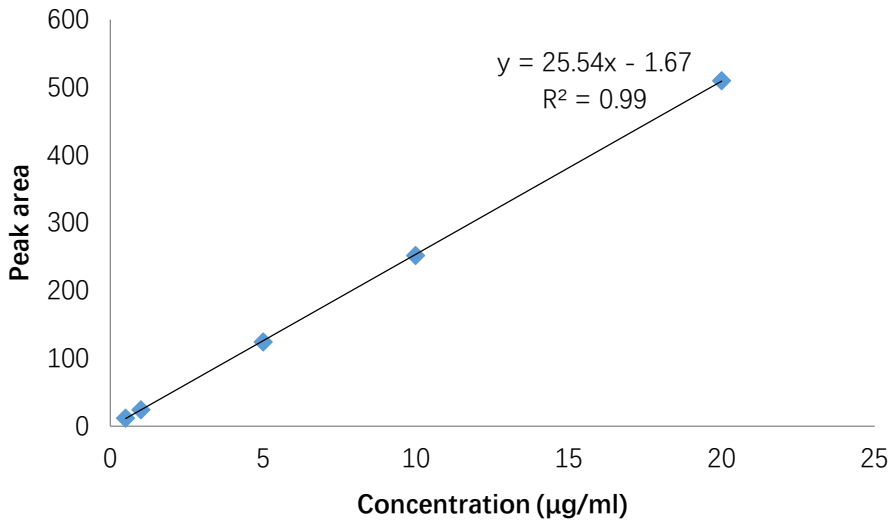

Figure 2.2 Calibration curve for caffeine

2.3.2.2 Accuracy

Table 2.10 shows the recovery values at the three different concentrations, were 107.52, 104.40 and 99.15% with R.S.D of 1.83, 1.00 and 0.06 % respectively. The mean value of recovery was 103.69 % and that of R.S.D was 0.96%. All the recovery values for the three selected concentrations and the mean values were in the range between 95% and 110%.

Table 2.10 Accuracy results for caffeine

Theoretical concentration (µg/ml)	**Measured concentration (µg/mL)***	**Recovery (%)**	**S.D (%)**
0.5	0.54 ± 0.01	107.52	1.83
1	10.4 ± 0.01	104.40	1.00
10	9.91 ± 0.03	99.15	0.06
		mean 103.69	mean 0.96

*mean ± SD (n=3)

2.3.2.3 Precision

As shown in Table 2.11, the % R.S.D values of the measurements ranged from 0.08 to 0.96% for intra-day variability studies while the results obtained from intermediate precision studies (Table 2.12) were 0.96, 0.20 and 0.12% respectively. In other words, the repeatability of precision obtained was less than 1% and hence meets the evaluation criterion that the RSD should be smaller than 2%. In addition, it is noticeable that the %R.S.D values decreased while the concentration of the solutions increased.

Table 2.11 Summary of intra-day (repeatability) variability data for caffeine

Theoretical concentration (µg/mL)	**Intra-day measured concentration(µg/mL)***					
	day 1		day 2		day 3	
	Mean ± SD	% R.S.D	Mean ± SD	% R.S.D	Mean ± SD	% R.S.D
1	1.04 ± 0.01	0.96	1.04 ± 0..1	0.96	1.05 ± 0.01	0.95
10	9.90 ± 0.02	0.20	9.94 ± 0.02	0.20	20.10 ± 0.04	0.30
50	50.13 ± 0.07	0.14	50.15 ± 0.06	0.11	50.12 ± 0.04	0.08

*mean ± SD (n=3)

Table 2.12 Summary of inter-day (intermediate precision) variability data for caffeine

Theoretical concentration (µg/mL)	**Inter-day measured concentrations(µg/ml)***	
	Mean ± SD	%R.S.D
1	1.04 ± 0.01	0.96
10	9.93 ± 0.02	0.20
50	50.13 ± 0.06	0.12

*mean ± SD (n=3); Inter-day precision was determined from three different runs over a 1-week period.

2.3.2.4 Robustness

Table 2.13 presents the results and chromatographic conditions selected as variables in each experiment. There were no obvious differences in the chromatograms when specific modifications were made in the given experiments. The correlation coefficients (R^2) of the calibration curves were 0.99, 0.99, 0.99 and 0.99 respectively and the recovery range was between 96.91 and 107.79%. The results demonstrated that the selected method is robust for caffeine.

Table 2.13 Robustness of HPLC conditions and obtained responses for caffeine

Method	Wavelength (nm)	Injection volume (µL)	Temperature (°C)	Mobile phase water : MeOH (v/v)	R^2	Recovery (%)
1	270	15	35	70:30	0.99	97.44
2	270	10	40	70:30	0.99	98.46
3	275	10	35	70:30	0.99	107.79
4	270	10	35	75:25	0.99	96.91

2.3.2.5 Limit of detection (LOD) and limit of quantification (LOQ)

Based on the standard deviation and slope determination, the LOD and LOQ of this HPLC method were determined to be 0.20 and 0.61 µg/mL respectively.

2.3.2.6 Suitability

Table 2.14 presents the results for the system suitability study for caffeine. The %R.S.D for peak area and retention time was 0.20 and 0.36 respectively. All these values met the required limits. In other words, the proposed method met the requirements to produce good resolution between the peaks of interest with high reproducibility.

Table 2.14 System suitability results for caffeine

	Mean ± SD*	**%R.S.D**
peak area	251.87 ± 0.51	0.20
retention time(min)	8.28 ± 0.03	0.36
symmetry	0.83 ± 0.01	1.20

*n=6

2.3.3 Validation of GC method for PG

2.3.3.1 Linearity

The peak areas of PG at different concentrations are illustrated in Table 2.15 and Figure 2.3.

Table 2.15 Peak areas of PG at different concentrations

Concentration(µL/mL)	Peak area
0.031	64.3
0.063	129.4
0.13	277.5
0.25	601.4

The representative linear equation was y=2473.60x – 21.76 (n=3, R^2=0.99). In this equation, 2473.60 represented the slope and – 21.76 was the intercept. The value of the determination coefficient of 0.99 showed good linearity for the calibration curve for the selected method.

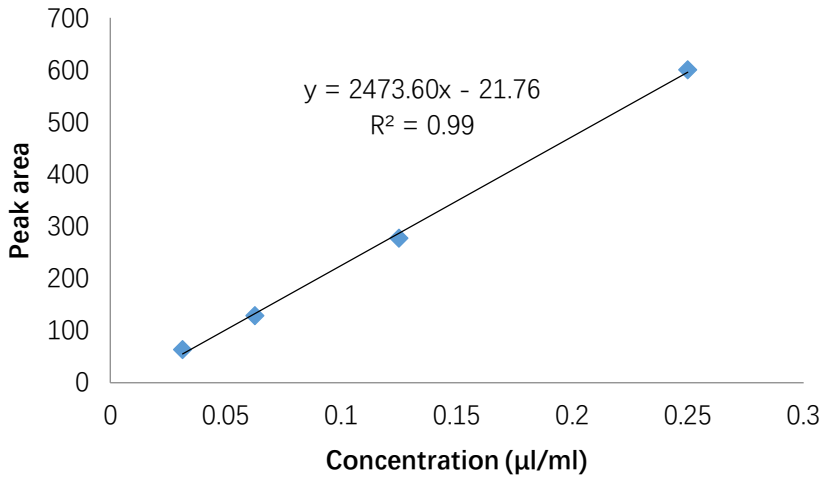

Figure 2.3 Calibration curve of PG

2.3.3.2 Accuracy

Table 2.16 shows the recovery values at the three different concentrations, were 109.80, 97.43 and 103.46% with R.S.D of 0.29, 0.85 and 2.45 % respectively. The mean value for recovery was 103.56 % and that of

R.S.D was 1.20 %. The results show that the recovery values for the three selected concentrations, except at 1 µg/ml, and the mean value, were in the range between 90% and 110%.

Table 2.16 Accuracy results for PG

Theoretical concentration (µL/mL)	**Measured concentration (µL/mL)***	**Recovery (%)**	**S.D (%)**
0.031	0.034 ± 0.03	109.80	0.29
0.063	0.062 ± 0.00	97.43	0.85
0.25	0.26 ± 0.01	103.46	2.45
		mean 103.56	mean 1.20

*mean ± SD (n=3)

2.3.3.3 Precision

As shown in Table 2.17, the % R.S.D values of the measurements ranged from 1.11 to 4.43% for intra-day variability studies while the results obtained from intermediate precision studies (Table 3.14) were 3.05, 3.50 and 2.94% respectively. Noticeably, the %R.S.D values decreased while the concentration of the solutions increased.

Table 2.17 Summary of intra-day (repeatability) variability data for PG

Theoretical concentration (µL/mL)	Intra-day measured concentration(µL/mL)*					
	day 1		day 2		day 3	
	Mean ± SD	% R.S.D	Mean ± SD	% R.S.D	Mean ± SD	% R.S.D
0.031	0.034 ± 0.00	3.82	0.034 ± 0.00	3.53	0.034 ± 0.01	1.79
0.063	0.061 ± 0.00	4.43	0.059 ± 0.00	3.62	0.061 ± 0.00	2.46
0.25	0.26 ± 0.01	3.85	0.26 ± 0.01	3.85	0.26 ± 0.00	1.11

*mean ± SD (n=3)

Table 2.18 Summary of inter-day (intermediate precision) variability data for PG

Theoretical concentration (µL/mL)	Inter-day measured concentrations(µg/mL) *	
	Mean ± SD	%R.S.D
0.031	0.034 ± 0.00	3.05
0.063	0.060 ± 0.00	3.50
0.25	0.26 ± 0.01	2.94

*mean ± SD (n=3); Inter-day precision was determined from three different runs over a 1-week period.

2.3.3.4 Limit of detection (LOD) and limit of quantification (LOQ)

Based on the standard deviation and slope determination strategy, the LOD and LOQ of this GC method were determined to be 0.01 and 0.03 µL/mL separately.

2.3.4 Solubility studies for ibuprofen

The solubility of ibuprofen in each solvent or solvent system at 32 ± 1°C is summarized in Table 2.19. The results also confirmed that PG and PBS could be respectively a vehicle and receptor phase for ibuprofen.

Table 2.19 Solubility of ibuprofen, n=3, mean ± SD

Solvent	Solubility (mg/mL)
PG	295.97±1.67
PEG 300	324.42±2.27
IPA	953.99±15.61
PBS	1.22±0.13

2.3.5 Solubility studies for caffeine

Because solubility of an active in a solvent/solvent system plays a significant role in permeation efficiency of the active from a formulation through membranes, the solubility of caffeine in each selected solvent as well as PBS was investigated at 32 ± 1°C before designing more complex formulations, which are binary and ternary solvent systems. In addition, in order to understand caffeine solubility in different solvents, the solubility parameters of each solvent as well as the log P values were calculated using Molecular Modelling Pro Plus® software (Chem SW, USA). The solubility parameter values of caffeine are 11 and 13.5 $(cal/cm^3)^{1/2}$ (Dias et al., 2007, Herrador and

González, 1997). The Log P of caffeine is -0.7. The solubility of caffeine in each solvent or solvent system is summarized in Table 2.20 and Figure 2.4.

Table 2.20 Solubility parameter and Log P of each solvent calculated using Molecular Modelling Pro® software and caffeine solubility in different solvent at 32 ± 1°C, mean ±SD, n=3.

Solvent	Solubility parameter $(cal/cm^3)^{1/2}$	Log P	Solubility of caffeine (mg/mL)
PBS	-	-	32.54±2.93
LIM	8.02	3.2	0.89±0.11
IPM	8.21	4.5	0.91±0.10
DPPG	8.72	4.5	1.89±0.04
1,3-BG	14.15	-0.1	6.71±0.45
PGML	9.44	3.2	7.26±0.21
GER	9.53	2.6	9.79±0.33
1,2-PENT	12.46	0.3	10.80±0.16
TPG	11.91	0.1	11.41±0.39
PEG-6-CCG	-	-	11.88±0.13
PGMC	9.89	2.5	12.00±0.57
1,2-BG	13.13	-0.1	13.77+0.28
TRANS	10.62	-0.1	14.89±0.14
PG	14.06	-0.5	15.02±0.13
T-BA	10.75		16.40±0.18
DMI	9.97	-1.0	21.83±0.20
Lina	9.58	2.6	24.40±0.35

According to Table 2.20 and Figure 2.4, it can be seen that caffeine is more soluble in hydrophilic solvents, which is consistent with is the fact that it is a hydrophilic chemical.

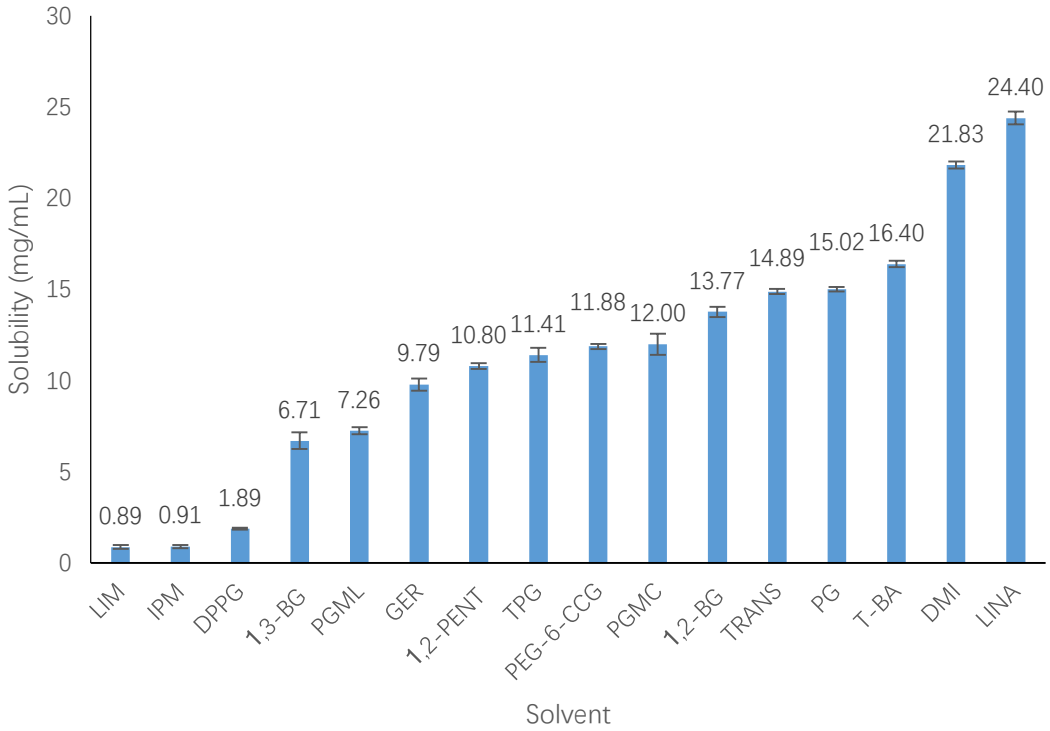

Figure 2.4 Solubility of caffeine in a range of different solvents at 32±1°C, mean ± SD; n=3.

Solubility parameter is also a factor that may influence the solubility of a compound. As shown in Table 2.20, when the solubility parameters of solvents are close to those of caffeine, which is either 11 $(cal/cm^3)^{1/2}$ or 13.5 $(cal/cm^3)^{1/2}$, the solubility of caffeine in these solvents is relatively high.

2.4 Conclusions

HPLC methods were developed for ibuprofen and caffeine. A GC method was also developed for the solvent PG, which acted as a significant vehicle for caffeine. According to the validation results, both the HPLC and GC methods were reliable, sensitive and effective within their related proposed measurement ranges.

Solubility studies were conducted for ibuprofen and caffeine in a range of selected solvents. The results from solubility studies provided valuable information for selecting suitable vehicles and receptor phase for *in vitro* permeation studies and developing optimum formulations. These formulations will be taken forward in the other chapters of this thesis.

2.5 References

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Chapter 3 *In vitro* permeation studies in silicone membrane and porcine skin

3.1 Introduction

In vitro strategies are developed to estimate the penetration of chemical molecules into and subsequent permeation across the skin into a fluid reservoir. Non-viable skin can be used in these studies to measure penetration and permeation only or fresh, metabolically active skin to simultaneously measure permeation and skin metabolism (OECD, 2004). As mentioned previously, the outermost layer of skin, the stratum corneum is the major barrier of drug permeation, and hence often acts as the rate-limiting step for percutaneous absorption (Dugard et al.; 1984, Lane, 2013). As SC is a non-living layer, its permeability properties remain the same after excision from the body. A plethora of literature has verified a good agreement between *in vivo* and *in vitro* studies with the same chemicals (Franz, 1975; Bronaugh et al., 1982; Ng et al., 1992; van de Sandt et al., 2004; van Ravenzwaay and Leibold, 2004). Consequently, *in vitro* studies to study skin permeation are used widely in both industry and academia.

Moreover, *in vitro* studies offer advantages over *in vivo* methods. For instance, permeation through the isolated skin is measured directly, for which

sampling is conducted immediately below the skin surface. The most common methods for the evaluation of *in vitro* skin permeation studies use diffusion cells. The major advantage of this kind of investigation is that the experimental conditions can be controlled precisely, such that the only variables are the skin and the test material. Therefore, in some ways, some form of *in vitro* diffusion cell experiment is often the most appropriate method for assessment of percutaneous penetration in a developmental drug-delivery program or in a dermal toxicology screen.

Conventional *In vitro* systems, diffusion cells, range in complexity from a simple two-compartment “static” cell (Franz, 1975) to multi-jacketed flow-through cells (Bronaugh and Stewart, 1985). Excised skin is always mounted as a barrier between the donor compartment and the receptor compartment. The amount of tested compound permeating from the donor to the receptor side is determined as a function of time. Efficient mixing of the receptor phase is essential, and sample removal should be straightforward (Walters et al., 1998).

The Franz diffusion cell is one of the most common systems for *in vitro* skin permeation studies (Friend, 1992). The design of this type of diffusion cell systems is relatively simple. The receptor phase under the skin may be simply manually sampled by removing aliquots periodically for analysis (Bronaugh, 2004). However, an important consideration in Franz diffusion cell systems is the solubility of the test compound in the receptor fluid, which may affect the skin conditions. Therefore, the receptor chamber volume, dimensions and

sampling frequency should be taken into account according to the solubility of test compound (Walters et al., 1998). A typical Franz diffusion cell is presented in Figure 3.1.

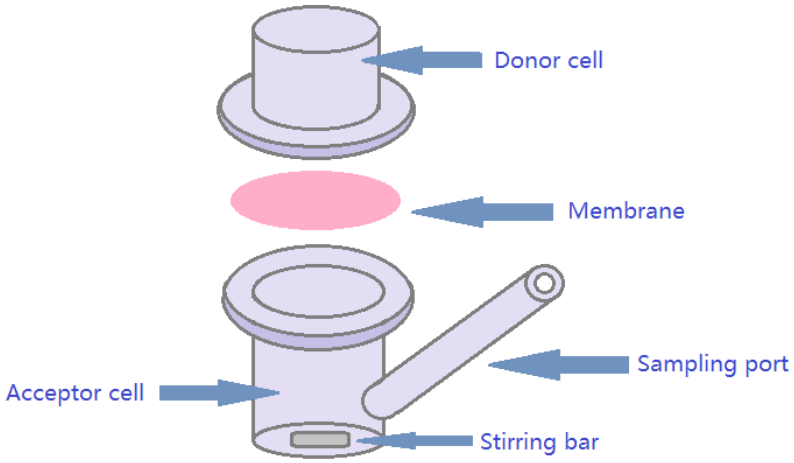

Figure 3.1 Design of a typical Franz diffusion cell

Due to the complexity of skin, it is often difficult to interpret experimental data. Mathematical models may be used as a strategy to help understand permeation data. However, all these mathematical models are based on some assumptions.

The most common assumptions in the diffusion equations that are applied to the *in vitro* situation are as follows (Walters, 2002):

1. The receptor phase is a perfect sink.

2. Depletion of the donor phase is negligible.

3. The membrane is a homogeneous slab.

It is obvious that none of these assumptions is wholly true in fact, so the potential significance of these imperfections must not be overlooked.

In mathematical models, two kinds of conditions are considered, namely infinite and finite dose conditions. The definition of an infinite dose is the amount of the tested chemicals applied alone or in a vehicle to the skin such that a maximum rate of absorption of the test substance (per unit area of skin) is achieved and maintained (OECD, 2004). In principle, the dose solution is applied in excess and the donor can be occluded during the experiments (Sartorelli et al., 2000). The depletion of the chemical is unlikely to occur. In the finite dose procedure, the maximum absorption rate may be reached for some of the time, but is not maintained, or it may not be achieved (OECD, 2004). The application of finite dose studies should be more representative of the actual “in-use” situations. The dose applied is in a volume sufficient to cover the skin and normally non-occluded. The concentration of the tested compounds in the donor phase changes (reduces) due to uptake of the compounds into the skin or evaporation. It may also change (increase) because of evaporation of the donor fluid. Figure 1.5 shows typical permeation profiles for an infinite dose and a finite dose study.

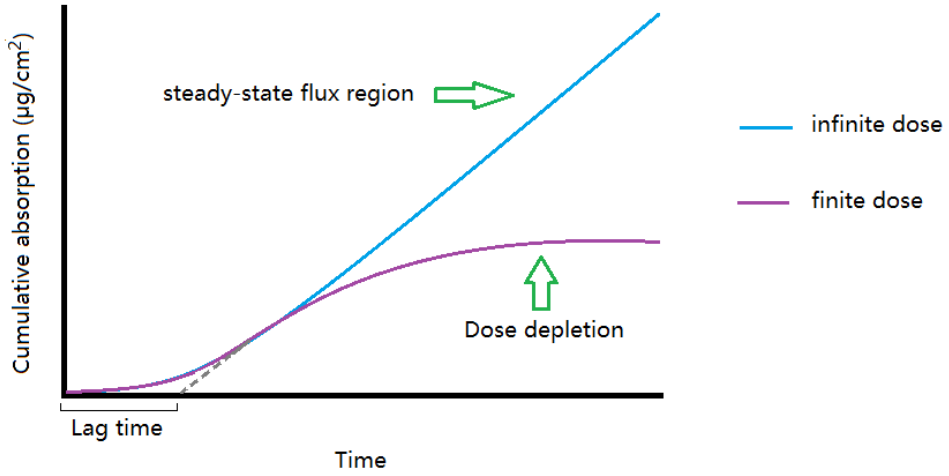

Figure 3.2 Typical permeation profiles for an infinite dose and a finite dose study

In permeation studies, the chemicals diffuse based on the concentration gradient between the donor and receptor phase. The amount of the chemical permeated through a unit area per unit time is the flux (J) of the permeation studies. This principle follows Fick's first law of diffusion, whereby the diffusion rate of a solute through unit area of a section is proportional to the concentration gradient (Katz and Poulsen, 1971). The equation used to describe this is as follows:

$$J = -D\frac{dc}{dx} \qquad \text{Equation 3.1}$$

Where J is the flux of permeation, D is the diffusion coefficient; dc/dx is the concentration over length of the diffusional field.

Another important permeation parameter is the permeability coefficient (k_p). This parameter can be expressed as:

$$k_p = \frac{K\,D}{h}$$ Equation 3.2

Where K is the partition coefficient of the permeant, D is diffusion coefficient, and h is the thickness of the membrane.

The aims of the studies in this chapter may be summarized as follows:

- To perform both *in vitro* infinite and finite dose permeation studies and mass balance studies on silicone membrane and porcine skin the Franz cell model to investigate the permeation behaviour of ibuprofen from different kinds of formulations;
- To perform both *in vitro* infinite and finite dose permeation studies and mass balance studies on porcine skin in the Franz cell model to investigate the permeation behaviour of caffeine from various formulations. As caffeine permeation behaviour in silicone membrane has been studied in detail in our research group (Dias et al., 1999; Dias et al., 2007), silicone membrane will not be employed in the present study for caffeine. However, as a common and significant permeation enhancer for caffeine (Luo and Lane, 2015), propylene glycol (PG) will be investigated as well in the studies for caffeine.

3.2 Materials and methods

3.2.1. Materials

3.2.1.1 Chemicals and tissues

Ibuprofen was a gift from Wyeth (Haversham, Hants., UK). Analytically pure standards of caffeine, polyethylene glycol (PEG) 300, propylene glycol (PG), and Isopropyl alcohol (IPA) were obtained from Sigma-Aldrich (UK).

HPLC grade water, acetonitrile (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade) and absolute ethanol were purchased from Fisher Scientific (UK). Phosphate buffered saline (pH 7.4 at 25^{o}C) was prepared using Dulbecco A Tablets (Oxoid, UK).

Silicone membrane (250 μm) was provided by Samco (Nuneaton, UK). This grade and thickness of silicone was selected because it has been used previously to examine the effects of a range of hydrophilic and lipophilic vehicles on ibuprofen permeations (Watkinson et al., 2009a; Watkinson et al., 2009b; Watkinson et al., 2011). Porcine tissue was obtained from a local abattoir.

The ibuprofen commercial formulations selected for evaluation were IBUGEL® (Ibuprofen 5% w/w, Dermal Laboratories, Hitchin, Hertfordshire, UK) and IBULEVE® Speed Relief 5% Spray (Dermal Laboratories, Hitchin,

Hertfordshire, UK). The two commercial formulations include a gel and a spray. They were selected in order to study permeation from different types of commercial formulations.

3.2.1.2 Instruments

A water bath (SUB Aqua 26) was obtained from Grant (UK), an electronic balance (Sartorius 1702) was from Sartorius (Germany), a sonicator (PDL 356) was obtained from Camlab Serving Science (UK). The HPLC system used consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. The software used to acquire and analyse the data was ChemStation® for LC 3D Rev. A. 09.03 (Agilent Technologies, U.S.A.). A water purification system with a 75 L reservoir (Elga Option 3) was obtained from Veolia Solutions and Technologies (UK). A rotator (SB2) was from Stuart Equipment (U.S.A.). A centrifuge (5415R) was from Eppendorf (U.S.A.).

3.2.2 Methods

3.2.2.1 Preparation of silicone membrane and porcine skin

Silicone membrane, with a measured thickness of 0.25 mm (Samco, USA) was cut into discs using a 16 mm cork-borer. Silicone discs were placed in distilled water and cleaned by ultrasonication for 5 min. Discs were submerged in phosphate buffered saline (PBS) pH 7.4 (Oxoid Ltd, UK) and ultrasonicated for a further 5 min. Membranes were finally rinsed in fresh PBS and left to hydrate in PBS overnight at 32 ± 1°C. Hydrated silicone discs were clamped in between the receptor and donor compartments of the Franz cells.

Porcine tissue was obtained from healthy animals from a local abattoir. Porcine ears were washed gently with distilled water to remove particulate contamination. Hairs were carefully trimmed from the surface and ears were rinsed using distilled water. A continuous cut approximately 10 mm from the edge was made on the back of the ear using a scalpel. The skin was then cut away from the cartilage avoiding damage to the underside of the tissue. Surgically removed porcine skin was mounted on aluminium foil, placed in polyethylene bags and stored at -20°C until required. Frozen porcine skin was rapidly cut into discs using a 16 mm cork-borer and when thawed, clamped between the receptor and the donor compartments of Franz cells.

3.2.2.2 Preparation of formulations

3.2.2.2.1 Preparation of ibuprofen formulations

As a common solvent in topical and transdermal formulations, PG was selected as a vehicle in the study for ibuprofen. In order to prepare a saturated formulation of ibuprofen in PG, an excess amount of ibuprofen was placed in a test tube with PG, shaken for 48 hours at 32 ± 1^{o}C, and then centrifuged. The upper clear layer was then used as the saturated formulation of ibuprofen in PG.

Other vehicles selected for the studies for ibuprofen include PEG 300 and IPA, which is a polymer and an easily evaporating solvent, respectively. As mentioned in section 2.3.4, the solubility of ibuprofen is 324.42 ± 2.27 mg/mL in PEG 300, and 953.99 ± 15.61 mg/mL in IPA, both of which are rather high. In order to compare ibuprofen permeation behaviour from simple solutions with commercial formulations, two ibuprofen formulations in binary solvent systems were prepared as PG: ibuprofen: IPA 15.6: 5.0: 79.4% (w/w) and PEG300: ibuprofen: IPA 15.3: 5.0: 79.7% (w/w). As for the selected commercial formulations, both of these solutions contained 5% ibuprofen. In addition, with these compositions, after the evaporation of IPA the residual phase (ibuprofen and vehicle) was 80% of the saturated solubility of ibuprofen in that vehicle.

3.2.2.2.2 Preparation of caffeine formulations

In order to prepare a saturated formulation of caffeine in PG, an excess amount of caffeine was placed in a test tube with PG, shaken for 48 h at 32 ± 1^{o}C, and then centrifuged. The upper clear layer was then used as the saturated formulation of caffeine in PG. Taking into account that caffeine has a solubility of 15.05 ± 0.12 mg/ml in PG at 32 ± 1^{o}C, in order to prepare 80%, 60% and 40% of the saturated levels of formulation, 120, 90 and 60 mg of caffeine were dissolved into 10 mL of PG separately and shaken. Caffeine formulations were also prepared as 50 and 20 mg/ml in water.

3.2.2.3 *In vitro* finite and infinite dose permeation studies in porcine skin

3.2.2.3.1 *In vitro* finite dose permeation studies in silicone membrane and porcine skin for ibuprofen

Permeation studies were conducted using glass Franz diffusion cells with an approximate diffusion area of 1 cm^2. The contact surfaces of the donor and receptor compartment were coated with a thin, even layer of silicone grease (Dow Corning, USA) and the cut membrane was clamped between both compartments. Assembled Franz cells were filled with PBS pH 7.4 which served as the receptor fluid, 0.02% sodium azide (w/v) was included for studies

that lasted for 48 h. Receptor volume was determined by difference in mass. In order to ensure uniform mixing of the receptor phase, a 7 mm polytetrafluoroethylene coated magnetic stir bar was placed in the receptor compartment and cells were placed in a custom made acrylic Franz cell holder positioned over a submersible magnetic stir plate (Variomag, Thermo Scientific, USA), accommodating 15 Franz cells. The sampling arm was capped to prevent evaporation of receptor fluid. Franz cells were left in a thermostatic water bath held at 36 ± 1°C (SUB 28, Grant, UK) for at least 1 h with stirring. Prior to dosing, the membrane surface temperature of five cells in different locations was measured using a digital thermocouple to ensure membrane surface temperature was 32 ± 1°C.

After 1 h, 10 µL of the prepared ibuprofen saturated solutions in PG were homogenously spread on the porcine skin in each donor compartment. 3.6 µL of IBULEVE Speed Relief Spray, 5% w/w ibuprofen solutions in either PEG 300 or PG, and 4 µL of IBUGEL were applied to either silicone membrane or porcine skin. IBUGEL was spread over the diffusion area using a glass Pasteur pipette with a melted tip. The experiment was conducted up to 6 h with silicone membrane and up to 24 h with porcine skin. At specified intervals, 200 µl samples were withdrawn from the receptor compartment and an equivalent amount of pre-warmed receptor phase was added in order to maintain a constant volume; all samples were then analysed by HPLC in order to quantify the amount of ibuprofen that permeated.

After permeation experiments a mass balance study was conducted to account for the applied dose. The skin surface was cleaned with cotton buds, which were weighed before and after to measure the amount of formulation remaining on the skin surface. Receptor fluid was discarded and using 90% (v/v) methanol in water the membrane surface was washed with five times with 1 mL of solution. Filter paper strips were used to remove any remaining liquid. Liquid from each wash and filter paper were transferred to 1.5 mL centrifuge tubes. Franz cells were then disassembled and the membrane placed into a 2 mL centrifuge tube with 1 mL of methanol which served as the extraction solvent. Tubes for extraction were sealed with Parafilm® , placed in an ultrasonic bath for 5 min and then transferred to a microplate shaker (PMS-1000i, Grant, UK) at 1200 rpm for 5 h at room temperature. After 5 h, skin was blotted dry and transferred to a clean 2 mL centrifuge tube and a second extraction performed for a further 19 h using 1 mL methanol. Samples obtained from washes and extractions were centrifuged at 13200 rpm for 15 min at 32°C before analysis by HPLC.

All the data were recorded by MS Excel® (Microsoft Corp., USA). The results are shown as mean ± standard deviation (SD). Statistical analysis was performed using MS Excel® and OriginPro® (OriginLab Corp., USA). One way analysis of variance (ANOVA) followed by a Tukey test was conducted (OriginPro®) for multiple comparison between the groups; $p < 0.05$ was considered as the statistical significance.

3.2.2.3.2 *In vitro* infinite dose permeation studies in porcine skin for ibuprofen

Vertical glass Franz diffusion cells were used to perform finite dose studies and the method used was as previously described in Section 3.2.2.3.1 except for the amounts of applied formulations.

For the infinite dose studies, 1 mL of the prepared formulation was homogenously spread on the skin in each donor compartment. The donors were then immediately covered with Parafilm® to prevent the evaporation of the constituents from the formulation. The experiment was conducted for 24 or 48 h. At specific time intervals, 200 µL samples were withdrawn from the receptor compartment and an equivalent amount of pre-warmed receptor phase was added in order to maintain a constant volume. Samples were then analysed by HPLC to quantify permeated ibuprofen.

Saturated solutions of ibuprofen in PG were selected to conduct the infinite dose studies in order to maximize the thermodynamic activities of ibuprofen in the solutions.

Mass balance studies were also conducted after permeation studies were completed. The method used was as previously described in Section 3.2.2.3.1 except for the amounts of applied formulations.

3.2.2.3.3 *In vitro* finite dose permeation studies in porcine skin for caffeine

Vertical glass Franz diffusion cells were used to perform finite dose studies and the method used was as previously described in Section 3.2.2.3.1 except the applied formulations and the amounts are different. For the finite dose study for caffeine on porcine skin, 10 µL of the prepared solutions were homogenously spread on the skin in each donor compartment. The experiment was conducted for 48 h with porcine skin. At specified intervals, 200 µL samples were withdrawn from the receptor compartment and an equivalent amount of pre-warmed receptor phase was added in order to maintain a constant volume, all samples were then analysed and quantified by HPLC and GC after extraction.

After permeation studies were completed, the formulation remaining on the skin surface was transferred into centrifuge tubes with cotton buds. The sample tubes and cotton buds were weighed before and after taking the formulation. The skin surface was then washed twice with 1 mL of water and then washed twice with 1 ml of methanol: water 90: 10 (v/v). After this, the Franz cells were dismantled and caffeine and PG were extracted with 2mL of methanol: water 90:10 (v/v) for 48 h with shaking. The skin was cut into small pieces prior to extraction to optimise the extraction from skin. All samples were analysed and quantified by HPLC and GC after extraction.

3.2.2.3.4 *In vitro* infinite dose permeation studies in porcine skin for caffeine

Vertical glass Franz diffusion cells were used to perform infinite dose studies and the method used was is described in Section 3.2.2.3.1. For the infinite dose study, 1 mL of the prepared solution was homogenously spread on the skin in each donor compartment. The experiment was conducted for 24 h with porcine skin. At specific time intervals, 200 µL samples were withdrawn from the receptor compartment and an equivalent amount of pre-warmed receptor phase was added in order to maintain a constant volume. All samples were then analysed by HPLC to quantify permeated caffeine.

Mass balance studies were also conducted after permeation studies are completed. The method used was as previously described in Section 3.2.2.3.3.

3.3 Results and discussion

3.3.1 *In vitro* finite and infinite dose studies in Franz cells in silicone membrane and porcine skin for ibuprofen

3.3.1.1 *In vitro* finite dose studies in Franz cells in silicone membrane and porcine skin for four selected ibuprofen formulations

Four ibuprofen formulations were selected for the finite dose studies. These formulations include two commercial formulations, IBULEVE® Speed Relief 5% Spray (Dermal Laboratories, Hitchin, Hertfordshire, UK) and IBUGEL® (Ibuprofen 5% w/w, Dermal Laboratories, Hitchin, Hertfordshire, UK), and two ibuprofen formulations in binary solvent systems prepared as 5% w/w solutions in isopropyl alcohol and either PEG 300 or PG.

Permeation profiles for ibuprofen for these formulations under finite dose conditions with silicone membrane are shown in Figure 3.1.

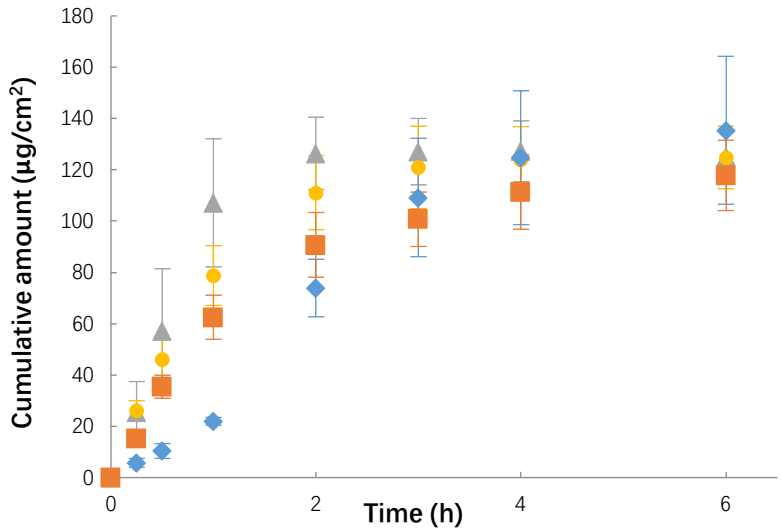

Figure 3.1 Cumulative amounts of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) for finite dose Franz cell studies with silicone membrane after 6 h at 32 ± 1°C. Each data point represents the mean ± SD (n=5).

Figure 3.2 illustrates the permeation profiles for ibuprofen under finite dose conditions with porcine skin.

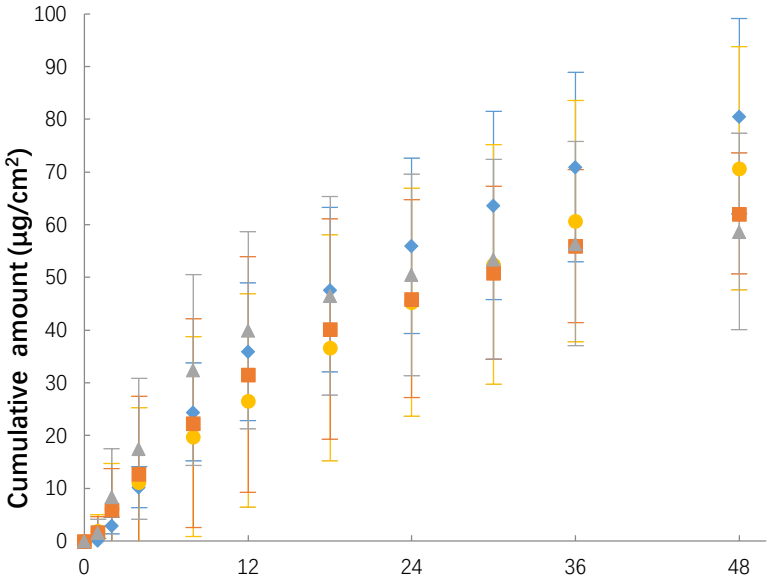

Figure 3.2 Cumulative amounts of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) during finite dose Franz cell studies with porcine skin after 24 h at 32 ± 1°C. Each data point represents the mean ± SD (n=5).

After 6 h, around 140 µg/cm^2 of ibuprofen had permeated in silicone membrane, while after 48 h maximum amounts of permeation were about 80 µg/cm^2 for porcine skin. Typical curvilinear permeation profiles for all formulations were observed with both membranes. However, no significant differences were observed for the amounts permeated in porcine skin and silicone membrane.

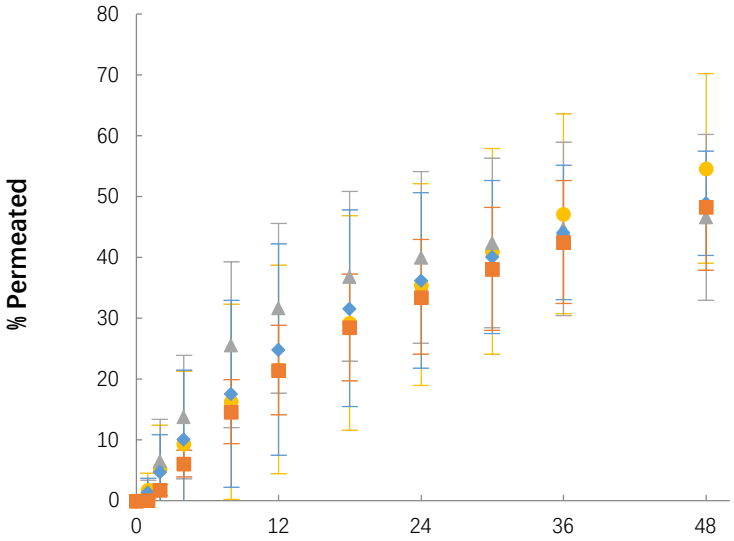

Figure 3.3 Percentages of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) during finite dose Franz cell studies in porcine skin. Each data point represents the mean ± SD (n≥5).

The percentage permeation was also analysed to assess the efficiency of the selected formulations. Figures 3.3 and 3.4 shows the percentage profiles for ibuprofen from all the tested formulations for studies on porcine skin and silicone membrane.

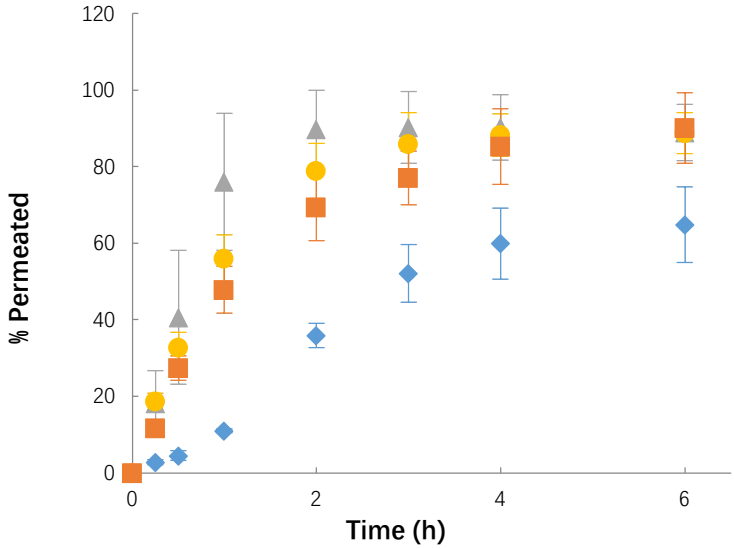

Figure 3.4 Percentage of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) for finite dose Franz cell studies in silicone membrane. Each data point represents the mean ± SD (n≥5).

At 24 h the percentage permeation was approximately 40% in porcine skin. Values for percent permeated at 6 h for the formulations for silicone membrane ranged from 60-100%.

Results for the mass balance study after the permeation using porcine skin are shown in Figure 3.5.

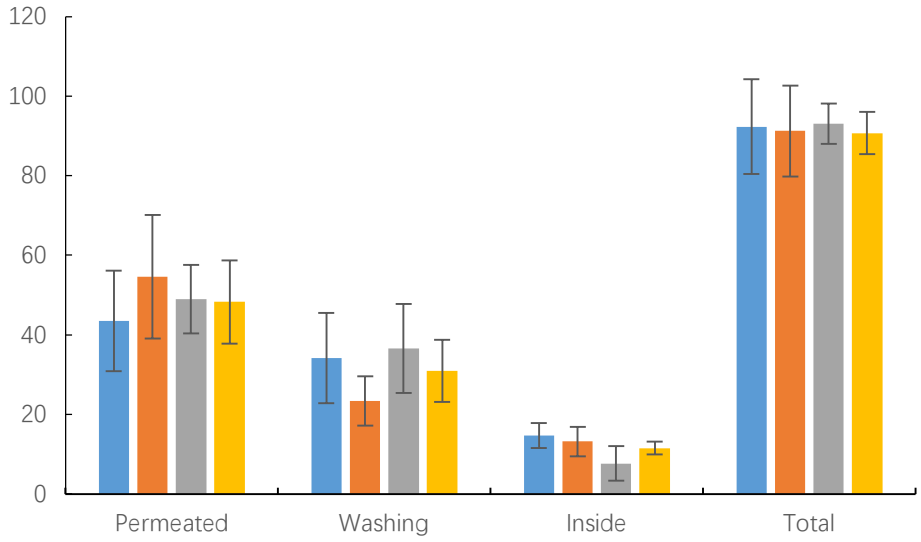

Figure 3.5 Mass balance results for ibuprofen for permeation from PG (■), PEG 300 (■), IBULEVE® (■), and IBUGEL® (■) for finite dose Franz cell studies on porcine skin. Each data point represents the mean ± SD (n≥5).

The recovery values of ibuprofen for all the selected formulations were above 90%, with the values of 92.32 ± 11.89 % for PG, 91.21 ± 11.44 % for PEG 300, 93.05 ± 5.13 % for IBULEVE®, and 97.7 ± 5.26 % for IBUGEL®, separately. About 40-50% of ibuprofen permeated through the skin and around 25-35% remained on the skin surface; approximately 10% of the active was extracted from the skin.

3.3.1.2 *In vitro* finite and infinite dose studies in Franz cells in porcine skin for ibuprofen in PG

Figure 3.6 presents the permeation profiles for the *in vitro* infinite and finite dose studies for the saturated solution of ibuprofen in PG.

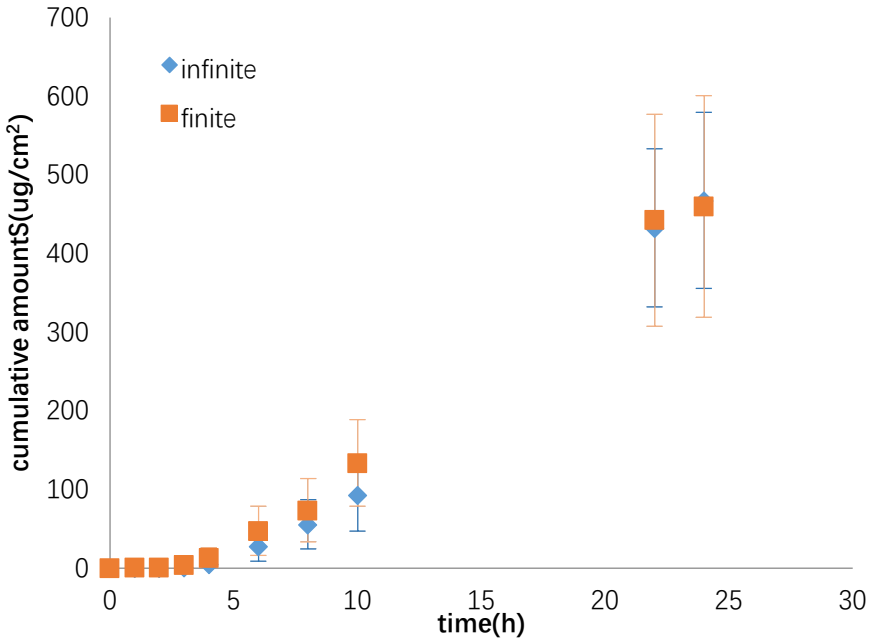

Figure 3.6 Cumulative amount of ibuprofen permeated from saturated formulation in PG through porcine skin after 24 h at 32 ± 1°C. Each data point represents the mean ± SD (n=5).

The steady-state flux obtained from the infinite dose study was 26.84 ± 5.03 µg. cm^{-2}. h^{-1}, and the lag time was 6.27 ± 1.00 hours. For the finite dose study, the steady-state flux was 25.53 ± 6.72 µg. cm^{-2}. h^{-1}, and the lag time was 4.96 ± 1.06 h. The permeability coefficients (k_p), estimated from the data for infinite and finite dose studies were calculated as 2.36 X 10^{-8} ± 0.35 X 10^{-8} and 2.40 X 10^{-8} ± 0.63 X 10^{-8} $cm.s^{-1}$, separately. At the end of the 24 h studies, the cumulative permeated amounts of ibuprofen were 467.53 ± 111.78 and

460.02 ± 140.80 µg. cm^{-2}. The results suggest that the permeation of ibuprofen remains nearly the same regardless of the applied dose.

3.3.2 *In vitro* finite and infinite dose studies in Franz cells in porcine skin for caffeine

3.3.2.1 *In vitro* finite dose studies in Franz cells in porcine skin for caffeine

In these in vitro studies, a caffeine formulation at 80% saturation solubility in PG was investigated in the Franz cell model using porcine skin. The concentration of each caffeine formulation in the present study was prepared at 80% of the saturated level in order to maintain the thermodynamic activity at the same level and to avoid drug crystallisation.

The permeation profile for the *in vitro* finite dose studies for the caffeine formulation in PG is shown in Figure 3.7.

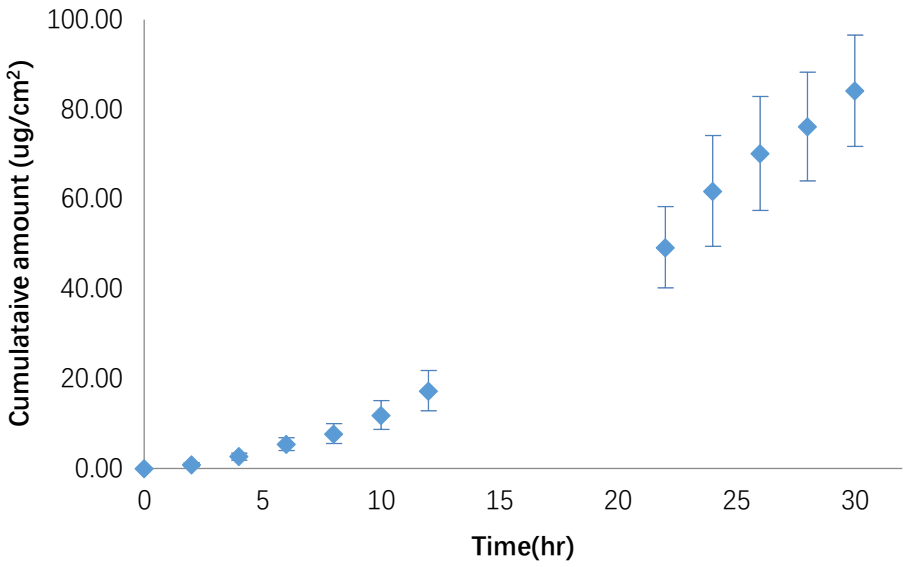

Figure 3.7 Cumulative amount of caffeine permeated from 80% saturation solution in PG in porcine skin after 30 h at 32±1°C, mean ± S.D, n=5.

The data show that up to 30 h, caffeine continued to permeate through the tissue. About 85 µg/cm^2 of caffeine permeated through porcine skin at 30 h. The steady-state flux, estimated from the data was calculated as 4.03 ± 0.53 µg.cm^{-2}.h^{-1}, and the lag time was 9.49 ± 2.76 h. Accordingly, the permeability coefficient (k_p) obtained for the porcine skin study was 9.33 X 10^{-8} ± 1.23 X 10^{-8}cm.s^{-1}, and the log k_p was -7.03 ± 0.05.

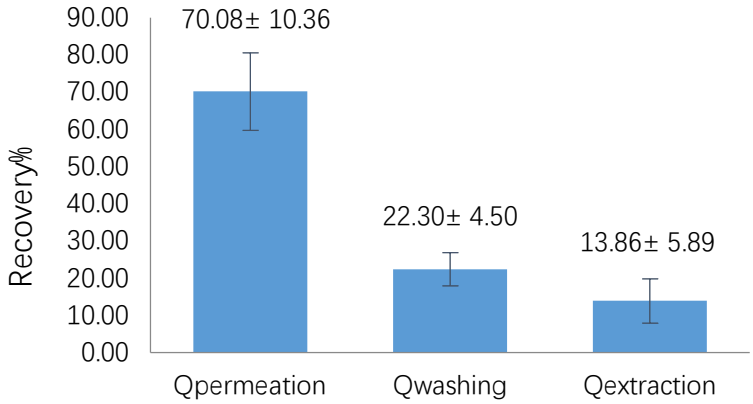

Figure 3.8 Distribution of caffeine 30 h post-application of 10 µL of 80% saturation solution in PG in porcine skin, mean ± S.D, n=5.

Figure 3.8 shows the distribution of caffeine in the receptor fluid (Qpermeation), on the skin surface (Qwashing) and in the skin (Qextraction). The data indicate that in the finite dose studies, 70.08 ± 10.36% of the applied active permeated through the skin, with 22.30 ± 4.50% remaining on the skin surface and 13.86 ± 5.89% being retained inside the skin. The mean total recovery percentage was 106.24%, which shows that the mass balance method (washing and extraction) used in this experiment met the requirements for mass balance studies (OECD, 2000; SCCP, 2006). It is interesting to note that, in this finite dose study, caffeine kept permeating up to after 30 h, when around 70% of the applied caffeine had gone through the skin. The data also confirmed that up to 30 h, the amount of PG which permeated through porcine skin was undetected by the GC method used. This may be due to loss from the samples or over dilution of the samples. However, the amounts of PG which were deposited on the membrane surface and which were retained in the skin membrane were quantified.

The recovery percentages of applied PG from the formulation are shown in Figure 3.9.

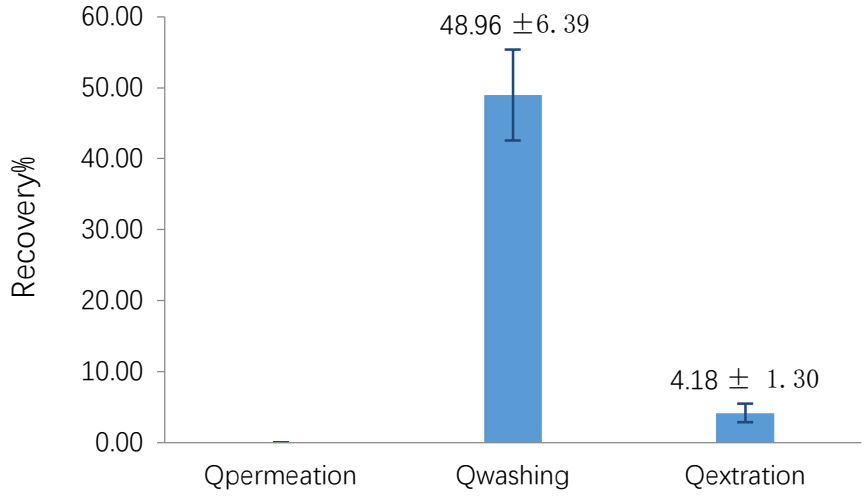

Figure 3.9 Distribution of PG 30 h post-application of a 10 µL formulation of caffeine at 80% saturation solubility in PG in porcine skin, mean ± S.D, n=5.

The mean total recovery percentage of PG was rather low with a value of 53.14 ± 7.47%. It is known that PG is a volatile solvent (Megrab et al., 1995). Therefore, one of the main reasons for the low recovery could be that around half of the PG applied had evaporated after 30 h. Moreover, it is noticeable that 48.96% of the applied PG remained on the skin surface at the end of the study. This meant that the concentration of caffeine in the formulation kept increasing during the experiment to a saturated condition, leading to a large concentration gradient between the donor and the receptor phase throughout. The persistent significant concentration gradient, consequently, resulted in caffeine continuing to permeate through the skin. This is possibly the main reason why the caffeine could continue to permeate in a linear fashion even when more than 70% of the applied dose had permeated. It is also interesting to note that 4.18% of the applied PG was extracted from the skin after the 30

h permeation study. PG is well-known as a penetration enhancer. Taking these factors into account, it is proposed that the formation of a skin depot of caffeine in PG within the skin may be possible (Morgan et al., 1998). However, to confirm the hypothesis of the stratum corneum reservoir effect of PG, further studies will have to be conducted, for example, on the clearance rate of PG from the skin.

The effect of the degree of drug saturation (DS) for a model drug, oxybutynin, on solvent permeation, octyl salicylate (OSAL) and PG, and drug permeation in silicone membrane and human skin has been investigated by Santos et al. (2009). Their results showed that in both human skin and silicone membranes, the PG permeation remained unaffected with the DS of the formulations. To understand the effect of the degree of DS for caffeine on PG permeation, *in vitro* studies in porcine skin were conducted by applying finite dose caffeine formulations with different DS, including 100%, 60% and 40% saturated solubility levels, for 48 h.

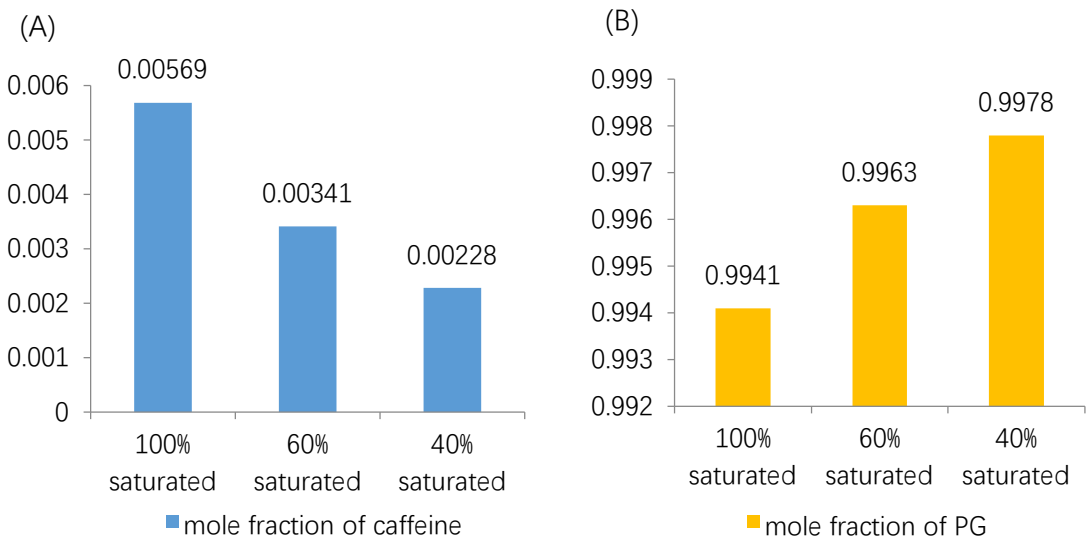

Figure 3.10 Mole fractions of caffeine and PG in formulations with 100%, 60% and 40% level of caffeine saturation in PG

Figure 3.10 shows the mole fraction of caffeine and PG in the corresponding formulations. It is clear that the mole fractions of caffeine drop with the increase of the PG mole fraction.

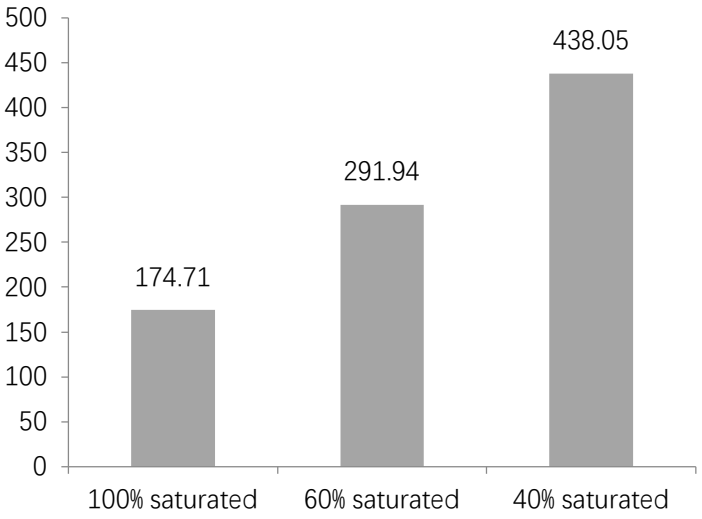

Figure 3.11 Ratios of caffeine mole fractions to PG mole fractions in formulations with 100%, 60% and 40% level of caffeine saturation in PG

The ratios of caffeine mole fractions to PG mole fractions are presented in Figure 3.11. The ratios of the three formulations were 174.71, 291.94 and 438.05, respectively.

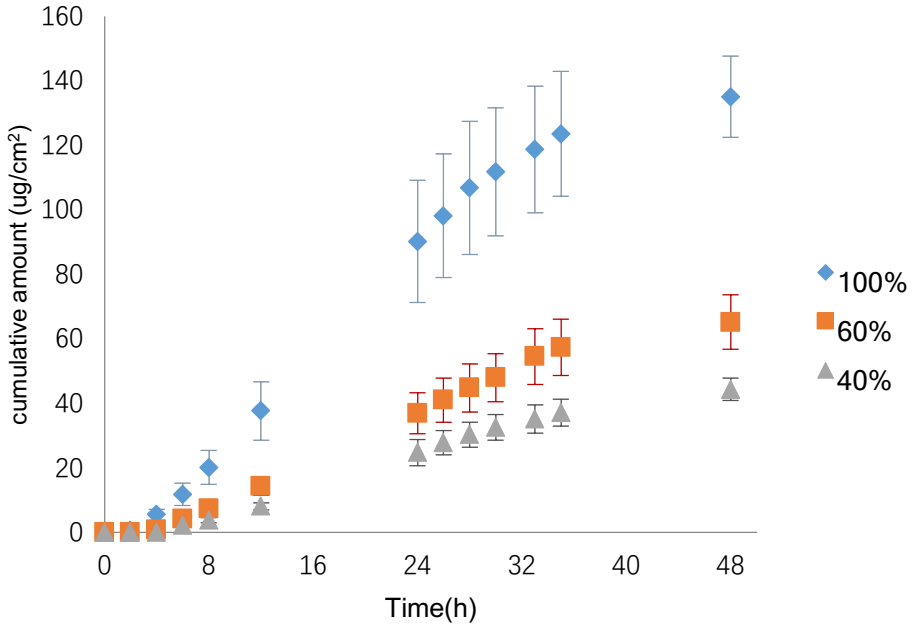

Figure 3.12 Cumulative amount of caffeine permeated from formulation at 100%, 60% and 40% saturation solubility in PG after 30 h at 32±1°C, mean ± S.D, n=5.

Figure 3.12 illustrates the permeation profiles for caffeine formulations at different saturation levels in PG. From this figure, the caffeine permeation increased with the DS of the formulation. The amount permeated after 48 hours from 100%, 60% and 40% saturated level formulations was 135.07 ± 12.59, 65.17 ± 8.42, and 44.39 ± 3.49 μg/cm^2, respectively. Also, the flux of caffeine went up with increased concentration in the formulations. The thermodynamic activity of caffeine in the formulations is related to its concentration. A high concentration of caffeine means high thermodynamic activity and hence an increase in the amount permeated.

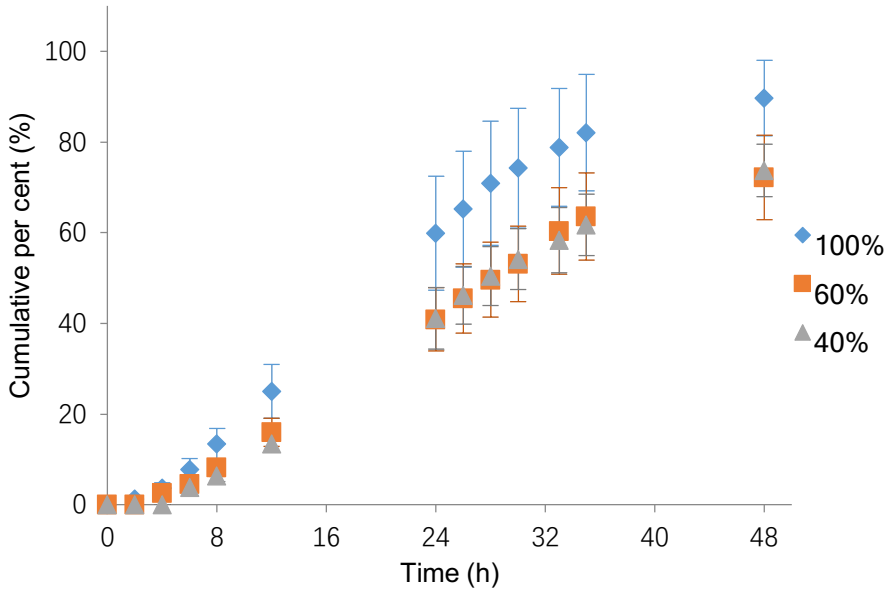

Figure 3.13 Cumulative percentage of the applied amount of caffeine permeated from formulation at 100%, 60% and 40% saturation solubility in PG after 30 h at 32±1°C, mean ± S.D, n=5.

Figure 3.13 illustrates the cumulative percentage of the applied amount of caffeine that had gone through the skin from the corresponding formulations. After 48 h, 89.75 ± 8.37%, 72.18 ± 9.33% and 73.74 ± 5.80% of the applied caffeine has permeated from the 100%, 60% and 40% saturated level formulations, respectively. The permeated percentages of formulations with 60% and 40% saturated level caffeine were similar, while that of 100% saturated formulation was significant higher ($P < 0.05$).

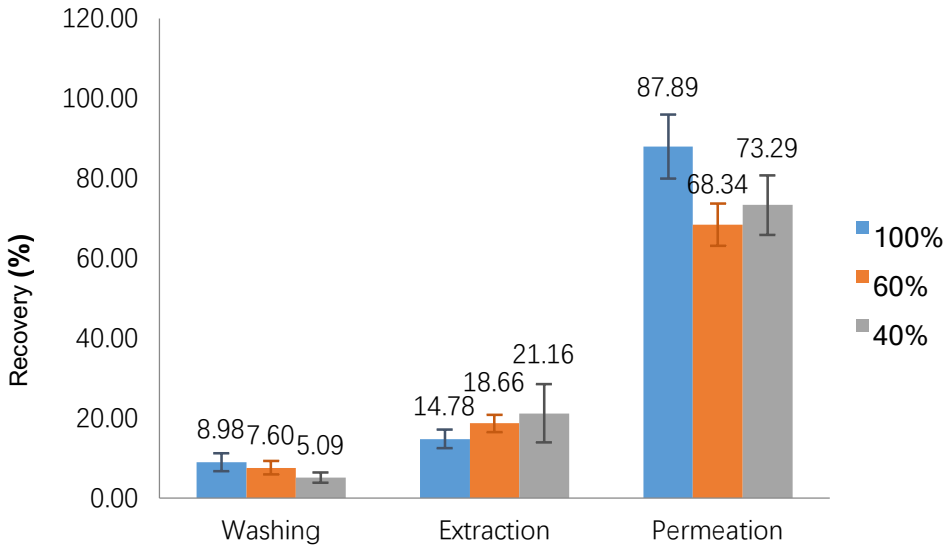

Figure 3.14 Distribution of caffeine 48 h post-application of a 10 µL for 100%, 60% and 40% saturation solubility in PG, mean ± S.D, n=5.

The washing, extracted amount, and amount of caffeine that permeated (%) at the end of these 48 h finite dose studies are shown in Figure 3.14. The total recovery values for the 100%, 60% and 40% formulations were 111.65% ± 6.53%, 94.61% ± 3.33%, and 99.54% ± 8.53%, respectively. All of these values were within the range of 80% and 120%, which corresponds to the OECD guidelines.

After 48 h permeation, the percentages of permeated amounts of caffeine from the PG formulations were rather high, ranging from 68.34% to 87.89%, with only around 20% getting through the stratum corneum to the deeper layers of the skin and less than 10% left on the skin surface. These results are in line with those from the study for the caffeine formulation at 80% saturation in PG.

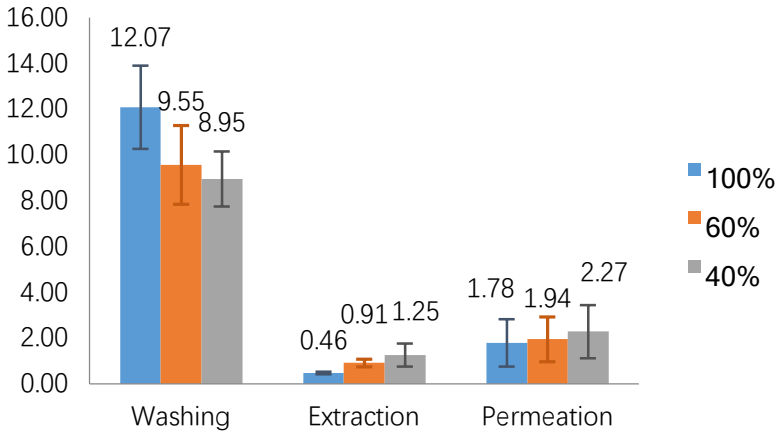

Figure 3.15 Distribution of PG 48 h post-application of 10 µL of caffeine at 100%, 60% and 40% saturation solubility in PG, mean ± S.D, n=5.

The washings, extracted amount, and permeated amounts of PG (%) at the end of these 48 h finite dose studies are shown in Figure 3.15. The total recovery values of PG for 100%, 60% and 40% formulations were 14.30% ± 2.15%, 12.87% ± 1.36%, and 13.39% ± 6.31%, respectively, which were much lower compared to the results after 30 h. Furthermore, about 10% of the applied PG remained on the skin surface. In other words, most of the PG applied had likely evaporated at the end of the studies. It is interesting that the permeation of PG was influenced by the caffeine concentration to a certain extent. As seen in Figure 3.15, with the decrease of caffeine concentration, the extracted and permeated amounts of PG increased from 0.46% to 1.25% and from 1.78% to 2.27%. The possible reason is that the increase of caffeine concentration enhanced the thermodynamic activity of caffeine molecules but altered the activity of PG also.

3.3.2.2 *In vitro* infinite dose studies in Franz cells in porcine skin for caffeine

In these *in vitro* infinite dose studies, a caffeine formulation at 80% saturation solubility in PG was investigated in the Franz cell model using porcine skin. The permeation profile of caffeine for this study is shown in Figure 3.17.

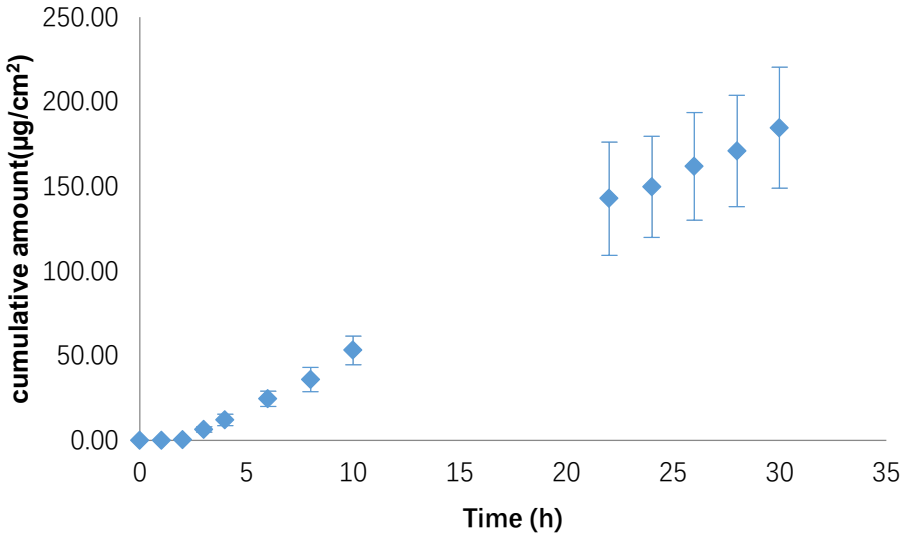

Figure 3.17 Cumulative amount of caffeine permeated after 30 h in porcine skin at 32±1°C, n=5, mean ± S.D.

After 30 h, approximately 185 µg/cm^2 of caffeine from the applied formulation permeated through the porcine skin. The steady-state flux determined from this study was 6.81 ± 1.38 µg. cm^{-2}. h^{-1}, and the lag time was 2.76 ± 0.48 h. Figure 3.17 shows that after 30 h, the permeation flux was at steady state, which is in line with the requirements of infinite dose studies. The

permeability coefficient (k_p), estimated from the data was calculated as 1.58 $X10^{-7} \pm 0.32\ X10^{-7}$ $cm.s^{-1}$.

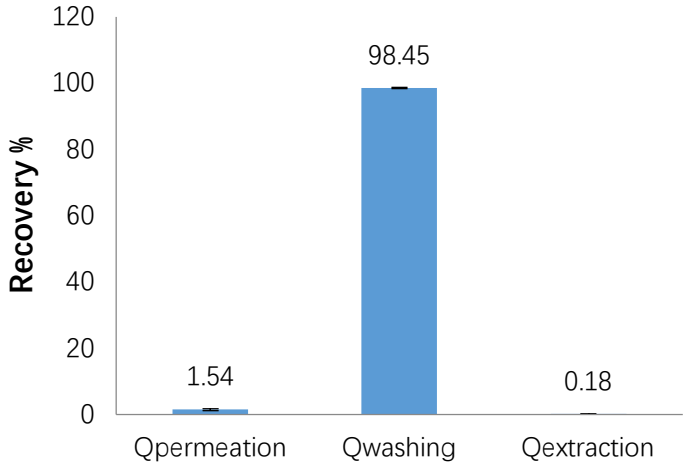

Figure 3.18 Distribution of caffeine 30 h post-application of 1 mL of formulation in porcine skin, mean ± S.D, n=5.

Figure 3.18 shows the distribution of caffeine in the receptor fluid (Qpermeation), on the skin surface (Qwashing) and in the skin (Qextraction). After 30 h, around 98.45 per cent of the applied formulation remained on the skin surface, while only about 1.54 per cent of the dose permeated through the skin and 0.18 per cent stayed in the deeper layers of the membrane. The results also show that in the infinite dose studies, the amount of the formulation in the donor did not affect the permeation behaviour during the length of the experiment. In addition, both the flux and cumulative permeated amounts of caffeine were much lower than those for ibuprofen (section 3.3.1), which indicates that ibuprofen can go through the skin more easily than caffeine. The reason is that the partition coefficient (Log P) of ibuprofen is 3.97 while this parameter for caffeine is –0.07 which means that caffeine does not have the ideal properties to go through the lipid layers of skin. Moreover, the melting point of caffeine is 238 ^{o}C while that of ibuprofen is 76^{o}C. According to Martin

(1993), the solubility of a solid in a vehicle can be expressed as the following equation:

$$-\ln X_2 = \frac{\Delta H_f}{RT}\left(\frac{T_0 - T}{T_0}\right) + \frac{V_2 \emptyset_1^2}{RT}(\delta_1 - \delta_2)^2 \qquad \textbf{Equation 3.3}$$

Where X_2: molar fraction solubility; Φ_1: volume fraction of solvent; V_2: molar volume of solute; R: gas law constant; T: temperature in degrees Kelvin; T_0: melting point of the solid; Hf: molar heat of fusion; δ_1: the solubility parameter of the solvent; δ_2: solubility parameter of drug. For a particular temperature and a particular vehicle, the solubility of a certain drug is depent on its melting point to a large extent. In other words, a drug with high melting point will have low solubility consequently. This is why the high melting point contributes to the limited ability of caffeine to permeate through the skin.

3.4 Conclusions

The *in vitro* permeation studies were conducted in conventional Franz cell models using silicone membrane and porcine skin for various ibuprofen and caffeine formulations.

As a lipophilic active, ibuprofen permeated rapidly though porcine skin and silicone membrane. After 24 h, more than 460 μg/cm^2 ibuprofen had permeated from PG solutions in porcine skin, for both infinite and finite dose studies. The ibuprofen percentage permeation values for silicone membrane were higher than corresponding values in porcine skin.

In both infinite and finite dose *in vitro* studies in porcine skin, caffeine permeated up to 30 h with more than 70% of the applied caffeine permeating through the skin in the finite dose studies. A possible reason could be that the vehicle PG, which remained in the skin, may form a stratum corneum reservoir. The evaporation of PG on the skin surface also contributed to maintain the saturated state of caffeine and maintained a concentration gradient of caffeine between the donor and receptor phases. It was also confirmed that the increase of concentration of caffeine in the formulations lead to a drop in PG permeation. This may be explained by the effects of the solute caffeine on the activity of the solvent, PG. In addition, compared with ibuprofen, caffeine went through the skin more slowly. More than 460 μg/cm^2 ibuprofen has gone through porcine skin after 24 h, while only around 180 μg caffeine permeated at the end of the 30 h infinite dose study.

In order to compare the permeability of the model drugs in different models, the permeation of both ibuprofen and caffeine will be investigated further in the Franz cell model using human skin and the Skin PAMPA model, which are going to be discussed in Chapter 4 and 5.

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Chapter 4 *In vitro* permeation studies in human skin

4.1 Introduction

As mentioned in Chapter 1, human tissues are the best possible models for *in vitro* studies, as the data collected from such studies should better reflect human *in vivo* performance (Netzlaff et al., 2005). However, there are legal and ethical issues associated with using human tissue. Firstly, human skin samples are usually obtained from skin banks or by donation from a patient undergoing a surgical procedure. Thus researchers have little control over the handling of the tissues. For example, before a surgical procedure, the skin is commonly swabbed to remove microorganisms to minimize potential infection. Such disinfectants usually contain alcohol, which might altering the membrane barrier properties, or cetrimide, which might damage the skin (Williams, 2003). Furthermore, the tissues will have been stored for a period of time before being shipped to researchers. This additional process may also result in damage to, or degradation of, the tissue samples.

Secondly, high data variability between skin tissues obtained from different donors or different sites of the body increases the difficulties of the studies, and raises the cost consequently (Batheja et al., 2009).

The aims of the studies in this chapter are as follows:

- To perform both *in vitro* permeation studies and mass balance studies in human skin in the Franz cell model to investigate the permeation behaviour of ibuprofen from different formulations. The selected formulations are IBUGEL® (ibuprofen 5% w/w), IBULEVE® Speed Relief 5% Spray (5% w/w), and two ibuprofen formulations in binary solvent systems prepared as 5% w/w solutions in isopropyl alcohol and either PEG 300 or PG. These formulations were also tested in porcine skin (as described in Chapter 3). Therefore the results from human skin can be compared with those from porcine skin, and hence there can be comparisons of the ibuprofen permeability between these two membranes;
- To perform *in vitro* permeation studies and mass balance studies in human skin in the Franz cell model to investigate the permeation behaviour of caffeine from various commercial formulations, namely a gel (L'Oréal® Perfect Firm Pro Intense Cellulite Massage System), a serum (Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum) and a hydrogel patch (L'Oréal® Sublime Slime Patch). These formulations will also be tested with the Skin PAMPA model. The results from the human skin Franz model will be compared with those from the Skin PAMPA model, in order to verify if the novel Skin PAMPA model may be used to predict passive permeability of caffeine from the various formulations.

4.2 Materials and methods

4.2.1. Materials

4.2.1.1 Chemicals and tissues

Ibuprofen was a gift from Wyeth (Haversham, Hants., UK). Analytically pure standards of polyethylene glycol (PEG) 300 and propylene glycol (PG) were obtained from Sigma-Aldrich (UK). Analytically pure standards of caffeine were purchased from Fisher Scientific (UK).

HPLC grade water, acetonitrile (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade) and absolute ethanol were purchased from Fisher Scientific (UK). Phosphate buffered saline (pH 7.4 at 25°C) was prepared using Dulbecco A Tablets (Oxoid, UK).

Excised abdominal human skin was obtained from the UK Human Tissue Bank and was stored in a freezer at -20°C until required (Research Ethics Committee reference 06/MRE04/37).

The ibuprofen commercial formulations selected for evaluation were IBUGEL® (Ibuprofen 5% w/w, Dermal Laboratories, Hitchin, Hertfordshire, UK) and IBULEVE® Speed Relief 5% Spray (Dermal Laboratories, Hitchin, Hertfordshire, UK). The caffeine commercial formulations selected were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum,

L'Oréal® Perfect Firm Pro Intense Cellulite Massage System, and L'Oréal® Sublime Slime Patch.

4.2.1.2 Instruments

A water bath (SUB Aqua 26) was obtained from Grant (UK), an electronic balance (Sartorius 1702) was from Sartorius (Germany), a sonicator (PDL 356) was obtained from Camlab Serving Science (UK). The HPLC system used consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. The software used to acquire and analyse the data was ChemStation® for LC 3D Rev. A. 09.03 (Agilent Technologies, U.S.A.). A water purification system with a 75 L reservoir (Elga Option 3) was obtained from Veolia Solutions and Technologies (UK). A rotator (SB2) was from Stuart Equipment (U.S.A.). A centrifuge (5415R) was from Eppendorf (U.S.A.).

4.2.2 Methods

4.2.2.1 Preparation of human tissue

Human female abdominal tissue, obtained with appropriate ethical approval, was removed from -20°C storage and thawed out at room temperature for 4 h. Excess subcutaneous fat was removed by sharp dissection and tissue was submerged for 45 seconds in approximately 4 L of distilled water heated to 60°C. Tissue was placed stratum corneum facing up and a corner of the upper surface was gently rubbed with forefinger to separate the epidermis. The heat separated epidermis was placed on a ridged surface, covered with filter paper and stored at -20°C until required. Prior to *in vitro* permeation studies sections of skin approximately 16 mm in diameter were mounted on a single piece of filter paper and clamped in between the donor and receptor compartments of the Franz cell.

4.2.2.2 *In vitro* finite dose Franz cell studies in human skin for ibuprofen

Vertical glass Franz diffusion cells were used to perform these finite dose studies and the method used was as previously described in Section 3.2.2.3.1 except for the amounts of applied formulations.

For the finite dose study on human skin, selected test formulations include IBULEVE® Speed Relief Spray, IBUGEL®, and two simple ibuprofen solutions. These two ibuprofen solutions were prepared as PG: ibuprofen: IPA 15.6: 5.0: 79.4% (w/w) and PEG300: ibuprofen: IPA 15.3: 5.0: 79.7% (w/w). As for the selected commercial formulations, both of these solutions contained 5% ibuprofen. In addition, with these compositions, after the evaporation of IPA the residual phase (ibuprofen and vehicle) was 80% of the saturated solubility of ibuprofen in that vehicle.

3.6 µL of IBULEVE® and the 5% w/w ibuprofen solutions in either PEG 300 or PG were applied to the skin surface. 4 µL of IBUGEL® were aapplied to the skin surface. IBUGEL® was spread over the diffusion area using a glass Pasteur pipette with a melted tip.

The experiment was conducted up to 48 h. At specified intervals, 200 µL samples were withdrawn from the receptor compartment and an equivalent amount of pre-warmed receptor phase was added in order to maintain a constant volume; all samples were then analysed by HPLC.

Mass balance studies were also conducted after permeation studies were completed. The method used was as previously described in Section 3.2.2.3.1 except for the amounts of applied formulations.

4.2.2.3 *In vitro* Franz cell studies in human skin for caffeine

4.2.2.3.1 *In vitro* Franz cell studies in human skin for semi solid caffeine formulations

4.2.2.3.1.1 Analysis of caffeine content of commercial formulations

The selected commercial formulations in this study were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum and L'Oréal® Perfect Firm Pro Intense Cellulite Massage System. In order to analyse the content of these two formulations, 10 µL of the formulation was diluted in methanol to 10 mL in volumetric flasks. The flasks were shaken to ensure all the caffeine was dissolved. The solutions were then analysed by HPLC to quantify the content of caffeine.

4.2.2.3.1.2 *In vitro* finite dose Franz cell studies in human skin for commercial caffeine formulations

Permeations studies were performed in human skin. Assembled Franz cells were filled with PBS pH 7.4 which served as the receptor fluid for studies that lasted for 48 h at 32 ± 1°C. 10 µL of selected formulations were applied to

the membrane surface. 200 µL of receptor fluid was removed at specified intervals and replaced with fresh receptor fluid.

The selected formulations were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum and L'Oréal® Perfect Firm Pro Intense Cellulite Massage System. After permeation experiments a mass balance study was conducted to account for the applied dose. The method used was as previously described in Section 3.2.2.3.3.

4.2.2.3.2 *In vitro* Franz cell studies in human skin for caffeine hydrogel patch

4.2.2.3.2.1 Analysis of the caffeine content for the commercial caffeine hydrogel patch

L'Oréal® Sublime Slime Patch is a hydrogel patch. In order to analyse the content of the hydrogel formulation, various sizes of the patch were cut and weighed individually. The weighed piece of the patch was placed in a plastic centrifuge tube (n=3) containing 1 ml of methanol, and the patch was cut into smaller pieces to ensure complete extraction of caffeine from the patch. The tubes for extraction were then sealed with Parafilm® , placed in a micro plate shaker (PMS-1000i, Grant, UK) at 1200 rpm for 24 h at room temperature.

Samples obtained from extractions were centrifuged at 13200 rpm for 15 min at 32°C and analysed by HPLC to quantify the content of caffeine.

4.2.2.3.2.2 *In vitro* Franz cell studies in human skin for the commercial caffeine hydrogel patch

The patch was cut into circles to cover the skin, and was placed on the surface of the skin. The patch and skin were sandwiched between the donor and the receptor chambers of the Franz cells. The Franz cells were then placed into a water bath with a temperature of 32 ± 1^{o}C. The receptor phase was PBS, pH 7.4. At specific intervals, 200 µL samples were taken, and 200 µL fresh receptor phase was replaced. Samples were analysed by HPLC.

4.3 Results and discussion

4.3.1 *In vitro* finite dose Franz cell studies in human skin for ibuprofen

The four ibuprofen formulations, namely IBUGEL® (ibuprofen 5% w/w) and IBULEVE® Speed Relief 5% Spray (5% w/w) and two ibuprofen formulations in binary solvent systems prepared as 5% w/w solutions in isopropyl alcohol and either PEG 300 or PG, were selected for the finite dose studies in human skin.

Cumulative amounts of ibuprofen permeated from the various formulations and the corresponding percentages permeated are shown in Figures 4.1 and 4.2, respectively.

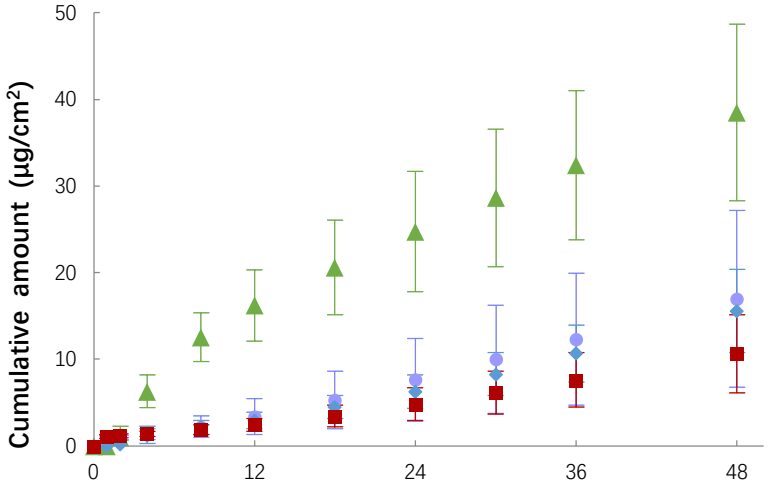

Figure 4.1 Cumulative amounts of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) for finite dose Franz cell studies in human skin. Each data point represents the mean ± SD (n≥5).

For the Franz cell studies in human skin, maximum amounts of permeation were ~ 40 µg/cm^2. Typical curvilinear permeation profiles for all formulations were observed. Significantly higher permeation was observed for the PG formulation ($p < 0.05$) in this case.

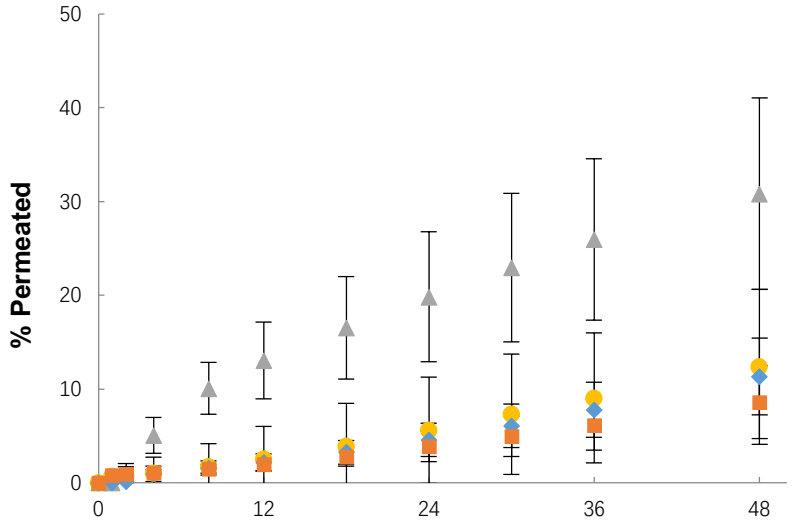

Figure 4.2 Percentages of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) during finite dose Franz cell studies on Human skin. Each data point represents the mean ± SD (n≥5).

At 24 h the maximum average percentage permeation of ibuprofen was only around 4% in human skin, with the exception of the PG formulation where 20% of the dose permeated (Figure 4.2). Although PG is also present in IBUGEL®, the amount used in the volatile PG solution has been adjusted to ensure optimal thermodynamic activity of ibuprofen (see Session 3.2.2.2). This likely explains the superior permeation of ibuprofen from this vehicle.

Results for the mass balance study after the permeation using human skin are shown in Figure 4.3.

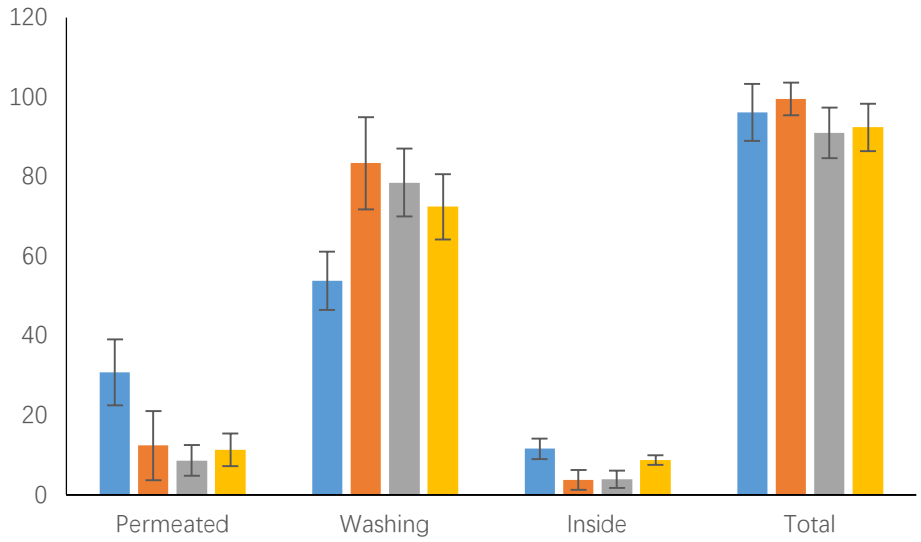

Figure 4.3 Mass balance results for ibuprofen for permeation from PG (■), PEG 300 (■), IBULEVE® (■), and IBUGEL® (■) formulations for finite dose Franz cell studies in human skin. Each data point represents the mean ± SD (n≥5).

The recovery values of ibuprofen for all the selected formulations were between 90-100%, with values of 96.12 ± 7.13 % for PG, 99.52 ± 4.14 % for PEG 300, 91.00 ± 6.40 % for IBULEVE®, and 92.4 ± 5.95 % for IBUGEL®, separately. The percentages of ibuprofen permeated through human skin were about 30, 13, 9 and 11% from the four formulations, separately. Around 70-85% remained on the skin surface, with the exception of PG formulation (~55%). Approximately 10, 4, 4 and 9% of the active was extracted from the skin from PG and PEG 300 formulations, IBULEVE® and IBUGEL®.

4.3.2 *In vitro* Franz cell studies in human skin for caffeine

4.3.2.1 *In vitro* Franz cell studies in human skin for semi-solid caffeine formulations

4.3.2.1.1 Analysis of caffeine content for commercial formulations

The selected commercial formulations in this study were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum and L'Oréal® Perfect Firm Pro Intense Cellulite Massage System. The amounts of caffeine in the two formulations are shown in Table 4.1.

Table 4.1 Content of caffeine in the two tested formulations, mean ± SD, n=3

	L'Oréal®	**EVELINE®**
Content of caffeine (w/w, %)	3.21±0.10	1.36±0.07
Ingredients	Aqua/water, alcohol, denat., cyclohexasiloxane, propylene glycol, glycerine, cyclopentasiloxane, ammonium polyacryloyldimethyl taurate, dimethicone, triethanolamine, silica, salicylic acid, hdi/trimethylol hexyllactone crosspolymer, sodium citrate, butylene glycol, PEG/PPG-18/18 dimethicone, dimethiconol, escin, menthe piperita/peppermint leaf extract, ruscus aculeatus/ruscus aculeatus root extract, pisum sativum/Pea extract, Ginkgo biloba/ginkgo biloba leaf extract, dioscorea opposite/wild yam root extract, PEG-6 caprylic/capric glycerides, T-butyl alcohol, potassium sorbate, methylparaben, chlorphenesin, ci 19140/yellow 5, ci 42053/green 3, parfum/fragrance, geraniol, hexyl cinnamal, limonene, linalool (FIL B12869/1).	Aqua/water, glycerine, butylene glycol, centella asiatica leaf extract, cetyl-alcohol, glycine soja oil, alcohol, haminaria hyperborean extract, propylene glycol, hedera helix leaf extract, ginkgo biloba leaf extract, glyceryl stearate SE, solium polyacrylate, dimethicone, sodium salicylate, lecithin, silica, hyaluronic acid, malus domestica fruit cell culture extract, Xanthan Gum, diamond powder, hydrolysed collagen, hydrolysed elastin, mentol, maris sal DMDM hydantoin, phenoxyethanol, methylparaben, butylparaben, ethylparaben, propylparaben parfum, limonene, CI 77019, CI 77891.

4.3.2.1.2 *In vitro* finite dose Franz cell studies in human skin for the two commercial caffeine formulations

Finite dose permeation studies were conducted for the two commercial caffeine formulations in Franz cells using human skin. Figure 4.4 shows the permeation profiles for these studies.

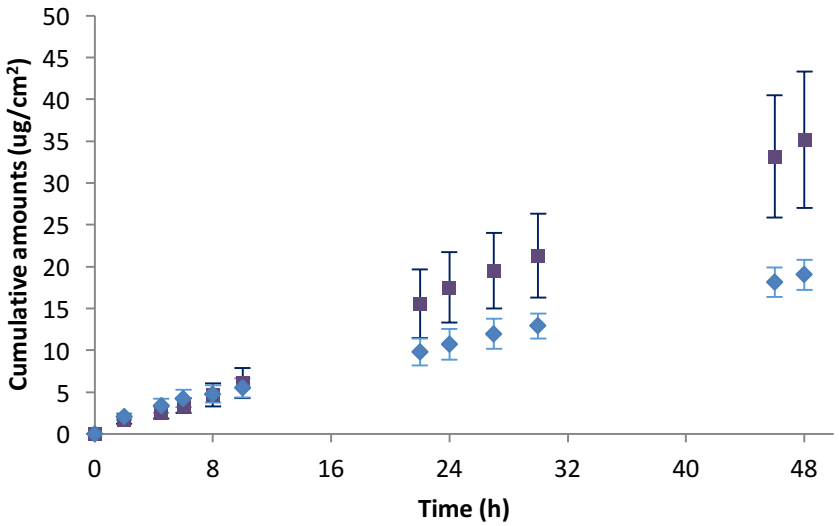

Figure 4.4 Cumulative amounts of caffeine permeated from EVELINE® (■) and L'Oréal® (◆) formulations for finite dose Franz cell studies with human skin after 48 h at 32 ± 1°C, mean ± S.D, n=5.

It can be seen from Figure 4.4 that comparing to the L'OREAL gel, the serum EVELINE® formulation results in higher permeation. After 48 h, around 35 μg/cm^2 of caffeine went through the skin from the EVELINE® serum, while significantly lower amounts of the active penetrated from the L'Oréal® formulation ($p < 0.05$), with a value of about 20 μg/cm^2. However, the content of caffeine in the L'Oréal® gel was 3.21 ± 0.10 %, while the value in EVELINE® serum was only about half, which was 1.36 ± 0.07 %. As shown in Table 4.1,

the ingredients of these two formulations were much different, so this may explain why they show different efficiency for delivering caffeine.

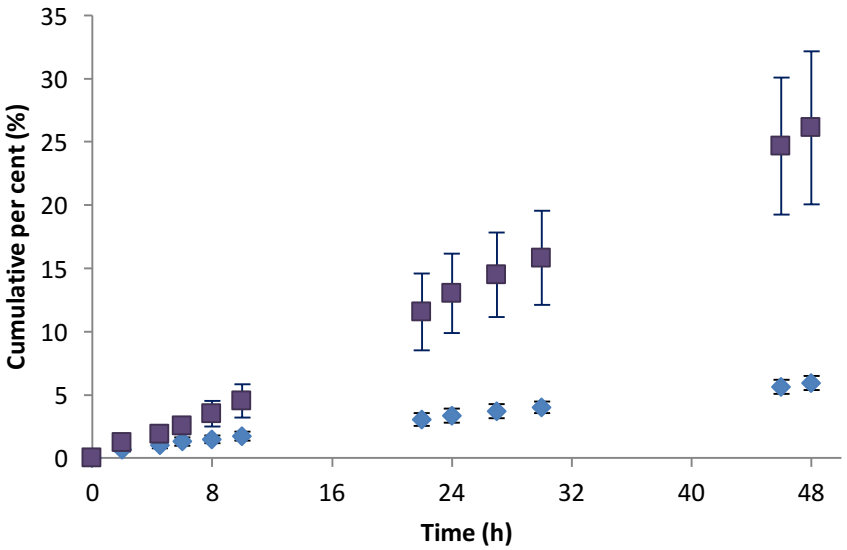

Figure 4.5 Cumulative percent of caffeine permeated from EVELINE® (■) and L'Oréal® (◆) formulations for finite dose Franz cell studies in human skin after 48 h at 32 ± 1°C, mean ± S.D, n=5.

Figure 4.5 shows the cumulative percentage permeation of caffeine from the two formulations Figure 4.5 shows that around 25% of caffeine permeated from the applied dose of EVELINE® serum, whereas only around 6% permeated from the dose of L'Oréal® gel, ($p < 0.05$).

Results for the mass balance study after the permeation studies using human skin are shown in Figure 4.6.

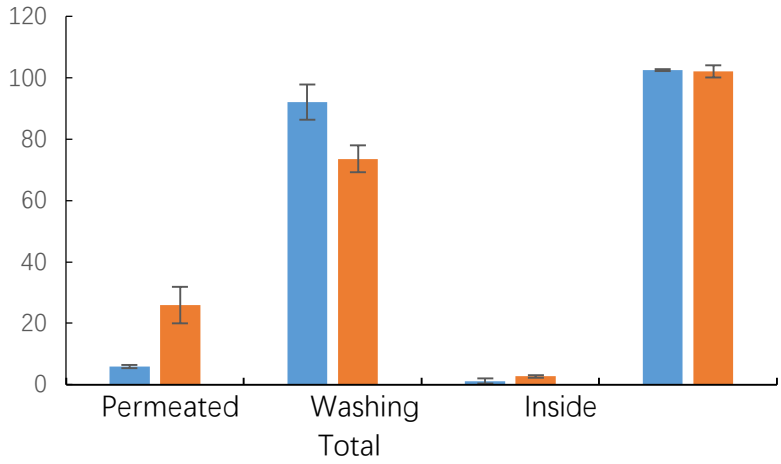

Figure 4.6 Mass balance results for caffeine for permeation from L'Oréal® (■) and EVELINE® (■) formulations for finite dose Franz cell studies in human skin, mean ± S.D, n=5.

The recovery values of caffeine for both of the selected formulations were between 95-105%, with values of 102.55 ± 0.89 % for L'Oréal® and 102.06 ± 1.14 % for EVELINE®. About 6 and 25% of caffeine permeated through the skin from L'Oréal® and EVELINE®, separately and around 90 and 75% remained on the skin surface; approximately 1-2% of the active was extracted from the skin.

4.3.2.2 *In vitro* Franz cell studies with human skin for caffeine hydrogel patch

4.3.2.2.1 Analysis of the content for the commercial caffeine hydrogel patch

The ingredients of the patch are listed in Table 4.2.

Table 4.2 The ingredients of the L'Oréal® Sublime Slime Patch

	L'Oréal® Sublime Slime Patch
Ingredients	Aqua/water, glycerin, sodium polyacrylate, polyacrylic acid, tartaric acid, methanol, polysorbate 80, caffeine, escin, disodium, rutinyl disulfate, ginkgo biloba/ ginkgo biloba leaf extract, butylene glycol, pisum sativum/ PEA extract, siloxanetriol alginate, phenoxyethanol, methylparaben, propylparaben, potassium sorbate, chlorphenesin.

The content of caffeine in the L'Oréal® Sublime Slime Patch was determined to be 0.29 ± 0.01 %.

4.3.2.2.2 *In vitro* Franz cell studies in human skin for the commercial caffeine hydrogel patch

Figure 4.7 and 4.8 show the permeation profiles of caffeine from the commercial patch and the related percentage permeation.

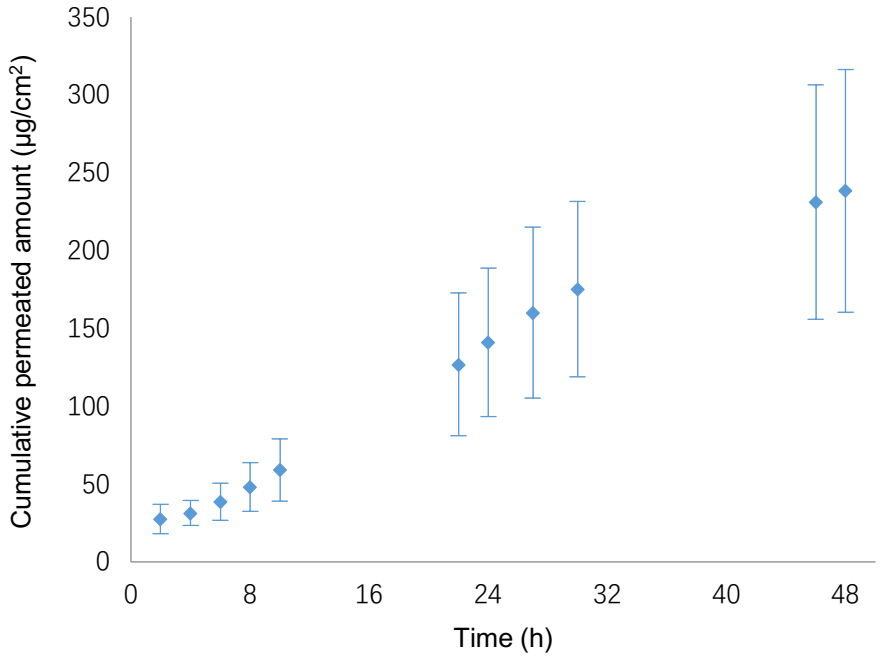

Figure 4.7. Permeation profile of caffeine from the L'Oréal® Sublime Slime?? in Franz cells using human skin, mean ± SD, n=6

At 48 h, approximately 250 µg/cm^2 of caffeine from the patch penetrated through human skin, which was about 8-10 times more than from the tested semi-solid commercial caffeine formulations. However, the applied dose of the selected formulations in the permeation studies were also much different. The dose for the EVELINE® serum and L'Oréal® gel was10 µL which approximates to around 0.32 and 0.14 mg/cm^2 of caffeine, separately, while the caffeine dose from the patch was about 0.96 mg/cm^2. Moreover, the different content may also mean different saturation levels, which would affect the thermodynamic activity of the active during permeation.

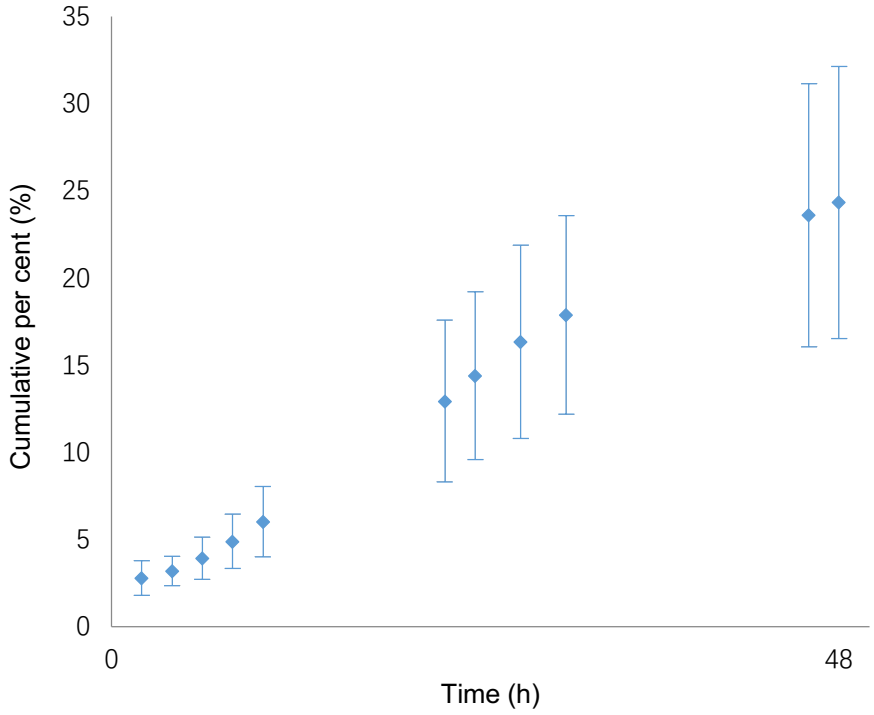

Figure 4.8 Percentage of cumulative permeated amounts of caffeine from the L'Oréal® patch, mean ± SD, n=6

Figure 4.8, shows that after 48 h, only about 25 per cent of the dose has been delivered through the skin.

4.4 Conclusions

In vitro permeation studies were conducted in conventional Franz cell models using human skin for various ibuprofen and caffeine formulations.

For the Franz cell studies in human skin, the maximum amounts of ibuprofen permeation were ~ 40 μg/cm^2. Typical curvilinear permeation profiles for all formulations were observed for finite dose studies. At 24 h the maximum average percentage permeation of ibuprofen was only around 4%, with the exception of the PG formulation where 20% of the dose permeated.

As expected, the ibuprofen percentage permeation values for human skin were much lower than corresponding values in porcine skin. Higher permeation in porcine skin compared with human skin has also been observed by other researchers (Dick and Scott, 1992, Singh et al., 2002, Barbero and Frasch, 2009).

In contrast to ibuprofen, caffeine is a hydrophilic active. After 48 h, around 35 μg/cm^2 of caffeine went through the skin from the EVELINE®, with about only 20 μg/cm^2 of the active penetrated from the L'Oréal® formulation. In addition, around 25% of caffeine permeated from the applied dose of EVELINE® serum, whereas only around 6% permeated from the dose of L'Oréal® gel, which shows that EVELINE® serum has better efficiency in delivering caffeine. The permeability of caffeine shown in human skin is going to be compared with that in the Skin PAMPA model which is going to be discussed in Chapter 5. The comparisons between these two models may verify if the novel Skin PAMPA model may be used as an effective screen tool to predict passive caffeine permeation.

4.5 References

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Chapter 5 *In vitro* permeation studies in the Skin PAMPA model

5.1 Introduction

Since the introduction of the Biopharmaceutics Classification System (BCS)(Amidon et al., 1995), it has been necessary to develop efficient and effective permeability measurement strategies to classify actives at the earliest stages of drug discovery.

In 1998, Kansy et al (1998) developed a simple but high throughput strategy, the Parallel Artificial Membrane Permeability Assay (PAMPA), to predict passive membrane permeability of drugs. With this strategy, the measurement efficiency of epithelial permeability was highly improved. Hundreds of drugs could effectively be analysed in a day.

Due to their high throughput and low cost, PAMPA models have attracted increasing industry interest in recent years. Several pharmaceutical companies have developed their own models. The current existing PAMPA models include GIT-PAMPA, which is used for the prediction of gastrointestinal absorption (Ruell et al., 2003; Avdeef et al., 2007; Avdeef and Tsinman, 2006), BBB-PAMPA, which is used to model the blood brain barrier (Tsinman et al., 2011) and Skin PAMPA, which is applied to estimate skin penetration (Ottaviani et al., 2006).

PAMPA is based on a 96-well microtiter plate technology (Thompson et al., 1980; Thompson et al., 1982), which is completely artificial and without pores and active transporter systems. Microtitre plate technology allows 96 permeation cells to be simultaneously studied, resulting in high throughput but low cost. The PAMPA studies are performed in a 2-chamber PAMPA Sandwich with microtitre plates (see later). It is similar to the device used in cell-based assays, such as Caco-2 transwells (Kansy et al., 2004).

The Skin PAMPA Sandwich consists of two 96 well plates, namely a top plate and a bottom plate. The Skin PAMPA membranes are pre-coated on the top plate to separate the two compartments (donor and receptor). A schematic of the Skin PAMPA Sandwich is shown in Figure 5.1.

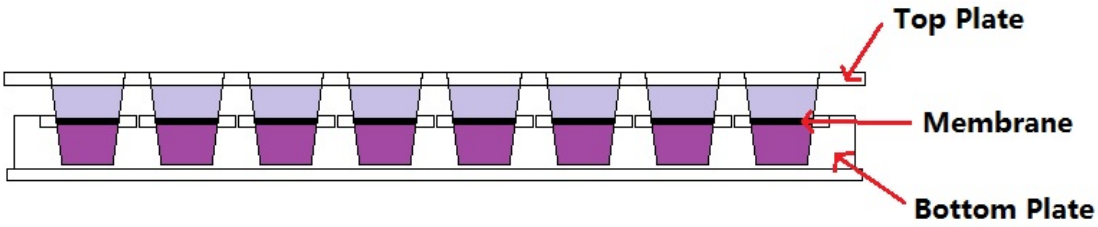

Figure 5.1 Schematic view of the Skin PAMPA Sandwich

The PAMPA membrane usually contains lipid mixtures. The original PAMPA method (Kansy et al., 1998) includes a filter-immobilized membrane by infusing a lipophilic microfilter with 10% (w/v) egg lecithin dissolved in n-dodecane. Sinkó et al. (Sinkó et al., 2012) developed a new Skin PAMPA model, in which the membrane components consist of ceramides, cholesterol

and free fatty acids, which is similar to the real human stratum corneum lipid matrix.

The aims of the studies in this chapter are as follows:

- To verify if the novel Skin PAMPA model may be suitable to classify and predict passive permeability of ibuprofen, a model lipophilic compound. The following assays will be designed for ibuprofen formulations to evaluate this model:
    1) Assay 1: to study ibuprofen permeation from an infinite dose (30 or 200 µL per well) PG solution and water solution on the Skin PAMPA model with the top plate designed to be a donor;
    2) Assay 2: to study ibuprofen permeation from an infinite dose (30µL per well) slurry of ibuprofen in DI water in the Skin PAMPA model with or without PBS in the empty donor well prior to adding formulation to investigate if empty donor wells may cause bubbles under the membrane;
    3) Assay 3: to study ibuprofen permeation from an infinite dose (200 µL per well) DI water, buffer or methanol solutions with different ibuprofen concentrations in the Skin PAMPA model with the bottom plate designed to be a donor;
    4) Assay 4: to study ibuprofen permeation from an infinite dose (200 µL per well) methanol solutions with different ibuprofen concentrations in the Skin PAMPA model with the top plate designed to be a donor;

5) Assay 5: to study ibuprofen permeation from an infinite dose (200 µL per well) different commercial formulations and PG solutions in the Skin PAMPA model with the bottom plate designed to be a donor;
6) Assay 6: to study ibuprofen permeation from an infinite dose (30 µL per well) PG solutions and two commercial formulations with different ibuprofen concentrations in the Skin PAMPA model with the top plate designed to be a donor;
7) Assay 7: to identify the amount of the ibuprofen formulation which best represents finite dose conditions by applying different doses, namely 1, 3, and 30 µL per well while using the top plate as a donor;
8) Assay 8: to study ibuprofen permeation from semi-finite dose commercial formulations in the Skin PAMPA model using the lip as donor compartment;
9) Assay 9: to study ibuprofen permeation from a finite dose of a commercial ibuprofen spray in a Skin PAMPA sandwich where the artificial membrane has been replaced by human skin.

- To evaluate if the novel Skin PAMPA model may be a suitable model to rank and predict passive permeability of caffeine, a model hydrophilic compound. The following assays will be designed for caffeine formulations for examination in this model
    1) Assay 1: to study caffeine permeation from infinite doses (200 µL per well), namely aqueous solutions with various caffeine

concentrations in the Skin PAMPA model with the bottom plate as the donor compartment;

2) Assay 2: to study caffeine permeation from infinite doses (200 µL per well), namely aqueous solutions with various caffeine concentrations in the Skin PAMPA model with or without stirring disks;
3) Assay 3: to study caffeine permeation from infinite doses (200 µL per well), namely PG solutions with various saturated caffeine concentrations in the Skin PAMPA model with the bottom plate as the donor compartment;
4) Assay 4: to study caffeine permeation from infinite doses (30 µL per well), namely PG solutions with various saturated caffeine concentrations in the Skin PAMPA model with the top plate as the donor compartment;
5) Assay 5: to study caffeine permeation from infinite doses (30 µL per well), namely commercial caffeine formulations in the Skin PAMPA model with the top plate as the donor compartment;
6) Assay 6: to study caffeine permeation from finite doses (3 µL per well), namely various commercial caffeine formulations in the Skin PAMPA model with the lip of the Skin PAMPA model as the donor compartment;
7) Assay 7: to identify the amount of caffeine formulation which best represents finite dose conditions by applying various doses, namely 1, 3, and 30 µL per well using the top plate as the donor compartment;

8) Assay 8: to study caffeine permeation from a commercial caffeine hydrogel patch in the Skin PAMPA model;

The purposes of each essay for ibuprofen formulations are summarised in Table 5.1.

Table 5.1 Summary of purposes of each PAMPA experiment for ibuprofen

Assay No.	Purposes
1	To study infinite dose permeation of ibuprofen from PG and water solutions with top plate as donors;
2	To study infinite dose permeation of ibuprofen from slurry in DI water and the effect of PBS in empty donors to avoid producing bubbles;
3	To study infinite dose permeation of ibuprofen from DI water, buffer and methanol solutions with bottom plate as donors, and the effect of different ibuprofen concentrations;
4	To study infinite dose permeation of ibuprofen from methanol solutions with top plate as donors, and the effect of different ibuprofen concentrations;
5	To study infinite dose permeation of ibuprofen from different commercial formulations and PG solutions;
6	To study infinite dose permeation of ibuprofen from two commercial formulations with different ibuprofen concentrations;
7	To identify finite dose conditions of ibuprofen formulations;
8	To study semi-finite dose permeation of ibuprofen with the lip of Skin PAMPA model as a donor;
9	To study finite dose permeation of ibuprofen in a Skin PAMPA where the artificial membrane replaced by human skin.

The purposes of each essay for caffeine formulations are summarised in Table 5.2.

Table 5.2 Summary of purposes of each PAMPA experiment for caffeine

Assay No.	Purposes
1	To study infinite dose permeation of caffeine from aqueous solutions with various concentrations;
2	To study the effect of stirring in the Skin PAMPA model;
3	To study infinite dose permeation of caffeine from PG solutions with different saturated levels of caffeine with the bottom plate as donors;
4	To study infinite dose permeation of caffeine from PG solutions with different saturated levels of caffeine with the top plate as donors;
5	To study caffeine permeation from various commercial formulations with the top plate as donors;
6	To study caffeine permeation from various commercial formulations with the lip of the Skin PAMPA model as a donor;
7	To identify finite dose conditions of caffeine formulations;
8	To study caffeine permeation from a commercial caffeine hydrogel patch.

5.2 Materials and methods

5.2.1. Materials

5.2.1.1 Chemicals and tissues

Ibuprofen was a gift from Wyeth (Haversham, Hants., UK). Analytically pure standards of polyethylene glycol (PEG) 300 and propylene glycol (PG) were obtained from Sigma-Aldrich (UK).

Analytically pure standards of caffeine, 1,2-pentanediol (1,2-pent), 1,3-butylene glycol (1,3-BG)/1,2-butylene glycol (1,2-BG) and propylene glycol (PG) were obtained from Sigma-Aldrich (UK). Geraniol (GER), limonene (LIM), t-butyl alcohol (T-BA), Linalool (Lina) and tripropylene glycol (TPG) were purchased from Fisher Scientific (UK). Isopropyl myristate (IPM), and dimethyl isosorbide (DMI) were gifts from Croda Ltd. (UK). 1, 2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), propylene glycol monocaprylate (PGMC), propylene glycol monolaurate (PGML), Transcutol® (TRANS) were received as gifts from Gattefossé (France). PEG-6-caprylic/capric glycerides (PEG-6-CCG) were a gift from Avon. These solvents are generally regarded as safe (GRAS) chemicals.

HPLC grade water, acetonitrile (HPLC grade), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade) and absolute ethanol were purchased from Fisher Scientific (UK). Phosphate buffered saline (pH 7.4 at 25^{o}C) was prepared using Dulbecco A Tablets (Oxoid, UK).

The commercial formulations selected for evaluation were IBUGEL® (Ibuprofen 5% w/w, Dermal Laboratories, Hitchin, Hertfordshire, UK), IBULEVE® Speed Relief 5% Spray (Dermal Laboratories, Hitchin, Hertfordshire, UK), IBULEVE® Speed Relief Max Strength Gel (Ibuprofen 10% w/w, Dermal Developments Ltd, Hitchin, Herts, UK), NUROFEN® Maximum Strength 10% Gel (Mercury Pharma Group Ltd, Croydon, UK), NUROFEN® 5% Gel (Mercury Pharma Group Ltd, Croydon, UK), IBUPROFEN Gel (Ibuprofen 5% w/w, Boots, Nottingham, UK), IBUPROFEN Gel (Ibuprofen 5%

w/w, Mentholatum Ltd, East Kilbride, UK), IBULEVE® Gel (Ibuprofen 5% w/w, Dermal Developments Ltd, Hitchin, Herts, UK), IBULEVE® Speed Relief Gel (Ibuprofen 5% w/w, Dermal Developments Ltd, Hitchin, Herts, UK), IBUPROFEN Gel (Ibuprofen 5% w/w, Mentholatum Ltd, East Kilbride, UK), and Deep Relief® (Ibuprofen 5%, Mentholatum Ltd, East Kilbride, UK).

The caffeine commercial formulations selected were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum, L'Oréal® Perfect Firm Pro Intense Cellulite Massage System, and L'Oréal® Sublime Slime Patch.

5.2.1.2 Instruments

An electronic balance (Sartorius 1702) was from Sartorius (Germany), a sonicator (PDL 356) was obtained from Camlab Serving Science (UK). The HPLC system used consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. The software used to acquire and analyse the data was ChemStation® for LC 3D Rev. A. 09.03 (Agilent Technologies, U.S.A.). A water purification system with a 75 L reservoir (Elga Option 3) was

obtained from Veolia Solutions and Technologies (UK). A specialised Gut-Box® stirring device was obtained from Pion Inc. (Billerica, USA).

5.2.2 Methods

5.2.2.1 Preparation of Skin PAMPA Plate for the assay

5.2.2.1.1 Hydration step

The membranes of the Skin PAMPA plate appear white originally. During hydration, the membranes become translucent.

To hydrate the membranes, 200 µL of the hydration solution was added into each well of the 96-well plate, which was used as a support plate. The Skin PAMPA top plate was submerged carefully in Hydration solution. The lid was placed on top of the top plate and Parafilm® was placed around the perimeter where the top plate met the bottom reservoir. The bottom part of the Skin PAMPA Sandwich was covered with a lid and set aside. The top plate with membranes was allowed to hydrate overnight. After the wells were hydrated, the top plate was removed from the Hydration solution reservoir and placed on the bottom part of the Skin PAMPA Sandwich. In order to avoid drying out of the membranes, this was completed in 4 - 5 min. The wells of the bottom part of the Sandwich were filled with sample solutions before the top plate was put in position.

5.2.2.1.2 Preparation of the bottom part of Skin PAMPA Sandwich

All sample solutions were maintained at skin temperature, 32 ± 1^{o}C, prior to starting the assay. For infinite dose studies, both the top plate and the bottom plate may be used as the donor compartment. However, if the formulation tested contains volatile ingredients, the donor compartment must be the top plate, otherwise the permeation area may change during incubation.

If the bottom plate is used as the receptor compartment, 200 µL of the receptor phase is transferred into each well of all columns of the bottom plate. If stirring is used, the stirring disks should be placed into the bottom plate before adding 180µL of receptor phase (Pion, 2013).

The hydrated top plate was then placed on the top of the bottom plate. This step was done in such a manner that the bottom row (H) or the last column (12) was used as a guide and the top plate was slowly and gently moved into its final position. The lid was placed on the top of the top plate once the sandwich was assembled. After this the Sandwich was placed into a humidity chamber with a wet sponge to maintain a high relative humidity in order to minimize evaporation.

Alternatively, where the bottom plate is used as the donor compartment, the solutions transferred into the wells are the tested formulations.

5.2.2.1.3 Preparation of formulations and stirring

The incubation time appears to be critical for a Skin PAMPA assay. As the bottom part of the Sandwich has been filled with either tested formulations or receptor phase, the assay will start once the wells of the top plate are filled. Therefore, the filling of the top plate wells needs to be done in a well-organised manner.

For example, provided that the top plate is the donor compartment and there are three different incubation times (20 min, 40 min, and 60 min), the procedure was as follows. To start the assay, the formulations were filled in column 1-4 (Figure 5.2). After filling columns 1-4, the sandwich was placed on the Gut-Box™ and stirring was started.

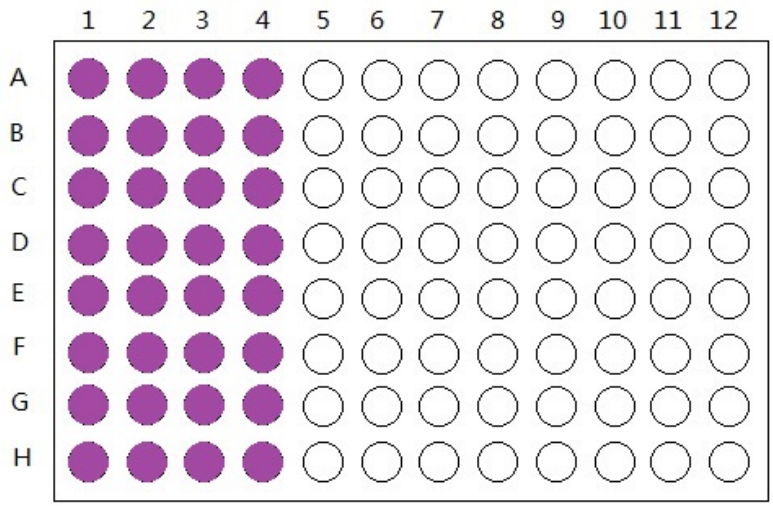

Figure 5.2 At the beginning of the assay, the formulations were filled in column 1-4

At the 20 min incubation time point, without separating the sandwich, the formulations were filled into columns 5-8 (Figure 5.3). The sandwich then was placed back on the Gut-Box™.

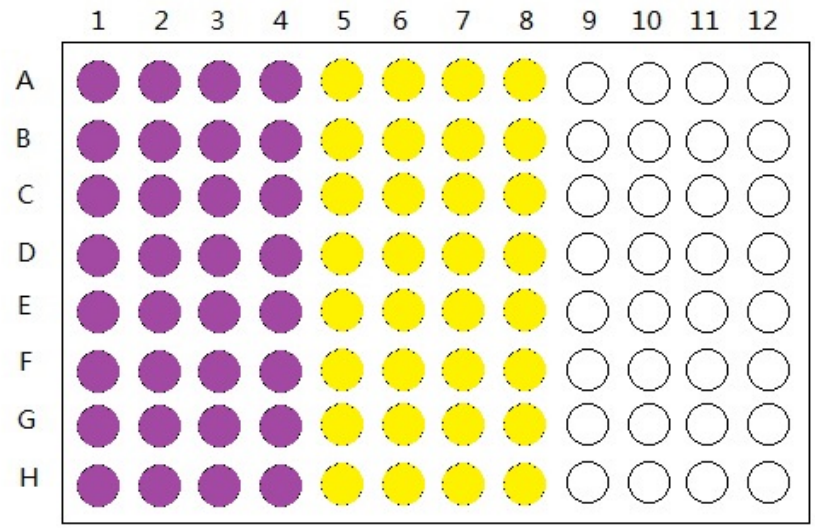

Figure 5.3 After 20 min, the formulations were filled in column 5-8

After another 20 min, the formulations were added into columns 9-12 (Figure 5.4). After all the wells were filled up with formulations, the sandwich was placed back onto the Gut-Box™ and stirring was continued until the end of the incubation time - 60 min in total.

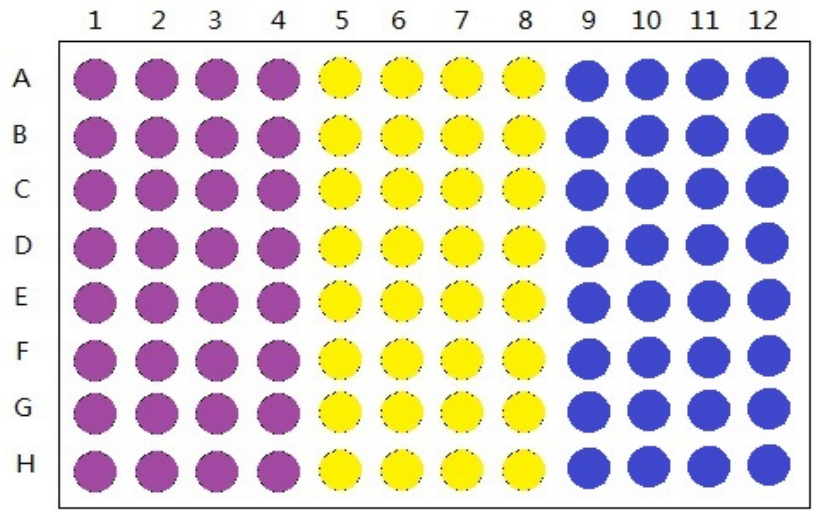

Figure 5.4 After another 20 min, the formulations were filled in column 9-12

After the incubation, the top plate was removed from the bottom receiver plate. A 150 µL sample of the receptor solution was analysed by HPLC. Also, where the tested formulations are solutions, the donor solutions were analysed by HPLC at the end of the assay as well.

The procedure above is an example to understand the Skin PAMPA Assay. In practice, the filling procedures should be designed according to the features of the tested formulations and the purposes of the assays.

5.2.2.2 Assays for ibuprofen

5.2.2.2.1 Assay 1 for ibuprofen

In this assay, two ibuprofen formulations were tested. The first formulation contained 5% ibuprofen, 15.6% PG and 79.4% IPA (Form A). The second formulation was a slurry of ibuprofen in DI water (Form B).

There were 5 different incubation times, namely 0.5, 1, 2, 4, 6 h. The top plate was the donor compartment while the bottom part was the receptor compartment. In this set up, both the finite dose and infinite dose studies could be conducted at the same time, depending on the applied doses on the top plate. Two doses, 30 or 200 µL of formulations were added into the corresponding wells as shown (Figure 5.5). Stirrer plates were placed in each of the wells of the bottom (receptor) plate.

Temperature has a significant effect on permeability, so in order to study whether the temperature of membranes at different locations of the plate were uniform, studies for each formulation were repeated on more than one line for the same plate. The temperature inside the Gut-Box® was controlled to be 32 ± 1 °C. The design of the assay is shown in Figure 5.5.

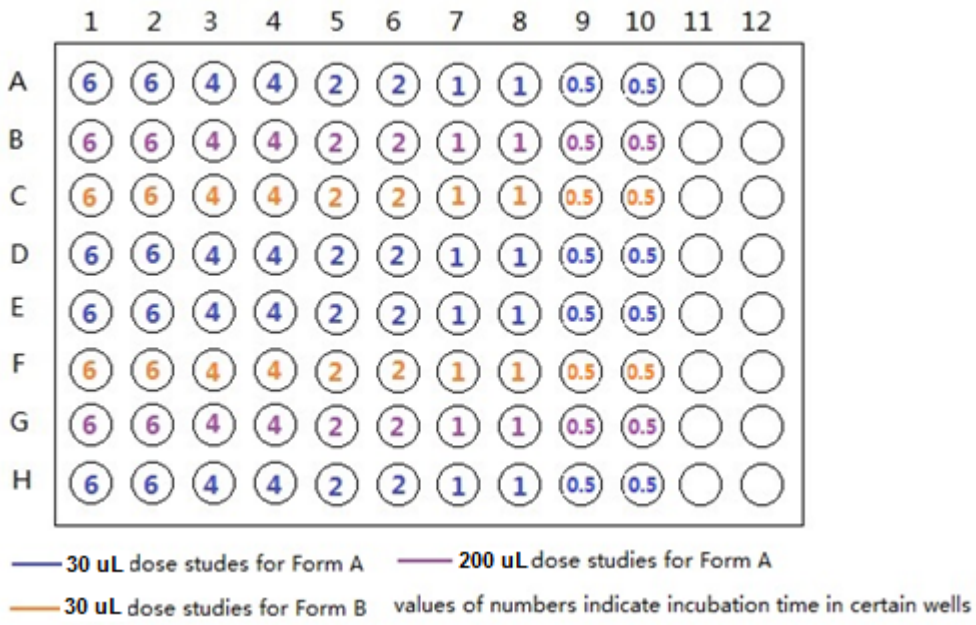

Figure 5.5 Design of Skin PAMPA assay 1 for ibuprofen

After the incubation step, the top plate was removed from the bottom receiver plate. A 150 µL sample of the receptor solutions was analysed by HPLC.

5.2.2.2.2 Assay 2 for ibuprofen

To minimize variables, only a simple formulation which was a slurry of Ibuprofen in DI water was studied in this assay. 6 different incubation times were studied, namely 10, 20, 30, 45, 60 and 120 min. Similar to Assay 1, the top plate was the donor compartment while the bottom part was the receptor compartment. 30 µL of the formulation was added into the corresponding wells as shown in the planned manner (Figure 5.6). Stirrer plates were placed in each of the wells of the bottom (receptor) plate.

Those wells without formulations were observed to cause evaporation problems of the receptor phase, for instance, bubbles were evident under the membranes after around 1 h incubation. Therefore, PBS was added into some of the wells before adding the formulation and compared with those wells without PBS. Prior to adding the formulation, PBS was removed from the wells.

The effects of temperature were also studied in this assay. The design of the assay is shown in Figure 5.6.

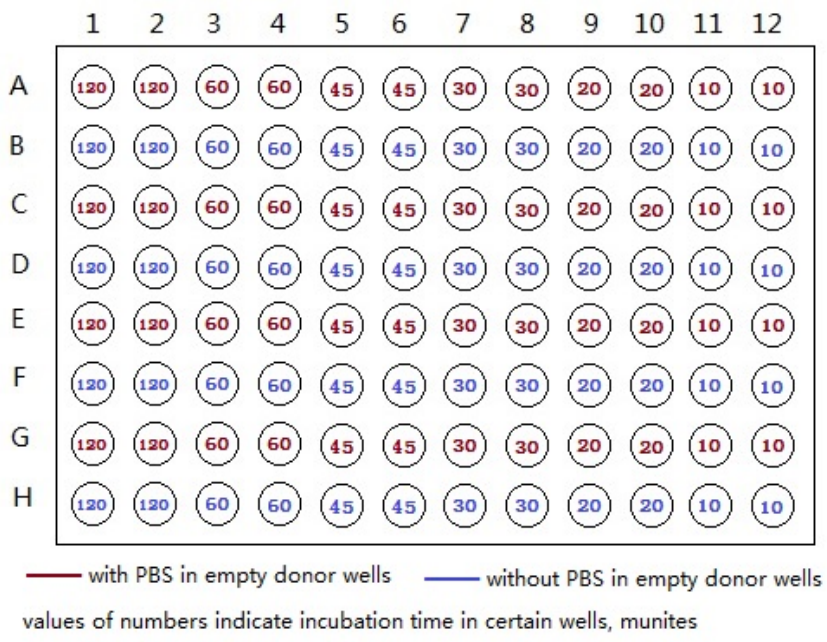

Figure 5.6 Design for Skin PAMPA assay 2 for ibuprofen

After the incubation, the top plate was removed from the bottom receiver plate. A 150 µL sample of the receptor solution was analysed by HPLC.

5.2.2.2.3 Assay 3 for ibuprofen

In this assay, the top plate was the receptor compartment while the bottom part was the donor compartment. Stirrer plates were placed in each of the wells of the bottom (donor) plate. 5 Ibuprofen formulations were studied for 6 incubation times. The formulations were a slurry of Ibuprofen in DI water (Form A), a slurry of ibuprofen in DI water: PEG mixture 50: 50 v/v (Form B), a slurry of ibuprofen in Prisma HT buffer (Form C), 80 µg/mL Ibuprofen in

methanol (Form D) and 20 μg/mL ibuprofen in methanol (Form E). Incubation times were 0.5, 1, 2, 3, 4 and 6 h.

Only 3 columns of the Skin PAMPA Sandwich were used in this case. 200 μL of the specific formulations was added into each of the wells of these columns in the donor compartment. 180 μL of receptor phase was added into corresponding wells in the receptor compartment. At each time point, 120 μL samples were withdrawn from each well in the receptor plate and an equivalent amount of pre-warmed receptor phase was added to maintain a constant volume. All samples were then analysed by HPLC.

After the incubation, the top plate was removed from the bottom receiver plate. All the data obtained from HPLC were then recorded by MS Excel® (Microsoft Corp., USA). Figure 5.7 shows the layout of the wells used in the Sandwich in this case.

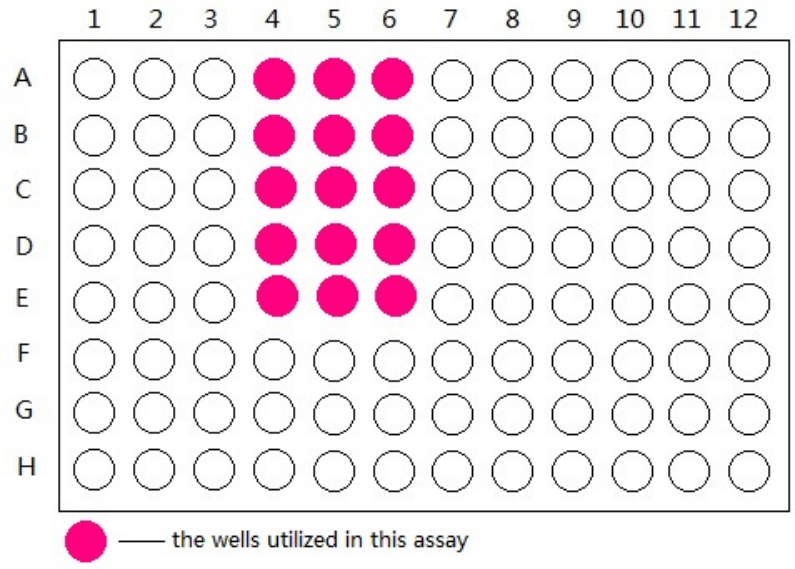

Figure 5.7 Utilization of the Sandwich in Skin PAMPA assay 3 for ibuprofen

5.2.2.2.4 Assay 4 for ibuprofen

Due to the obvious evaporation of the volatile compositions in the formulations in the previous assay, which led to separation between the donor phase and membrane, the top plate was used as the donor compartment and the bottom plate was the receptor compartment this time. Stirrer plates were placed in each of the wells of the bottom (receptor) plate. All the formulations tested were ibuprofen / methanol formulations, which were 80 µg/mL Ibuprofen in methanol, 40 µg/mL ibuprofen in methanol, 20 µg/mL ibuprofen in methanol, and 10 µg/mL ibuprofen in methanol.

Incubation times were 0.5, 1, 2, and 3 hours. 180 µL of receptor phase were added into each well in the bottom plate before the Sandwich was assembled. 200 µL of specified formulations were added into corresponding wells in the top plate as planned manner (Figure 5.8). The layout of the wells for this assay is shown in Figure 2.8.

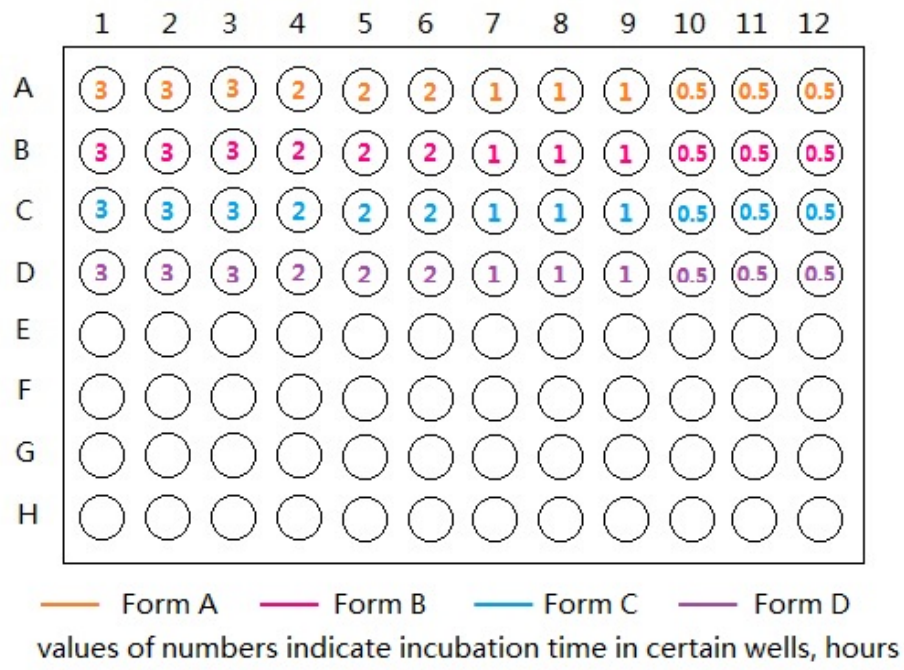

Figure 5.8 Design for Skin PAMPA assay 4 for ibuprofen

After the incubation step, the top plate was removed from the bottom receiver plate. 150 µL of the receptor solutions were analyzed by HPLC. All the data obtained with HPLC were then recorded by MS Excel® (Microsoft Corp., USA).

5.2.2.2.5 Assay 5 for ibuprofen

In this assay, the top plate was the receptor compartment while the bottom part was the donor compartment. Stirrer plates were placed in each of the wells of the bottom (donor) plate. 10 Ibuprofen commercial formulations and 3 simple ibuprofen solutions in PG with different concentrations were studied for 6 h incubation times. The formulations studied are listed in Table 5.3. Incubation times are 0.5, 1, 2, 3, 4 and 6 h.

Table 5.3. Tested formulations in assay 5 for ibuprofen

	formulations	manufacturer	Active% w/w
Form A	IBULEVE GEL	DDD Ltd(40g)	10
Form B	NUROFEN GEL	Reckitt Benckiser Healthcare(40g)	10
Form C	NUROFEN GEL	Reckitt Benckiser Healthcare(35g)	5
Form D	IBUPROFEN GEL	BOOTS(50g)	5
Form E	IBUPROFEN GEL	Superdrug	5
Form 1	IBULEVE GEL	DDD Ltd(50g)	5
Form 2	IBULEVE GEL	DDD Ltd(40g)	5
Form 3	IBUPROFEN GEL	Mntholatum(50g)	5
Form 4	Deep Relief GEL	Mentholatum(50g)	5
Form 5	IBUGEL GEL	DERMAL(100g)	5
Form 6	50% saturated IBU in PG	Prepared in lab	-
Form 7	80mg/ml IBU in PG	Prepared in lab	-
Form 8	80ug/ml IBU in PG	Prepared in lab	-

10 columns of the Skin PAMPA Sandwich were used in this case. Around 200 μL of the specific gel formulations was added into each of the wells of the planned columns in the donor compartment. 200 μL of each solution of ibuprofen in PG was also added (Figure 5.9). 180 μL of receptor phase was

added into corresponding wells in the receptor compartment. At each time point, 120 µL samples were withdrawn from each well in the receptor plate and an equivalent amount of pre-warmed receptor phase was added to maintain a constant volume. All samples were then analysed by HPLC. The layout of the wells for this assay is shown in Figure 5.9.

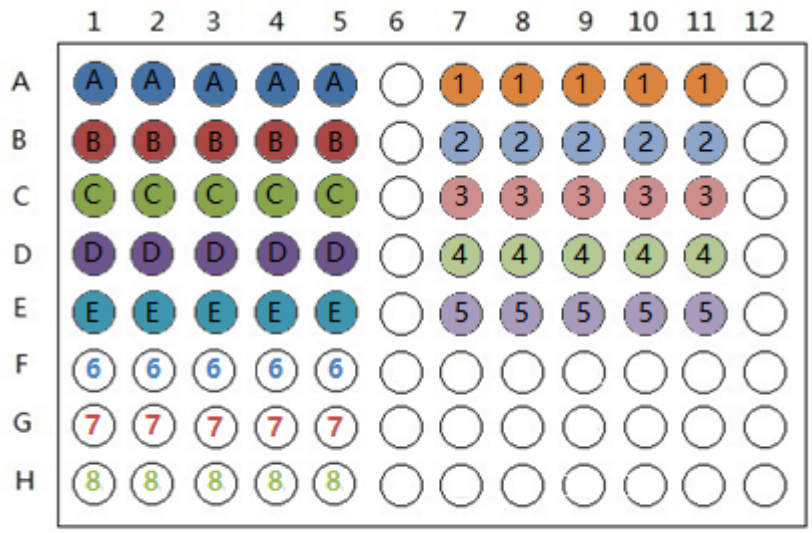

Figure 5.9 Design for Skin PAMPA assay 5 for ibuprofen

5.2.2.2.6 Assay 6 for ibuprofen

5 columns of the Skin PAMPA Sandwich were used in this assay. The dose was designed to be 30 µL per well, and the top plate was used as the donor compartment while the bottom part was the receptor compartment. Stirrer plates were placed in each of the wells of the bottom (receptor) plate, as well as 180 µL of the PBS (receptor phase). Ibuprofen solutions in PG at three different concentrations, namely 296 mg/ml (saturated solution, Form IBU1), 149 mg/ml (50% saturated solution, Form IBU2) and 80 mg/ml (Form

IBU3), were studied for 8 incubation periods. Formulations B and C which were studied in assay 5 were also tested in this assay. The incubation times were 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h. The bottom plate was replaced by a new plate with fresh receptor phase during the specified intervals. The advantages for this operation are that sink conditions were maintained throughout the experiment, and the incubation in all the wells started and stopped at the same time. Figure 5.10 illustrates the layout of the wells for this assay.

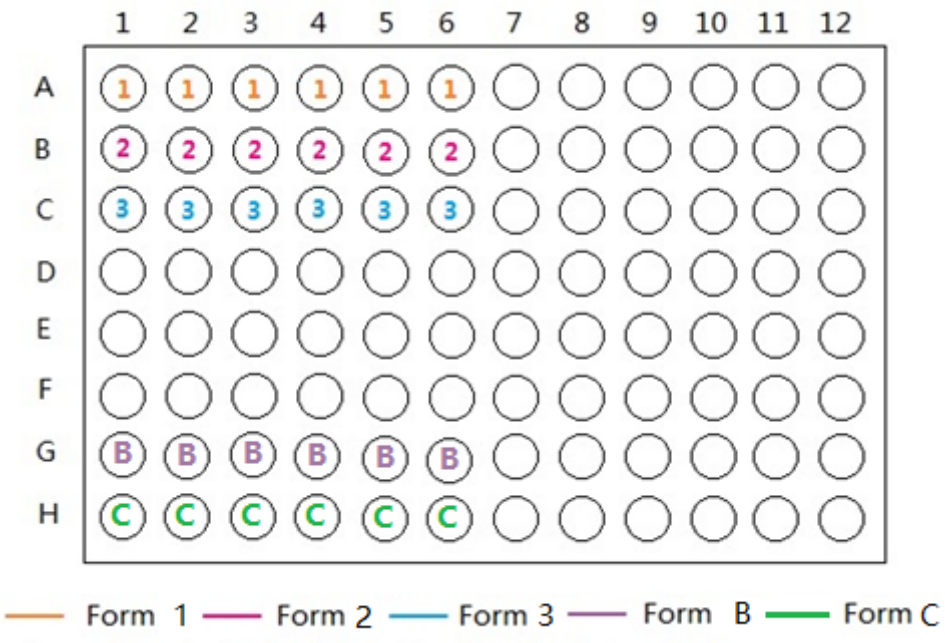

Figure 5.10 Design for Skin PAMPA assay 6 for ibuprofen

5.2.2.2.7 Assay 7 for ibuprofen

Two commercial ibuprofen formulations, IBUGEL® (ibuprofen 5% w/w) and IBULEVE® Speed Relief 5% Spray (5% w/w), and two 5% ibuprofen solutions in isopropyl alcohol and either PEG 300 or PG were studied in this assay. The top plate was used as the donor compartment. Three different doses, 1, 3, or 30 µL per well, were investigated in order to determine the amount of formulation which best represented finite dose conditions. These doses correspond to 3.3, 9.9 and 99 µL/cm^2. The bottom plate was replaced by a new plate with fresh receptor phase during the specified intervals, including 0.5, 1, 2, 3, 4 and 6 h. Figure 5.11 shows the layout of the wells for this assay.

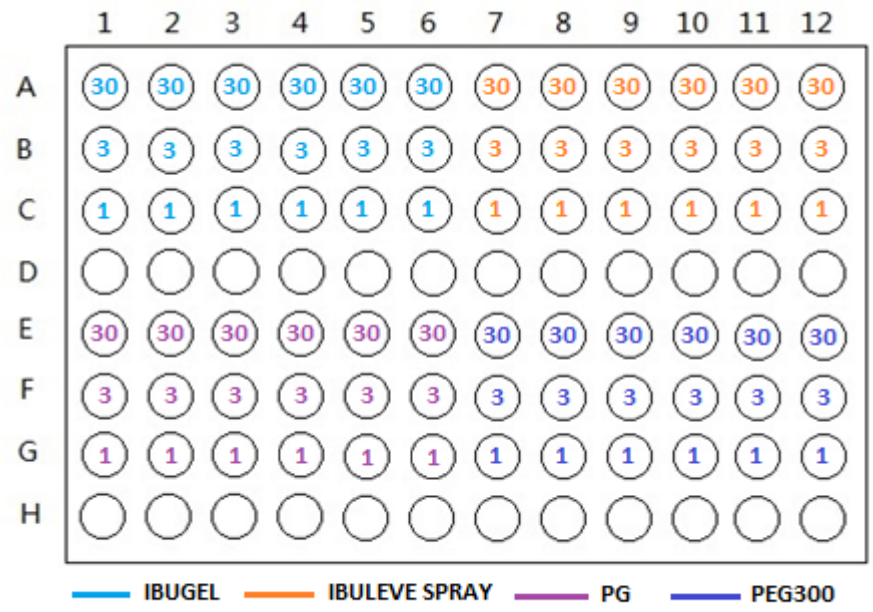

Figure 5.11 Design for Skin PAMPA assay 7 for ibuprofen

5.2.2.2.8 Assay 8 for ibuprofen

Instead of the top plate of the Sandwich, the lip may also act as the donor in the Skin PAMPA model in a finite dose study. In this assay, the tested formulations were applied on the bottom of the membrane, and the top plate was held by a Skin PAMPA lip. The schematic view of this Skin PAMPA set-up is illustrated in Figure 5.12.

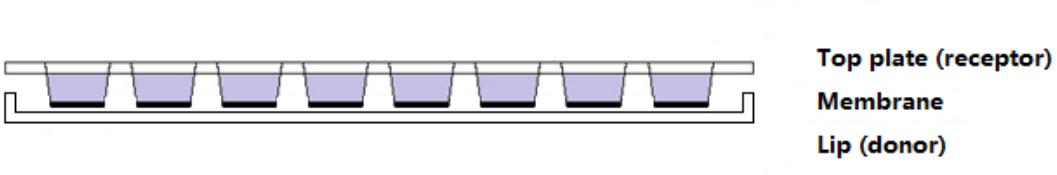

Figure 5.12 Schematic view of the Skin PAMPA with lip as the donor

Two commercial ibuprofen formulations, IBUGEL® (ibuprofen 5% w/w) and IBULEVE® Speed Relief 5% Spray (5% w/w) were selected for study in this assay. 3 µL of each tested formulation was applied under the membrane on the top plate. The top plate was then used as the receptor. Incubation times were 0.25, 0.5, 1, 2, 3, and 4 h. 250 µL of PBS, pH 7.4, was added into corresponding wells of the top plate as the receptor phase. The lip was used as the bottom plate. At each time point, 200µL of the receptor phase was sampled and replaced by the same amount of fresh receptor phase. Stirring was always done in the donor. Figure 5.13 shows the layout of the wells for this assay.

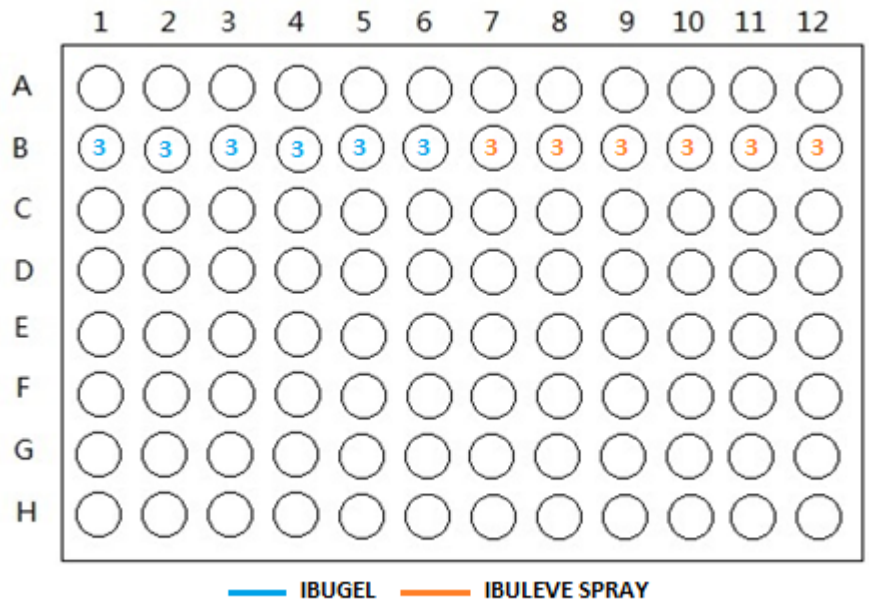

Figure 5.13 Design for Skin PAMPA assay 8 for ibuprofen

5.2.2.5.9 Assay 9 for ibuprofen

The Skin PAMPA obtained from Pion Inc. (Billerica, USA) was pre-coated with an artificial membrane, which mimics normal human skin stratum corneum. However, in this study, the original artificial membrane was removed and then replaced by human skin, in order to study how the Sandwich structure design performed compared with conventional *in vitro* human skin studies.

Human skin was cut to an appropriate size to cover the bottom of 8 wells on the top plate. The bottom plate was used as the receptor compartment. 180 µL of PBS, pH 7.4 was added into each corresponding well as the receptor phase. The receptor plate was replaced by a new bottom plate filled with the fresh receptor liquid at the designated intervals. Stirring was always done in

the receptor compartment and the membrane was kept at 32 ± 1°C. All samples were analysed by HPLC at the end of the experiments.

The tested formulation was IBULEVE® Speed Relief 5% Spray, which contained 5% ibuprofen (w/w). 1 µL of the formulation was applied to each of the Skin PAMPA wells on the donor plate. The layout of the selected wells for this assay is shown in Figure 5.14.

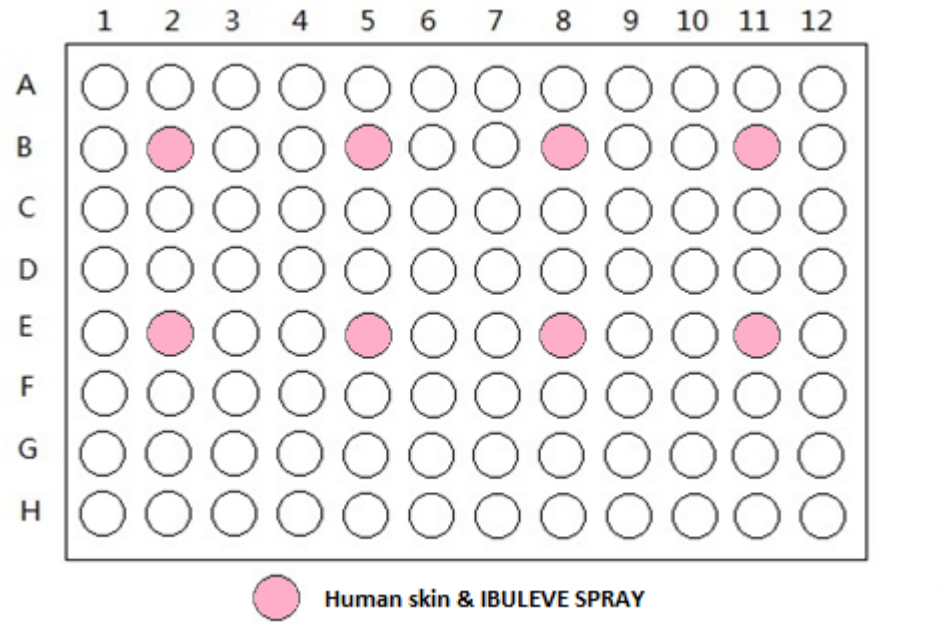

Figure 5.14 Design for Skin PAMPA assay 9 for ibuprofen

5.2.2.3 Assays for caffeine

5.2.2.3.1 Assay 1 for caffeine

This assay was conducted in the same manner as for assay 3 for ibuprofen (session 5.2.2.2.3). In this assay, two caffeine formulations were tested. One contained 50 µg/mL caffeine in water (Form A). The other

contained 20 μg/mL caffeine in water (Form B). As for assay 3 for ibuprofen, the top plate was the receptor compartment and the bottom plate was the donor compartment. Stirrer plates were placed in each of the wells of the bottom (donor) plate. There were 6 incubation times, namely 0.5, 1, 2, 3, 4 and 6 h. The layout of the wells in the Sandwich in this assay is shown in Figure 5.15.

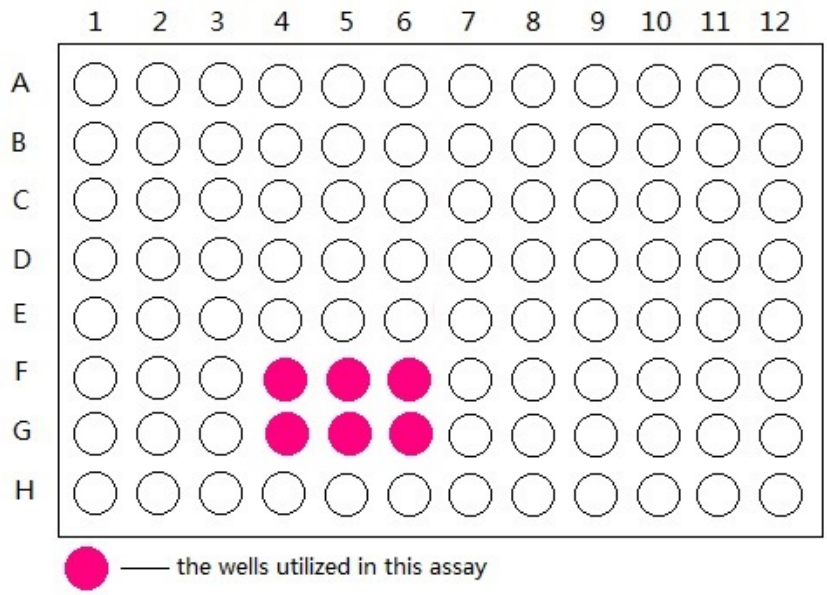

Figure 5.15 Utilization of the Sandwich in Skin PAMPA Assay 1 for caffeine

200 μL of the formulations were added into each of the wells of these columns in the donor compartment. 180 μL of receptor phase was added into the corresponding wells in the receptor compartment. At each time point, 120 μL samples were withdrawn from each well in the receptor plate and an equivalent amount of pre-warmed receptor phase was added to maintain a constant volume. All samples were then analysed by HPLC.

After the incubation, the top plate was removed from the bottom receiver plate. All the data obtained from HPLC were then analysed by PAMPA Explorer Command Software®.

5.2.2.3.2 Assay 2 for caffeine

In this assay, the top plate was the receptor compartment and the bottom plate was the donor compartment. To test whether the stirring disks could influence permeation, stirrer plates were placed in each of the wells of both the bottom (donor) plate and the top (receptor) plate.

Three caffeine formulations were studied, namely, 800 µg /ml caffeine in water, 40 µg/mL caffeine in water, and 20 µg/mL caffeine in water. 6 different incubation times were selected in this case, namely 1, 2, 3, 4, 6 and 8 h. The volume of formulations in the wells with stirrer plates was 180 µL, while the application amount in those wells without the stirrer plates was 200 µL. The layout of wells in the Sandwich in this assay is shown in Figure 5.16.

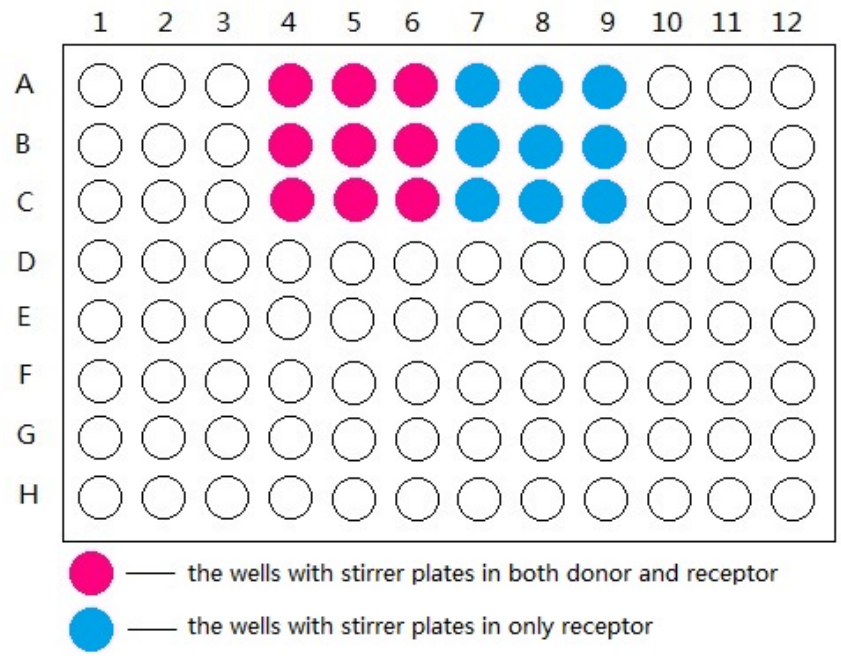

Figure 5.16 Utilization of the Sandwich in Skin PAMPA Assay 2 for caffeine

At each time point, 120 µL samples were withdrawn from each well in the receptor plate and an equivalent amount of pre-warmed receptor phase was added to maintain a constant volume. All samples were then analysed by HPLC. After the incubation, the top plate was removed from the bottom receiver plate. All the data obtained from HPLC were then analysed by PAMPA Explorer Command Software®.

5.2.2.3.3 Assay 3 for caffeine

In assay 3, the top plate was the receptor compartment while the bottom part was the donor compartment. Stirrer plates were placed in each of the wells of the bottom (donor) plate. 3 caffeine solutions in PG, namely 60, 80 and 100

per cent saturation, were studied for 6 h incubation times. The layout of the wells for this assay is illustrated in Figure 5.1. Incubation times are 0.5, 1, 2, 3, 4 and 6 h.

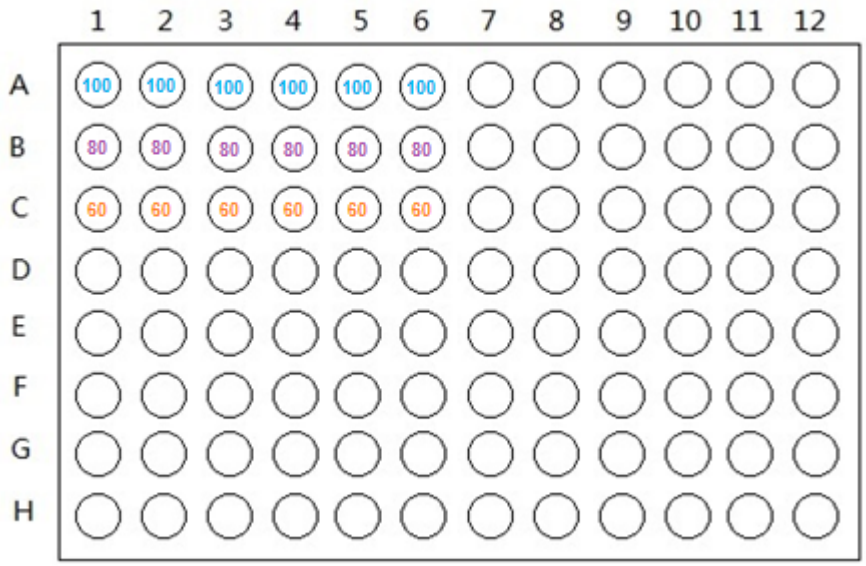

Figure 5.17 Utilization of the Sandwich in Skin PAMPA Assay 3 for caffeine

200 µL of each PG formulation was added as shown. 180 µL of receptor phase was added into corresponding wells in the receptor compartment. At each time point, 120 µL samples were withdrawn from each well in the receptor plate and an equivalent amount of pre-warmed receptor phase was added to maintain a constant volume. All samples were then analysed by HPLC. The samples from the simple formulations in PG were then analysed by GC.

5.2.2.3.4 Assay 4 for caffeine

In this assay, the tested formulations included a 100% saturated solution of caffeine in PG (15 mg/mL), 80% saturated solution of caffeine in PG (12 mg/mL) and 60% saturated solution of caffeine in PG (9 mg/mL).

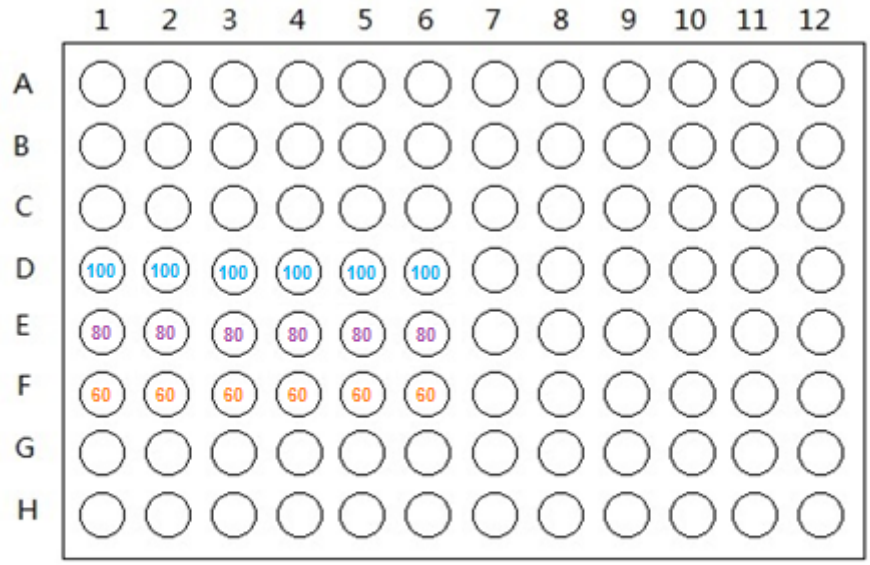

Figure 5.18 Utilization of the Sandwich in Skin PAMPA Assay 5 for caffeine

30 µL of each formulation were applied on the top plate as shown (Figure 5.18). Incubation times were 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h. 180 µL of PBS, pH 7.4, was added into corresponding wells as receptor phase. At each time point, the receptor plate (the bottom plate) was replaced by a new bottom plate filled with fresh receptor phase. Stirring was always conducted in the receptor phase.

5.2.2.3.5 Assay 5 for caffeine

The selected commercial formulations in this assay were Eveline® Cosmetics-Slim Extreme 4D Anti-cellulite Diamond Slimming Serum and L'Oréal® Perfect Firm Pro Intense Cellulite Massage System.

30 µL of the two tested formulations were applied on the top plate as shown in Figure 5.19.

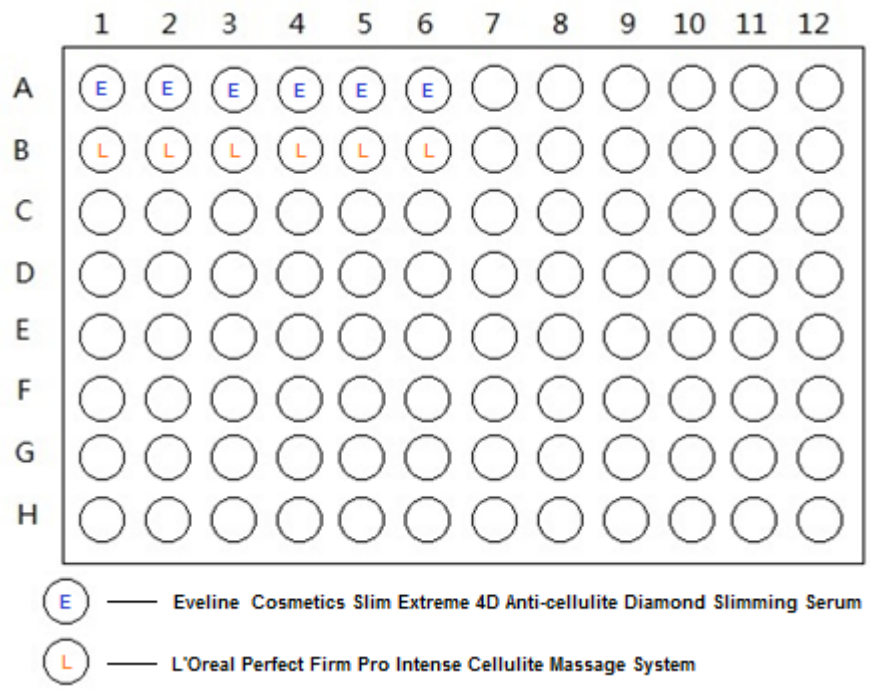

Figure 5.19 Utilization of the Sandwich in Skin PAMPA Assay 5 for caffeine

Incubation times were 0.5, 1, 2, 3, 4, and 6 h. 180µl of PBS, pH 7.4, was added into the corresponding wells as receptor phase. At each time point, the receptor plate (the bottom plate) was replaced by a new bottom plate filled with fresh receptor phase. The receptor phase was stirred.

5.2.2.3.6 Assay 6 for caffeine

The lip of the Skin PAMPA was used as the donor compartment in this assay. The set-up for this Skin PAMPA is outlined in Figure 5.12.

The selected formulations in this assay were the commercial formulations Eveline® and L'Oréal®. 3 µL of each tested formulation was applied under the membrane on the top plate as shown in Figure 5.20.

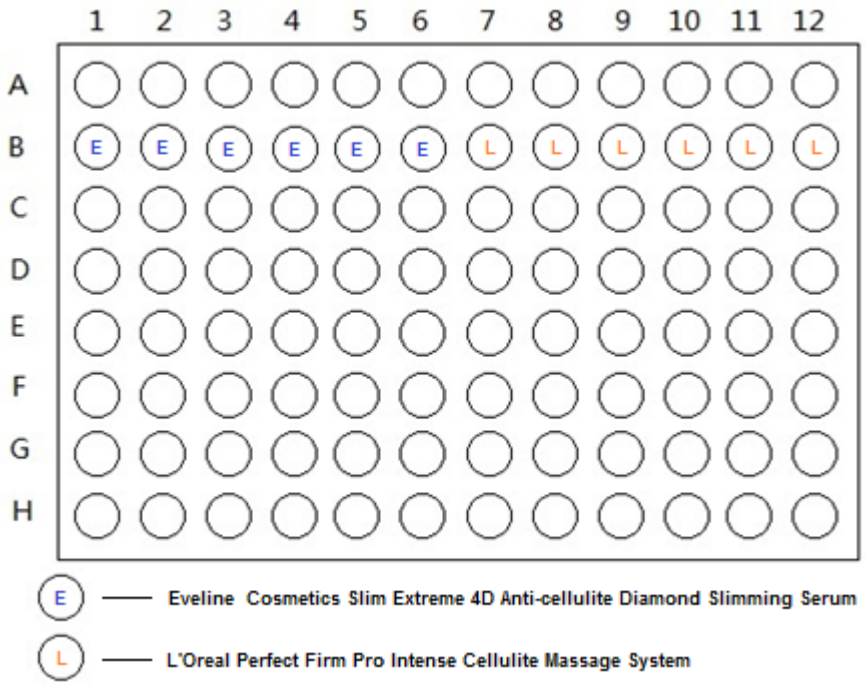

Figure 5.20 Utilization of the Sandwich in Skin PAMPA Assay 6 for caffeine

The top plate was used as the receptor plate. Incubation times were 0.25, 0.5, 1, 2, 3, and 4 h. 250 µL of PBS, pH 7.4, was added into corresponding wells of the top plate as receptor phase. The lip was used as the bottom plate. At each time point, 250 µL of the receptor phase was sampled and replaced by the same amounts of fresh receptor phase. Stirring was maintained in the donor phase.

5.2.2.3.7 Assay 7 for caffeine

Two commercial caffeine formulations, Eveline® and L'Oréal® were studied in this assay. The top plate was used as the donor compartment. Three different doses, 1, 3, or 30 µL per well, were investigated in order to determine the amount of formulation which best represented finite dose conditions. These doses correspond to 3.3, 9.9 and 99 µL/cm^2. Figure 3.8 shows the layout of the wells for this assay.

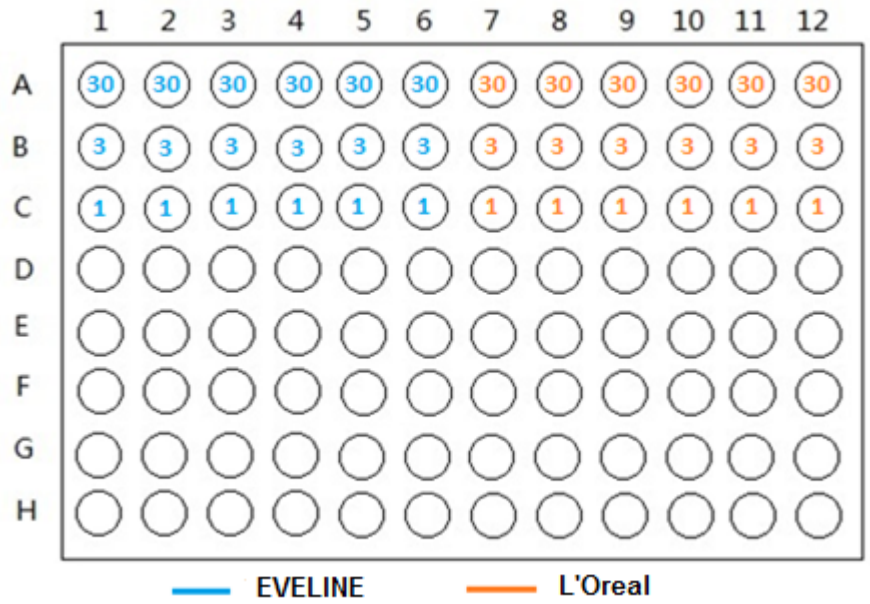

Figure 5.21 Utilization of the Sandwich in Skin PAMPA Assay 7 for caffeine

The bottom plate was replaced by a new plate with fresh receptor phase during the specified intervals, including 0.5, 1, 2, 3, 4 and 6 h.

5.2.2.3.8 Assay 8 for caffeine

L'Oréal® Sublime Slim Patch was also selected as a model patch to be investigated in the novel Skin PAMPS model. The top plate acted as the receptor phase in this assay. The patch was cut into small sizes to cover all membranes on the wells of column 1 and 2 (Figure 5.22).

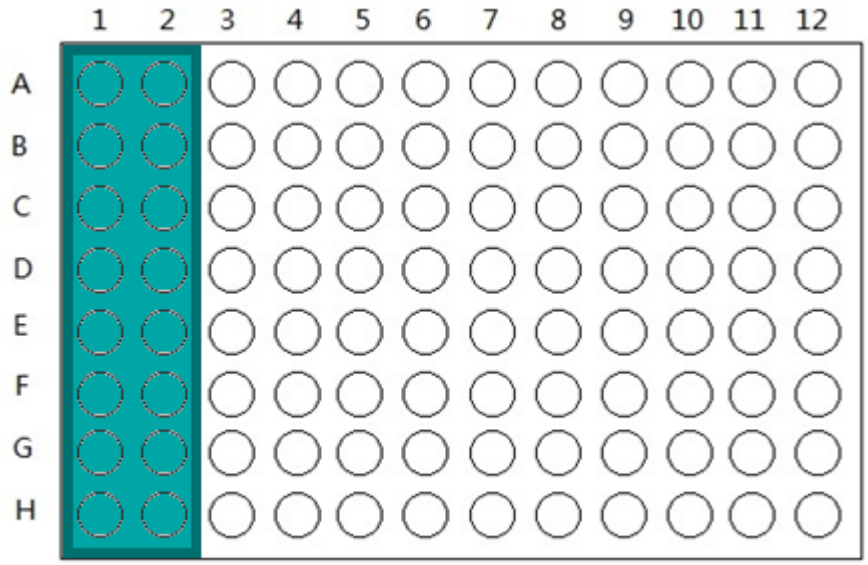

Figure 5.22 Utilization of the Sandwich in Skin PAMPA Assay 8 for caffeine

The lip was used as the bottom plate to hold the patch. Incubation times were 0.25, 0.5, 1, 2, 3 and 4 h. 200 µL of PBS, pH 7.4, was added into corresponding wells in the top plate as receptor phase. At each time point, 150 µl samples were taken from each receptor well and the same amounts of fresh receptor phase were replaced. Stirring was maintained in the receptor phase, and the membrane was kept at 32 ± 1^{o}C. All samples were analysed by HPLC.

5.3 Results and discussion

5.3.1 Assays for ibuprofen

5.3.1.1 Assay 1 for ibuprofen

Two ibuprofen formulations were tested. The first formulation contained 5% ibuprofen, 15.6% PG and 79.4% IPA (Form A). The second formulation was a slurry of ibuprofen in DI water (Form B). The receptor phase in this case was PBS, pH 7.4. The solubility of ibuprofen in PBS, pH 7.4 is 1.22 mg/ml. In order to maintain sink conditions, the concentration of ibuprofen in the receptor phase should be less than 0.12 mg/ml throughout the incubation times. The permeation results of Assay 1 for ibuprofen are shown in Figure 5.23.

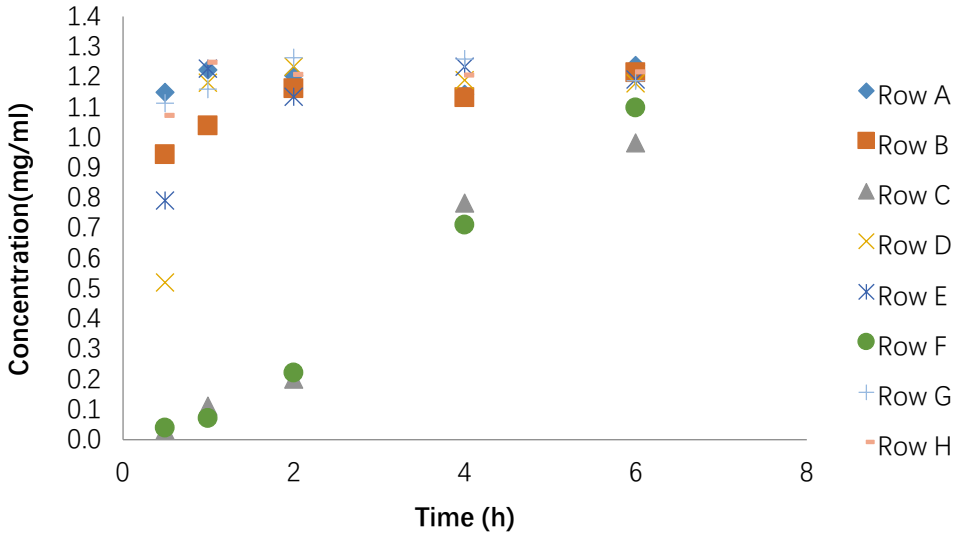

Figure 5.23 Concentration of ibuprofen in receptor phase in Skin PAMPA assay 1 for ibuprofen

As shown in Figure 5.23, in the studies carried out in Row A, B, D, E, G and H, where the tested formulations were Form A, the concentrations of

ibuprofen in the receptor phase were all higher than 0.5mg/ml even just after 0.5 h, thus the concentrations were near saturated levels. In other words, sink conditions in the receptor phase could not be maintained during the whole incubation, which caused the permeation of the tested compound to be suboptimal. For the studies in Row C and F, where the tested formulation was a slurry of ibuprofen in water, the concentrations of ibuprofen increased with incubation time. The data show a linear correlation between the concentration of ibuprofen and time. However, after 2 h, the concentrations of ibuprofen were also higher than 10% of saturated solubility, thus sink conditions were not maintained.

Moreover, the results for the different rows for the same formulation did not show significant difference ($P > 0.05$). The most likely explanation for this phenomenon is that the temperature across the whole Sandwich was homogeneous.

5.3.1.2 Assay 2 for ibuprofen

In this assay, only 30 µL of a slurry of ibuprofen in DI water was applied into each well of the donor (top) plate. This meant that even if all the ibuprofen applied permeated through the membrane, the receptor phase could maintain sink conditions throughout the experiment.

When the Skin PAMPA Sandwich was assembled, it was ensured that there were no air gaps under the membranes. However, when the experiments were running, small bubbles were generated, especially under those membranes without formulations from the beginning. Therefore, PBS was added on some of those membranes prior to applying the formulation, in order to compare with those membranes open to the air. The result was that obviously fewer bubbles were generated under those membranes with PBS.

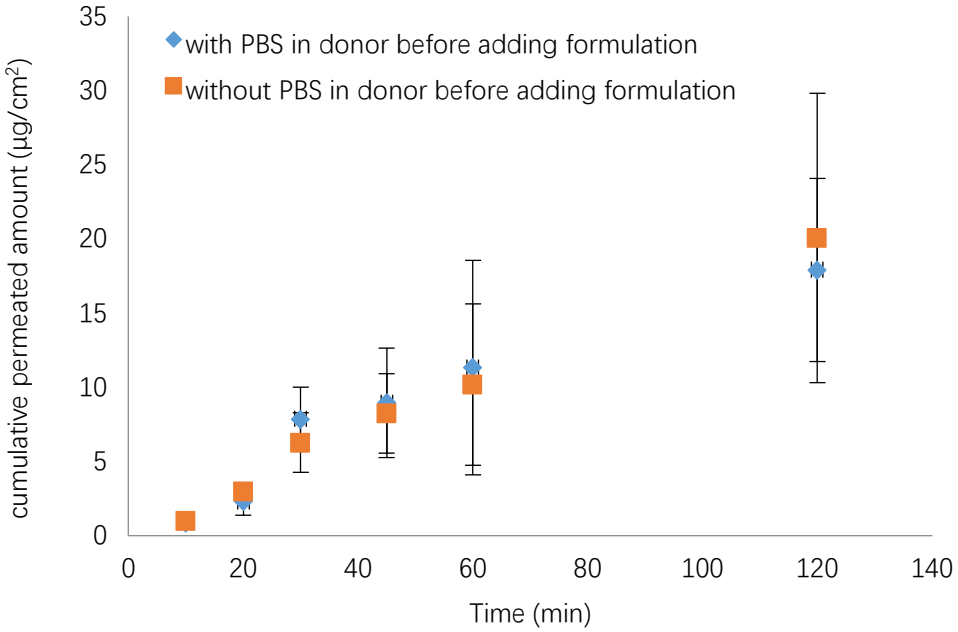

Figure 5.24 Concentration of ibuprofen in receptor phase in the Skin PAMPA model at different incubation times at 32 ±1°C. Each data point represents the mean ± SD (n=3).

However, according to Figure 5.24, the amount of ibuprofen permeated through all the membranes was around 20 µg/cm^2 after 120 min, and the permeation between the two membranes did not show significant differences ($p<0.05$). It was also confirmed that the temperature was the same across the whole PMPA model during incubation.

5.3.1.3 Assay 3 for ibuprofen

As in this case, the top plate was the receptor compartment, 120 µL of fresh receptor phase was replaced in each receptor well after withdrawing the samples, which meant that sink conditions were maintained even in such infinite dose studies.

The tested formulations included a slurry of Ibuprofen in DI water (Form A), a slurry of ibuprofen in mixture of DI water and PEG (50: 50 v/v, Form B), a slurry of ibuprofen in Prisma HT buffer (Form C), 80 µg/mL ibuprofen in methanol (Form D) and 20 µg/mL ibuprofen in methanol (Form E).

Figure 5.25 shows the permeation profile of Skin PAMPA Assay 3 for ibuprofen. All the studies in this assay were infinite dose studies. The concentrations of ibuprofen in each formulation were different.

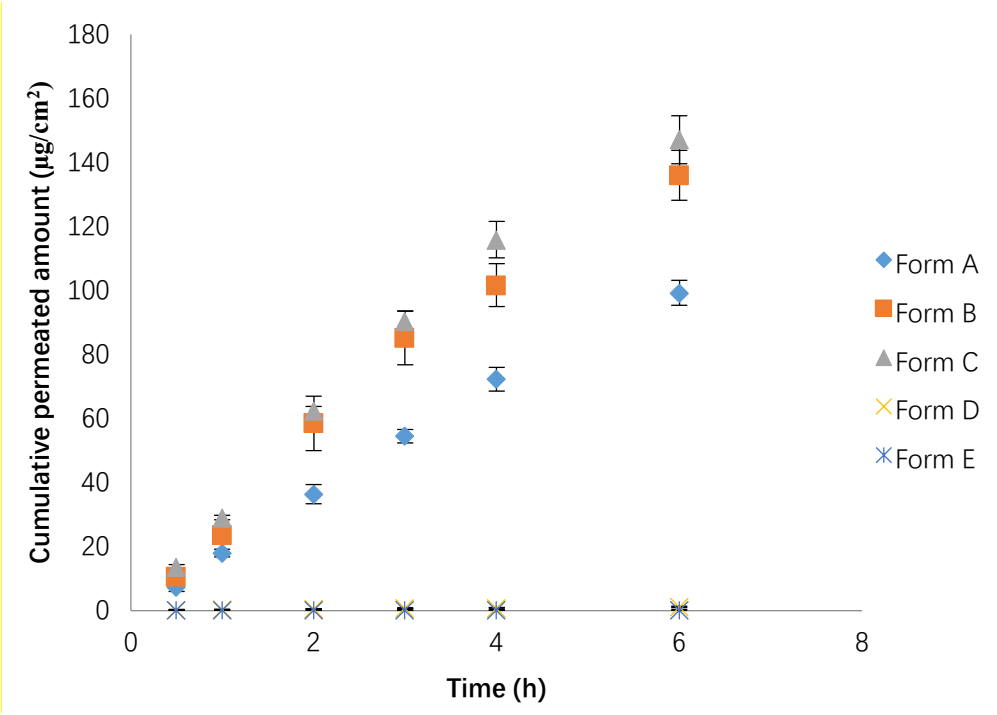

Figure 5.25 Cumulative permeated amount of ibuprofen in Skin PAMPA assay3 for ibuprofen. Each data point represents the mean ± SD (n=3).

According to Figure 5.25, 484.13 ± 24.95 and 447.43 ± 25.67 µg/cm^2 of ibuprofen permeated from Form C and B after 6 h, separately, while the permeation of the active from Form C was much lower ($p<0.05$), with a value of 326.56 ± 12.89 µg/cm^2. It can be seen that the rate of permeation of the tested compound increased with ibuprofen concentration in the formulations.

However, because Form D and E were methanol formulations, the contact area between donor phase and membrane decreased due to the evaporation of methanol, which affected permeation of ibuprofen to a great extent. As can be seen in Figure 5.25, little or no permeation of ibuprofen could be detected in the receptor phases for Form D and E. Therefore, if the formulation contains volatile components, the donor compartment in the Skin PAMPA Sandwich can only be the top plate, which would ensure that the

contact area between formulation and membrane would not change during incubation.

Table 5.4 shows the cumulative amount of ibuprofen permeation from formulation A, B, and C after 6 h.

Table 5.4 Cumulative permeation of ibuprofen, n=3, mean ± SD

Formulation	Cumulative permeated amounts (μg/cm^2)
A	326.56 ± 12.89
B	447.43 ± 25.67
C	484.13 ± 24.95

In the previous Franz cell studies on porcine skin (section 3.2.2.3), the cumulative permeation of ibuprofen was 467.53 ± 111.78 μg.cm^{-2}. A previous Franz cell study on human skin has also been conducted in the Skin Research Group (UCL). In this study, the tested formulation was a saturated PG solution of ibuprofen. 288 μg.cm^{-2} ibuprofen penetrated through human skin after 24 h (Vieira., 2012). It has been noted by Dick and Scott (1992) that porcine skin is more permeable than human skin. In other words, the data obtained from the Skin PAMPA assay was consistent with those from Franz cell studies on both porcine skin and human skin.

5.3.1.4 Assay 4 for ibuprofen

To avoid the evaporation of methanol formulations, the top plate of the Sandwich was used as the donor compartment in this assay. All the formulations tested in this assay were ibuprofen / methanol formulations,

including 80 µg/mL ibuprofen in methanol (Form A), 40 µg/mL ibuprofen in methanol (Form B), 20 µg/mL ibuprofen in methanol (Form C), and 10 µg/mL ibuprofen in methanol (Form D). The permeation profiles are shown in Figure 5.26.

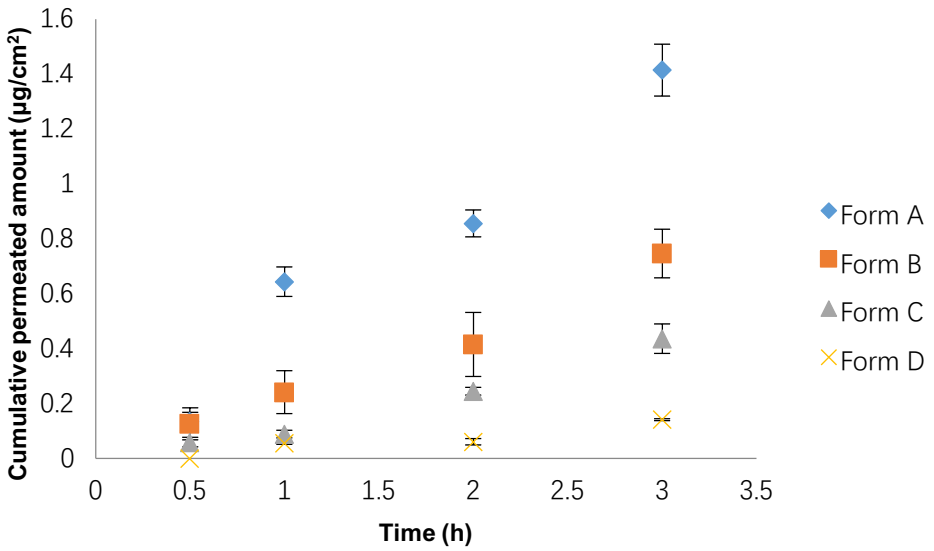

Figure 5.26 Cumulative permeated amount of ibuprofen in Skin PAMPA assay 4 for ibuprofen. Each data point represents the mean ± SD (n=3).

According to Figure 5.26, the rate of permeation of ibuprofen also increased with its concentration in the formulations. After 3 h, permeation of ibuprofen from Form A was 1.4 µg/cm^2, and the amount permeated from Form B was 0.7 µg/cm^2. Form C and D delivered much lower amounts of ibuprofen ($p<0.05$) through the membrane, with values of 0.4 and 0.1 µg/cm^2. The results show significant differences ($p<0.05$) in ibuprofen permeation for all four tested formulations, and the order was the same as the ibuprofen concentrations, which was Form A > B > C > D. This may be explained by the increase of the drug concentration leading to the increase of thermodynamic activity of the

drug in delivery system. As a result, the permeation rate of the drug increased correspondingly.

5.3.1.5 Assay 5 for ibuprofen

Ten different ibuprofen commercial formulations were selected to be investigated in this assay. Figure 5.27 illustrates the permeation profiles of ibuprofen from the 10 commercial formulations evaluated.

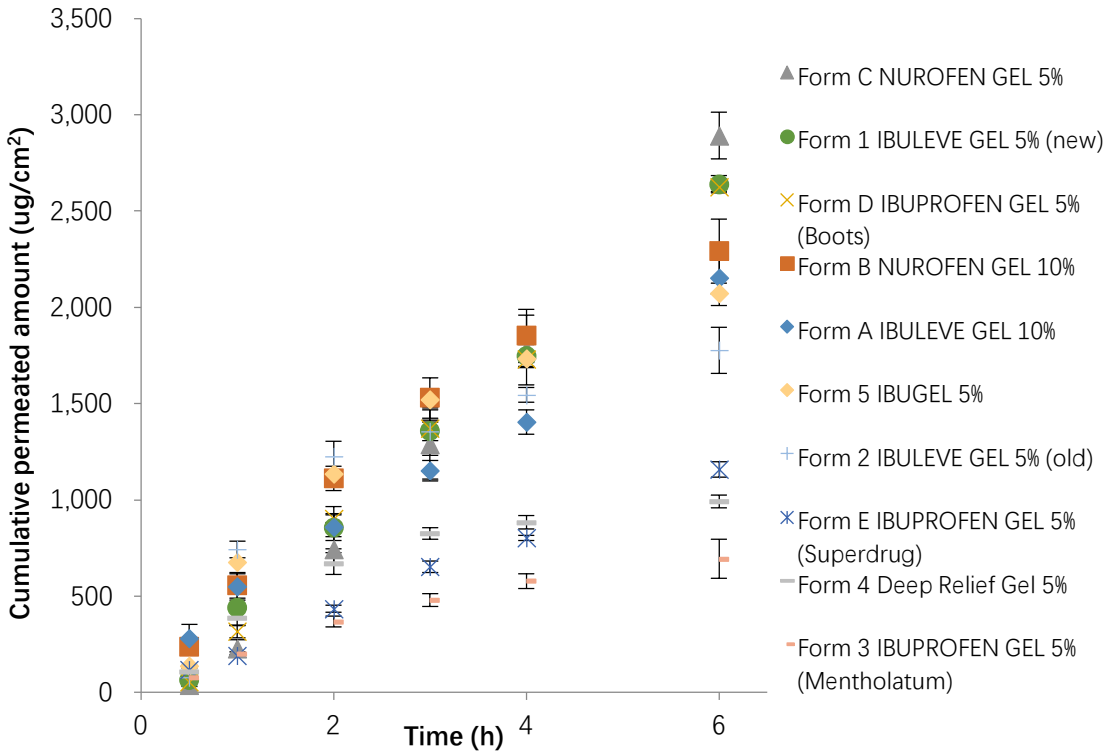

Figure 5.27 Cumulative permeated amount of ibuprofen from the tested ibuprofen commercial formulations on Skin PAMPA model. Each data point represents the mean ± SD (n=5).

Of the 10 tested commercial formulations, only Form A and B contained 10% (w/w) ibuprofen, while all the others contained 5% (w/w). However,

according to Figure 5.27, the permeated amounts of ibuprofen from Form A and B were not significantly greater than the other formulations ($P>0.05$).

Interestingly, compared with the other formulations, the amounts of ibuprofen that permeated from Form E, 3 and 4 were much less ($p<0.05$) after 6 h, with the values ranging from 600 to 1250 μg/cm^2, while the corresponding values for the other tested formulations were greater than 1700 μg/cm^2. The main components of the tested formulations are listed in Table 5.5. From the table, it could be seen that one of the common characteristics of Form E, 3 and 4 is that all of them contain diisopropanolamine, a pH adjuster.

Table 5.5 Main excipients of the tested commercial formulations

	industrial methylated spirit (IMS)	carbomers	diethylamine	purified water	isopropyl alcohol	ethylhydroxycellulose	hydroxyethylcellulose	benzyl alcohol	sodium hydroxide	ethanol	propylene glycol (PG)	diisopropanolamine
Form A IBULEVE GEL 10%	x	x	x	x								
Form B NUROFEN GEL 10%				x	x		x	x	x			
Form C NUROFEN GEL 5%				x	x		x	x	x			
Form D IBUPROFEN GEL 5% (Boots)				x	x	x		x	x			
Form E IBUPROFEN GEL 5% (Superdrug)		x		x						x	x	X
Form 1 IBULEVE GEL 5% (new)	x	x	x	x							x	
Form 2 IBULEVE GEL 5% (old)	x	x	x	x							x	
Form 3 IBUPROFEN GEL 5%		x		x						x	x	X
Form 4 Deep Relief Gel 5%		x		x						x	x	X
Form 5 IBUGEL 5%	x	x	x	x							x	

Table 5.5 lists the main excipients of all the tested commercial formulations. Except for Form A, B, C and D, all the other formulations include PG. However, Form A, B, C and D were in the top six formulations, showing best efficacy ($p<0.05$) in delivering ibuprofen through the membrane than the others. In contrast to Form A, Form B, C and D do not contain Carbomer but benzyl alcohol and sodium hydroxide.

The permeation profiles of ibuprofen from the three simple ibuprofen formulations in PG are shown in Figure 5.28. It is clear that the concentration of ibuprofen in the formulations affected its permeation.

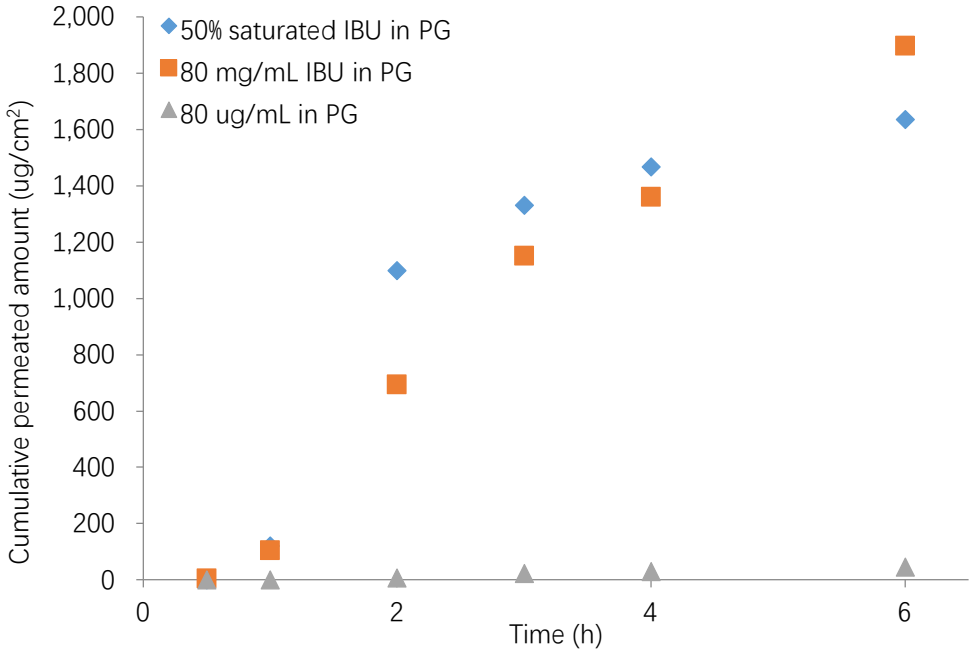

Figure 5.28. Cumulative permeated amount of ibuprofen for ibuprofen formulations with PG on the Skin PAMPA model. Each data point represents the mean ± SD (n=5).

Figure 5.28 illustrates the permeation profiles of ibuprofen from a slurry of ibuprofen in water.

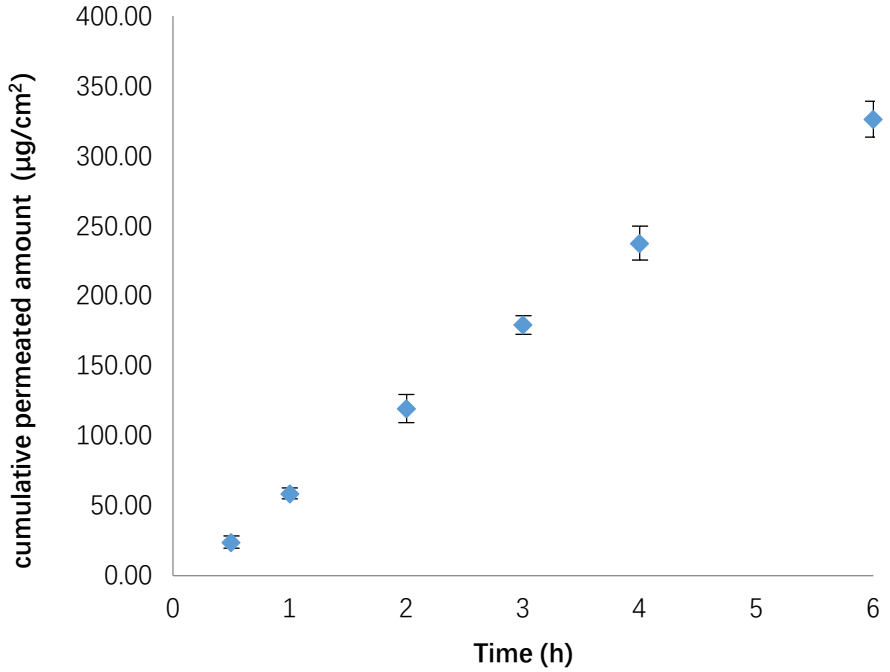

Figure 5.29 Cumulative permeated amount of ibuprofen for a slurry of ibuprofen in water on the Skin PAMPA model. Each data point represents the mean ± SD (n=5).

Compared with the permeation of ibuprofen from a slurry in water (Figure 5.29), ibuprofen in PG formulations (Figure 5.28) demonstrated higher permeation ($p<0.05$).

5.3.1.6 Assay 6 for ibuprofen

Three PG solutions with different ibuprofen concentrations and two commercial ibuprofen formulations with different ibuprofen content were tested in the Skin PAMPA in this assay. The dose was decreased to 30 µL per well.

The permeation profiles of ibuprofen from the PG formulations are illustrated in Figure 5.30.

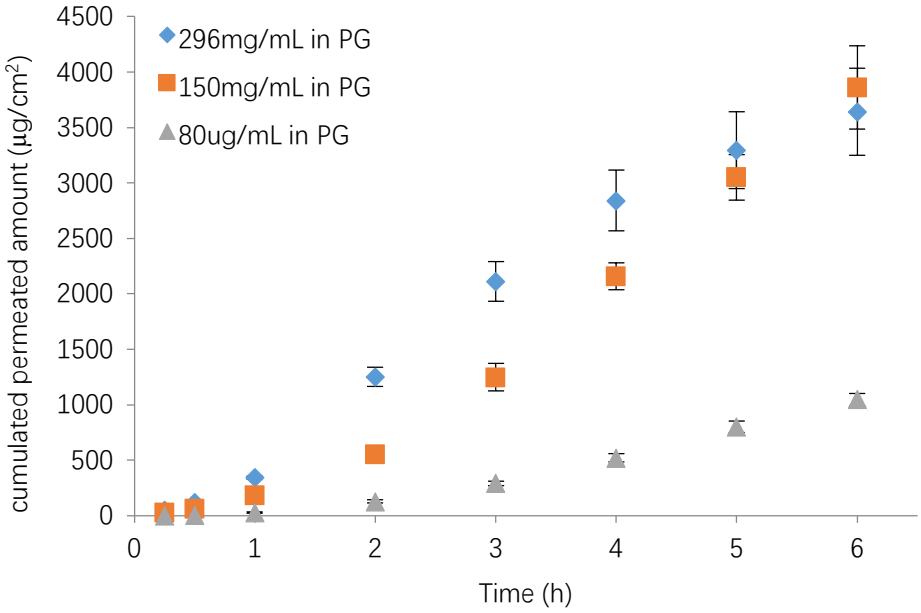

Figure 5.30 Cumulative permeated amount of ibuprofen formulations in PG in the Skin PAMPA model. Each data point represents the mean ± SD (n=5).

According to Figure 5.30, the permeated amounts of ibuprofen in this assay were 3800, 3600 and 1000 µg/cm^2, after 6 h from the three formulations. These values are much higher compared with the previous infinite dose studies (Section 5.3.1.5). Therefore, this underlines the importance of maintaining sink conditions in the receptor phase for permeation studies. Otherwise, the permeability would be underestimated to a great extent. However, although the doses applied were much smaller and the receptor phase was replaced by fresh PBS in this study, there were still quite a lot wells in which the concentrations exceeded sink conditions. Therefore, other methods to maintain sink conditions need to be explored.

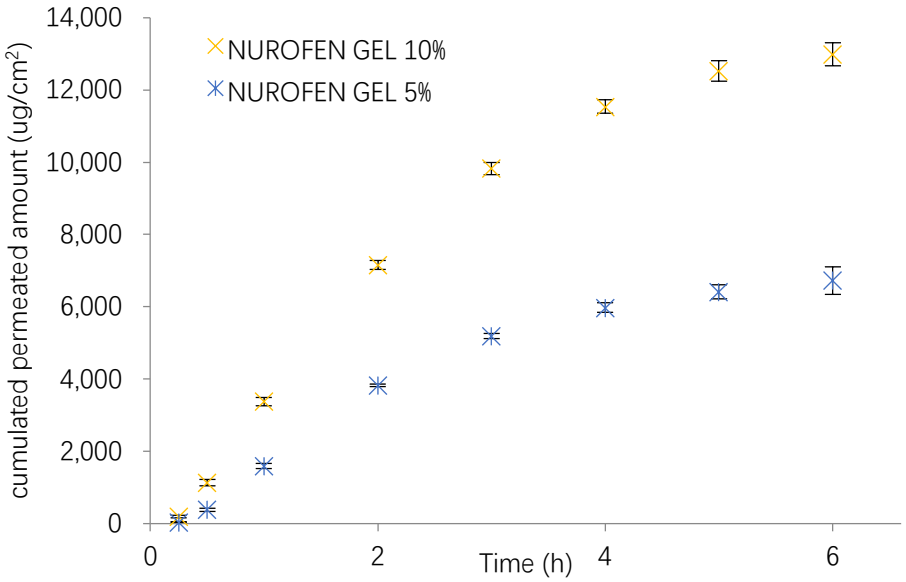

Figure 5.31 Cumulative permeated amount of ibuprofen in the Skin PAMPA model. Each data point represents the mean ± SD (n=5).

From Figure 5.31, it is clear that the steady state flux and the amount of ibuprofen that permeated from NUROFEN GEL 10% were significantly higher ($p<0.05$) than the corresponding values for NUROFEN GEL 5%. The main components of these two formulations are listed in Table 5.6.

Table 5.6 Main components of the tested commercial formulations

	ibuprofen	purified water	isopropyl alcohol	hydroxyethylcellulose	benzyl alcohol	sodium hydroxide
NUROFEN GEL 10%	x	x	x	x	x	x
NUROFEN GEL 5%	x	x	x	x	x	x

However, it was unknown if the amounts of the other components in NUROFEN GEL were the same in both of these two formulations, so it may not be concluded that NUROFEN GEL 10% delivers a higher steady flux of permeated ibuprofen only because of the higher concentration of this active.

5.3.1.7 Assay 7 for ibuprofen

In the current assay, two commercial ibuprofen formulations, IBUGEL® (ibuprofen 5% w/w) and IBULEVE® Speed Relief 5% Spray (5% w/w), and two 5% ibuprofen solutions in isopropyl alcohol and either PEG 300 or PG were studied in order to compare their efficiency in delivering ibuprofen. Three different doses, 1, 3, or 30 µL per well, corresponding to 3.3, 9.9 and 99 µL/cm^2 separately, were investigated in order to determine the amount of formulation which best represented finite dose conditions. Figure 5.32 compares the permeation profiles of ibuprofen from each tested formulation in the skin PAMPA model following different applied doses.

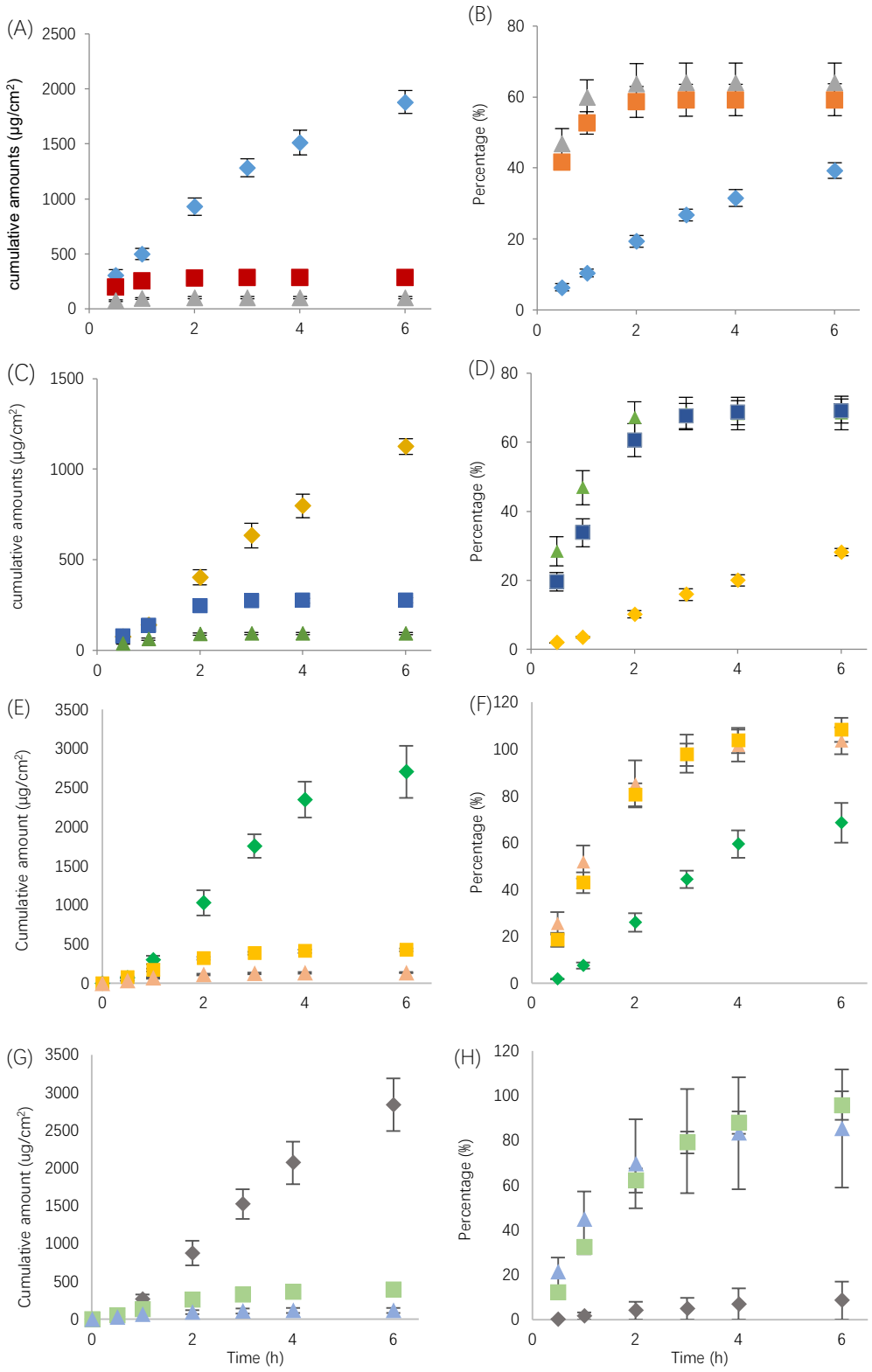

Figure 5.32 Left: (A), (C), (E), and (G) Cumulative amounts of ibuprofen from IBUGEL® in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, from IBULEVE® in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, from PG solution in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, and from PEG 300 solution in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well; Right: (B), (D), (F) and (H) Percentages permeated from IBUGEL® in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, from IBULEVE® in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, from PG solution in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well, and from PEG 300 solution in Skin PAMPA following application of 30 µL (◆), 3 µL (■) and 1 µL (▲) per well. Each data point represents the mean ± SD (n=6).

According to Figure 5.32, it is clear that, for each formulation, cumulative permeated amounts of ibuprofen are lower for the 1 and 3 µL doses compared with the 30 µL application at 2 h. However, the permeation percentages indicate the reverse, where significantly lower percentage permeation was observed for the 30 µl application. This is consistent with an exaggerated influence of formulation excipients on membrane transport at the higher 30 µL dose. As previously noted data from infinite dose studies may not be extrapolated to finite dose conditions (Santos et al., 2011; Goh and Lane, 2014; Luo and Lane, 2015; Hadgraft and Lane, 2016).

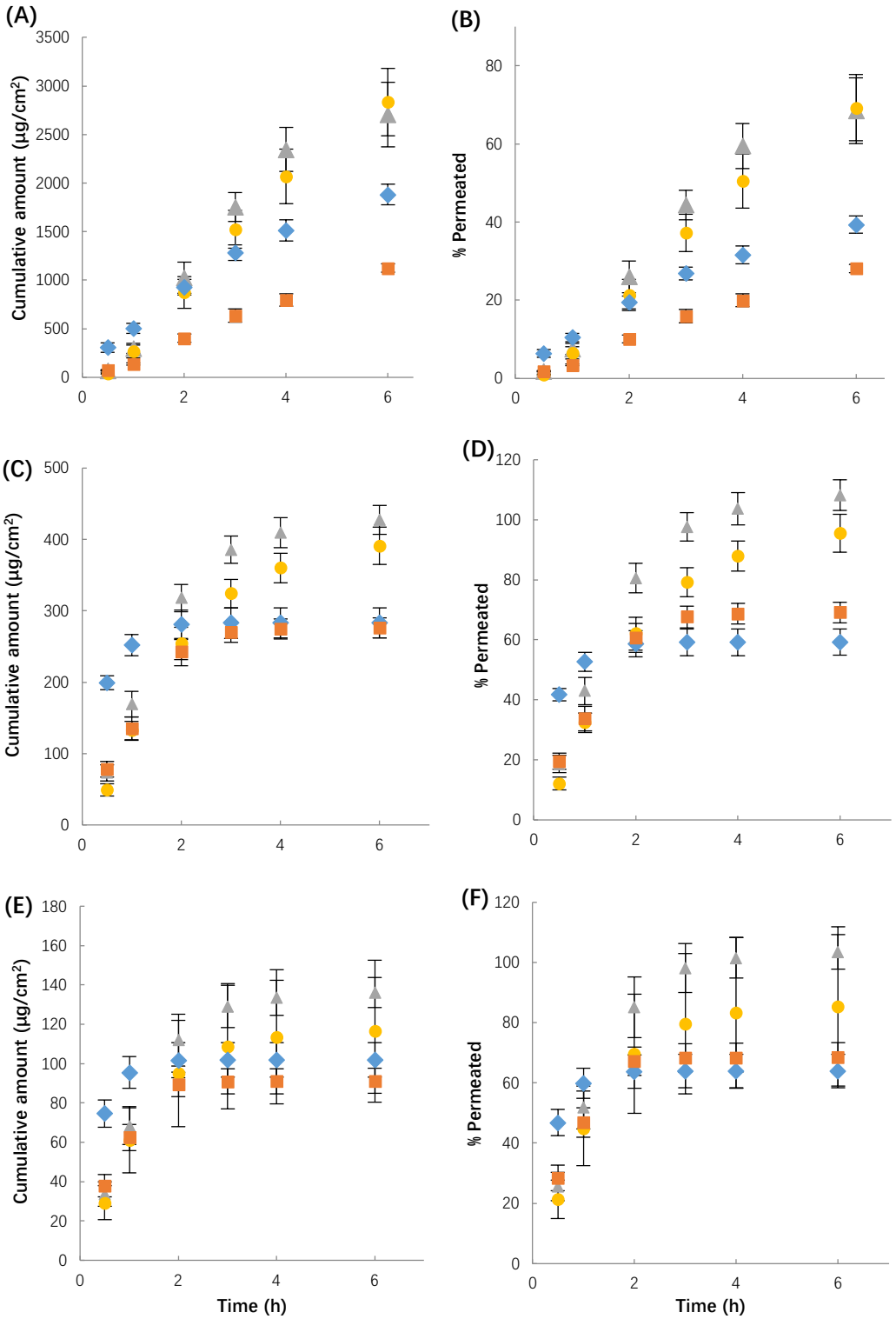

Figure 5.33 Cumulative amounts of ibuprofen and percentages permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) in Skin PAMPA following application of 30 µL (A) and (B), 3 µL (C) and (D), and 1 µL (E) and (F) per well. Each data point represents the mean ± SD (n=6).

Figure 5.33 compares the cumulative amounts of drug and percentages permeated for different formulations.

For the 30 µL applications (Figures 5.33A, 5.33B), the profiles for all the formulations are generally linear, consistent with these amounts representing infinite doses. At 6 h, there are significant differences in ibuprofen permeation from the two commercial formulations and between the gel and all other formulations ($p < 0.05$), but not between the PG and PEG 300 formulations. Overall ibuprofen permeation from the PG and PEG 300 alcoholic solutions is higher than for the commercial formulations in Skin PAMPA ($p < 0.05$). For the commercial formulations the permeation differences may reflect the influence of various excipients on ibuprofen permeation. Both formulations contain industrial methylated spirit (IMS) or denatured alcohol. The gel contains PG whereas the spray does not. The volatile nature of the spray formulation should also result in a shorter residence time of this formulation on the Skin PAMPA membrane compared with the gel. Differences between the commercial spray and the simple solutions may also reflect differences in the absolute content of the volatile components. However, further studies with individual excipients and the Skin PAMPA lipid mixture will be needed to interpret these data. These specific excipients and the functions previously proposed for them are detailed in Table 5.7.

Table 5.7 Excipients included in commercial and experimental formulations and proposed functions reported in the literature.

Excipient	Functions
IMS	Penetration enhancer[a], solvent[b]
PEG 300	Solvent[c]
PG	Penetration enhancer, solvent[b]

[a] Hadgraft et al., 2003.
[b] Lane, 2013.
[c] Rowe et al., 2012.

For the 3 µL dose, ibuprofen permeation is significantly ($p < 0.05$) higher from the formulations containing PG and PEG 300 when compared with both commercial formulations. However, after the application of 1 µL, only permeation from the PG formulation is statistically higher ($p < 0.05$) than the commercial formulations. Overall, a greater percentage of each formulation permeates in the Skin PAMPA model at these lower doses compared with the 30 mL dose. At 6 h approximately 280 µg/cm^2 of ibuprofen had permeated following application of 3 µL doses of the commercial formulations, accounting for 60–70% of the applied dose. For the 1 µL application the cumulative amounts permeated and percentages of ibuprofen delivered were 90–100 mg/cm^2 and 64–69% respectively. Clearly the amounts permeated for the 1 µL dose approach values for porcine skin and silicone membrane (session 3.2.2.3); however percentage permeation is closest to values for the silicone membrane.

5.3.1.8 Assay 8 for ibuprofen

In the skin PAMPA model, a bottom plate is not suitable to act as the receptor compartment while the applied doses need to be small, especially in finite dose studies. This is because the volume of each well is fixed, which is 200 μL. However, while the dose is small, for example 3 or 1μL per well, a lip may be used as the donor. In this case, as there is not border on a lip, the possible contamination of the applied formulations between the neighbouring wells needs to be taken into account. It is therefore necessary to ensure the applied dose would not spread outside the bottom of the membrane.

Figures 5.34 and 5.35 show the cumulative amounts of ibuprofen and percentages permeated from the gel and spray formulations for Assay 8. The top plate was the receptor while the lip was the donor.

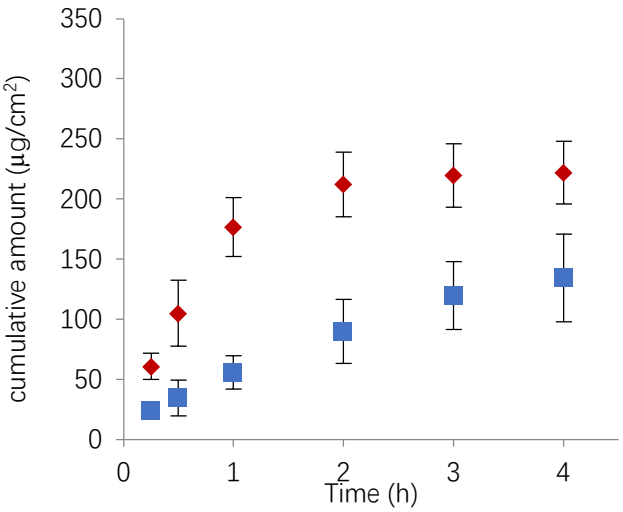

Figure 5.34 Cumulative permeation of ibuprofen from IBUGEL® (◆) and IBULEVE® (■) in Skin PAMPA model following application of 3 µL per well, when the top plate is the receptor. Each data point represents the mean ± SD (n=6).

According to Figure 5.34, it is clear that significantly more ibuprofen permeated through the membrane from the gel formulation than the spray ($p<0.05$).

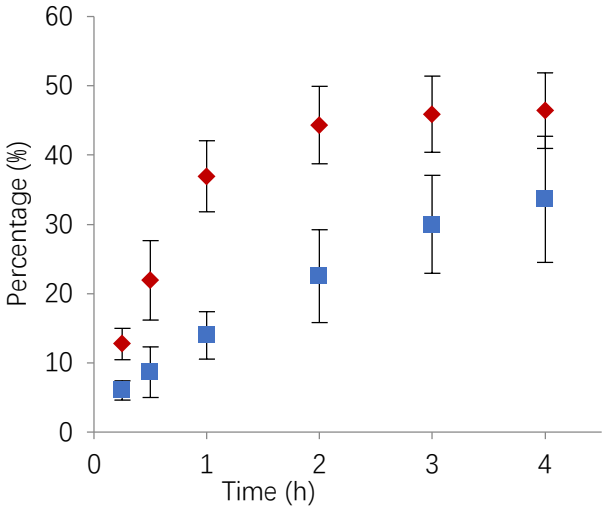

Figure 5.35 Permeated percentage of ibuprofen from IBUGEL® (◆) and IBULEVE® (■) in Skin PAMPA model following application of 3 µL per well, when the top plate is the receptor. Each data point represents the mean ± SD (n=6).

For comparison, the results for the assay in which the bottom plate was the receptor are illustrated in Figures 5.36 and 5.37.

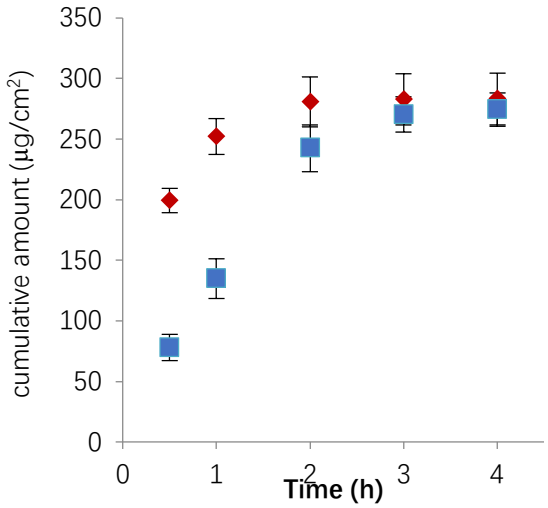

Figure 5.36 Cumulative permeated amounts of ibuprofen from IBUGEL® (◆) and IBULEVE® (■) in Skin PAMPA model following application of 3 µL per well, when the bottom plate is the receptor (mean ± SD, n=6).

For ibuprofen permeation from IBUGEL®, the amount was about 280 µL/cm^2 at 2 h when the bottom plate was the receptor. When the top plate acted as the receptor, the corresponding value dropped to only around 210 µL/cm^2. After 4 h, the amount of ibuprofen permeated from the gel formulation (~280 µL/cm^2) shown in Figure 5.36 was still higher than that illustrated in Figure 3.54, which was about 220 µL/cm^2.

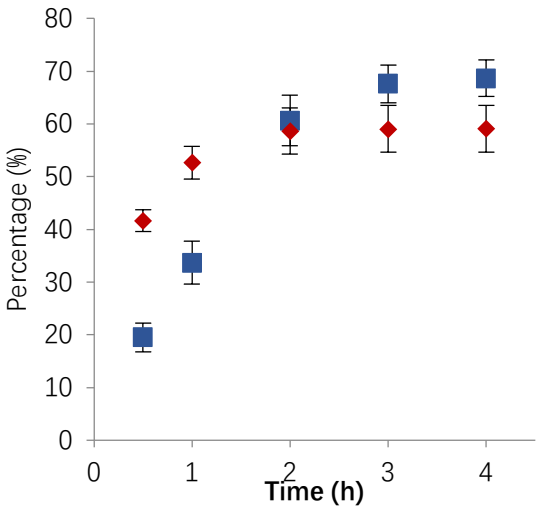

Figure 5.37 Permeated percentage of ibuprofen from IBUGEL® (◆) and IBULEVE® (■) in Skin PAMPA model following application of 3 µL per well, when the bottom plate is the receptor (mean ± SD, n=6).

For the spray formulation, significantly more ibuprofen ($p<0.05$) permeated through the membrane from the top donor compared with those amounts from the bottom donor throughout the experiments. In contrast to the results in Figure 5.34, there was no significant difference ($p<0.05$) between ibuprofen permeation from IBUGEL® and IBULEVE® after 4 h shown in Figure 5.36. Moreover, the top donor assay also results in low variability in the permeation data compared with the assay in which the donor compartment was a lip.

5.3.1.9 Assay 9 for ibuprofen

In this assay, the pre-coated artificial membrane on the Skin PAMPA sandwich was replaced by human skin in order to see if the sandwich model is comparable to human skin permeation. The tested formulation was IBULEVE® Speed Relief 5% Spray (5% w/w).

Because of the small membrane area in the Skin PAMPA model (0.304 cm^2), it was difficult to cut human skin to the suitable size to cover adequately the well bottom of the top plate. Another challenge was how to fix the skin on the plate and avoid leakage between the skin and both the top and bottom plate. In this assay, some DOW CORNING® high vacuum grease was applied between the skin and bottom of top plate to fix the skin. Grease was also applied on the top of each receptor plate and around the outer wall of each well of the top plate, in order to avoid leakage in each mini “Franz cell” in the Sandwich. The grease was applied carefully in order not to cover the permeation area.

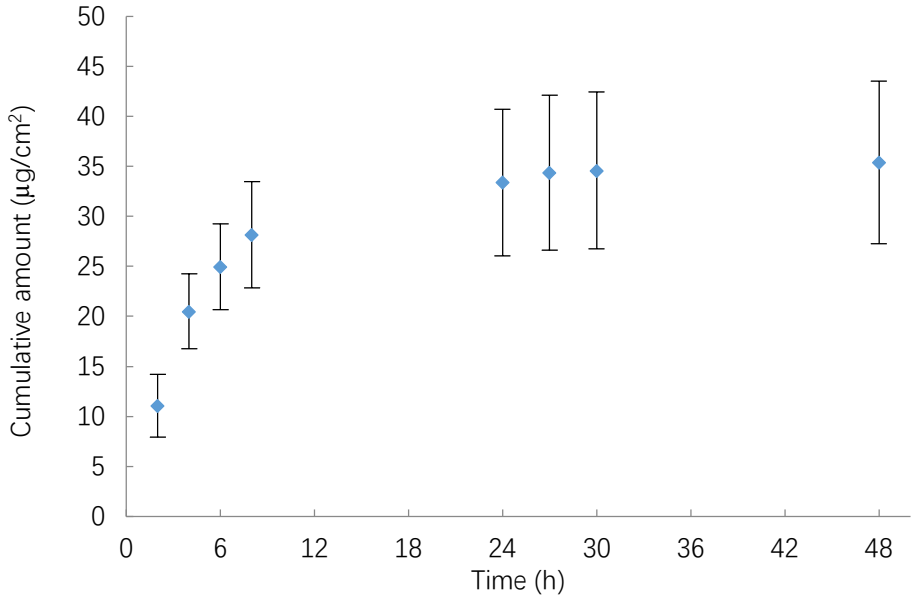

Figure 5.38. Permeation profiles of ibuprofen permeated from IBULEVE® Speed Relief 5% Spray (5% w/w) to the human skin PAMPA studies, mean ± SD, n=8

Figure 5.38 shows the permeation profiles of ibuprofen from the IBULEVE® spray through the human skin in the Skin PAMPA studies. Compared with the permeation amounts from the Skin PAMPA model and the artificial silicone membrane, the ibuprofen permeation is much lower (~35 µg/cm^2), but approached values for Franz cell studies with human skin (Figure 4.1).

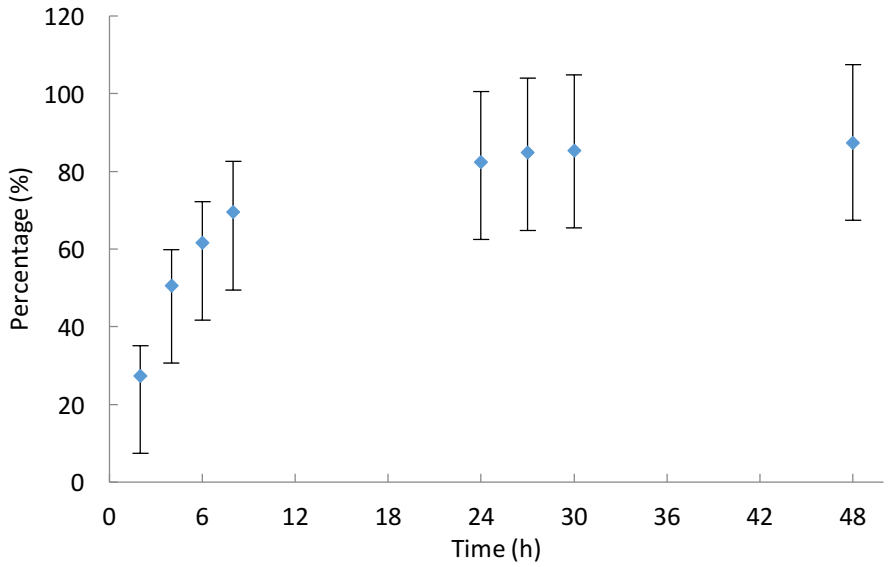

Figure 5.39. Percentage permeation of ibuprofen from IBULEVE® Speed Relief 5% Spray (5% w/w) to human skin in the PAMPA model, mean ± SD, n=8

After 48 hours, about 80 per cent of applied ibuprofen permeated through the skin as shown in Figure 5.39. The corresponding value permeated from IBULEVE® Speed Relief 5% Spray (5% w/w) in the Skin PAMPA model with the artificial silicone membrane was ~ 70 % after 6 h.

5.3.1.9 Comparative studies of *in vitro* permeation of ibuprofen in human skin, porcine skin, silicone membrane and the Skin PAMPA model

Cumulative amounts of ibuprofen permeated from the four selected formulations (IBULEVE®, IBUGEL®, and the two ibuprofen solutions in binary solvent systems prepared as 5% w/w solutions in isopropyl alcohol and either

PEG 300 or PG) for the four different models (human skin, porcine skin, silicone membrane, skin PAMPA with artificial membrane) are compared in Figures 5.40 and 5.41, respectively.

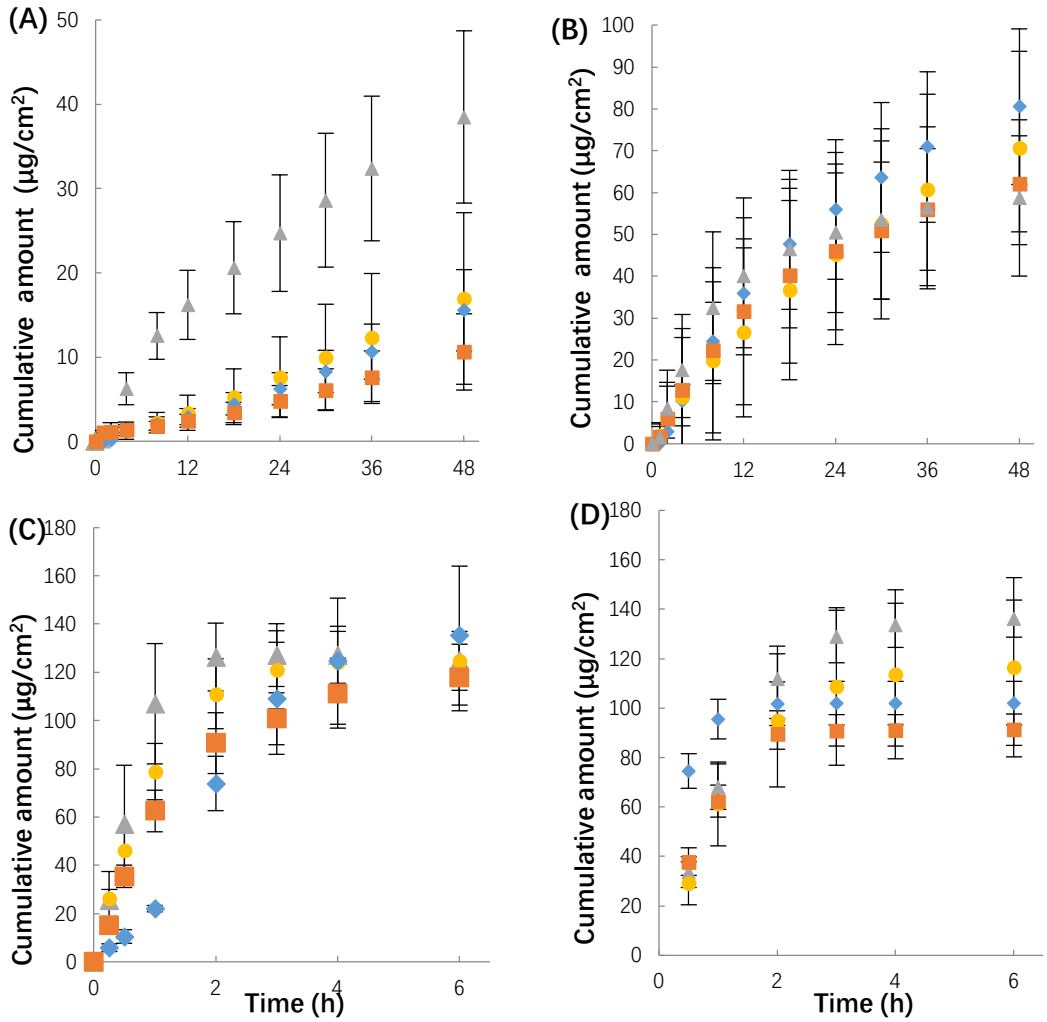

Figure 5.40 Cumulative amounts of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) during finite dose Franz cell studies; Human skin (A), Porcine skin (B), Silicone membrane (C) and Skin PAMPA (D). Each data point represents the mean ± SD (n≥5).

After 6 h, similar amounts of ibuprofen had permeated in silicone membrane and in Skin PAMPA (about140 µg/cm^2). After 48 h, maximum amounts of permeation were ~80 µg/cm^2 for porcine skin and ~40 µg/cm^2 for human skin. Typical curvilinear permeation profiles for all formulations were observed with all the membranes studied. Higher permeation in porcine skin

compared with human skin has also been observed by other researchers (Dick and Scott, 1992; Singh et al., 2002; Barbero and Frasch, 2009).

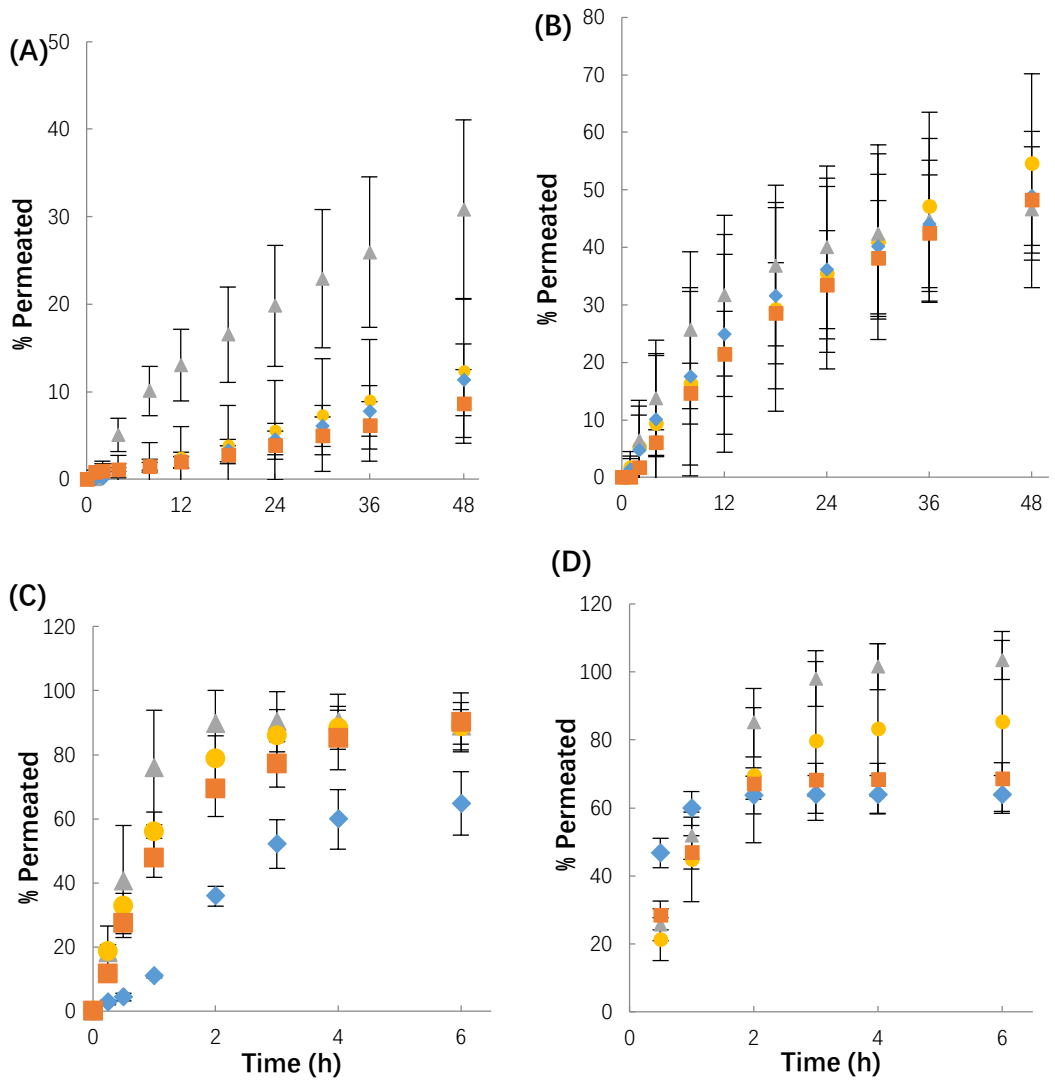

Figure 5.41 Percentages of ibuprofen permeated from IBUGEL® (◆), IBULEVE® (■), PG (▲) and PEG 300 (●) during finite dose Franz cell studies; Human skin (A), Porcine skin (B), Silicone membrane (C) and Skin PAMPA (D). Each data point represents the mean ± SD (n≥5).

As noted, at 24 h the percentage permeation was ~ 40% in porcine skin compared with much lower per cent (~4%) in human skin, with the exception of the PG formulation where 20% of the dose has permeated. Comparatively

higher percentages of active permeated at 6 h for the Skin PAMPA and silicone membrane modes with values ranging from 60 to 100%.

5.3.2 Assays for caffeine

5.3.2.1 Assay 1 for caffeine

Two caffeine formulations, namely 50 µg/mL and 20 µg/mL of caffeine in water were investigated in this assay. In contrast to ibuprofen formulations (section 2.3.4.3), the lag time for caffeine is longer according to the results of this assay, which is in line with the *in vitro* Franz cell studies in porcine skin. After 3 h, only caffeine from Form A (50 µg/mL) permeated through the Skin PAMPA membrane. The permeation profile of caffeine from Form A is shown in Figure 5.42.

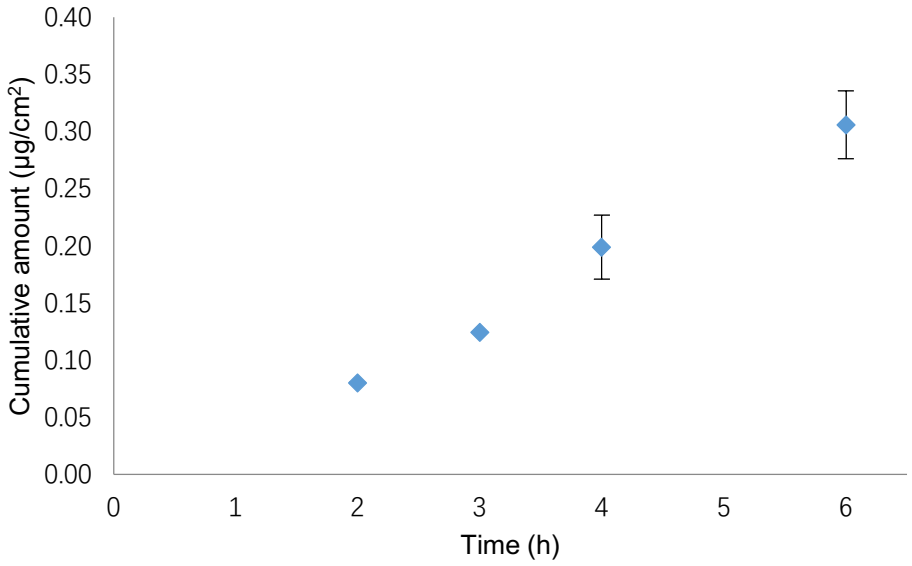

Figure 5.42 Cumulative permeated amount of caffeine permeated from the formulation with 50 µg/mL caffeine in water in the PAMPA assay 1 for caffeine, mean ± SD, n=3

The $logk_p$ obtained from the Skin PAMPA model for caffeine from the formulation with 50 µg/mL of caffeine in water was -6.49 ± 0.01, while the $logP_a$ calculated by the PAMPA Explorer® for caffeine in the same formulation was -6.02 ± 0.04. In addition, the $logk_p$ obtained for caffeine in the *in vitro* Franz cell studies in porcine skin was -7.03 ± 0.05 (session 3.3.5.2). Moreover, the $logk_p$ obtained human skin using diffusion cells for caffeine was reported as 7.56 according to Mitragotri et al.(1995). This indicates that all these data are consistent with other studies, indicating that PAMPA seems to be a promising model for rapid prediction of permeation through the stratum corneum for caffeine.

5.3.2.2 Assay 2 for caffeine

Three formulations were investigated in this assay, namely 800 µg/mL (Form A), 40 µg/mL (Form B) and 20 µg/mL caffeine in water (Form C). The permeation profiles of caffeine in this assay are shown in Figure 5.43.

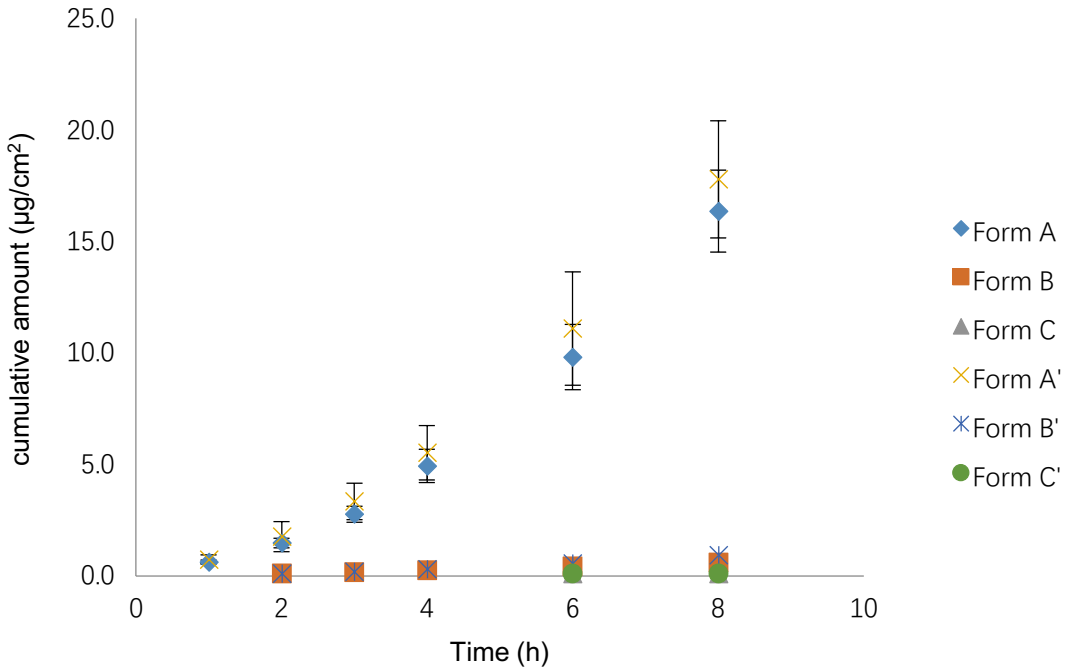

Figure 5.43 Cumulative permeated amounts of caffeine permeated from 800 µg/mL (Form A), 40 µg/mL (Form B) and 20 µg/mL caffeine in water (Form C) in the PAMPA assay 2

Form A, B and C were studied with wells with stirring disks in both donor and receptor compartments while form A', B' and C' were formulations in those wells where there were only stirrer plates in receptor compartments. It was hypothesised that stirrer plates could influence permeation. However, the results show that any improvement was not significant. At the end of 8 h, around 17 µg/cm^2 caffeine went through the membrane from Form A (800 µg/mL caffeine in water), while less than 1 µg/cm^2 of the active was delivered into the receptors from Form B (40 µg/mL caffeine in water) and C (20 µg/mL caffeine in water).

5.3.2.3 Assay 3 for caffeine

Three caffeine formulations in PG (100, 80 and 60% saturated solubility) were studied in assay 3 for caffeine. The permeation profiles are illustrated in Figure 5.44.

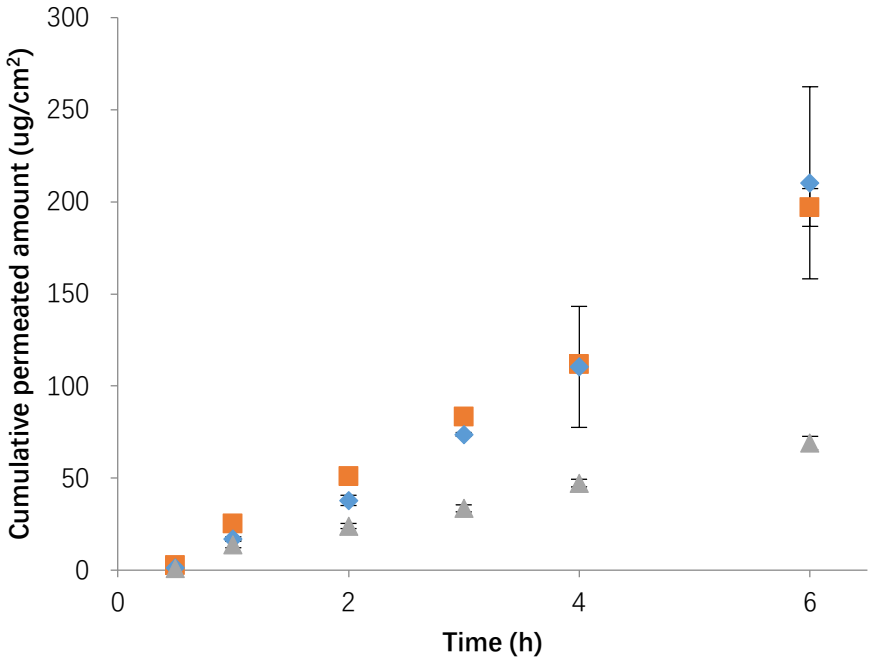

Figure 5.44 Cumulative permeated amounts of caffeine for in PG with different saturated solubility levels, namely 100% (■), 80% (◆) and 60% (▲), mean ± SD, n=5.

After 6 h, the permeation of caffeine from the formulations with 100 and 80% saturated solubility levels were similar, with values of about 200 μg/cm^2. The corresponding value for the formulation with lower saturation (60%) was significantly lower ($p<0.05$), approximately 70 μg/cm^2.

Compared with data from the previous study, caffeine permeation from PG was greater ($p< 0.05$) than from the aqueous formulation (Figure 5.42).

Figure 5.45 shows the permeation profiles of PG from the tested caffeine formulations with PG.

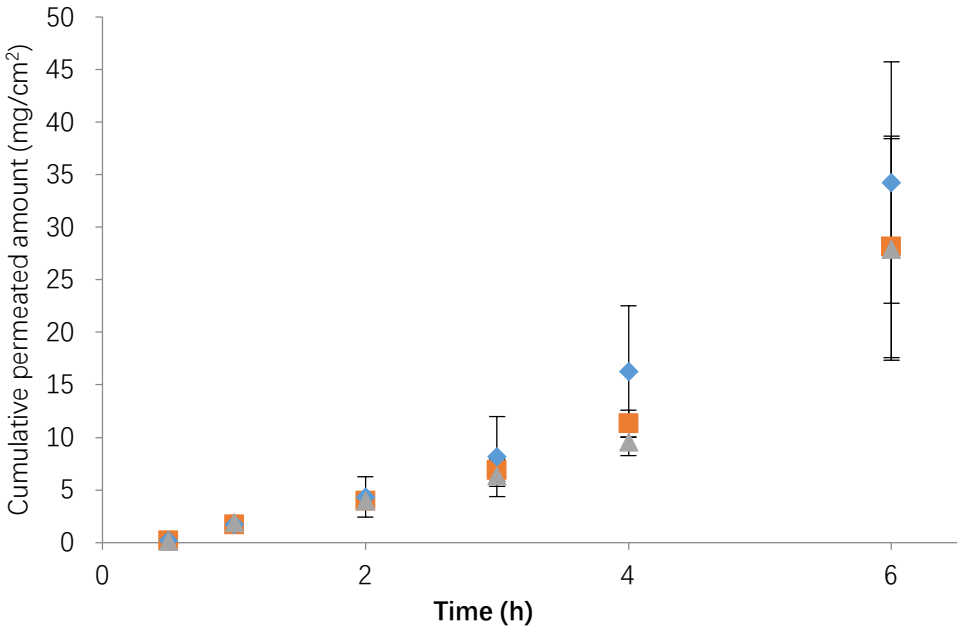

Figure 5.45. Permeation profile of PG from caffeine formulations at different saturated solubility levels, namely 100% (■), 80% (◆) and 60% (▲), mean ± SD, n=5.

In contrast to the active, there was no significant difference in the permeation of the solvent PG ($p > 0.05$), regardless of the concentration of caffeine. At 6 h, the amounts of PG that permeated were about 30 µg/cm^2 for all the three tested formulations. As mentioned in section 3.3.5.2, the effect of the degree of drug saturation (DS) for a model drug, oxybutynin, on solvent permeation, octyl salicylate (OSAL) and PG, and drug permeation in silicone membrane and human skin has been investigated by Santos et al. (2009). Their results showed that in both human skin and silicone membranes, the PG permeation remained unaffected with the DS of the formulations, which is in line with the result shown in this assay for caffeine by the Skin PAMPA model.

5.3.2.4 Assay 4 for caffeine

Three caffeine formulations in PG with different saturated levels (100, 80 and 60% saturated solubility) were studied in this assay. The difference here compared with assay 3 was that the top plate was used as the donor compartment and the dose was 30 µL per well. Figure 5.46 shows the permeation profiles of caffeine from the PG formulations for this assay.

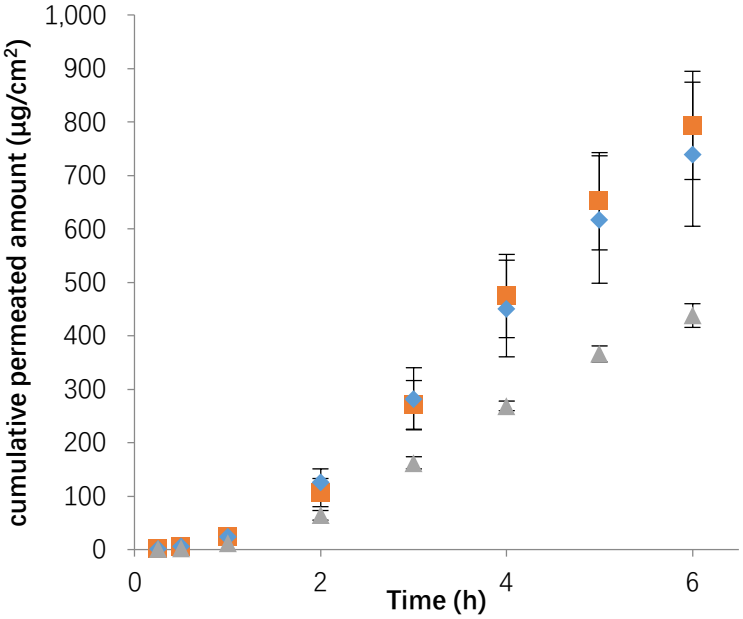

Figure 5.46 Cumulative permeated amounts of caffeine from PG formulations with different caffeine saturated solubility levels, namely 100% (■), 80% (◆) and 60% (▲), mean ± SD, n=5.

After 6 h, about 750-800 µg/cm^2 caffeine penetrated through the membrane from the 100 and 80% saturated solubility formulations; the amount permeated from the 60% saturated solution was 440 µg/cm^2. According to the results, the permeation of caffeine was much higher compared with the infinite dose studies, where about 200 µg/cm^2 of active permeated from the 100 and

80% saturated solubility solutions and the value for the 60% saturated formulation was 70 μg/cm^2 (Figure 5.44). This indicates that keeping sink conditions in the receptor phase is critical. Otherwise, the permeability may be underestimated to a significant extent. However, although the doses applied in this assay were much smaller than in assay 3, and the receptor phase was replaced by fresh PBS at specific intervals, there were still quite a lot of wells in which the caffeine concentrations were higher than the requirements for sink conditions. Therefore, other solvents will be necessary in the receptor phase systems for caffeine, or the applied dose will need to be decreased.

5.3.2.5 Assay 5 for caffeine

The formulations which were studied in this assay were the two commercial caffeine formulations, EVELINE® and L'Oréal®. The dose was 30 μL per well and the bottom plate acted as the receptor. Figure 5.47 shows the permeation profile for caffeine from the tested formulations.

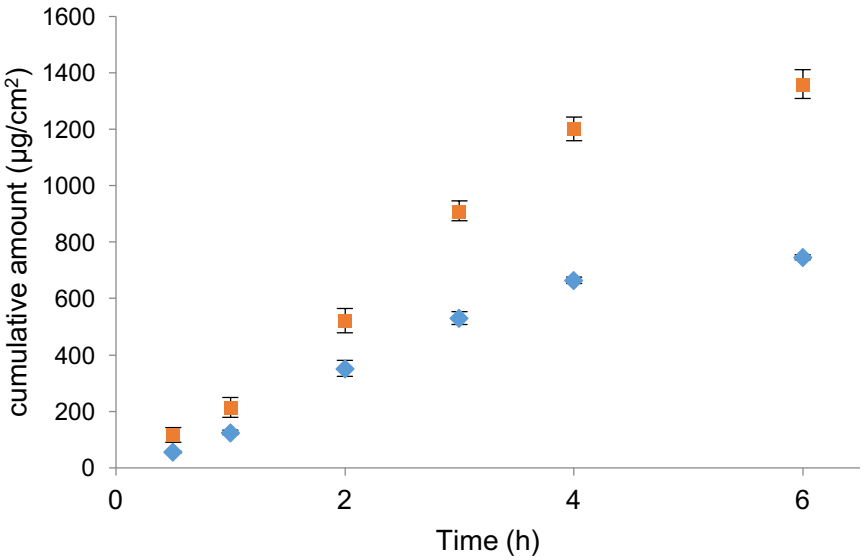

Figure 5.46 Skin PAMPA cumulative permeated amount of caffeine from EVELINE®(■) and L'Oréal®(◆), mean ± SD, n=6.

At 6 h, approximately 1360 µg/cm^2 caffeine permeated from the EVELINE® formulation which was significantly higher ($p<0.05$) than the corresponding caffeine permeation from the L'Oréal® formulation (~745 µg/cm^2). The cumulative percentages of caffeine that permeated from the two formulations are shown in Figure 5.48.

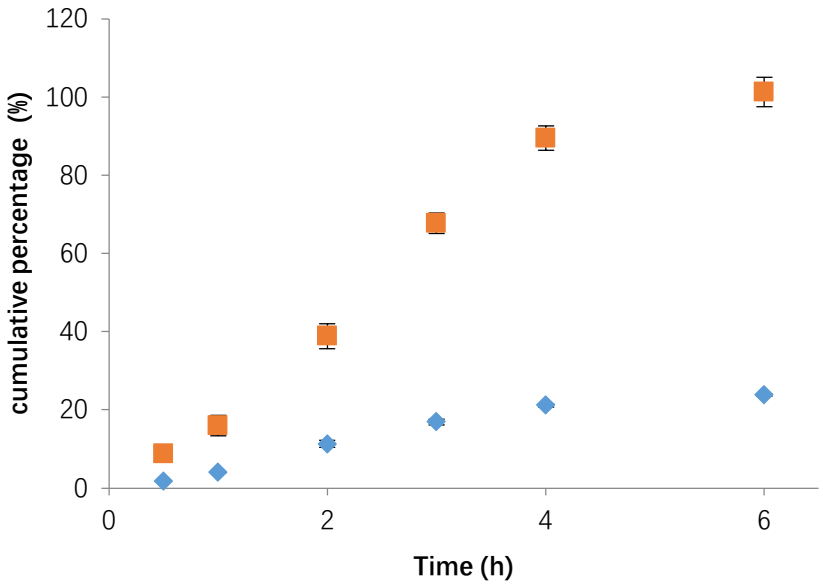

Figure 5.48 Skin PAMPA cumulative percentages permeated of caffeine from EVELINE®(■) and L'Oréal®(◆), mean ± SD, n=6.

All the caffeine permeated from the applied dose of EVELINE® through the membrane after 6 h, while a significantly lower percentage permeated ($p<0.05$) from the L'Oréal® formulation, which was only about 23%. These results are consistent with those from the *in vitro* Franz cell studies in human skin (section 4.3.2).

5.3.2.6 Assay 6 for caffeine

The commercial caffeine formulations, EVELINE® and L'Oréal® were investigated in this assay again, but the dose was decreased to 3 µL per well and the lip was used as the donor compartment here to better simulate finite dose studies. Figure 5.49 illustrates the cumulative permeation of caffeine from the two formulations.

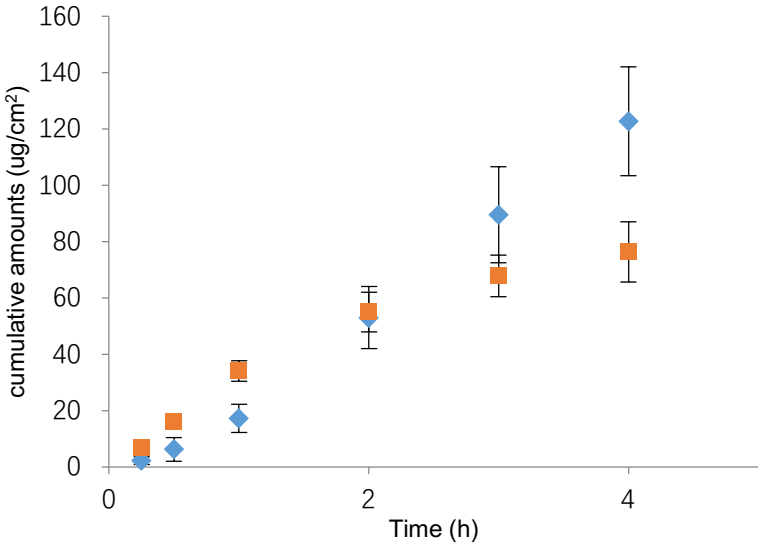

Figure 5.49 Cumulative permeated amounts of caffeine from EVELINE®(■) and L'Oréal®(◆) formulations in the Skin PAMPA, mean ± SD, n=6.

After 4 h,122 μg/cm^2 caffeine permeated from the L'Oréal® formulation from the bottom of the membrane to the receptor (top plate). The corresponding value from the EVELINE® formulation was 76 μg/cm^2. In contrast to the results in assay 5, the permeation of caffeine from L'Oréal® is greater than that from EVELINE® after 4 h in this assay.

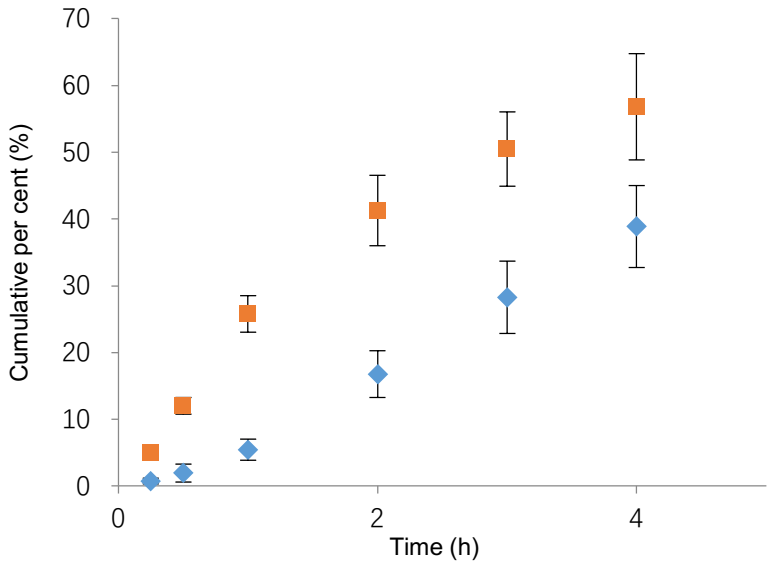

Figure 5.50 Cumulative amounts of caffeine permeation from EVELINE®(■) and L'Oréal®(◆) formulations , mean ± SD, n=6

The cumulative percentages permeated are shown in Figure 5.50. It is clear that more than 50 per cent of the applied caffeine in EVELINE® went through the membrane in the Skin PAMPA while the percentage penetrated from the L'Oréal® was less than 40 per cent. The results also illustrate that caffeine permeates to a higher extent from EVELINE® even though the content of caffeine in L'Oréal® was nearly twice that in EVELINE®.

5.3.2.7 Assay 7 for caffeine

Three different doses, 1, 3, or 30 µL per well, were investigated in this assay for the two commercial formulations L'Oréal® and EVELINE®, in order to determine the amount of formulation which best represented finite dose conditions. The top plate was the donor and the bottom plate acted as the receptor, which meant the structure of the Skin PAMPA in this assay was comparable to that of a conventional Franz cell. The permeation profiles for EVELINE® are illustrated in Figure 5.51.

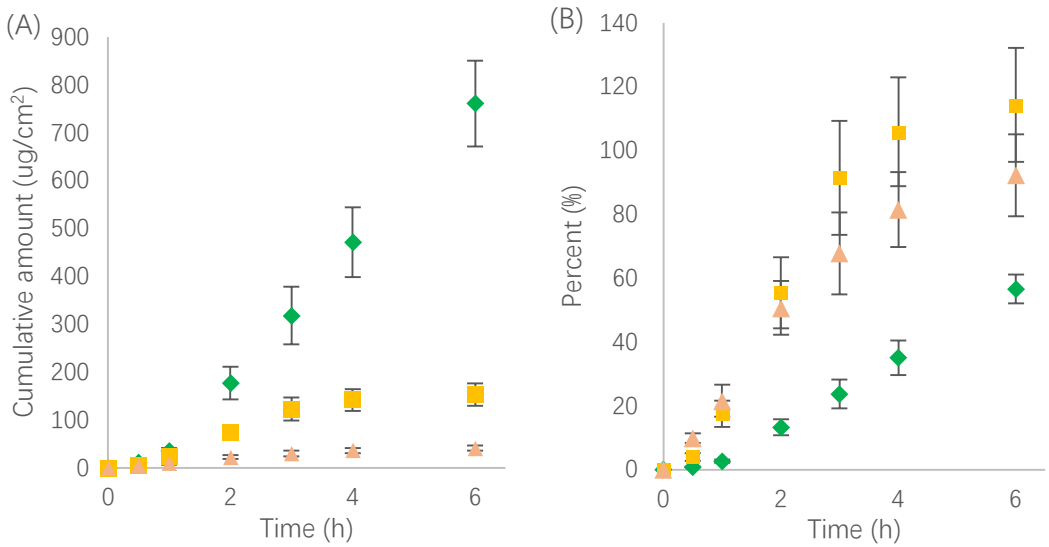

Figure 5.51. Cumulative permeation profiles of caffeine from EVELINE®, 30µL (◆), 3 µL (■) and 1 µL (▲) per well. Each data point represents the mean ± SD, n=6

It is clear that after 6 h, the amounts of caffeine permeated from EVELINE® increased with the applied doses. The caffeine permeation was about 760, 240 and 42 µg/cm^2, respectively, from the 30, 3 and 1 µL dose. However, there was no significant difference between the per cent of permeated active for the 3 and 1 µL dose.

Figure 5.52 shows the permeation profiles for L'Oréal®.

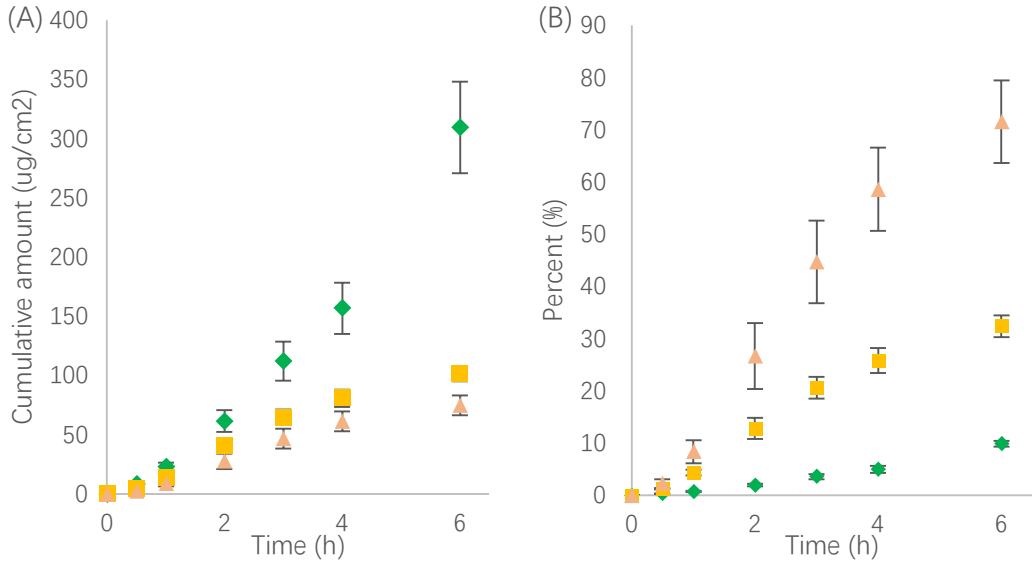

Figure 5.52 Cumulative permeation profiles of caffeine from L'Oréal®, 30µL (◆), 3 µL (■) and 1 µL (▲) per well. Each data point represents the mean ± SD, n=6

Consistent with the results for EVELINE®, the permeation amounts of caffeine from L'Oréal® increased with the applied doses, with values of about 310, 100 and 75 µg/cm^2, separately. The percentage permeation of caffeine was significantly higher when the dose was smaller, namely about 72% for the 1 µL dose, about 32% for the 3 µL dose and about only 10% for the 30 µL dose.

Figure 5.53 compares the permeation profiles of caffeine for both of the formulations from the 30 µL dose (A) and 1 µL dose (B).

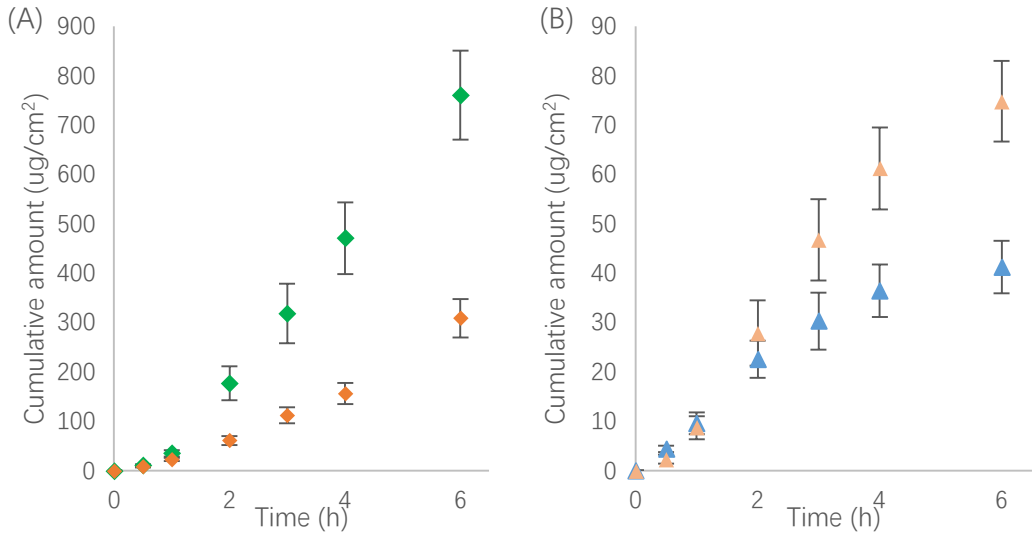

Figure 5.53 Cumulative permeation profiles of caffeine from EVELINE® dosed at 30 µL (◆) and 1 µL (▲) per well, and L'Oréal® dosed at 30 µL (◆) and 1 µL (▲) per well. Each data point represents the mean ± SD, n=6

For the 30 µL applications, the profiles for both of the formulations are generally linear, consistent with these amounts representing infinite doses. The amount permeated for the 1 µL dose approaches values for human skin (section 4.3.2), which suggests realistic finite dose conditions in Skin PAMPA for caffeine should be 1 µL (3.3 µL/cm^2).

5.3.2.8 Assay 8 for caffeine

Figure 5.54 shows the permeation profile of caffeine from the L'Oréal® Sublime Slim patch.

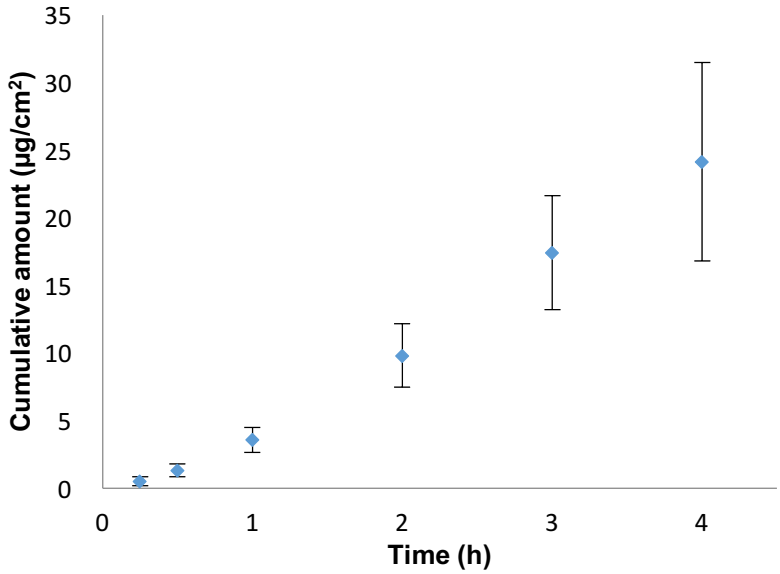

Figure 5.54 Cumulative permeated amounts of caffeine in the Skin PAMPA model for the hydrogel patch. Each data point represents the mean ± SD, n=16.

As the area of each well in the Skin PAMPA sandwich was quite small (about 0.3 cm^2), cutting the patch to fit each well may damage the patch. Therefore, the patch was just cut to cover all the wells on column 1 and 2, and hence separate doses on each well and the cumulative percentage permeation could not be determined.

Comparing the results with those for human skin (section 4.3.2), caffeine was more permeable in human skin in Franz cell studies than the Skin PAMPA membrane. However, the patches in the Franz cells became thicker after the 48 h experiment, even though silicone was applied to seal the skin and patch between the donor and receptor apartments of Franz cells. The absorption of water might increase the mobility of caffeine in the patch and then increase the caffeine permeation. In the Skin PAMPA model, the studies ran for a much shorter time, so the absorption of water by the patch may not be so obvious.

5.4 Conclusions

The *in vitro* permeation studies were conducted in the novel skin PAMPA model, for various ibuprofen and caffeine formulations.

For ibuprofen, 9 different assays were designed with the Skin PAMPA in order to study the permeation from different commercial formulations and simple solutions. Based on the Skin PAMPA assays conducted, the performance of the model may be summarised as follows:

- ✓ Firstly, due to the small volume (200 µL) of the well in the Skin PAMPA Sandwich, it was difficult to conduct permeation studies for formulations with high concentrations. If the active could permeate through the membrane rapidly and its applied amount is high, for example 200 µL per well, it is challenging to maintain sink conditions in the receptor phase throughout the studies. Hence, only those formulations with relatively low concentrations of actives, rather than saturated formulations, are appropriate candidates for Skin PAMPA models, especially in infinite dose studies. However, the application of unsaturated formulations may cause other problems, for instance, when comparing the permeability of an active in different formulations as thermodynamic activity of the active is not standardised.

- ✓ Secondly, it has been shown that the temperature across the whole Sandwich plate was uniform. Therefore, in the Skin PAMPA studies in the same plate, temperature control is achieved.
- ✓ Thirdly, evaporation of the phase from bottom plate (either acting as a donor or receptor plate) was observed, which could be a critical problem in the permeation studies. When the bottom plate was used as the receptor plate, adding PBS in those wells without formulation from the beginning of the experiment did not resolve the problem of evaporation. Also due to this problem, when formulations contain volatile ingredients, such as alcohols, the bottom plate could not be used as the donor compartment. This is because with the evaporation of the liquid in the bottom plate, the contact area between the formulation and the membrane changed during incubation.
- ✓ For the comparative study of ibuprofen permeation conducted using human and porcine tissue, a skin PAMPA model and silicone membrane, ibuprofen was generally more permeable in Skin PAMPA than human skin after 6 h and Skin PAMPA data were comparable to results in silicone membrane. For individual formulations permeation is also higher in Skin PAMPA compared with porcine skin. As for silicone membrane, the composition of the Skin PAMPA membrane is considered to be homogeneous and inert. The low variability of results obtained from Skin PAMPA and silicone may be attributed to these membrane characteristics. With porcine and human skin, the results are naturally more

variable due to the added complexity of biological membranes. Although the time for permeation studies in Skin PAMPA was not varied, shorter experimental times may be more appropriate considering the relatively high percentage of ibuprofen permeation in Skin PAMPA.

- ✓ Application conditions in Skin PAMPA which approach realistic finite dose conditions were confirmed to be 1 μL (3.3 μL/cm^2) however it is important to note that this may be specific to a particular active. Interestingly, the formulation which demonstrated the highest delivery in human skin was also the formulation which performed best for the 1 μL application conditions in the Skin PAMPA. The Skin PAMPA model also appears to be more sensitive to differences in formulation composition i.e. gel versus solution. Further studies expanding the range of molecules and formulations which may be suitable for screening using Skin PAMPA are currently underway. This should provide insight into which formulations are best suited to evaluation using this model.

A series of assays PAMPA model and allow the following conclusions to be drawn:

- ✓ Assay 1: The logk_p obtained from the Skin PAMPA model in this assay was comparable with the values acquired for Franz cell models using both porcine and human skin, which indicated that

the Skin PAMPA is a promising model for rapid prediction of molecular permeation through the stratum corneum for caffeine.

- ✓ Assay 2: The introduction of stirring disks in the donor for infinite dose studies for liquid formulations did not improve the permeation significantly ($p > 0.05$).
- ✓ Assay 3: Caffeine permeated from PG formulations to a greater extent than from aqueous solutions. Moreover, similar to the results from the studies in Franz cells using porcine skin, the formulations with high caffeine concentrations (100 and 80% saturated solubility levels) lead to higher active permeation than the formulation with low saturated levels (60%) in the Skin PAMPA model. However, the permeation of PG did not change significantly ($p > 0.05$) regardless of the concentration of caffeine.
- ✓ Assay 4: When the dose was decreased to 30 µL per well from 200 µL per well, the permeation of caffeine became much higher, which again indicated that sink conditions played a critical role in the permeation studies in the PAMPA model for caffeine, the same as for ibuprofen.
- ✓ Assay 5: For the two selected commercial caffeine formulations, EVELINE® shows better efficiency in delivering caffeine than L'Oréal® in infinite dose studies in the Skin PAMPA model. The results were in line with those from Franz cell studies in human skin.
- ✓ Assay 6: The lip was not an ideal donor compartment in the Skin PAMPA model even when the dose was as small as 30 µL per

well, because there was no border on it, which may result in contamination between applied formulations.

- ✓ Assay 7: Similar to ibuprofen, the application conditions for caffeine in Skin PAMPA which approach realistic finite dose conditions were confirmed to be 1 μL (3.3 μL/cm^2).
- ✓ Assay 8: For the hydrogel patch caffeine was not as permeable as for Franz cell studies using human skin. The possible reason might be that during a 48 h study on Franz cell, the patch absorbed a certain amount of water, which improved the mobility of caffeine in the patch and then improved caffeine permeation. However, the study in Skin PAMPA was much shorter (4 h), and the water absorption in the patch would be much less.

Even though the Skin PAMPA technique seems to have some limitations, the results from the various assays conducted confirm that the Skin PAMPA model discriminated between different formulation types and different solvent systems compared with other models, with low variability in the permeation data. The advantages associated with this model also include ease of standardization, high throughput of samples and low cost compared with experiments which require human or animal tissue.

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Chapter 6 Formulation studies and *in vivo* studies for caffeine

6.1 Introduction

In the development of a topical or transdermal formulation, skin permeation assessment of the actives or excipients in a critical step. Current skin permeation assessment methods are mainly divided into *in vitro* and *in vivo* methods. Common *in vitro* methods involve infinite and finite dose studies in the conventional Franz cell models using human skin, animal skin or non-animal membranes, and the novel Skin PAMPA model. These methods have been described in detail in the previous chapters. There are also a number of techniques which are used in *in vivo* percutaneous absorption studies. Those techniques which were applied in the present studies are examined in this chapter.

6.1.1 Trans-epidermal Water Loss (TEWL)

The Trans-epidermal Water Loss (TEWL) is the total amount of water loss through the skin. This water loss can be in two ways, namely liquid water loss by passive diffusion passing through the stratum corneum and/or water vapour loss due to sweating (Blank, 1952; Rogiers and Group, 2001). TEWL

is a non-invasive method which elucidates the barrier properties of the skin and the effect of formulations on the skin barrier integrity. The TEWL value increases when the skin barrier is disrupted and corresponds to the water vapour that evaporates from the skin to the skin surface with the condition that the skin surface remains dry during the measurement (Imhof et al., 2009).

The measurement of TEWL can be conducted with three types of instruments, namely, the open-chamber system, unventilated-chamber system and condenser-chamber system.

In the open-chamber device, both the top and lower ends of the cylinder are open. The lower end has an orifice, which is placed on the skin surface, and the top end acts as an exhaust. The problem in this approach is that the interference of ambient air flow may affect the measurements (Imhof et al., 2009). This interference from the ambient air flow can be avoided by using closed-chamber devices, either the unventilated-chamber apparatus or the condenser-chamber apparatus. In both devices, the upper end of the cylinder is closed and the lower end consists of an orifice that is placed on the study surface. However, with the unventilated-chamber method, the measurement cannot be taken continuously because of the accumulation of water vapour inside the chamber. This means that after each reading, the water vapour needs to be purged from the system (Pinnagoda et al., 1990; Imhof et al., 2009). A closed-chamber system was introduced to overcome this limitation. This system is equipped with a condenser inside to remove the water vapour and therefore no purging is needed in between the measurements

(Farahmand et al., 2009). In this closed measurement chamber, the open end is in contact with the skin surface and the water vapour from the skin surface diffuses towards the top end of the cylinder. The water vapour is removed by freezing onto the condenser on the closed end at the top of the chamber. This condenser creates a diffusion vapour density gradient and at steady state conditions, the flux density (TEWL) may be described by Fick's first law of diffusion in Equation 6.1(Imhof et al., 2009).

$$J = -D\frac{\Delta c}{\Delta z} \qquad \text{Equation 6.1}$$

where, J = water vapour flux density-TEWL ($kg.m^{-2}.s^{-1}$)

D = diffusion coefficient of water in the stratum corneum ($m^2.s^{-1}$)

Δc = positive concentration difference across the membrane ($kg.m^{-3}$)

Δz = membrane thickness (m)

6.1.2 Tape stripping

Tape stripping is an efficient method to evaluate the quality and efficacy of topical formulations (Moser et al., 2001; Lademann et al., 2009). In this manner, the stratum corneum is removed from the skin using adhesive tapes. The amount of stratum corneum removed is influenced by the formulations

applied on the skin, therefore this method can not only provide information on the amount of actives permeated through the skin, but also about the homogeneity and the distribution of actives in the different depths of the stratum corneum (Lademann et al., 2009). The amount of removed stratum corneum can be determined by quantifying the amount of protein content, because protein is the primary material in the stratum corneum (Diffey, 1983).

6.1.3 Confocal Raman Spectroscopy (CRS)

Confocal Raman Spectroscopy (CRS) is a technique with considerable potential for the non-invasive study of biological tissues and skin samples *in vivo* and *in vitro* (Forster et al., 2011). This technique can be applied to investigate skin physiology and possible pathological conditions and to examine the molecular composition and the structure of skin. The application of this technique in the determination of the composition of skin layers *in vivo* and *in vitro* was pioneered by Caspers et al. in the late 1990s (Caspers et al., 1998; Caspers et al., 2001; Caspers et al., 2002; Caspers et al., 2003; Pudney et al., 2007).

This technique is now regarded as the “gold standard” for the measurement of SC thickness. In 2013, a report was published about the utility of this technique for probing drug disposition *in vivo* (Mateus et al., 2013). It was confirmed that ibuprofen distribution profiles in the SC were in line with

previous tape stripping studies and hence Mateus et al. proposed CRS as a non-invasive strategy in dermatopharmacokinetic evaluation of topical formulations.

In the present study, CRS was applied in *in vivo* studies to investigate distribution of caffeine. and the results will be compared with those from *in vitro* Franz diffusion cells and PAMPA experiments.

6.1.4 Aims

The aims of the studies in this chapter are as follows:

- To conduct *in vitro* formulation studies for caffeine in the conventional Franz cell model and the novel Skin PAMPA model to investigate if binary and ternary solvent systems improve caffeine permeation;
- To investigate how *in vitro* formulation studies compare with *in vivo* studies and to verify if Confocal Raman Spectroscopy (CRS) may be suitable to predict passive permeability of caffeine by comparing *in vivo* studies with CRS with the conventional tape stripping technique and with *in vitro* Franz cell studies.

6.2 Materials and methods

6.2.1. Materials

6.2.1.1 Chemicals and tissues

Analytically pure standards of caffeine, Sudan III dye, 1,2-pentanediol (1,2-pent), 1,3-butylene glycol (1,3-BG)/1,2-butylene glycol (1,2-BG) and propylene glycol (PG) were obtained from Sigma-Aldrich (UK). Geraniol (GER), limonene (LIM), t-butyl alcohol (T-BA), Linalool (Lina) and tripropylene glycol (TPG) were purchased from Fisher Scientific (UK). Isopropyl myristate (IPM), and dimethyl isosorbide (DMI) were gifts from Croda Ltd. (UK). 1, 2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), propylene glycol monocaprylate (PGMC), propylene glycol monolaurate (PGML), Transcutol® (TRANS) were received as gifts from Gattefossé (France). PEG-6-caprylic/capric glycerides (PEG-6-CCG) were a gift from Avon. These solvents are generally regarded as safe (GRAS) chemicals.

HPLC grade water, acetonitrile (HPLC grade), trifluoroacetic acid (HPLC grade) and absolute ethanol were purchased from Fisher Scientific (UK). Phosphate buffered saline (pH 7.4 at 25^{o}C) was prepared using Dulbecco A Tablets (Oxoid, UK).

6.2.1.2 Instruments

A water bath (SUB Aqua 26) was obtained from Grant (UK), an electronic balance (Sartorius 1702) was from Sartorius (Germany), and a sonicator (PDL 356) was obtained from Camlab Serving Science (UK). The HPLC system used consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. The software used to acquire and analyse the data was ChemStation® for LC 3D Rev. A. 09.03 (Agilent Technologies, U.S.A.). A water purification system with a 75 L reservoir (Elga Option 3) was obtained from Veolia Solutions and Technologies (UK). A rotator (SB2) was from Stuart Equipment (U.S.A.). A centrifuge (5415R) was from Eppendorf (U.S.A.).

6.2.2 Methods

6.2.2.1 *In vitro* formulation studies for caffeine

6.2.2.1.1 Miscibility studies

Miscibility studies were conducted for both binary and ternary systems at room temperature. For binary systems, the solvents were mixed at different proportions as shown in Table 6.1.

Table 6.1 Proportions of solvents investigated in miscibility studies of binary systems

Solvent A (%)	Solvent B (%)
10	90
20	80
30	70
40	60
50	50
60	40
70	30
80	20
90	10

Table 6.2 shows the solvent mixtures at different proportions for ternary systems.

Table 6.2 Proportions of solvents investigated in miscibility studies of ternary systems

Solvent A (%)	Solvent B (%)	Solvent C (%)
10	10	80
10	20	70
10	30	60
10	40	50
10	50	40
10	60	30
10	70	20
10	80	10
20	10	70
20	20	60
20	30	50
20	40	40
20	50	30
20	60	20
20	70	10
30	10	60
30	20	50
30	30	40
30	40	30
30	50	20
30	60	10
40	10	50
40	20	40
40	30	30
40	40	20
40	50	10
50	10	40
50	20	30
50	30	20
50	40	10
60	10	30
60	20	20
60	30	10
70	10	20
70	20	10
80	10	10

When a clear and transparent mixture is visualized, it is considered as miscible. On the contrary, if the mixtures form separate layers or the whole mixture is cloudy, then the mixture would be considered as immiscible. If it is difficult to determine whether the mixture is miscible or not, Sudan III dye, which is absorbed more into the lipophilic layers in the mixture, may be added. Therefore, with the dye, miscible mixtures would have one uniform and homogeneous mixture, while the immiscible mixtures would show separate layers with a distinct redness.

6.2.2.1.2 Solubility studies

Solubility studies of caffeine in each selected solvent and solvent system were conducted. The method for solubility studies is described in Section 2.2.2.2.

6.2.2.1.3 Single solvent systems

As a potential skin permeation screening model, the Skin PAMPA was compared with the conventional Franz cell model. In the single solvent system studies, sixteen solvents were selected and these solvents were 1,2-pentanediol (1,2-pent), 1,3-butylene glycol (1,3-BG), 1,2-butylene glycol (1,2-BG), propylene glycol I (PG), limonene (LIM), t-butyl alcohol (T-BA),

tripropylene glycol (TPG), isopropyl myristate (IPM), dimethyl isosorbide (DMI), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), propylene glycol monocaprylate (PGMC), propylene glycol monolaurate (PGML), PEG-6-caprylic/capric glycerides (PEG-6-CCG), and Transcutol® (TRANS).

Caffeine formulations were prepared in all these selected solvents as single solvent systems. The concentration of each formulation was prepared at 80% of the saturated solubility level of the solutions in order to maintain the thermodynamic activity at the same level and to avoid drug crystallisation. The skin permeation of caffeine from each of these systems was screened by the Skin PAMPA model initially. The membrane for the Skin PAMPA was hydrated by filling 200 µL of the hydration solution in each single well of the bottom plate overnight. After removing the hydration solution, 3 and 30 µL of each tested formulations were applied on the membrane surface in each well of the top (donor) compartment of the PAMPA Sandwich. Two Skin PAMPA sandwiches were used for all sixteen systems. The various assays are shown in Figure 6.1.

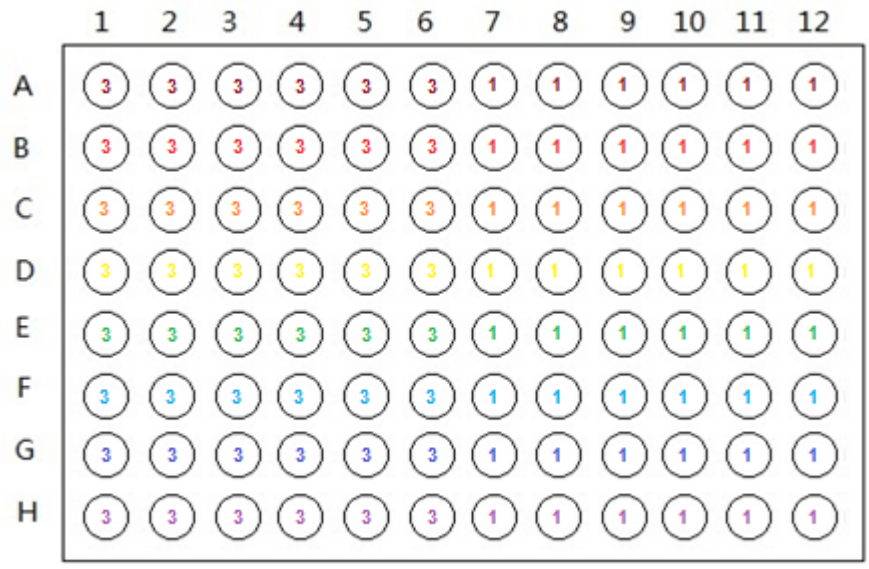


Figure 6.1 Utilization of the Skin PAMPA sandwiches for caffeine single solvent system studies, 3 in the circle indicates the dose was 3µL per well and indicates the dose was 1µLper well.

The corresponding wells in each bottom (receptor) plate were pre-filled with 180 µL of phosphate-buffered saline (PBS), pH 7.4 and a stirred disk in each well. After preparation the resulting PAMPA Sandwich was incubated in the Gut-Box^{TM} at 32 ± 1^{o}C. At 0.5, 1, 2, 3, 4 and 6 h, the bottom (receptor) plates were replaced with plates prefilled with fresh receptor phase and stirrer disks. Samples were then analysed by HPLC to quantify the permeation of actives from the tested formulations. After screening with the Skin PAMPA model, finite dose permeation studies were also performed on human skin for a number of the single solvent systems. Vertical glass Franz diffusion cells were used to perform the studies and the method used is described in Section 4.2.2.2. 3.6 µL of each formulation were applied to the skin surface and the studies were run up to 48 h. Mass balance studies were also conducted after permeation studies. The method used is described in Section 4.2.2.3.

6.2.2.1.4 Binary solvent systems

The Skin PAMPA was also used as a skin permeation screening tool for the caffeine binary solvent systems. Finite dose Skin PAMPA studies were conducted for these systems which were selected according to the results from the single solvent systems and miscibility studies. The concentration of each formulation was prepared at 80% of the saturated solubility level of the formulations in order to maintain the thermodynamic activity at the same level and to avoid drug crystallisation. The method was the same as that for the single systems (section 6.2.2.1.3) and the dose in this assay was 1 µL per well.

Finite dose permeation studies on Franz cells using human skin and mass balance studies were carried out for all the selected binary systems. The methods are described in section 4.2.2.2 and 4.2.2.3.

6.2.2.1.5 Ternary solvent systems

Based on the results from the previous caffeine solvent studies and the miscibility studies, two ternary solvent systems in which the solvents were miscible with each other and with high efficiency in delivering caffeine were selected to conduct *in vitro* permeation studies in Franz cells using human skin. Mass balance studies were also run afterwards. The experiments were carried out using the methods described in section 4.2.2.2 and 4.2.2.3.

6.2.2.2 *In vivo* studies for caffeine formulations

6.2.2.2.1 Ethical approval and volunteer recruitment

Ethical approval was obtained from the North London REC1 Central Middlesex Hospital Research Ethics Committee (10/H0717/100). 6 volunteers were recruited from the UCL School of Pharmacy. The volunteers were 6 Asian females, between the ages of 20 – 30 years old, with no history of any skin problems. All the volunteers were provided with a participant information leaflet entitled “An in vivo study for topical caffeine formulations”, before they signed the consent form to participate in this study. Apart from daily washing, the participants were requested to refrain from applying topical cosmetic products to the volar surface of the forearms 24 h prior to taking part in the study and during the study.

6.2.2.2.2 Application of formulations

Two caffeine formulations were selected for the *in vivo* studies. These two formulations were selected based on the permeation data obtained from the formulation studies in single, binary and ternary solvent systems. The selected formulations were those two shown the best promoting effect of caffeine to be delivered through the skin in the Franz cell model (session 6.3.1.4.2). A template (Figure 6.2) was used on volunteers in order to have a

constant application site area and to ensure the two treatment sites were well separated throughout the studies. After treatment for the designated time (8 h), the sites were wiped gently with tissue before analysis.

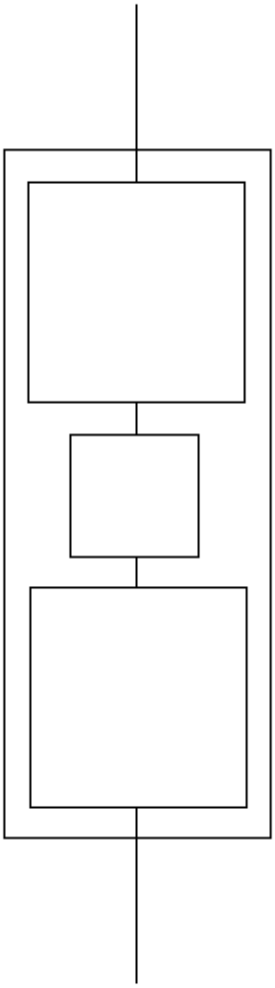


Figure 6.2 The template used for formulation application for the *in vivo* studies

57.6 µL of the formulations were applied on the site (16 cm^2) using a pipette early in the morning. The dose of 57.6 µL was applied to 16 cm^2 of the treatment site because this was the same dose applied previously in *in vitro* studies. 3.6 µL/cm^2 of formulations were applied in *in vitro* finite dose permeation studies on Franz cells using human skin. The formulation was

spread homogenously and left to air dry before the volunteers resumed any activities. The sites were non-occluded as for *in vitro* studies.

6.2.2.2.3 Trans-epidermal water loss (TEWL)

The volunteers were allowed to acclimatize to ambient conditions at a temperature of 20 ± 1°C and a humidity of 45 ± 1% for 15 min prior to the study. It is critical to avoid any interference from excessive sweating or perspiration that may affect the TEWL reading (Pinnagoda et al., 1989, Rogiers and Group, 2001). These measurements were performed prior to tape stripping on control and each treatment site, and after each series of four tape strippings.

6.2.2.2.4 Tape stripping

A standard D-Squame® tape (2.2 cm in diameter, 3.8 cm^2 area) obtained from CuDerm Corporation (Dallas, TX, U.S.A.) was applied on the control or treatment sites. A D-Squame pressure instrument was used to press the tape for 5 s. The tape was gently removed with a forceps and arranged on a standard D-Squame® tape rack (CuDerm). This step was repeated until all twenty tape strippings were completed. Tapes were handled carefully to avoid protein contamination.

6.2.2.2.5 Measurement of protein content

Protein content was measured directly from the tape strips. The method has previously been validated by the conventional bicinchoninic acid colorimetric approach to determine protein content (Voegeli et al., 2007). Protein absorption was determined at 850 nm using an infrared densitometer SquameScan 850A (Heiland Electronic). The amount of protein was then quantified by the method reported by Voegeli et al. (2007) using the following equation:

$$C_{protein}\ (\mu g/cm^2) = 1.366\ X\ absorption\ (\%) - 1{\cdot}557 \qquad \textbf{Equation 6.2}$$

6.2.2.2.6 Extraction of caffeine

All the tape strips were individually placed in a 1 mL eppendorf tube. Caffeine was extracted using 1 mL of methanol:water mixture (50:50 v/v). All the Eppendorf tubes were then shaken in an Orbital incubator SI50 (Stuart Scientific, Stone, U.K.) at 32 ± 1°C for 24 h. The samples were then analysed by HPLC.

6.2.2.2.7 Confocal Raman Spectroscopy (CRS) studies

The *in vivo* study for PG was performed using Confocal Raman spectroscopy (CRS). The CRS device is equipped with two fibre-coupled diode pumped lasers with two different wavelengths: 671 nm and 785 nm. The 671nm laser collects data in the high fingerprint region (2500-4000 cm^{-1}) and the 785 nm laser collects the spectrum in the low fingerprint region (400-2000 cm^{-1}). The laser light focuses on the skin through an oil-immersion microscope objective at a well-defined depth by a high precision piezoelectric focusing drive. The tissue scattered light focuses onto the core of an optical fibre acting as a pinhole, rejecting signals from out of focus regions of the skin. The scattering signal is detected with an air-cooled high sensitivity back-illuminated deep depletion CCD camera (Crowther et al., 2008). The details of formulation application are described in section 6.2.2.2.2. 6 volunteers were recruited from the UCL School of Pharmacy to participate the studies. Data were acquired by RiverIcon® software v.2.5.2 and Skin tools® software Version 2.0 (River Diagnostics, Netherlands) was then used to analyse the data. Raman spectra profiles (n≥8) were collected from the outer layers of the skin and with a 2 μm increment step up to 40 μm inside the skin for all treatment and control sites. The caffeine signals required for the fitting algorithm were acquired from the signals for the caffeine saturated solution, by subtracting the signals of the corresponding solution without caffeine.

6.2.2.2.8 Statistical analysis

Parametric statistical tests (one-way between-group ANOVA and paired t-test to compare means) were used to investigate statistical differences between treated and untreated sites. A probability of $p < 0{\cdot}05$ was considered statistically significant. All results are presented as mean ± standard deviation.

6.3 Results and discussion

6.3.1 Formulation studies for caffeine

6.3.1.1Single solvent systems

6.3.1.1.1 Skin PAMPA

According to the previous studies, the Skin PAMPA model is a promising screening tool for permeation assessment. Therefore, in the formulation studies for caffeine, the Skin PAMPA studies were conducted first on all the selected single solvents. The formulations tested in the permeation studies were 80% saturated solubility solutions of caffeine in the selected solvent systems. Finite dose studies (1 µL per well) were conducted for each solvent separately. Figure 6.3 shows the permeation profiles of caffeine from all the tested formulations in the Skin PAMPA model.

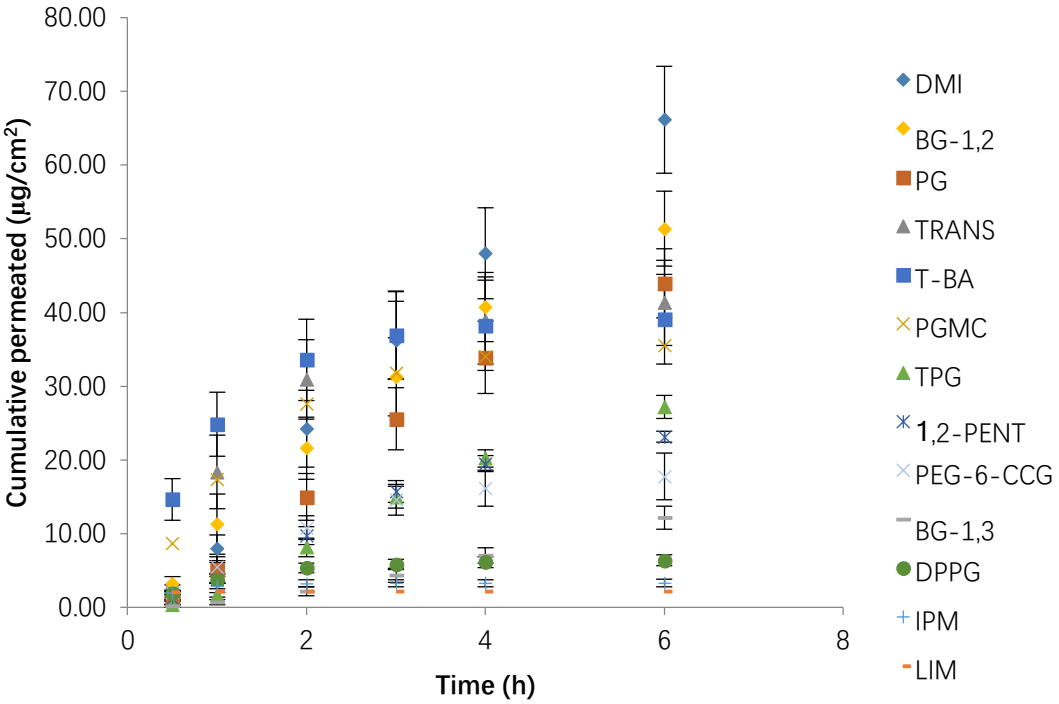


Figure 6.3 Permeation profiles of tested formulations on PAMPA model, 1 uL each well. Each data point represents the mean ± SD, n=5

From Figure 6.3, the amounts of caffeine which permeated from the selected solvents at 6 h ranged from 2–65 µg/cm^2 in PAMPA. The formulation with the best efficiency in delivering caffeine was the DMI solution, which delivered around 66 µg/cm^2. Both DMI and 1, 2-BG delivered significantly higher amounts of caffeine ($p<0.05$) than PGMC, TPG, 1, 2-PENT, PEG-6-CCG, BG-1, 3, DPPG, IPM and LIM. PG was also significantly more efficient ($P<0.05$) than TPG, 1,2-PENT, PEG-6-CCG, 1, 3-BG, DPPG, IPM and LIM. DPPG, IPM and LIM shown significant lower efficiency ($p< 0.05$) in this case than all the other solvents.

6.3.1.1.2 Human skin Franz cell permeation studies

Based on the screening results from Skin PAMPA studies, caffeine formulations in DMI, 1, 2-BG, PG, TRANS, PGMC, TPG, 1,2-PENT and PEG-6-CCG were selected for further studies in Franz cells using human skin. The studies were set up as non-occluded and with finite dose studies, 3.6 μL/cm^2, which mimic "real life" application of a topical formulation. As mentioned previously, the concentration of each formulation was prepared at 80% of the saturated level of the solubility in the formulations in order to maintain the thermodynamic activity at the same level and to avoid drug crystallisation. Figure 6.4 illustrates the permeation profiles of caffeine from these test solutions.

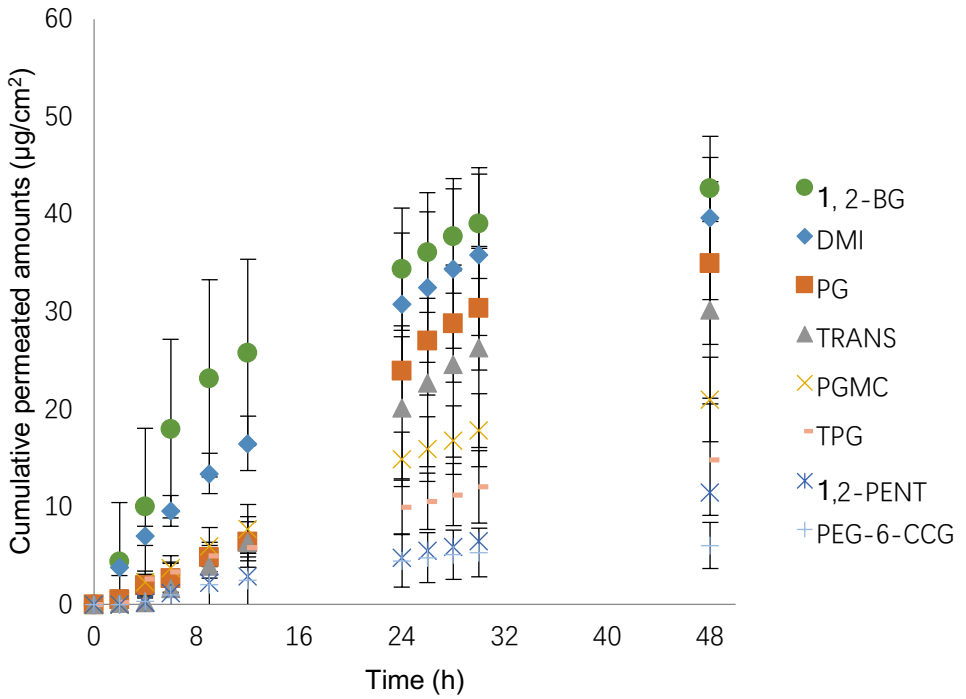


Figure 6.4 Permeation profiles of caffeine from selected formulations in Franz cells studies using human skin. Each data point represents the mean ± SD, n=6

Figure 6.4 shows that the amounts of caffeine which permeated from the selected solvents at 48 h ranged from 6 to 45 μg/cm^2 in Franz cells with human skin. This is comparable to the corresponding permeation observed in the Skin PAMPA model ($p<0.05$), where the values ranged from 18 to 65 μg/cm^2 at 6 h. Both DMI and 1, 2-BG delivered significantly higher amounts of caffeine ($p<0.05$) than PGMC, TPG, 1, 2-PENT and PEG-6-CCG in both the PAMPA and human skin models. PG was significantly more efficient ($p<0.05$) than TPG, 1, 2-PENT and PEG-6-CCG in both models.

Mass balance studies were conducted to quantify the amounts of caffeine after the 48 h permeation from the selected caffeine single solvent systems. The distribution of caffeine for the different formulations is shown in Fig 6.5.

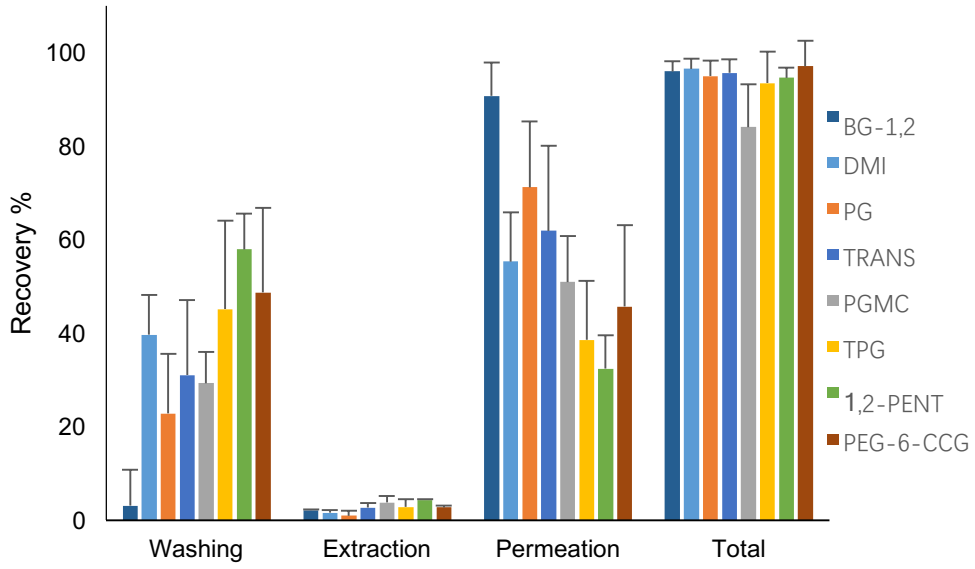


Figure 6.5 Mass balance profiles of caffeine for the selected formulations. Each data point represents the mean ± SD, n=6

All of the total recovery values of caffeine from the studies were between 80% and 100%. This indicates that the mass balance method (washing and extraction) used in this experiment was optimised. In order to mimic the “real

life” application of a topical caffeine formulation, the experiments were set up as non-occluded. Therefore, the evaporation may contribute to the depletion of some of the solvents.

6.3.1.2 Binary solvent systems

6.3.1.2.1 Miscibility studies for binary systems

Table 6.3 summarises the miscibility study results for all binary combinations of selected solvents.

Table 6.3 Miscibility studies for binary solvent systems (M=completely miscible; IM=completely immiscible; IM+M= partially miscible)

	LIM	IPM	DPPG	GER	PGMC	PGML	TRANS	1,2-Pent	DMI	PG	BG	T-BA	Lina	TPG	PEG-6-CCG
LIM		M	M	M	M	M	M	M+IM	M	IM	IM	M	M	IM	M
IPM	M		M	M	M	M	M	M	M	IM	IM	M	M	IM	IM
DPPG	M	M		M	M	M	M	M	M	IM	IM	M	M	M	M
GER	M	M	M		M	M	M	M	M	M	M	M	M	M	M
PGMC	M	M	M	M		M	M	M	M	M	M	M	M	M	M
PGML	M	M	M	M	M		M	M	M	M	M	M	M	M	M
TRANS	M	M	M	M	M	M		M	M	M	M	M	M	M	M
1,2-Pent	M+IM	M	M	M	M	M	M		M	M	M	M	M	M	M
DMI	M	M	M	M	M	M	M	M		M	M	M	M	M	M
PG	IM	IM	IM	M	M	M	M	M	M		M	M	M	M	M
BG	IM	IM	IM	M	M	M	M	M	M	M		M	M	M	M
T-BA	M	M	M	M	M	M	M	M	M	M	M		M	M	M
Lina	M	M	M	M	M	M	M	M	M	M	M	M		M	M
TPG	IM	IM	M	M	M	M	M	M	M	M	M	M	M		M
PEG-6-CCG	M	IM	M	M	M	M	M	M	M	M	M	M	M	M	

6.3.1.2.2 Solubility studies for caffeine in selected binary systems

Based on the results for the single solvent systems studies for caffeine, a number of binary solvent systems were selected for further investigation. The selected binary systems were PGMC: TRANS 50:50 (v/v), PEG-6-CCG: TRANS 50:50 (v/v), PEG-6-CCG: PG 50:50 (v/v), DMI: PGMC 50:50 (v/v), DMI: PEG-6-CGG 50:50 (v/v), and 1, 2-BG: PGMC 50:50 (v/v).

Table 6.4 summarises the caffeine solubility studies results for the binary solvent systems.

Table 6.4 Caffeine solubility in selected binary solvent systems at 32 ± 1°C, mean ±SD, n=3.

Solvent systems	Solubility of caffeine (mg/ml)
1,2-BG : PGMC 50:50 (v/v)	13.49 ± 0.07
DMI: PEG-6-CGG 50:50 (v/v)	14.84 ± 0.49
PGMC: TRANS 50:50 (v/v)	15.07 ± 0.34
DMI: PGMC 50:50 (v/v)	15.34 ± 0.18
PEG-6-CCG: TRANS 50:50 (v/v)	17.62 ± 0.70
PEG-6-CCG: PG 50:50 (v/v)	17.91 ± 0.48

From Table 6.4, it is evident that the solubility of caffeine was improved by the combination of solvents in the binary systems compared to solubility in single solvents (Table 3.16), except for the DMI solution. The caffeine solubility in DMI was 21.83 ± 0.20 mg/mL. However, in the binary systems DMI: PEG-

6-CGG 50:50 (v/v) and DMI: PGMC 50:50 (v/v), the caffeine solubility was only 14.84 ± 0.49 and 15.34 ± 0.18 mg/ml, separately.

6.3.1.2.3 Skin PAMPA permeation study

Permeation of caffeine from the selected binary solvent systems was firstly screened in the Skin PAMPA model. Finite dose studies (1 µL per well) were conducted. Again, the concentration of each formulation was prepared at 80% of the saturated solubility level of the formulations. Figure 6.6 shows permeation profiles of caffeine from the selected binary systems in the Skin PAMPA model after 6 h.

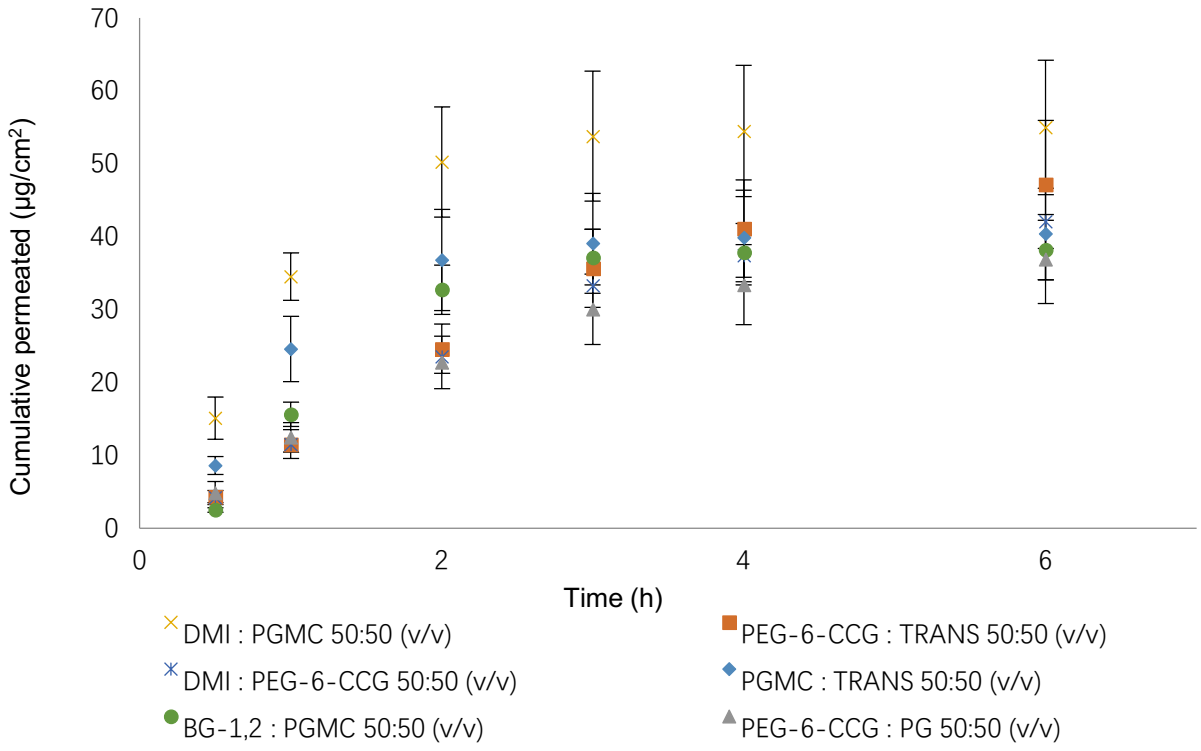


Figure 6.6. Permeation of selected formulations in Skin PAMPA. Each data point represents the mean ± SD, n=5.

At 6 h, caffeine permeation from the tested binary systems ranged from about 35 to 55 μg/cm^2, which was comparable to the values for the corresponding single systems. The difference in efficiency between the binary formulation was not significant ($p<0.05$). This was consistent with the solubility studies results when comparing single and binary solvent systems.

6.3.1.2.4 Human skin Franz cell permeation studies

All the selected binary systems were investigated in Franz cells using human skin for 48 h. Figure 6.7 shows permeation profiles of caffeine from the selected binary systems investigated in Franz cells using human skin.

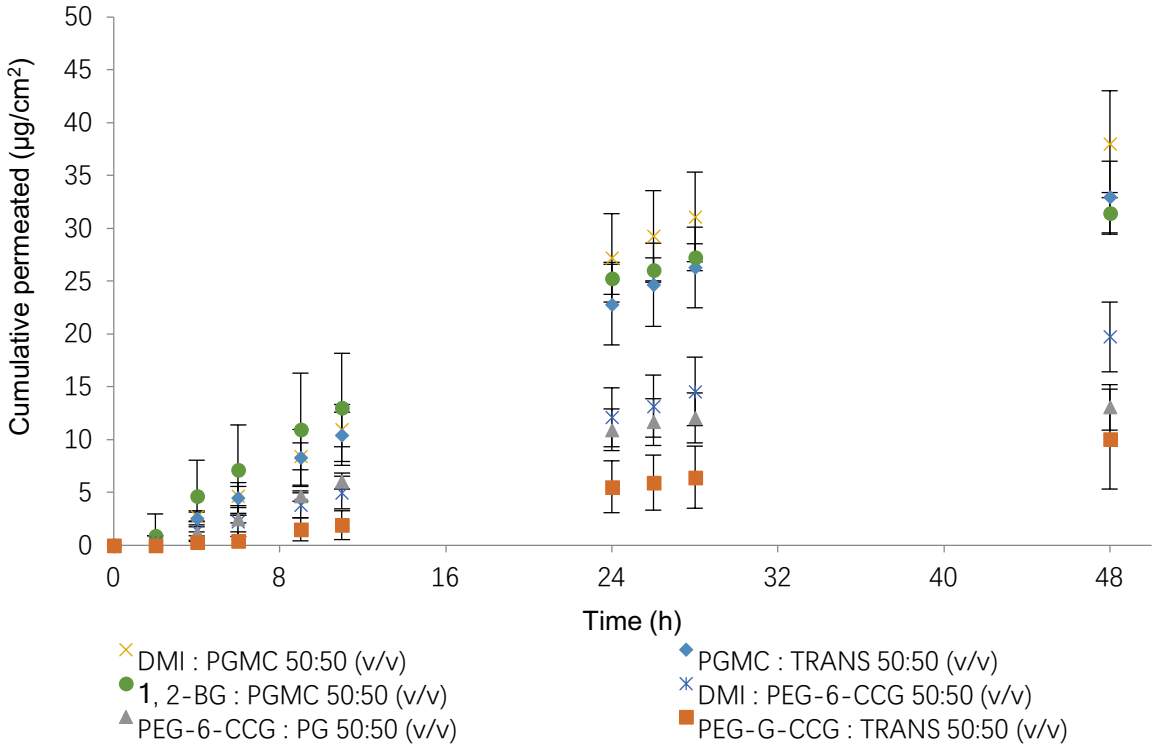


Figure 6.7 Permeation of selected formulations on Franz cells with human skin. Each data point represents the mean ± SD, n=5.

As can be seen from Figure 6.7, around 10 to 40 μg/cm^2 of caffeine was delivered through human skin from the binary systems after 48 h in the Franz cell model. These amounts were also comparable to the corresponding values in the Skin PAMPA model studies for the same formulations. However, it is interesting that the efficiency of DMI: PGMC 50:50 (v/v), PGMC: TRANS 50:50 (v/v), and 1, 2-BG: PGMC 50:50 (v/v) was significantly higher ($p < 0.05$) than that of the other three systems. However, none of these formulations show significant higher efficiency ($p<0.05$) than the single system, 1, 2-BG, (as shown in Figure 6.4), in delivering caffeine.

The distribution of caffeine from different formulations in mass balance studies for the selected binary systems investigated in Franz cells using human skin is shown in Figure 6.8.

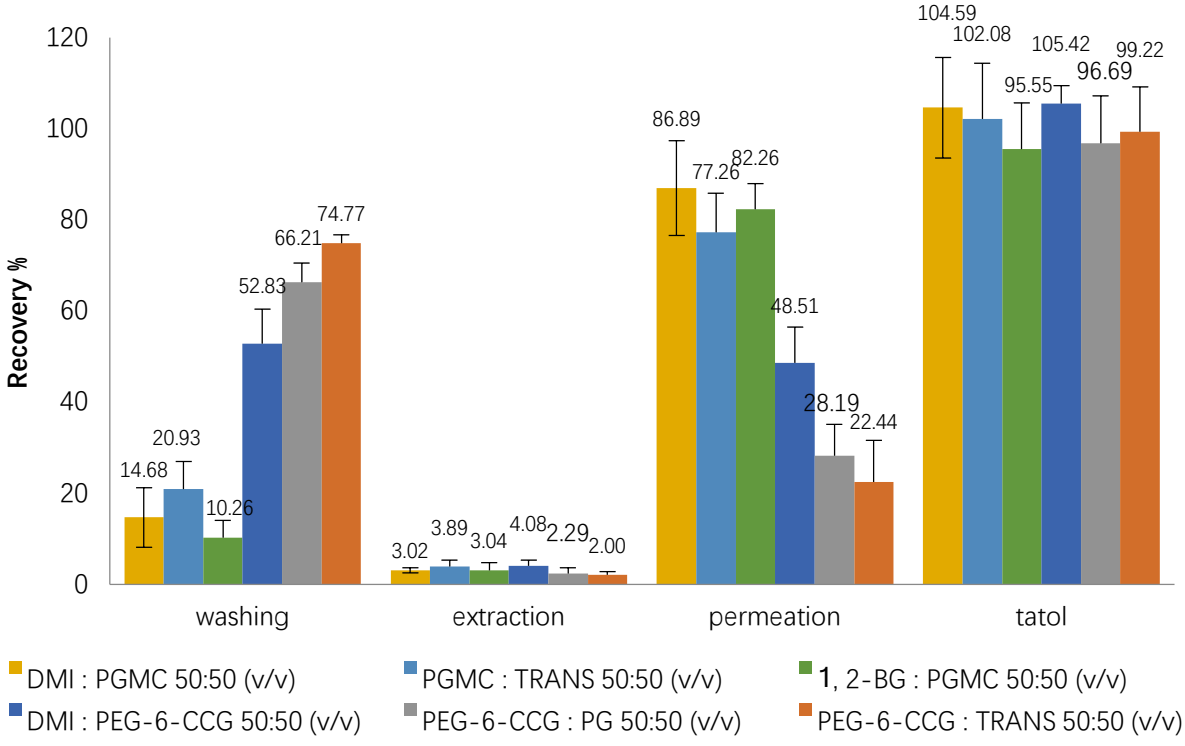


Figure 6.8 Mass balance studies of selected formulations in Franz cells with human skin. Each data point represents the mean ± SD, n=5.

All of the total recovery values of caffeine from these studies were between 90% and 110%, which confirms that the mass balance method (washing and extraction) used in this experiment was also optimised. For all the tested systems, the amounts of caffeine which were delivered to the deeper layers of human skin were approximately between 2 and 4% of the applied dose. The formulations which illustrated relatively high percentage permeation show corresponding low percentages for the amounts that remained on the skin surface.

6.3.1.3 Ternary solvent systems

6.3.1.3.1 Selection of ternary caffeine formulations

Taking the results of miscibility studies and both single and binary solvent system studies into account, the solvents DMI, PGMC, PG and TRANS show promising efficiency in skin delivery of caffeine and deserve further investigation in ternary solvent systems. DMI and its combinations with other solvents have been studied comprehensively in the UCL skin group previously (HADI, 2012), and their excellent capacity in delivering caffeine was demonstrated. IPM was also selected for further studies even though as a single system, the delivered amount of caffeine was relatively low. The main reason is that IPM is a non-polar solvent. The combination of polar and non-polar solvents may result in synergistic enhancement effects in permeation.

Therefore, two combinations, PG: PGMC: TRANS and PG: PGMC: IPM were selected for the ternary system studies for caffeine.

Miscibility studies were conducted for the two combinations before deciding the composition. The results show that PG, PGMC and TRANS are completely miscible with each other. The ternary phase diagram of PG: PGMC: IPM is shown in Figure 6.9.

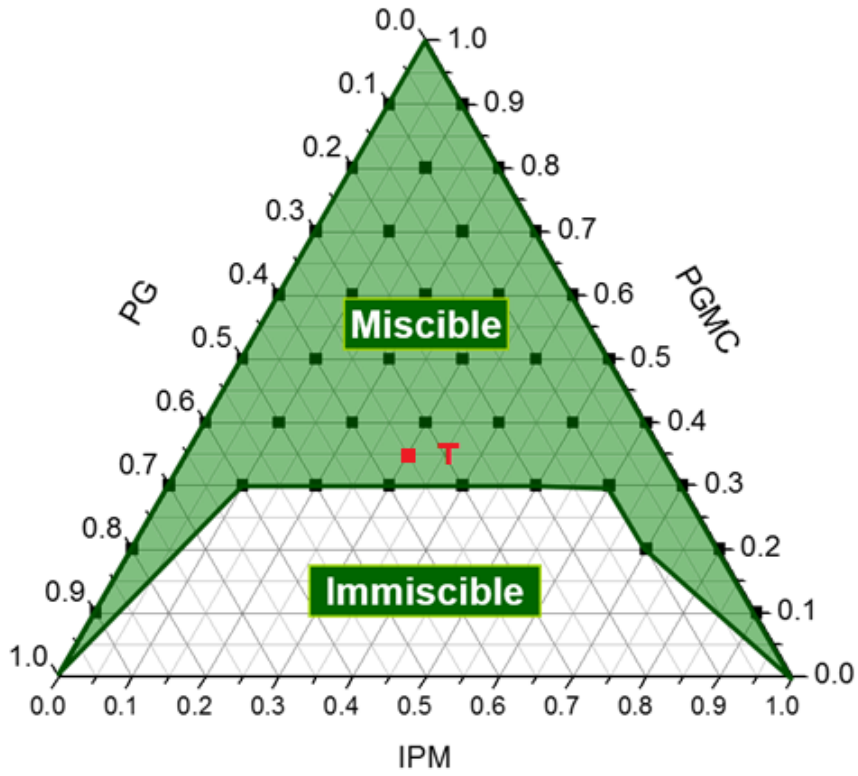


Figure 6.9 Ternary phase diagram of PG: PGMC: IPM system [T-PG: PGMC: IPM (35:35:30 v/v)]. The original ternary phase diagram with other selected compositions was constructed by Hadi (2012).

The selected combination was marked as T, which was PG: PGMC: IPM 35:35:30 (v/v). This combination was selected based on their distance from the boundary of the system. According to Davis et al (2002), the permeation of actives and enhancers may be influenced by the boundary distance of a partially miscible system. If a formulation can produce a chemical potential

gradient at the moment of interfacial skin contact, and preserve the gradient for the appropriate time fame, then the formulation may have the potential to deliver high amounts of actives or enhancers into the stratum corneum. In order to compare the efficiency of the two selected systems, the combination of PG: PGMC: TRANS was also selected as 35:35:30 (v/v).

6.3.1.3.2 Solubility studies for caffeine in selected ternary solvent systems

The results for solubility studies are shown in Table 6.5.

Table 6.5 Caffeine solubility in selected ternary solvent systems at 32 ± 1°C, mean ±SD, n=3.

Solvent systems	Solubility of caffeine (mg/mL)
PG: PGMC: TRANS 35:35:30 (v/v)	15.36 ± 0.32
PG: PGMC: IPM 35: 35: 30 (v/v)	10.66 ± 0.46

It is clear that, the solubility of caffeine in PG: PGMC: TRANS (35:35:30 v/v), with a value of 15.36 ± 0.32 mg/mL, was better than in PG: PGMC: IPM (35: 35: 30 v/v), where the value was 10.66 ± 0.46 mg/mL. The results show that the combination of polar and non-polar solvents did not result in obvious enhancement effects of solubility of caffeine in the PG: PGMC: IPM (35: 35: 30 v/v) system.

6.3.1.3.3 Human skin Franz cell permeation studies

As only two formulations were selected to study ternary solvent system efficiency, the Skin PAMPA model was used as a screening tool in this case. The permeation profiles of caffeine from these two formulations are shown in Figure 6.10.

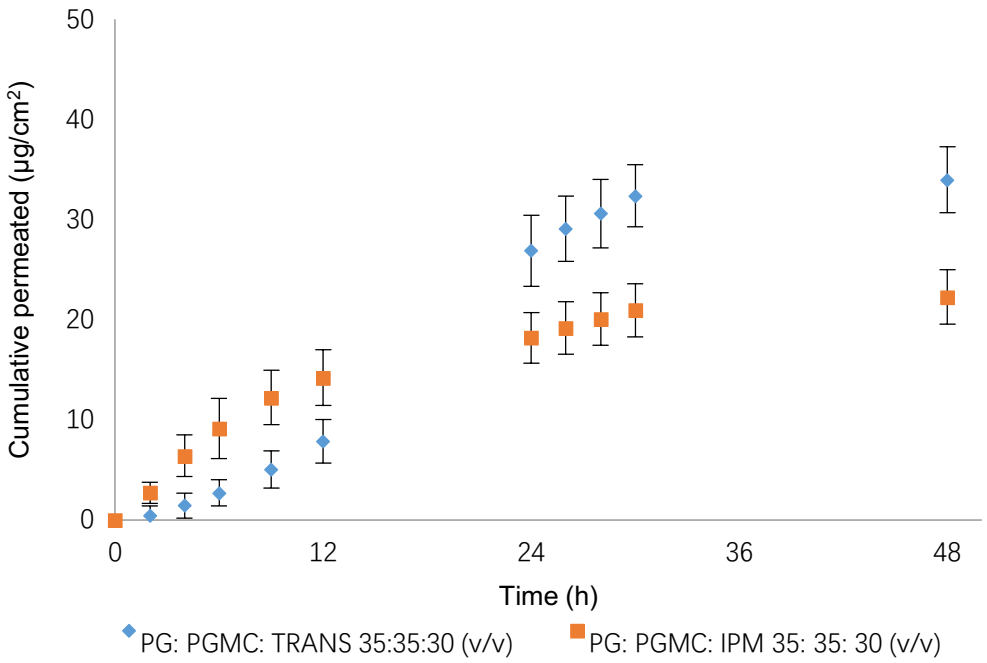


Figure 6.10 Permeation of selected formulations in Franz cells with human skin. Each data point represents the mean ± SD, n=5.

After 48 h, about 34 µg/cm^2 of caffeine from PG: PGMC: TRANS was delivered through human skin. This value is significantly higher ($p < 0.05$) than that from PG: PGMC: IPM where the amount was around 22-34 µg/cm^2. The

comparison of caffeine permeation from selected single, binary and ternary solvent systems is discussed in section 6.3.1.4.

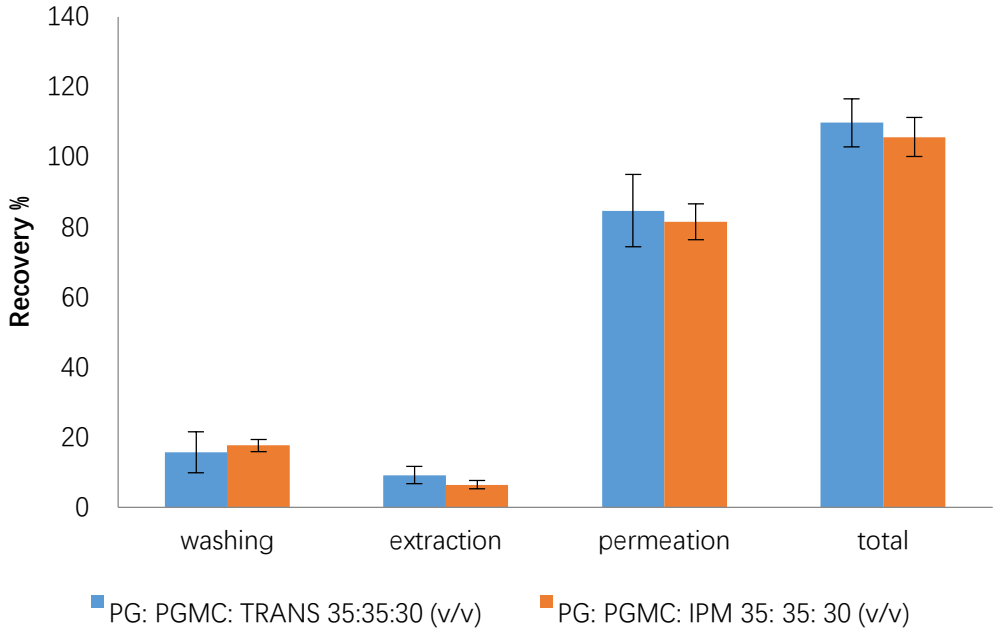


Figure 6.11 Mass balance studies of selected formulations in Franz cells with human skin. Each data point represents the mean ± SD, n=5.

Figure 6.11 illustrates the distribution of caffeine for mass balance studies after 48 h permeation. Both of the total recovery values of caffeine from these studies were between 100% and 110%. This is in line with the OECD and SCCP guidelines (OECD, 2000; SCCP, 2006). More than 80% of the applied doses permeated into the receptor compartment, with 15-18% remaining on the skin surface, and about 6-9% were delivered to deeper layers of skin.

6.3.1.4 Comparison of caffeine permeation from different single, binary and ternary solvent systems

6.3.1.4.1 Caffeine solubility in the selected binary, ternary and some of the single systems

In order to examine the improvement of caffeine solubility by using binary and ternary solvent systems, the solubility data of caffeine in the selected binary, ternary and some of the single systems are compared in Figure 6.12.

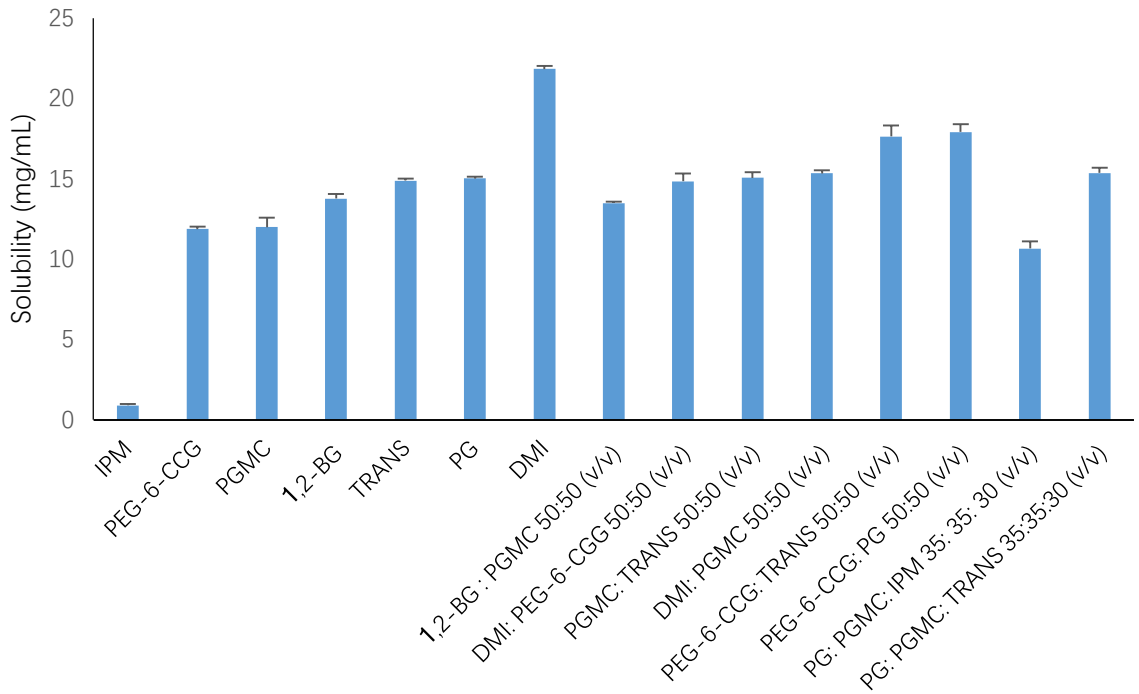


Figure 6.12 Solubility of caffeine in a range of different solvents and solvent systems at 32±1°C. Each data point represents the mean ± SD, n=5.

In single systems, caffeine has a high solubility in DMI and a low solubility in IPM. The solubility of caffeine in IPM was improved by using a mixture with PG and PGMC. However, the solubility of caffeine in PGMC, 1, 2-BG, PEG-6-

CCG, TRANS, PG and DMI was not improved by using binary or ternary mixtures as shown in Figure 6.12

6.3.1.4.2 Caffeine permeation in the selected binary, ternary and some of the single systems

In order to compare permeation of caffeine from the selected binary, ternary and some of the single systems, all the permeation profiles are illustrated in Figure 6.13.

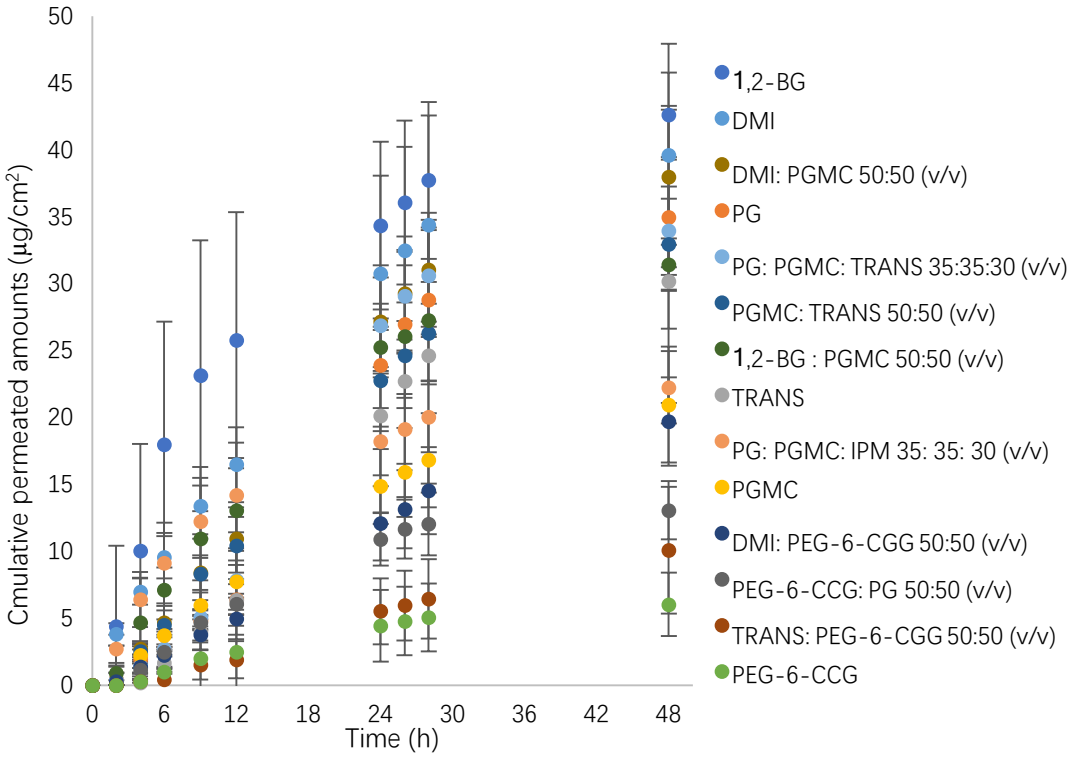


Figure 6.13 Permeation profiles of caffeine from a range of different solvents and solvent mixtures in Franz cells with human skin. Each data point represents the mean ± SD, n=5.

From Figure 6.13, it is evident that caffeine permeation in single solvents was not improved by the combination of binary or ternary solvent systems. For example, after 48 h, the cumulative amounts of caffeine that permeated from PEG-6-CCG: PG and TRANS: PEG-6-CCG were not significantly higher ($p>0.05$) than from PEG-6-CCG. However, the permeation from DMI: PEG-6-CCG was significantly higher ($p< 0.05$) than the single solvent PEG-6-CCG, but not DMI. Also, the permeation of caffeine from binary and ternary systems including PG was not significantly more ($p >0.05$) than the PG solution alone. However, significantly more caffeine ($p <0.05$) permeated from the single solvent 1, 2-BG than the binary system 1, 2-BG: PGMC through human skin in the studies. 1, 2-BG also shown significant better efficiency in delivering caffeine than the ternary systems PG: PGMC: TRANS 35: 35: 30 (v/v) and PG: PGMC: IMP 35: 35: 30 (v/v) ($p<0.05$).

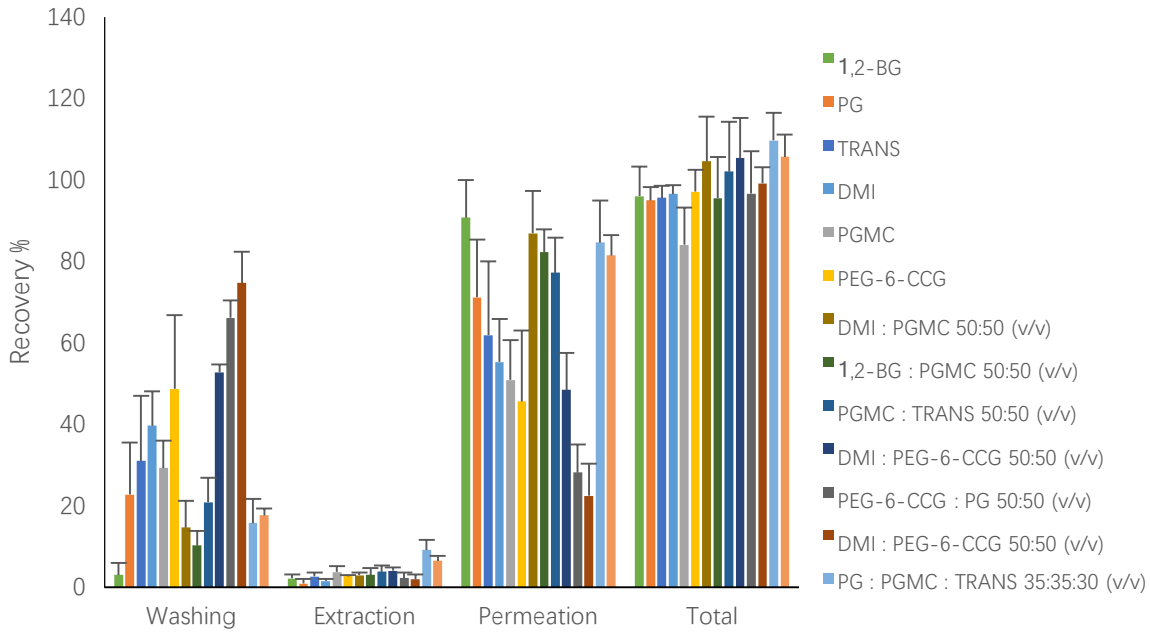


Figure 6.14 Mass balance studies of caffeine from a range of different solvents and solvent systems on Franz cells with human skin. Each data point represents the mean ± SD, n=5.

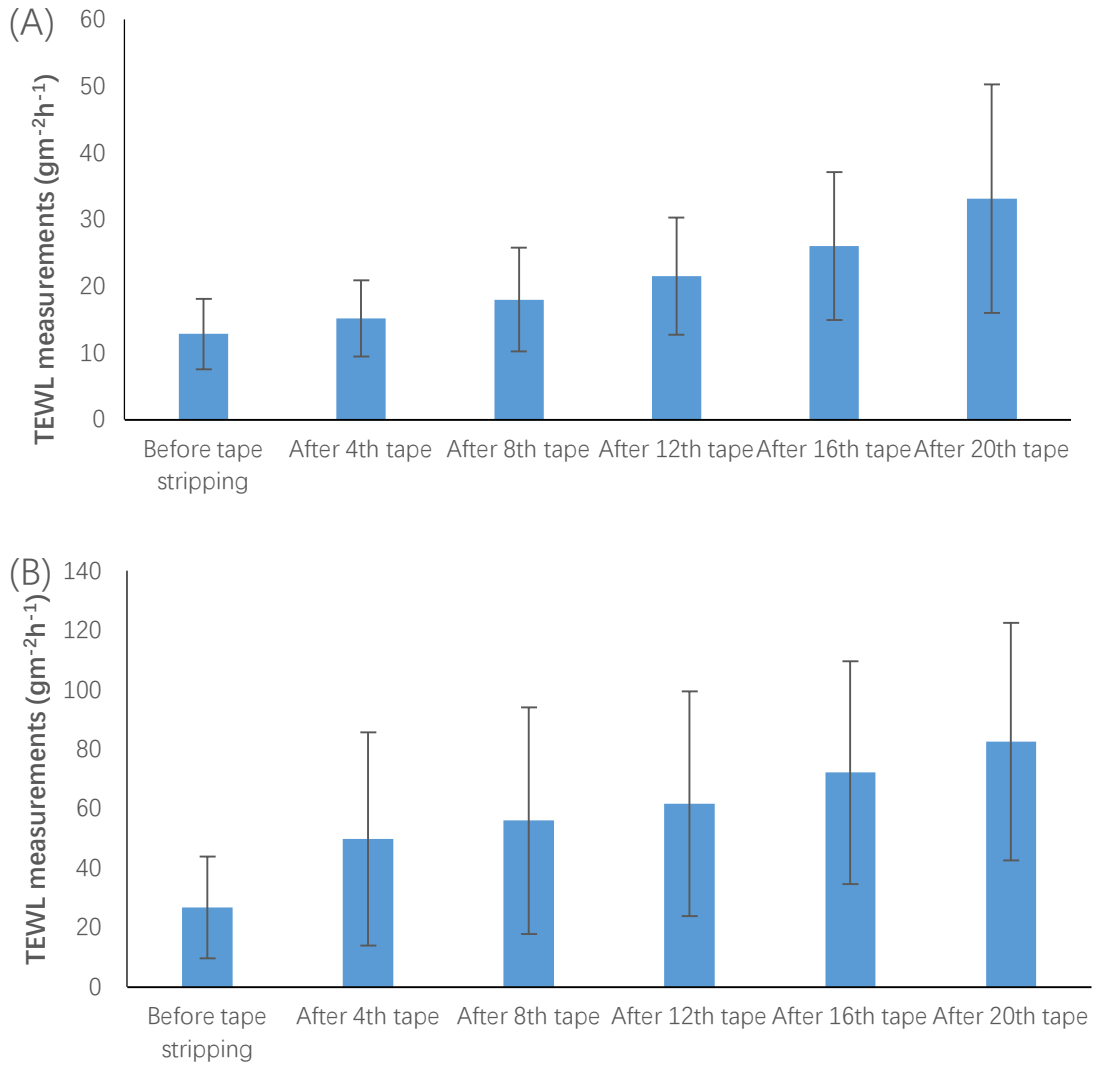
Figure 6.14 illustrates the distribution of caffeine for mass balance studies after 48 h permeation of the selected single, binary and ternary formulations. It is interesting that the percentage permeation of caffeine from the applied doses from the two ternary systems, PG: PGMC: TRANS and PG: PGMC: IPM were relatively high when compared with corresponding percentages for the single solvents PG, PGMC and TRANS. Even though the permeation of caffeine from the ternary systems was not obviously improved compared with values from the single solvents, the permeation efficiency from ternary systems was higher than neat solvents.

6.3.2 *In vivo* permeation studies for caffeine

In the *in vivo* permeation studies, the conventional methods, trans-epidermal water loss (TEWL) and tape stripping, were used for comparison with the novel method of Confocal Raman Spectroscopy (CRS). The selected formulations in the studies were 80% per cent saturation solubility of caffeine in the single solvent 1, 2-BG and the ternary solvent system PG: PGMC: TRANS (35: 35: 30 v/v).

6.3.2.1 Trans-epidermal water loss (TEWL)

As mentioned in the introduction, the TEWL value is influenced by the integrity of the skin barrier, which may be altered by excipients in formulations. TEWL values before and after the removal of tape strips 4, 8, 12, 16 and 20 are shown in Figure 6.15.



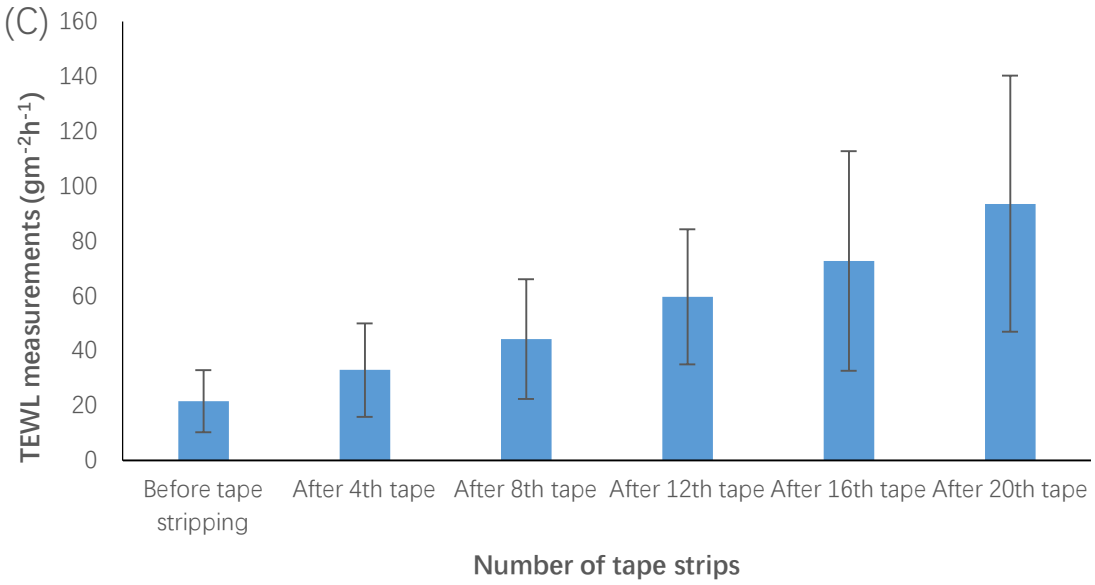


Figure 6.15 TEWL measurements for control (A), 1, 2-BG treated site (B) and PG: PGMC: TRANS treated site (C). Each data point represents the mean ± SD, n=6.

As expected, the TEWL values increased with increasing number of tape strips (Russell et al., 2008; Mohammed et al., 2011a) for both control and treated sites. However, the inter-individual variation was relatively high even though all the volunteers were Asian, female and around the same age. From Figure 6.15, the initial TEWL values for the two investigated sites were of a similar magnitude to the values for the control site, which was 13 g m^{-2} h^{-1}. The corresponding values reported in literatures were also between 10-15 g m^{-2} h^{-1} (Mohammed et al., 2011a; Mohammed et al., 2011b). Initially, only a slight increment was observed in the TEWL values with the increasing number of tape strips for control and all treated sites ($p > 0.05$). For control and 1, 2-BG treated sites, the TEWL measurement after 16 and 20 tape strips shows a significant increase compared with initial values ($p < 0.05$), while for the PG: PGMC: TRANS treated site a significant increase is evident after 12 tape strips

($p < 0.05$). Figure 3.55 compares the TEWL measurement for the control and two treated sites for the same number of tape strips.

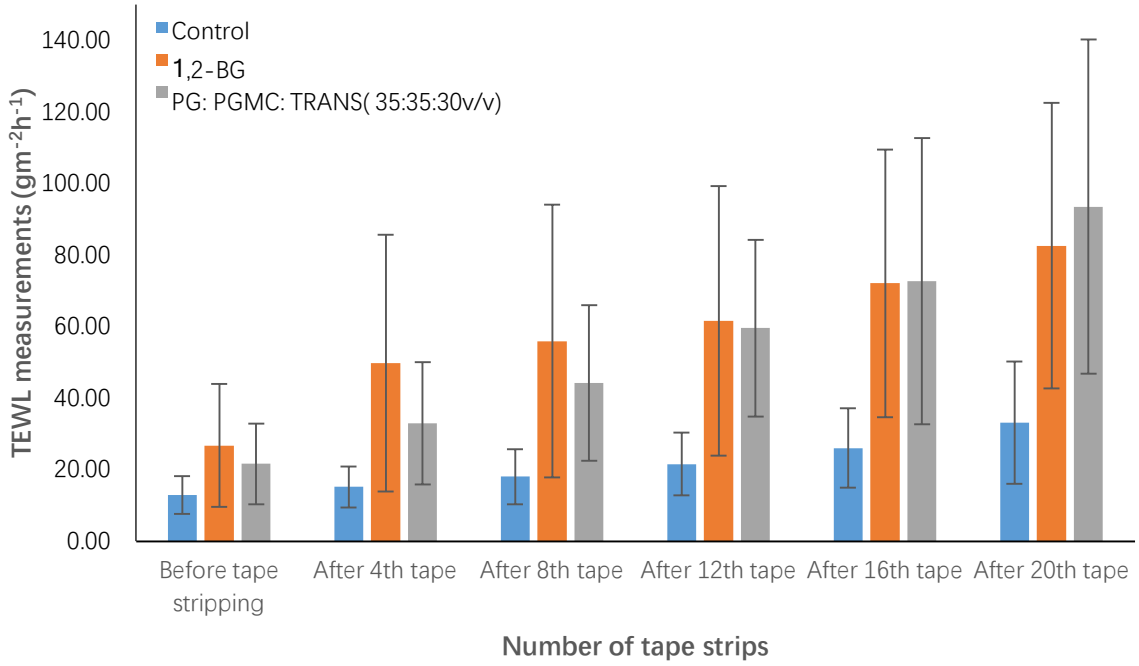


Figure 6.16 TEWL measurements for control (A), 1,2-BG treated site (B) and PG: PGMC: TRANS treated site (C). Each data point represents the mean ± SD, n=6.

After the removal of 12 tape strips, the control site showed significant differences compared with the site treated with the 1,2-BG formulation ($p<0.05$). Significant differences were observed for the site treated with PG: PGMC: TRANS only after 8 tape strips ($p<0.05$) compared with the control site. However, the TEWL values for the two treated sites were not significantly different after removal of 20 tape strips ($p> 0.05$). All the volunteers reported that after a couple of hours, the sites treated with 1, 2-BG and PG: PGMC: TRANS formulations were slightly red (erythema) and dry. Such discomfort was probably due to the irritation caused by these excipients. Therefore, the

high TEWL values observed from the formulation treated sites may be due to this irritation, which would result in an impairment of the skin barrier function.

6.3.2.2 Protein content measurement

The conventional tape stripping methods provide not only the information of the integrity and cohesion of the stratum corneum, but also a measurement of the protein content. Protein content measurement has been applied to evaluate the skin health status and to study the effects of topical treatments on skin (Bornkessel et al., 2005). The amounts of protein removed for each individual tape strip from the control and treated sites are shown in Figure 6.17.

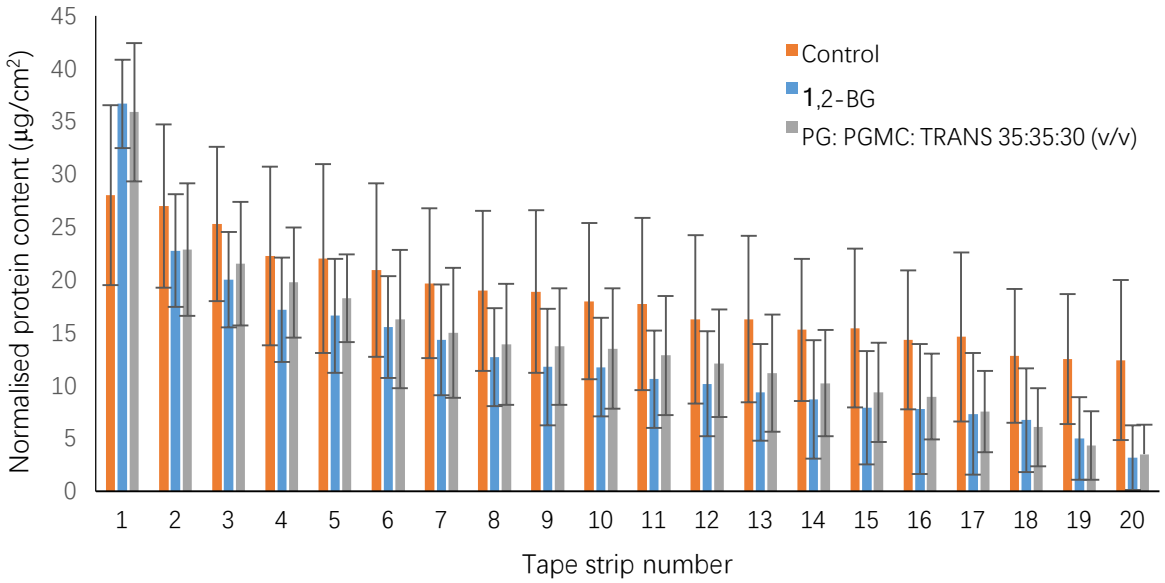


Figure 6.17 Stratum corneum protein content removed from different sites on the mid-ventral forearm with depth. Each data point represents the mean ± SD, n=6.

In general, the highest protein content was removed from the outermost layer of the stratum corneum according to Figure 3.56. With more tape

stripping the amount of protein removed decreased. The results were consistent with reports by Dreher et al (1998) and Bashir et al (2001), that the amount of protein removed decreases with increasing depth into the SC, which suggests an increase in corneocyte cohesiveness in the deeper layers of the stratum corneum. It can be seen from Figure 6.17 that except for the first tape strip, the control site generally showed a higher amount of protein removed than the treated sites. However, there was not a significant difference between the amounts of protein removed from the treated sites compared to the control site ($p>0.05$). Figure 6.18 compares the cumulative protein amount removed from the control and treated sites.

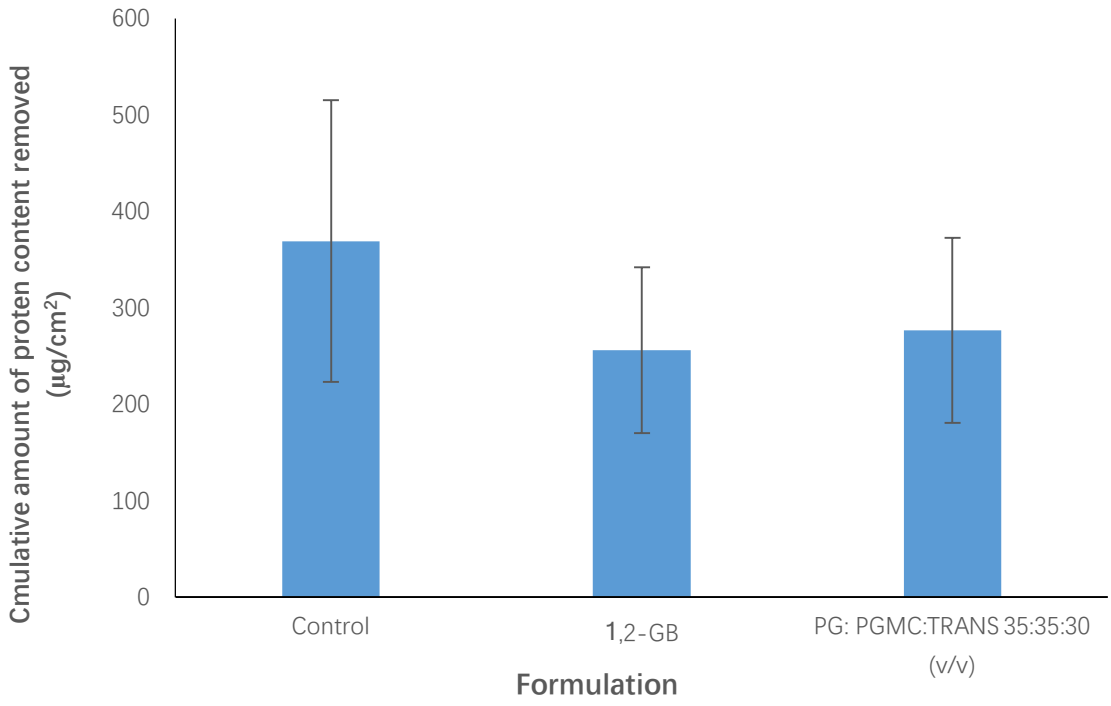


Figure 6.18 Cumulative amount of protein removed from the volar forearm. Each data point represents the mean ± SD, n=6.

According to Figure 6.18, the amount of protein removed from the formulation treated sites is not significantly different compared with control ($p > 0.05$) but this may reflect the high individual variation between volunteers.

6.3.2.3 Effect of excipients on permeation of caffeine

As mentioned, the stratum corneum is the main barrier to penetration of compounds through the skin. Therefore, it has been assumed that the amount of active that permeates to the underlying viable epidermis should be correlated with the amount extracted from the stratum corneum (Elias, 1983; Herkenne et al., 2008). The tape strips were extracted to quantify and recover the absorbed active. The total amounts of caffeine extracted from the two formulation treated sites are shown in Figure 6.19.

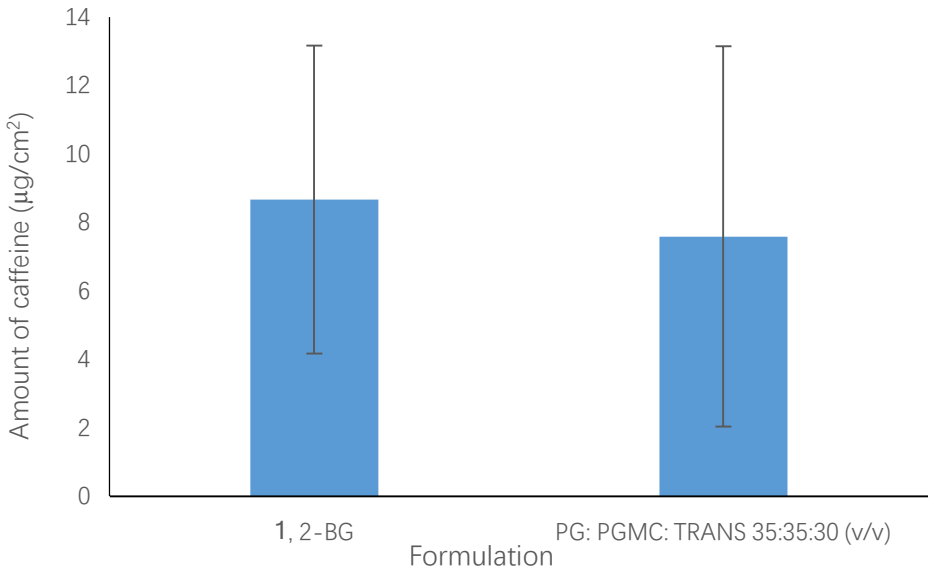


Figure 6.19 Total amount of caffeine extracted from treated sites. Each data point represents the mean ± SD, n=6.

As shown in Figure 6.19, the total amount of caffeine extracted from tape strips from both treated sites was around 8 μg/cm^2. There was no statistical difference between the two tested formulations ($p > 0.0.5$). The amounts of caffeine extracted from each tape trip from both of the treated sites are shown in Figure 6.20.

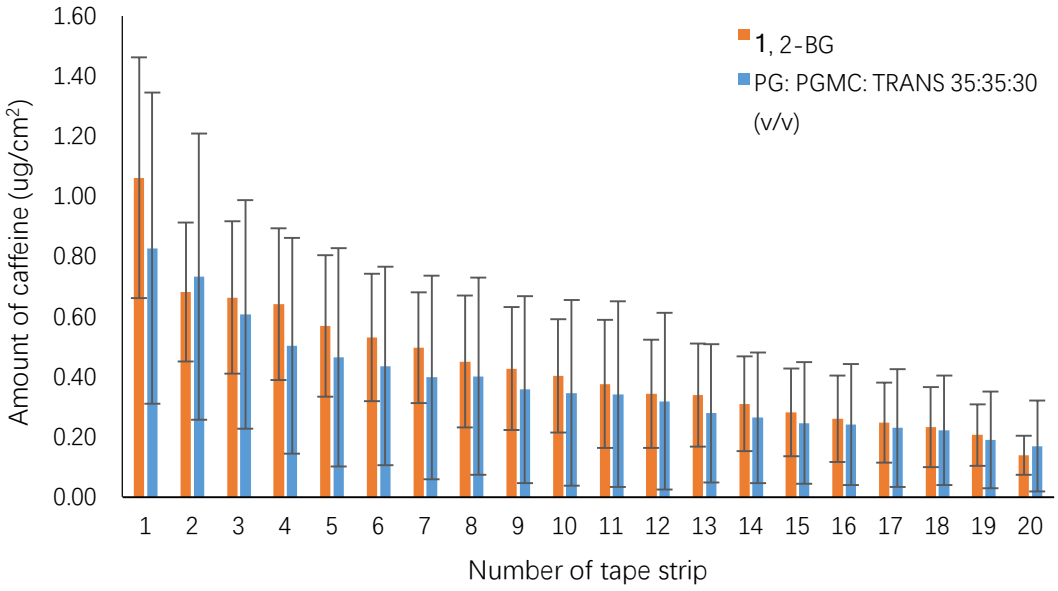


Figure 6.20 Amount of caffeine extracted from each tape strip. Each data point represents the mean ± SD, n=6.

According to Figure 6.20, more caffeine stayed in the outer layers of skin. The amount of caffeine extracted decreases with increasing depth into the stratum corneum.

6.3.2.4 Confocal Raman Spectroscopy (CRS) studies

The caffeine signal was extracted from the Raman spectrum of the skin after the treatment of the two selected formulations (described in session

6.2.2.2.2). The caffeine depth profiles in the human volar forearm after finite dose applications of 1, 2-BG and PG: PGMC: TRANS formulations are shown in Figure 6.21.

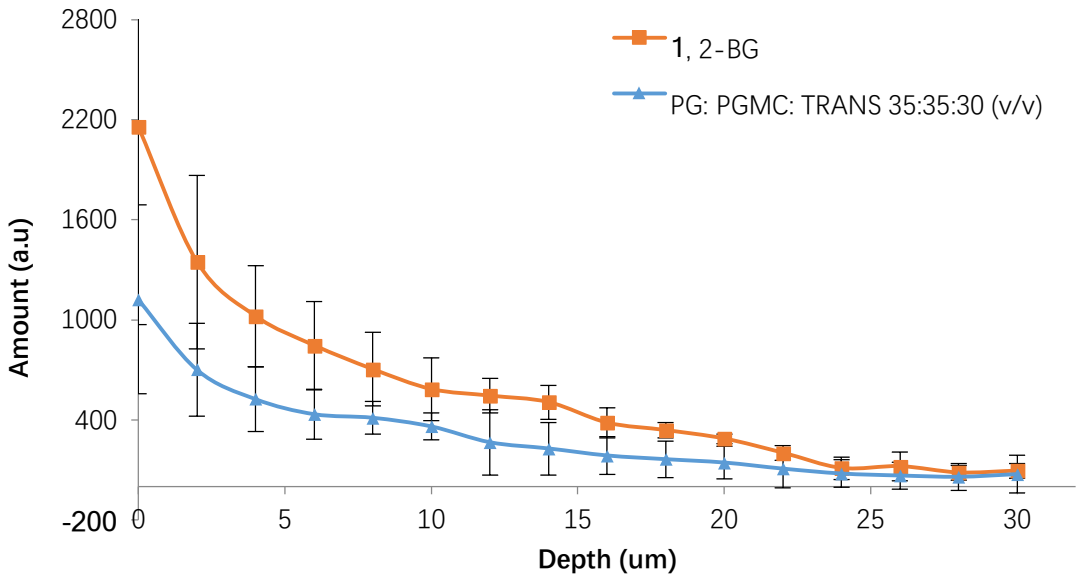


Figure 6.21 Caffeine depth profiles in the human volar forearm obtained after 8 h of application of the tested formulations. Each data point represents the mean ± SD, n>8.

Figure 6.21 clearly indicates that more caffeine was detected in the outer layers of skin. With the increasing depth into the stratum corneum, the amount of caffeine decreased. Significantly more caffeine was detected from the 1, 2-BG treated site, especially in the outermost layers of the stratum corneum ($p<0.05$). These profiles are consistent with those for the corresponding formulations when evaluated by tape stripping (session 6.3.2.3). The results also follow the same trend reported in the *in vitro* caffeine permeation studies using human skin that more caffeine was delivered from 1, 2-BG than PG: PGMC: TRANS (v/v) solution ($p<0.05$, section 6.3.1.4). The solubility of caffeine in 1, 2-BG is 13.77± 0.28 mg/mL, while the values PG: PGMC: TRANS (v/v) solution is 15.36 ± 0.32 mg/mL. It is clear that even though caffeine

solubility in the single solvent was lower than that in the ternary systems, it still delivered more caffeine through the skin than the ternary system.

6.4 Conclusions

Following *in vitro* finite dose studies in both the Franz cell model and the Skin PAMPA, caffeine permeation was not improved from binary or ternary solvent systems compared with single solvents. However, the cumulative percentage permeated of caffeine indicates that the permeation efficiency from ternary systems was higher than single solvent solutions.

In the *in vivo* studies, the results from the novel Confocal Raman Spectroscopy (CRS) technique follow the same trends reported in the *in vitro* caffeine permeation studies using human skin and the conventional tape stripping technique. As a completely non-invasive technique, CRS has the ability to track actives and solvents with a Raman signal, inside the skin and simultaneous alteration of the skin components, which allows real time drug concentration profiling *in vivo* and could be used as supplementary means to trace the active in *in vitro* studies. This technique allows a better understanding of the interaction of formulation components with the stratum corneum and delivery of substances to the skin.

6.5 References

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Chapter 7 General Conclusions

7.1 General conclusions

It is well known that the skin is the largest and a complicated living organ of the body. In our daily lives, many agents are applied to the skin either deliberately or accidentally, with either beneficial or harmful outcomes. Therefore, the assessment of skin permeation is critically important in many fields and a suitable and robust model that may be used to quantify and predict percutaneous penetration is necessary.

Human tissues from cadaver skin, biopsy material or cosmetic surgery are considered to be the best possible models at this point because they best reflect *in vivo* performance (Netzlaff et al., 2005). However, there are legal and ethical issues associated with obtaining human tissue. In addition, the high data variability between different donors or different body sites increases the variability in data, complicates analysis, and can be significantly costly.

Animal tissues, for example porcine skin, have been recommended as acceptable alternatives because of their more extensive availability. However, differences between human skin and animal skin are evident and the related ethical problems have also been controversial.

For these reasons, some non-animal models have been researched intensively. Studies focused on non-animal models have focused on include

synthetic membranes such as lipid mixtures impregnated in filter paper or synthetic polymers such as polydimethyl siloxane or silicone membrane. These membranes are useful to probe the thermodynamic activity of actives in specific formulations, but they cannot provide any insight into specific excipient interactions with skin. Model based on cultures of normal human keratinocytes and/or fibroblasts have also been developed. Although they are reported to over-estimate human skin permeation, they are routinely used for irritation and toxicity tests.

Recently, the Skin Parallel Artificial Membrane Permeation Assay, namely the Skin PAMPA has been proposed as a simple but high throughput screening system that may be suitable to study skin permeation. The Skin PAMPA model consists of two 96-well plate arrays. The top plate is pre-coated with an artificial membrane. The membrane consists of synthetic ceramides, cholesterol and free fatty acids, which mimic human skin stratum corneum. The structure of an individual cell of the Skin PAMPA is similar to the conventional permeation system, the Franz cell. In other words, in a PAMPA sandwich, there are 96 mini-Franz cells with a homogeneous membrane sandwiched between two compartments.

In the present study, a lipophilic active, ibuprofen and a hydrophilic active, caffeine were selected to conduct *in vitro* permeation studies in the conventional Franz cell models using silicone membrane, porcine skin and human skin, and the novel Skin PAMPA model. The overarching aim was to

determine the utility of Skin PAMPA for routine *in vitro* skin permeation testing with reference to topical formulations.

In chapter 2, HPLC methods were developed for both ibuprofen and caffeine. A GC method was also developed for the solvent PG, which appeared to promote effective skin delivery of caffeine. According to the validation results, both the HPLC and GC methods were reliable, sensitive and effective within their related proposed measurement ranges. Solubility studies were also conducted for ibuprofen and caffeine in a range of selected solvents and the solubility results provided valuable information in selecting suitable vehicles and receptor phase for *in vitro* permeation studies and developing optimum formulations.

Chapter 3 described the *in vitro* permeation studies in Franz cell models using silicone membrane and porcine skin for various ibuprofen and caffeine formulations. As a lipophilic active, ibuprofen permeated rapidly though porcine skin and silicone membrane. After 24 h, more than 460 μg/cm^2 ibuprofen had permeated from PG solutions in porcine skin, for both infinite and finite dose studies. The ibuprofen percentage permeation values for silicone membrane were higher than corresponding values in porcine skin. In both infinite and finite dose *in vitro* studies in porcine skin, caffeine permeated up to 30 h with more than 70% of the applied caffeine permeating through the skin in the finite dose studies. The evaporation of PG on the skin surface, suggested by the mass balance studies, would be expected to contribute to maintenance of the saturated state of caffeine and a concentration gradient of

caffeine between the donor and receptor phases. It was also confirmed that the increase of concentration of caffeine in the formulations lead to a drop in PG permeation. This may be explained by the effects of the solute caffeine on the activity of the solvent, PG. In addition, compared with ibuprofen, caffeine went through the skin more slowly. More than 460 μg/cm^2 ibuprofen had permeated through porcine skin after 24 h, while only around 180 μg/cm^2 caffeine permeated at the end of the 30 h infinite dose study.

In vitro permeation studies in conventional Franz cell models using human skin for various ibuprofen and caffeine formulations are reported in Chapter 4. For the Franz cell studies in human skin, the maximum amounts of ibuprofen permeation were about 40 μg/cm^2. As expected, the ibuprofen percentage permeation values for human skin were much lower than corresponding values in porcine skin. Higher permeation in porcine skin compared with human skin has also been observed by other researchers (Dick and Scott, 1992; Singh et al., 2002; Barbero and Frasch, 2009).

In contrast to ibuprofen, caffeine is a hydrophilic active. After 48 h, around 35 μg/cm^2 of caffeine permeated through the skin from the EVELINE® formulation, with about only 20 μg/cm^2 of the active being delivered from the L'Oréal® formulation. In addition, around 25% of caffeine permeated from the applied dose of EVELINE® serum, whereas only around 6% permeated from the dose of L'Oréal® gel, confirming the more efficient performance of the EVELINE® serum.

Various *in vitro* permeation studies were conducted in the novel skin PAMPA model, for different ibuprofen and caffeine formulations, which were discussed in detail in Chapter 5. In the initial studies for ibuprofen, the results showed that the temperature across the whole Sandwich was uniform while the Sandwich was placed in the stirring and temperature control unit, i.e. the Gut box®. Thus temperature control is not a factor that appears to be problematic for permeation assessment when using PAMPA. However, due to the small volume (200 µL) of the well in the Skin PAMPA Sandwich, it was difficult to conduct permeation studies for formulations with high concentrations. If the active permeates through the membrane rapidly and the applied dose is high, for example 200 µL per well, it is challenging to maintain sink conditions in the receptor phase throughout the studies. Hence, only those formulations with relatively low concentrations of actives, rather than saturated formulations, are appropriate candidates for Skin PAMPA models. In addition, because of this problem, when formulations contain volatile ingredients, such as ethanol, the bottom plate could not be used as the donor compartment. This is because with the evaporation of the liquid in the bottom plate, the contact area between the formulation and the membrane changed during incubation.

For the comparative study of ibuprofen permeation conducted in the Franz cell model using silicone membrane, porcine skin and human, and the skin PAMPA model, ibuprofen was generally more permeable in Skin PAMPA than human skin after 6 h and Skin PAMPA data were comparable to results in silicone membrane. For individual formulations permeation is also higher in Skin PAMPA compared with porcine skin. As for silicone membrane, the

composition of the Skin PAMPA membrane is considered to be homogeneous and inert. The low variability of results obtained from Skin PAMPA and silicone may be attributed to these membrane characteristics. With porcine and human skin, the results are naturally more variable due to the added complexity of biological membranes. Although the time for permeation studies in Skin PAMPA was not varied, shorter experimental times may be more appropriate considering the relatively high percentage of ibuprofen permeation in Skin PAMPA.

The Skin PAMPA studies with caffeine confirmed that the log k_p obtained from the Skin PAMPA model was comparable with the values acquired from Franz cell models using both porcine and human skin, which indicated that the Skin PAMPA is a promising model for rapid prediction of molecular permeation through the stratum corneum for caffeine. Similar to the results from the studies in Franz cells using porcine skin, the formulations with high caffeine concentrations (100 and 80% saturated solubility levels) showed higher active permeation than the formulation with low saturated levels (60%) in the Skin PAMPA model. However, the permeation of PG did not change significantly ($p > 0.05$) regardless of the concentration of caffeine. For the two semi-solid commercial caffeine formulations, Eveline® serum showed better efficiency in delivering caffeine than the L'Oréal® gel in infinite dose studies in the Skin PAMPA model. The results were in line with those from Franz cell studies in human skin. For the hydrogel patch in the Skin PAMPA, caffeine was less permeable than in Franz cell studies using human skin. The possible reason might be that during a 48 h study on Franz cell, the patch absorbed a certain amount of water, which improved the mobility of caffeine in the patch and then

improved caffeine permeation. However, the study with Skin PAMPA was much shorter (4 h), and the water absorption in the patch would be expected to be much less. For both ibuprofen and caffeine, the application conditions in the Skin PAMPA which approached realistic finite dose conditions were confirmed to be 1 µL (3.3 µL/cm^2).

In conclusion, the Skin PAMPA model did discriminate between different formulation types and different solvent systems compared with other models, with low variability in the permeation data. The more permeable nature of the PAMPA, silicone membrane and porcine tissue models to ibuprofen compared with human skin was also demonstrated, while the permeation of caffeine, a hydrophilic compound, in the PAMPA model was comparable to that in human skin.

The Skin PAMPA model, as well as the Franz cell model with human skin were then employed in the formulation studies for caffeine, which was described in Chapter 6. From conducting *in vitro* finite dose studies in both the Franz cell model with human skin and the Skin PAMPA model, caffeine permeation was not improved from binary or ternary solvent systems compared with single solvents. However, the cumulative percentage permeated of caffeine show that the permeation efficiency from ternary systems was higher than single solvent solutions. Further *in vivo* studies were conducted for a selected single solution and a ternary solvent system, namely 80% per cent saturation solubility of caffeine in the single solvent 1, 2-BG and the ternary solvent system PG: PGMC: TRANS (35: 35: 30 v/v). The results from the Confocal Raman Spectroscopy (CRS) technique showing that the

single solvent 1, 2-BG delivered significant more caffeine than the ternary system PG: PGMC: TRANS (35: 35: 30 v/v) ($p < 0.05$), which follows the same trends reported in the *in vitro* caffeine permeation studies using human skin. The conventional tape stripping technique also shown that more caffeine stayed in the outer layers of skin, however the total amounts of caffeine extracted from the two formulation treated sites were not statistically different ($p > 0.05$). As a completely non-invasive technique, CRS has the ability to track actives and solvents with a Raman signal, inside the skin and simultaneous alterations of the skin components, which allows real time drug concentration profiling *in vivo* and could be used as supplementary means to trace the active in *in vitro* studies.

7.2 Future work

Optimisation of the Skin PAMPA experimental conditions is ongoing, including further refinement of the dose applied, the formulation application procedure and sampling intervals. In addition, the range of actives that are suitable for study using the Skin PAMPA model also needs to be delineated. Therefore, a wider range of drugs, as well as actives used in cosmetics should be studied in the future. A wider range of different types of formulations should also be taken into consideration.

Following the initiatory *in vivo* studies by tape stripping and the CRS technique, more volunteers with different ages, and different ethnic groups should be studied. It would also be interesting to probe any differences

between genders in terms of formulation effects on skin barrier integrity. Validation of the CRS experiments should still be accomplished by conventional *in vitro* skin permeation testing and tape stripping.

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