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Original Research Article

Effect of the Various Solvents on the *In Vitro* Permeability of Vitamin B_{12} through Excised Rat Skin

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Abstract

Purpose: *To investigate the effect of different solvents on the in vitro skin permeability of vitamin B_{12}.*
Method: *Vitamin B_{12} (B_{12}) permeability experiments through rat skin pretreated with various solvents namely, propylene glycol, oleoyl macrogol-6-glycerides, propylene glycol monocaprylate and oleic acid, were performed in Franz diffusion cells and compared with hydrated rat skin as control. The permeability parameters evaluated include steady-state flux (J_{ss}), lag time (T_{lag}), permeability coefficient (Kp) and diffusion coefficient (D). The solvents' permeability enhancement mechanisms were investigated by comparing of changes in peak position and their intensities of assymmetric (Asy) and symmetric (Sym) C-H stretching, and C=O stretching absorbance using Fourier transform infrared spectroscopy (FTIR), as well as by comparing mean transition temperature (Tm) and their enthalpies (ΔH) using differential scanning calorimetery (DSC).*
Results: *All the solvents significantly decreased diffusion coefficient ($p < 0.05$), with capryol showing the greatest enhancement ratio (ER_D) based on diffusion coefficient followed by labrafil, oleic acid and propylene glycol. Flux enhancement ratio (ER_{flux}) for all the solvents was < ER_D. The solubility of B_{12} in stratum corneum was the rate-limiting step in partitioning. All solvents with different lipophilic properties decreased drug solubility in the stratum corneum and hence lowered partitioning and flux. FTIR and DSC results showed lipid fluidization and extraction by labrafil and capryol, disruption of lipid structure and fluidization by oleic acid, and interaction with stratum corneum keratin by propylene glycol.*
Conclusion: *Water is a suitable topical vehicle for B_{12} as it can increase partitioning and diffusion through rat skin.*

Keywords: *Percutaneous absorption, Enhancer, Vitamin B_{12}, Skin permeation, Diffusion coefficient, Flux, Enthalpy*

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INTRODUCTION

The use of a topical drug for dermal and transdermal drug delivery depends on the ability of the drug to penetrate via skin in sufficient quantities to achieve the desired effect [1]. Transdermal delivery has many advantages such as avoidance of first pass metabolism, controlled and continuous drug delivery, reduction of dose frequency, enhances patient compliance, facilitation of drug localization at target site, non-invasive, and reduction in toxic levels of drugs [2-6].

Drug permeation across different skin layers is affected by various factors such as physicochemical properties of the drug, vehicle, and formulation components. Many substances such as surfactants and solvents used as formulation ingredients can alter the permeability- properties of skin [6]. Passive permeation of drug through skin often depends on two major physicochemical properties, viz, solubility and partition coefficient. The reversible enhancement effect of solvent and modifications of the thermodynamic activity of drug are two strategies to improve permeation of drug[7]. Therefore, a suitable solvent mixture may greatly affect drug delivery from topical preparations. Several solvents (water, propylene glycol, oleic acid) are known to enhance drug permeations by different mechanisms, which include disruption of the organized intercellular lipid structure of the stratum corneum, fluidizing stratum corneum (SC) lipids, altering cellular proteins, and extracting intercellular lipids by mostly non-polar solvents. Increase in the diffusity of stratum corneum has been reported for polar solvents, e.g., dimethyl sulfoxide (DMSO) and dimethyl formamide (DMFA) [6-8].

B_{12} is the most common and most complex vitamin with a molecular weight of 1355.4 Da. It is poorly absorbed from the gastrointestinal tract; Gastrointestinal absorption of vitamin B12 depends on the presence of sufficient intrinsic factor . Percutaneous absorption of B_{12} in guinea pig and rat has been investigated and it was demonstrated that absorption was more efficient from an ethanol solution than from water or dimethyl sulfoxide, and also it was independent of the quantity of vitamin applied to a given area [9].

The aim of this study was to investigate the effect of several hydrophilic and lipophilic solvents on the *in vitro* skin permeability of B_{12} with a view to designing and developing a suitable transdermal drug delivery system for vitamin B_{12}.

EXPERIMENTAL

Materials

B_{12} was purchased from Iran Hormone Company (Tehran, Iran) and Capryol 90(Propylene glycol monocaprylate), Labrafil M 1944 CS (oleoyl macrogol-6-glycerides) were obtained as gift samples (Gattefosse, Saint-Priest, France). Water, deionized and filtered in-house, was used. PG and oleic acid were purchased from Fluka .All other materials used were of analytical grade.

Solubility studies

The solubility of B_{12} was investigated in water, propylene glycol, oleoyl macrogol-6-glycerides, propylene glycol monocaprylate and oleic acid. An excess amount of B_{12} was added to 5 ml of the solvent. The mixture was immersed in a water bath for 24 h at 37 ^{0}C and allowed to equilibrate. Then, suspension was centrifuged for 10 min at 3000 rpm, filtered, diluted and the dissolved drug measured by a validated UV spectrophotometric method at 362 nm[10].

Animal experiments

Male adult Wistar rats (weighing 100 - 150 g) and aged 10 - 12 weeks were purchased from Animals Laboratory, Jundishapur University of Medical Sciences, Ahvaz, Iran. The hair on the abdominal skin was removed with an electric clipper, taking care not to damage the skin. The rats were anaesthesized with ether prior to sacrificing them. Abdominal full-thickness skin was removed and any extraneous subcutaneous fats cleaned from the dorsal side using cooled pure acetone solution with 4 ^{0}C. Whole skin thickness was measured using a digital micrometer (AAOC, France). The animals were treated according to the principles for the care and use of laboratory animals, and approval for the animal studies was obtained from the Ethical Committee of Ahvaz Jundishapur University of Medical Sciences (ref no. 5931). The procedures followed complied with standard international guidelines [11].

DSC studies

The changes in structure of whole skin induced by solvents were examined using a DSC (Mettler Toledo DSC1 system) equipped with a refrigerated cooling system (Hubert Tc45). Approximately 5 – 10 mg of sample was placed in hermetically sealed aluminum pans. Simultaneously an empty hermetically sealed pan was used as a reference. Skin samples were exposed to heat ranging from 20 to 200 ^{0}C (scan rate: 5 ^{0}C/min). All experiments were at least in triplicate. In order to ensure accuracy and repeatability of data, DSC analyzer was calibrated and checked with indium standard. Enthalpy (ΔH) values were calculated from endothermic and exothermic transitions of the thermograms as in Eq 1.

ΔH = peak area/sample weight…… (1)

FT-IR spectroscopy

The excised rat skin samples were treated with Propylene glycol, oleoyl macrogol-6-glycerides, Propylene glycol monocaprylate and oleic acid for 24 h, vacuum-dried (650 mm Hg, 25 ± 1 ^{0}C) for 1 h and the stored in desiccators to remove traces of solvent [12]. The samples scanned in the range 4000 to 500 cm^{-1} using an FT-IR facility (Uker, Vertex70, Germany) .

***In-vitro* skin permeation studies**

In-vitro permeation studies were carried out using vertical glass diffusion cells fabricated in house with an effective diffusion area of approximately 3.46 cm^2. The volume of the receptor compartment was 22 ml .Whole skin sample, hydrated prior to use, was mounted between the donor and receptor compartments of the cell with the stratum corneum facing the donor medium. B_{12} (0.07 %w/v), dissolved in the test solvent, was in the donor compartment and the receptor cell was filled with phosphate buffer (pH 7). The diffusion cell was placed and clamped in a water bath 37 ± 0.05 ^{o}C placed on a magnetic stirrer with a heater.

The receptor medium was stirred with a small magnetic bead at 200 rpm. At predetermined time intervals (0.5, 1, 2, 3,……,80 h), a 2 ml sample was withdrawn from the medium and immediately replaced with an equivalent volume of fresh phosphate buffer to maintain sink condition. The samples filtered and the permeated amount of B_{12} was determined by UV spectroscopy method at 362 nm. Aqueous solution of B_{12} was used as blank.

Permeation data analysis and statistics

B_{12} concentration was corrected for sampling effects according to Eq 2 [7,8].

$$C^1_{n1} = C_n(V_T/V_T-V_S)/C^1_{n-1}/C_{n-1} \quad \text{…….. (2)}$$

where C^1_n is the corrected concentration of the n^{th} sample, C_n is the measured concentration of vitamin B_{12} in the n^{th} sample, C_{n-1} is the measured concentration of vitamin B_{12} in the $(n-1)^{th}$ sample, V_T is the total volume of the receptor fluid and V_S is the volume of the withdrawn sample.

The corrected B_{12} concentration divided by the area of the skin exposed to donor solution to calculate the cumulative amount of B_{12} permeated per unit area which was plotted against time. Steady-state flux (µg/cm^2/h) was computed from the linear portion of the slope of the permeation curve. Permeability coefficient (Kp, cm/h) of B_{12} through the skin was calculated as in Eq 3 [7,8]:

$$Kp = Jss/Cv \quad \text{…….. (3)}$$

where Jss is steady-state flux and Cv the initial concentration of B_{12} in the donor compartment. The permeation enhancing effect of the solvent on skin permeation of B_{12} was calculated in terms of enhancement ratio (ER) as in Eq 4 [8].

$$ER = Kps/Kpw \quad \text{…….. (4)}$$

Statistical comparison was made using one-way ANOVA and $p < 0.05$ was considered statistically significant. Correlation analysis was performed by least square linear regression method and correlation coefficient examined for significance by Student's t-test. All statistical analyses were conducted using SSPS software (version 13.0 for Windows, SPSS Inc, Chicago, IL, USA).

RESULTS

Solubility of B_{12}

The solubility of B_{12} in various solvents is shown in Table 1. The results indicate the highest solubility in water followed by propylene glycol, labrafil, capryol and oleic acid in that order.

Table1: Solubility of B_{12} in various solvents at 37 ^{o}C

Solvent	Solubility(mg/ml)
Water	79.780±0.195
Propylene glycol	52.162±0.169
Oleoyl macrogol-6-glycerides	0.4785±0.121
Propyleneglycolmonocaprylate	0.1507±0.027
Oleic acid	0.1071±0.003

FT-IR Spectroscopy

Spectral analysis has been done involved examination of change in peak position and their intensities from 4200cm^{-1}-500cm^{-1} (Fig 1 and Table 2). In our study, untreated whole rat skin (control) showed bands at 3610.46, 3417.59, 3310.20, 3255.39, 3200.08, 3105.44, 2948.02, 2821.56, 1723.62, 1652.78 and 1572.27 cm^{-1}. The bands observed in the range of 3000 - 3600cm^{-1} represent O-H and N-H stretching from lipid, protein and water while the peaks near 2948.02 and 2821.65 cm^{-1} represent asymmetric and symmetric stretching bands of the terminal methyl groups of lipids in rat skin. The lipid ester carbonyl stretching in SC showed at 1723.62 cm^{-1} position while the bands observed at 1652.78 and 1572.27 cm^{-1} represent amide I (C=O

stretching) and amide II (C-N stretching) linkage of the helical secondary structure found in epidermal keratin [9,11]. The spectra of PG-treated whole rat skin induced the smallest decrease in peak height at 2929.500, 2855.7 and 1749.003 cm^{-1}, while bands of amide I and amide II (1652.78 and 1572.27 cm^{-1}) disappeared.

The IR spectra of whole skin rat treated with Labrafil M 1944CS displayed significant decrease in peak height of the wave numbers (2926.60 and 1745.31 cm^{-1}) and disappearance of the peaks in 2850, 1652.7, and 1572.2 cm^{-1} positions. The spectrum for whole skin rat treated with Capryol 90 presents an intermediate blue or red shift at 2869.46 cm^{-1}. At the same band position, a decrease in intensity of the signal was observed, suggesting lipid extraction [13].

Differential scanning calorimetery (DSC)

Thermotropic behavior of skin treated with various vehicles evaluated by comparing for mean transition temperature (Tm) and their enthalpies (ΔH). The temperature transition and enthalpy amounts after solvent treatment are shown in Table 3 while the thermograms are displayed in Figure 3.

Two endothermic transitions were obtained at around 67.5 (T_1) and 112 ^oC (T_2) were obtained in thermogram of hydrated whole rat skin. T_1 and T_2 transitions appeared to be due to melting of lipids and irreversible denaturation of intracellular keratin, respectively. Previous studies on the thermotropic behavior of rat skin indicates three

Table 2: Decrease in mean peak height (± SD), compared with control (untreated skin) of assymmetric (Asy) and symmetric (Sym) C-H stretching and C=O stretching absorbance of abdominal hydrated whole skin rat following treatment with different solvents (mean ± standard deviation, n = 3)

Band stretching	solvents								
	Control	***Propylene glycol***		***Oleic acid***		***Capryol 90***		***Labrafil***	
	Peak height	Peak height	%D	Peak height	%D	Peak height	%D	Peak height	%D
Asy	4.8±0.02	4.6±0.1	5±0.3	2.6±0.3	45.7±0.7	-	-	3.7 ±0.7	22.2±0.5
Sym	5.1±0.3	4.1±0.1	19.8±0.4	2.3±0.3	53.2±0.6	3.1±0. 4	37.9±0.7	-	-
C=O	4.9±0.06	4.1±0.1	17.7±0.4	2.2±0.9	55.6±0.7	4.5± 0.5	9.1±0.4	3.5±0.1	29.5±0.1

Note: %D = decrease in peak height (%)

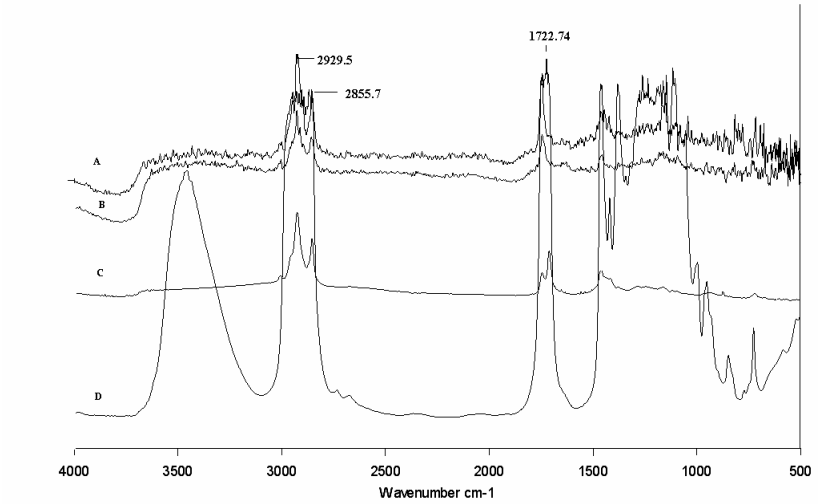

Fig 1: FT-IR spectra of whole skin abdominal rat after treatment with (A) propylene glycol (B) Labrafil M 1944CS (C) oleic acid, and (D) Capryol 90

Table 3: Effect of solvents on the thermal properties of hydrated rat skin (mean ± SD, n = 3)

Treatment	**Transition temperature (°C)**		**Transition enthalpy (j/g)**	
	T_{m1}	T_{m2}	ΔH_1	ΔH_2
Hydrated control	67.5±2.1	112±6.6	7.01±0.4	552.1±19 .5
Propylene Glycol	77.5±4.2	132±5.9	6.05±0.6	16.4± 2.2
Oleic acid	53±3.6	122±8.6	1.36±0.6	59.9 ± 5.1
Capryol 90	72±4.1	122±8.2	95.4±4.2	2.2± 0.1
Labrafil	87.5±3.5	116±4.6	61.9±5.3	45.5± 3.7

Tm_1 = mean transison temperature of lipids; $SCTm_2$ = mean transition temperature of irreversible denaturation of intracellular stratum corneum keratin; ΔH_1 = transition enthalpy of lipid phase $SC\Delta H_2$ = transition enthalpy of keratin phase SC

endothermic transition at 62, 79, and 95 ^{o}C [14], and four endothermic transitions at 34, 82, 105, and 114 ^{o}C [15]. In another study on the SC of human skin [16], three endothermic transition peaks were obtained at temperatures 59 - 63 ^{o}C (Tm_1), 75 - 82 ^{0}C (Tm_2) and 99.5 - 120 ^{o}C (Tm_3) and they did not report a low transition temperature peak (35 – 40 ^{o}C) because their samples were desiccated before DSC examinations and 15 % moisture content is required for detection of low temperature transition [16]. They showed that Tm_1 corresponds to lipid transformation from a lamellar to disordered state, Tm_2 is due to the melting of lipid – protein(keratin) complex [2,16], or the disruption of polar head groups of lipids [14-17], Tm3 is known to occur during the irreversible denaturation of proteins in the SC [16].

It was observed that T_1 was shifted by Capryol 90 and Labrafil M1944CS to higher melting points and ΔH_1 increased in comparison with control. In addition, T_1 was shifted by oleic acid and PG to lower and higher melting points, respectively, while ΔH_1 decreased in comparison with control. On the other hand, all the solvents caused T_2 shift to higher melting points and lower enthalpy, suggesting possible denaturation of protein.

In vitro permeation of B_{12}

The effect of solvents on B_{12} permeability (hydrated skin as control) is expressed in Table 4 as ER_{flux} (ratio of drug flux after and before skin pretreatment with solvent) and ER_D (drug diffusion coefficient after and before skin pretreatment with solvent). The results indicate that all solvents decreased permeability across rat skin compared with control. Labrafil provided the best effect on B_{12} flux, followed by oleic acid, capryol and propylene glycol in that order. All the solvents exerted significant decrease in diffusion coefficient ($p < 0.05$), with Capryol 90 showing the highest ER_D followed by Labrafil, oleic acid and propylene glycol. ER_{flux} for all the solvents was less than ER_D. This means that solvents decreased flux more than they did diffusion coefficient.

Table 2: Effect of solvents on derived permeation parameters for B_{12} (mean ± standard deviation, n = 5)

Solvent	ER_{flux}	ER_D
Propylene glycol	0.0010±0.0002	0.009±0.001
Oleic acid	0.0060±0.0004	0.020±0.001
Capryol 90	0.0050±0.0002	0.074±0.005
Labrafil	0.0200±0.0001	0.068±0.008

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

DISCUSSION

The results indicate that all solvents decreased drug flux through skin more than they did diffusion. The high solubility of B_{12} in water suggests that drug solubility stratum corneum is the rate-limiting step in partitioning. Therefore, all solvents with different lipophilic properties decreased drug solubility in strateum corneum

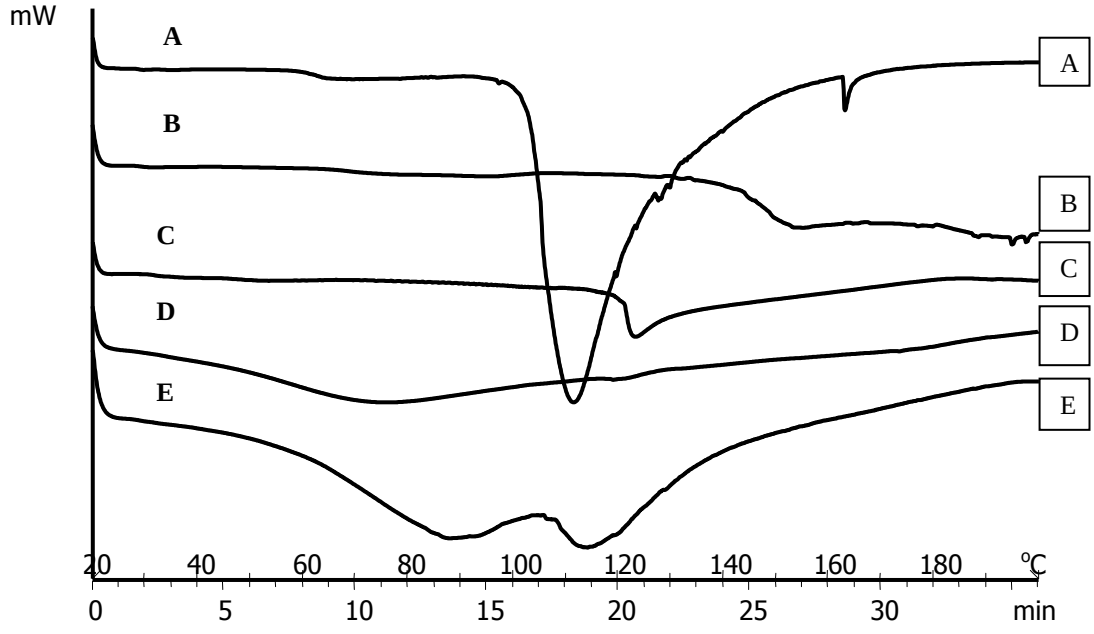

Fig 2: DSC thermograms of abdominal rat skin after treatment with (A) water, (B) propylene glycol, (C) oleic acid, (D) Capryol 90 and (E) Labrafil M 1944CS.

and hence also decreased partitioning and flux. The lack of significant correlation between drug solubility in PG and ER_{flux} indicates that the high hydrogen bonding capacity of PG decreased affinity of skin for bonding with B_{12} during treatment with this solvent. Therefore, the effect of solvents on drug solubility in strateum corneum and hydrogen bonding between drug and skin are determining factors for drug partitioning.

ER_D results after skin treatment with PG correlate with FT-IR and DSC results as they indicate that PG interacts mostly with SC keratins, and does not alter significantly SC lipid organization. The shift of 2929.5cm^{-1} band to a lower wave number (red shift) indicates reorientation of lipid groups leading to strengthening of SC barrier properties and therefore, leading to retardation of B_{12} transport across the skin by PG. The relative red shift observed in 1722.7cm^{-1} band indicates formation of strong hydrogen bonds within the lipid molecules. Treatment with penetration modifiers might have led to extraction of membrane lipids from SC as usually occurs with enhancers, or might have increased the intensity at the particular band representing retardation in the case of retardants [13].

Lipid fluidization and extraction by Labrafil and Capryol, as indicated by FT-IR, is the main factor for higher ER_D ratio compared with PG and oleic acid. Increase in enthalpy and transition T_{m1} by Labrafil and Capryol may due to bilayer cohesion [20], which is in contrast to lipid extraction and fluidization. The shift in Tm to lower temperatures can be interpreted as disruption of the lipid bilayer and the irreversible denaturation of proteins in the SC layer of skin, while decrease in ΔH is related to fluidization of lipid in lipid bilayer and lipid – protein complex [14-19].

Therefore, the effect of labrafil and capryol (being oily phases) on lipid structure is a combination of two opposite effects that limit their enhancement efficiency. Oleic acid is oily and hence miscible with lipid, thus resulting in decrease in the melting point of the strateum corneum lipids. Capryol 90 interacts mostly with SC keratins and changes the lipid structures of the rat skin. The skin treated with oleic acid causes disorder in SC due to lipid extraction. Furthermore, the relative red shift obtained in 1710.86 cm^{-1} band indicates formation of strong hydrogen bonds within the lipid molecules in the skin which is in contrast to disruption of lipid structure and fluidization by oleic acid.

CONCLUSION

The results obtained indicate that all the solvents tested decreased permeability across rat skin Lipid fluidization and extraction by Labrafil and Capryol is the main factor for higher ER_D compared to propylene glycol and oleic acid. Disruption of lipid structure and fluidization are the main modes of action exerted by oleic acid. Overall, the results indicate that aqueous base formulations would be suitable topical vehicles for delivering B_{12} across skin.

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