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Simultaneous determination of deflazacort metabolites II and III, cortisol, cortisone, prednisolone and prednisone in human serum by reversed-phase high-performance liquid chromatography

Hiroko Hirata*, Tooru Kasama, Yoneichi Sawai, Robert R. Fike

Pharmacokinetics, Development Laboratories Hirakata Center, Marion Merrell Dow, 11, Shodai-Tajika 3-Chome Hirakata-shi, Osaka 573, Japan

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Abstract

A new high-performance liquid chromatographic method for the analysis of human serum from subjects administered prednisolone or deflazacort has been established. Two deflazacort metabolites (metabolites II and III), prednisolone and its metabolite (prednisone), as well as cortisol and cortisone in human serum were analyzed simultaneously. These six compounds and the internal standard, betamethasone, were extracted from 1 ml of human serum using a Sep-Pak Plus Environmental C_{18} column and then separated on a Hypersil ODS (25 × 0.46 cm I.D.) column using a gradient system. The lower limit of quantitation for each compound was 10 ng/ml based on signal-to-noise ratio of 5 and calibration curve linearity. Within-day and between-day validations gave a coefficient of variation of less than 10% and a relative error of *ca.* 10% ($n = 11$).

1. Introduction

Glucocorticoid therapy is very important in many acute and chronic inflammatory conditions; however, frequent and sometimes severe side effects have been reported [1]. One of the side effects is the suppression of cortisol (CRS) which is secreted from the adrenal gland and metabolized to cortisone (CRN) mainly in the liver [2,3]. Simultaneous determination of the serum levels of administered glucocorticoid and endogenous CRS can be very useful for monitoring this side effect. Therefore, a method which can analyze CRS quantitatively in the serum of patients subjected to glucocorticoid therapy became necessary.

Classical glucocorticoid therapy includes the administration of prednisolone (PDS) or its metabolite prednisone (PDN). Deflazacort (DFC), 11ß,21-dihydroxy-2′-methyl-5′ß,H-pregna-1, 4-dieno[17, 16-d]oxazole-3, 20-dione-21-acetate (Fig. 1) is a new glucocorticoid which is marketed in Europe and is presently subject to clinical trials in Japan and the United States of America. In order to compare the effect of administered PDS and DFC on endogenous CRS

* Corresponding author.

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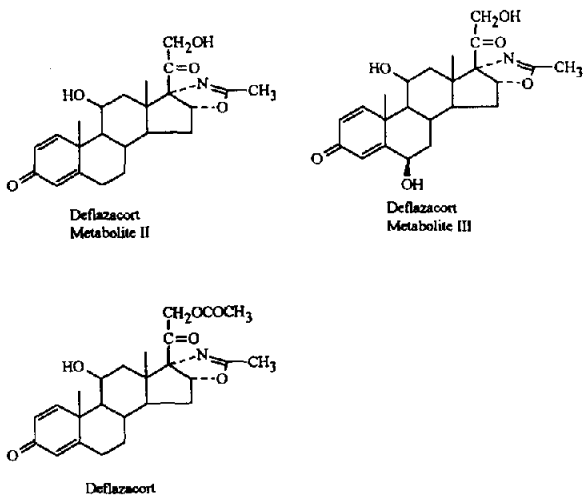


Fig. 1. Chemical structures of deflazacort and its metabolites.

concentrations, a high-performance liquid chromatographic (HPLC) method was needed. Several methods for the analysis of PDS and CRS have been reported [4–9]. However, none provided sufficient resolution to allow the quantitation of PDS, PDN, CRS, CRN, and metabolites of DFC [10,11] (Fig. 1).

Both liquid–liquid and solid-phase extractions have been used to separate steroids from serum [4–9]. The solid-phase extraction procedure reported by Prasad *et al.* [9] is very complicated and gave a slightly lower recovery than liquid–liquid extraction. Recently, Cannell *et al.* [6] reported another solid-phase extraction in which the recovery of PDS was satisfactory. However, they used perfusion media as matrix and their study did not include CRS.

Therefore, the present paper describes a new method which combines solid-phase extraction and reversed-phase HPLC for monitoring the CRS levels in serum of subjects receiving PDS or another glucocorticoid, DFC.

2. Experimental

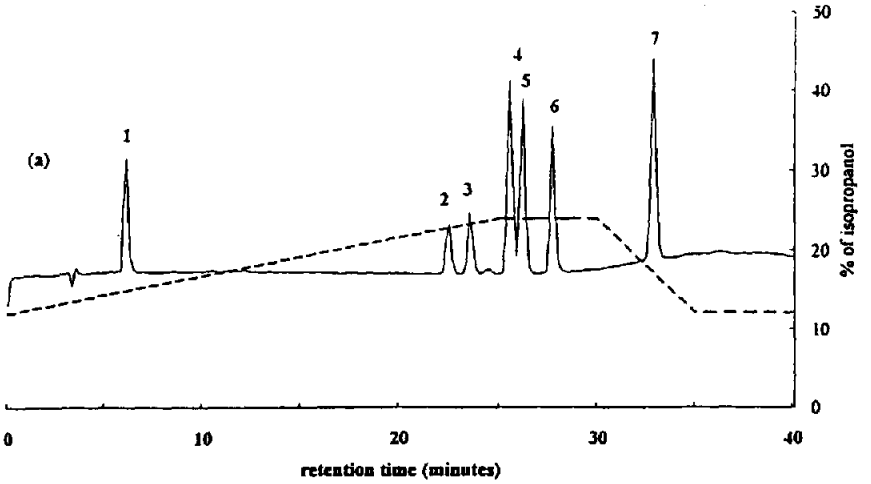
2.1. Reagents

CRN, PDS and PDN were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and CRS from Wako Pure Chemical (Osaka, Japan). The standard of metabolite II of DFC (Met II), 11ß,21-dihydroxy-2′-methyl-5′ß,H-pregna-1,4-dieno [17,16-d]oxazole-3,20-dione (Fig. 1), was provided by Groppo Lepetit S.p.A. (Gerenzano, Italy). The standard of metabolite III (Met III), 6ß,11ß,21-trihydroxy-2′-methyl-5′ß,H-pregna-1,4-dieno[17,16-d]oxazole-3,20-dione (Fig. 1), which is metabolized from Met II, was purified from rat urine in our Kansas City Laboratory, Marion Merrell Dow. Betamethasone (Sigma) was used as internal standard (I.S.). Methanol, acetonitrile and isopropanol (Wako) and acetic acid (Kishida Chemical, Osaka, Japan) were of HPLC or spectroscopic grade and used without further purification. Sodium acetate trihydrate (Wako) was of guaranteed grade. Reagent-grade water was generated with a Milli-Q reagent water system (Millipore Corporation, Bedford, MA, USA). High purity grade nitrogen was used for sample evaporation.

2.2. Apparatus

The chromatographic system from Waters (Milford, MA, USA) consisted of a Model 600E pump, Model 700 Satellite WISP automated injector, Model 484 tunable absorbance detector

Fig. 2. (a) Chromatogram of standard steroids with internal standard. The injection amounts are 20 ng each of (1), (4), (5) and (6), 7.5 ng each of (2) and (3), and 30 ng of (7). (b) Chromatogram of extracted blank serum (1 ml) spiked with 300 ng/ml of internal standard. The concentrations of endogenous cortisone and cortisol in the blank serum are 18.0 ng/ml and 51.9 ng/ml, respectively. (c) Chromatogram of extracted blank serum spiked with the standard steroids and internal standard. The concentrations are 200 ng/ml each of (1), (5) and (6), 75 ng/ml of (2), 300 ng/ml of (7). (3) and (4) are spiked with 75 ng/ml and 200 ng/ml, respectively, in addition to the endogenous glucocorticoids. Chromatographic conditions are described in the text. Dashed line indicates the gradient profile of isopropanol (%). Peaks: 1 = metabolite III; 2 = prednisone; 3 = cortisone; 4 = cortisol; 5 = prednisolone; 6 = metabolite II; 7 = betamethasone (internal standard).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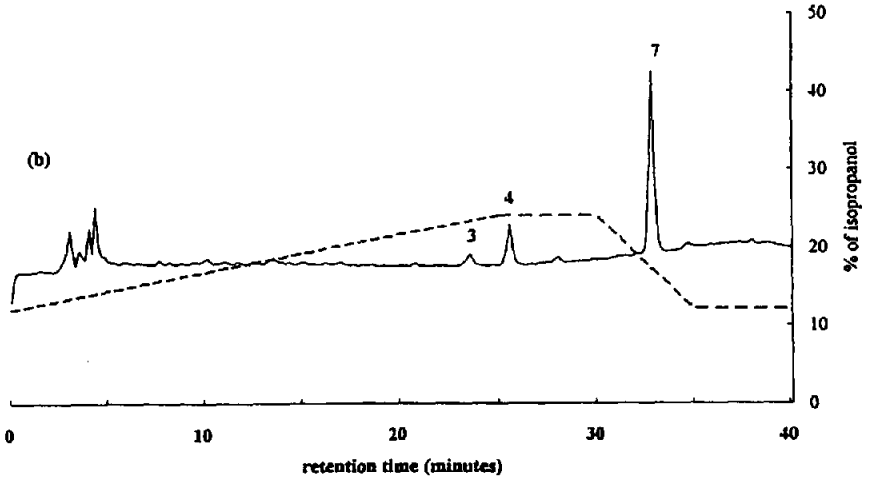
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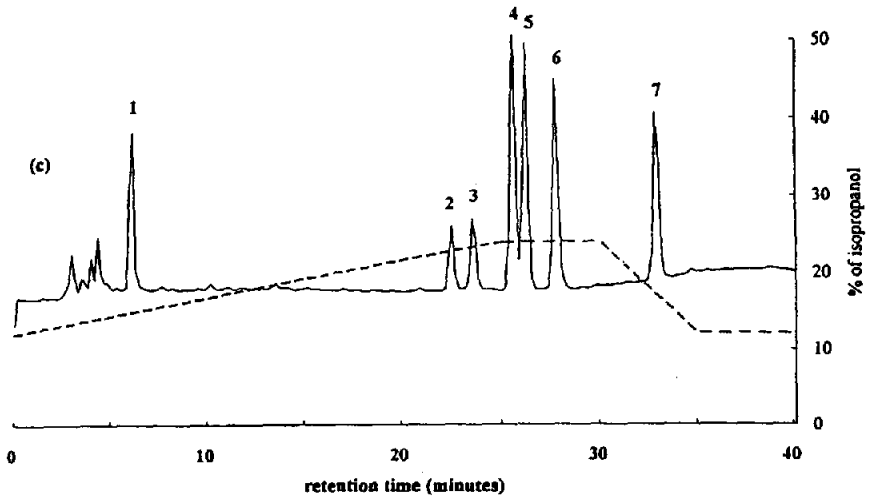
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and System interface module. The analytical column was a 25 × 0.46 cm I.D., 5 μm Hypersil ODS (Chemco Scientific Co., Osaka, Japan) equipped with a μBondapak C_{18} guard column (Waters).

2.3. Chromatographic conditions

The mobile phase A (in which samples were injected) was isopropanol–0.05 *M* acetate buffer pH 4.5 (10:90, v/v) and the 6 steroids and I.S. were separated at 40°C using a linear gradient of isopropanol–0.05 *M* acetate buffer pH 4.5 (30:70, v/v) as the mobile phase B at a flow-rate of 1.0 ml/min. The mobile phase A composition was decreased from 90% to 30% for 25 min and held for 5 min. Then it was increased to 90% for 5 min and held for 15 min. The acetate buffer was filtered through MF Millipore filter HA 0.45-μm (Millipore) before mixing with isopropanol. Mobile phases were degassed by sonication for 10 min prior to use and helium gas was used during measurement. The eluate was monitored by UV absorbance at 254 nm and the system pressure was 0.218–0.319 Pa.

2.4. Preparation of calibration standards

All solutions were made in isopropanol–acetonitrile (1:1, v/v). Stock solutions of each steroid were prepared at 30 μg/ml. The series of solutions I containing Met II, Met III, PDS and PDN were prepared at the concentrations of 200, 500, 1000 and 2000 ng/ml for Met II, Met III and PDS, and 200, 500, 1000, 1500 and 2000 ng/ml for PDN. The series of solutions II containing CRS and CRN were prepared at the concentrations of 200, 500, 1000 and 2000 ng/ml for CRS, and 200, 500, 1000, 1500 and 2000 ng/ml for CRN. I.S. was dissolved at a concentration of 3000 ng/ml. To prepare the samples for the calibration curve, 100 μl of I.S. and 50 μl each of the solutions I and II were added to 1 ml of blank serum. Final concentrations in the serum were 10, 50, 100, 200 and 300 ng/ml for Met II, Met III, PDS and CRS, and 10, 25, 50, 75 and 100 ng/ml for PDN and CRN. When analyzing the serum sample, 100 μl of I.S. was added and 100 μl of the solvent was added instead of the solutions I and II. The serum was immediately thoroughly mixed when the organic solvent was added.

2.5. Extraction procedure

The extraction column (Sep-Pak Plus Environmental C_{18}, Waters) was pretreated with a 15-ml volume of methanol followed by a 15-ml volume of distilled water. Serum samples were loaded onto the extraction columns and washed with 10 ml of water followed by 5 ml of 20% methanol. The steroids were eluted with 3 ml methanol. The methanol was evaporated under a stream of nitrogen at 50°C and the residue was reconstituted in 200 μl of mobile phase A, 20 μl of which was injected onto the HPLC system.

2.6. Accuracy and precision

The within-day accuracy and precision were assessed from a calibration curve (determined in duplicate) and five replicate assays at three control levels (25, 100, 250 ng/ml of Met II, Met III, PDS and CRS, and 15, 50, 85 ng/ml of PDN and CRN). The between-day accuracy and precision were estimated from the results of three replicate assays at three control levels (see above) on two different days, using a calibration curve in duplicate and the results of the within-day study.

2.7. Calculations

Concentrations were determined using peak-height ratios and a weighted least squares linear regression calculation [12]. Since both CRS and CRN are endogenous in the blank serum, the calibration curve was determined from the slopes.

3. Results

Shown in Fig. 2 are chromatograms of the standards (a), blank serum (b), and blank serum containing all steroids of interest (c). The steroid

Table 1
Absolute recovery percents of glucocorticoids including I.S. ($n = 5$)

Compound	Concentration added (ng/ml)			Mean
	25	100	250	
Met II	92.8 ± 6.4	91.3 ± 1.2	93.8 ± 3.4	92.6 ± 4.1
Met III	87.2 ± 6.6	88.0 ± 2.5	88.0 ± 2.5	89.5 ± 4.9
PDS	90.4 ± 7.3	87.2 ± 3.1	92.6 ± 3.9	90.1 ± 5.3
CRS	102.7 ± 13.9	85.9 ± 4.4	90.4 ± 5.7	93.0 ± 11.1
	15	50	85	
PDN	93.8 ± 8.1	82.7 ± 5.0	86.5 ± 5.7	87.6 ± 7.6
CRN	98.4 ± 12.8	83.5 ± 4.7	87.4 ± 9.0	89.8 ± 10.9
I.S.				89.7 ± 4.4

Data are presented as mean ± S.D.

Table 2
The results of within-day and between-day validations

Compound	Concentration added (ng/ml)	Within-day ($n = 5$)		Between-day ($n = 11$)	
		Concentration found (ng/ml)	Relative error (%)	Concentration found (ng/ml)	Relative error (%)
Met II	25	25.0 ± 0.4 (1.8)	−0.1	25.9 ± 1.3 (4.9)	3.4
	100	104.0 ± 3.4 (3.3)	4.0	106.4 ± 4.4 (4.1)	6.4
	250	264.6 ± 3.7 (1.4)	5.9	270.5 ± 6.6 (2.4)	8.2
Met III	25	23.9 ± 0.9 (3.7)	−4.6	24.7 ± 1.0 (4.2)	−1.1
	100	101.6 ± 4.4 (4.4)	1.6	103.2 ± 4.0 (3.9)	3.2
	250	263.6 ± 4.1 (1.6)	5.5	266.9 ± 5.1 (1.9)	6.8
PDS	25	26.3 ± 1.5 (5.5)	5.2	26.2 ± 1.1 (4.8)	4.8
	100	102.1 ± 3.3 (3.2)	2.1	105.1 ± 4.4 (4.1)	5.1
	250	260.7 ± 2.5 (1.0)	4.3	269.1 ± 9.0 (3.4)	7.6
PDN	15	17.4 ± 0.7 (4.0)	15.8	16.9 ± 0.7 (4.1)	12.6
	50	50.5 ± 2.6 (5.0)	−0.3	52.0 ± 2.5 (4.8)	3.9
	85	88.5 ± 2.6 (2.9)	4.2	92.1 ± 4.5 (4.9)	8.3
CRS	25	27.6 ± 1.4 (5.1)	10.3	26.3 ± 1.7 (6.4)	5.3
	100	98.6 ± 1.6 (1.6)	−1.4	101.3 ± 3.8 (3.7)	1.3
	250	245.0 ± 5.5 (2.2)	−2.0	247.6 ± 8.3 (3.4)	−1.0
CRN	15	16.3 ± 1.2 (7.4)	8.7	16.3 ± 1.0 (6.0)	8.5
	50	49.8 ± 0.6 (1.3)	−0.3	52.5 ± 3.1 (5.9)	5.0
	85	81.6 ± 4.9 (6.0)	−4.0	84.3 ± 4.9 (5.9)	−0.9

Data are presented as mean ± S.D. The coefficient of variation (%) is given in parentheses.

standards are resolved well from each other. As Met III has a high polarity, it is eluted much faster than the other compounds. The chromatogram of blank serum showed some interfering peaks as well as endogenous CRN and CRS peaks. These interferences did not prevent us from calculating the concentration from the peak height. The linearity of the calibration curve of each steroid was confirmed in the range of the concentrations tested. Each steroid peak had a signal-to-noise ratio of more than 5 at the lowest concentration. At 10 ng/ml for six replicate assays the coefficients of variation (C.V.) ranged between 5.1% and 8.6%, and the relative errors (mean ± S.D.) were 6.9 ± 9.9, -4.8 ± 8.7, -4.1 ± 5.9, -0.0 ± 5.8, 1.0 ± 10.2 and 1.2 ± 5.1% for Met II, Met III, PDS, PDN, CRS and CRN, respectively. Therefore, the lower limit of

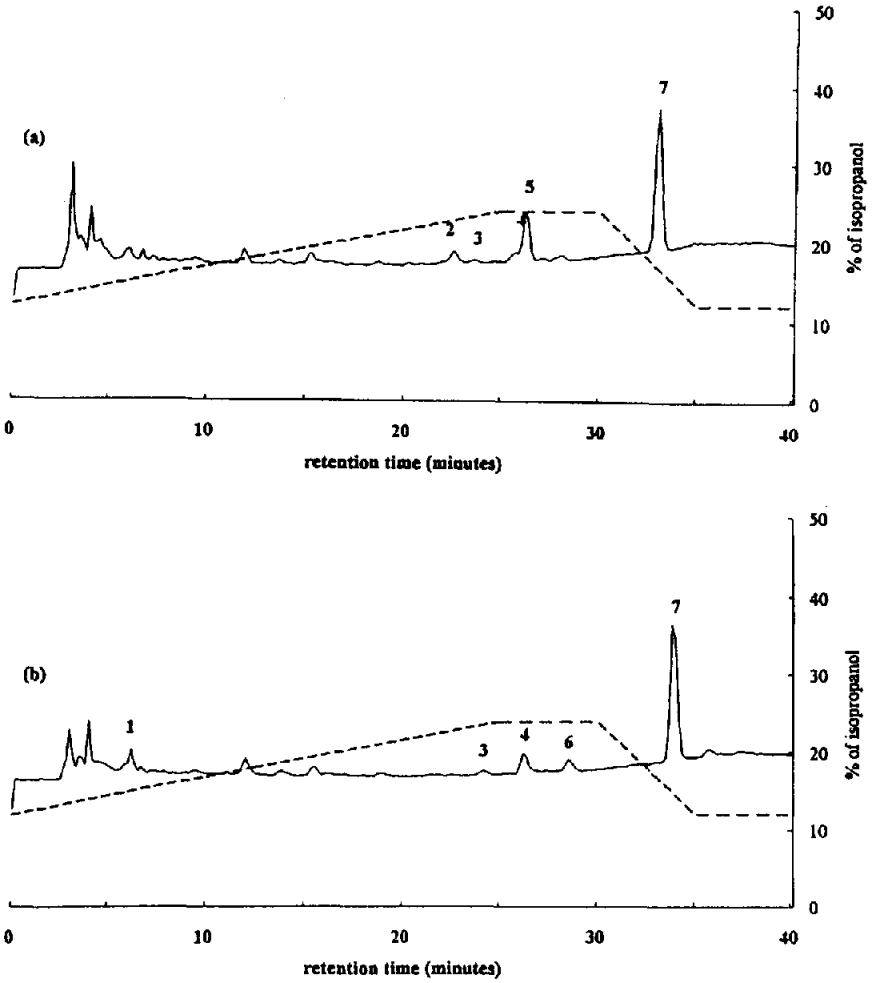

Fig. 3. (a) Chromatogram of the serum from the subject administered prednisolone. Detected concentrations are (2) 20.4 ng/ml, (3) below the lower limit of quantitation, (4) 12.5 ng/ml and (5) 79.3 ng/ml. The serum is spiked with 300 ng/ml of internal standard (7) before extraction. (b) Chromatogram of the serum from the subject administered deflazacort. Detected concentrations are (1) 25.9 ng/ml, (3) below the lower limit of quantitation, (4) 32.7 ng/ml and (6) 20.7 ng/ml. The serum is spiked with 300 ng/ml of internal standard (7) before extraction. The chromatographic conditions are described in the text. Peaks; 1 = metabolite III; 2 = prednisone; 3 = cortisone; 4 = cortisol; 5 = prednisolone; 6 = metabolite II; 7 = betamethasone (internal standard).

quantitation for each steroid was set at the lowest concentration on the calibration curve, 10.0 ng/ml. Absolute recoveries from serum were calculated from within-day validation data. The recoveries ranged between 82.7 and 102.7%, and the S.D. ranged from 1.2 to 13.9% (Table 1). The I.S. recovery was 89.7 ± 4.4% (mean ± S.D.). The within-day and between-day results are shown in Table 2. The relative error ranged from −4.6 to 15.8% but most values were within ± 10%. The C.V. in the analysis was below 7.4%.

Fig. 3 shows chromatograms of clinical samples of serum of subjects receiving either PDS (a) or DFC (b). The former sample contained PDS, PDN, CRS, CRN and I.S., the latter DFC Met III, Met II, CRS, CRN and I.S. The CRS suppression was observed especially in the samples from the subject receiving PDS.

4. Discussion

The solid-phase extraction method described is able to extract CRS, CRN, PDS, PDN, Met II, Met III and I.S. from human serum. This procedure is very simple compared with the previous one [9] and liquid–liquid extraction [4,5,7,9]. The recovery is also satisfactory.

As shown in Figs. 2 and 3, there is good resolution of the steroids, including the DFC metabolites. Referring to the structures shown in Fig. 1, DFC is immediately de-acetylated to Met II when administered to humans [10] and Met II is further metabolized to Met III [11]. Therefore, DFC was excluded from the analytes. The chromatogram of the sample from the subject administered DFC showed no significant later-eluting peak than the I.S. peak where DFC is expected to appear (Fig. 3b).

When the samples were analyzed using the procedures stated above, each intercept of the calibration curves was not significantly different from zero except for CRS and CRN whose intercepts reflected their endogenous levels. Furthermore, the analytical data were consistent with previously reported results which showed that levels of PDS and CRS were about ten times higher than those of their respective metabolites, PDN and CRN [13–15].

In conclusion, this simple and straightforward method has been shown to be useful for the simultaneous determination of these steroids in human serum.

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