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Research paper

Reversible effects of permeation enhancers on human skin

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Abstract

This study outlines a systematic approach for investigating a desired characteristic of chemicals used to facilitate the permeation of drugs across the skin that is, the reversibility of the permeation enhancement effect. This implies that the vital skin barrier function is restored and not permanently impaired after the application of these enhancers. The reversible effects of two terpene enhancers, (*R*)-(−)-carvone and eucarvone, on excised human skin were evaluated by *in vitro* permeation and extraction studies on normal (untreated) and enhancer-pretreated epidermis, respectively. For the permeation studies on normal epidermis, the donor solutions were the model drug, haloperidol (HP, 3 mg/ml), in propylene glycol (PG) with or without 5% (w/v) enhancer and for the extraction studies using epidermis pretreated with enhancer, a solution of HP (3 mg/ml) in PG was used. The solubilities of the enhancers in 0.03% lactic acid (receptor solution) and of HP in PG (donor solution) were determined to demonstrate that the sink and saturated conditions were maintained in the respective compartments of the flow-through cells throughout the *in vitro* experiments. (*R*)-(−)-Carvone cleared out of the skin faster than eucarvone. This could be due to the 4-fold higher skin permeability of (*R*)-(−)-carvone compared to that of eucarvone. The amount of HP deposited in the epidermis was much lower in the eucarvone-pretreated epidermis than that pretreated with (*R*)-(−)-carvone. The permeation profile of HP across the enhancer-pretreated skin was 4-fold greater than in the vehicle alone (control), but similar to that across untreated skin with enhancer present in the donor solution, indicating that permeation across the enhancer-pretreated skin did not change. The enhancing effects of both terpenes on the skin were found to be reversible and the permeability of the skin was left intact after the passage of the drug in the vehicle with these enhancers.

Keywords: Skin permeation; Chemical enhancer; Reversibility; Transdermal drug delivery; Haloperidol; Terpene

1. Introduction

An ideal skin penetration enhancer is effective, nonirritating, and reversible [1–3]. As stratum corneum (SC) regeneration takes 25–30 days, the loss of barrier function will persist [4]. Therefore, the effect of chemicals, in particular enhancers, on the skin is important. Some enhancers cause permanent epidermal damage that can only be repaired by SC regeneration [5–7]. On the other hand, the increased permeability of SC can return to its normal state when other enhancers are used and then removed. This temporary effect is attributed to the transient interactions between the enhancers and SC, mainly the SC lipids, which is the major diffusion passage of most small chemicals [8].

Terpenes and terpenoids are suitable skin penetration enhancers with low toxicity and irritancy [9]. They are components of essential oils, defined and classified by the 'isoprene rule' [10,11]. Some are designated as generally recognized as safe (GRAS) by FDA [12]. These chemicals have been widely used in perfumery and other cosmetics. A number of monoterpenes were reported to enhance the permeation of various drugs in transdermal drug delivery [13]. Carvone and eucarvone are ketone monoterpene

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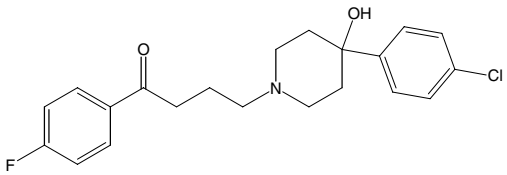
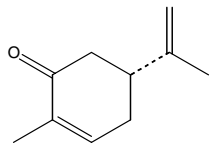
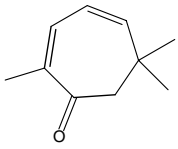
and terpenoid, respectively (Fig. 1). The hexagonal-ring carvone can be converted to heptagonal-ring eucarvone through simple chemical process [14]. Carvone has two enantiomers, of which the (*R*)-form smells of spearmint and the (*S*)-form smells of caraway seeds [15]. The (*S*)-carvone is a skin-irritant, so the (*R*)-form is a better candidate as a skin permeation enhancer [16]. Carvone is an important flavoring agent that is widely used in chewing gum, toothpaste, toiletries, food, drinks and other products [17]. It has been reported that carvone can enhance the skin permeation of 5-fluorouracil, tamoxifen and zidovudine [9,18,19]. Eucarvone is found in sugar mango, spearmint leaf, blackcurrant buds, Zieria and some Chinese herbal medicinal plants like Asari Herba and Asiasari Radix [15,20–22]. Asari Herba was reported to be used as a skin penetration enhancer for administration of buprenorphine [23]. The aim of this study is to investigate the reversibility of their enhancing effects on excised human skin with *in vitro* permeation methods. Propylene glycol (PG), a common solvent in cosmetics and pharmaceuticals, is used as the diffusion vehicle to dissolve the model drug, haloperidol (HP) [24]. HP is a hydrophobic molecule with low molecular weight (Fig. 1). As an anti-psychotic drug, its daily maintenance dose is 3–10 mg. The only available long-acting formulation is its ester for intramuscular injection, the haloperidol decanoate, which, however, has some disadvantages such as injection pain, marked inter-individual variations and complex administration regime [25,26]. A transdermal formulation of haloperidol could provide an alternative solution.



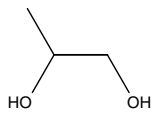
Haloperidol [52-86-8] (MW = 375.86, Log P = 4.06)



(R)- (-)-Carvone [6485-40-1] (MW = 150.22, Log P = 2.27)



Eucarvone [503-93-5] (MW = 150.22, Log P = 2.21)



Propylene glycol [57-55-6] (MW = 76.09, Log P = -1.34)

Fig. 1. The molecular structures of haloperidol, (*R*)-(−)-carvone, eucarvone and PG. The log *P* values were from SciFinder Scholar®, calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67 (© 1994–2005 ACD/Labs).

2. Materials and methods

2.1. Materials

Haloperidol, (*R*)-(−)-carvone (98%), DL-lactic acid, antibiotic antimycotic solution (100×), PG (99.9%) and sodium di-hydrogen phosphate monohydrate were purchased from Sigma–Aldrich (Germany). Eucarvone (96.6%) was obtained from TCI (Japan). All other chemical reagents were of at least reagent grade and all materials were used as supplied. The molecular structures of the chemicals are shown in Fig. 1. Water purified by the Milli-Q system was used.

2.2. Solubility study

2.2.1. Haloperidol in PG

Excess of HP was added to PG solution with or without enhancers in an amber bottle and stirred for 72 h at 37 °C in a magnetic heater-stirrer (PermeGear, US). These samples were filtered through 0.2 μm Teflon membrane filter units (Waters, UK) using a gas-tight syringe. Saturated drug concentrations were determined by HPLC assay in triplicate after appropriate dilution with 0.03% (v/v) lactic acid solution.

2.2.2. Enhancers in 0.03% (v/v) lactic acid solution

The enhancer was added to 1 ml of 0.03% (v/v) lactic acid in plastic cuvettes. Continuous stirring was performed for 72 h at 37 °C on a heater-stirrer (PermeGear, US). The solution was then centrifuged at 2000 rpm for 5 min and the aqueous phase was carefully withdrawn using a 1.2 × 38 mm metal needle attached to a gas-tight syringe. The solution was diluted appropriately with 0.03% (v/v) lactic acid before being subjected to HPLC assay.

2.3. Analytical method

2.3.1. Haloperidol

Drug concentrations were determined by a reversed phase HPLC method (C_{18} column, Hewlett–Packard) [24]. A photodiode array (PDA) detector was used to obtain the chromatographs corresponding to the wavelengths ranging from 170 to 800 nm. Mobile phase consisted of 0.05 M phosphate buffer (pH adjusted to 3) and acetonitrile with a ratio of 50:50. Droperidol was used as an internal standard. Flow rate was 1.3 ml/min and injection volume was 100 μl. Retention times of the internal standard and drug were approximately 4.9 and 6.7 min at 254 nm, respectively. Mean peak area ratios of the drug

and internal standard in 0.03% (v/v) lactic acid were linearly related to the drug concentrations for the samples containing 20–1000 ng/ml ($r^2 = 0.9990$).

2.3.2. (R)-(−)-Carvone and eucarvone

The same HPLC method was used. Retention times of carvone and eucarvone were 5.1 and 5.6 min at 240 and 306 nm, respectively. External standard method shows the peak areas were linearly related to the enhancer concentrations in 0.03% (v/v) lactic acid ranging from 0.1 to 4 mg/ml ($r^2 = 0.9999$) for (*R*)-(−)-carvone and eucarvone, respectively.

2.4. Preparation of human epidermis

Abdominal skin was obtained from a Chinese female donor with informed consent after she underwent plastic surgery at the Singapore General Hospital, Singapore. Epidermis was prepared by immersing the whole skin in 60 °C water for 2 min, followed by careful removal of the epidermis from the connective tissues [27]. Samples were stored in plastic bags at −80 °C until use. Prior to permeation experiments, these membranes with the stratum corneum side facing up were floated over 0.9% (w/v) sodium chloride solution containing antibacterial antimycotic solution (1 in 100 dilution) at 22 ± 1 °C for 2 h to equilibrate.

2.5. In vitro permeation study using normal human epidermis

Flow-through type diffusion cells were used for permeation studies [28]. Human epidermis was mounted between donor and receptor compartments and excessive skin at the sides was trimmed off to minimize lateral diffusion. Stratum corneum surface orientated towards the donor compartment and the circular skin area for permeation was 0.785 cm^2. Since the solubility of HP in 0.03% (v/v) lactic acid solution is approximately 1 mg/ml [29], the receptor solution of 500 ml of 0.03% (v/v) lactic acid solution containing 1% (v/v) antibacterial antimycotic solution was placed in the reservoir bottle and allowed to flow through the receptor compartment at 0.75 ml/h. The pH of the receptor solution was approximately 3 but that did not affect the integrity of the epidermis [24,30]. Receptor solution was thoroughly degassed to prevent the formation of bubbles beneath the membrane. An antibacterial and antimycotic solution was added to the receptor solutions to maintain the integrity of the skin throughout the experiment and to minimize the microbial contamination in samples during analysis. HP (3 mg/ml) in PG solutions with 5% (w/v) of either enhancer and without enhancer (control) were prepared. The concentration of HP in PG with 5% (w/v) (*R*)-(−)-carvone is 2.43 mg/ml (Table 1). A 1-ml solution was added to the donor compartment and covered with Parafilm® to minimize the contamination of the solution. Ambient temperature of the cells was controlled at 37 °C by a heater/circulator (Haake, Germany). The receptor solution is pumped by a 16-channel peristaltic cassette pump (Ismatec, Switzerland) continuously through the receptor compartment and drained into sample bottles in the fraction collector (ISCO Retriever IV, US). Cumulated receptor liquid samples were taken at 6-h intervals for HPLC assay.

Table 1
Solubility study of HP in PG and enhancers in 0.03% (v/v) lactic acid at 37 °C

Solute and solvent	Concentration (mg/ml)
HP in PG	3.08 ± 0.280
HP in PG with 5% (w/v) (*R*)-(−)-carvone	2.43 ± 0.185[a]
HP in PG with 5% (w/v) eucarvone	5.47 ± 0.0189[a]
(*R*)-(−)-Carvone in 0.03% (v/v) lactic acid	0.729 ± 0.051[b]
Eucarvone in 0.03% (v/v) lactic acid	0.566 ± 0.0171

[a] One-way ANOVA, Tukey's method comparing to control, $p < 0.05$.
[b] Two-sample *t*-test comparing (*R*)-(−)-carvone with eucarvone, $p < 0.05$.

2.6. In vitro permeation study using pretreated human epidermis

Epidermis was cut into smaller pieces before treatment. An enhancer solution, 50 μl of 5% (w/v), or PG (as control) was applied onto the SC of the epidermis floating on 100 ml of 0.9% (w/v) sodium chloride solution containing 1% (v/v) antibacterial antimycotic solution (hydration solution). The epidermis was then kept at room temperature for 24 h. The skin samples were rinsed five times with fresh hydration solution to remove excess enhancers left on the skin surface prior to *in vitro* permeation study. The donor solution was 3 mg/ml HP in PG without enhancers. The rest of the permeation setup is as for the study on untreated epidermis.

2.7. Recovery of enhancer from epidermis using ethanol

Epidermis was soaked in hydration solution for 2 h, then dabbed dry before it was cut into round pieces of 1 cm in diameter and immersed in 100 μl of hydration solution in a glass bottle with the SC facing upwards. Enhancer solution, 10 μl of 5% (w/v), was applied onto the SC which was then kept in the refrigerator for 24 h. Three repeated extractions were performed on the prepared epidermis over a period of 1 h at refrigerator temperature. Three milliliters of ethanol was used in the first and second extractions followed by 4 ml in the final extraction. The resulting solutions were subjected to vortex for 20 s after each extraction before transfer into amber glass bottles. Solutions from the three extractions were then combined and mixed prior to HPLC analysis in triplicate. In the control study, the same protocol was executed without the epidermis. Some 67.92% (±6.76) and 74.42% (± 9.64) of (*R*)-(−)-carvone were recovered from the solutions with immersed epidermis and the control solutions, respectively ($p > 0.05$, two-sample *t*-test).

2.8. Extraction of the pretreated epidermis after 48-h permeation

The pretreated human epidermis was removed from the flow-through cells after subjecting it to 48-h *in vitro* permeation and trimmed to the exact area of permeation (0.782 cm^2). Skin was washed once with fresh hydration solution to remove any traces of enhancers and drug on the epidermal surface. Then the epidermis was floated with the SC facing upwards on 100 μl of hydration solution in glass bottles. Repeated extraction was done hourly over a period of 3 h at 50 °C with pure ethanol. Three milliliters of ethanol was used in the first and second extractions followed by 4 ml in the final extraction. The resulting solutions were subjected to vortex for 1 min after each extraction before transferring into amber glass bottles. Solutions from each series of extractions were combined and mixed before subjecting to HPLC assay.

2.9. Permeation parameters and nonlinear regression

The following nonlinear model was used to estimate the permeability coefficient K_p and the lag-time Lt, from which $K_p = K'D'$ and $Lt = 1/(6D')$ [31,32].

$$Q = AK'C_0\left[D't - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\,e^{(-D'n^2t)}\right].$$

The parameters are Q, cumulative amount of permeated drug; A, the area of permeation; K' or D', the intermediate parameter defined by K, the partition coefficient between skin and donor solution, D, the diffusion coefficient and l, path length of diffusion ($K' = Kl$ and $D' = D/l^2$, respectively); C_0, the concentration of the drug; time t. Nonlinear regression analysis was carried out with the statistical software, JMP®. The K_p was estimated with two different methods. One of the methods discards the statistical errors from the nonlinear regression analysis [32]. The other takes all the errors generated from the nonlinear regression analysis [33,34]. The two sets of estimates are shown in Table 2.

3. Results

The solubility results are shown in Table 1. With the addition of 5% (w/v) (*R*)-(−)-carvone, the solubility of HP in PG dropped from 3.08 to 2.43 mg/ml and when 5% (w/v) eucarvone was added, it increased to 5.47 mg/ml. Eucarvone should have greater permeation enhancing potential than (*R*)-(−)-carvone. For the enhancers in 0.03% (v/v) lactic acid, (*R*)-(−)-carvone has a higher

Table 2
The point estimates (means ± SD) of K' and D' obtained from the nonlinear regression, and their 90% confidence intervals

Enhancer groups	K'	D'	K_p (cm/h) * 10^4
HP without enhancer	0.0128 ± 0.00404 0.00656, 0.0246	0.00932 ± 0.00175 0.00651, 0.0143	1.12 ± 0.185 0.546, 2.70 1.19 ± 0.199
HP with 5% w/v (*R*)-(−)-carvone	0.0863 ± 0.0533 0.0356, 0.225	0.00519 ± 0.00144 0.00353, 0.00806	3.72 ± 1.88 1.26, 18.1 4.77 ± 0.896[a]
HP with 5% w/v eucarvone	0.0471 ± 0.00484 0.0381, 0.0582	0.00997 ± 0.000627 0.00879, 0.0114	4.67 ± 0.196 3.34, 6.63 4.71 ± 0.544[a]
(*R*)-(−)-Carvone 5% w/v in PG (50 mg/ml)	0.0591 ± 0.0267 0.0192, 0.141	0.00811 ± 0.00233 0.00494, 0.0152	3.59 ± 1.31 1.58, 7.83 4.40 ± 0.217[a]
Eucarvone 5% w/v in PG (50 mg/ml)	0.0247 ± 0.0064 0.0153, 0.038	0.00387 ± 0.00038 0.00312, 0.0046	0.932 ± 0.158 0.732, 1.24 0.966 ± 0.121
HP without enhancer Skin pretreated with PG	0.0134 ± 0.0102 0.00522, 0.0301	0.0127 ± 0.0065 0.00775, 0.0257	1.04 ± 1.03 0.404, 7.74 1.71 ± 0.729
HP without enhancer Skin pretreated with carvone	0.0187 ± 0.0154 0.00775, 0.0462	0.0076 ± 0.00339 0.00489, 0.0119	0.902 ± 0.916 0.379, 5.96 1.44 ± 0.614
HP without enhancer Skin pretreated with eucarvone	0.0142 ± 0.00175 0.0108, 0.0185	0.01 ± 0.000757 0.00853, 0.0119	1.41 ± 0.0716 0.921, 2.20 1.42 ± 0.262

The point estimate (mean ± SD) of permeability coefficient and its 90% confidence interval, given by $K_p = K'D'$. For the column K_p, each cell contains three estimates, of which the first and second are the point and interval estimates from pooled data ($n = 24$) with estimation errors generated by the nonlinear regression, respectively, and the third is the point estimate from individual data set ($n = 8$) discarding the estimation errors generated by the nonlinear regression.

[a] One-way ANOVA, Tukey's method comparing all the pairs, $p < 0.05$.

solubility of 0.729 mg/ml than that of eucarvone at 0.566 mg/ml. Both enhancers have relatively lower solubility compared to HP with a solubility of approximately 1 mg/ml in 0.03% (v/v) lactic acid.

The permeation profiles of both drugs and enhancers, from permeation studies using either normal or pretreated epidermis, are shown in Fig. 2. For the permeation study using pretreated epidermis, the permeation profile of eucarvone was obtained but that of (*R*)-(−)-carvone was not, since (*R*)-(−)-carvone was washed out prior to the permeation. The permeation profile of eucarvone was distinctly different from other exponential curves because, for all the others, the donor concentration was constant but, for eucarvone, its amount was finite in the epidermis. The estimated values and confidence intervals of the permeability coefficient of K_p are given in Table 2, except for the finite-dosed eucarvone. It was demonstrated that the K_p of HP with 5% w/v (*R*)-(−)-carvone, HP with 5% w/v eucarvone, and (*R*)-(−)-carvone 5% w/v in PG (50 mg/ml) were comparable to one another but significantly larger than the rest. (One-way ANOVA and Tukey's method comparing all pairs, $p < 0.05$.)

In the extraction study, 3.82 ± 0.0521 μg of eucarvone was extracted from the eucarvone-treated epidermis but no (*R*)-(−)-carvone was extracted from the (*R*)-(−)-carvone-treated epidermis after the 48-h permeation period. HP of 295.27 ± 60.62, 242.45 ± 31.98 and 48.94 ± 13.90 μg was extracted from the control, (*R*)-(−)-carvone-pretreated and eucarvone-pretreated epidermis, respectively.

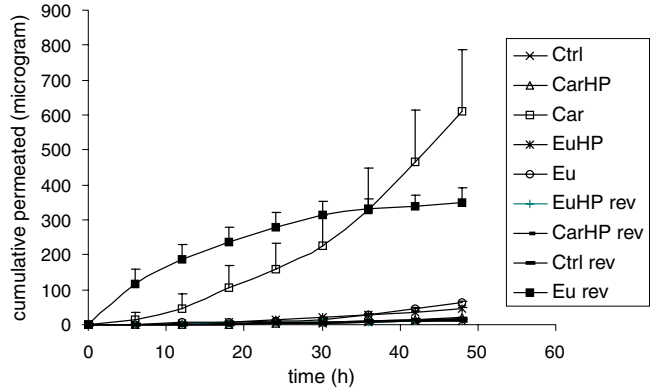


Fig. 2. Time course of mean cumulative amounts of HP permeated through 0.786 cm^2 of human epidermal membrane in the PG solutions. Each point represents mean value ($n = 3$). In the study using normal epidermis, three permeation experiments with different donor solutions gave five permeation curves: (a) the control of which HP (3 mg/ml) was in pure PG gave the permeation profile of HP (Ctrl), (b) HP (3 mg/ml) in PG with 5% (w/v) of eucarvone solution gave the permeation profiles of HP (EuHP) and eucarvone (Eu), and (c) HP (2.43 mg/ml) in PG with 5% (w/v) of (*R*)-(−)-carvone gave the permeation profiles of HP (CarHP) and (*R*)-(−)-carvone (Car). In the study using pretreated epidermis, the three permeation experiments using the same donor solutions (HP in PG, 3 mg/ml, w/v) gave four permeation curves: (a) the epidermis treated with pure PG gave the permeation profile of HP (Ctrl rev), (b) the study with eucarvone solution (5%, w/v)-pretreated epidermis gave the permeation profiles of HP (EuHP rev) and eucarvone (Eu rev), and (c) the study with (*R*)-(−)-carvone (5%, w/v)-pretreated epidermis gave the permeation profile of HP (CarHP rev).

4. Discussion

In the study using normal epidermis, three permeation experiments with different donor solutions gave five permeation curves (Fig. 2). Both enhancers can enhance the permeation of HP to similar level, about 4-fold higher than the control (Table 2). The permeability coefficient of (*R*)-(−)-carvone was also four times greater than that of eucarvone. It appears that the permeation of HP was independent of enhancer's permeability through skin. In the study using pretreated epidermis, three permeation experiments using the same donor solutions (HP 3 mg/ml, w/v in PG) yielded four permeation curves (Fig. 2). For the three HP permeation results, their K_p values were not significantly different from one another, or different from the control resulting from the untreated epidermis (one-way ANOVA, $p > 0.05$).

Combined with the results from skin extraction study, it was found that only about 1% (w/w) of the trapped eucarvone remained in the epidermis after the 48-h permeation, which showed that eucarvone could have been washed out by PG. Unlike eucarvone, (*R*)-(−)-carvone was not detected in the receptor solution or in the epidermis under the same conditions. Most of the (*R*)-(−)-carvone within epidermis was probably rinsed off by the washing solution prior to the permeation study. This is consistent with the findings from the solubility study, which showed that the solubility of eucarvone was lower than (*R*)-(−)-carvone in 0.03% lactic acid and eucarvone permeated much slower than (*R*)-(−)-carvone (Table 2).

Comparing the drug permeation from the two permeation studies, it was found that when the enhancers were removed, the HP permeability coefficients returned to normal from a 4-fold increase in the presence of enhancers. In addition, results from the permeation study with pretreated epidermis also showed that the permeability coefficients of HP were comparable among all groups. Therefore, the effects of both enhancers on skin permeability to HP were reversible.

The amounts of HP extracted from pretreated epidermis after 48-h permeation were 295.27 ± 60.62, 242.45 ± 31.98 and 48.94 ± 13.90 μg from the PG, (*R*)-(−)-carvone and eucarvone treatments, respectively. The eucarvone-pretreated epidermis retained much less HP than that treated with (*R*)-(−)-carvone or PG (one-way ANOVA, Tukey's method, $p < 0.05$). If all the (*R*)-(−)-carvone were rinsed off the epidermis prior to mounting it for the permeation study, then it should not have much influence on HP deposition in epidermis. But this is not the case as the eucarvone-pretreated epidermis retained only about 20% of the amount in PG-pretreated or 5% (w/v) (*R*)-(−)-carvone-pretreated epidermis. As the permeability coefficients of HP through the three types of pretreated skin were similar, the trapped eucarvone probably did not facilitate the permeation of HP and could have decreased the diffusion path length and partition coefficient proportionally.

To estimate K_p, two different methods were used. In the first method, all replicates were pooled for nonlinear

regression, which gave point and interval estimates. In the second method, each replicate was used as individual data set for nonlinear regression. Consequently, one point estimate was obtained from each replicate and the error term was dropped. These clean estimates, therefore, became the sampled K_p, subject to further statistical comparisons. In the first method K_p was estimated from the original permeation data but in the second method the quality of estimation was compromised because of the arbitrary omission of the error term. When the data variance is small, effective confidence interval can be obtained by the first method and pairwise comparisons can be conducted by comparing the variable's confidence intervals. But when the data variation is large the confidence intervals tend to be so wide that comparisons become too conservative. In this case, the second method was selected at the expense of reduced data quality.

The mechanism for this reversible enhancement could be attributed to the insertion of these enhancers within the SC intercellular lipid lamella [24,35]. The disruptions in the lipid lamella eased the permeation of the lipophilic drug through the tortuous pathway, hence resulting in enhancement of drug permeation [36]. Likewise, once the enhancers were removed, bonds between the lipids would start to reform and the depletion of the enhancers would allow the packing of the lipids to revert back to its original alignment.

5. Conclusion

From the results of this study, the permeability of the pretreated epidermis was comparable to that of the control, so the insult to the barrier function of the skin caused by the enhancers was restored. As an *in vitro* study was performed, the recovery of the epidermal barrier function would not be due to the cellular regeneration at the horny layer, thereby restoring its physical barrier.

(*R*)-(−)-Carvone yielded a much faster elution profile out of the epidermis than eucarvone. The results also showed that (*R*)-(−)-carvone, rather than eucarvone, retained more HP within the epidermis. This suggests that (*R*)-(−)-carvone could have a better potential as an enhancer for depot HP therapy than eucarvone. In conclusion, both (*R*)-(−)-carvone and eucarvone were shown to be effective and reversible enhancers for the *in vitro* permeation of HP through human epidermis.

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