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Diclofenac metabolic profile following *in vitro* percutaneous absorption through viable human skin

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SUMMARY

The extent of metabolism of diclofenac sodium in excised viable human skin was investigated using combination HPLC and radioactivity assay. In an earlier diffusion experiment using an *in vitro* flow-through diffusion system, radiolabelled diclofenac sodium in either lotion (Pennsaid[PR]) or aqueous solution was applied to viable human skin, either as single dose or multiple dose (8 times over 2 days). In this study, the receptor fluid samples from the diffusion experiment were subjected to extraction and the aliquot was analysed using HPLC to separate diclofenac and authentic metabolites. Based on the radioactivity of each HPLC fraction, the collection time of the fractions was compared with the retention time of diclofenac and metabolites in standard solutions. The samples from a single or multiple dose application of lotion showed radioactivity in mainly one fraction, whose retention time corresponded with diclofenac. Other HPLC fractions showed none or only small amounts of radioactivity within the error range of the assay. The same results were obtained with the pooled samples from the application of the lotion or of aqueous solution. The results suggest that diclofenac sodium does not undergo metabolism in viable human epidermis during percutaneous absorption *in vitro*. Hence, with topical application to human skin *in vivo*, diclofenac will be delivered with minimal, if any, metabolism.

INTRODUCTION

Diclofenac sodium (*o*-[2,6-dichloroanilino] phenyl acetic acid, sodium salt) is a phenyl derivative that is widely used as a potent non-steroidal anti-inflammatory drug (NSAID) for the treatment of both inflammation and pain of arthritis and other painful conditions in general. The common route of administration is oral, but this route entails systemic distribution which also brings adverse side effects, including gastric or duodenal ulceration and bleeding, hypertension, diarrhea, renal dysfunction and fluid retention. As an alternative route of administration, transdermal delivery of diclofenac has been studied (1–4). Since diclofenac sodium is not extensively absorbed through the skin due to its hydrophilic nature (5), this involves the use of permeation enhancers or co-solvents to increase penetration (6,7). More recently, a patented carrier lotion including dimethyl sulfoxide (DMSO) has been used in Pennsaid[PR] lotion and showed an increase in the *in vitro* permeation through viable skin (8). *In vivo* pharmacokinetic data have also shown

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minimal systemic absorption of diclofenac from topical application of Pennsaid[PR] lotion (9).

Following human absorption, diclofenac sodium is extensively metabolized, as determined by the analysis of urine and plasma (10,11). The metabolism of the drug occurs in two ways: by direct conjugation; or by hydroxylation of the aromatic rings, with or without subsequent conjugation. About 60–70% of an oral dose of diclofenac sodium is excreted in the urine as conjugates of mono- or di-hydroxylated metabolites (11). The metabolism upon topical administration showed a similar pattern (3,9). However, it is not known yet whether metabolism occurs systematically or during drug passage through the skin.

This study investigates the extent of metabolism of diclofenac in excised, viable human skin after topical application from solution or lotion. The skin used in the experiments was handled to maintain the viability (8) according to the literature (12). Analyses were performed using high-performance liquid chromatography (HPLC) on the organic extracts of the receptor fluid diffusates, to detect any possible metabolites.

MATERIALS AND METHODS

Test material

[^{14}C]-Diclofenac sodium (phenylacetic acid ring-U-^{14}C-diclofenac sodium) code CFQ7204, light yellow solid powder was provided by Amersham Life Sciences and purified by Wizard Laboratories (Sacramento, CA, USA). The specific activity was 9.76 mCi/mmol and radiochemical purity was 99%. The patented carrier lotion (lot no. 977132) for Pennsaid[PR], without diclofenac sodium, was provided by Dimethaid Research, Inc. (Markham, Ontario, Canada). Non radioactive compounds were used as standard for chromatographic analysis: Diclofenac, sodium salt was purchased from Sigma Chemicals, St Louis, MO, USA. The authentic metabolites: 3′-hydroxydiclofenac (CGP 13 294), 4′-hydroxydiclofenac (GP 47 766), 5-hydroxydiclofenac (GP 47 852), 3′-hydroxy-4′-methoxydiclofenac (CGP 30 890) and 4′,5-dihydroxydiclofenac (CGP 14 217) were gifts from Novartis. The compounds were dissolved in methanol and diluted with a mixture of acetonitrile:water (50:50). Perfusate fluid consisting of minimum essential media (MEM) Eagles with Earle's BSS was prepared at the UCSF Cell Culture Facility. Prior to use, 50 µg/ml gentamycin sulfate (Sigma Chemicals) was added in MEM to ensure sterility throughout the experiments.

Previous diffusion experiment

In the previous experiment (8), dermatomed human skin was clamped into a continuous flow-through diffusion cell system, made of glass, with 1 cm^2 surface area of exposed skin. The cell temperature was maintained at 32°C. The donor solutions were either: (i) aqueous solution containing 6 mg/400 µl dose [^{14}C]-diclofenac sodium; or (ii) lotion (Pennsaid[PR] formulation) containing 6 mg/400 µl dose [^{14}C]-diclofenac sodium. Each test solution was applied as a single dose and as multiple doses (at times 0, 4 h, 8 h, 12 h – daily for 2 days) to 1 cm^2 skin surface area. The perfusate fluid, MEM Eagles with Earle's BSS and 50 µg/ml gentamycin sulfate, was pumped at a rate of 3 ml/h. Receptor fluid timed-samples were collected every 4 h of a 48 h study.

Extraction of organic phase

The method to extract the organic phase from the receptor fluid timed-samples was a modification of one from the published literature (13). To a 1.0 ml sample in a centrifuge tube, 0.2 ml 20% aqueous ascorbic acid, 1.0 ml acetate buffer (pH 4.7–5.0) and 5.0 ml diethylether:dichloromethane (2:1) were added. The mixture was shaken for 24 h in a horizontal shaker (Gyrotory® Water Bath Shaker, New Brunswick Scientific, Edison, NJ, USA) at room temperature. Then, the mixture was centrifuged for 2 min at 2500 *g*. The organic phase was allowed to separate from the aqueous phase and then was transferred to a clean tube.

Sample preparation for HPLC

After extraction, 1.0 ml of the organic phase of each receptor fluid sample from the same skin donor and the same treatment was combined and evaporated to dryness under a nitrogen stream. Then, 2.0 ml mobile phase was added. The mixture was shaken for 15 s in a vortex mixer and was ready for HPLC analysis.

To enhance the chance of detecting low-level metabolites, 1.0 ml of every sample that was collected from the topical application of the lotion (from all donors) was pooled (labelled PS) to provide sufficient sample and prepared, as mentioned above, for HPLC analysis. No distinction was made between samples collected after single or multiple doses. The same procedure was performed on samples (of all donors) collected from the topical application of aqueous solution (labelled AQ).

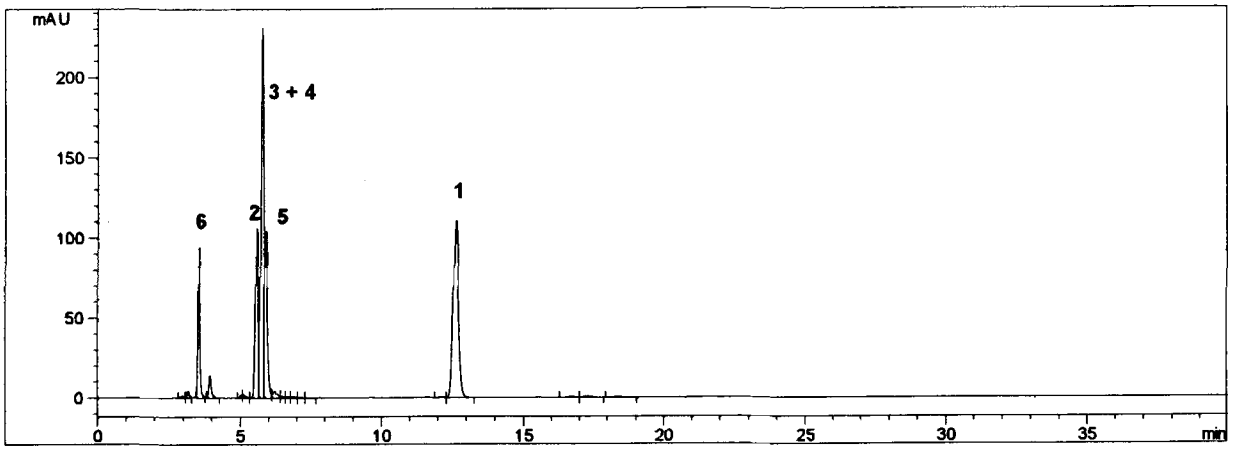

Fig. 1 : Chromatogram of a mixture containing diclofenac sodium (DFN) (1) and its authentic metabolites: 3′-hydroxydiclofenac (2), 4′-hydroxydiclofenac (3), 5-hydroxydiclofenac (4), 3′-hydroxy-4′-methoxydiclofenac (5) and 4′,5-dihydroxydiclofenac (6). HPLC condition: HP-1100 system; column, Luna C18(II) (Phenomenex); mobile phase, acetonitrile:aqueous acetic acid (pH 3.4) 55:45 (isocratic); at room temperature (~20°C); flow rate: 1.0 ml/min; run-time: 40 min; detection, 280 nm.

HPLC analysis

An aliquot (500 µl) of test sample was transferred to a vial for injection into the HPLC system. Analysis, separation and isolation were performed on an HPLC (HP 1100, Hewlett-Packard, USA) comprising of a vacuum degasser, a quaternary pump, an autosampler with an variable-volume injector (0.1–100 µl), and a variable wavelength UV detector. The data acquisition and analysis were performed using a computer running the HP ChemStation 2D software.

Chromatographic separations are performed using a modification of the published procedure (14): column, Luna C18(II) (Phenomenex); mobile phase, acetonitrile: aqueous acetic acid (pH 3.4) 55:45 (isocratic); temperature, room (~20°C); flow rate, 1.0 ml/min; time: 40 min; and detection, 280 nm.

Preparative HPLC

After preliminary study with UV detection, time intervals were defined for collection of fractions. Each separation was performed using the same HPLC system. The separation was repeated 5–10 times to obtain a sufficient amount of radioactivity in the suspected fractions.

Radioactivity assay

An aliquot (1.0 ml) of each separated fraction was transferred to a scintillation vial. Then, 10.0 ml of scintillation cocktail (Universol, ICN, Costa Mesa, CA, USA) was added. Radioactivity was analysed by liquid scintillation counting.

Data analysis

Results from control and test sample counts (expressed as dpm) were transferred to a computer spreadsheet (Quattro Pro 7.0 for Windows 95) and the percent of radioactivity recovered from the applied dose was determined. The percentage of the total radioactivity was calculated from these values.

RESULTS

Feasibility study of HPLC separation

The standard solutions of diclofenac and metabolites were analysed by HPLC, either alone or in combination. The analysis showed that diclofenac can be separated from its metabolites. There is sufficient chromatographic separation for isolation of diclofenac from other compounds. The metabolites can also be distinguished among themselves. The chromatogram of diclofenac and its metabolites is presented in Figure 1.

Validity of extraction procedure

Five samples and their organic phase extracts were analysed by HPLC and the peak areas of diclofenac were compared. This procedure extracted more than 95% of

Table I: Validation of extraction procedure: area of the diclofenac peak in chromatograms before and after sample extraction

	Area (mAu.s)		Percent extracted
	Before extraction	After extraction	(%)
Sample 1	11.8908	11.8411	99.58
Sample 2	2.9432	2.8255	96.00
Sample 3	2.4690	2.4037	97.35
Sample 4	53.2854	51.3677	96.40
Sample 5	2.1282	2.1223	99.72
Average			97.81

Percent extracted = area after extraction/area before extraction x 100.

diclofenac from all samples (Table I). Hence, the extraction method is valid.

Optimization of HPLC separation procedure

The optimization was performed by controlling the flow rate of the mobile phase and the time interval to collect the fractions. The fractions were analysed using a radioactivity assay.

Analysis of a sample from single-dose application of lotion

A series of receptor fluid samples collected from a single-dose application of the lotion was extracted, combined and dissolved in 500 µl of mobile phase of HPLC. The separation of the solution by HPLC was performed 4 times and the fractions were analysed. Based on its retention time, diclofenac was collected between 11–14 min. However the fraction 7–11 min contained about 4% of the total radioactivity (Fig. 2A). Therefore, more fractions were collected over the earlier time prior to the appearance of diclofenac. Analysis showed that these early fractions contained less than 3% of the total radioactivity (Fig. 2B). A third analysis was performed with a narrower time interval, but the radioactivity found in fractions other than that containing diclofenac did not differ significantly from background (data not shown).

Analysis of a sample from multiple dose application of lotion

A series of receptor fluid samples collected from a multiple dose application of the lotion was extracted, combined and dissolved in 500 µl of mobile phase of HPLC. The separation of the solution by HPLC was

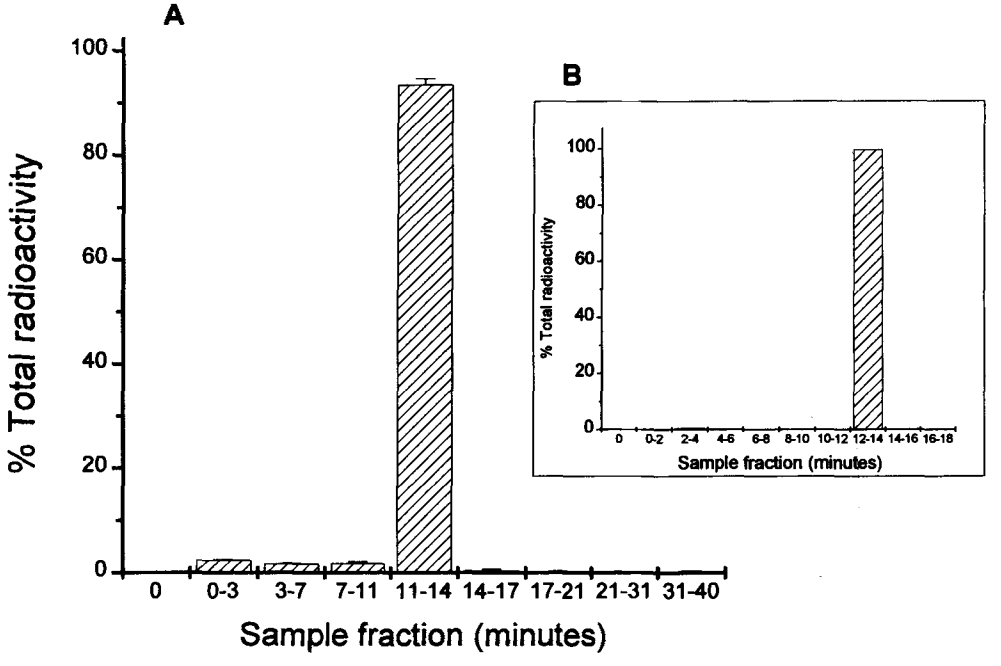

Fig. 2: The proportion of radioactivity found in the HPLC separated fractions of the samples from a single dose application of Pennsaid^{PR} lotion on excised human skin.

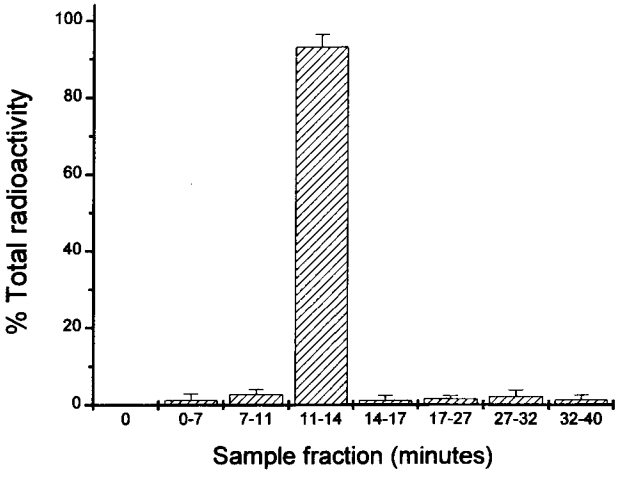

Fig. 3 : The proportion of radioactivity found in the HPLC separated fractions of the samples from a multiple dose (8 times with a 4 h interval) application of Pennsaid[PR] lotion on excised human skin.

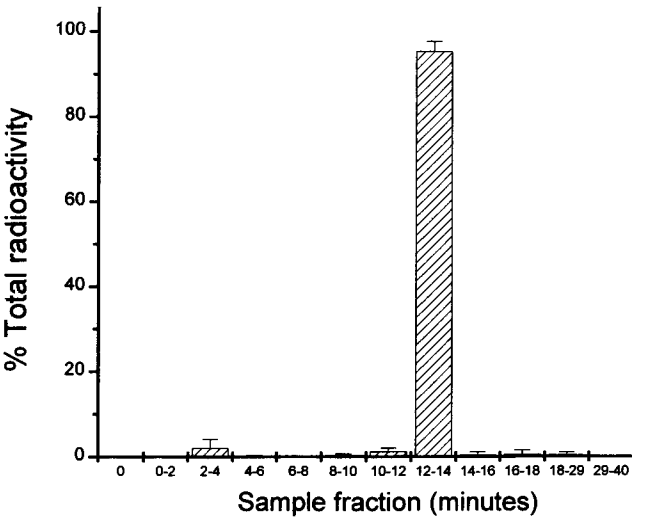

Fig. 4 : The proportion of radioactivity found in the HPLC separated fractions of the combined samples from the application of Pennsaid[PR] lotion on excised human skin.

performed 4 times and the fractions were analysed. Based on its retention time, diclofenac was collected between 11–14 min. The radioactivity found in other fractions did not differ significantly from background (Fig. 3).

Analysis of the pooled sample from the application of lotion

The pooled sample PS was evaporated to dryness and dissolved in 500 µl of mobile phase of HPLC. The separation of the solution by HPLC was performed 10 times and the fractions were analysed. Based on its retention time, diclofenac was collected between 12–14 min. The radioactivity found in other fractions did not differ significantly from background (Fig. 4). Analysis of a second aliquot showed a similar result (data not shown).

Analysis of the pooled sample from the application of aqueous solution

The pooled sample AQ was evaporated to dryness and dissolved in 500 µl of mobile phase of HPLC. The separation of the solution by HPLC was performed 10 times and the fractions were analysed. Based on the retention time, diclofenac was collected between 12–14 min. A small amount of radioactivity (6%) was found in the fraction collected between 4–6 min (Fig. 5). This time range corresponds to the retention times of the hydroxy

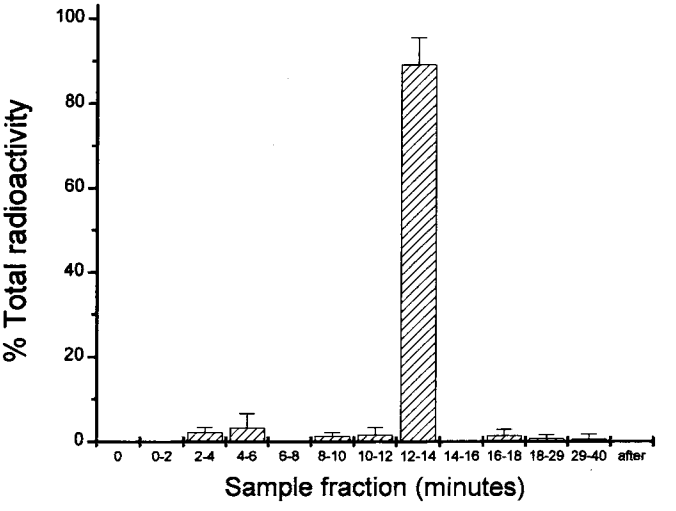

Fig. 5 : The proportion of radioactivity found in the HPLC separated fractions of the combined samples from the application of aqueous diclofenac solution on excised human skin.

metabolites of diclofenac. However, when analysis of a second aliquot was performed, the radioactivity of this fraction was below the background. The radioactivity found in other fractions did not differ significantly from background.

DISCUSSION

As published in the literature, diclofenac sodium is extensively metabolised in the body upon topical application (3,9), but it is not yet known whether this compound is metabolised in viable epidermis during its transit time in the skin. In the systemic circulation, two main processes occur: hydroxylation and conjugation. Since skin probably lacks the ability to form conjugates, the metabolites we might expect would be the products of hydroxylation only.

HPLC is a versatile method to separate diclofenac and authentic metabolites (14–17). In this study, HPLC was optimized to provide sufficient intervals between retention times of the parent compound diclofenac and its authentic metabolites. The dimension of the column was chosen to be 250 mm x 4.6 mm to provide an optimal efficiency in both analysis and separation. Hypersil ODS column (Hewlett Packard) was used in the preliminary experiments to determine the feasibility of separation, the quality of extraction and the optimal composition of mobile phase. However, upon consultation with the manufacturer, it was decided to discontinue the use of this column, because the column material cannot withstand a pH below 4. Another column with a similar separation specificity, Luna C18(II), is known to withstand low pH, and was used instead.

The separation was performed manually, since there is no existing protocol specifying the time interval. Although each separation procedure was performed with great care, nevertheless variation can still occur due to technical factors including inadequate equilibration of the mobile phase in the column, especially during the first separation. A sufficient time is needed to flush the column with mobile phase to avoid a change in composition, for example when a new batch of mobile phase is used. Therefore, it is possible that the retention time of a certain compound will shift slightly and a part of the compound then be collected in an adjacent fraction. This seems to have occurred with in our work with one sample. However, repeated separation with a second aliquot showed that the small amount of distinct radioactivity in some fractions was not reproducible. There were small traces of radioactivity in the fractions predicted to contain some metabolites, but further analyses showed that the amount fell within the error range of 5% and was not statistically significant.

The stability of biological samples containing diclofenac sodium or its metabolites can be maintained for 5 months in storage at –20°C and is not influenced by repeated freezing-thawing cycles during this period of time (15).

CONCLUSIONS

The results indicate that during absorption of diclofenac by viable human skin under *in vitro* conditions, diclofenac sodium does not undergo metabolism. Therefore, with topical application of aqueous solution or Pennsaid^{PR} lotion to human skin, *in vivo*, diclofenac will be absorbed with minimal, if any, metabolism and is available for local and systemic activity.

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