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Note

Chromatographic determination of percutaneous absorption of topical non-radiolabelled prednisolone in vivo, and preliminary application to transdermal pharmacokinetics

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Recent investigations of topical steroid percutaneous absorption [1–3] have been carried out using animal or human skin and radiolabelled molecules. Early studies using live human skin were also carried out using [^{14}C]hydrocortisone [4–6]. Since the amounts of steroid absorbed transdermally are very small, measurement of blood and/or urine steroids without radioactivity should be quite difficult if not impossible by conventional analytical techniques such as chromatography. It is well known that topical steroids cause diverse pharmacological effects similar to those resulting from their oral administration [7]. Thus, it may be of clinical significance to investigate the pharmacokinetics of topical steroid percutaneous absorption using live human skin.

This paper reports the development of a novel chromatographic technique that would be sufficiently sensitive for steroid detection without the use of radiolabelled molecules. A profile of prednisolone urinary excretion corresponding to prednisolone 17-valerate 21-acetate percutaneous absorption in a healthy subject was obtained and examined.

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EXPERIMENTAL

Assay

Our high-performance liquid chromatographic (HPLC) method for the measurement of prednisolone in urine was conducted in the following manner. A 5-ml urine aliquot was spiked with 10 μg of dexamethasone and made basic with 100 μl of 5% sodium carbonate solution. This mixture was shaken vigorously with 1 ml of diethyl ether for 1 min using a vortex mixer followed by separation of the organic layer. The aqueous layer was washed four times with 1-ml aliquots of diethyl ether. The combined organic layer was passed through a short diatomaceous earth column (70 mm×4 mm I.D., Kusano Scientific, Tokyo, Japan) previously pretreated with 100 μl of 4% sodium hydroxide solution. The eluent was collected in a centrifuge tube and evaporated to dryness. The resulting residue was redissolved in 20 μl of 10% methanol in dichloromethane and analysed by HPLC. At this particular time, it was important to inject the entire sample solution into the injector port since the sample solution contained no internal standard for quantification.

The HPLC apparatus included a Model 635 reciprocal pump (Hitachi, Tokyo, Japan) and an SPD-2A UV detector (Shimadzu, Tokyo, Japan) set at 240 nm. The silica gel column, LiChrosorb Si-100, 10 μm, 250 mm×4 mm I.D., from Merck (Darmstadt, F.R.G.), was washed with 0.5% sulphuric acid and then with distilled water until the eluent became neutral and finally with methanol before the analysis. The chromatographic solvent system consisted of 0.1% water, 4% methanol and 30% dichloromethane in *n*-hexane; the flow-rate was set at 2.0 ml/min. Following the appearance of the standard peak of dexamethasone, the eluate was collected for 2–3 min, then spiked with 10 ng of authentic cortisol as the internal standard for prednisolone quantitation. The mixture was evaporated to dryness and the resulting residue, after being redissolved in 20 μl of 10% methanol in dichloromethane, was chromatographed a second time, using a solvent system containing 0.1% water, 10% ethanol and 30% dichloromethane in *n*-hexane. The fraction that eluted 3–4 min after the injection was collected and evaporated to dryness. The residue was redissolved in 20 μl of 10% methanol in dichloromethane and chromatographed for the third time for the quantitative determination of prednisolone. The solvent system was 0.1% water and 5% methanol in dichloromethane, at a flow-rate of 1.0 ml/min. A calibration curve was obtained by adding known amounts of prednisolone to prednisolone-free (control) urine followed by the analytical procedures mentioned above.

Application of drug

A 15-g sample of a commercially available cream preparation for topical use, containing 3% prednisolone 17-valerate 21-acetate (Ridomex-Kowa®, Kowa Pharmaceutical, Tokyo, Japan) was applied to an area (1000 cm^2) of the trunk

skin surface of a healthy male volunteer. The cream was uniformly spread over the restricted area with the fingers. A plastic film (Saran Wrap) was placed over the creamed area and taped in place so as to facilitate occlusive dressing (ODT) [8]. After 6 and 12 h, the cream was gently removed with a stainless-steel spatula and replaced with 15 g of the same fresh cream so that the original drug concentration on the skin surface would be maintained. After 24 h, the cream was removed completely, and the skin surface was cleaned with a warm wet towel. The total amount of drug applied in topical form was equivalent to 1000 mg of prednisolone. A control urine specimen was collected prior to administration of the cream. The 72-h timed urine collection was carried out following removal of the cream. All urine specimens were stored at $-20°C$ until analysis.

Pharmacokinetic analysis

The area under the urinary drug excretion rate–time curve (AUC), the area under the moment curve (AUMC) [9] and the mean residence time (MRT) [10] were calculated, respectively, as follows:

$$\text{AUC} = \int (dX_u/dt)dt \tag{1}$$

$$\text{AUMC} = \int (t dX_u/dt)dt \tag{2}$$

$$\text{MRT} = \text{AUMC}/\text{AUC} \tag{3}$$

where dX_u/dt is the urinary excretion rate. The values for AUC and AUMC were obtained by the trapezoidal method using urinary excretion rate–time and (time)·(excretion rate)–time data, respectively.

RESULTS AND DISCUSSION

As part of the process of cleaning up the urine extract, a short diatomaceous earth column was used to eliminate possible interfering substance(s) that would have a stronger polarity than prednisolone. Through this pretreatment the diethyl ether extracts were further concentrated, thus affording a fraction enriched with prednisolone. A representative chromatogram is shown in Fig. 1A. It was necessary to add dexamethasone to the urine specimens to assist in locating the prednisolone peak. Thus, the fraction eluting 2–3 min after dexamethasone contained a measurable amount of prednisolone, together with several contaminants. These contaminants appeared at or near the peak of prednisolone, whereas peaks of other glucocorticoids such as cortisol were entirely absent. Authentic cortisol was added to this fraction as the internal standard for quantitation of prednisolone; the resulting mixture was introduced into the same silica gel column with a different solvent system for further elimination

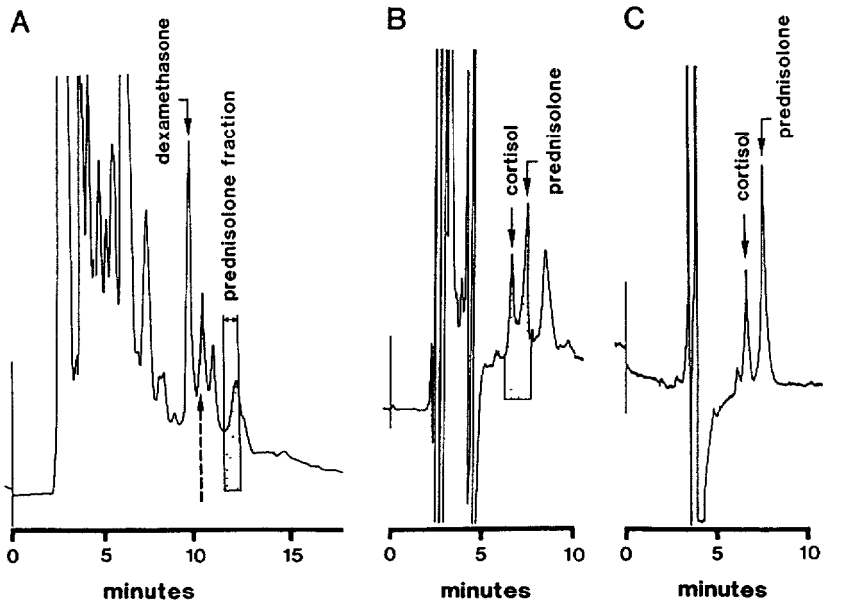

Fig. 1. Chromatograms of urine extracts. (A) The first chromatogram for prednisolone quantitation, in which 10 μg of dexamethasone and 13 ng of prednisolone were added to prednisolone-free urine. The dashed arrow indicates the position at which the cortisol peak should appear. The dotted area represents the eluent fraction collected. This fraction was spiked with 10 ng of cortisol for the subsequent chromatography. The solvent system was a mixture of 0.1% water, 4% methanol and 30% dichloromethane in *n*-hexane; the flow-rate was 2.0 ml/min; the detector range was set at 0.04 a.u.f.s. (B) The second chromatogram for fractionation of prednisolone and the internal standard. The dotted area represents the eluent fraction collected for the final chromatography. The solvent system consisted of 0.1% water, 10% ethanol and 30% dichloromethane in *n*-hexane at a flow-rate of 1.0 ml/min with the detector range set at 0.005 a.u.f.s. (C) The final chromatograms for quantitating prednisolone. The detection limit was 1 ng at a signal-to-noise ratio of 4 at $k' = 1.15$. The solvent system was a mixture of 0.1% water and 5% methanol in dichloromethane; the flow-rate was 1.0 ml/min and the detector range was set at 0.005 a.u.f.s. All chromatograms were obtained with a silica gel column (LiChrosorb Si-100, 10 μm, 250 mm $\times$ 4 mm I.D.) pre-washed with dilute sulphuric acid.

of contaminants. A representative chromatogram is shown in Fig. 1B. The eluent containing cortisol and prednisolone was recovered and chromatographed to obtain the analytical chromatogram shown in Fig. 1C, where baseline separation of the two glucocorticoids was sufficient for the accurate quantitation of prednisolone.

The correlation coefficient of the calibration curve was 0.999, with a detection limit of 1 ng at a signal-to-noise ratio of 4. The linear regression equation was expressed as $y = 11.652x + 0.516$, where y is the prednisolone concentration (ng/ml) and x is the peak-height ratio. The inter-assay coefficients of variation were less than 3%.

Excretion data for prednisolone in the timed urine specimens following the

topical application of prednisolone 17-valerate 21-acetate are plotted as a function of time in Fig. 2. The urinary excretion of prednisolone began ca. 2.5 h after application (82.04 ng/h in the 2.5–3.0 h interval) and increased to 150.78 ng/h at the 3.5–4.0 h interval. Thereafter, the amount excreted per hour decreased to and remained at a stable value despite the two successive changes of cream (Fig. 2). The amount excreted during this period ranged from 26.40 to 53.01 ng/h. The urinary excretion of prednisolone tended to increase shortly before cream removal (201.30 ng/h at 22.0–24.0 h). However, a dramatic increase in urinary excretion was observed after the final removal: the excreted amount was 356.3 ng/h at 25.0–25.5 h and 250.8 ng/h at 25.5–26.0 h, with a peak of 627.13 ng/h at 26.0–27.0 h followed by value of 116.20 and 182.09 ng/h at 28.0–30.0 and 32.0–35.0 h, respectively (Fig. 2). The excretion rapidly decreased thereafter, becoming almost zero at 14 h after removal. The total value of AUC of prednisolone in the urine was calculated to be 5.306 μg (ca. 0.0005% of the applied dose) of which ca. 70% came from the AUC component

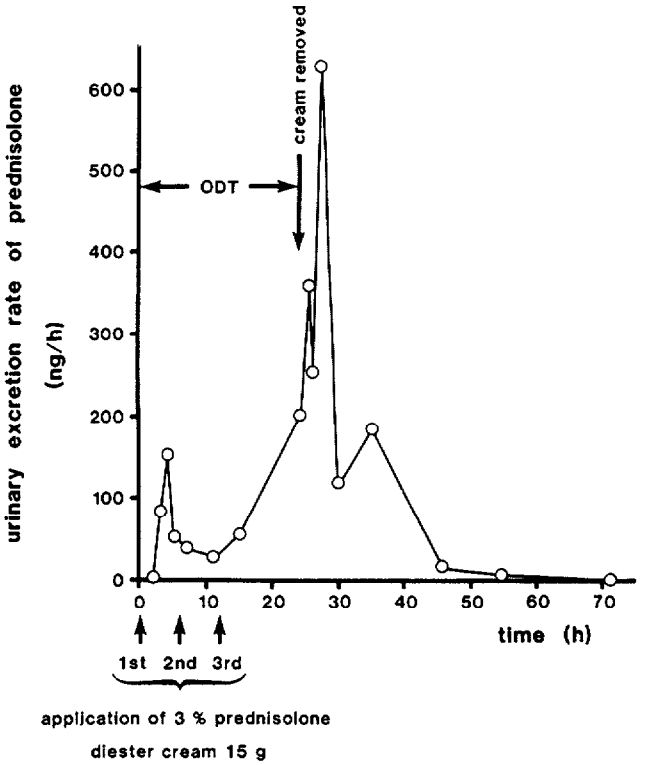

Fig. 2. Extraction pattern of prednisolone in urine following application of prednisolone 17-valerate 21-acetate to the trunk skin surface (1000 cm^2) of a male subject. The total amount of drug applied was equivalent to 1000 mg of prednisolone. The area under the excretion curve was 5.306 μg, corresponding to ca. 0.0005% of the applied dose. ODT, occlusive dressing technique.

after removal. Using eqn. 3, the overall MRT was calculated to be 25 h. Although an attempt was made to determine the mono- and diester forms of prednisolone in the urine, neither could be detected by the present method.

The therapeutic effects of topical corticoids depend on the penetration through the skin, the antiinflammatory activity and the time of contact with the skin. The mono- and diester of corticosteroids are now recognized as having a high capacity for penetrating the skin [11]. Since the plasma concentrations of prednisolone and its mono- and diesters were below the detection limit of the present HPLC method, the rate at which prednisolone was eliminated in the urine was measured. Our method was sufficiently sensitive for determining prednisolone excreted in the urine following its application in topical dosage form: 5.3 μg of the drug were detected in the urine 24 h after application of 3% prednisolone 17-valerate 21-acetate to healthy skin.

The urinary excretion data in Fig. 2 may reflect vasoconstriction effects of applied corticosteroids. This is because, following its initial appearance in the urine 2.5–3.0 h after topical application of the cream, prednisolone excretion decreased and continued to do so until complete removal of the cream 24 h later. The skin became pale while the cream was in contact with it, possibly due to the vasoconstriction activity of the glucocorticoid [12,13]. However, during the hour following removal of the cream, prednisolone excretion in the urine increased to substantially more than that observed during the period of skin contact. The reason for this is unclear, but it may have been due to the particular manner by which the drug is percutaneously absorbed through follicles overlying the epidermis [14]. Also, in contrast to initial vasoconstriction, there may be a relationship between the manner of prednisolone urinary excretion and increased skin capillary clearance or regional blood flow due to telangiectasia caused either by elastic capillary fatigue from long-term application or certain physical stimuli (e.g. a warm wet towel as used in this study), or both.

In the present study, an accurate estimation of the metabolic rate of prednisolone in the blood was not possible and, consequently, its actual percutaneous absorption rate remains to be determined. However, the urinary excretion data derived from non-radiolabelled ointment dosage, using an assay method sensitive enough for its detection, may be of clinical significance for the study of the topical therapeutic corticoids. For instance, percutaneous absorption and the bioavailability of topical steroids in live human skin can be estimated from steroid urine concentration–time data. Furthermore, application of the statistical moment theory [10] to evaluate various kinetic parameters of percutaneous drug absorption, as in the present study, may facilitate studies on percutaneous drug absorption.

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