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Short Communication

Extraction and high-performance liquid chromatographic separation of deflazacort and its metabolite 21-hydroxydeflazacort Application to urine samples

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Abstract

Two HPLC methods for the separation of a mixture of corticoids including the oxazolinic corticoid deflazacort and its metabolite 21-hydroxydeflazacort using different water–tetrahydrofuran mobile phases were developed. Both separations allowed the detection and determination of fifteen out of sixteen corticoids using different C_{18} columns. Extraction data for deflazacort and its metabolite using different extraction procedures are also reported. These separation conditions were applied to urine samples from two male volunteers administered Dezacor, with both doping control and clinical purposes.

1. Introduction

Deflazacort (DF) is a recently developed synthetic glucocorticoid with the structure shown in Fig. 1. As a result of its structure, it has the same clinical and therapeutic properties as other corticoids, but has a smaller influence on phosphate–calcium metabolism [1,2]. DF is hydrolysed to the active metabolite 21-hydroxydeflazacort (DFM) (Fig. 1) by seric sterases in blood, and the latter is further transformed into 6-β-hydroxydeflazacort. DF is mainly excreted into urine in the DFM form [3]. Some sportsmen use corticoids to improve performance. Therefore, the determination of corticosteroids is necessary for both clinical and doping control purposes.

In a previous study, optimization of the HPLC separation of a mixture of fourteen corticoids was carried out [4] by using different reversed-phase C_{18} columns and water–tetrahydrofuran as the mobile phase. In another paper, different extraction procedures with different solvents and different solid-phase materials for fourteen corticoids were reported [5].

In this paper, two improved HPLC separations for a mixture of sixteen corticoids that contains DF, DFM and the above-mentioned corticoids are described. One was performed with a Hypersil column and the other with a Spherisorb C_{18} column, giving rise to different selectivities. One

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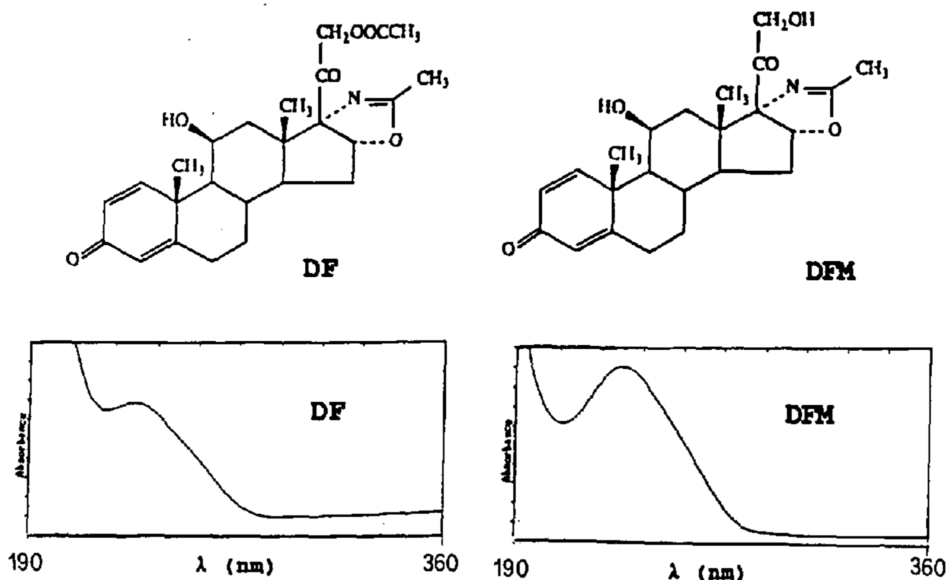

Fig. 1. Structures and UV spectra of deflazacort (DF) and 21-hydroxydeflazacort (DFM).

of these separations was used to obtain extraction data from urine samples spiked with DF and DFM, by applying solvent extraction (SE) and solid-phase extraction (SPE) procedures for these compounds. With these improved HPLC conditions, a more rapid separation only for DF and DFM in the presence of cortisol and cortisone was developed and applied to urine samples from volunteers administered the pharmaceutical preparation Dezacor for both doping control or clinical purposes.

2. Experimental

2.1. Chemicals

Deflazacort (DF), (11β,16β)-21-acetyloxy-11-hydroxy - 2′ - methyl - 5′*H* - pregna - 1,4 - diene-[17,16-*d*]oxazole-3,20-dione, and their metabolite 21-hydroxy-DF (DFM) were used and methanolic solutions were prepared. These compounds were a gift from Marion Merrell Dow España (Madrid, Spain). Other corticoids used in this work and their nomenclature were the same as those used in previous papers [4,5]. HPLC-grade tetrahydrofuran was purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore (Bedford, MA, USA) 0.45-μm nylon filters were also used. C_{18} Extrasep cartridges (2.8 ml and 500 mg) from Phenomenex (Torrance, CA, USA) were used. Serdolit AD-2 resin (0.1–0.2 mm) (Serva, Heidelberg, Germany) was prewashed with acetone, methanol and demineralized water and used as a water slurry. Other chemicals were of analytical-reagent grade.

2.2. Apparatus

The chromatographic components and other materials used in this work were the same as those reported in previous papers [4,5]. Reversed-phase Hypersil 5-ODS (200 × 4.6 mm I.D.; 5 μm) and Spherisorb 5-ODS (150 × 4.6 mm I.D.; 5 μm) columns from Phenomenex were also used.

2.3. Mobile phase

The mobile phase was prepared by mixing Milli-Q-purified water with tetrahydrofuran in the required volume ratio, by programming the pump. Tetrahydrofuran and water were first

filtered under vacuum through nylon filters and degassed using helium sparging.

2.4. Sample preparation

SE and SPE with C_{18} cartridges and with Serdolit AD-2 for DF and DFM spiked urine samples were performed according to a previously described procedure [5].

2.5. Chromatographic analysis

Once the column had been conditioned with the mobile phase, 20 μl of methanolic solutions containing a single corticoid or an appropriate mixture of them (5 μg/ml) including DF and DFM were injected. The separation conditions used are given in Table 1. Corticoids were identified by comparison of the UV spectra of the chromatographic peaks with those of reference compounds previously registered using diode-array detection (DAD). Absorption spectra of DF and DFM are shown in Fig. 1.

2.6. Drug administration

Two male volunteers (subject A aged 41 and B aged 25 years), having given informed consent, each received 30 mg of dezacor tablets (Merrel Dow Spain). Several urine samples from both subjects were collected during 24 h and stored at 4°C until analysis.

3. Results and discussion

3.1. Corticoid screening in the presence of DF and DFM

In a previous study paper [4], optimization of the HPLC separation of a mixture of fourteen corticoids was carried out. Different separation series of thirteen of the fourteen compounds were obtained by using different reversed-phase C_{18} columns and a mobile phase composed of water–tetrahydrofuran (72:28, v/v). In this work, DF and DFM were introduced into the above mixture and a study of the separation of these compounds was carried out using the above-described Hypersil 5-ODS and Spherisorb 5-ODS columns.

Corticoids were eluted from these columns using different mobile phases composed of water–tetrahydrofuran with a similar composition to that used previously [4]. Under the optimum conditions, each column separated fifteen out of sixteen compounds: using the Spherisorb 5-ODS column (separation A) DFM and prednisone (PS) co-eluted, whereas using the Hypersil 5-ODS column (separation B) betamethasone (BM) and triamcinolone acetonide (TRA) co-eluted (Fig. 2). The capacity factors of corticoids obtained and the experimental conditions for these separations are given in Table 1. These separation conditions were applied to spiked urine samples with subsequent solvent extraction (the chromatograms were very similar) and showed the possibility of solving different problems related to the separation and determination of these compounds in biological fluids or other materials. Separation B was finally chosen for further investigation.

3.2. Recoveries of DF and DFM

Under the experimental conditions described, SE and SPE of DF and DFM using disposable C_{18} cartridges and the non-ionic polymeric resin Serdolit AD-2 were performed.

The recoveries were assessed by analysing urine samples spiked with DF and DFM and using the chromatographic conditions given in Table 1 (B). For this purpose, two calibration graphs were prepared using 5 μg/ml of methylprednisolone (MPL) as an internal standard (I.S.). The first calibration graph was obtained by direct injection of 20 μl of standard solutions of DF of DFM in the range 2–10 μg/ml. The plot of the ratio of the DF or DFM peak-area response to that of the I.S. (*PAR*) *versus* the concentration of DF or DFM gave the following calibration equations: $PAR = a + s_1x$, $PAR = 0.181 + 0.595x$ for DF and $PAR = -0.137 + 0.221x$ for DFM. Both calibration graphs were linear (the correlation coefficients

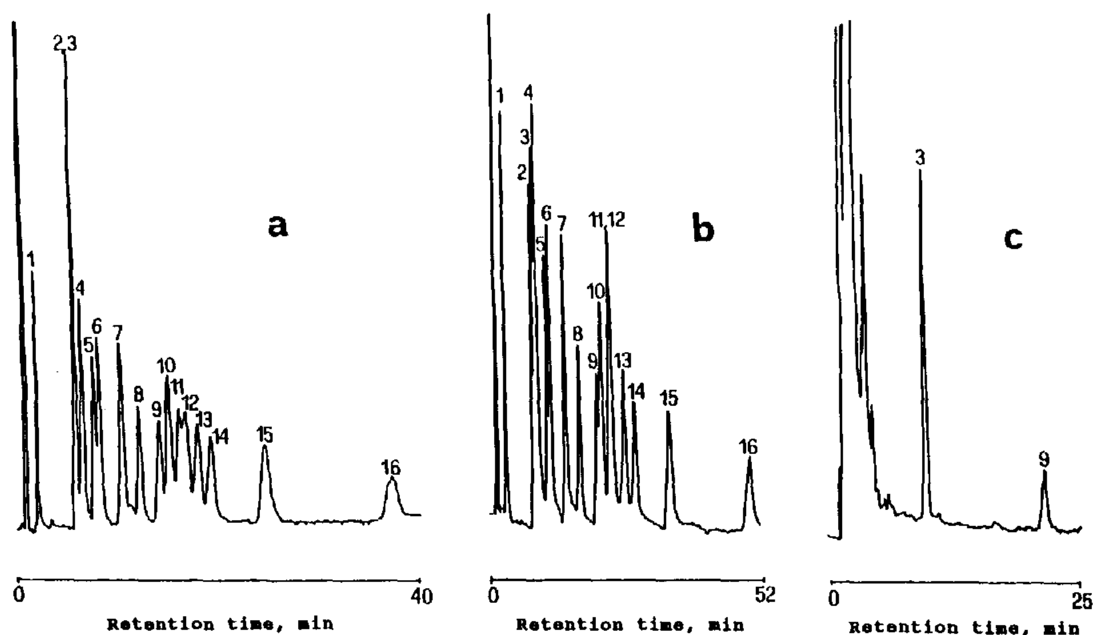

Fig. 2. HPLC of a mixture of corticoids. Chromatograms (a) and (b) were obtained with a standard mixing of corticoids (5 μg/ml). Conditions (a) A and (b) B and peak numbers as in Table 1. (c) Chromatogram obtained with a urine sample from subject B under conditions B.

Table 1
Capacity factors (k') for corticoids obtained under different conditions

No.	Compound	A	B
1	Triamcinolone	1.00	1.28
2	Prednisone	3.75	4.28
3	21-Hydroxydeflazacort	3.75	4.57
4	Cortisone	4.29	4.81
5	Prednisolone	5.11	5.88
6	Cortisol	5.51	6.25
7	Fudrocortisone	7.13	7.87
8	Corticosterone	8.46	9.43
9	Methylprednisolone	9.98	11.36
10	11α-Hydroxyprogesterone	10.72	11.86
11	Betamethasone	11.49	12.84
12	Triamcinolone acetonide	11.98	12.84
13	Dexamethasone	12.85	14.28
14	Deflazacort	13.84	15.45
15	Deoxycorticosterone	17.89	19.18
16	Fludrocortisone acetate	27.34	28.04

(A) Spherisorb 5-ODS column, mobile phase water–tetrahydrofuran (80:20, v/v), flow-rate 1.2 ml/min and 20°C and (B) Hypersil 5-ODS column, mobile phase water–tetrahydrofuran (77:23, v/v), flow-rate 1.0 ml/min and 30°C; UV detection 245 nm.

were higher than 0.999). In both instances the intercepts were not significantly different from zero. The detection limits (DL) for DF and DFM were assessed at a signal-to-noise ratio (S/N) of 3 by means of the calibration graphs. The values obtained were 0.12 ng for DF and 0.15 for DFM. The day-to-day and within-day precisions were examined by analysing ten different samples of DF and DFM containing 5 μg/ml each by means of the calibration graphs. The R.S.D.s were 3.1 and 2.2% for DF and 3.4 and 2.8% for DFM, respectively.

A second calibration in the range 0.13–0.66 μg/ml was obtained with urine samples containing DF and DFM under SE or SPE conditions (see procedures), and the slope, s_2, was also calculated. This calibration graph (the correlation coefficients were above 0.990) was used for the determination of DF and DFM in urine samples.

The recoveries (R) were calculated using the equation $R\ (\%) = (s_2/s_1) \cdot 100$, and are independent of the concentration range of DF and DFM

Table 2
SE and SPE recoveries (% ± R.S.D., $n = 5$) for DF and DFM added to urine samples

Compound	SE	C_{18} cartridges	Serdolit AD-2
DF	101.3 ± 4.2	90.8 ± 3.3	94.2 ± 4.3
DFM	91.3 ± 4.5	93.4 ± 3.5	91.0 ± 4.3

investigated. The recoveries obtained were above 90% (Table 2).

This extraction study completes a previous study of other corticoids [5]. For further work SPE with C_{18} cartridges was used because the samples are cleaner than with other extraction processes.

3.3. Separation of DF and DFM in the presence of cortisol and cortisone

Screening of drugs in urine is sometimes not needed in clinical applications because of the time of analysis and the solvent involved. For this reason, a study of the HPLC separation of DF and DFM in the presence of cortisol (CL) and cortisone (CS) was carried out. Different separations using mixtures of water and tetrahydrofuran were performed. The optimum separation is shown in Fig. 3a, together with the chromatographic conditions. The calculated capacity factors, k', for DFM, CS, CL, the I.S. (methylprednisolone) and DF were 2.47, 2.88, 3.51, 5.56 and 7.44, respectively. A calibration graph was obtained in a similar way to that above with analogous characteristics.

3.4. Application to urine samples

Several applications were carried out.

The chromatographic separation in Table 1 (B) was applied to urine samples from subject B. Fig. 2c shows an example of the chromatogram obtained. DFM was identified and confirmed using UV–DAD. Under the test conditions no interfering endogenous compounds were observed.

Under the chromatographic separation conditions (Fig. 3a), a chromatogram of a urine sample from one healthy male volunteer administered Dezacor (tablets containing 30 mg of DF) was obtained (Fig. 3b). The results confirm that deflazacort is excreted mainly in the urine in the DFM form. In this urine sample, CL and CS were also detected and confirmed using UV–DAD.

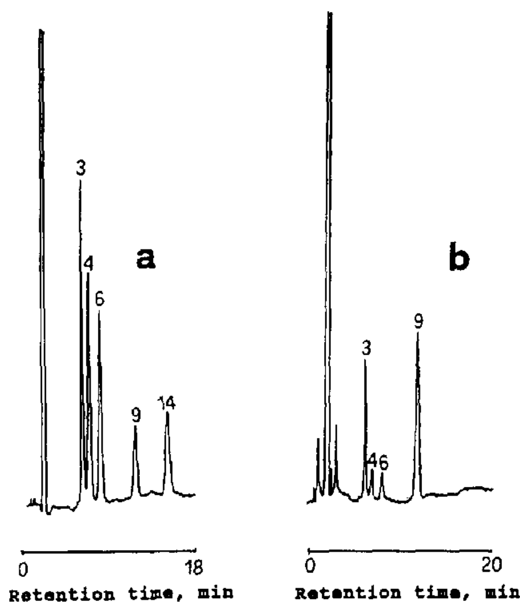

Fig. 3. (a) HPLC of a standard mixture of DF, DFM, CL, CS and the I.S. (methylprednisolone), all at 5 μg/ml. (b) HPLC of a urine sample from subject B with subsequent SFE extraction with C_{18} cartridges. The eluate was reconstituted with 50 μl of I.S. solution. Conditions: column, Hypersil 5-ODS; mobile phase, water–tetrahydrofuran (73:27, v/v); flow-rate, 1.0 ml/min; temperature, 30°C; UV detection at 245 nm. Peak numbers as in Table 1.

The urinary excretion curves for volunteers A and B obtained by measuring the DFM in each urine sample collected (Fig. 4) showed very similar qualitative shapes. However, there are quantitative differences. The maximum of the curves is observed in both instances at the same time of sample collection. However, the amounts excreted are influenced by individual factors such as age, height and body mass. The total elimination was slower for the older subject, who required an additional 6 h for total elimination of deflazacort. In Fig. 4c and d are shown the cumulative curves for DFM excreted into urine.

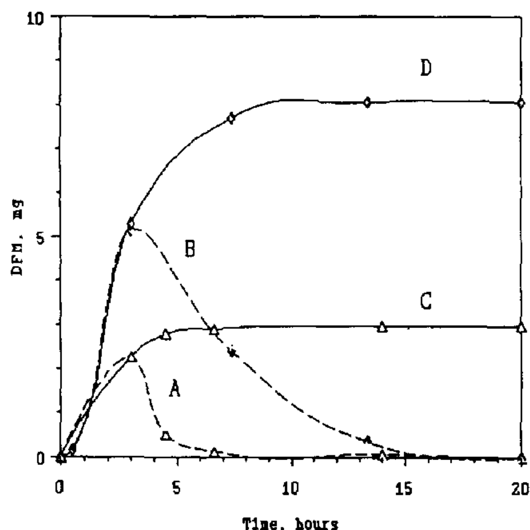

Fig. 4. Urinary excretion of deflazacort from two male volunteers, (A) 41 and (B) 25 years old. C and D are cumulative curves.

A correlation between the curves obtained in urine and plasma [3] was found, allowing extrapolation of the results obtained for urine. Hence it is not necessary to carry out blood analyses.

For doping control purposes and only considering the younger subject and the amount administered to the pharmaceutical product Dezacor, positive doping can be observed during the 5 h following drug administration. The calculated detection limit of the above method is 0.12 ng of DFM. This amount is equivalent to 1.2 ng in a urine sample (3 ml). Hence the concentration equivalent to the detection limit in urine is 0.4 ng/ml.

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