In-vitro percutaneous absorption of losartan potassium in human skin and prediction of human skin permeability

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ABSTRACT

This study describes the feasibility of transdermal controlled administration of Losartan potassium (LP) across human cadaver skin. Study also defines the influence of capsaicin, sex and site of application on permeation characteristics and determined an appropriate animal model for human skin permeability. The permeation of LP of various formulations was studied using Keshary-Chein diffusion cell. Optimized controlled formulation (without capsaicin) released 42.17% (±1.85) of LP in 12 hr whereas treatment formulation (with capsaicin 0.028 % w/v) released 48.94% (±1.71) of LP with significant difference on null hypothesis. Influence of sex showed statistically significant difference for permeation of LP through male and female rats, as well as male and female mice across both the abdominal and dorsal sides of the skin ($p<0.05$). Similarly statistically significant differences were noted for permeation of LP across male and female mice abdomen-dorsal, but not for male rat abdomen-dorsal and female rat abdomen-dorsal. Furthermore, *in-vitro* permeation of LP across human skin was compared with the permeation across rat and mice skins. Male rat and male mice dorsal skin was found to have closer permeability characteristics to human than other skin membranes, but the Factor of Difference values were < 3 for all membranes which were used suggesting the membranes are good models for human skin permeability. In conclusion simple transdermal adhesive patches formulations incorporating high molecular weight of LP can deliver a dose *in-vivo* and proposed model skin membranes can be utilized for future pharmacokineic and toxicokinetic studies as well as metabolism studies of LP

Keywords: Transdermal, Losartan potassium, Human, rat and mice skin, Eudragit, Enhancer.

INTRODUCTION

Losartan potassium (2-butyl-4 chloro-1 (2-(1H tetrazol-5yl) (1, 1' biphenyl- 4 yl) methyl)-1H-imdazole-5-methanol) (1) is the first angiotensin receptor antagonist which is extensively used for treatment of hypertension (2). However, the drug is subjected to high degree of hepatic metabolism, (3) and meal has also effects on its absorption. It increases level of liver enzymes usually alanine aminotransferase (4-5) which necessitates drug withdrawal after oral administration and as a result alternative drug delivery modes are required. Previously various approaches like sustained release dosage form (6) and injectable spray dried micorsphere (7) for improved therapy has been developed but no systematic investigation on transdermal delivery of LP to minimize meal effects and elevated level of liver enzymes has been reported. Moreover to understand the effectiveness and phramacokinetic properties of the substances, human *in-vivo* percutanneous absorption study is the most preferred approach to obtain the data pertaining to man. However, human volunteer studies are often difficult to justify particularly in the case of toxic or irritant active ingredients or components of formulation. Therefore, skin absorption has been examined using *in-vitro* techniques and good agreement with *in-vivo* experimental data has supported further use of this approach (8, 9). However for regular use in *in-vitro* studies, a reliable supply of human skin is required which is often difficult to procure. For this reason more readily available animal models have been employed for prediction of chemical absorption across human skin. Various animal skin alternatives have been used in the absorption studies of which two species viz. rat and mice are most widely used even though their permeability properties do not closely resemble to human skin.

In the following *in-vitro* experiment, LP was choosen to evaluate the feasibility of its controlled administration via transdermal delivery from ammoniomethacrylate copolymeric patch formulation. This system may improve its systemic bioavailability and therapeutic efficacy by avoiding first pass metabolism, effects of meals and increased levels of liver enzyme following oral administration as well as decrease in the dosing frequency. Moreover, study defines the use of capsaicin as penetration enhancer and influence of sex and site of application using rat

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and mice skin to characterize human skin permeability. Thus the objectives of these studies were to formulate LP in transdermal dosage form, to evaluate capsaicin as penetration enhancer, to determine permeation of LP across the skin of various species (i.e. hairless rat, mice of both sexes - male and female across both the sides dorsal and abdominal and human cadaver skin), to evaluate the influences of sex and site of applications, and to find appropriate animal model for future pharmacokinetic and toxicokinetic studies as well as to determine the extent of metabolism of LP by viable human skin.

MATERIAL AND METHODS

Losartan potassium was obtained as a gift from the Orchid Laboratory, Chennai, INDIA. Eudragit (Eu) RL100 and Eudragit (Eu) RS 100 were from Rohm Pharma, West Germany. Capsaicin was kindly provided by USV Ltd., Mumbai, India. Human cadaver skin was obtained from Krishna Institute of Medical sciences, Karad, India. Rat and mice of both sexes were from Animal house of government college of pharmacy, Karad, India and used as per the institutional guidelines. All other reagents were HPLC grades.

Keshary-Chein diffusion cells with a volume of 21 ml and diffusional area of 6.15 cm^2 were designed and employed in this study.

Preparation of model patches

Solvent casting using mercury substrate technique was followed for preparation of patches. A 5% w/v solution of polymer Eu RL100: Eu RS100 was prepared in the ratio of 4:1 using methanol as solvent. Methanol was the solvent of choice as it was fully miscible with the adhesive system and LP. PEG 400 (40% w/w) was incorporated as plasticizer. The drug was dissolved in solvent and added to the adhesives (Control formulation). The total area of the patches was 13.85 cm^2. It was found that 2 ml of the solution containing 20 mg of LP and 5% w/v of adhesives mixture was required to produce a patch thickness of approximately 0.1mm after evaporation of the solvent. Capsaicin (0.028 % w/v) was separately dissolved in methanol and added gradually to the mixture of drug and adhesive with constant stirring and the resulting mixture was used as treatment formulation. Respective solutions were then poured into glass rings placed on mercury substrate. Matrices were removed after controlled evaporation, backed with aluminum foil and used for further experiments (an optimized formulation is presented here).

Analytical methods

Samples were analyzed at 254 nm using Shimadzu UV/VIS spectrophotometer (spec 1700) and quartz cuvettes (10). A set of standard solutions of LP in methanol (2-12 µg/ml) was prepared and absorbance values were determined to construct the calibration curve, which was then used to determine the drug content.

Similar procedure was followed to determine the amount of drug diffused across different membranes but area between 208-202 nm were used instead of absorbance and solvent phosphate buffer pH 7.4 was used as solvent instead of methanol (11).

Preparation of skin

For the preparation of the skin, rats and mice were sacrificed by placing them in chloroform saturated chamber. The Stratum Corneum (SC) of human cadaver skin (HCS), rat and mice skin was isolated by trypsin treatment (12). The dermis side of the whole skin was placed on a filter paper saturated with 1% w/w trypsin solution in phosphate buffered saline (PBS pH 7.4) at 37 °C in a sealed petri dish for 8 hours. At the end of this period, SC was carefully separated from the viable epidermis by removal of the subcutaneous tissues and lipids using surgical blade and forceps, rinsed thoroughly with distilled water. The skin was stored until used (13).

Skin Permeation Studies

The SC of HCS was thawed to room temperature floated on deionised water and taken up onto filter paper support. Excess water was removed from the surface of the skin by gently dabbing with lint free tissue paper.

About 6.15 cm^2 area patches were excised, firmly affixed to the centre of the specimen on stratum corneum side and mounted on individually calibrated Keshary-Chein diffusion cells. The donor chamber with pre-greased flange was placed over the patch onto the membrane. Receptor compartments were filled with phosphate buffer pH 7.4 and a magnetic needle was immersed so as to mix the solution properly like agitation. Temperature of the assembly was maintained at 37±1°C. At predetermined time intervals aliquots of 1ml were withdrawn from each receptor compartment and immediately analyzed by Shimadzu UV/VIS spectrophotometer with proper dilution. The receptor compartments were replenished with receptor solution, which was maintained at 37 ±1 °C. Similar procedure was followed for all skin preparations (Male Rat Abdomen – MRA; Female Rat Abdomen - FRA; Male Rat Dorsal - MRD; Female Rat Dorsal - FRD; Male Mice Abdomen - MMA ; Female Mice Abdomen - FMA; Male Mice Dorsal - MMD ; Female Mice Dorsal – FMD) as that of HCS.

Stability Study

Stability study was conducted on patches by storing at 58%, 79%, 98% relative humidity at room temperature for six weeks (14). The samples were withdrawn at weakly intervals and analyzed for drug content and specific decomposition (15-16).

Kinetics of drug release

The *in-vitro* diffusion data was analyzed quantitatively to describe the kinetics of drug release from patches using zero order kinetics equation and Korsemeyer's equation to determine the release profile and release mechanism (17)

Zero order equation: $X = kt + cons.$ (1)

Where, X = amount of drug release, k rate constant, t = time.

Korsemeyer equation: $M_t / M\text{ø} = ktn$ (2)

Where, $M_t / M\text{ø}$ = fraction of drug release, k = kinetic constant, *t* = release time and

n = the diffusional exponent for drug release. The value of 'n' gives an indication of the release mechanism. When n = 1, the release rate is independent of time (zero order = case II transport); n = 0.5 for Fickian diffusion; and when 0.5 < n < 1 diffusion and Non-Fickian transport are implicated. Lastly when n > 1 super case II transport is apparent.

Data analysis

From the concentration profiles of LP in the receptor solution, steady state skin permeation flux, J ($\mu g/cm^2/h$) was calculated using a modified Fick's law equation.

$$J = V\left(\frac{dc}{dt}\right) / A.. \quad (3)$$

Where, dc/dt is the steady state slope of the concentration time plot ($\mu g/cm^{-3}/h^{-2}$), V is the volume of receptor compartment (cm^3), and A is the diffusional area of the diffusion cell (cm^2). The permeability coefficient P_T ($cm\ h^{-1}$) was calculated by the steady state flux and the donor concentration ($mg\ ml^{-1}$) using the following relationship (18).

$$P_T = J/Cd \quad (4)$$

Furthermore, J was used to calculate permeation rate by following equation (19).

$$\textit{Permeability Rate} = J \times h \quad (5)$$

Where, *J* is flux; and *h* denotes thickness of the membrane.

Statistical analysis

All the *in-vitro* data are reported as ± SE of the mean. The *in-vitro* data for influences of sex and site of application was tested for statistical significance using analysis of variance (ANOVA) and difference was determined on the basis of F value *(P<0.05).*

RESULTS AND DISCUSSION

Various permeation profiles were obtained and optimized formulation is presented as cumulative percent of drug release versus time. Good consistency was found indicating integrity of the skin specimen over the duration of the experiment. A very low degradation rate constant *(k)* was observed (0.000467, 0.000569, 0.006767) on performing the stability studies in relative humidities of 58, 79, 98% respectively at room temperature indicating good stability of the patches.

The *in-vitro* percutaneous absorption of LP applied as a patch in two different formulations (control and treatment) was studied in human skin. Permeation of LP from control patch (formulation without capsaicin) through and into human skin was extensive after a single dose, reached approximately to 42.17% (± 1.85) of the applied dose which demonstrated good transdermal delivery of LP. In the case of treatment patch (with capsaicin 0.028 % w/v) formulation, the mean percent LP diffused for 12 hour was 48.94% (± 1.71). The mean percent of LP diffused from control and treatment formulations are presented in Figure 1, which show significant differences ($p<0.05$) in drug release from patches with the increase in drug release from treatment patch formulation. It is reported that capsaicin stimulates the blood circulation, (20) which could be a possible reason for the increased permeation of LP from treatment patch formulation. Moreover it is an effective antihypertensive agent, which might results in additional effects.

Results indicated that the plots of formulations were fairly linear as indicated by their high regression values. Therefore it is ascertained that the drug release from formulation could follow either near zero order or zero order kinetics. In order to confirm the exact mechanism of drug release form the patches, the data were computed and graphed according to Korsemeyer's equation (Figure 2). The 'n' value suggested that the formulation F1 and F1Ca followed super case II transport (n = 1.01 and 1.10 respectively). Thus the result of permeation study obtained for F1 was very close to zero order and therefore more extensive study on the formulation development may results in zero order permeation.

Table 1. *In-vitro* Diffusion parameters

Membran	%Permeation (±S.E.)	J ($mg/cm^2/h$)	P (mg/h)	Log P_T ($cm.\ h^{-1}$)	R^2	Skin thickness(mm)
HCS	42.17(±1.85)	$5.23x10^{-2}$	$3.40x10^{-3}$	-1.6964	0.9980	0.65
F1Ca	48.94 (±1.71)	$6.47x10^{-2}$	$4.20\ x10^{-3}$	-2.1375	0.9825	0.65
MRA	58.62(±1.62)	$8.54x\ 10^{-2}$	$5.55x10^{-3}$	-2.0169	0.9297	0.2
MRD	57.03(±1.14)	$6.64x\ 10^{-2}$	$4.30x10^{-3}$	-2.2162	0.9857	0.2
FRA	68.89(±1.90)	$8.88x\ 10^{-2}$	$5.78x10^{-3}$	-2.0039	0.9481	0.15
FRD	65.89(±3.47)	$8.79x\ 10^{-2}$	$5.70x10^{-3}$	-2.0044	0.9742	0.16
MMA	65.60(±1.83)	$8.13x\ 10^{-2}$	$5.28x10^{-3}$	-2.0385	0.9771	0.14
MMD	57.92(±0.96)	$7.14x\ 10^{-2}$	$4.64x10^{-3}$	-2.09478	0.9959	0.2
FMA	72.76(±1.78)	$8.94x\ 10^{-2}$	$5.81x10^{-3}$	-1.9973	0.9947	0.12
FMD	65.02(±1.65)	$7.55x\ 10^{-2}$	$4.90x10^{-3}$	-2.0705	0.9959	0.14

J = Flux; P = Penetration Rate; Log P_T = Permeability coefficient; R^2 = Regression value. (± S.E. = Standard error, n =5)
HCS – Human Cadaver Skin; MRA - Male Rat Abdomen; FRA - Female Rat Abdomen ; MRD - Male Rat Dorsal; FRD - Female Rat Dorsal; MMA - Male Mice Abdomen ; FMA -Female Mice Abdomen; MMD - Male Mice Dorsal ; FMD - Female Mice Dorsal.

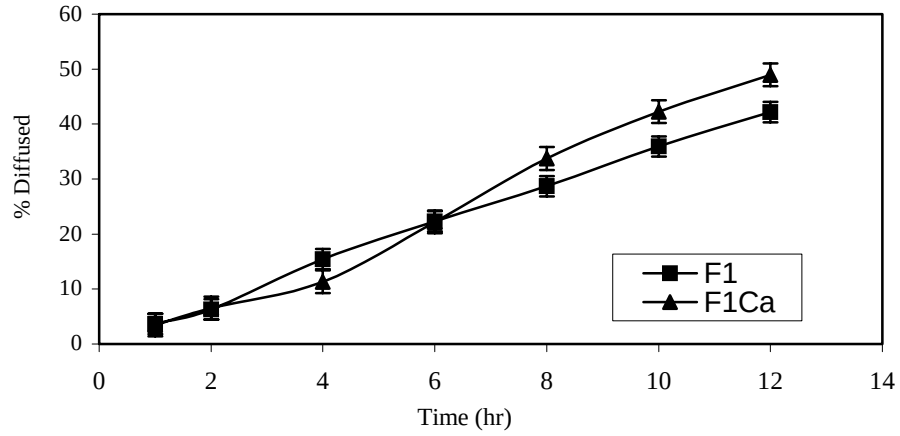

Figure 1. Permeation Data of LP; Control patch (F1) and Treatment patch (F1Ca).

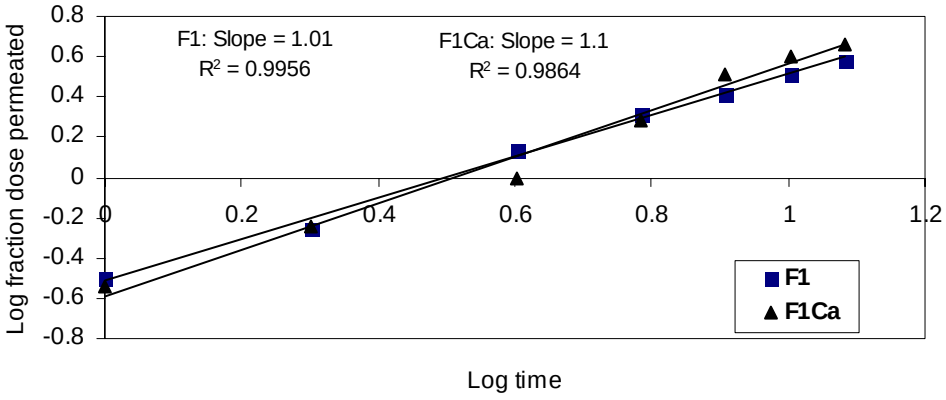

Figure 2. Korsemeyer's Data Curve of Control patch (F1) and Treatment patch (F1Ca).

Influence of sex on skin permeation of LP

The summary of LP skin permeation characteristics across various species is reported in Table 1. The highest LP permeation was noted in hairless female mice abdomen, 72.76(±1.78) which possessed the thinnest skin (0.12 mm) of the all tested species. The second highest LP permeation was noted with female rat abdomen 68.89(±1.90) which had the second thinnest skin (0.14 mm). However male species of both rat and mice showed less permeation (58.62 (±1.62)) and (65.60 (±1.83)) respectively as compared to female animals (Figure 3).

A significant differences in the percent permeation was found between males and females of both rat and mice (Table 1). In the similar manner, calculated permeation rate indicated that thinnest skin presented drug with the higher rate as compared to thicker one. These results made it clear that both the rate and extent of absorption of LP across skin may be dependent on the thickness of the skins (Table 1), which could be a major deciding factor in the permeation of the tested drugs.

Influence of site of application on skin permeation of LP

Influence of site of application was determined using dorsal and abdominal side of the skin of both genders of rat and mice. Result indicated that abdominal side of rat skin of both male and female showed slightly higher extent of permeation but no significant differences ($P < 0.05$) was noted when compared to dorsal side. However in the case of mice, results were opposite, showing significant differences ($P < 0.05$) between abdominal and dorsal sides of both male and female, where abdominal sides of the skin permeated higher amount of drug than dorsal side (Table 1; Figures 4,5). From the results observed, differences in the extent of permeation of LP across male and female and abdomen and dorsal sides of the skin may be due to differences in the thickness of the skin.

Comparison of permeation profiles across various species

The summary of LP skin permeation characteristics across various species is reported in table 1. The highest mean steady state permeation was noted in female mice abdomen (72.76±1.78), which possessed the thinnest skin of all species tested. The second highest mean steady state permeation was noted with female rat abdomen (68.89±1.90), which had the second thinnest skin. The human skin was 0.65 mm thick and showed least permeation among all tested species (42.17±1.85). There were significant differences between male and female rats, as well as male and female mice from both abdominal and dorsal sides of the skin. Similarly, significant differences were observed between the permeation of LP from male mice abdomen-dorsal, female mice abdomen- dorsal. Based on the cumulative 12 hr permeation data, male rat dorsal and male mice dorsal skin had closer permeation properties to human skin, so these skins could be a reasonable model for *in-vitro* permeation of LP across human skin. Furthermore LP permeation across tested species was compared with permeation across HCS, which showed significant differences, therefore FoD value was calculated to determine an appropriate animal model for human skin permeability.

Calculation of Factor of Difference (21)

A comparison of rat and mice skin permeability relative to human skin is displayed in Table 2. FoD values were calculated for LP using following equation.

$$FoD = \frac{\text{Rate of penetration through animal skin}}{\text{Rate of penetration through HCS}} \qquad (6)$$

Calculated FoD values indicated that human skin had closer permeability properties to male rat and mice through dorsal side of skin. Variations in FoD values for all species were 1.26-170 where 1.26 was the smallest FoD value obtained from male rat (dorsal) skin. The second smallest FoD value was from mice (dorsal) skin.

In order to determine the usefulness of an animal model to predict human percutaneous absorption, it is necessary to find satisfactory degree of human skin permeability. It is reported that the permeability may differs by up to threefold between cadavers of same species and this factor could be considered as typical inter experimental variations (8). It is proposed therefore, that an animal model may be predictive of human skin permeability if the FoD value in the permeability between species is less than 3. In this study by applying these criteria, FoD values of < 3 were obtained from all species (Table 2). However, there was FoD value as high as 1.70 for male rat abdomen and female mice abdomen, which were not much different.

Degree of correlation between animals and human skin has been observed for many animal models and appear to vary as the physicochemical nature of the chemicals involved varies. Hawkins and Reifenrath (21) suggested that the FoD values between pig and human skin increases as the lipophilicity of the penetrant increases. They demonstrated good agreement between *in-vivo* human and *in-vitro* pig skin absorption data for hydrophilic penetrants (FoD value ~ 1: caffeine,

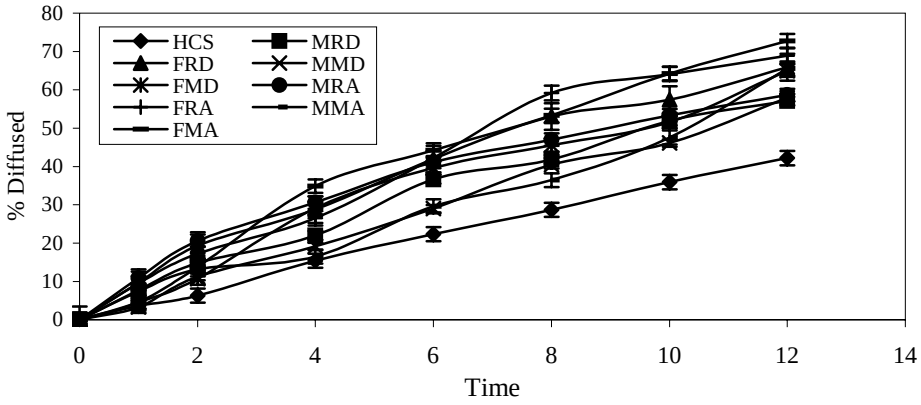

Figure 3. Comparison of permeability and Influence of sex
HCS – Human Cadaver Skin; MRA - Male Rat Abdomen; FRA - Female Rat Abdomen ; MRD - Male Rat Dorsal; FRD - Female Rat Dorsal; MMA - Male Mice Abdomen; FMA -Female Mice Abdomen; MMD - Male Mice Dorsal ; FMD - Female Mice Dorsal.

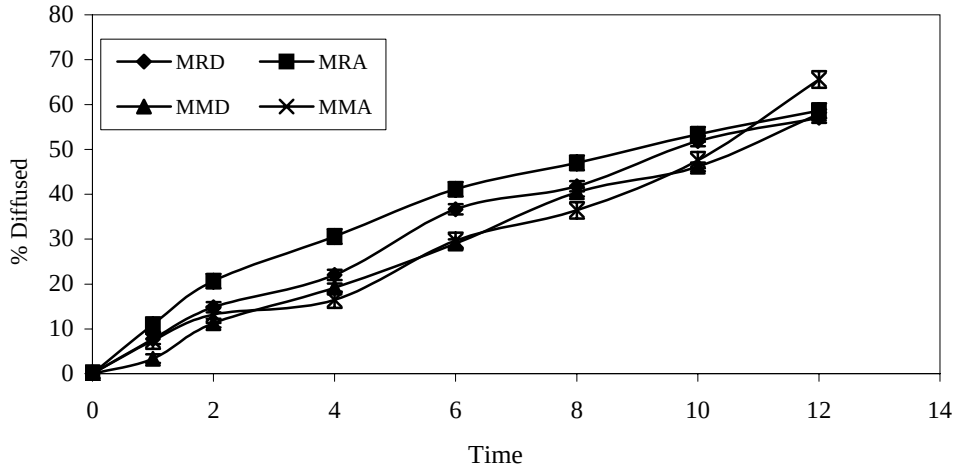

Figure 4. Influence of site of application (Male rat and mice).
MRD: Male Rat Dorsal, MRA: Male Rat Abdominal, MMD: Male Mice Dorsal and MMA: Male Mice Abdomen.

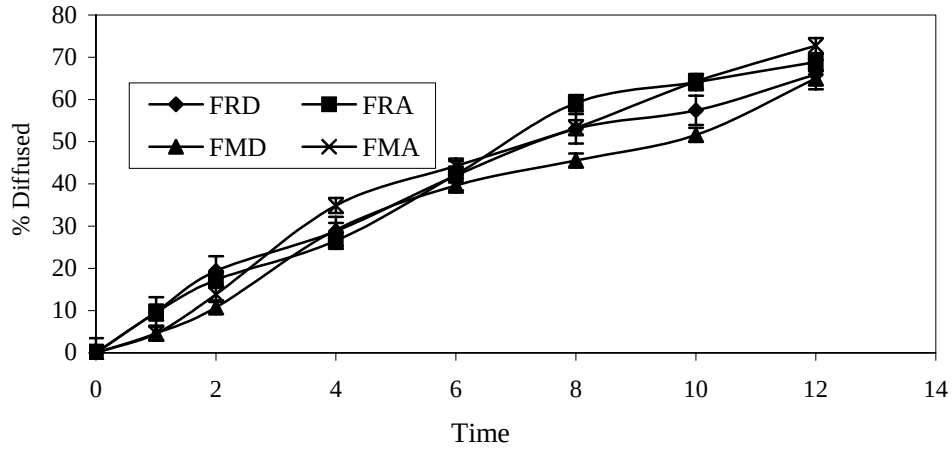

Figure 5. Influence of site of application (Female rat and mice).
FRD: Female Rat Dorsal, FRA: Female Rat Abdomen, FMD: Female Mice Dorsal and FMA: Female Mice Abdomen.

benzoic acid etc.) but loss of correlation was observed as the lipophilic character of the penetrant increased. Similarly in this study the drug which employed was hydrophilic and the calculated FoD values were found to be in the range of 1.26-1.70 (FoD value ~ 1) for all tested skin membranes. Thus FoD values of < 3 demonstrated the feasibility of all skin membranes as an alternative model to human skin for the tested drug.

Table 2. Factor of difference (FoD) values calculated from permeability data of human, rat and mice skin.

Membrane used	Man	FoD values
Male Rat Abdomen (MRA)	1	1.63
Female Rat Abdomen (FRA)	1	1.70
Male Rat Dorsal (MRD)	1	1.26
Female Rat Dorsal (FRD)	1	1.67
Male Mice Abdomen (MMA)	1	1.55
Female Mice Abdomen (FMA)	1	1.70
Male Mice Dorsal (MMD)	1	1.36
Female Mice Dorsal (FMD)	1	1.44

Correlation of in-vitro flux to in-vivo delivery of drug

The pharmacokinetic properties of LP have been studied and are well established (1). Based upon the flux achieved from best formulation F1, it is possible to estimate the magnitude of a patch that could delivers a therapeutic dose *in vivo* (22) Following equation describes flux, pharmaco-kinetic parameters and body weight in relation to the dimensions of a patch required to deliver a therapeutic dose.

$$JA = Cl \times Cp \times W \qquad (7)$$

Where, J is the flux (µg/cm^2/hr), A is the area of application (cm^2), Cp is the plasma concentration (µg/ml) and W is the body weight (average male, 70 kg), Cl is the clearance.

From the findings of this work and considering 70 kg as body weight, a patch with a calculated area of 13.85 cm^2, formulated in the same manner as the prototype area of 6.15 cm^2 would be expected to deliver a therapeutically useful dose *in vivo.*

CONCLUSION

Primary results of this study demonstrate the feasibility of developing transdermal drug delivery of LP. Capsaicin may be useful to increase permeation. The data in this study provides further evidences that male rat dorsal and male mouse dorsal, whilst not a perfect model for human skin permeability, but have closer permeability properties than several other species tested, thus could be used for future pharmacokinetic and toxicokinetic studies as well as to determine the extent of metabolism of LP by viable human skin.

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