American Journal of Agricultural and Biological Sciences 3 (2): 444-450, 2008
ISSN 1557-4989

Comparative Permeation Studies between Scale Region of Shed Snake Skin and Human Skin *In vitro*

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Abstract: Scales of shed king cobra (*Ophiophagus hannah*) skin from the dorsal portions, (SSS) and human breast epidermis (HE) were used as barrier membranes for comparison in an *in vitro* drug permeation study of nine active compounds (MW range 150-300 g mol^{-1}, pK_a 3-10). Each compound, saturated in a donor solution at pH 4.0 or 5.6, permeated through the barrier membrane, fully hydrated. A receptor solution at pH 7.4 was sampled for quantification at its λ_{max} by UV-Visible spectrophotometry and/or HPLC. The permeability coefficients of nine compounds were correlated to the n-octanol/water partition coefficients of these compounds. The permeability coefficient of these compounds using HE and SSS was correlated. Scales of shed skin from the dorsal portions of king cobras were shown to be well correlated to the human breast epidermis in this *in vitro* aqueous permeation study of these compounds.

Key words: Scale region, shed king cobra skin, human skin, permeation, barrier membrane

INTRODUCTION

Permeation of drugs through the skin barrier has been the basis of transdermal delivery[1]. The human skin is an effective and selective barrier to chemical permeation[2]; however, it is not always possible to do actual skin permeation studies in humans. *In vitro* permeation studies and model barrier membranes using excised skins (human cadaver and animals) or artificial materials have been employed. Animal skins generally gave higher permeation profiles than the human skin because of the differences in thickness, stratum corneum, density of hair follicles, sweat glands and appendages[3]. The idea of using shed snake skin as the barrier membrane in *in vitro* permeation studies was introduced by Higuchi and Kans[4]. It is a non-living tissue which can be obtained without sacrifices, lacks hair follicles, and gives less variation than human or animal skin. Shed skins of several snakes, such as *Elaphe obsoleta*[3,5,6,7,8], *Python reticulatus*[9] and *Ophiophagus hannah*[10], have been investigated. Haigh *et al*.[7] emphasized the effect of species, sites and regions of the shed snake skin used. Good correlation of results to human skin suggested the use of shed snake skin as a model membrane for permeation studies despite the differences in anatomical and chemical compositions[5,11,12]. Haigh *et al*.[7] suggested that the differences in lipid contents, a vital component of the skin, between shed snake and human skin could affect the permeability of compounds.

Shed snake skin usually consists of three distinctive layers; beta, mesos and alpha-layers[3]. Major lipid compositions of shed snake skins are phospholipids while that of the human skin are ceramides[13]. Shed snake skin is composed of two very different regions; scales and separating these, hinges. The scales are rigid while the hinge is elastic. The scales on the dorsal part are much smaller and usually thinner than the scales on the ventral part. Most of the studies related to the use of shed snake skins employed whole skin which included the scales and hinges, except that of Harada *et al*.[9] which used only the scales of *Python reticulatus* for *in vitro* permeability of salicylic acid.

Factors affecting the permeation of different compounds include ionization[14], partition, and molecular weight[14]. Takahashi *et al*.[6] used six basic compounds with low pK_a (0.6-5.3) and found that non-ionized forms permeated at a greater rate ($0.01\text{-}33.3\times10^{-3}$ cm h^{-1}). Potts and Guy[15] selected more than 90 compounds from various reports with molecular weights ranging from 18 to 750 and n-octanol/water partition coefficients (log $K_{o/w}$) ranging from ~3 to 6 to predict skin permeability using multiple linear regressions.

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In this study, weak acidic compounds and/or some of their salt forms, with molecular weights ranging from 150 to 300 and with different degrees of water solubility and partition coefficients, were selected to compare the permeabilities through scales of the shed king cobra skin (SSS) and human epidermis (HE). Each compound permeated from a saturated donor solution at the skin pH of 4.0 (for salicylic acid) or 5.6 (for the others) into a receptor phase containing phosphate solution, pH 7.4 representing subdermal conditions. To evaluate the possibility of SSS as a model barrier membrane, permeation of these model compounds across SSS and HE were compared.

MATERIALS AND METHODS

Materials: Methyl Paraben (MP) was purchased from Fluka (Buchs, Switzerland); Propyl Paraben (PP), Butyl Paraben (BP), Diclofenac Sodium (DS), ibuprofen (Ibu), Paracetamol (Para) and Methyl Salicylate (MeSA) from SigmaAldrich (Steinheim, Germany); Salicylic Acid (SA) from Ajax Finechem (Seven Hills, Australia); sodium salicylate (NaSA), n-octanol and methanol (HPLC grade) from BDH Laboratory Supplies (Poole, England). Other reagents used were analytical grade and used as received.

Apparatus: Side-by-side diffusion cells, with an effective area of 0.694 cm^2 and a capacity volume of 3 ml each (Crown Glass Company Inc., U.S.A.), a microprocessor thickness gauge (Minitest 600B Typ 1210306, Cologne, Germany), UV-Vvisible spectrophotometer (Shimadzu UV1700; Pharmaspec, Japan), and high performance liquid chromatography (HPLC; Agilent 1100 series, Waldbronn, Germany) were used in this study.

Partition coefficient determination: Partition coefficients between n-octanol and McIlvaine citrate-phosphate buffer solution at pH 5.6 (CPS) were determined. A mixture of an equal volume of n-octanol and CPS was pre-equilibrated for at least 1 h at 32±1°C. A specified amount of each drug was dissolved in the pre-equilibrated n-octanol and CPS at an equal concentration of 50-100 µg/ml each. A volume of 5 ml each of the n-octanol and CPS was thoroughly mixed, then shaken to equilibrate at 32±1°C for 12 h. Samples from both n-octanol and CPS were analyzed spectrophotometrically for drug contents. Apparent partition coefficient between n-octanol and CPS of each compound was calculated, as follows:

$$K_{o/w} = \frac{C_o}{C_w} \quad (1)$$

where:

$K_{o/w}$ = Partition coefficient.
C_o = Concentration of drug in n-octanol phase.
C_w = Concentration of drug in CPS phase.

Preparation of donor suspension and solubility studies: To calculate the apparent permeation parameters of each compound, the total solubility was determined under the same conditions as the permeation studies and represented by C_d for the concentration of the donor solution. An excess amount of each compound was added to provide an oversaturated solution in 30 ml CPS in a tightly-closed container. The suspension was stirred overnight at temperature of 32°C. Filtrated samples (0.45 µm Nylon filter; Whatman International Ltd., England) were then analyzed at their maximum wavelengths by using UV-Visible spectrophotometry. Samples of the saturated solutions were introduced into the donor compartments to determine permeation fluxes across SSS and HE.

Sources of skins: Human Epidermis (HE) and shed king cobra scale (SSS): Human skin was obtained after breast plastic surgery in Srinagarind Hospital, Khon Kaen University, Thailand. Each volunteer donated the skin by following the protocol approved by the Ethics Committee with a signed informed consent. The donors were non-smoking Asian females (n = 11). Immediately after excision, subcutaneous fat was removed with tweezers and surgical scissors. The epidermis of the skin samples was gently separated from the underlying dermis after removal of subcutaneous fat and immersion in water at 60°C for 45 sec and then gently peeling off the epidermis[16]. The resultant epidermal sheets were thoroughly dried on a sheet of filter paper (no.1, Whatman International Ltd., England) and stored flat in aluminium foil at 4-8°C until use.

Shed king cobra skins (n = 4) were collected at Khok Sanga King Cobra Club, Khon Kaen, Thailand. Each sheet of the skin was cleaned prior to storage in a tightly-closed container at 0±1°C until use. Each scale of the dorsal skin with a diameter of larger than 1 cm was used for one experiment after overnight hydration in CPS at room temperature. One sheet of the shed king cobra skin gave approximately 30-40 scales for the experiment.

Average thicknesses of HE and SSS, measured by using a microprocessor thickness gauge, were 24.0±3.0 and 18.7±2.0 µm (n = 25), respectively.

***In vitro* permeation studies:** A piece of hydrated barrier membrane was mounted between the two compartments of the side-by-side diffusion cells with the outer membrane surface facing towards the donor

Table 1: HPLC systems for the determination of the compounds

Compounds	Mobile phase (volume ratio)	Wavelength (nm)	Approx. retention time (min)
MP	Methanol : 0.02M acetate buffer pH 4.0 (25 : 75)	256	13.0
PP	Methanol : 0.02M acetate buffer pH 4.0 (25 : 75)	256	48.6
BP	Methanol : 0.02M acetate buffer pH 4.0 (25 : 75)	256	9.2
Para	Methanol : deionized (DI) water (80 : 20)	243	7.8
Ibu	Methanol : 0.1% phosphoric acid in DI water (80 : 20)	225	8.0
DS	Methanol : 0.1% phosphoric acid in DI water (75 : 25)	276	11.5
SA	Acetonitrile : DI water (60 : 40)	295	3.0
MeSA	Acetonitrile : DI water (60 : 40)	295	7.0
NaSA	Isopropanol : 1% formic acid in 0.1M potassium hydrogen orthophosphate (34.2 : 65.8)	295	13.4

compartment. The donor compartment was filled with 3.0 mL of saturated (about 2-5 times more solute than the amount required for saturation to ensure constant thermodynamic activity throughout the experiment) with each compound in CPS to keep the concentration of the compound constant at its solubility limit. The receptor compartment was filled with 3.0 mL of Sørensen, modified phosphate buffer solution pH 7.4 (PBS). Both compartments were stirred at 600 rpm and temperature controlled at 32±0.1°C by water jacket circulation. A sink condition was always maintained in the receptor compartment. Samples (2.7 ml) were taken from the receptor compartment and immediately replaced with an equal volume of fresh PBS. UV-Visible spectrophotometry was used to quantify the permeated compounds, except for diclofenac sodium where HPLC was used due to the detection limit of UV-Visible spectrophotometry. The concentration of the compound in the receptor solution was corrected for previous sample removal and cumulative permeated amount (Q, μg cm^{-2}) calculated.

Quantitative analysis

UV-Visible spectrophotometry: The λ_{max} of each compound, obtained by scanning using a UV-Visible spectrophotometer, was as follows: methyl paraben, propyl paraben and butyl paraben 256 nm, paracetamol 243 nm, diclofenac sodium 276 nm, ibuprofen 225 nm, salicylic acid, methyl salicylate and sodium salicylate 295 nm.

Standards of the compounds were freshly prepared at appropriate concentrations in the relevant solvents. Validations were performed by 10 replicates of the standards of within-day and more than 3 replicates of between-days. Linear correlation ($r^2 \geq 0.998$) was used to obtain calibration curves for quantitative analysis.

HPLC analysis: HPLC was used to check and ensure the qualitative analysis of the permeated compounds. The systems used are shown in Table 1. The HPLC systems used, shown in Table 1, employed a HIQ SIL C_{18} column (250 mm×4.6 mm i.d.), 20 μl injection loop volume and isocratic mobile phase with a flow rate of 1.0 ml/min at ambient temperature. The mobile phase was filtered through 0.45 μm nylon membrane filter (Millipore, U.S.A.) and degassed before use. Quantification of the compounds was carried out by integration of peak area compared with the relevant standards. Using paired *t*-test to compare with UV-spectrophotometry of the same samples, analysis of diclofenac sodium was the only compound which was found significant different which might be explained by the findings of Llinàs *et al*.[17]. Therefore, the analysis of permeated diclofenac sodium in our study was performed by HPLC. Standards for diclofenac sodium at 0.01 to 30 μg mL^{-1} in PBS were used.

Data treatment and statistics: Steady-state permeation profiles of the drugs, and solubility or C_d, were used to estimate permeation rate or flux (J) and permeability coefficient (P).

Linear regression was used to analyze the relationship between the cumulative permeation amount of each compound per unit surface area (M, μg cm^{-2}) and time (t, h). For each compound, a correlation coefficient of more than 0.99 with an average of 6 replicates was used to determine its slope, i.e. steady-state flux (J, μg cm^{-2} h^{-1}) according to Fick's first law as shown in Eq. 2 and also lag time (t_L, h). The permeation coefficient (P, cm h^{-1}) was calculated by using Eq. 3, as follows:

$$J = \frac{dM}{Sdt} = PC_d \quad (2)$$

$$D = \frac{Ph}{K_{o/w}} \quad (3)$$

where:

dM/dt = Amount of compound permeated at time interval (h).
S = Effective diffusional area of the diffusion cells (cm^2).
C_d = The concentration of each compound in the donor solution which was controlled at its saturation, i.e. solubility.
D = Diffusion coefficient (cm^2 h^{-1}).
h = Thickness of barrier membrane (cm).

Statistical analysis at a significant level of $p<0.05$ was performed, as follows: comparison of UV-Visible spectrophotometric and HPLC analysis of the same compound by paired *t*-test, cumulative permeated amounts across HE and SSS by Student's *t*-test, and a relationship of P, $K_{o/w}$ and MW of the compounds by multiple linear regression (SPSS program for MS Windows, release 11.5) at a significant level of 0.05.

RESULTS AND DISCUSSION

Table 2 summarizes some physicochemical properties of the compounds used in this study which are a range of drugs with molecular weights (MW) of about 150-300 g mol^{-1}, $K_{o/w}$ about 0.2-10,000 and poorly to highly soluble and/or ionized in aqueous solutions. Steady-state permeation profiles of the nine compounds through SSS, (Fig. 1, 2, 3a) and HE (Fig. 1, 2, 3b), into an aqueous receptor resulted in linear relationships over the sampling period of 24 h.

Table 2: Physicochemical properties of the compounds used

Compound	MW (g mol^{-1})	pK_a	C_d^* (mg/ml)	log $(K_{o/w})^{**}$
MP	152.2	8.17[25]	2.14±0.11	2.0±0.1
PP	180.2	8.35[25]	0.34±0.00	2.1±0.1
BP	194.2	8.37[25]	0.24±0.02	3.0±0.1
MeSA	152.1	-	0.69±0.04	2.2±0.3
SA	138.1	3.0[26]	15.0±1.94	-0.5±0.0
NaSA	160.1	3.0[21]	682±61.8	-0.7±0.0
Ibu	206.3	5.3[9]	0.88±0.29	0.7±0.1
DS	318.1	4.2[9]	0.11±0.05	2.5±0.0
Para	160.1	9.5[26]	13.4±0.11	0.4±0.0

* n = 6, ** n = 3

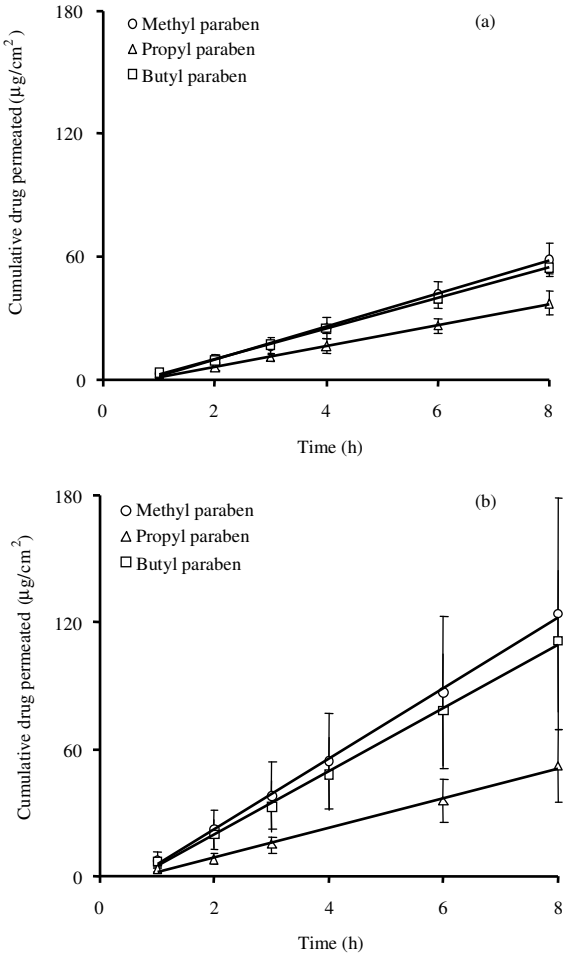

Fig. 1: Steady state permeation profiles of methyl paraben, propyl paraben and butyl paraben paraben through (a) SSS and (b) HE at 32°C. Each point represents the mean ± SD (n = 6). Lines are best fitted by linear regression analysis

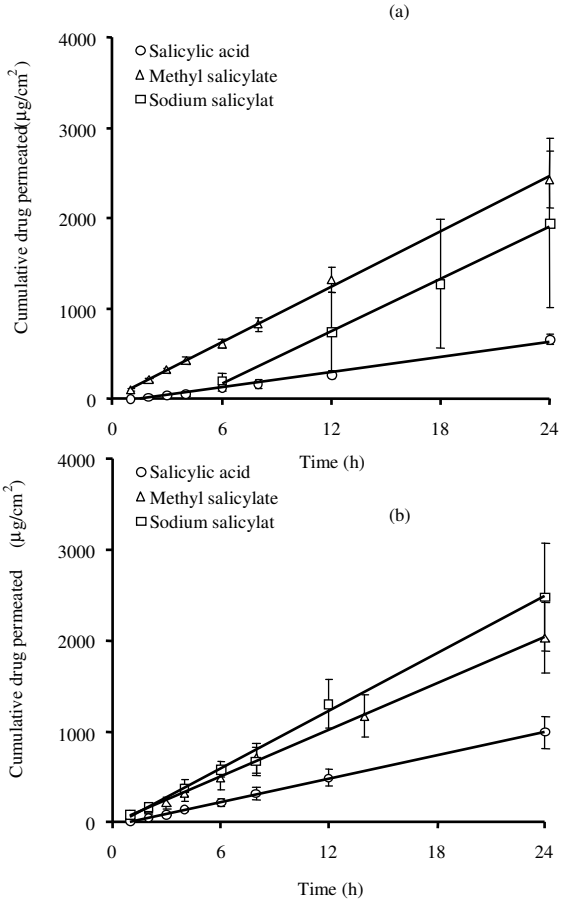

Fig. 2: Steady state permeation profiles of salicylic acid, methyl salicylate and sodium salicylate through (a) SSS and (b) HE at 32°C. Each point represents the mean ± SD (n = 6). Lines are best fitted by linear regression analysis

Table 3: Mean flux, lag time, permeability coefficient and diffusion coefficient of the compounds permeated through scales of shed king cobra skin (SSS) and human epidermis (HE) (n = 6)

	Mean flux (μ cm^{-2} h^{-1})		Lag time (h)		Permeability coefficient ($\times10^{-3}$ cm h^{-1})		Diffusion coefficient ($\times10^{-6}$ cm^2 h^{-1})	
Compound	HE	SS	HE	SSS	HE	SSS	HE	SSS
MP	16360±7.15	7.96±1.96	0.65±0.04	0.74±0.48	7.76±3.34	3.72±0.92	0.14±0.06	0.07±0.02
PP	7.00±1.80	5.05±1.02	0.72±0.11	0.70±0.61	20.49±5.27	14.78±2.99	0.30±0.08	0.21±0.05
BP	14.90±4.75	7.40±0.72	0.66±0.16	0.62±0.17	62.41±19.87	31.01±3.00	0.12±0.044	0.06±0.01
MeSA	85.49±16.00	99.30±13.99	0.26±0.54	0.00	124.55±23	144.65±0.203	1.36±0.26	1.58±0.32
SA	43.23±7.73	28.43±5.17	0.92±0.28	1.51±0.65	2.88±0.58	1.89±1.62	15.39±3.10	10.12±1.84
NaSA	105.62±1.33	96.17±20.22	0.50±1.08	4.20±3.16	0.15±0.03	0.14±0.00	1.28±0.28	1.17±0.17
Ibu	10.82±3.99	5.04±1.22	0.21±0.34	0.01	12.29±4.01	5.37±1.38	4.34±1.42	2.02±0.49
DS	0.53±0.19	0.40±0.04	3.54±0.64	5.57±0.61	4.96±1.28	3.76±0.43	0.03±0.01	0.02±0.15
Para	0.69±0.29	1.01±0.29	0	0.19	0.05±0.02	0.08±0.02	0.04±0.02	0.06±0.02

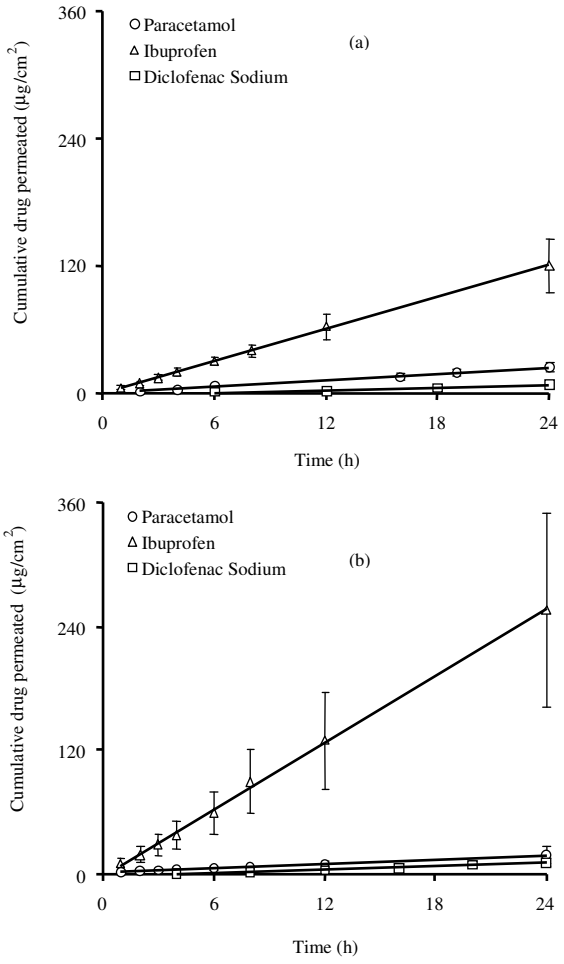

Fig. 3: Steady state permeation profiles of paracetamol, ibuprofen and diclofenac sodium through (a) SSS and (b) HE at 32°C. Each point represents the mean ± SD (n = 6). Lines are best fitted by linear regression analysis

Calculation of the results from these figures and parameters from Table 2 results in steady-state mean fluxes, lag times, permeability coefficients (P) and diffusion coefficients (D) of each compound through SSS and HE, shown in Table 3.

Methyl paraben gave a higher permeation than propyl paraben and butyl paraben. This was found when using both SSS and HE as shown in Fig.1a and b, respectively. As expected, an increase in the ester chain length of parabens decreased the permeation flux but increased permeability coefficient. The results were similar to those conducted by using whole shed skin of *Elaphe obsoleta*[3,18] and comparable to the results obtained by using the human skin[19,20].

Comparison of permeation profiles of methyl salicylate, salicylic acid and sodium salicylate through SSS and HE, were shown in Fig. 2a and b, respectively. Sodium salicylate, a salt form with a pK_a of 3.0[21], ionized in the donor solution and was found to permeate after an initial period of the lag time. Methyl salicylate, on the other hand, permeated at a higher rate than sodium salicylate and salicylic acid because of its hydrophobicity and high partition coefficient. The pH of the donor solution which was suspended by salicylic acid was 4.0, the same pH that reported by Harada *etal*.[9]. At this pH, salicylic acid showed precipitation due to saturation in the donor, its concentration used was 3 times higher than that of Harada *et al*.[9], and as a result, our permeability coefficient was also 3 times higher.

The permeation profiles of ibuprofen, diclofenac sodium, and paracetamol through SSS and HE are demonstrated in Fig. 3a and b, respectively. The permeability coefficient of paracetamol was lower than that of diclofenac sodium and ibuprofen due to higher aqueous solubility and lower partition coefficient.

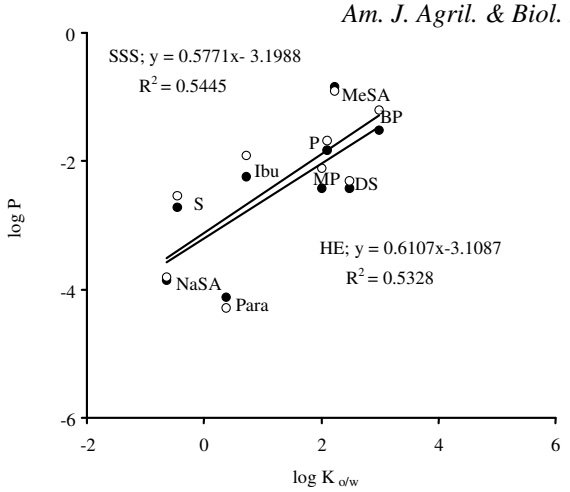

Fig. 4: Relationship between Log P and Log $K_{(o/w)}$ at 32±1°C of all model drugs used. (o) scale of shed king cobra skin (SSS) and (•) human epidermis (HE)

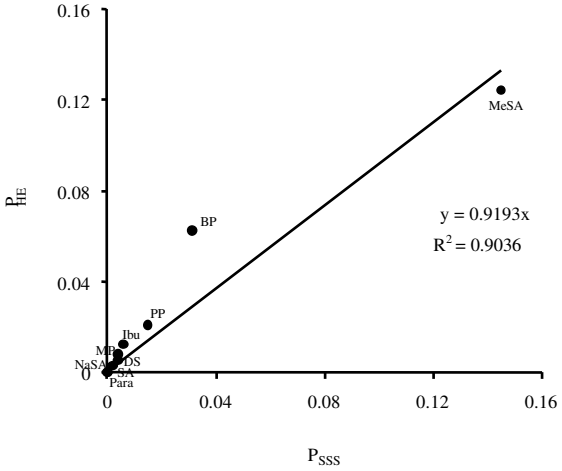

Fig. 5: Correlation between permeability of the model drugs through the human epidermis (P_{HE}) and the scales of shed king cobra skin (P_{SSS})

The solubility of paracetamol in the donor phase was higher than that of ibuprofen, while $K_{o/w}$ was lower, as shown in Table 2. In comparison with ibuprofen, paracetamol distributed in the donor phase rather than partitioned through the barrier membrane into the receptor medium, resulted in lower permeability coefficient and diffusion coefficient. This finding was different from that reported by Bhatt *et al*.[11] which employed a mixture of propylene glycol and methanol as the donor solution of ibuprofen and paracetamol. The donor concentrations of both compounds in this present study, much less than those reported by Bhatt *et al*.[11].

Diclofenac sodium with its high $K_{o/w}$ and low C_d was poorly soluble in aqueous solution but highly distributed into the SSS. Its permeation was, thus, rather low and slow and its lag time was found to be approximately 4 h which is similar to that reported by Morimoto *et al*.[22]. Additionally, when comparison to salicylic acid, diclofenac sodium permeated much slower, similar to the results of Gabboun *et al*. [23].

Figure 4 displays a linear relationship between log (P) and log ($K_{o/w}$) of the compounds using SSS and HE as the barrier membranes with correlation coefficient (r^2) of 0.544 and 0.534, respectively. This suggests that the overall profiles followed Fick's first law[24]. Further analysis of the relationship of permeability and $K_{o/w}$ and MW by multiple regressions could display by these following equations:

$$\text{SSS: } \log P = (0.575)\log K_{o/w} + (-0.066)\log MW - 3.046 \quad (r^2 = 0.544) \quad (4)$$

$$\text{HE: } \log P = (0.611)\log K_{o/w} + 0.336\log MW - 3.863 \quad (r^2 = 0.534) \quad (5)$$

Multiple regression analysis of both indicate no significant difference ($p<0.1$). Both SSS and HE gave similar increase in permeability coefficients of drugs and related to lipophilicity and MW.

Our findings demonstrated the similarity between 2 different types of barrier membranes, HE and SSS, in the permeation of nine compounds which occurred by a zero order process in accordance with Fick's first law. The correlation between permeability of the compounds across HE and SSS by a plot between P_{HE} and P_{SSS}, as shown in Fig. 5, gave a linear line with a slope = 0.9193 and $r^2 = 0.9036$

CONCLUSIONS

The dorsal scale of shed king cobra skin behaves as a barrier membrane in this series of *in vitro* permeation studies using nine compounds of varying molecular weights, aqueous solubility and $K_{o/w}$. The higher the molecular weights of the compounds, the slower the permeation rate, and the greater the partition coefficient, the greater the permeability of the compounds. Drugs with molecular weight between 150-320 g mol^{-1} and $K_{o/w}$ about 0.2-10,000 permeated through HE and SSS under sink conditions from the donor solution at pH 5.6 into receptor solution at pH 7.4, mimicking percutaneous absorption. The scales of shed king cobra gave similar results to the human epidermis. Dorsal scales of king cobra can provide a

model for human epidermis and permeability of the compounds studied gave good linear correlation.

ACKNOWLEDGMENTS

Khok-Sanga King Cobra Club and the volunteers and staff at Srinagarind Hospital of Khon Kaen University are thanked for their cooperation and provision of samples. Thailand National Metal and Materials Technology Center (MTEC; MT-B-47-BMD-12-097-G), National Science and Technology Development Agency, Thailand Research Fund Master Research Grants (TRF-MAG), and the Graduate School, Khon Kaen University are gratefully thanked for their financial support.

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