TRANSDERMAL DELIVERY OF GENISTEIN AS A CHEMOPROTECTIVE DRUG FOR MELANOMA

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Gurkishan S. Chadha

Certificate of Approval:

Daniel L. Parsons, Co-Chair
Professor
Pharmacal Sciences

Jayachandra Ramapuram, Co-Chair
Assistant Professor
Pharmacal Sciences

William R. Ravis
Professor
Pharmacal Sciences

Muralikrishnan Dhanasekaran
Assistant Professor
Pharmacal Sciences

George T. Flowers
Dean
Graduate School

TRANSDERMAL DELIVERY OF GENISTEIN AS A CHEMOPROTECTIVE DRUG FOR MELANOMA

Gurkishan S. Chadha

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TRANSDERMAL DELIVERY OF GENISTEIN AS A CHEMOPROTECTIVE DRUG FOR MELANOMA

Gurkishan S. Chadha

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THESIS ABSTRACT

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Gurkishan S. Chadha

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Genistein is a soy isoflavone and it is a chemo preventative agent against various cancer cell lines including melanoma. The percutaneous absorption of genistein has been scantly investigated. Also, genistein has very poor skin permeability characteristics. The main objective of this study was to formulate genistein as a topical gel, and incorporate various penetration enhancers like terpenes and glycol derivatives, in it to increase the retention and the permeation of genistein through the skin. Methocel® E4M was found to formulate a stable and homogenous gel with genistein. It had a better skin permeation and retention as compared to the gels formulated from Pluronic® F-127 NF and Carbopol® 934 NF polymers.

Genistein is a highly lipophilic drug and its log octanol-water partition coefficient (*log* P) was determined to be 2.94. The drug was solubilized in ethanol, but when the gel was made, the drug was precipitated as fine particle dispersion. With the drug in the form of dispersion, it was possible to determine the solubility of genistein in the various formulations. It was observed that formulations containing terpenes had similar solubility for genistein as compared to Methocel gel, except for Carvone which had increased solubility (1.5 fold). Although the solubility was similar, yet the permeability constants were significantly higher than the control formulation ($P < 0.05$). Menthol and limonene showed 9 and 7 fold increase, respectively. On the other hand, the formulations containing various glycol derivatives had decreased solubility for genistein (6-8 folds), yet had much larger permeability constant ($P < 0.05$). This can be associated with the fact that glycol derivative formulations had a much lesser amount of alcohol as compared to the Methocel gel, thus the solubility of Methocel gel containing ethanol was higher.

For use in percutaneous absorption experiments, the HPLC method of genistein was validated for its precision and accuracy. The percentage coefficient of variance (%CV) for intra-day and inter-day precision was 0.44% and 0.67%, respectively. Furthermore, forced degradation studies were performed on genistein to determine its stability under various stress conditions. Genistein was found to be stable in neutral and 1.5% w/w H_2O_2 solution, while it had 80% degradation in the presence of 1N NaOH (24 hours), and 1N HCl (5 days).

In conclusion, genistein was formulated with various penetration enhancers for increasing the permeation and drug retention in human skin. Based on the permeation and

skin retention data, it was found that menthol, Lauroglycol® 90, Labrasol® and Transcutol® P were most efficient in enhancing the skin permeation and retention of genistein. The gel having sufficient skin retention for genistein, would act as a promising chemoprotective agent against melanoma. These formulations also will have a potential for the use as anti-aging products.

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"*If the only prayer you ever say in your entire life is thank you, it will be enough.*"

-Meister Eckhardt

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I. LITERATURE REVIEW

Introduction

According to the American Cancer Society, there were 565,650 deaths due to cancer in the USA in 2008 along with a total of 1,437,180 new cancer cases. The United States is ranked 14th in the world in female breast cancer incidence whereas Japan was ranked 43rd and China was ranked 44th (Jemal et al. 2008). Among the Japanese, the incidence of cancer is less for those living in Japan compared to the newer generation of Japanese who have immigrated to Hawaii from Japan (Persky and Van Horn 1995). Such a disparity in the occurrence of cancer in Asian and American cultures may be due to fundamental differences in diet. Consumption of soybean products, such as soya milk, soya sauce, tofu, miso, and tempeh, as well as low fat foods is common in Asian cultures. Specifically, the typical Asian diet contains 10 to 12 g of isoflavone rich soy protein per day which is roughly 20 to 50 times that of the average western diet (Hawrylewicz et al. 1995).

Over the last decade, Genistein (4',5,7-trihydroxyisoflavone) has garnered significant attention from the scientific and medical communities alike for its antioxidative properties.

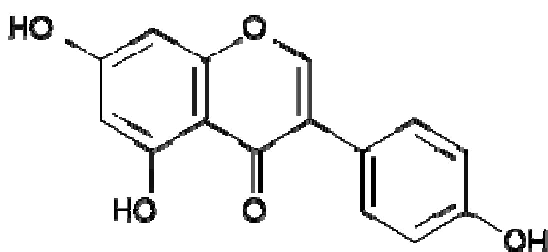


Figure 1.01: The molecular structure of Genistein

Genistein is the most abundant isoflavone of the soy derived phytoestrogen compounds. It is the aglycone form of the soybean isoflavone genistin (5,7,4'-trihydroxyisoflavone-7-glucoside). The majority (99%) of the isoflavones in soybeans are present in their glucoside form. Glycosylated genistin is believed to be readily hydrolyzed by the acidic environment in the stomach and/or converted to the more active genistein in the gastrointestinal tract by bacterial galactosidase (Chang et al. 1995).

The in vitro antioxidative properties of isoflavones are influenced by the number of hydroxyl groups present on the molecule; the addition of a glucose molecule to the aglycone decreased the antioxidative property of those isoflavones (Naim et al. 1974). A hydroxyl group present on carbon-5 of genistein may be responsible for its greater antimutagenic property compared to daidzein, which has a hydrogen atom on carbon-5.

Genistein containing soya complex and supplement preparations are commercially available as tablets and capsules for oral use. Although genistein is well absorbed, it has poor oral bioavailability because it undergoes extensive Phase II metabolism in the intestine (Mallis et al. 2003; Rowland et al. 2003; Zubik et al. 2003; Cave et al. 2007; Nielsen et al. 2007). Thus, if genistein delivery is to be modeled as

chemopreventive agent, its direct application as a topical formulation would be beneficial.

A review of the literature revealed genistein to be an effective moiety against skin cancer, but limited data is available regarding in vitro topical and transdermal delivery of genistein. Topically applied genistein was shown to reduce the incidence and multiplicity of skin tumors in the dimethylbenz[a]anthracene (DMBA) initiated and 12-O-tetradecanoyl phorbol-13-acetate (TPA) promoted mouse models (Wei et al. 1998; Khan et al. 2008). In the UVB light-induced complete carcinogenesis model, topical pretreatment of mice with 10 μmol genistein significantly reduced the formation of H_2O_2 and 8-hydroxy-2-deoxyguanosine (Wei et al. 1998, 2002; Khan et al. 2008). Since these are the precursors for free radicals, their attenuation will be a significant step for chemoprevention.

The present study was designed to formulate genistein as a topical gel for enhanced skin permeation and retention in human skin, along with identification of certain permeation enhancers that increase its retention and delivery into the skin. This chapter provides a brief review on:

i) Genistein for topical delivery,

ii) Permeation pathways of skin and various vehicular effects on percutaneous absorption,

iii) Various penetration enhancers.

Genistein

Genistein is an isoflavone first isolated from soyabeans in 1931. Isoflavones represent 0.25% of the weight of soybeans, with genistein occurring as the primary isoflavone (Niam et al. 1974). Genistein has been found to have multiple biofunctional activities. It shows fungistatic (Niam et al. 1974), antioxidative (Valachovicova et al. 2004; Park et al 2004; Moore et al 2006), antimutagenic (Valachovicova et al. 2004; Park et al 2004; More et al 2006; Banerjee et al. 2008), chemopreventive activity (Banerjee et al. 2008; Warri et al 2008) and activity against osteoporosis (Valachovicova et al. 2004; Horiuchi 2008). Due to such multi-functional abilities, genistein has undergone intense and extensive research with over 8000 citations in the NCBI database. The major portion of all this research has been oriented around biological activity, and the multiple-targeted potential of this compound.

Topical application of genistein will have advantages such as local delivery into the skin for its chemopreventive effects against UV induced skin damage including melanoma and systemic delivery for its protective effects against various types of cancer. Topical delivery will also help to overcome the discrepancies associated with the relative bioavailability of genistein after oral administration, which has been observed in certain studeis (Setchell et al. 2001). These studies have shown genistein to have a non-linear relationship for dose and absorption, suggesting a saturable mechanism of absorption (Setchell et al. 2001, Setchell et al. 2003a, 2003b). Thus, frequent doses throughout the day, rather than a single high dose, would help to achieve the optimum steady-state plasma concentration (Setchell et al. 2003a). Accordingly, transdermal administration

would be a viable route, since this would provide prolonged release of this compound at a controlled rate.

Increasing the retention of the drug in the skin will lead to local therapeutic effects. Genistein is known to be a potent antioxidant (Wei et al. 1995; Afaq and Mukhtar 2006), and this property can be used for the prevention of photocarcinogenesis and photoaging, by providing effective photoprotection to the skin. Its usefulness has also been demonstrated in rejuvenating the skin, by exhibiting anti-aging properties (Sudel et al. 2005).

Solar ultraviolet (UV) radiation is known to cause oxidative damage to skin leading to sunburn, skin cancer and photoaging. UV radiation is divided into three regions, depending on the wavelength. These include short-wave UV-C (200-280 nm), mid-wave UV-B (280-320 nm), and long-wave UV-A (320-400 nm). Of the three, UV-C is mostly blocked by the ozone layer and thus has minimal effect on the skin. Almost 90% of the radiation reaching the earth's surface is UV-A. UV irradiation to the skin results in erythema, edema, hyperplasia, immunosuppression, sunburn and photocarcinogenesis (F'guyer et al. 2003). Epidemiologic investigations of immunocompromized renal transplant patients and immunologic studies with skin cancer patients have provided evidence that immune suppression via general UV radiation may play an important role in the development of skin cancers (Fisher et al. 1982; Penn 1984; Yoshikawa et al. 1990). Although the amount of UV-B is much less compared to UV-A, its higher energy has a huge detrimental effect on the skin too. UV-B radiation on the skin has been shown to cause the formation of cyclobutane pyrimidine dimers (CPDs)

and pyrimidine pyrimidone photodimers, photoisomerization of trans- to cis-urocanic acid and generation of reactive oxygen species (ROS) (Lu et al. 1999; Afaq et al. 2005). These effects of UV-B may result in a variety of skin disorders including skin cancer.

Genistein can scavenge hydroxyl and superoxide anion radicals (Rimbach et al. 2003), which can help protect against lipid peroxidation (Ruiz-Larrea et al. 1997; Arora et al. 1998), along with protecting DNA from oxidative damages caused by hydrogen peroxide (Sierens et al. 2001). Additionally, genistein protects mouse-skin from oxidative stress (Wei et al. 2002) and photoprotects pig skin from UV-induced sunburn and erythema (Lin et al. 2008). When genistein was used along with human keratinocyte cell line NCTC 2544, UV-induced enhancement of the DNA-binding activity of signal transducer and activator of transcription (STAT) was prevented. It was thought to act as a tyrosine kinase inhibitor, thus limiting lipid peroxidation and increase in ROS formation (Maziere et al. 2000). Also, topical application of genistein before UV-B radiation reduced c-fos and c-jun expression in SENCAR mouse skin in a dose-dependent manner (Wang et al. 1998). Genistein can inhibit UV-induced DNA damage, which was evaluated with pyrimidine dimers (PD). A dose-dependent inhibition of UVB-induced PD formation was observed relative to increasing genistein concentrations (Moore et al. 2006).

With the advantages associated with genistein, it has become increasingly popular as dietary supplementation. But over the time, certain concerns about potential detrimental or other genotoxic effects have risen. Genistein is reported to induce micronucleus formation in L5178Y mouse lymphoma cells, in the concentrations of 1.5-

100 nM, cause DNA-damage in the comet assay at concentration of 29.6-118.9 nM (Boos and Stopper 2000). Genistein was also shown to induce a dose dependent micronucleus increase in V79 cells starting at concentrations ≥5 μM (Snyder and Gillies 2003; Virgilio et al. 2004). Genistein induced tumors in some experimental models, but did not induce tumors in the 6-months study in transgenic p53-knockout mice, when given in the diet (Misra et al. 2002). Genistein is also reported to decrease the androgen biosynthesis in rat Leydig cells by interference with luteinizing hormone-dependent signaling (Hancock et al. 2009). Some review in the literature has focused on other such toxicological issues (Stopper et al. 2005; Klein and King 2007).

While a variety of genotoxic effects of genistein have been reported in vitro, the concentrations at which such effects occurred are often much higher than the physiologically relevant doses achievable by dietary or pharmacologic intake of soy foods or supplements. The levels of dietary genistein uptake and bioavailability utilized for in vitro studies are mostly in the concentrations >5 μM. These concentrations are deemed much higher as compared to normally physiological relevant levels. In doing so, many of the often-cited genotoxic effects of genistein, including apoptosis, cell growth inhibition, topoisomerase inhibition and others become less obvious (Klein and King 2007). Thus, the balance between the genotoxic versus potentially beneficial in vitro effects of genistein can be defined according to the individual application, condition and the concentrations used.

Skin

The skin is the outer covering of the body, covering 2 m^2 in area, and is the largest organ of the integumentary system (Hadgraft 2001). Topical and transdermal drug delivery has a wide range of therapeutic applications but the desired delivery is restricted by the strong barrier nature of the skin. The skin is composed of three major components: the epidermis, dermis, and subcutaneous fat layer (hypodermis). Of these three layers, the epidermis is the most impermeable one. The epidermis is itself a complex multiple layered membrane, yet varies in thickness from approximately 60 μm on the eyelids to approximately 800 μm on the load-bearing palms and soles of the feet. It is non-vascular in nature and hence nutrients and waste products must diffuse across the dermo-epidermal layer in order to maintain tissue integrity. The epidermis contains five histologically distinct layers, which, from the inside to the outside, are the stratum germinativum, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The stratum corneum is composed of large, flat, polyhedral, plate-like envelopes filled with keratin, which is made up of dead cells that have migrated up from the stratum granulosum. Although the stratum corneum is only between 20 and 25 μm thick, it provides a very effective barrier towards penetration of drugs and chemical substances. The impermeability of skin is a considerable problem in the delivery of drugs both to and through the skin (Hadgraft 2001; Trommer and Neubert 2006). The stratum lucidum lies below the stratum corneum. It is a layer of the epidermis found only in palmoplantar skin (the thicker skin of the palms and soles) and is composed of three to five layers of dead, flattened keratinocytes. This is followed by the stratum granulosum and spinosum, while the last layer of the epidermis is called the stratum germinativum (or

basal layer, stratum basale). It is the deepest layer of the epidermis, and is a continuous layer of cells often described as one cell thick, though it may be two to three cells thick in glabrous skin and hyperproliferative epidermis. The basal cells of this layer can be considered the "stem cells" of the epidermis, undifferentiated, proliferating, and creating daughter cells that migrate upward, beginning the process of differentiation (Hadgraft 2001; McGrath et al. 2004).

The exact nature of the barrier function, for drug permeation, has been investigated over many years and recent advances in biophysical techniques have provided interesting insights into the mechanisms of absorption at a molecular level (Guy and Hadgraft 1989; Roberts and Walters 1998; Hadgraft 2001). There are a number of ways in which the drug can cross the stratum corneum. These include intercellular, transcellular and the route through the pores. The transcellular and intercellular route together is known as transepidermal route. The transcellular route is more direct, with the drug passing directly across the skin through both the lipid structure of the stratum corneum, and the cytoplasm of the dead keratinocytes. Although this is the shortest route for the molecules, the drug molecules encounter significant resistance to permeation because they have to cross both lipophilic and hydrophilic structures (Hadgraft, 2001). The intercellular route is more common as the molecules are able to overcome the stratum corneum by passing between the corneocytes (Trommer and Neubert 2006). Skin appendages like hair follicles and the sweat and sebaceous glands also contribute to the permeation, but the amount is small as they only occupy 0.1% of the total skin surface. Recently, research regarding follicular absorption has shown to be of more

significance. This is more for micro- and nanoemusions which have the ability to be absorbed into the hair shaft and reside there to provide a significant local effect. UV protective agents like zinc oxide (ZnO) and titanium dioxide (TiO_2) are known to penetrate through this route (Lademann et al. 2004, 2007).

Thus, for overcoming this barrier and for the topical delivery system, the vehicle has a more pronounced influence than on any other membrane. Having the ideal vehicle will cause the desired delivery and effect at the target site.

There is a broad spectrum of polymers for use as vehicles for such delivery including gels, emulsions, creams and ointments. Each comes with its own pros and cons and thus the delivery system used depends on the drug's activity, compatibility and stability. Emulsions and creams consist of two immiscible phases of lipids and water. The composition of the emulsion and cream can be adjusted according to the individual affinity of the drug for the phase. These final formulations are easy to produce and have good patient compliance. Similarly, gels are also very popular delivery vehicles and have a good aesthetic appeal. In contrast to emulsions, gels generally do not consist of two immiscible phases of opposite lipophilicity. Thus, the polarity, and therefore solubility characteristics of the incorporated substances are either hydrophilic in hydrogels or lipophilic in lipogels. The consistency of gels is the result of gelating agents, which are mainly polymers. The solvent molecules in this polymeric network are bound, by intermolecular forces, leading to the formation of a three dimensional network. Due to the reduced mobility of these polymer molecules in structured systems with increased viscosity, gels exhibit viscoelastic properties. Depending on whether the drug is required

for local or systemic action; and on the physical-chemical properties of the drug, the drug formulation can be made so as to be more retained on the skin or have a higher penetration so as to reach the vascular dermis and get absorbed into the systemic circulation. The vast majority of drugs incorporated in gels do not pass the skin, but act locally, mainly on the skin surface or in the whole epidermis (Valenta and Auner 2004).

Gels, as vehicles, are further classified as 'Single-phase' systems and 'Two-phase' systems. When the gel mass consists of a network of small discrete particles, it is classified as a Two-phase system. If the particle size of the dispersed phase is relatively large (> 0.5 μm), it is called as a 'magma'. On the other hand, single phase gel systems consist of organic-macromolecules distributed uniformly throughout the liquid in such a manner that there is no visible boundary between the two phases. Single phase gels are more commonly used in topical and cosmetic application because they provide certain advantages like a semi-solid state, higher clarity, and easy of application and removal. These also provide a faster release of drug substance, independent of the water solubility of the drug, as compared to the creams and lotions (Nairn 1995). These gels can be made from different pharmaceutical agents like xanthan gum (1-5%), tragacanth (2-5%), sodium alginate (2-10%), methyl-cellulose (3-5%), sodium carboxymethylcellulose (2-5%), carbomer (0.3-5%), and chitosan (1-5%) (Collett 1990; Souto 2004). All of these find wide usage in topical and cosmetic applications. The choice of the polymer depends largely upon the desired final characteristics of the formulation and drug compatibility. For the current study, the polymers used for the gel vehicles were poloxamer, hydroxypropylmethylcellulose and carbomer.

1. Hydroxypropylmethylcellulose (HPMC):

HPMC is also known as hypromellose. Chemically it is cellulose hydroxypropyl methyl ether. It is described as a partly O-methylated and O-(2-hydroxypropylated) cellulose. It is available in several grades that vary in viscosity and extent of substitution (Rowe et al. 2006). Commercially, HPMC is available as 'Methocel®' from Dow Chemical Company (Midland, Michigan, USA). This polymer is available in different grades which are distinguished by appending a number indicative of the apparent viscosity and character of the substituted polymer. The viscosity of the Methocel® products ranges from 3 to over 200,000 millipascal-seconds (mPa.s). This helps to formulate the gel according to the required consistency. Methocel® product grades are defined by an initial letter, thus, 'A' identifies methylcellulose products, while 'E', 'F', 'J' and 'K' identify different HPMC products (Table 1.01; Figure 1.02). The number that follows identifies the viscosity in mPa.s of the product measured at a 2% concentration in water at 20º C. In designating viscosity, the letter 'C' represents 100 while 'M' represents 1,000. Thus, with Methocel® E4M in the current study, E signifies HPMC polymer having 4000 mPa.s viscosity. Other suffixes include 'P' for Premium grade, 'LV' for Low Viscosity, 'G' for Granular product, 'S' for Surface Treated products, 'CR' for Controlled-release grade and 'FG' for Food grade (*Methocel Cellulose Ethers: Technical Handbook*).

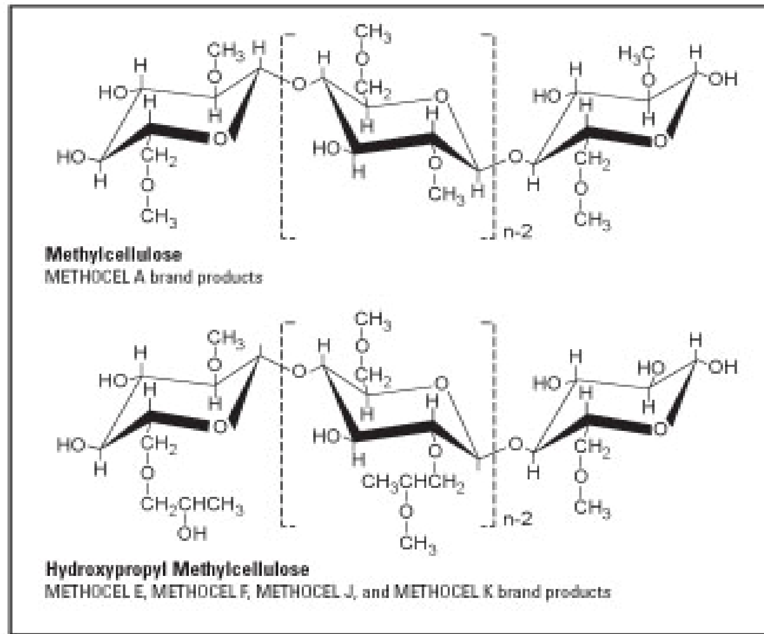


Figure 1.02: Chemical Structures of Methocel® Products
(*Methocel Cellulose Ethers: Technical Handbook)*

Table 1.01: Different grades of Methocel®
(Methocel Cellulose Ethers: Technical Handbook)

Product	Methoxy Degree of Subsitution	Methoxyl %	Hydroxypropyl Molar Subsitution	Hydroxypropyl %
METHOCEL® A	1.8	30	0	0.0
METHOCEL® E	1.9	29	0.23	8.5
METHOCEL® F	1.8	28	0.13	5.0
METHOCEL® J	1.3	18	0.82	27.0
METHOCEL® K	1.4	22	0.21	8.1
METHOCEL® 310 Series	2	25	0.80	25.0

Methocel® has the advantage over the other polymer gels in that it forms a well dispersed, homogenous gel in an aqueous system. It also has solubility in binary organic solvent/water systems, thus providing a unique combination of organic solubility and water solubility. These cellulose ethers are non-ionic and

do not interact with metallic salts or ionic species to form insoluble complexes. Also, they are stable over a wide pH range (2.0-13.0).

2. Carbomer:

Carbomer is the generic name for various Carbopol homoploymers. These are the polymers of acrylic acid cross-linked with polyalkenyl ethers, allyl sucrose or divinyl glycol. The Carbopol polymers are produced in a cosolvent mixture with a proprietary polymerization aid, and then the resins are crosslinked with a polyalkenyl polyether. Depending upon the cross linking degree and manufacturing conditions, different grades of Carbopol are available (Noveon Company Technical Literature, 2005; Rowe 2006). These grades, so produced, depend upon the primary polymer particles, which range in size of about 0.2 to 6.0 micron average diameter. Each primary particle can be viewed as a network structure of polymer chains interconnected by crosslinks, which results in polymers with molecular weights of up to 3 to 4 billion daltons. The cross linking chemically binds the linear polymer chains. Carbopol polymers have the ability to absorb large volumes of water. These polymers swell up to 1,000 times their original volume and 10 times their original diameter to form a gel when exposed to water at a pH above their pKa of 6. This leads to the ionization of the carboxylate groups on the polymer backbone, which further results in repulsion between the negative particles, and causes the polymer to swell. The gel contains a large amount of swollen microgel particles, with interstitial spaces in which insoluble particles can be entrapped (suspended). In addition to their hydrophilic

nature, the cross-linked structure and essentially insolubility in water makes Carbopol a potential candidate for use in controlled release drug delivery systems (Carnali and Naser 1992; Rowe et al 2006).

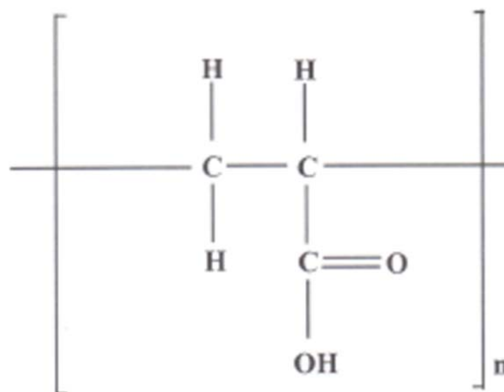


Figure 1.03: General structure of Carbomer polymer (Rowe et al. 2006)

The carboxyl groups provided by the acrylic acid backbone of the polymer are responsible for many of the product benefits. Carbopol polymers have an average equivalent weight of 76 per carboxyl group.

Carbopol polymer microgels are easily moved by shear, but once the shear stops, the macro-gel structure immediately forms again. This enables highly viscous suspensions to be stirred or pumped easily, with instantaneous recovery of viscosity once the stirring or pumping ceases (Lubrizol Pharmaceutical Bulletin 22, 2008).

Carbomers have been adapted to topical delivery formulations. Carbopol® 934P NF was designed for the pharmaceutical industry in the 1960's as a high purity grade of Carbopol® 934 NF polymer. These formulations have been found

to be safe and effective, are known to have extremely low irritancy properties and are non-sensitizing with repeated usage. Since these have an extremely high molecular weight, they do not penetrate the skin nor affect the activity of the drug. Various products with a wide range of viscosities and flow properties can be formulated as topical gels and creams using carbomers (Hosmani 2006).

3. Poloxamer 407 NF:

Commercially, this is known as Pluronic® F-127 NF. It has been effectively used to deliver hydrophilic and lipophilic drugs topically and transdermally (across the stratum corneum). This is an ABA type block copolymer (Figure 1.04), containing 70% of the polyoxyethylene fraction with a molecular weight of 12,500 Da (Franckum et al. 2004; Rowe et al. 2006). It is characterized by the property of reversible thermal gelation, being liquid when cold (<10 °C) and forming a semisolid gel at physiologic temperatures. Thus, when it is applied to the skin, it forms a gel and facilitates proper inunction and adhesion (Franckum et al. 2004; Richards et al. 2006). Poloxamer polymers are available in different grades having different characteristics. Poloxamer 407 has the advantage of being freely soluble in water and ethanol and forms a clear, stable gel. It has been found to be nonirritating and nonsensitizing even at 10% concentrations on the skin (Rowe et al. 2006).

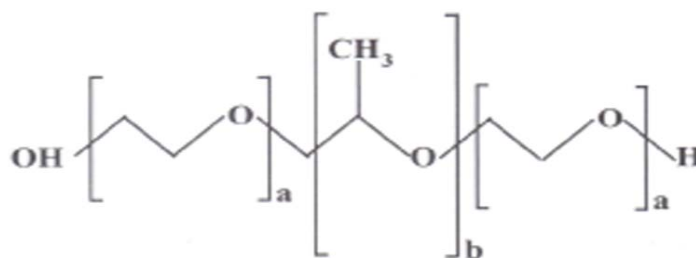


Figure 1.04: General structure of Poloxamer
For Poloxamer 407, a= 101; b= 56 (Rowe et al. 2006)

Penetration Enhancers

It has been observed that a drug formulation applied topically does not always have the desired or effective permeation. This is due to the strong barrier, the stratum corneum, of the skin. Most topically administered drugs are unable to permeate the stratum corneum, so modulations of the skin penetration profile of drugs and skin manipulations become necessary. Permeation enhancers are compounds that facilitate such a modulation so as to increase the absorption of the drug through the skin. This change in skin properties is temporary, so as not to cause any permanent damage. Ideally, these materials should be compatible with the drug, pharmacologically inert, nonirritating, nontoxic and have good solvent properties. Furthermore, the enhancer should not have any permanent effect on the skin or cause the loss of body fluids, electrolytes or other endogenous materials (Sinha and Kaur 2000). The ability of the enhancer to increase the permeation greatly depends on the physicochemical and molecular properties of the drug.

The percutaneous absorption of a drug is a passive diffusion process, which is described by Fick's first law of diffusion (Barry 1985).

$$J = (DKC)/h$$

Where J is the amount of drug transported through a unit area of skin per unit time; D is the diffusion coefficient of the drug in the skin; K is the skin-vehicle partition coefficient of the drug; C is the drug concentration in the vehicle and h is the skin thickness. If we can increase the drug diffusity and drug solubility in the skin, the penetration of the drug would be enhanced. This can be explained as:

$$J = PC$$

Where P is the skin permeability (Pfister and Hsieh 1990). Thus, by modulating the skin permeability, the transport can be affected. The mechanism by which such modulation is caused depends upon the type of chemical enhancer used. When there is an interaction of the enhancers with the polar head groups of the lipids, the stratum corneum lipid-lipid packing is disturbed and there is facilitated diffusion of hydrophilic drugs. This further leads to an increased content of free water molecules, thus leading to augmentation of the cross-section for polar drug diffusion. Water is known to be the safest and most effective penetration enhancer. If the normal content of water in the skin is increased from 5-10% to about 50%, there will be a vast improvement in hydrophilic drug diffusion. This can be achieved by occluding the skin, via foil, plastic wrap or occlusive vehicles.

Similarly, through polar penetration enhancers, the headgroup of lipids can be disturbed to affect the hydrophobic parts of the lipids, which causes penetration

enhancement of lipophilic drugs (Trommer and Neubert 2006). Some penetration enhancers increase the drug solubility in the vehicle and thus improve the drugs partition coefficient. Another mechanism is extraction of lipids so as to fluidize the stratum corneum. These can also affect the desmosomes which maintain the cohesion between corneocytes. Certain enhancers like Azone and oleic acid show a "pooling" effect. They modify the intercellular lipid domains, which leads to a decrease in barrier resistance of the bilayer lipids. The enhancer then distributes within the complex domains of the bilayer lipids (Williams and Barry 2004). Indirectly, certain enhancers like ethanol modify the thermodynamic activity of the vehicle. Rapid permeation of some enhancers can lead to supersaturation of the drug in the vehicle. Another indirect effect is by solubilizing the drug in the vehicle, mainly in the case of drugs with low solubility (steroids). This reduces depletion effects and prolongs drug permeation (Williams and Barry 2004).

Various classes of permeation enhancers include (Trommer and Neubert 2006):

- Alcohols and Glycols
- Alkyl-N,N-Disubstituted Aminoacetates
- Azone and its derivatives
- Esters
- Fatty acids
- Propylene glycol
- Pyrrolidones
- Sulphoxides

- Surfactants
- Terpenes and Terpenoids
- Urea and its derivatives

Alcohols, glycols and terpenes classes of enhancers were used in the present study. A brief review of each class of enhancers is provided below:

Terpenes as Penetration Enhancers

Terpenes are usually constituents of volatile oils, having a chemical structure of repeating isoprene (C_5H_8) units which are classified according to the number of isoprene units. The classifaction includes Monoterpenes, having two isoprene units (C_{10}), sesquiterpenes having three (C_{15}), and diterpenes with four isoprene (C_{20}) units (Sinha and Kaur 2000). Terpenes are highly lipophilic compounds having a high octanol/water partition coefficient. They are a very safe and effective class of penetration enhancers, obtained from natural sources, and are included in the list of GRAS agents issued by the US FDA (Williams and Barry 1991; Aqil et al. 2007). They cause no skin toxicity or, if any, only mild irritation.

The activity of terpenes is governed through their chemical structure along with the physical-chemical properties of the drug. Lipophilic drugs better permeate with lipophilic terpenes. Thus, hydrocarbon, nonpolar terpenes (e.g., limonene) are more potent enhancers for lipophilic drugs than are oxygen-containing polar terpenes (1,8-cineole, carvone) and vice versa (Okabe et al. 1989). In terms of size, smaller terpenes are more effective enhancers than larger ones. In the case of the boiling point, the penetration enhancing effect is inversely proportional to the boiling point. Such an

inverse relation also exists with the energy of vaporization. Terpenes with lower energies of vaporization show greater permeation enhancement for hydrophilic drugs. For degrees of unsaturation, terpenes with a minimal degree of unsaturation (menthol and cineole) are good sorption promoters for polar and water soluble drugs. It was also reported that formulations containing 40% ethanol along with terpenes had a synergistic effect (Aqil et al. 2007).

Menthol (Figure 1.05) is a monoterpene ($C_{10}H_{20}O$), obtained from the flowering tops of *Mentha piperita*. It is said to be the most effective permeation enhancer in its class, and has been successfully formulated as an enhancer with drugs such as imipramine hydrochloride (Jain et al. 2002), propranolol hydrochloride (Amnuaikit et al. 2005), caffeine, triamcinolone and hydrocortisone (Godwin and Michniak 1999). It has been observed that menthol enhances permeation by a dual mechanism. It forms an eutectic compound with some of the drugs, thereby increasing its solubility; and by altering the barrier properties of the stratum corneum. It has also been suggested that menthol preferentially distributes into the intracellular spaces of the stratum corneum which leads to reversible disruption of lipid domains, thus enhancing the permeation of drugs (Sinha and Kaur 2000).

Limonene (Figure 1.05) is a hydrocarbon monoterpene ($C_{10}H_{16}$) which is lipophilic in nature. It is obtained from lemon peel of *Citrus limon*. Limonene enhances the permeation of lipophilic (butyl paraben) and amphiphilic (6-mercaptopurine) compounds, but is ineffective for hydrophilic compounds such as mannitol (Koyama et al. 1994). When 4% (w/w) limonene was formulated in 2% (w/w) hydroxypropyl

cellulose with nicardipine hydrochloride, an increased bioavailability and prolonged steady state concentration were observed (Krishnaiah et al. 2002).

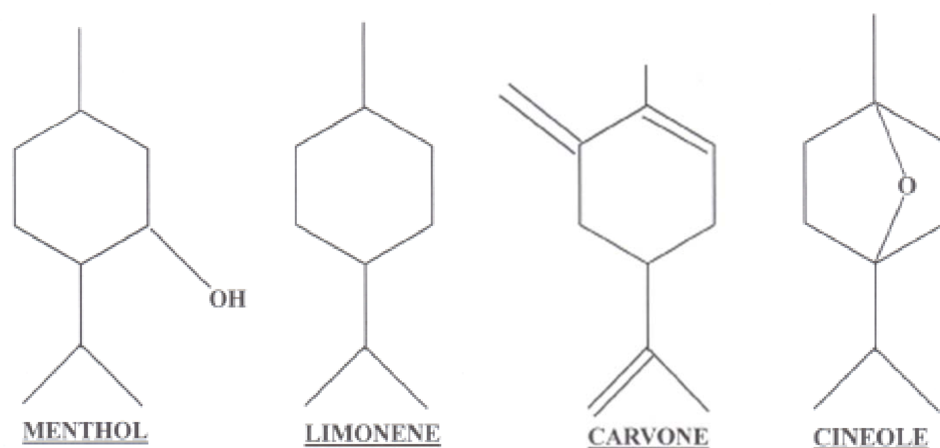


Figure 1.05: Structures of various Terpenes

Cineole (Figure 1.05) is the primary chemical constituent of *Eucalyptus globulus* and other species of Eucalyptus. It is a cyclic ether and a monoterpene ($C_{10}H_{18}O$). It is also known as 1,8-cineole, limonene oxide, cajeputol, 1,8-epoxy-p-menthane, and 1,8-oxido-*p*-menthane. The mechanism for penetration enhancement by cineole was unknown for a long time. Recently, DSC and ATR–FT-IR analysis revealed that the 1,8-cineole enhanced permeation of zidovudine by transforming stratum corneum lipids from a highly ordered orthorhombic perpendicular subcellular packing to a less ordered, hexagonal subcellular packing. A reduction in transition temperature (Tm) and a blue shift in nonhydrogen bonded amide I stretching frequency indicated the effect on alkyl tails and polar head groups, respectively. This further suggested that cineole acts at polar head groups and breaks interlamellar and intralamellar hydrogen bonding networks (Narishetty and Panchagnula 2005).

Carvone (Figure 1.05) belongs to the class of ketone terpenes ($C_{10}H_{14}O$). It is abundantly found in caraway seed (*Carum carvil*) oil. It has shown to increase the bioavailability of nicardipine hydrochloride three fold, when incorporated with hydroxypropyl cellulose gel (Krishnaiah et al. 2003). The suggested mechanism for carvone is by disruption of the highly ordered intercellular lipid structure of the stratum corneum (Aqil et al. 2007).

Alcohols and Glycols

Alcohols, propylene glycol (PG), polyethylene glycol and their derivatives have been long used as penetration enhancers. Ethanol is the most commonly used alcohol. It extracts large amounts of stratum corneum lipids and increases the number of free sulphydryl groups of keratin in the stratum corneum proteins (Sinha and Kaur 2000). Propylene glycol is known to solvate the keratin of the stratum corneum, and occupies the hydrogen bonding sites. Various derivatives of propylene glycol have been formulated and have been successfully employed as enhancers for cutaneous delivery.

Transcutol® P (diethylene glycol monoethyl ether) is a hygroscopic liquid that is freely miscible with both polar and non-polar solvents. It has been recognized as a potential transdermal permeation enhancer due to its non-toxicity, biocompatibility with skin, and unique solubilizing properties. Transcutol® has the ability to form an intracutaneous depot for topical drugs. This increases the drug efficiency and reduces its systemic absorption (Ritschel et al. 1991; Transcutol® Technical bulletin from Gattefosse). Transcutol® has been incorporated into various pharmaceutical topical formulations. These include Antralgon® baume (Saunier-Daguin) containing Amyleine

in an ointment, Dextrarine phenylbutazone® (Synthelabo) containing Phenylbutazone in an ointment, and Xenar® gel (Alfawassermann) containing Naproxen.

Labrasol® is composed of a well-defined mixture of mono-, di-, and tri-glycerides and mono- and di-fatty acids esters of polyethylene glycol. Chemically it is Caprylocaproyl macrogolglyceride (Polyoxylglycerides). Labrasol has been successfully incorporated in topical formulations to enhance the permeation of drugs like cyclosporine A (Liu et al. 2006), mechlorethamine (Ritschel et al. 2008).

Lauroglycol® 90 is propylene glycol monolaurate (Type II). Propylene glycol monolaurate is a mixture of the propylene glycol mono- and di-esters of lauric acid. It comes in two types (Type I and II) which differ in their percentage of monoesters and diesters (USP/NF, 2006; Brüsewitz et al. 2007).

Capryol® PGMC is propylene glycol monocaprylate (Type I). Propylene glycol monocaprylate is a mixture of the propylene glycol mono- and di-esters of caprylic acid. It comes in two types (Type I and II) which differ in their percentage of monoesters and diesters (USP/NF, 2007; Singh et al. 2009).

Labrafil® M 1944 CS is composed of a well-defined mixture of mono-, di-, and tri-glycerides and mono- and di-fatty acids esters of polyethylene glycol. Chemically it is oleoyl polyoxylglycerides. It is a non-ionic amphiphilic excipient and has been successfully used as a permeation enhancer in topical formulations (Technical bulletin from Gattefosse; Lee et al. 2005).

Polyethylene glycol 400 (PEG 400) is the addition polymer of ethylene oxide and water. PEG occurs in different grades and has been used in topical formulations for a

very long time. It is known to be a very safe and reliable penetration enhancer (Fruijtier-Pölloth 2005; Gong et al. 2006). PEG has been shown to have penetration enhancement activity for both hydrophilic and hydrophobic drugs. It is known to influence the penetration enhancing effects of the block copolymers and increases the partition coefficient at the skin surface rather than through a change in the drug diffusion coefficient of the skin (Asbill and Michniak 2000).

II. MATERIALS AND METHODS

Genistein was obtained from Alexis Biochemicals (Carlsbad, CA). HPLC grade water and methanol were from J.T.Baker (Phillipsburg, NJ) and EMD Chemicals (Gibbstown, NJ), respectively. Methocel® E4M, Carbopol® 934P and Pluronic® F-127 NF were gifts from Dow Chemical Company (Midland, MI), Noveon (Cleveland, OH) and BASF Chemicals (Mt. Olive, NJ), respectively. Polyethylene glycol 400, Menthol, Limonene, Cineole and Carvone were from Sigma-Aldrich (St. Louis, MO). Transcutol® P, Labrasol®, Lauroglycol® 90, Capryol® PGMC and Labrafil® M 1944 CS were gifts from Gattefosse (Paramus, NJ). All other chemicals were from Fisher Scientific (Fair Lawn, NJ).

Instrumentation

A High-Performance-Liquid-Chromatograph (HPLC) equipped with a 717plus auto-sampler, 1525 Binary HPLC pumps and 2998 Photodiode Array detector (Waters Corporation, Milford, MA) was used in this study. The system was interfaced with a computer (Winston-Salem, NC) and Waters Empower2 software.

A Waters Symmetry® C18 column (150 mm x 3.9 mm; 5 micron-diameter) was used. The mobile phase was used at a gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid and was pumped at a flow rate of 1.5 ml/min. The injection volume was 50 µL and the detector was set at 262 nm. All chromatographic procedures were performed at room temperature.

HPLC Method Validation

To validate the HPLC method, the accuracy and the precision of the method was determined. This was accompanied by performing Intra- and Inter-Day assays of fixed and varied concentrations of genistein.

The precision of the method was assayed by determining the peak areas of injections (4 replicates) 3 times in a single day and for 5 different days. The Coefficient of Variation (% CV) of the peak areas was determined to represent the precision of the HPLC assay.

The accuracy of the method was determined by assaying known concentrations of genistein (1.0, 2.5, 5.0, 10.0, and 25.0 μg/mL). The study was repeated three times in 3 consecutive days to represent the variation of the assay of the compound. The % CV of the concentration of genistein was determined to represent the accuracy of the HPLC assay.

Genistein Standard Curve

A standard curve was prepared to determine the concentrations of genistein in the various studies. A 1 mg/mL stock solution of genistein was prepared by adding 5 mg of genistein to a 5 mL volumetric flask and diluting to volume with an ethanol-water (1:1) solution. This stock solution was further diluted into a series of 10 mL solutions containing 1, 2.5, 5, 10 and 25 ug/mL of genistein. Samples were analyzed by HPLC as described earlier. A plot of peak area under the UV absorbance vs concentration of genistein was constructed.

Forced Degradation Studies on Genistein

An initial stock solution of 500 µg/ml of genistein was prepared in methanol. This was diluted separately with aqueous solutions of NaOH, HCl or H_2O_2 to obtain a final concentration of 50 µg/ml of genistein in 1N NaOH, 1N HCl, and 1.5% v/v H_2O_2 (methanol:water,1:9), respectively. These were stored in glass containers, protected from light, at 60ºC and subjected to the conditions listed in Table 2.01. Samples were periodically analyzed by HPLC to determine the extent of degradation. For HPLC analyses, 1.0 ml aliquots of the solutions were transferred to 10 ml volumetric flasks, neutralized as needed, and diluted to volume with methanol to obtain a final maximum concentration of 5 µg/ml.

Table 2.01: Forced degradation study for genistein

Stress Condition	Solvent	Time
Neutral	Water:Methanol (9:1)	30 days
Basic*	1N NaOH	1 Day
Acidic**	1N HCl	5 Days
Oxidizing***	1.5% H2O2	30 days

*1N NaOH solution in Water:Methanol (9:1) mixture.
**1N HCl solution in Water:Methanol (9:1) mixture.
***1.5%H_2O_2 solution in Water:Methanol (9:1) mixture.

Determination of Partition Coefficient

The partition coefficient of genistein was determined using an n-octanol/water solution. Equal volumes (30 ml) of *n*-octanol and water were placed in a screw-capped test tube, shaken vigorously and then stored for 24 h. After the equilibration, the aqueous phase saturated with n-octanol and oil phase saturated with water were separated. To 20 mL n-octanol, 20 mg of genistein was added and dissolved, which was then mixed with the aqueous phase in a separatory funnel. The funnel was vigorously shaken for 1 hour

and was then stored undisturbed for 24 hours. The concentration of the drug in each phase was then determined through UV absorbance using a Jasco V-630 spectrophotometer (Tokyo, Japan).

The partition coefficient was calculated as $log\ P_{(octanol/water)}$

$$log\ P_{(octanol/water)} = log\ ([Conc]_{octanol} / [Conc]_{water})$$

Gel Formulations

Three basic gel formulations consisting of Methocel® E4M, Pluronic® F-127 NF or Carbopol® 934P NF were made.

Table 2.02: Gel Formulations (%w/w)

Ingredients	Methocel Gel	Pluronic Gel	Carbopol Gel
Genistein	0.50%	0.50%	0.50%
Ethanol (99.5%)	50%	41%	
Water	49%	41%	98.5%
Methocel® E4M	0.50%		
Pluronic® F-127 NF		17.5%	
Carbopol® 934P NF			0.50%
N-methyl-pyrrolidone			0.50%

1. F1: Genistein-Methocel Gel

   Genistein was dissolved in 99.5% ethanol. To this, Methocel® E4M was added and dispersed. Finally, hot water (70-80 ºC) was added and the formulation was stored overnight to allow the polymer to swell properly.

2. F2: Genistein-Pluronic Gel

   Genistein was dissolved in 99.5% ethanol. To this, Pluronic® F-127 NF was added and dispersed. Finally, chilled water (1-5 ºC) was added and the formulation was stored overnight in a refrigerator to allow the polymer to swell properly.

3. F3: Genistein-Carbopol Gel

Genistein was dissolved in N-methyl-pyrrolidone. To this, Carbopol® 934P NF was added and dispersed. Water was added to this mixture and it was sonicated for up to 10 minutes until it became homogenous. The gel was then neutralized with 1N NaOH to form a gel.

Gel Formulations with Terpenes as Penetration Enhancers (Table 2.03):

Table 2.03: Contents of the gels with various terpenes (%w/w)

Ingredients	**F1 I**	**F1 II**	**F1 III**	**F1 IV**
Genistein	0.50%	0.50%	0.50%	0.50%
Ethanol	50.00%	50.00%	50.00%	50.00%
Methocel®	0.50%	0.50%	0.50%	0.50%
Water	45.00%	45.00%	45.00%	45.00%
Menthol	4.00%			
Limonene		4.00%		
Cineole			4.00%	
Carvone				4.00%

Procedure

Genistein was dissolved in ethanol. To this, the terpene was added and mixed. Then Methocel® E4M was added and dispersed. Finally, hot water (70-80 ºC) was added and the formulation was stored overnight to allow the polymer to swell properly.

Gel Formulations with Glycols as Penetration Enhancers (Table 2.04)

Table 2.04: Contents of Gels with various Glycol derivatives (%w/w)

Ingredients	F1 A	F1 B	F1 C	F1 D	F1 E	F1 F
Genistein	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%
Ethanol	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%
Water	73.50%	73.50%	68.50%	83.50%	68.50%	68.50%
Methocel®	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%
Transcutol® P	25.00%		22.00%		22.00%	22.00%
PEG 400		25.00%				
LauroGlycol® 90			8.00%			
Labrasol®				15.00%		
Capryol® PGMC					8.00%	
Labrafil® M 1944 CS						8.00%

Procedure

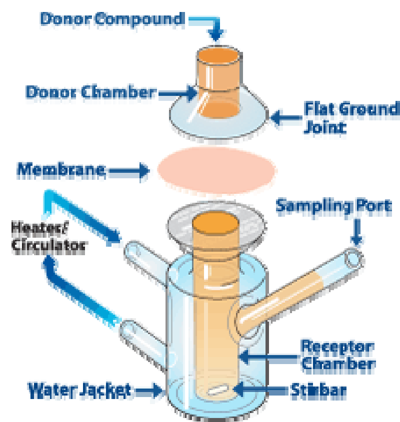
For each formulation, genistein was dissolved in the mixture of ethanol and the glycol derivatives. To this, Methocel® E4M was added and dispersed. Finally, hot water (70-80 ºC) was added and the formulation was stored overnight to allow the polymer to swell properly.

Permeation Studies

Human skin dermatomed to a thickness of ~ 0.35 mm was obtained from a tissue bank (International Institute for the Advancement of Medicine (IIAM), Exton, PA). The skin was collected within 8 h of donor death (Male Caucasian, died from cancer) and frozen at -70 ºC until use. The frozen skin was thawed by keeping it at ambient temperature for about 20 minutes. It was then washed with pH 7.4 buffer solution. Each experiment was carried out with each formulation at least 3 times using the skin from one donor.

The transdermal Franz diffusion cell apparatus used in this study (PermeGear, Bethlehem, PA) holds up to 6 diffusion cells in series (Figure 2.01). In vitro percutaneous absorption of various formulations was studied quantitatively with human skin mounted horizontally between the donor and receptor halves of the diffusion cell. The surface area of the skin exposed to the formulation in the donor chamber was 0.64 cm^2, and the receptor cell volume was 5 ml.

The receptor compartment was filled with 0.01 M phosphate buffered saline pH 7.4 (PBS) and ethanol (8:2). The fluid was stirred by a magnetic bead at 600 rpm. A water circulation jacket (37.0 ºC) surrounded the receptor cell to maintain the skin temperature at a physiologic level.



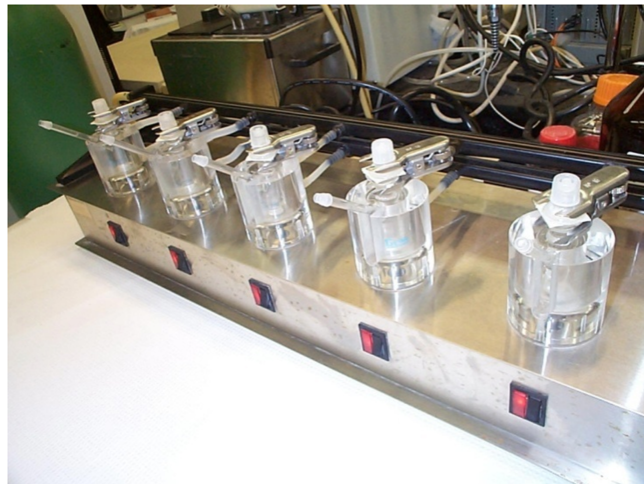


Figure 2.01: Franz Diffusion Cell Setup

The washed skin was mounted on the cells approximately 30 minutes before the application of the formulations. The formulation (200 mg) was applied over the surface of the epidermis gravimetrically using a syringe. The donor chamber was protected from the external environment, thus preventing the exposure of the drug formulation to the surrounding air. Samples (0.4 ml) were taken from the receptor cell to measure the amount of drug transported across the skin at 0, 1, 2, 4, 8, 12, and 24 h. The samples were replaced by fresh solution to maintain a constant volume after sampling. A correction factor was applied, to cover the loss of drug removed during the sampling.

At the end of the experiment (24 h), the residual drug formulation remaining on the surface of the skin was removed using cotton swabs by alternate swabbing and dabbing the surface with 200 µl of a solution of ethanol and water (1:1) for 6 times. The active diffusion area of the skin was then collected using a circular 10 mm diameter biopsy punch for determining the drug content in the skin. The skin of the active diffusion area was weighed, cut into small pieces, placed in glass vials and 1 ml of a solution of ethanol and water (1:1) was added. The samples were sonicated for 15 minutes and allowed to stand overnight. The vials were sonicated again for 15 minutes and the supernatant was filtered using 0.22 µm syringe filters into HPLC vials.

Human Tissue Handling:

Tissues from the International Institute for the Advancement of Medicine, Exton, PA, were shipped and received through Federal Express by Priority Overnight. The excess human tissue after the permeation study and the analysis samples was placed into Ziploc bags and then into secondary containment and transported to the designated medical waste accumulation site in room 4215 of the Walker Building. The work bench

was covered with a protective liner, and this liner was changed at periodically. The liner, hand gloves, the human tissue were disposed carefully at the medical waste accumulation site at 4215, Walker building. Disposable blades were used for cutting the skin for the experiment. The glassware was disinfected by sodium hypochloride solution (Clorax® solution) after the experiment.

Solubility of genistein in various gel formulations

All the formulations were made in triplicates. These formulations were centrifuged at 14,000 rpm for 90 minutes and the supernatant were then filtered using Whatmans cellulose acetate filters (0.45 µm, 25 mm). The filtrates were analyzed by HPLC and the solubility (µg/mL) of genistein in the individual formulations was determined.

Data analysis

For each experiment, three replicates were performed by using the skin from the same donor. The cumulative amount of genistein permeated through the skin was plotted as a function of time. The slope of the linear portion of the plot was calculated as flux (µg/cm^2/h). Permeability coefficient was calculated by dividing the flux by the mean solubility of genistein in the vehicle. The flux data were subjected to One-way Analysis of Variance (ANOVA) followed by Tukey's test to determine the level of significance between various groups. The data was considered significant at $p < 0.05$.

III. RESULTS AND DISCUSSIONS

The HPLC method

The HPLC method used in the current study was a modified method of the one developed by Fukutake et al. (1996) for quantification of genistein. To confirm the linearity of the method, a five point calibration curve (Figure 3.01) was plotted from 1.0 µg/ml to 25.0 µg/ml, which had 0.1 µg/ml as the lower limit of detection. The plot was linear with an R^2 value of 0.9998.

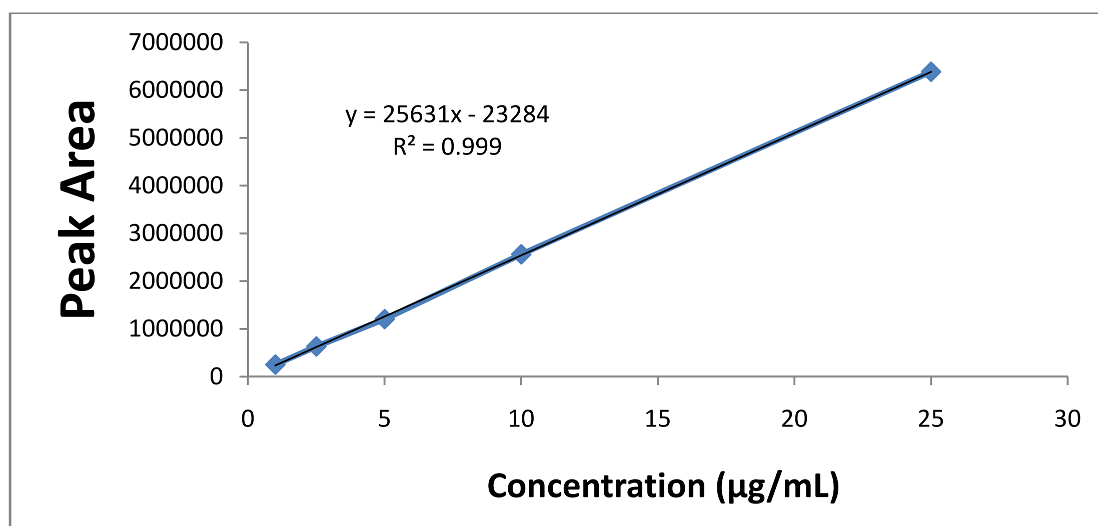


Figure 3.01: The standard curve for genistein absorbance.

The intra-day and inter-day variability of the method was determined by injecting a standard solution of 5 µg/ml genistein at 3 different times (six hours apart) in a day and on 5 different days, respectively (Table 3.01, 3.02 and 3.03). Precision is an expression of

method reproducibility and is represented by Coefficient of Variation (CV) of the peak areas, which were < 0.44 and < 0.67 for intra-day and inter-day variability, respectively (Table 3.03). The data clearly demonstrates a reproducible and accurate HPLC assay of genistein.

Table 3.01: Intraday Variability

Injection	Set-1 ($x 10^6$)	Set-2 ($x 10^6$)	Set-3 ($x 10^6$)
1	1.211	1.217	1.217
2	1.218	1.215	1.222
3	1.215	1.207	1.220
4	1.197	1.191	1.213
MEAN	**1.210**	**1.207**	**1.218**
SD	**0.00942**	**0.0119**	**0.00402**
CV	**0.78 %**	**0.99 %**	**0.33 %**

Table 3.02: Interday Variability

Injection	Day 1 ($x 10^6$)	Day 2 ($x 10^6$)	Day 3 ($x 10^6$)	Day 4 ($x 10^6$)	Day 5 ($x 10^6$)
1	1.214	1.218	1.218	1.224	1.226
2	1.212	1.209	1.206	1.221	1.226
3	1.211	1.214	1.192	1.203	1.225
4	1.198	1.185	1.195	1.197	1.219
MEAN	**1.209**	**1.207**	**1.203**	**1.211**	**1.224**
SD	**0.00721**	**0.01487**	**0.01182**	**0.01304**	**0.00318**
CV	**0.60%**	**1.23%**	**0.98%**	**1.08%**	**0.26%**

Table 3.03: Mean Variability

INTRADAY (3 Sets)		INTERDAY (5 Days)	
MEAN	**1.212×10^6**	**MEAN**	**1.211×10^6**
SD	**0.00536×10^6**	**SD**	**0.00816×10^6**
CV	**0.44%**	**CV**	**0.67%**

Stress testing of Genistein

Stress testing can provide insight into the formulation effect on the stability of a drug. Drug decomposition often results in loss of potency and possible adverse effects due to the formation of degradation products. The tests were performed on 50 µg/ml solutions of genistein using various stresses, including hydrolytic and oxidizing conditions. Analyses revealed the drug stability, as summarized in Table 3.04.

Table 3.04: Genistein stability under various stress condition

Stress Condition	Solvent	Time	Temperature	Result
Neutral	Water:Methanol (9:1)	30 days	60 ºC	Stable
Basic*	1N NaOH	1 Day	60 ºC	80% degradation
Acidic**	1N HCl	5 Days	60 ºC	80% degradation
Oxidizing***	1.5% H_2O_2	30 days	60 ºC	Stable

*1N NaOH solution in Water:Methanol (9:1) mixture.
**1N HCl solution in Water:Methanol (9:1) mixture.
***1.5%H_2O_2 solution in Water:Methanol (9:1) mixture

Under neutral conditions, the samples were stable even after exposing the drug to 60 ºC for 30 days. On the other hand, there was rapid decomposition of genistein under basic conditions of 1N NaOH, with 80% decomposition in one day (Figure 3.02-3.06). This decomposition resulted in highly polar products, which were eluted out along with the void volume. Although the drug also decomposed under acidic conditions, it was not as rapid as with the basic conditions. It took approximately 5 days for 80% degradation of the drug in 1N HCl. This degradation, unlike that in 1N NaOH, produced relatively more non-polar products (Figure 3.07-3.10). The drug solution was also stored as a 1.5% H_2O_2 solution to determine if oxidative degradation occurred. The genistein solution was found to be stable at 60 ºC even after 30 days. For future studies, the identification of these

degradation products through LC-MS (Liquid chromatography-mass spectrometry) can be studied.

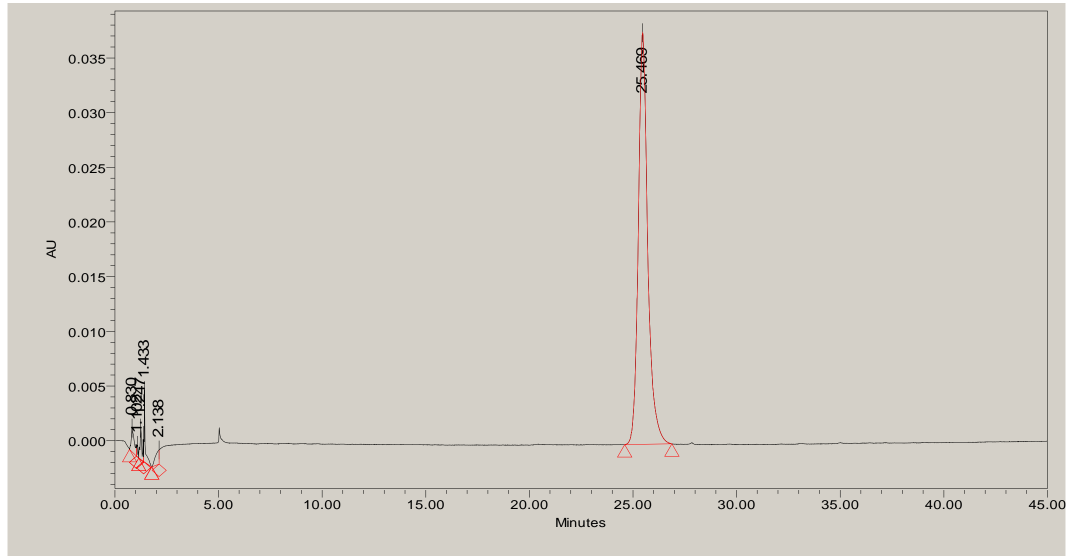


Figure 3.02: Representative Chromatogram showing the genistein peak in the presence of 1N NaOH at **0 Hour**

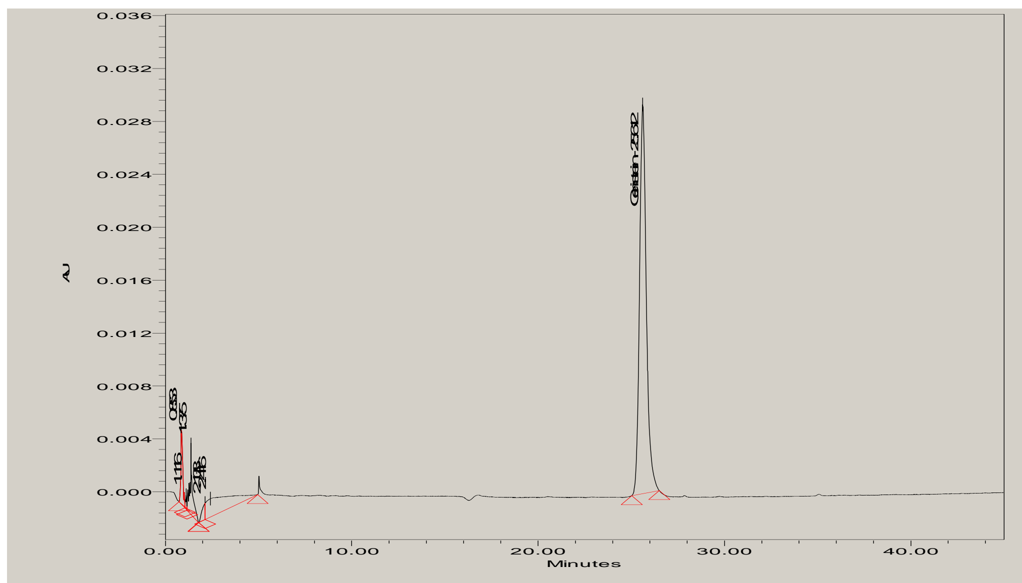


Figure 3.03: Representative Chromatogram showing the genistein peak in the presence of 1N NaOH after **4 Hours**

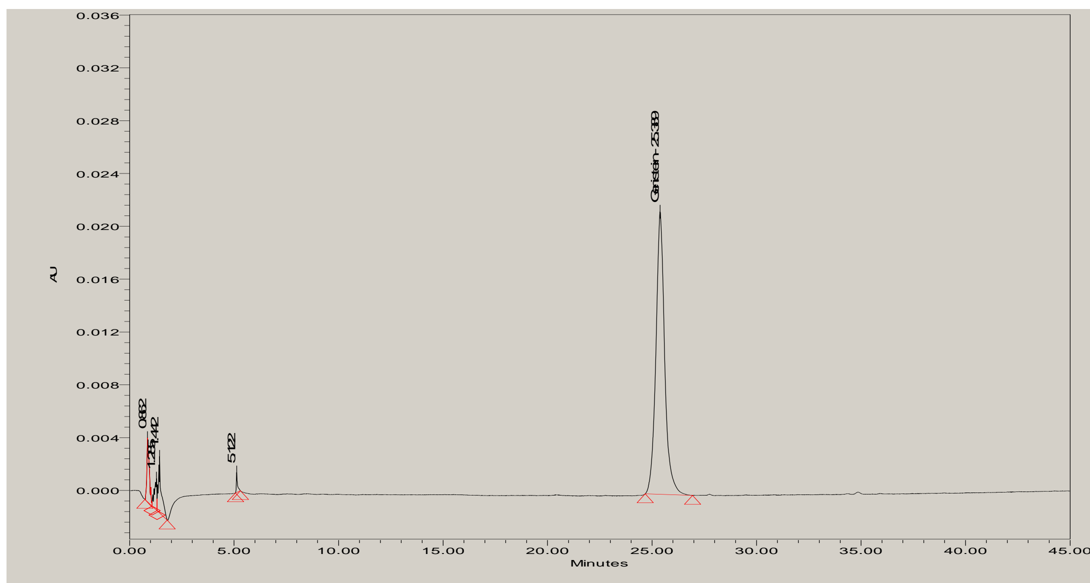


Figure 3.04: Representative Chromatogram showing the genistein peak in the presence of 1N NaOH after **12 Hours**

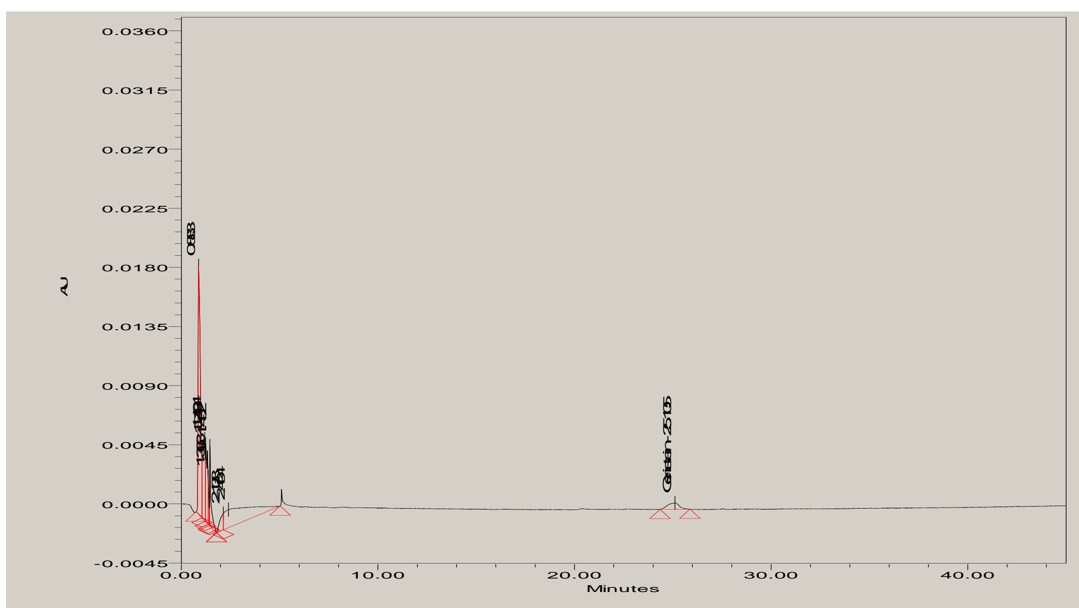


Figure 3.05: Representative Chromatogram showing the genistein peak in the presence of 1N NaOH after **24 Hours**

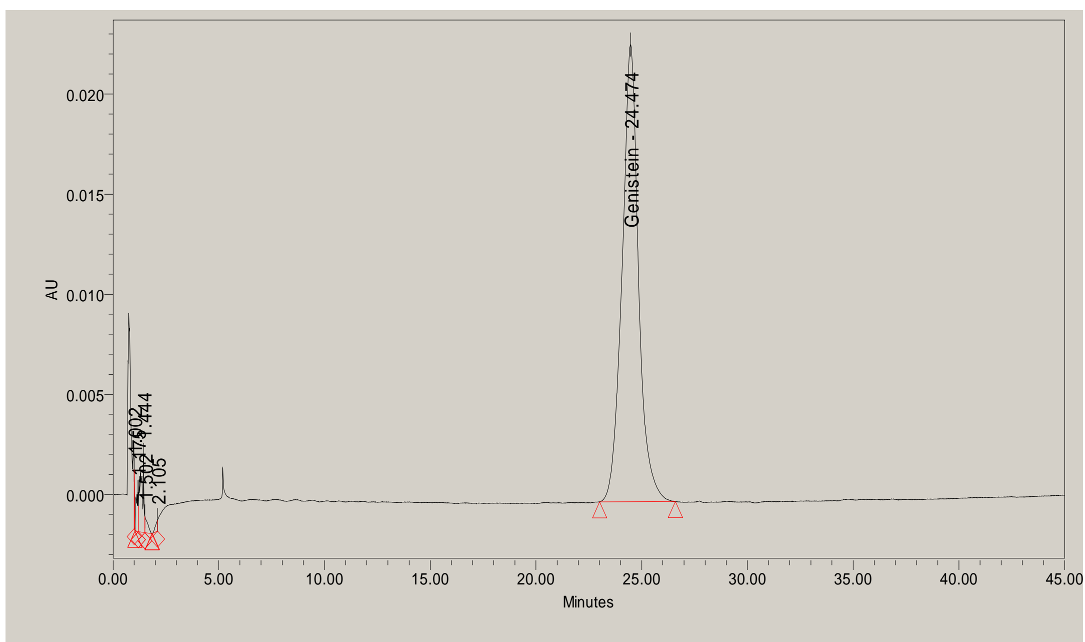


Figure 3.06: Representative Chromatogram showing the genistein peak in the presence of 1N HCl after **1 Day**

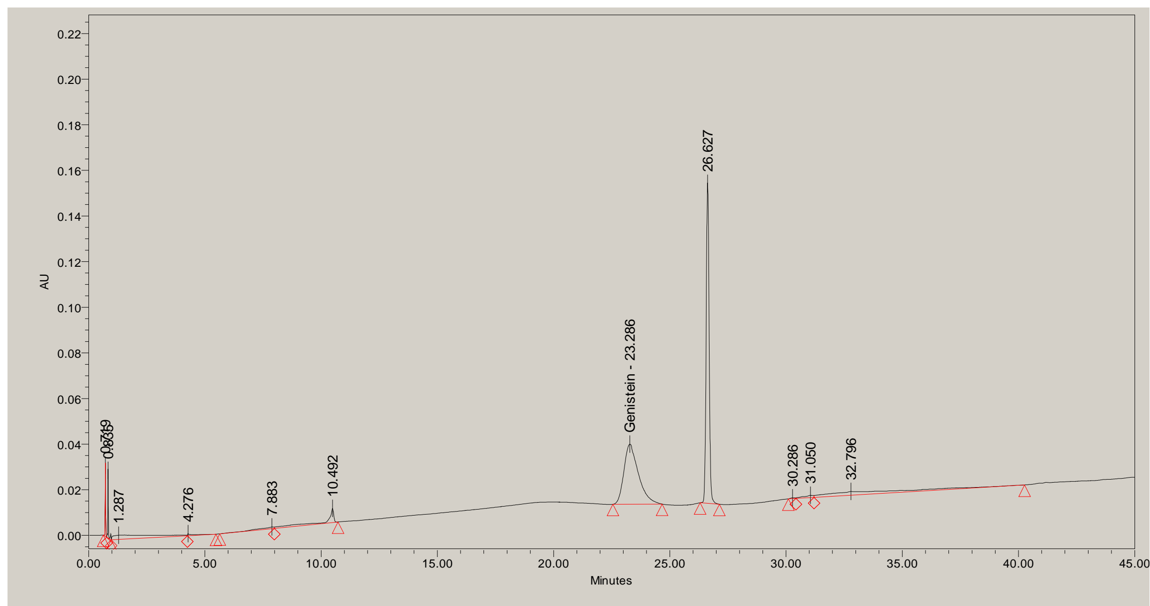


Figure 3.07: Representative Chromatogram showing the genistein peak in the presence of 1N HCl after **3 Days**

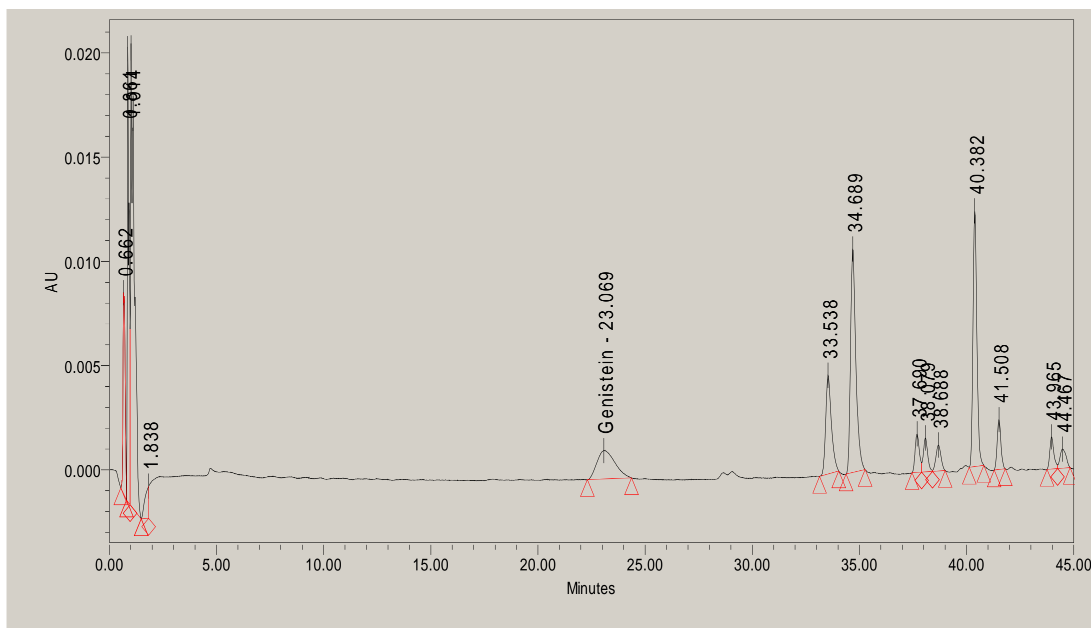


Figure 3.08: Representative Chromatogram showing the genistein peak in the presence of 1N HCl after **5 Days**

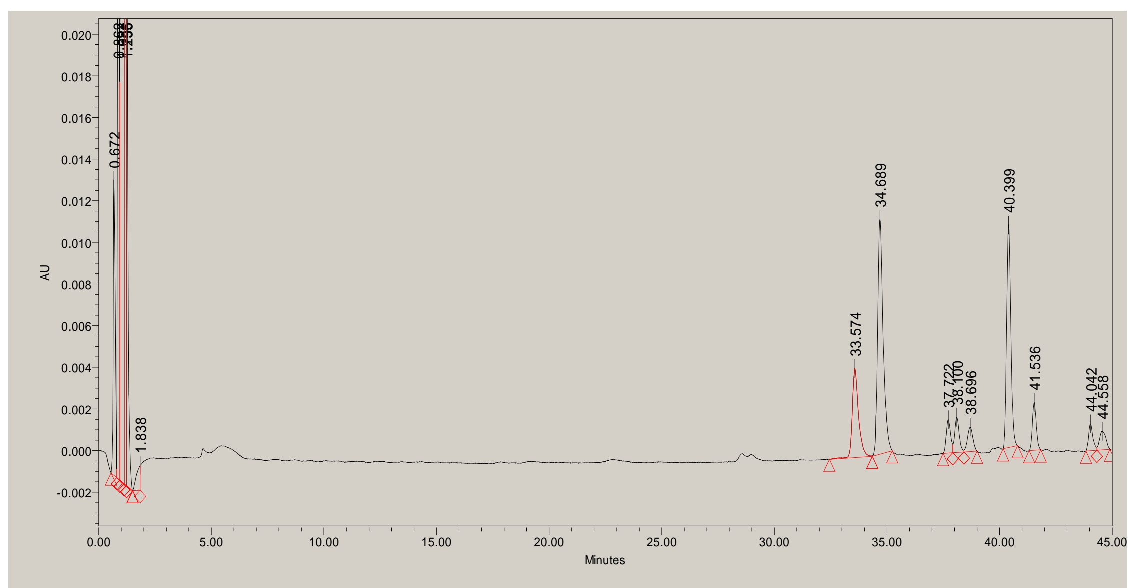


Figure 3.09: Representative Chromatogram showing the genistein peak in the presence of 1N HCl after **7 Days**

Formulation of gels

Since genistein is insoluble in water, it was first solubilized in ethanol, followed by the addition of the gelling agent. Addition of water to this led to swelling of the gel along with precipitation of the drug, thus forming a gel with a fine particle dispersion. Ethanol, along with being a good solubilizer, also acts as a permeation enhancer (Liu et al. 2006). It has been effectively used upto 60% v/v in different formulations as a penetration enhancer (Sinha and Kaur 2000). Thus, ethanol was identified as a solubilizer for genistein as well as a permeation enhancer in the Methocel Gel and Pluronic Gel. In the Carbopol gel, the drug was solubilized in N-methyl-pyrrolidone instead of ethanol. It was observed that with ethanol, the Carbopol gel formulated was a suspension rather than a fine dispersion. On the other hand, only 0.5% w/w N-methyl-pyrrolidone was required to solubilze the drug which also produced the final formulation as a fine particle dispersion. Although N-methyl-pyrrolidone is known to be an efficient penetration enhancer, its use is limited because of its irritantation potential (Sinha and Kaur 2000).

There is limited literature regarding the transdermal formulation of genistein. Minghetti et al. (2006) studied the ex-vivo human skin permeation of genistein from soy extract saturated solutions of different vehicles. The effects of the vehicles on the in vitro topical delivery of genistein, from saturated solutions such as aqueous buffers (pH 6.0, pH 10.8) and soyabean oil, have been investigated (Huang et al. 2008). Apparently, genistein in its non-ionized form had a higher skin deposition than in its ionized form (at pH 10.8). Our accelerated (stress) degradation studies demonstrated that genistein is highly unstable in alkaline medium (1N NaOH), compared to neutral medium

(water:methanol, 9:1). There are no literature reports, to our knowledge, on the formulation of genistein as a topical gel for use as a chemoprotective agent in skin disorders. Based on the literature data (Motlekar et al. 2003; Minghetti et al. 2006), the gels for the current study were formulated with 0.5% w/w genistein. The gels were made in the form of dispersions instead of solutions. The excess drug in the dispersion can then replace the absorbed drug to maintain the concentration gradient for drug diffusion into the skin. In addition, this provided the advantage that the dispersion can be filtered and the actual concentration of the drug involved in permeation (dissolved) can be calculated. From this the permeability coefficient can be determined.

In steady-state kinetics, Fick's first and second law of diffusion can be represented as (Motlekar et al. 2003):

$$J = dM/dt = (DK/h)C_d$$

Where J is the flux of the drug, M is the mass of drug permeated as a function of time, t; D and K are, respectively, the diffusion coefficient and partition coefficient of the drug, C_d is the dissolved drug concentration in the donor cell, and h is the thickness of the membrane. The use of this equation is based on Fick's law of diffusion with the following assumptions: (i) a steady state is reached in the membrane; (ii) the membrane area and the soluble drug concentration in the donor cell (C_d) are constant; and (iii) the drug concentration in the receptor cell is much lower than C_d .

The apparent permeability coefficient (P_{app}) of genistein can be calculated as:

$$P_{app} = (1/AC_0)J$$

Where A is the surface area of the membrane (0.64 cm^2 in the current study) and C_0 is the initial drug concentration in the solution. The slope of the cumulative amount of the drug permeated versus time curve gave the flux, J (Motlekar et al. 2003).

HPMC (Methocel® E4M), Carbomer (Carbopol® 934P NF) and Poloxamer (Pluronic® F127 NF), based on their ionic properties, were identified as the polymers to formulate the gels. Transdermal permeation studies of genistein from these gels through cadaver skin showed that Methocel® E4M had a better skin permeation and retention for genistein, as compared to the other gel formulations (Figure 3.10, 3.11 and 3.12).

Since the aim of the current study was to provide the chemopreventive and antioxidant effect of genistein, greater retention in the skin would mean a higher local therapeutic effect. Along with better skin retention, Methocel® E4M has the advantage that it does not need to be treated or neutralized during formulation, as is the case for carbomers. This can provide a reproducible viscosity in the formulation. Also, Methocel® E4M gels are more homogenous in apperance and more temperature stable than poloxamer gels, which transit to a liquid when refrigerated and are gels at room temperature only. Thus, for these reasons, Methocel® E4M was identified as the polymer for formulation of genistein gel for further studies.

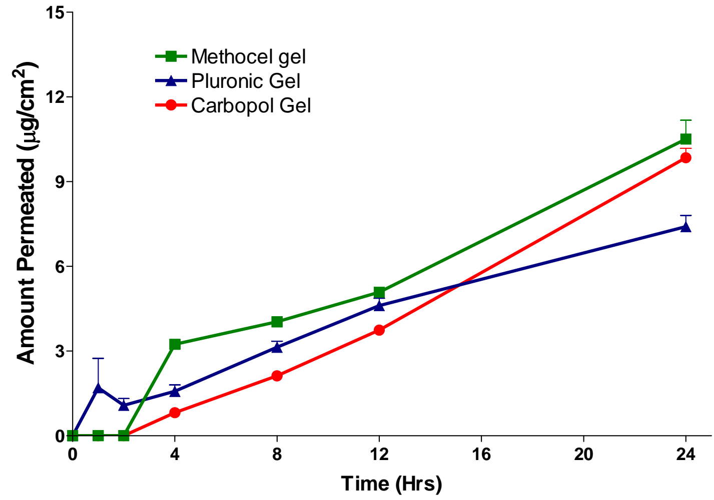


Figure 3.10: Release profile of genistein in various formulations

Bar represents Mean ± S.D. (n=3)

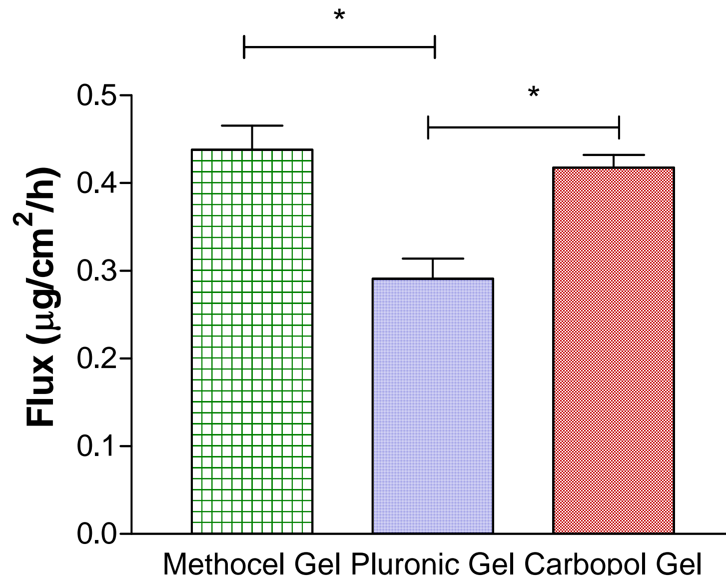


Figure 3.11: Flux of genistein through various gels

Bar represents Mean ± S.D. (n=3)

*$p < 0.05$ Tukey's multiple comparison test (P = 0.0071)

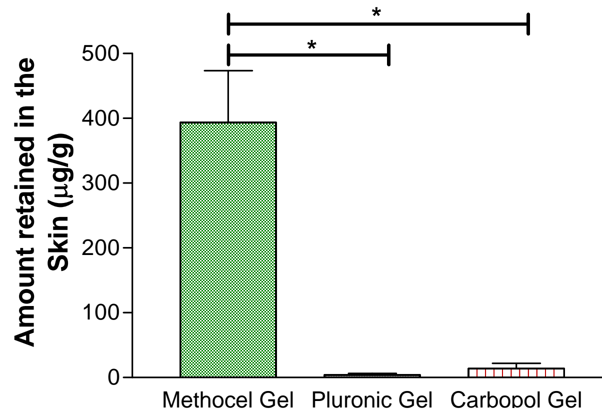


Figure 3.12: Retention of genistein in the skin with various gels
Bar represents Mean ± S.D. (n=3)
*$p < 0.05$ Tukey's multiple comparison test (P=0.0015)

To further increase the skin permeation and retention of genistein, various penetration enhancers were incorporated into the Methocel® formulation. Based on the initial formulation studies, terpenes and glycol derivates were identified as permeation enhancers for the current study. Terpenes have been classified as GRAS by FDA and they are also known to be non-toxic and non-irritants. Although some terpenes are known to be mild irritants, they do not cause any lasting erythema (Okabe et al. 1990; Asbill et al. 2000). Various terpenes have been used in different proportions for their enhancement effect. Based on the literature, menthol, limonene, cineole and carvone were formulated into the gels, at a concentration of 4% w/w (Aqil et al. 2007). It was also reported that terpenes have a synergistic effect when ethanol is incorporated along with them (Aqil et al. 2007). Thus, the formulations with terpenes also included 50% w/w ethanol, which was also necessary to solubilize genistein.

Table 3.05: Permeation parameters of various terpenes

*$p < 0.05$ Tukey's multiple comparison test ($P < 0.0001$)

Formulation	Flux (µg/cm²/h)	Mean Solubility (µg/mL)	Permeability Constant * 10^{-4} (cm/h)
Methocel® E4M	0.41 ± 0.05 *	820.10 ± 124.68 *	4.99 ± 0.74 *
Menthol	3.93 ± 0.68 *	839.51 ± 143.64	46.81 ± 7.55 *
Limonene	0.71 ± 0.21	752.51 ± 102.06	9.43 ± 1.38 *
Cineole	3.04 ± 0.42 *	831.10 ± 140.11	36.57 ± 6.97 *
Carvone	1.96 ± 0.68 *	1173.42 ± 181.29 *	16.70 ± 2.69 *

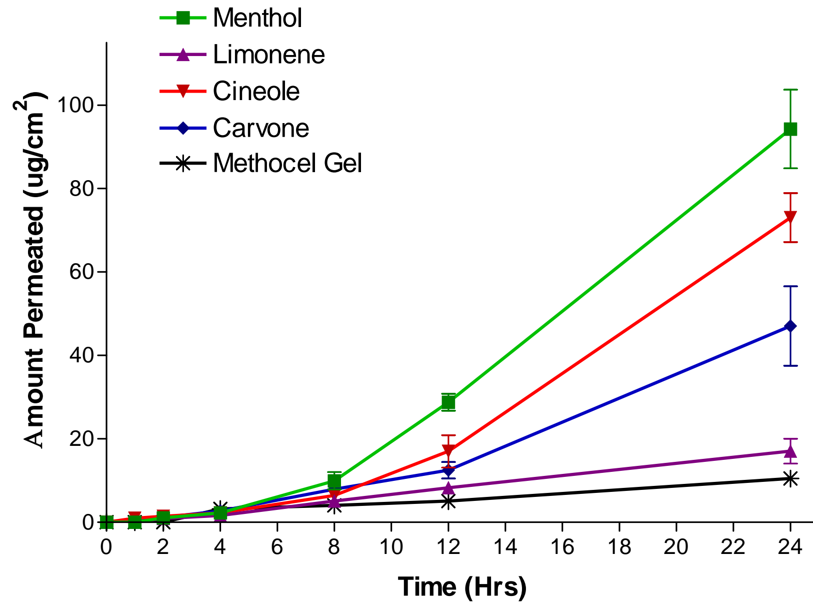


Figure 3.13: Release profile of genistein with terpenes

Bar represents Mean ± S.D. (n=3)

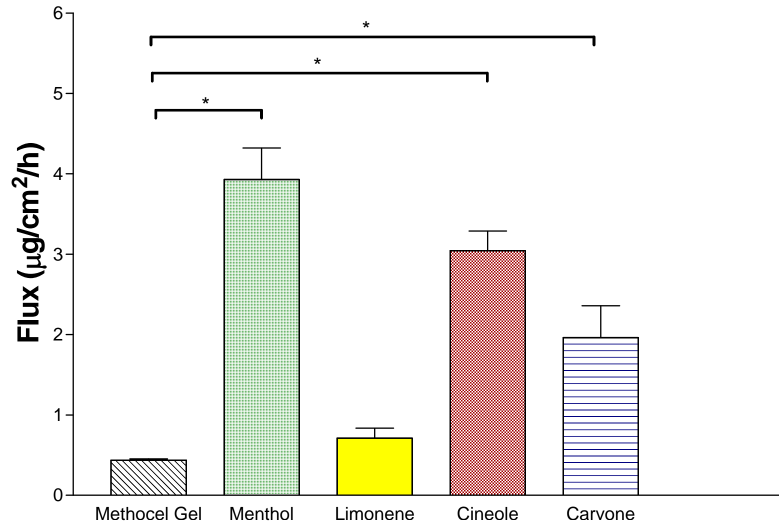


Figure 3.14: Flux of genistein with terpenes

Bar represents Mean ± S.D. (n=3)

* $p < 0.05$ Tukey's multiple comparison test. ($P < 0.0001$)

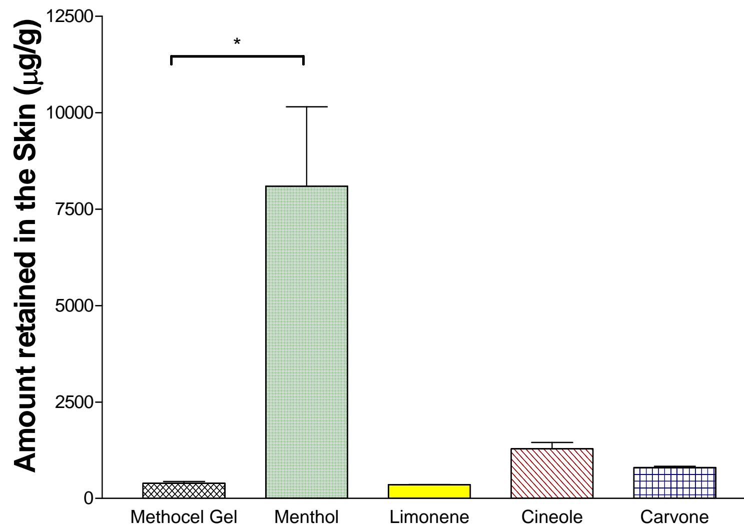


Figure 3.15: Retention of genistein in the skin with terpenes

Bar represents Mean ± S.D. (n=3)

*$p < 0.05$ Tukey's multiple comparison test ($P = 0.0006$)

Menthol, cineole and carvone showed a significant increase ($p<0.05$) in the permeation of genistein, as compared to the Methocel gel without the terpenes as penetration enhancer. However, only menthol showed a significant increase in the skin retention of genistein.

For all the formulations containing terpenes, the mean solubility of genistein was similar to that in the base formulation of Methocel gel. Only Carvone showed a higher solubility (1.5 fold) as compared to the base formulation (Table 3.05).

Menthol showed a 9 fold increase in the flux and a 22 fold increase in the retention of genistein by the skin after 24 hours of application (Figures 3.13, 3.14 and 3.15). Menthol does not significantly affect the solubility of genistein in Methocel® E4M, but has a 9 times higher permeability constant (Table 3.05). Thus, menthol is increasing the genistein flux through an effect on the skin permeability. It was suggested that menthol disrupts the bilayer of the stratum corneum and forms pools in it (Sinha and Kaur 2000; Liu et al. 2006). This suggestion correlates with the current study findings as both retention and flux will be increased if the lipid bilayer is disrupted. Menthol disrupts the hydrogen bond network in the head of ceramides in the lipid bilayer, as determined by differential scanning calorimetry (Jain et al. 2002; Zhao and Singh 1998, 1999). Menthol has also been reported to be a better penetration enhancer than other enhancers such as terpineol, menthone, pulgeone and Carvone for imipramine HCl (Jain et al. 2002), propranolol (Kunta et al. 1997) and indomethacin (Fuji et al. 2004). Menthol (2-5%) shows a synergistic effect when it is formulated with around 40% ethanol (Sapra et al. 2008) and such an enhancement was seen in this study. Cineole and carvone showed an

approximately 7 and 4 fold increase in flux, respectively, but the skin retention of genistein showed only an insignificant increase. These also have a higher permeability constant, although their mean solubilities are similar, as compared to the Methocel® E4M base formulation (Table 3.05). This suggests that the limonene, cineole and carvone interact with skin to cause the observed increase in the genistein flux. These interactions can be through the disruption of the highly ordered lipid structure of the stratum corneum, or by increased drug diffusivity in the stratum corneum, or by increased drug partitioning into the stratum corneum (Aqil et al. 2007). Of the four terpenes examined, only limonene did not significantly affect the flux of genistein through the skin.

Various glycol derivatives are known to show a permeation enhancement effect. When these are used together, they tend to be synergistic in their effect.

Table 3.06: Permeation parameters of various glycol derivatives

*$p < 0.05$ Tukey's multiple comparison test ($P < 0.0001$)

Formulation	Flux ($\mu g/cm^2/h$)	Mean Solubility ($\mu g/mL$)	Permeability Constant * 10^{-4} (cm/h)
Methocel® E4M	0.41 ± 0.05 *	820.10 ± 124.68 *	4.99 ± 0.74 *
Transcutol P	1.15 ± 0.07 *	130.12 ± 35.09 *	88.37 ± 21.59 *
Lauroglycol® 90	3.27 ± 0.08 *	537.82 ± 68.01 *	60.98 ± 7.35 *
Labrasol®	1.38 ± 0.27 *	232.38 ± 25.38 *	43.41 ± 4.51 *
PEG 400	0.52 ± 0.14	122.06 ± 12.94 *	59.38 ± 6.69 *
Capryol® PGMC	0.31 ± 0.04	142.08 ± 12.36 *	21.11 ± 1.88 *
Labrafil® M 1944 CS	0.16 ± 0.05	101.72 ± 18.18*	15.72 ± 3.13 *

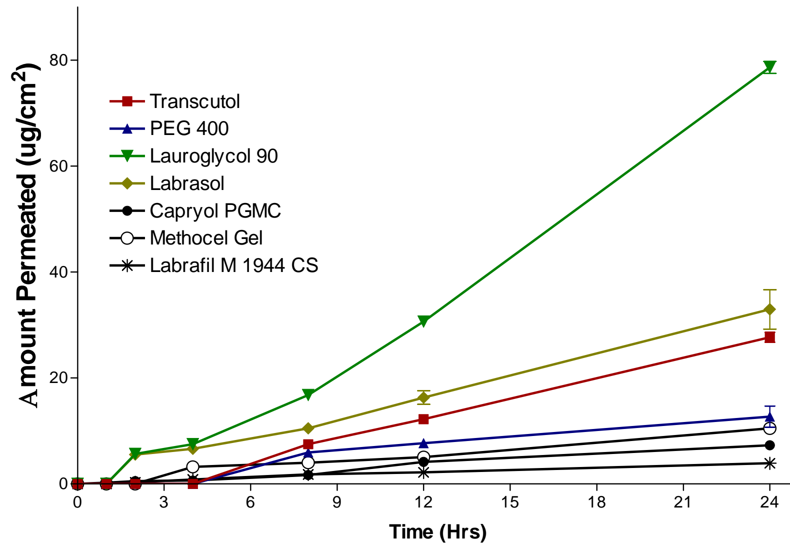


Figure 3.16: Release profile of genistein with glycol derivatives

Bar represents Mean ± S.D. (n=3)

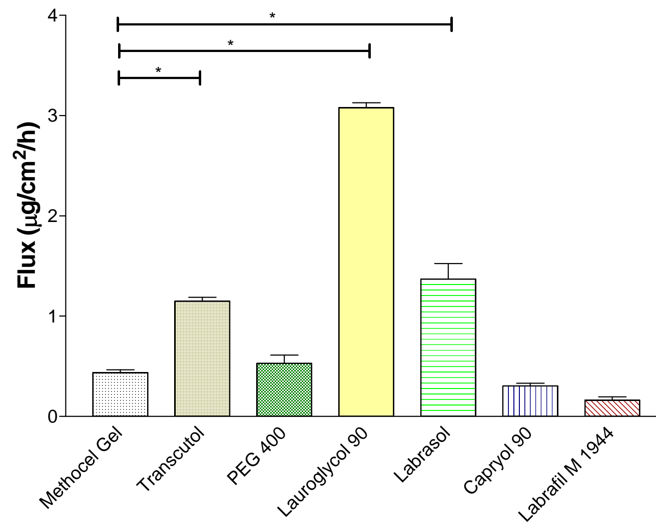


Figure 3.17: Flux of genistein with glycol derivatives

Bar represents Mean ± S.D. (n=3)

*$p < 0.05$ Tukey's multiple comparison test (P <0.0001)

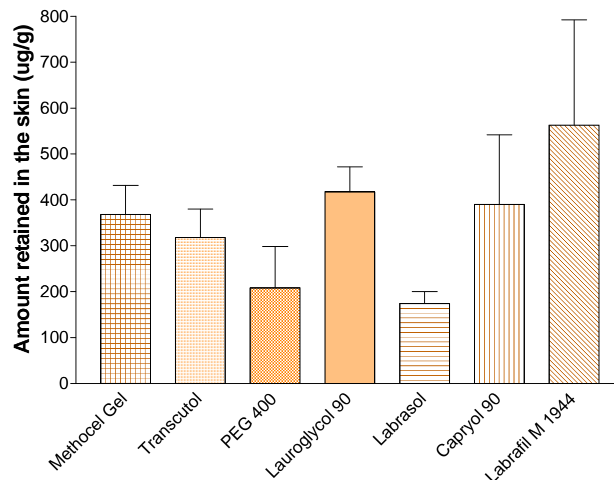


Figure 3.18: Retention of genistein in the skin with glycol derivatives
Bar represents Mean ± S.D. (n=3)

All the glycol derivatives used in the current study are known to be good solubilizers for several drugs. Since the final formulations require genistein to be in the dispersed state as fine particles, the concentration and ratio of ethanol and the glycol derivatives in the various formulations was altered as compared to the base Methocel gel formulation. Unlike the base formulation, where ethanol was 50% w/w, in the glycol derivative formulations ethanol was reduced to 0.50% w/w. Such a reduction in ethanol concentration provided the opportunity to incorporate a higher percentage of the penetration enhancer along with keeping the gel formulation as a fine particle dispersion.

It was observed that the mean solubility of genistein in the glycol formulations was much lower compared to the Methocel gel formulation (Table 3.06). For Transcutol® P, Lauroglycol® 90, Capryol® PGMC and Labrafil® M 1944 CS this decrease in solubility was by 6-8 fold, while in the case of PEG 400 and Labrasol the decrease in solubility was

3.5 fold and 1.5 fold, respectively. Obviously, this decrease in solubility greatly affected the flux of genistein.

Transcutol® has been used as a penetration enhancer and a solubilizer in varying concentrations. It enhanced the permeation of genistein and diadzein (Minghetti et al. 2007), clorazepam and lorazepam (Puglia and Bonina 2008), and bromocriptine (Degim et al. 2003). It was also suggested that a combination of glycol derivatives provided a synergistic effect (Baboota et al. 2007; Shakeel et al. 2007; Ritschel et al. 2008). For this reason, Transcutol® was mixed with other glycol derivatives in some of the formulations.

As compared to the base formulation, not all glycol derivatives were capable of increasing the flux and none increased the skin retention significantly. Transcutol® increased the flux of genistein 2 fold, but had a similar skin retention as compared to the base formulation (Figure 3.16, 3.17 and3.18). Genistein solubility in the Transcutol® gel was much lower than that in the base formulation, but had an almost 18 times higher permeability constant. This suggests the ability of Transcutol® to partition the drug into the skin and increase its penetration.

Transcutol® showed a relatively higher permeability coefficient for diclofenac, as compared to other enhancers (Minghetti et al. 2007). Transcutol® is a powerful solubilizing agent used in several dosage forms and is very attractive as a penetration enhancer due to it being non-toxic, biocompatibility with the skin, and its miscibility with polar and non-polar solvents (Watkinson et al. 1991). Transcutol® is assumed to increase skin penetration by one or more of the following mechanisms: by increasing the drug solubility in the formulations, formation of a drug depot in the skin, and by strong

interactions with the barrier structure of the stratum corneum (Watkinson et al. 1991). However, compared to the Methocel® formulation with 50% w/w ethanol, the only effect observed was an interaction with the barrier structure of the stratum corneum.

When Transcutol® was formulated with Lauroglycol® 90, there was a 6 fold increase in the flux of genistein, while the retention increased insignificantly. Lauroglycol® 90 is known as a solubilizer, but shows a lower solubility of genistein than the base formulation containing 50% w/w ethanol. Like Transcutol®, Lauroglycol® 90 has a higher permeability constant, suggesting its ability to partition the drug into the skin more than the Methocel® base formulation leading to greater flux. Like Lauroglycol® 90, Capryol® PGMC and Labrafil M® 1944 CS are also derivatives of propylene glycol and have a similar solubility like that of the base formulation around 800 µl/mL), for genistein. But, unlike Lauroglycol® 90, these showed no change in the flux compared to the Methocel® base formulation.

Finally, the formulation with PEG 400 and Labrasol showed no significant change in the flux or retention for genistein when compared to the base formulation. The main reason for this may be that the ethanol in the base formulation acts as a better penetration enhancer (Liu et al. 2006). Although PEG 400 and Labrasol are known to be good solubilizers, the solubility of genistein is less in these gels. In spite of the lower solubility of genistein, the permeability constant for PEG 400 and Labrasol was significantly higher by 12 and 9 fold, compared to the Methocel gel. This suggests that the flux of genistein may be lesser due to the lower total concentration of the dissolved drug in the individual formulations.

IV. CONCLUSIONS

The super saturated state and higher thermodynamic activity of genistein in Methocel gel provided enhanced skin permeation and retention of the drug. This augmented the idea for topical gel based delivery. Based on the amount of drug retained and permeated, several enhancers like Transcutol® along with Capryol® PGMC and Labrafil® M 1944 CS, menthol and Labrasol® have been identified as permeation enhancers for topical delivery. These formulations would help to localize the drug into the stratum corneum and epidermis of the skin, where the drug would provide its therapeutic effect as a chemoprotective and antioxidant. Regular application of such a gel based formulation would go a long way in preventing melanoma and wrinkles on the skin.

It may be possible to achieve better skin permeation and retention if terpenes and glycols are combined for synergistic effect. Combination of menthol and Transcutol®, with Capryol® PGMC and Labrafil® M 1944 CS may act as relatively better delivery formulations. But we should identify any significant interactions between different classes of the penetration enhancers, which may either affect the stability or skin permeation of the gel formulation. Similarly, future studies on cellular effects of genistein, in vitro, should be undertaken with critical considerations of genistein dose, and with careful assessment and reporting of long-term cell survival at the doses chosen.

Future research is directed towards studying the chemo preventive effects of genistein formulations in a mouse model of melanoma.

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