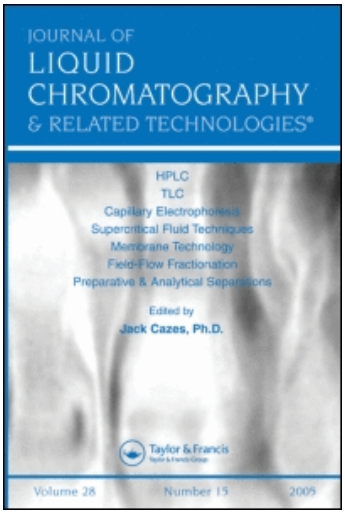

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USE OF AN INTERNAL STANDARD TO ASSAY 6 β-HYDROXYCORTISOL IN URINE

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ABSTRACT

Currently 6 β-hydroxycortisol is assayed by radioimmunoassay or high performance liquid chromatography techniques. We have developed an HPLC method, utilizing gradient elution and an internal standard (Δ 4-pregnenetetrol-3-one). In this way, accuracy and sensitivity of the assay were greatly improved and allowed the application of this modified method for monitoring the time-course of hepatic microsomal enzyme activity.

INTRODUCTION

A polar metabolite of cortisol, 6 β-hydroxycortisol (6 β-OHF) is formed in the liver during the phase I of biotransformation reactions. The binding of this metabolite to plasma proteins is low and it is excreted in the unconjugated form in urines. Measurement of 24h urinary 6 β-OHF excretion is a non invasive method and would appear to be one of the best endogenous index of hepatic oxidation of drugs in vivo (1). It is particularly useful to assess the influence of inducers (barbiturates, rifampicine, antiepileptic drugs) or inhibitors (cimetidine) of drug metabolism on the enzyme activity of the microsomal system.

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Therefore it was of great interest to develop an accurate and easy method for quantitative determination of urinary 6 β-OHF.

EXPERIMENTAL

Reagents. All reagents were of analytical grade and were used without any further purification.

6 β-hydroxycortisol (Δ 4-pregnene-6β, 11β, 17α, 21 tetrol-3,20 dione) was supplied by Steraloids Inc. (Pawling, NY, USA) and the internal standard (Δ 4-pregnene-11β, 17α, 20α, 21 tetrol-3-one) by Sigma (St-Louis, MO, USA) abbreviated as Δ-4). 16 α-hydroxy hydrocortisone was kindly supplied by Roussel Uclaf (Romainville, France). Stock solutions of 6 β-OHF and the internal standard were prepared in absolute ethanol and when stored at +4°C, were stable for at least 4 months.

Equipment. The HPLC system was from Dupont Instruments with a pump module 841, a variable-wavelength UV/VIS detector (837 model) operating at 243 nm, and a programmable gradient 838 model (Wilmington, DE, USA). The detector was connected to a Shimadzu C-R1A Chromatopac integrator (Shimadzu Corporation, Kyoto, Japan). The column was a Rsil C 18 HL (10 µm or 5 µm), 25cm x 4.6mm i.d. stainless steel (RSL, Eke, Belgium). The injection valve was a Rheodyne 7010 model fitted with a 200 µl sample loop.

Sample preparation. Urines were stored frozen at -20°C prior to analysis. Extraction of 6 β-OHF was performed as described previously by Frantz and al. (1), from 10 ml portions of urine after addition of 200 µl of the internal standard (5 mg Δ-4 in 50 ml of absolute ethanol).

Calibration curve. The calibration curve was established as follows : 10 ml portions of distilled water, instead of urine, containing increasing concentrations of 6 β-OHF were subjected to the extraction procedure des-

cribed for urine and the data were plotted on the calibration curve (peak area ratio method). The curve obtained by the least-squares was linear over the range used (1 µg to 15 µg per 10 ml). The linear regression value was : r = 0.995 (n = 6).

Analysis. The mobile phase consisted of a solution of KH_2PO_4 0.01 M which was freshly prepared in distilled water and to which 0.05 % trichloroacetic acid 1.7 N was added. Subsequently acetonitrile (Baker UV grade) was added to the prepared solution in the proportions : 9/1 buffer/acetonitrile (v/v) for reservoir A and 7,5/2,5 buffer/acetonitrile (v/v) for reservoir B (Eluent A and B respectively).

The analysis was performed by using a linear elution gradient flow over a period of 10 minutes. The gradient flow was started 3 minutes after the injection of the sample.

The flow-rate was about 1.5 ml min^{-1}, corresponding to a pressure of 2000 psi. After addition of 500 µl of eluent A to the dry extract, 200 µl were injected through the sample loop of the HPLC system. The concentration of the urinary 6 β-OHF was calculated from the peak area ratio compared to the calibration curve.
All procedures were carried out at room temperature.

RESULTS

Our first results were obtained with a 10 µm particles column packing. The retention times of 6 β-OHF and of the internal standard (Δ-4) were 10.0 min and 18.5 min respectively.

Sharper peaks but longer retention times were obtained if the column was packed with 5 µm particles (15.7 and 28.5 min for 6 β-OHF and the internal standard respectively). A typical chromatogram of a urine sample obtained with this last column is shown in the Fig. 1.

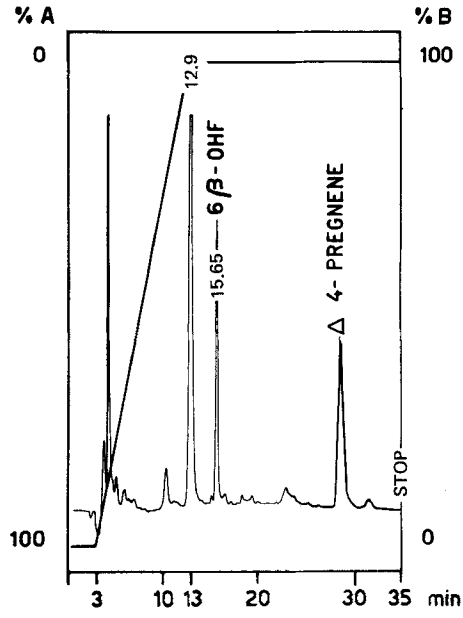


FIGURE 1. Typical chromatogram of a urine sample containing the internal standard (Δ 4), with illustration of the time-course crossing from eluent A to eluent B

Assuming the magnitude of the signal to noise ratio to be at least of the order of three, the detection limit of 6 β-OHF corresponds to a concentration of 10 ng ml^{-1}. This limit could be increased still further by carrying out the extraction step on larger volumes of urine.

The reproducibility of the method was investigated with aliquots of urine containing low, moderate and high concentrations of 6 β-OHF (Table 1). When urines were kept frozen, no significant decrease in 6 β-OHF values was observed after a period of 6 months.

Recovery of the internal standard (Δ-4) from urine was found to be above 95 %. In addition to Δ-4, the use of 16 α-hydroxycortisol as an internal standard was investigated.

Table 1. Reproducibility and accuracy of the method expressed as the coefficient of variation on 6 samples (for high values, the samples were different) ; 6 β-OHF concentrations were given in µg per 10 ml of urine.

Concentration range of β-OHF	Intra-assay	C.V. %	Inter-assay	C.V. %
Low	2.74 ± 0.09	7	2.73 ± 0.07	5
Moderate	5.38 ± 0.11	6	5.29 ± 0.24	9
High	13.13 ± 0.43	6	9.87 ± 0.19	5

A blank and a spiked urine containing both standards are shown in Fig. 2.

DISCUSSION

For clinical purposes, measurements of urinary 6 β-hydroxycortisol from a 24h collection require a simple, rapid and reproducible method. Others have described methods utilizing radioimmunoassay (2,3,4) and high performance liquid chromatography (5,6). A comparison of the two methods was done by Gerber-Taras and al. (7). Both gave identical values and reliable intra-assay variation. Those authors who utilized HPLC technique measured the concentrations of urinary 6 β-OHF by comparison with a standard solution run daily or with spiked urine samples.

To our knowledge, a true internal standard in order to eliminate the losses during the processing of the urines has not yet been described as so far.

We have improved the HPLC method for quantitative determination of urinary 6 β-OHF by including Δ4-pregnene-tetrol-3-one in the samples analyzed.

The use of a gradient elution allowed a fairly good separation of 6 β-OHF and to elute two internal standards

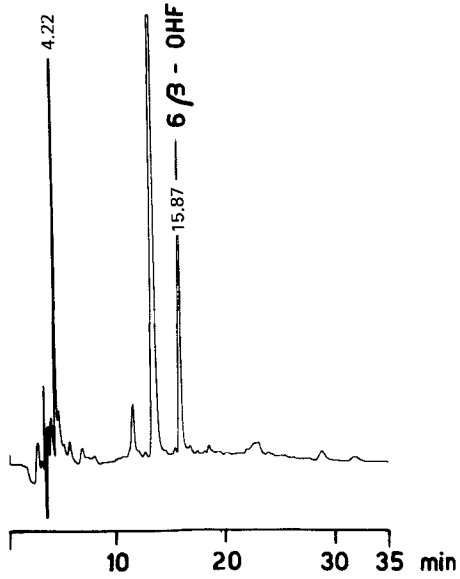
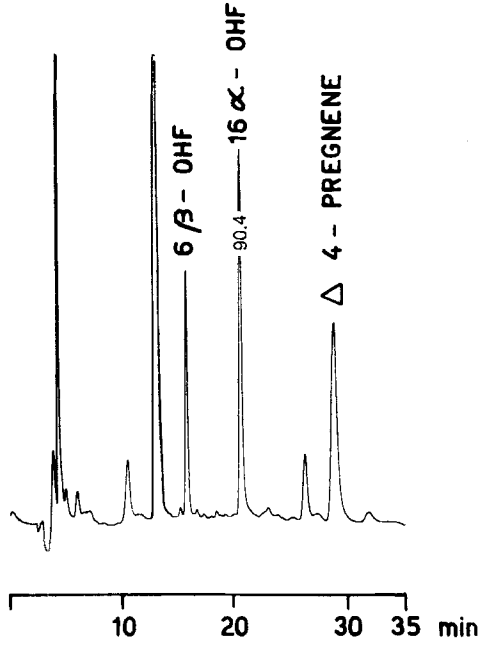


FIGURE 2a) Chromatogram of a blank urine



2b) Chromatogram of a urine spiked with 16 β-OH cortisol and Δ4-pregnene

from the corticosteroids family which are normally absent from urines. Unfortunately, 16 α-OHcortisol was unstable in ethanol solution (evidenced by the additional peak eluting just before Δ4-pregnene, in Fig. 2b).

There is little hope to speed-up the analysis with the 5 μm particles column packing unless we added a higher proportion of acetonitrile to eluent B, but increasing the risk to contaminate the 6 β-OHF peak.

Mean values of urinary 6 β-OHF in 23 healthy volunteers of both sexes aged 18 to 25 years were 199 μg ± 17/24h (M ± SEM). There was no significant difference between male and female volunteers.

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