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Development and in vitro evaluation of furosemide transdermal formulations using experimental design techniques

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Abstract

The in vitro skin permeation of furosemide, a commonly used loop diuretic, through human epidermis, as a preliminary step towards the development of a transdermal therapeutic system, was examined. A screening study was carried out, in order to estimate the effects of the type, the concentration of enhancer and the concentration of gelling agent on the cumulative amount of furosemide permeated through human epidermis, using a 3^3 factorial design. The type and the concentration of enhancer were further evaluated as they were found to affect significantly furosemide permeation. In order to further increase the amount of the drug permeated, the combination of two enhancers, Azone® and oleyl alcohol, at three concentration levels was employed, using an optimization technique. The results indicated that higher amounts of furosemide permeated were observed when Azone® was used at 5.0–6.5% (v/v) and oleyl alcohol at 7.5–9% (v/v), in the gels used. These formulations seem to be suitable for possible transdermal delivery of furosemide for pediatric use.

Keywords: Furosemide; Transdermal; Experimental design; Penetration enhancer; Gel

1. Introduction

Furosemide (5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid) is a potent diuretic agent that induces a powerful diuresis, followed by the loss of sodium, potassium and chloride into the urine, by acting on the thick ascending limb of the loop of Henle (Giebisch, 1985). Its usual daily dose for adults is 20–80 mg, while for pediatric use ranges from 1 mg/kg up to a maximum of 40 mg daily. It is commonly used in the treatment of cardiac and pulmonary disorders in premature infants and neonates. The half-life of furosemide is about 2 h and its oral bioavailability has been reported to be about 60–70% (Micromedex, 2001).

Furosemide is administered per os or parenterally although its physicochemical and pharmacokinetic characteristics (e.g. low molecular weight, lipid solubility, elimination half-life, low melting point) are in agreement with the ideal properties of a molecule for effective penetration of the stratum corneum (Barry, 2001a). This renders furosemide an appropriate candidate for transdermal drug delivery. The selection of the appropriate vehicle is important for the percutaneous absorption of drugs. Moreover, transdermal penetration enhancers can increase the permeation of drugs through the stratum corneum (Ghosh and Banga, 1993; Barry, 2001b). In the present study, the effects of the type, the concentration of enhancer and the concentration of gelling agent were examined,

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using experimental design techniques (factorial design and response surface methodology), in order to find the most appropriate formulations for possible transdermal administration of furosemide.

2. Materials and methods

2.1. Materials

The materials used were furosemide (batch: 279/01, Teva-Tech, Israel), oleyl alcohol and oleic acid (Merck, Germany), laurocapram (Azone®, ChemPacific, USA), glycerol Ph. Eur., hydroxypropyl cellulose (Klucel® H.F., Aqualon, USA), absolute ethanol (Chromasolv, Germany), methanol HPLC (LabScan Analytical Sciences, Ireland), KH_2PO_4 (Panreac Quimica, Spain). All the materials were used as received without further purification.

2.2. Methods

2.2.1. Solubility studies

Solubility studies were conducted by adding excessive amounts of furosemide into sealed glass vials containing 10 ml of vehicle, consisting of ethanol 60% (v/v), glycerin 15% (v/v), the appropriate amount of penetration enhancer and water (q.s.). The vials were then placed on a rotating disk for 24 h at 25 °C. The content of the vials was then transferred into test tubes and centrifuged at 3000 rpm for 10 min, using a centrifugal device (Sigma 202 MK, Sigma, Germany). Samples from each vial were taken and filtered through a 0.45 μm filter (Type FP-450, Gelman Sciences, USA). Appropriate amounts of the filtrate were diluted in methanol and the concentration of furosemide was determined according to a modified high performance liquid chromatography (HPLC) method (Mills et al., 1997). All experiments were performed in triplicate ($n = 3$).

2.2.2. HPLC method

The HPLC system consisted of a high-pressure pump (P1000, Spectra Physics, USA), an autosampler (AS1000, Spectra Physics, USA), equipped with a Hypersil® BDS C18 column (150 mm × 4.6 mm, 5 μm, ThermoQuest Hypersil, Division®, UK), a variable-wavelength detector (Spectra System 2000 UV–vis detector, Thermo Separation Products, USA), set at 229 nm. The data analysis was performed using the ChromQuest Chromatography Data System (ThermoQuest, USA). The mobile phase consisting of 0.01 M KH_2PO_4 (pH = 5.5) and methanol 70/30 (v/v), was pumped through the column at a flow rate of 1.2 ml/min (Mills et al., 1997). The injection volume was 50 μl.

2.2.3. Preparation of standard solutions

The mother solution was prepared by dissolving a known amount of furosemide in methanol. From this solution, seven standard stock solutions were prepared with appropriate dilutions. Calibration curves, constructed on the basis of peak area versus concentration, were found to be linear (correlation coefficients ranged from 0.9996 to 0.9999) over the concentration range studied (0.1–2 μg/ml).

2.2.4. Preparation of furosemide vehicles

Furosemide was formulated in gels. The amount of furosemide incorporated in the gels was based on its saturation solubility in the appropriate vehicles (Tables 1, 6 and 8) in order to obtain maximum skin permeation (Zatz, 1991; Barry, 2000). This amount was dissolved in 10 ml of the solvent system. These solutions were then gelled by adding hydroxypropyl cellulose as gelling agent. The gels were left stirring overnight at ambient temperature and stored at 25 °C.

2.2.5. Quantative determination of furosemide in the gels

Accurately pre-weighed amount of each gel was transferred in volumetric flasks and diluted to an ap-

Table 1
Saturation solubility of furosemide in the solvent system consisting of ethanol (60%), glycerin (15%), enhancer (5 or 10%) and water (q.s.) for the formulations used in 3^3 factorial design

Enhancer	Concentration of enhancer (%, v/v)	Saturation solubility ± S.D. (mg/ml), $n = 3$
–	0	8.48 ± 0.39
Oleic acid	5	10.88 ± 0.20
Oleic acid	10	12.45 ± 0.13
Oleyl alcohol	5	8.92 ± 0.35
Oleyl alcohol	10	12.16 ± 0.21
Azone®	5	11.26 ± 0.05
Azone®	10	14.85 ± 0.05

propriate volume with methanol. The samples were filtered through a 0.45 μm filter (Type FP-450, Gelman Sciences, USA) and analyzed with the previously described HPLC method (Mills et al., 1997). All experiments were performed in triplicate ($n = 3$).

2.2.6. In vitro skin permeation studies

The in vitro skin permeation studies were carried out using modified Franz diffusion cells, made of amber glass, with 6.275 ml volume and 0.636 cm^2 diffusion surface area. In the donor compartment, 100 μl of furosemide gel were applied on the skin epidermis and covered with impermeable film (CoTran 9720, 3M, USA) to prevent evaporation of the solvents. The receptor fluid was a phosphate buffer pH = 8, to ensure sink conditions and stability of the drug (Bundgaard et al., 1988). Human epidermal membrane from back region, taken from full thickness cadaver skin using heat separation technique (Kligman and Christophers, 1963), was mounted between the donor and receptor compartments. Two different skin donors were used (epidermis A and epidermis B). Temperature was maintained at 32 ± 0.5 °C, using a water bath. Five cells were used for each formulation. Samples were taken at predetermined time intervals (6, 12, 24 and 48 h) and analyzed by HPLC. In every series of experiments, a gel without enhancer was used as a control formulation.

2.2.7. Experimental design

Factorial design is an experimental design technique by which the factors involved in a process can be identified and their relative importance assessed. It is thus a means of separating those factors that are important from those that are not. Additionally, this technique identifies the interactions, if any, between the factors chosen. Thus, the construction of a factorial design involves the selection of parameters and the choice of responses. In our study a 3^3 factorial plan was used (Armstrong and James, 1990; Montgomery, 1997). The three factors (independent variables), their corresponding levels and the responses are shown in Table 2. The experimental field should not be too large, leading to non-realistic experiments and not too small, far from an optimal region (Giannakou et al., 2002).

The purpose of Response Surface Methodology is to obtain a model allowing to understand as fully as possible the effects of the factors and their levels, over the whole of the experimental domain, and also to predict the response inside this domain. Moreover, it can be used for optimizing a process (i.e. maximizing one or more of the responses, keeping the remainder within a satisfactory range), carrying out simulations with the model equation and plotting the responses (Lewis et al., 1999).

To describe the response surface curvature of a 3^2 experimental design, a quadratic model can be extracted and calculated by Eq. (1):

$$Z = A_1 + A_2X + A_3Y + A_4X^2 + A_5Y^2 + A_6XY \quad (1)$$

where Z is the response variable, X, Y the independent variables, A_1 the constant and A_2, A_3, A_4, A_5, A_6 the regression coefficients.

2.2.8. Data analysis

Statistical evaluation of the factors effects on the transdermal permeation of furosemide was performed applying one-way analysis of variance at 0.05 level, using a commercially available software package Design Expert® V. 6.0.4 (Stat-Ease, USA).

Table 2
Factors and their corresponding levels implemented for the construction of 3^3 factorial design

Factors	Levels		
	Low	Medium	High
Type of penetration enhancer	Oleic acid	Oleyl alcohol	Azone®
Concentration of penetration enhancer (%, v/v)	0	5	10
Concentration of gelling agent (%, w/v)	1.00	1.25	1.50
Response	Amount of furosemide permeated (Q, μg/cm^2), at 24 and 48 h		

3. Results and discussion

3.1. Solubility studies

Solubility studies were performed to determine the maximum amount of furosemide dissolved in the vehicles (Table 1). Based on the saturation solubility results, gels of furosemide were prepared for use in the in vitro skin permeation studies.

3.2. Quantative determination of furosemide in gels

In all the formulations the concentration of furosemide was found between 95 and 105% of the theoretical value.

3.3. In vitro skin permeation studies

A 3^3 factorial design was used, in order to study the permeation of furosemide through human epidermis. The factors (independent variables) and the corresponding levels were (Table 2): the type of permeation enhancer (oleic acid, oleyl alcohol, Azone®), the concentration of permeation enhancer (0, 5, 10%, v/v) and the concentration of gelling agent (1.00, 1.25, 1.50%, w/v). The response (dependent variable) was the cumulative amount of furosemide permeated human epidermis per unit area, Q ($\mu g/cm^2$), after 24 and 48 h.

Using the combinations of the three factors and three levels, formulations were prepared and placed on Franz diffusion cells, as described previously. The results of the in vitro skin permeation studies are shown in Table 3. The data were analyzed to identify the factors that affect statistically significant the responses and the results are shown in Table 4. It is obvious that the type and the concentration of penetration enhancer affect significantly the permeation of furosemide from the human epidermis ($P < 0.05$). The concentration of gelling agent did not have a significant effect on the transdermal permeation ($P > 0.05$); in other words, the viscosity of the gel, within the levels studied, does not influence significantly the penetration of the drug through the membrane. Similar results have been previously reported (Giannakou et al., 1998; Kim et al., 2001). Additionally, no significant interactions between the factors were found.

From the data obtained is obvious that the gels containing Azone® 5% (v/v) resulted in higher permeation

Table 3
Amount of furosemide permeated per unit area (epidermis A) from gels used in 3^3 factorial design

Enhancer	Concentration of enhancer (%, v/v)	Concentration of gelling agent (%, w/v)	24 h, $Q \pm$ S.D. ($\mu g/cm^2$), $n = 5$	48 h, $Q \pm$ S.D. ($\mu g/cm^2$), $n = 5$
–	0	1.00	5.61 ± 0.58	13.62 ± 1.27
–	0	1.25	4.23 ± 0.74	10.37 ± 1.85
–	0	1.50	3.46 ± 0.65	9.90 ± 1.51
Oleic acid	5	1.00	91.30 ± 15.48	167.17 ± 27.10
Oleic acid	5	1.25	92.19 ± 6.13	157.23 ± 22.50
Oleic acid	5	1.50	93.45 ± 8.83	145.11 ± 21.80
Oleic acid	10	1.00	71.46 ± 9.72	95.26 ± 16.50
Oleic acid	10	1.25	51.56 ± 9.11	96.90 ± 19.30
Oleic acid	10	1.50	69.65 ± 12.77	95.13 ± 18.51
Oleyl alcohol	5	1.00	105.47 ± 21.15	164.69 ± 31.10
Oleyl alcohol	5	1.25	103.25 ±19.65	155.75 ± 30.88
Oleyl alcohol	5	1.50	98.59 ± 19.20	149.87 ± 28.30
Oleyl alcohol	10	1.00	124.06 ± 11.90	191.13 ± 25.10
Oleyl alcohol	10	1.25	105.02 ± 7.97	185.59 ± 19.87
Oleyl alcohol	10	1.50	127.95 ± 20.53	189.10 ± 26.39
Azone®	5	1.00	156.63 ± 10.48	281.99 ± 27.40
Azone®	5	1.25	140.23 ± 11.54	264.39 ± 12.90
Azone®	5	1.50	149.03 ± 14.82	263.14 ± 24.60
Azone®	10	1.00	80.75 ± 15.01	165.01 ± 31.50
Azone®	10	1.25	89.34 ± 17.38	165.93 ± 28.09
Azone®	10	1.50	84.55 ± 13.27	158.68 ± 23.30

Table 4
Analysis of variance for the in vitro skin permeation studies

Factors	24 h		48 h	
	F	*P*	*F*	*P*
Type of enhancer	9.62	0.0007*	15.79	<0.0001*
Concentration of enhancer	85.61	<0.0001*	65.35	<0.0001*
Concentration of gelling agent	0.029	0.8667	0.26	0.6117

* Significant at $P < 0.05$.

of furosemide, causing an about 30-fold increase to the amount of furosemide permeated per unit area, compared to the formulations without enhancer. Azone® at 10% (v/v) concentration caused an about 18 times increase to the permeation of furosemide. For the explanation of the mode of action of Azone®, which is composed of two moieties, a lipophilic chain and a more polar cyclic lactam group, it is generally accepted that the lactam group interacts with the polar region of the structured lipid array of the stratum corneum, whereas the alkyl chain inserts into the more lipophilic region of the lipid domain (Büyüktimkin et al., 1997). Disruption of the lipids of stratum corneum results in lipid fluidization, followed by reduction in the diffusional resistance for the penetrating substances. The decrease in the permeation of furosemide with an increase in Azone® concentration from 5 to 10% (v/v) in the vehicle may be attributed to increased lipophilicity of the gel in combination with lipophilicity of furosemide (Hadgraft et al., 1993; Diez-Sales et al., 1996). Oleyl alcohol at 5% (v/v) increased about 20-fold the permeation of furosemide compared to the formulations without enhancer, while at 10% (v/v) caused an about 25-fold increase. For oleyl alcohol it has been proposed that the double bond in its structure forms kinks in the lipid structure to allow water permeation across the skin. It is known that hydration increases the permeability of the stratum corneum probably because water that is being absorbed acts like a plasticizer in its bond state (Agenda et al., 2001). Oleic acid increased about 17-fold the permeation of furosemide at 5% (v/v) compared to the formulations without enhancer and about 8-fold at 10% (v/v). It is suggested (Francoeur et al., 1990; Hadgraft and Walker, 1991) that it may be heterogeneously dispersed and not clustered within the lipids of the stratum corneum, forming fluid-like channels, also described as permeable pores, at physiological temperatures. Defects associated with water at the interfaces between the solid and the liquid areas could be responsible for the enhanced transport of polar or ionized molecules. It is believed that the decrease in the permeation of furosemide with an increase in oleic acid concentration from 5 to 10% (v/v) is due to an increase in the lipophilicity of the

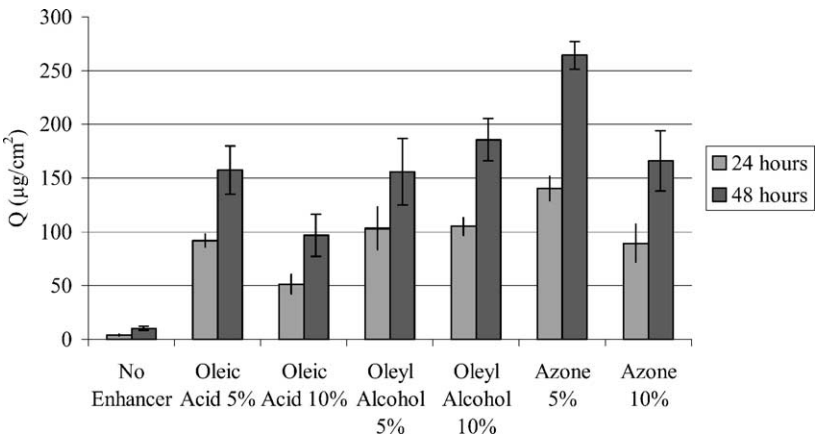

Fig. 1. Amount of furosemide permeated per unit area vs. type and concentration of the enhancers.

Table 5
Factors and their corresponding levels for 3^2 optimization technique

Factors	Levels		
	Low	Medium	High
Azone® concentration (%, v/v)	0	5	10
Oleyl alcohol concentration (%, v/v)	0	5	10

Table 6
Saturation solubility of furosemide in the solvent system consisting of ethanol (60%), glycerin (15%), the appropriate combination of enhancers and water (q.s.) for the formulations used in 3^2 optimization technique

Oleyl alcohol (%, v/v)	Azone® (%, v/v)	Saturation solubility ± S.D. (mg/ml), $n = 3$
5	5	13.83 ± 0.02
5	10	16.38 ± 0.07
10	5	14.02 ± 0.20
10	10	18.27 ± 0.52

gel that leads to a reduction of the enhancing effect (Santoyo et al., 1995).

The effects of the type and the concentration of penetration enhancer on the amount of furosemide permeated per unit area are shown in Fig. 1. It is obvious that Azone® and oleyl alcohol resulted in higher furosemide skin permeation.

3.4. Optimization

Based upon the above-mentioned results, a 3^2 optimization technique was employed, in order to investigate if the combination of the penetration enhancers that showed higher furosemide permeation (Azone® and oleyl alcohol) leads to a further increase. The factors and the corresponding levels are shown in Table 5. Concentration of gelling agent was maintained at its middle level (1.25%).

For the needs of this phase of the study, four new gels were prepared as previously described. The experimentally found saturation solubility of furosemide in the vehicles is shown in Table 6. After conducting the in vitro skin permeation studies the amount of furosemide permeated per unit area of epidermal membrane (Q, μg/cm^2) was measured at 24 and 48 h. The results are shown in Table 7. Based on these data, the effects of the type and concentration of enhancer as well as the response surface model were then calculated by one-way analysis of variance.

The constant, the regression coefficients and the statistical parameters for each response variable, were the following:

24 h:

$$Z_1 = 29.24 + 22.57 \times X_1 + 32.07 \times Y_1 - 1.32 \times X_1^2 - 2.62 \times Y_1^2 - 0.21 \times X_1 \times Y_1 \quad (R^2 = 0.92,\ F = 15.35,\ P = 0.0012) \qquad (2)$$

48 h:

$$Z_2 = 47.88 + 39.24 \times X_2 + 75.50 \times Y_2 - 2.37 \times X_2^2 - 6.10 \times Y_2^2 + 0.078 \times X_2 \times Y_2 \quad (R^2 = 0.93,\ F = 18.08,\ P = 0.0007) \qquad (3)$$

where Z is the amount of furosemide permeated per unit area of epidermal membrane (μg/cm^2), X the con-

Table 7
Experimental data, Q (μg/cm^2) ± S.D., obtained from gels used in 3^2 optimization technique, after 24 and 48 h (epidermis B)

Oleyl alcohol (%, v/v)	Azone® (%, v/v)	24 h, Q ± S.D. (μg/cm^2), $n = 5$	48 h, Q ± S.D. (μg/cm^2), $n = 5$
0	0	8.06 ± 1.39	20.24 ± 3.04
5	0	117.80 ± 14.60	181.34 ± 36.24
10	0	129.44 ± 12.04	220.70 ± 28.86
0	5	141.13 ± 10.62	295.79 ± 12.90
0	10	86.10 ± 14.57	184.61 ± 36.09
5	5	200.55 ± 16.05	415.41 ± 50.90
5	10	136.98 ± 19.40	319.60 ± 54.24
10	5	178.61 ± 14.71	391.39 ± 41.29
10	10	180.48 ± 12.22	379.40 ± 25.56

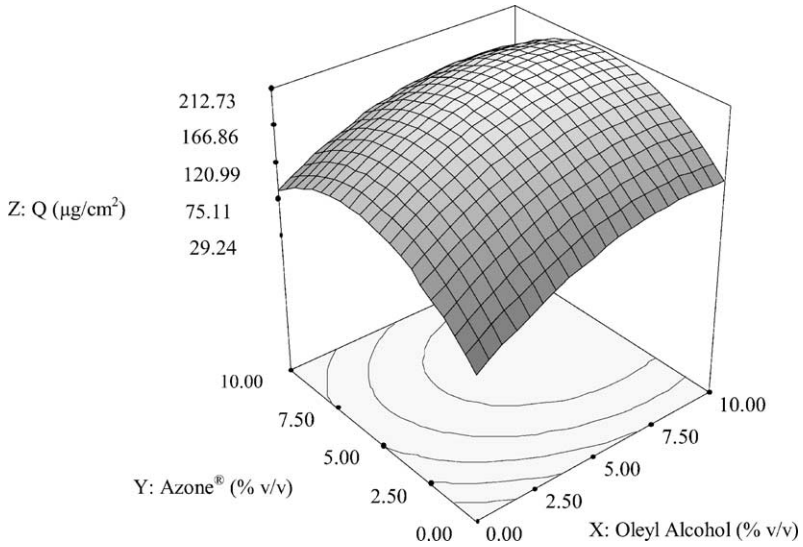

Fig. 2. Estimated response surface, illustrating the relationship between the concentration of Azone® (%, v/v) and oleyl alcohol (%, v/v) and the amount of furosemide permeated per unit area, *Q*, at 24 h.

centration of oleyl alcohol and *Y* the concentration of Azone®.

The response surfaces for *Z*1 and *Z*2 are shown in Figs. 2 and 3, respectively.

By analyzing Figs. 2 and 3, it is obvious that the gels containing combination of oleyl alcohol and Azone® cause an increase in the transdermal permeation of furosemide, compared to the gels that contain a single enhancer. This may be attributed to a synergistic effect (Hadgraft, 2001) between these two enhancers. Although they both act at the lipid domain of stratum corneum (Ghosh and Banga, 1993), their specific

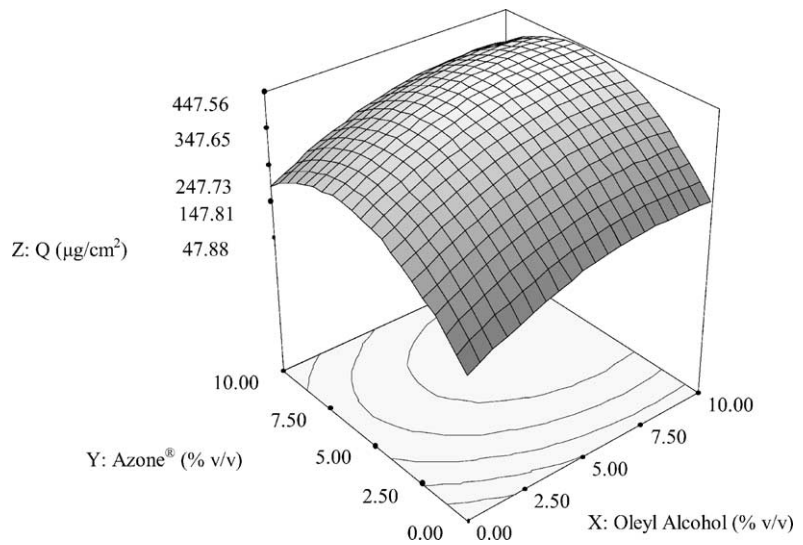

Fig. 3. Estimated response surface, illustrating the relationship between the concentration of Azone® (%, v/v) and oleyl alcohol (%, v/v) and the amount of furosemide per unit area, *Q*, at 48 h.

Table 8
Saturation solubility of furosemide in the solvent system consisting of ethanol (60%), glycerin (15%), the appropriate combination of enhancers and water (q.s.) for the formulations used to evaluate the reliability of the response surface model

Oleyl alcohol (%, v/v)	Azone® (%, v/v)	Saturation solubility ± S.D. (mg/ml), $n = 3$
2.5	7.5	15.09 ± 1.12
7.5	2.5	10.11 ± 0.22
7.5	7.5	14.45 ± 0.51

Table 9
Predicted vs. experimental data of Q used to evaluate the reliability of the response surface model (epidermis B)

Oleyl alcohol (%, v/v)	Azone® (%, v/v)	Predicted Q (μg/cm^2)	Experimental Q ± S.D. (μg/cm^2), $n = 5$	Variation (%)
24 h				
2.5	7.5	166.31	157.23 ± 10.99	5.46
7.5	2.5	183.80	172.13 ± 29.19	6.35
7.5	7.5	204.91	197.96 ± 9.71	3.39
48 h				
2.5	7.5	353.03	331.01 ± 47.41	6.24
7.5	2.5	357.95	347.67 ± 13.87	2.87
7.5	7.5	427.71	404.92 ± 50.69	5.33

mode of action within this domain is different. Probably, oleyl alcohol forms kinks in the lipids (Agenda et al., 2001), causing a primary disruption and Azone® inserts deeper, in the hydrophobic region of the lipids, to cause a further disorganization (Büyüktimkin et al., 1997).

Furthermore, the enhancing effect of Azone® increases with an increase in its concentration from 0% (v/v), reaches a maximum in a concentration area of 5–6.5% (v/v) and then its effect on furosemide permeability decreases. As far as oleyl alcohol is concerned, it shows an increase in furosemide permeation with an increase in its concentration from 0% (v/v) up to a maximum in a concentration area of 7.5–9% (v/v) and then seems to have a decreasing tendency. The greater Q values at all time intervals (24 and 48 h) can be achieved with a combination containing 8.37% (v/v) oleyl alcohol and 6.25% (v/v) Azone®.

A series of experiments were conducted in order to challenge the reliability of the response surface model. Three points of the surface were selected corresponding to intermediate levels of the factors studied and the additional gels were prepared (Table 8). In vitro skin permeation studies were conducted in order to calculate the experimental Q values for Z_1 and Z_2. The experimental data were then compared with the predicted values derived from Eqs. (2) to (3). The results presented in Table 9 indicate an acceptable agreement between the predicted values and the experimental data.

Taking into account the pharmacokinetic data and the oral dose of furosemide, the desired transdermal permeation rate per day was calculated, assuming a patch surface of 40 cm^2 (Cleary, 1991) and was found to be 300 μg/cm^2 for adults and 60 μg/cm^2 for children. From our results, summarized in Tables 3, 7 and 9, it can be concluded that many formulations could meet the desired daily transdermal permeation rate for pediatric treatment. Furosemide is used in cardiac and pulmonary disorders in neonates and premature infants (Micromedex, 2001). Since this target group appears to be rather problematic with furosemide administration via the conventional dosage forms (Evans and Rutter, 1989), the transdermal route of administration appears to be feasible.

4. Conclusions

Furosemide was, indeed, able to permeate human epidermis, in vitro. The type and the concentration of permeation enhancer affected significantly this process. Oleyl alcohol and Azone® resulted in higher permeation of furosemide through human epidermis, while their combination led to higher cumulative

amounts of the drug permeated, compared to those incorporating a single enhancer. A mathematical model that correlated concentration of oleyl alcohol and Azone® with the amount of furosemide permeated per unit area was formed and was able to predict furosemide permeation. The highest drug permeation occurred when concentration of oleyl alcohol was 8.37% (v/v) and concentration of Azone® was 6.25% (v/v). These formulations could be suitable for possible transdermal delivery of furosemide for pediatric use.

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