Iranian Journal of Pharmaceutical Sciences
2020: 16 (2): 87-104
www.ijps.ir

Original Article

The Effect of Various Penetration Enhancers on the Octyl Methoxycinnamate Permeability: Mechanisms of Action Study

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Abstract

Octyl methoxycinnamate is one of the ingredients in sunscreen products. The main aim of this study was to investigate the effect of different enhancers of in vitro skin permeability of Octyl methoxycinnamate. Octyl methoxycinnamate permeability parameters were evaluated through the whole skin of the rat with and without chemical enhancers including eucalyptus oil, urea, menthol and olive oil by Franz cell diffusion. The effects of enhancers on skin structure were also studied using DSC and FT-IR techniques. The skin prevented the permeability of Octyl methoxycinnamate so that after 24 hours less than 3% of the substance passed through the skin. The results of this study showed that by increasing the time, it is possible to increase the skin permeation and the highest rate of skin absorption were corresponded to olive oil (ER_{flux}=63.074), eucalyptus oil (ER_{flux}=48.78) and menthol (ER_{flux}=33.5), respectively while the least amount of skin absorption was related to urea (ER_{flux}=29.53). Chemical penetration enhancers are substances that interfere with the complex structure of the skin and protein lipids. Two endothermic transitions were obtained at about 67 (Tm1) and 112 ° C (Tm2) in thermogram of the hydrated whole rat skin. Tm_1 and Tm_2 seems to be due to the melting of the lipids and the irreversible intracellular keratin or melting of the lipid-protein (keratin) complex, respectively. The amount of Tm1, ΔH1 and ΔH2 were decreased by all penetration enhancers compared to the hydrated skin. The FT-IR results suggested the mechanism of increasing absorption effect by lipid fluidization and lipid extraction. All of penetration enhancers used in this study significantly increased the skin permeability of Octyl Methoxycinnamate.

Keywords: Differential Scanning Calorimetry, FT-IR, Octyl methoxycinnamate, penetration enhancers, skin permeability.

1. Introduction

Ultraviolet radiation may be absorbed by sunscreens. The use of sunscreens has increased due to increased concerns about burns caused by the mentioned [1]. Octyl methoxycinnamate is a chemical compound

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Cite this article as: Salimi E, Moghimipour E, Kogani P, Mohammad Soleymani S, The Effect of Various Penetration Enhancers on the Octyl Methoxycinnamate Permeability: Mechanisms of Action Study, 2020, 16 (2): 87-104.

found in many types of cosmetics and personal care products (such as soaps, shampoos, and colognes). It is extracted from petroleum and is added to cosmetics, lotions and moisturizers by manufacturers [2]. It is often used as an active ingredient in sunscreen products in combination with oxybenzone and titanium oxide for protecting against UV-B rays [3]. The material has several advantages in terms of sun protection, also has some potential health risks for people who do not turn out well. However, this combination is widely used in sunscreen products [2]. Octyl methoxycinnamate has C18H23O3 chemical formula and molecular weight of 209.397 g / mol.

The skin is a protective coating layer that carries out various tasks. The main barrier for crossing the skin is the stratum corneum that is the outmost layer of the epidermis, and consists of 10 to 25 layers of dead cells in a lipid layer. Under the usual conditions, the main route of transport is the intercellular spaces or lipid bilayer [4,5]. The main limitation in the use of medications through the skin is the intolerance of the human body's epithelial to external material. Therefore, today the most important challenge for topical formulation is to provide an appropriate increase in the penetration of the drug into the skin so that no significant skin irritation is induced [6].

Two determinant factors in the passage of a drug from a membrane are the drug solubility in the carrier and the membrane and the drug's release in the membrane. The relative solubility and partition coefficient of the drug determines the distribution of the drug between the carrier and the stratum corneum. Also, the solubility of the drug in this category affects its distribution between the stratum corneum and the deeper layers of the skin [7]. The process of propagation indicates the ability of drug to across a membrane, influenced by several factors such as the viscosity of the environment, the strength of transplantation between the drug and skin, the porosity of the passageway, lipophilicity and molecular weight of the drug, and other factors [8]. The distribution of drug molecules between the carrier and the stratum corneum is dependent on various factors such as drug solubility in the carrier and the stratum corneum, the energy of converting solid particles into molecular form and the dissolution energy in the carrier and the stratum corneum [9]. There are several mechanisms to increase the permeability of the material into the skin including the use of absorption enhancing additives, the use of supersaturated drug delivery systems, the electrical conduction of molecules into or through the skin using iontophoresis, physical degeneration of the skin structure by electroporation or sonophoresis [10, 11, 12].

The study of lipids intercellular microstructure in the underlying skin is essential for the development of dermal delivery systems. Various techniques such as Differential Scanning Calorimetry (DSC) and Fourier Infrared Spectroscopy (FT-IR) are recently utilized to reveal the fat structure and microstructure of the skin. [9, 13]. FT-IR analysis of the skin can be used to evaluate the interaction between the enhancers and skin and provides the band in a number of different waves [14].

The FT-IR analysis of skin after exposure to enhancers is a practical way to study the interactions between the chemical enhancers and stratum corneum. These interactions are revealed by the formation of absorption bands at different wavelengths. The bands in FT-IR absorption spectra represent vibrations of lipid and protein molecules in the stratum corneum layer [15].Molecular vibration of lipids is a good indicator of the evaluation of lamellar lipid microstructures in the intercellular region of the stratum corneum. These vibrations include the Symmetric CH2- (near area 2850 cm^{-1}) and Asymmetric CH2- (near area 2920 cm^{-1}).

Also, tensile vibrations produce a portion of the stratum corneum protein includingAmid I (near area 1650 cm^{-1}) and Amide II (region near 1550 cm^{-1}).

Changes in the protein curvature of the stratum corneum cause changes in the frequency of amides I and II, and as a result of its displacement to frequencies greater or less. Amide I band (C=O stretching) shows secondary structure of the protein [16]. If the absorption band is transitted to higher wavelengths (blue shift), this indicates the fluidity of the bilayers in the hermetic membrane and the impairment of the barrier properties and possibly the increase in the passage of the drug from the stratum corneum. On the other hand, if the displacement of the absorption band toward the lower wavelength (red shift) is indicative of reordering lipid bilayer stratum corneum groups, which ultimately prevents the introduction of drug into the skin [17] . If the enhancer affects the lipid structure of the skin, lipid transfer phase transitions occur in the form of increasing or decreasing the signal wavelengths in the 2920 cm^{-1}, 2850 cm^{-1} and 1738 cm^{-1}. Since the height (or intensity) or position of the absorbing bands shows the amount of lipids or proteins in the stratum corneum, the increase in peak intensity, indicating the strengthening of the lipid structure and the effect of drug retardation, and the reduction of peak intensity, indicates a weakening of the lipid structure The lipid is in the stratum corneum, which increases the permeability of the drug [18].

The DSC method is widely used to determine lipid melting points, phase transition of lipid bilayers, and denaturation of proteins present in the stratum corneum [19]. This method examines the average phase transition temperature (Tm) and its enthalpy in the skin . The displacement of the phase transition temperature to lower temperatures indicates a lipid layer disorder and an irreversible denaturation of the protein structure. While the decrease in enthalpy indicates lipid

fluidization in lipid bilayers and lipid-protein complexes in the stratum corneum [20].

Therefore, the aim of the present study was to develop a topical delivery system of Octyl methoxycinnamate and to evaluate the effect of the penetration enhancers on the drug dermal permeability characteristics.

2. Materials and Methods

2.1. Materials

Octyl methoxycinnamate was purchased from ACT Cosmetics Company (Tehran, Iran). Eucalyptus oil and Menthol were obtained from Barij Essence (Kashan, Iran). Olive oil was purchased from the manufacturer of hygienic and cosmetic products, Sinuhe co. Urea, potassium phosphate monobasic and dibasic were purchased from Merck Company. The used water was deionized and filtered in-house.

2.2. Animal Experiments

Male Wistar rats (weighing 200-250 g) and 8-10 weeks' old were purchased from the Animal Laboratory of Ahvaz Jundishapur University of Medical Sciences. The rats were anesthetized with Ketamine and xylazine mixture before sacrificing [21]. After being sacrificed under the anesthesia, the skin of the abdominal skin was carefully removed with an electric clipper without damaging. Subcutaneous fat was removed from the skin and cooled using a 4° C cool acetone solution. The thickness of the sample skin was measured by a digital micrometer (AACO Company, France) [22] . Animals were treated according to the principles of care and use of laboratory animals and confirmed by the ethical committee (with Gp-96035 code) of Ahvaz Jundishapur University of Medical Sciences.

2.3. Octyl Methoxycinnamate Assay

The amount determination of Octyl methoxycinnamate was carried out by UV spectroscopy method at λmax= 211 nm [23].

2.4. In Vitro Skin Permeation Study

In-vitro permeation studies were performed using Franz diffusion cells with an effective contact area of approximately 3.46 cm^2. The volume of the receptor phase was 25 ml. Skin specimens were hydrated prior to use and then were placed between the donor and receptor compartments of the epidermis against the donor medium [24].

Octyl methoxycinnamate (1% w/v), dissolved in distilled water, was poured in donor compartment and the receptor portion was filled with methanol-phosphate buffer solution (pH=7)3:1. The diffusion cell was placed in a bath of water at 37 ± 0.05 ° C and derived from a small magnetic bead at 300 rpm. At specified intervals (0.5, 1, 2, ... , 8, 24 hours), a 2 ml of samples were removed from receptor compartment and immediately replaced with a fresh volume of methanol-phosphate buffer solution (pH=7, 3:1) to maintain sink condition [25, 26]. The samples were filtered and permeated Octyl methoxycinnamate was determined by UV spectroscopy at 211 nm.

2.5. FT-IR Spectroscopy

Skin samples were treated by Eucalyptus, Menthol, Urea and Olive oil for 4 hours, then dried (650 mm Hg, 25 ± 1 °C) for 1 h and finally stored in desiccators to remove any traces of enhancers. The skin samples were scanned from 500 to 4000 cm^{-1} and analyzed in the maintained range using an FT-IR facility [9, 27].

2.6. Differential Scanning Calorimetry (DSC)

The Mettler -Toledo DSC was used to examine the thermal behavior of skin samples. For this purpose, a small amount of specimens was weighed in aluminum pan, and then the doors were completely closed so that they did not exchange any outside with the environment. The DSC was performed in cooling mode [28]. In cooling mode, the samples were exposed to a temperature of 20-200 ° C and scan speed (5°C / min) [29]. To ensure the accuracy and repeatability of the data, the DSC analyzer was calibrated using the standard extraction [27].

2.7. Pretreatment of Skin Samples with Chemical Penetration Enhancers

Before exposure to penetration enhancers skin samples were completely hydrated, 2 ml of the enhancer solution was placed on the skin two hours prior to the experiments. The donor and receptor chambers were then washed with water and filled with Octyl methoxycinnamate and methanol-phosphate buffer (pH 7, 3:1). The effect of chemical enhancers on Octyl methoxycinnamate penetration through whole skin specimens was studied using eucalyptus oil, urea, menthol and olive oil as enhancers. The blank fully hydrated specimens were used as controls. To minimize biodiversity-based empirical errors, Also a piece of blank skin was used as control [30].

2.8. Permeation Data Analysis

The permeability of Octyl methoxycinnamate from the whole rat skin was studied by calculating parameters including steady state flux (J_{ss}), permeability coefficient (p), lag time (T_{lag}) and apparent diffusion coefficient (D_{app}).

Enhancement ratios (ER) were calculated from equation 1.

$$ER = \frac{\text{Permeability parameter after treatment}}{\text{Permeability parameter before treatment}} \text{ (Equation 1)}$$

The permeability coefficient (p) was calculated from equation 2 [31].

$$J_{SS} = P.C \text{ (Equation 2)}$$

Where Jss and C are steady state flux and initial concentration of octyl methoxycinnamate in receptor compartment, respectively. The D value was calculated from equation 3 [32].

$$D = \frac{h^2}{6Tlag} \text{ (Equation 3)}$$

Because h does not represent the actual length of the pathway, D parameter was calculated from equation 3. Since all calculations are based on the Steady State region, the cumulative flow rate of the drug has been measured.

3. Results and discussion

3.1. Effect of Enhancers on Permeation of Octyl Methoxycinnamate

The profile of Octyl methoxycinnamate permeability after exposure to each of the enhancers, in the mentioned intervals is shown in Figure 1. Permeability parameters after pretreatment with penetration enhancers in comparison to control are shown in Table 1. Table 2 shows the effect of penetration enhancers on permeation parameters for Octyl methoxycinnamate through whole skin. It was shown that the highest penetration rate of the drug was observed in the 4th hours of study as compared to the control group for all enhancers.

The results showed that all enhancers increased the permeability through the skin significantly compared to Octyl methoxycinnamate alone. Olive oil increased the permeability of Octyl methoxycinnamate to 63.074 times.

Eucalyptus oil contains 75% of 1, 8-cineol, which is a cyclic terpene. Several enhancing mechanism have been proposed for terpens including increased diffusion coefficient, drug solubility enhancement(i.e., increase in thermodynamic activity of the drug) improvement of, dispersion coefficient and fat extraction (e.g., disruption of the fatty protein range) [33]. Eucalyptus has an acceptable uptake of Octyl methoxycinnamate, with an increase of 48.78 times of drug permeability. The increase of penetration of menthol was 33.5 times higher than saturated solution.

Urea (a 1% urea aqueous solution) with an increase of 29.53 times the permeability of the drug, has the greatest effect on the Octyl methoxycinnamate penetration after 24 hours.

The two main steps are skin penetration, diffusion through stratum corneum, partitioning and release into acceptable epidermis, dermal passage and, ultimately, systemic absorption or penetration into deeper tissues.

Poor solubility of Octyl methoxycinnamate in water indicates a rate- limiting step for penetration through the whole skin rat. The results obtained indicate that the used enhancers increased the drug permeability through the excised rat skin

3.2. FT-IR Spectroscopy

Figure 2, Table 3, Table 4, and Table 5 show the analysis of the results of FT-IR samples with respect to any change in the position of the peaks as well as their severity from the intensity of 500 – 4000 cm^{-1}.

The FT-IR results from the enhancing effect of urea on the skin of the rat indicate that this enhancer has produced redshifts in absorption regions 2915.13 and 2840.7 cm^{-1}, which indicate that the lipid groups are redirected and ultimately weaken the stratum corneum strong properties. This enhancer has increased the wave number in the area (blue shift), which reflects the weakening of hydrogen bonds between lipid molecules. This enhancer eliminates absorption regions in wave numbers that interfere with stratum corneum keratin supplements. The results of DSC and FT-IR show the effect of more urea on the protein content of the skin.

The FT-IR results from the effect of Menthol on the skin of the rat show that it has an irreversible decrease in the absorption region 2915.13 cm^{-1} and also induces a red shift in region 2840.74 cm^{-1}, which indicates the reordering of lipid bilayer groups in the stratum corneum layer and eventually it may slow down the entry of the drug into the skin. It has also increased the absorption wavelength in the 1690.2 cm^{-1} region, indicating a weakening of the hydrogen bond between lipid molecules. The results indicate that the combination has increased the wavelengths of 1642.5 and 1535.9 cm^{-1} (blue shift), which indicates the effect of this enhancer on the stratum corneum skin layer keratin. The compound appears to have the most effect of increasing absorption on the transfer of drug to the skin through the effect on skin keratin.

The results obtained from FT-IR study showed that the absorption peak in wave of eucalyptus oil is removed in 2915.13 cm^{-1} and the absorption peak of the regions 2840.7 cm^{-1}m and 1690.2 cm^{-1} has a blue shift indicating that the stratum corneum lipid is fluidal and disturbance in the properties of the barrier and an increase in the passage of the drug. These results also indicate that eucalyptus has significantly reduced the height of the peaks (2915.13 cm^{-1} and 2840.72 cm^{-1}). Eucalyptus FT-IR results also indicate a red shift in the area of 1642.58 cm^{-1} and 1535.9 cm^{-1} in the blue shift. This decrease has been considerable in the courtyard height in this area, and may also interfere with the keratin in the stratum corneum. The FT-IR results of the effect of olive oil on the skin indicate that this enhancer has increased the absorbance wavelength in regions 2915.13, 2840.74 and 16.90.2 cm^{-1} (blue shift). However, the elevation of peak height in the first region has been shown to indicate the fluidity of the lipid layers of the stratum corneum and disturbance of the skin barrier properties. This enhancer also has removed absorption wavelengths in the area of 1643.5 and 1535.9 cm^{-1} and the peak height decreased by 100%, indicating the effect of eucalyptus on the skin protein segment, as well as the disruption of keratin in the dermal horny layer and the change in skin lipid structures of the rat skin. It seems to affect the lipid and the skin protein part.

3.3. Differential Scanning Calorimetry (DSC)

The DSC method is widely used to characterization of lipid melting points, phase transition of lipid bilayers, and protein denaturation in SC layer [20].

This method investigates the pre-invasive Thermogenic behavior of various proximal adsorbents in terms of average phase transition temperature (Tm) and their enthalpy. To obtain more detailed information about lipid components and protein conformational stability of the whole skin rat treated with enhancer, a DSC study was programmed. By comparing mean transition temperature (Tm) and enthalpies (H), thermotropic behavior of treated skin was assessed. Any transition in Tm to lower degrees may be due to lipid disruption in bilayer and irreversible protein denaturation in SC [20].

Kaushik et al. reported human dermal DSC graphs and observed three endothermic transition peaks at temperatures 59-63°C (Tm1), 75-82°C (Tm2), and 99.5-120°C (Tm3). They suggested that Tm_1 was due to transformation of lipid forms from a lamellar to disordered state, Tm_2 corresponds to protein-lipid or the disruption of polar head groups of lipids and Tm3 is known to occur during the proteins irreversible denaturation, respectively .But a low temperature transition peak (35-40 ° C) has not been observed due to the drying of skin samples prior to DSC testing [33].

In this study, skin rats were contacted by various absorbing adsorbents, in which the transition temperature of the phase in the amount of variation (ΔH) corresponding to each absorption is given in Table 6.

The DSC thermograms resulting from the effect of water absorption on the entire full thickness rat skin are shown in Figure 3.

The thermograms of skin pretreated with menthol are presented lower Tm_1 and ΔH_1, ΔH_2 decreased in comparison with hydrated rat skin.

This finding indicates that menthol induced structural changing, increased lipid fluidity in intercellular region, lipid disruption in bilayer and irreversible protein denaturation in SC. The DSC results obtained from skin pretreated with eucalyptus oil shows lower Tm_1, and ΔH_1, ΔH_2 decreased in comparison with hydrated rat skin. This finding indicates that eucalyptus oil can increase skin permeation by more of mechanisms i.e.; lipid disruption in bilayer and irreversible protein denaturation in SC layer.

The thermograms of skin pretreated with urea indicated $Tm_1$and Tm_2 shifted to lower melting points and ΔH_1, ΔH_2 decreased compared with control. These findings show lipid- protein complex fluidity in SC layer.

The thermograms of skin pretreated with olive oil are presented lower Tm_1 and ΔH_1, ΔH_2 decreased in comparison with hydrated rat skin and serve as menthol.

4. Conclusion

According to the results, it is clear that all chemical enhancers significantly increased Octyl methoxycinnamate penetration through skin compared skin samples to control. Bilayer lipid membrane disorder and lipid fluidity in bilayer and also in lipid - protein are probably the main cause of increased permeability. It is suggested that, the combination of Olive oil, Eucalyptus oil, Menthol and Urea in cosmetic products to enhance the drug transdermal permeation.

Acknowledgements

This article is derived from the pharm D thesis of Payam Kogani and Ahvaz Jundishapur University of Medical Sciences is acknowledged for providing financial support (NO. 1110).

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Tables:

Table 1. Permeability parameters after pretreatment with penetration enhancers compared to control. (Mean ± SD, n = 3).

	Flux ($mg.cm^{-2}.h^{-1}$)	D ($cm^2.h^{-1}$)	T_{lag} (h)	P(cm/h)
Control (Water)	0.0004±0.00004	0.115±0.010	2.287±0.31	0.00002±0.000001
Eucalyptus Oil	0.0134±0.005	0.102±0.015	2.883±0.36	0.00133±0.0005
Olive oil	0.0176±0.003	0.771±0.055	0.168±0.105	0.00175±0.0002
Menthol	0.0114± 0.007	0.118±0.034	0.504±0.070	0.00114±0.0007
Urea	0.0092±0.003	0.104±0.018	1.804±0.43	0.00092±0.0001

Table 2. Effect of penetration enhancers on permeation parameters for Octyl methoxycinnamate through whole skin rat. (Mean ± SD, n = 3).

	ER_{flux}	ER_D	ER_p
Eucalyptus Oil	48.78± 8.92	10.24± 2.57	66.67± 6.07
Olive oil	63.074±8.34	45.109± 3.26	87.83±4.153
Menthol	33.5± 2.52	5.49± 1.130	56.83± 4.57
Urea	29.53± 3.63	4.64± 1.609	46± 3.402

***ER_{flux}** = ratio of flux after and before treatment with enhancer; **ER_D** = ratio of diffusion coefficient after and before treatment with enhancer.*

Table 3. Decrease in mean peak height, compared with control of asymmetric and symmetric C-H stretching and C=O stretching absorbance of abdominal hydrated whole skin rat following treatment with different enhancers. (Mean ± SD, n = 3).

	Asymmetric C-H stretching		Symmetric C-H stretching		C=O stretching of lipid ester	
	Peak height	D%	Peak height	D%	Peak height	D%
Control	0.395	_	0.242	_	0.015	_
Eucalyptus	0	100	0.001	99.58	0.038	Not seen
Menthol	0.242	38.73	0.434	N.S	0.864	Not seen
Olive oil	0.104	73.67	0.043	82	0.041	Not seen
Urea	0.021	94.68	0.023	90.42	0.327	Not seen

*%Decrease in peak height(%D) = (peak height from untreated whole skin - peak height from enhancer treated whole skin)/ peak height from untreated whole skin x 100

Table 4. Decrease in mean peak height, compared with control (untreated skin) of C=O stretching (Amide I) and C-N stretching of keratin (Amide II) absorbance of abdominal hydrated whole skin rat following treatment with different enhancers. (Mean ± SD, n = 3).

	C=O stretching Of keratin		C-N stretching Of keratin	
	Peak height	% D	Peak height	% D
Control	0.012	_	0.414	_
Eucalyptus	0.002	83.3	0.008	98.06
Menthol	0.392	Not seen	0.159	61.59
Olive oil	0	100	0	100
Urea	0	100	0	100

Table 5. FT-IR Peak wave numbers (cm^{-1}) changes compared with control (untreated skin) and abdominal hydrated whole skin rat following treatment with different enhancers. (Mean ± SD, n = 3).

	C-H stretching asy	C-H stretching sym	C=O stretching Of lipid ester	Amid I	Amid II
Control	2915.13±0.2	2840.74±0.4	1690.2±1	1642.58±0.9	1535.9±0.2
Eucalyptus	_	2856.57±0.1	1725.03±0.1	1631.07±0.1	1546.34±0.1
Menthol	2908.58±0.1	4772.51±0.1	1758.18±0.1	1672.45±0.2	1570.73±0.2
Olive oil	2923.39.13±0.2	2853.6±0.3	1742.11±0.2	_	_
Urea	2594.97±0.1	2467.94±0.1	1774.9±0.1	_	_

Table 6. Effect of penetration enhancer on the thermal properties of hydrated rat skin. (Mean ± SD, n = 3).

	Transition temperature (°C)		**Transition enthalpy (mj/mg)**	
	Tm1	**Tm2**	**ΔH1**	**ΔH2**
Control (Water)	67.5±2.1	112±6.6	7.01±0.4	555.1±19.5
Olive oil	39±0.4	118±1.8	1.76±0.01	3.24±0.02
Urea	37±0.3	89±2.1	2.4±0.3	3.86±0.5
Menthol	37±0.8	119±2.8	1.85±0.8	2.71±0.1
Eucalyptus	41±1.8	146±2.1	2.77±0.8	42.25±2.9

Tm1 = mean transition temperature of lipids; SC **Tm2** = mean transition temperature of irreversible denaturation of intracellular stratum corneum keratin**;** $\mathbf{\Delta H_1}$ = transition enthalpy of lipid phase SC $\mathbf{\Delta H_2}$ = transition enthalpy of keratin phase SC

Figures:

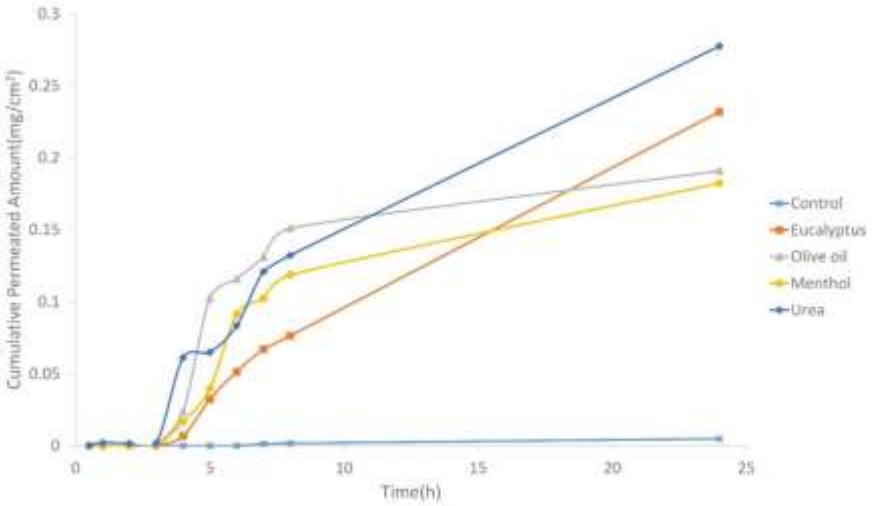
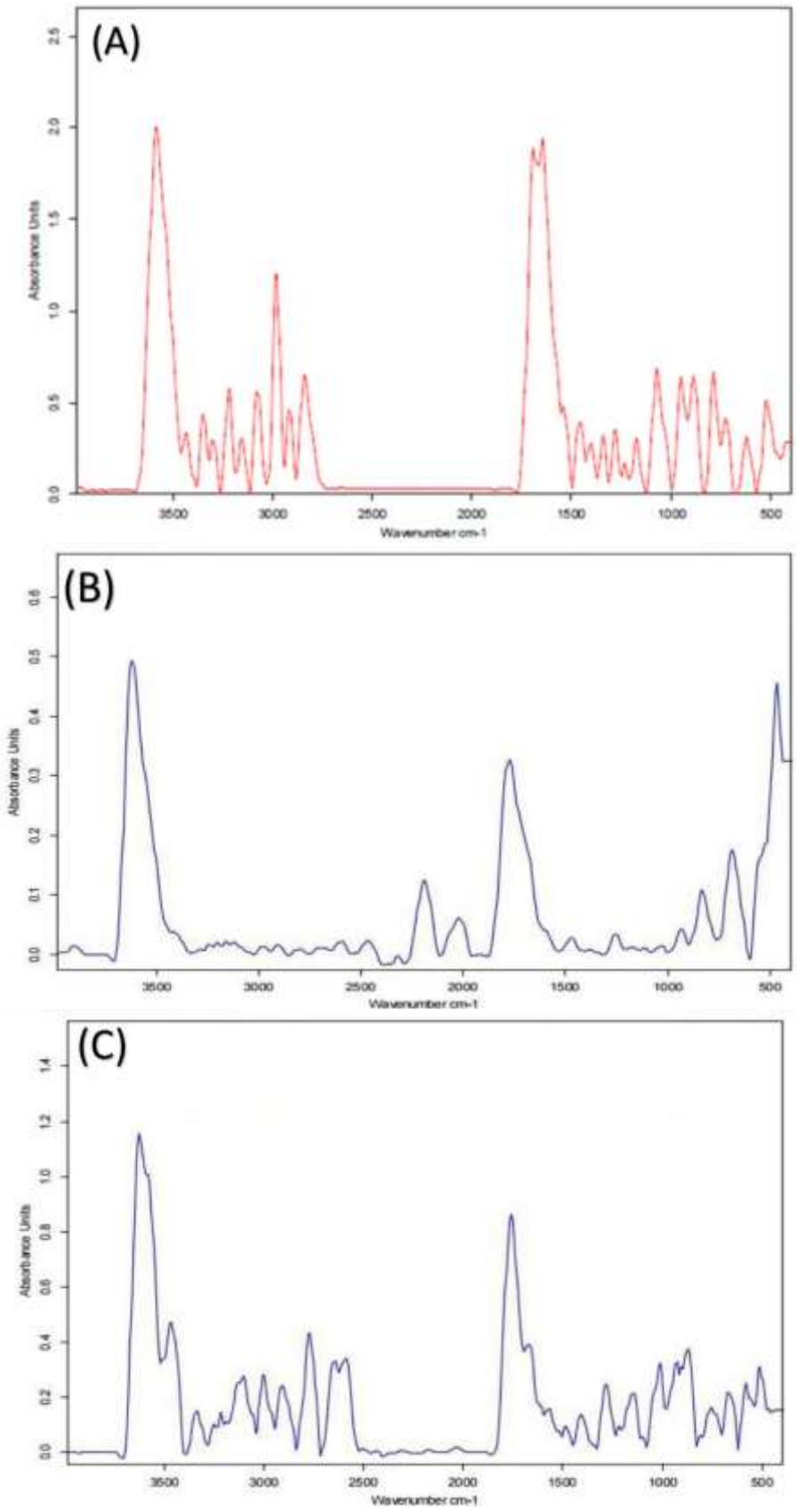


Figure 1. The amount of Octyl methoxycinnamate permeated from various pretreatment rat skins with various chemical enhancers.


(A)
Absorbance Units
Wavenumber cm-1
(B)
Absorbance Units
Wavenumber cm-1
(C)
Absorbance Units
Wavenumber cm-1

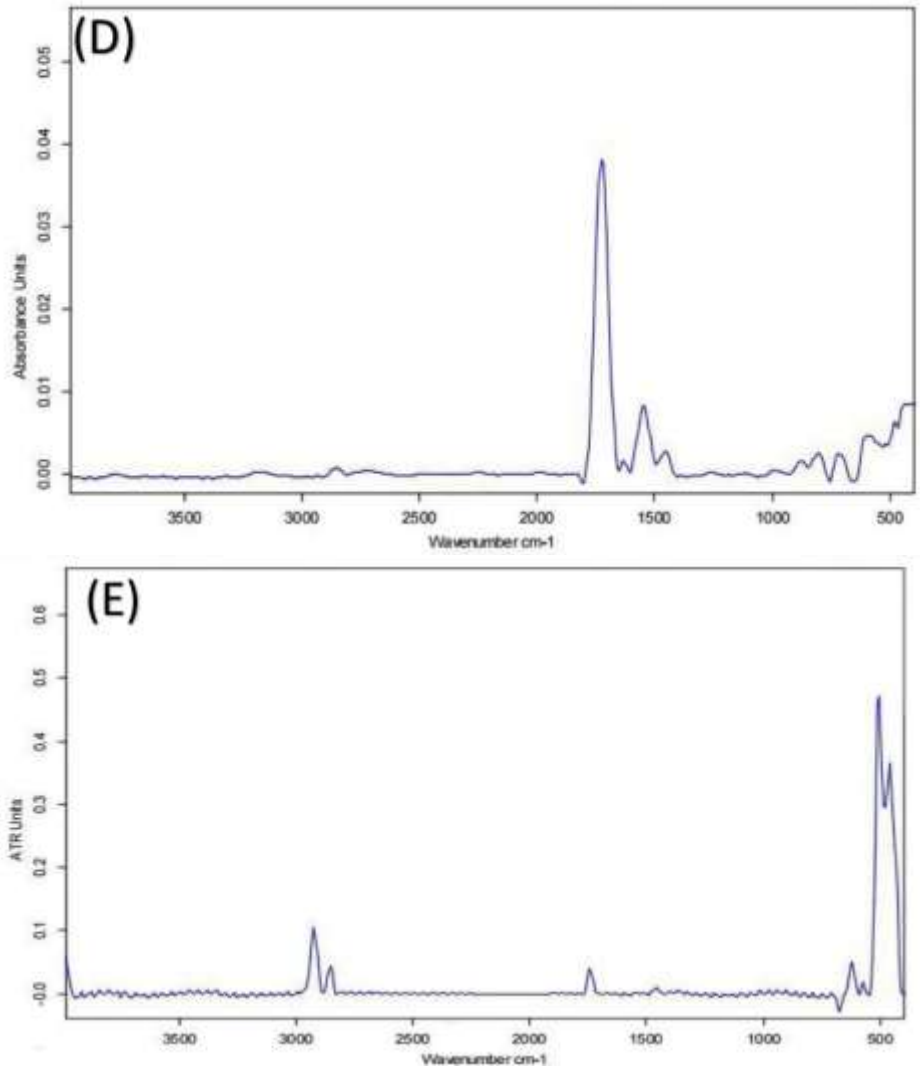
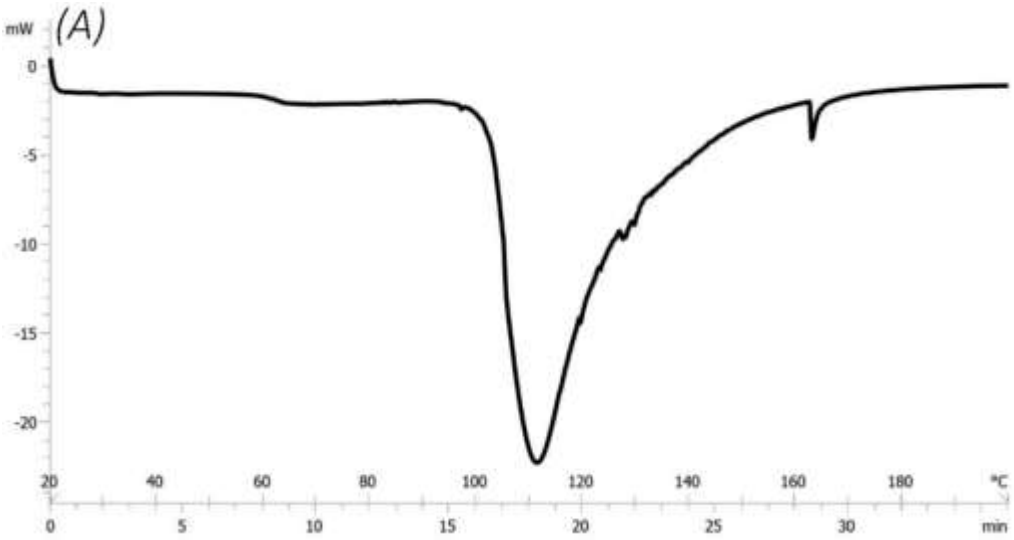
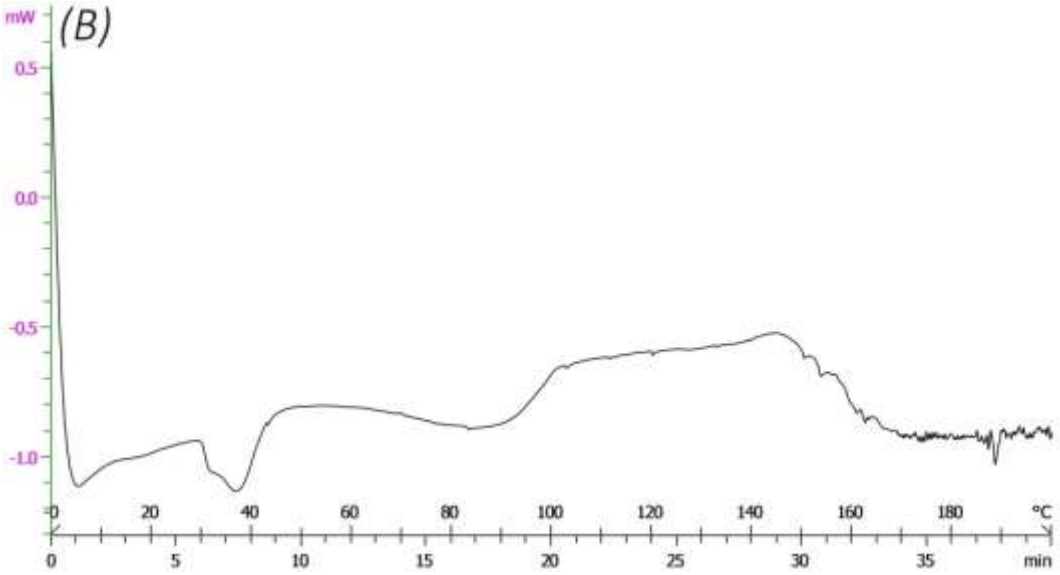


Figure 2. FT-IR spectra of pretreatment skin rat with (A): Water(control), (B): Urea, (C): Menthol, (D): Eucalyptus, (E): Olive oil.


(A)
mW
0
-5
-10
-15
-20
20
40
60
80
100
120
140
160
180
°C
0
5
10
15
20
25
30
min


(B)
mW
0.5
0.0
-0.5
-1.0
0
20
40
60
80
100
120
140
160
180
°C
0
5
10
15
20
25
30
35
min

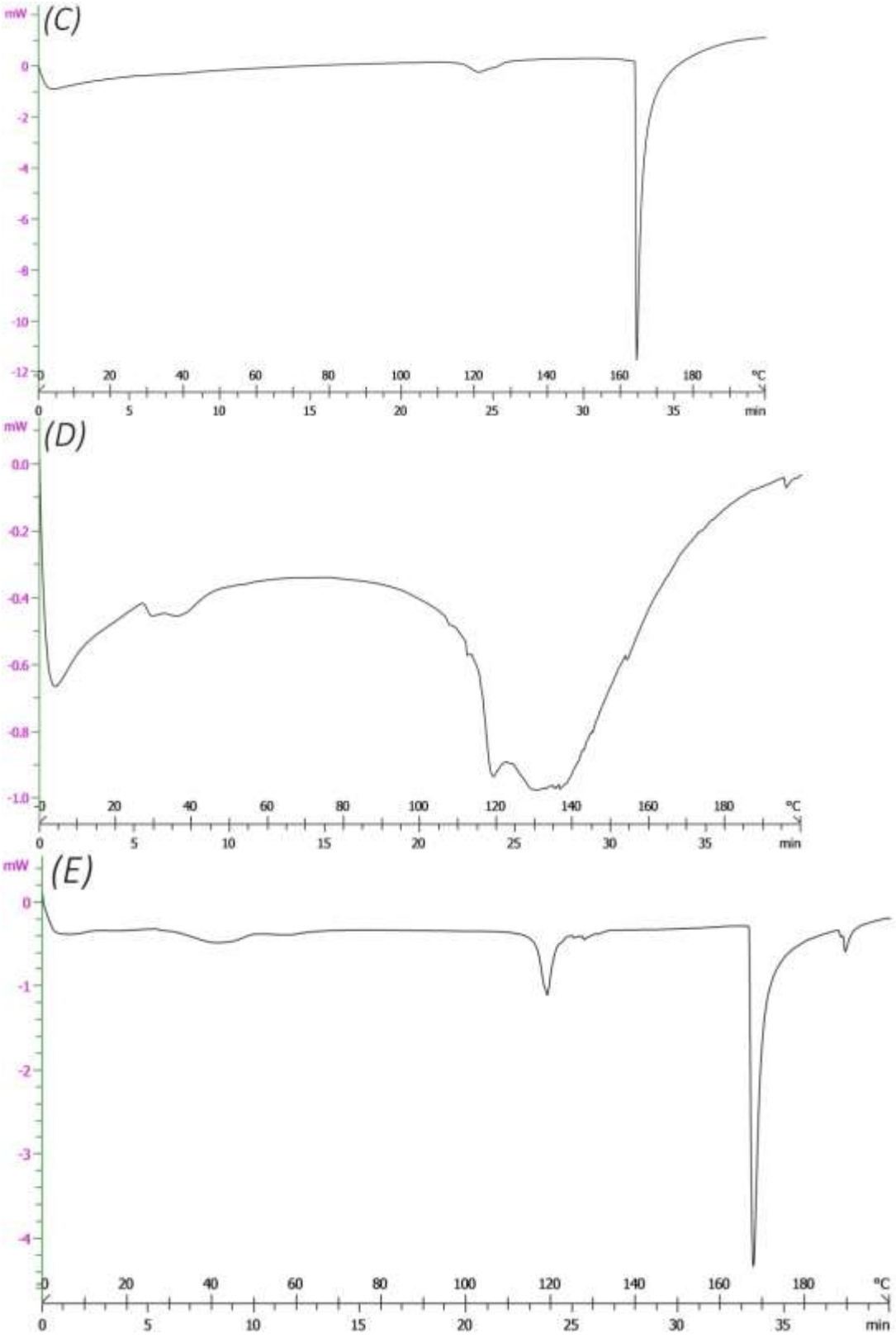


Figure 3. DSC Thermograms of pretreatment skin rat with (A): Water (control), (B): Urea, (C): Menthol, (D): Eucalyptus, (E): Olive oil.

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