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Journal of Chromatography B, 660 (1994) 23–29

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of urinary free cortisol and 6β-hydroxycortisol by high-performance liquid chromatography to measure human CYP3A activity

Jens Lykkesfeldt*, Steffen Loft, Henrik E. Poulsen

Department of Pharmacology, The Panum Institute, University of Copenhagen, 3 Blegdamsvej, DK-2200 Copenhagen N, Denmark

First received 15 November 1993; revised manuscript received 9 May 1994

Abstract

The ratio of the hydrophilic metabolite 6β-hydroxycortisol to its parent compound cortisol has recently been demonstrated to be a specific marker for human CYP3A oxygenase activity. We have developed a sensitive and simple single-run high-performance liquid chromatographic method for the quantification of urinary free cortisol and 6β-hydroxycortisol using dexamethasone as internal standard. The urine samples (1 ml) are applied to Sep-Pak cartridges, which are washed with water and eluted with ethyl acetate–diethyl ether (4:1, v/v). The organic extracts are washed sequentially with alkaline and acidic solutions saturated with sodium sulfate and subsequently concentrated to dryness. After reconstitution in ethanolic water, the samples are analyzed on a reversed-phase gradient system using ultraviolet absorbance detection at 254 nm. The within- and between-day coefficients of variation (C.V.) for the assay where both in the range of 5–10%. The reference interval for the 6β-hydroxycortisol/cortisol ratio of eleven healthy non-smoking subjects was 2.77–26.88 with an average of 10.09 ± 6.89 (S.D.). The method constitutes an improvement over previous methods and is suitable for routine assessment of the 6β-hydroxycortisol/cortisol ratio requiring only 1 ml of urine or less.

1. Introduction

The hepatic mixed-function oxygenases (cytochrome P450s, CYPs) are responsible for the metabolism of many xenobiotics including drugs, and therefore changes in the activity of these enzymes may lead to changes in biological effects and toxicity [1]. 6β-Hydroxycortisol (6β-OHF) is a hydrophilic metabolite and the major unconjugated urinary product of cortisol accounting for approximately 1% of the total daily cortisol secretion [2]. The urinary excretion of 6β-OHF has for some time been considered a useful non-invasive index of the induction of these enzymes, as 6β-OHF is a polar metabolite of cortisol (F) formed in the endoplasmic reticulum [3–6]. Recently, the excretion of 6β-OHF was identified as a specific marker of the induction of CYP3A [7,8]. This makes the 6β-OHF/F ratio an interesting addition to the specific assays of individual human CYP activities [9].

The use of 6β-OHF excretion as a marker of enzyme activity requires correction for the vari-

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00265-7

able production of the substrate, cortisol, which can be measured as such or in terms of 17-ketosteroids [10,11], the former method now being considered the most precise. Moreover, usually 24-h urinary excretion has been used [12,13]. The ratio of 6β-OHF to F in spot urine, however, appears to remain constant and reflect the 24 h value [8,14], presumably because F excretion into urine and metabolism to 6β-OHF follows first order kinetics. Obviously, the use of spot urine samples for the assay greatly facilitates large scale clinical studies.

The measurement of 6β-OHF and F is usually based on enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and/or high-performance liquid chromatography (HPLC). Due to cross-reaction between the metabolite and parent compound in the commercially available immunoassay kits, the use of these methods often results in higher concentrations of 6β-OHF and F compared to the equivalent HPLC measurements [15–17]. UV absorbance detection following HPLC separation is a well established method for the selective and sensitive quantification of steroids (for review see Ref. [18]). Previously published HPLC methods for the assessment of the 6β-OHF/F ratio have included mixed analysis (HPLC/RIA) [7], separate analysis of the two steroids [8], or partial rechromatography of a pooled fraction on a different chromatographic system [19].

In the present study we describe a sensitive and simplified single-run HPLC method for the direct assessment of the 6β-OHF/F ratio from human urine using a low sample volume.

2. Experimental

2.1. Materials and reagents

Water for all applications, ethyl acetate, and ethanol were of SpS (Super purity Solvent) quality and acetonitrile was of 190 (far UV) SpS quality; all purchased from Romil Chemicals (Shepshed, Loughborough, Leics, UK). F (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione) and dexamethasone (9α-fluoro-11β,17α, 21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) were obtained from Sigma (St. Louis, MO, USA), and 6β-OHF (6β,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione) was from Steraloids (Wilton, NH, USA). All other solvents and chemicals were at least of analytical grade and purchased from Merck (Damstadt, Germany). Spot urine samples were collected from 11 healthy non-smoking subjects (6 female, 5 male), who to the best of our knowledge did not receive any drug treatment.

Stock solutions of the steroids were made in ethanol (40 mg/100 ml), and working solutions were prepared by dilution with water to concentrations of 4 μg/ml. The working solutions were kept at 5°C and regularly freshly prepared. Daily injections of the standard solutions showed no change or decomposition for at least one month.

2.2. Sample preparation

Urine samples were prepared using a modification of a previously described method [11], which we optimized for the target compounds in this study. Urine samples were kept at −20°C until analysis. Following gentle thawing, the urine sample was centrifuged (4000 *g*, 10 min, 4°C) to remove possible turbidity and 1.0 ml of urine was transferred to a sample vial to which dexamethasone [internal standard (I.S.), 80 ng/ml] and 9 ml water were added and vortex-mixed. The mixture was allowed to pass through a preconditioned (3.0 ml methanol, 6.0 ml water) Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA, USA), using a vacuum line to maintain a flow-rate of approximately 1 drop per second at all times. The Sep-Pak was washed with 10.0 ml of water and, following removal of the aqueous phase using an air stream, the steroids were eluted with 5.0 ml of ethyl acetate–diethyl ether (4:1, v/v), as optimized for yield and resolution. The organic extract was washed with 2.0 ml of 1.0 *M* NaOH saturated with Na_2SO_4 followed by 2.0 ml of 1.0% acetic acid saturated with Na_2SO_4 and finally concentrated to dryness using a mild air stream. The residue was dissolved in 100 μl of water and vortex-mixed after which 20 μl of ethanol was added.

After subsequent additional vortex-mixing, 100 μl was used for HPLC analysis. Peak areas of F, 6β-OHF, and I.S. were measured.

2.3. Chromatographic analysis

Urine samples were analyzed with a fully automated HPLC system consisting of the following Merck-Hitachi Instruments (San Jose, CA, USA) units: 655A-40 autosampler (cooled to 3°C), L-6200 intelligent pump, L-6000 pump, T-6300 column thermostat operated at 30°C, D-6000 interface unit, and a L-4000 UV detector operated at 254 nm. All units were connected to a personal computer for control as well as for collection and analysis of data (HPLC-Manager version 2, Merck-Hitachi). The column was a Nova-Pak C_{18} (particle size 4 μm, pore size 60 Å, 300 × 3.9 mm I.D., Waters) operated at 1.0 ml/min using the following gradient profile: $t = 0$ min, 15% B; $t = 20$ min, 82% B; $t = 22.5$ min, 100% B; $t = 27.5$ min, 100% B; $t = 30$ min, 15% B; $t = 40$ min, 15% B, as indicated in Fig. 1. The retention times of the steroids were approximately 9.4 min (6β-OHF), 16.8 min (F), and 19.1 min (I.S.) in the present system. The gradient was formed using high-pressure mixing and the gradient delay was approximately 5 min. Mobile phase A was a 50 m*M* KH_2PO_4–10 m*M* acetic acid solution in water, while mobile phase B consisted of 65% (v/v) acetonitrile in mobile phase A. Mobile phases were filtered through a 0.45-μm filter under vacuum and degassed prior to use.

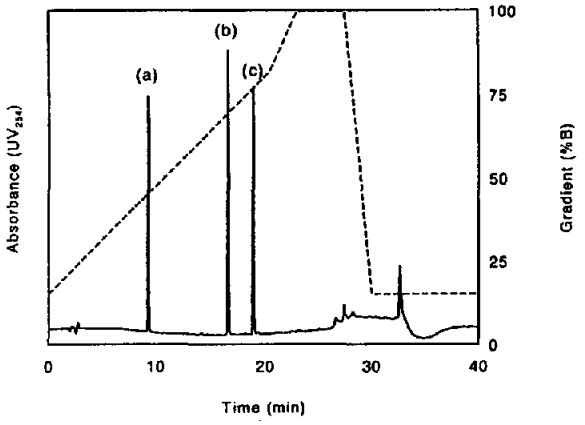

Fig. 1. Separation of 6β-OHF (a), F (b), and dexamethasone (c, I.S.) by HPLC. The amount injected was approx. 100 ng for each steroid. The gradient used is indicated as % mobile phase B in mobile phase A. Displayed without blank subtraction.

2.4. Calculations

6β-OHF and F concentrations were calculated from peak areas of the internal standard and 6β-OHF and F, respectively. The calculations were done essentially as described in Ref. [16] using the following formula exemplified for F:

$$F_{conc.}(ng/ml) = \frac{F_{peak\ area}}{I.S._{peak\ area}} \cdot I.S._{conc.} \cdot R \cdot \frac{120\ \mu l}{100\ \mu l}$$

where $I.S._{conc.} = 80$ ng/ml urine and R = relative response $(I.S._{UV}/F_{UV}) \cdot$ relative recovery $(I.S._{recov}/F_{recov})$. The relative response and relative recovery of 6β-OHF and F, respectively, to the I.S. were calculated from the slopes of standard curves obtained as described in the Results section. If only the 6β-OHF/F ratio is considered, the calculations can be simplified to:

$$\text{Ratio} = \frac{6\beta\text{-OHF}_{peak\ area} \cdot R_{6\beta\text{-OHF}}}{F_{peak\ area} \cdot R_F}$$

The R values obtained were recalculated regularly (see below under Standard curves).

3. Results

3.1. Chromatography

Figs. 1 and 2 show typical chromatograms of a standard mixture consisting of 6β-OHF, F, and I.S. and a urine sample, respectively. The coefficient of variation (C.V.) of the retention times of 6β-OHF, F, and I.S. was less than 0.31% ($n = 40$) within a series of runs and less than 1.11% ($n = 3$) between series of runs. The retention time ranges observed for the present system were 9.23–9.59 min (6β-OHF, $n = 86$), 16.59–17.02 min (F, $n = 86$), and 18.90–19.35 min (I.S., $n = 86$) on a day-to-day basis. A blank

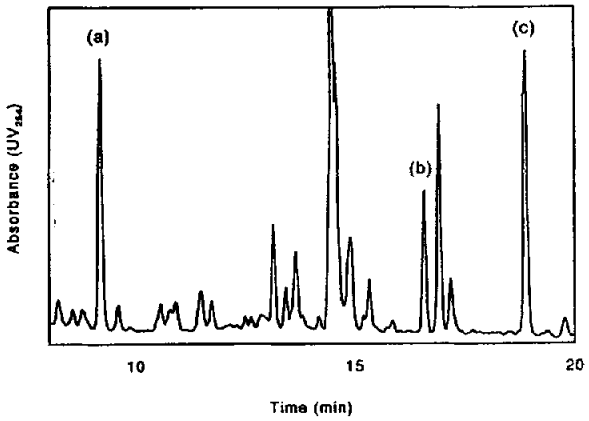

Fig. 2. Typical chromatogram of a urine sample. Peaks: 6β-OHF (a), F (b), and dexamethasone (c, I.S.). Displayed without blank subtraction.

injection of 100 μl of water was routinely subtracted from each chromatogram to compensate for the varying baseline resulting from the gradient profile. Confirmation of peak identities was performed by injection of the collected lyophilized peaks on different chromatographic systems. As shown in Table 1, the relative phase capacity ratios (k') of 6β-OHF and F in urine were identical to those of the authentic standard mixture. The peak-area ratios of 6β-OHF and F, respectively, to the internal standard were almost identical under four different HPLC conditions and in no case additional peaks were observed. Moreover, no peak interference was observed after inclusion of *N*-desmethylcitalopram, citalopram, fluoxetine, norfluoxetine, sertraline, litoxetine, paroxetine or fluvoxamine. These results imply that the present method is not subject to interference by coexisting substances and is suitable for the determination of 6β-OHF and F.

3.2. Standard curves

Standard curves were obtained from solutions prepared and processed by three different methods: direct injection of known concentrations of the steroids onto the HPLC system, normal sample preparation of aqueous solutions of known concentrations of the steroids, and finally normal sample preparation using a typical urine sample to which various amounts of 6β-OHF and F had been added prior to work up. From the standard curves obtained in these experiments, the relative response factors and relative recoveries could be calculated.

Standard solutions containing known amounts of 6β-OHF (1.0–2000 ng/sample), F (1.0–2000

Table 1
k' Values and peak-area ratios of 6β-OHF and F in urine relative to internal standard (I.S.)

Compound	k' Value				Peak-area ratio			
	I	II	III	IV	I	II	III	IV
Standard								
6β-OHF	0.41	0.06	0.07	0.22				
F	0.86	0.46	0.78	0.83				
Urine								
6β-OHF	0.41	0.06	0.07	0.22	1.55	1.53	1.53	1.54
F	0.86	0.46	0.78	0.83	1.44	1.43	1.45	1.43

Conditions: (I) Nova-Pak C_{18} (4 μm, pore size 60 Å, 300 × 3.9 mm I.D., Waters), 1.0 ml/min, gradient: $t = 0$ min, 15% B; $t = 20$ min, 82% B; $t = 22.5$ min, 100% B; $t = 27.5$ min, 100% B; $t = 30$ min, 15% B; $t = 40$ min, 15% B. A: 50 m*M* KH_2PO_4, 10 m*M* acetic acid in water, B: 65% (v/v) acetonitrile in A. (II) Zorbax ODS (5 μm, 250 × 4.6 mm I.D., Du Pont), 1.0 ml/min, isocratic, 30% acetonitrile in 50 m*M* KH_2PO_4 containing 10 m*M* acetic acid. (III) Ultrasphere ODS (5 μm, 250 × 4.6 mm I.D., Beckman), 1.0 ml/min, gradient: $t = 0$ min, 60% B; $t = 30$ min, 100% B. A: H_2O, B: methanol–water (4:1, v/v). (IV) Nucleosil 100-5C_{18} (5 μm, 150 × 4.6 mm I.D., Mikrolab Århus, Århus, Denmark), 1.0 ml/min, step-wise isocratic: $t = 0$–5 min, methanol–water (35:65); $t = 5$–20 min, methanol–water (56:44).

ng/sample), and I.S. (20–2000 ng/sample) were injected directly. Excellent linear correlations ($r = 0.9999$ in all cases) between the concentration of the standard solutions of 6β-OHF, F, and I.S. and their respective peak area as measured by the computer were confirmed over the entire measured range. The relative response factors of I.S. to 6β-OHF or F were obtained from the slope of I.S. to those of 6β-OHF and F, respectively, from such standard curves and found to be 1.03 ± 0.01 (S.D.) (I.S./6β-OHF, $n = 5$) and 0.72 ± 0.003 (S.D.) (I.S./F, $n = 5$).

Different overall recoveries were obtained for the individual steroids. This necessitated the additional incorporation of the relative recoveries in the calculations. Consequently, standard curves similar to those mentioned above were prepared for urine to which known amounts of the steroids had been added prior to sample preparation. The recoveries from experiments using different volumes of standard solutions diluted to 10 ml with water were also measured. Both measurements displayed a linear correlation between the originally added amount of standard and the peak area over the entire range. However, the urine samples showed a larger deviation than the aqueous samples. Thus, it was tested whether the recoveries of the aqueous standard solutions were comparable to or different from those of the urine standard solutions. Because of the previously established linear correlation in both cases, recoveries were only estimated for one selected concentration of each steroid representing an approximate average of the normal urine sample content. The recoveries of the aqueous and urine standard solutions were not significantly different, the actual difference being 1.49% (95% confidence interval: −32.6% to 35.6%, 6β-OHF), 4.88% (−31.5% to 21.7%, F), and 2.84% (−23.8% to 18.1%, I.S.), $n = 8$. These results demonstrate that the recoveries are not affected by the matrix used. Therefore routine control of the R values can be performed more simply and accurately by employing aqueous standards. The recoveries were found to be 70.8% (6β-OHF), 90.6% (F), and 91.6% (I.S.) resulting in relative recoveries of 1.29 (I.S./6β-OHF) and 1.01 (I.S./F) to be incorporated in the equation presented above, re-estimated on a regular basis.

3.3. Precision

The within-day and between-day precision of the assay with respect to the 6β-OHF/F ratio were calculated from series of experiments made with 1.0-ml aliquots of an average urine sample. The within-day coefficient of variation (C.V.) was 5.1% ($n = 7$), while the between-day C.V. was found to be 9.5% ($n = 19$). These determinations reflect the complete assay, including sample prepurification with Sep-Pak columns.

The results of the analyses mentioned below showed good correlation with results obtained similarly with 10.0 ml of urine ($y = 1.01 \times - 0.32$, $r = 0.990$), an amount that would normally offer a higher precision.

3.4. Reference range

We analyzed urine samples of 11 apparently healthy subjects. Due to the use of spot urines, the steroid concentrations fell within a wider range when calculated as ng/ml urine (Table 2). Only the ratio range should be considered as a reference range. The ratios ranged from 2.77 to 26.88 with an average of 10.09 ± 6.89 (S.D.).

4. Discussion

The isolation of steroids from urine has traditionally involved extraction procedures. Extractions have been performed using organic solvents, but solvent extraction alone for urinary cortisol clean-up prior to liquid chromatography was found to be inadequate in one study [20]. More recently, solid-phase extraction cartridges have been incorporated, i.e. products like Sep-Pak, Bond-Elut, etc., normally containing octadecylsilane-bonded phase packing. In the present study we found that the level of interfering compounds varied up to 100-fold depending on the composition of the solvent used to elute the Sep-Pak columns. Thus we tested several different solvents and combinations and amounts

Table 2
Quantification of 6β-OHF and F in spot urines of eleven healthy volunteers

Urine Sample	6β-OHF (ng/ml)	F (ng/ml)	6β-OHF/F
1	54.82	19.80	2.77
2	122.72	28.15	4.36
3	151.14	29.70	5.09
4	165.75	25.52	6.50
5	209.65	26.52	7.91
6	74.07	7.89	9.39
7	259.60	27.31	9.51
8	265.47	25.97	10.22
9	86.44	8.40	10.29
10	188.59	10.46	18.04
11	848.31	31.56	26.88
Mean	220.60	21.93	10.09
S.D.	219.79	8.87	6.89

of these. Methanol, ethanol, tetrahydrofuran, and acetone and combinations with water were all found to be insufficiently selective for the purpose although the internal standard was almost quantitatively eluted with all solvents. Diethyl ether has previously been used successfully in an extraction procedure for F [16]. We too found that it is highly selective for F, but unfortunately most of the 6β-OHF was retained on the Sep-Pak. Inclusion of one or more washing steps prior to elution as used for F in Ref. [16] was not successful as the increased level of purification was accompanied by loss of 6β-OHF. Ethyl acetate was found to be less selective for F but more selective for 6β-OHF. Experiments using varying concentrations of diethyl ether in ethyl acetate to optimize the column recovery for all three compounds resulted in the use of 5.0 ml ethyl acetate–diethyl ether (4:1, v/v) mixture as described above. It is important to note that the use of high-purity water, especially in the mobile phases, proved to be important for the overall precision of the assay as experienced in many assays involving gradient chromatography.

In the present study a 1.0-ml sample volume has been used. Experiments with different volumes up to 10.0 ml of urine have also been performed, but the marginally higher precision obtained in these experiments does not balance the problems with e.g. storage capacity in large scale studies. We also used urine volumes of less than 1.0 ml and found that 0.5 ml will work perfectly in most cases. However, the wider concentration range of spot urines favors 1.0 ml as the routine assay volume.

An other category of methods used for the quantification of steroids include RIA and ELISA. In the case of cortisol, previous investigations indicate that these assays overestimate the concentration of cortisol [15–17]. This is due to cross-reactivity with interfering compounds or metabolites of cortisol, which cannot be removed by solvent extraction procedures [6,21]. HPLC is therefore the most specific and suitable method for the quantification of these compounds, especially in studies concerned with minor differences in the steroid excretion correlated to e.g. lifestyle, diet, etc. Moreover, the requirement of only a 1.0-ml volume of urine or less for an accurate measurement takes away one of the few remaining advantages of RIA and ELISA assays which both can be performed with low sample volumes.

The mean 6β-OHF/F ratio presented here [10.09 ± 6.89 (S.D.)] is similar to previously published data obtained by other specific techniques, 9.34 ± 4.50 (spot urines) and 8.50 ± 3.76 (24-h urines) [8], 8.4 ± 4.1 (HPLC/RIA method) [7]. The present HPLC method uses easily accessible spot urine samples and only a single HPLC run for the analysis, and thus it constitutes an improvement compared to previous methods. The method is furthermore applicable in large scale cohort studies.

Acknowledgements

Birgit Lindquist is thanked for excellent technical assistance. This work was supported by The Danish Environmental Research Programme.

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