*Journal of Chromatography*, 613 (1993) 132–136
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6680

# Short Communication

# Determination of urinary 18-hydroxycortisol by isocratic normal-phase high-performance liquid chromatography

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(First received August 12th, 1992; revised manuscript received November 9th, 1992)

ABSTRACT

A method for the determination of urinary 18-hydroxycortisol by high-performance liquid chromatography is described. Urinary samples were first mixed with an internal standard, 18-hydroxyprednisolone. 18-Hydroxycortisol and 18-hydroxyprednisolone, extracted by a Bond Elut column, were dehydrated by 1% (w/v) *p*-toluenesulphonic acid to the 11,18-epoxides. The epoxides were separated into two distinct peaks on a Resolve Silica column with a mobile phase of chloroform–methanol (100:2.5, v/v). The detection wavelength was 248 nm. The urinary 18-hydroxycortisol concentration was calculated from peak-height ratio of 11,18-epoxycortisol to 11,18-epoxyprednisolone. The linearity of the ratio was satisfactory in the range 12.5–300 ng per injection of 11,18-epoxycortisol. A specific increase of urinary 18-hydroxycortisol in patients with primary aldosteronism was demonstrated.

## INTRODUCTION

18-Hydroxycortisol (I) is called a "hybrid steroid", because of its 17α- and 18-hydroxyl groups, known to be characteristic of glucocorticoid and mineralocorticoid, respectively. The unconjugated form of this steroid was first discovered in urine from patients with primary aldosteronism caused by adrenocortical adenoma [1] and later in patients with a rare congenital glucocorticoid-suppressible hyperaldosteronism [2]. Previous studies show that compound I is a good

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0378-4347/93/$06.00 

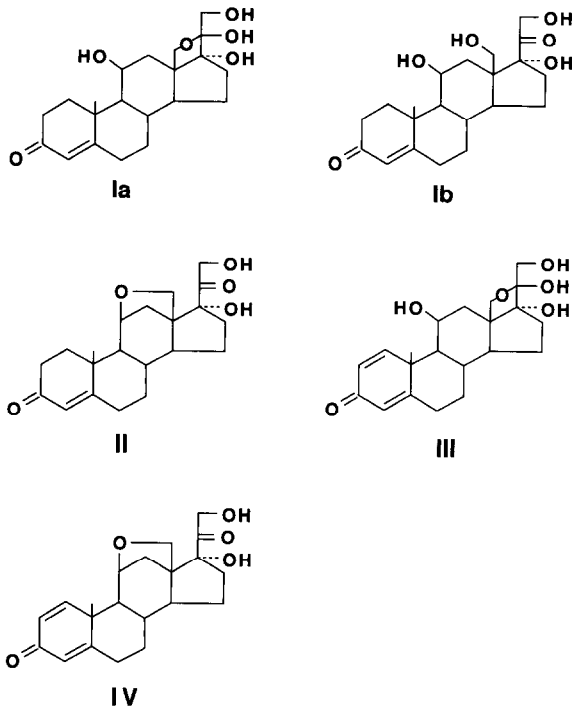


Fig. 1. Molecular structures of steroids.

discriminator of these disorders [3,4]. Although the use of high-performance liquid chromatography (HPLC) for separation of I in the pretreatment of radioimmunoassay has been reported [5], few studies are available describing the measurement of this compound by HPLC. In our attempt to measure I by reversed-phased HPLC [6], the retention time of this compound was found to vary, presumably owing to its transformation from the 18,20-hemiacetal form (Ia), the major form in physiological conditions, to the 20-ketone form (Ib) (Fig. 1) [1], requiring preliminary separation by thin-layer chromatography.

This paper describes a novel method for the measurement of I in urine, in which compound I was dehydrated into 11,18-epoxycortisol (II) (Fig. 1), to give a distinct and reproducible peak on isocratic normal-phase HPLC.

## EXPERIMENTAL

### *Steroids*

Compound I and 18-hydroxyprednisolone (III) (Fig. 1) were synthesized by a photochemical reaction, as previously described [7]. Compound II was synthesized as follows. The mixture of 18-iodocortisol 17α,21-acetonide (340 mg), prepared as previously described [7], and silver acetate (108 mg) in dioxane–water (30:3, v/v) was refluxed for 4 h with stirring. The reaction mixture was then evaporated, and the residual oil, dissolved in ethyl acetate, was filtered. The filtrate was washed with water, dried and evaporated. The crude product was purified by silica gel column chromatography (benzene–diethyl ether, 3:1, v/v) and was recrystallized from methanol to give 11,18-epoxycortisol 17α,21-acetonide. Acid hydrolysis of the acetonide was performed as described previously [7]. The crude product was recrystallized from methanol to give II as colourless prisms (110 mg, 48%): m.p. 238–240°C; $^1$H NMR $\delta$ ($CDCl_3$) 1.25 (3H,s), 3.29 and 3.69 (each 1H, d, $J$ = 8 Hz), 4.40 (1H, bs), 4.43 and 4.56 (each 1H, dd, $J$ = 5 and 15 Hz), 5.70 (1H, s); IR (Nujol) 3525, 1710, 1665 and 1612 cm$^{-1}$. 11,18-Epoxyprednisolone (IV, m.p. 214–217°C) (Fig. 1) was synthesized from 18-iodoprednisolone 17α,21-acetonide [7] by the method described for II.

### *Apparatus and chromatographic conditions*

The HPLC system consisted of a Model 6000A pump (Waters Assoc., Milford, MA, USA), a Model VL-611 injection valve (Jasco, Tokyo, Japan) and a UVIDEC 100 II UV detector (Jasco). The column, Resolve Silica (5-μm spherical silica, 150 mm × 3.9 mm I.D., Millipore, Bedford, MA, USA), was eluted with a mobile phase of chloroform–methanol (100:2.5, v/v), at a flow-rate of 0.5 ml/min at room temperature. The absorbance was continuously monitored at 248 nm.

### *Sample preparation*

Patients with primary aldosteronism ($n$ = 7) and those with secondary aldosteronism (renal tubular acidosis, $n$ = 1; renovascular hypertension, $n$ = 1; and diuretic-induced secondary aldosteronism, $n$ = 5), and normal subjects ($n$ = 10) collected a 24-h urine while consuming a normal diet. All of the patients with secondary aldo-

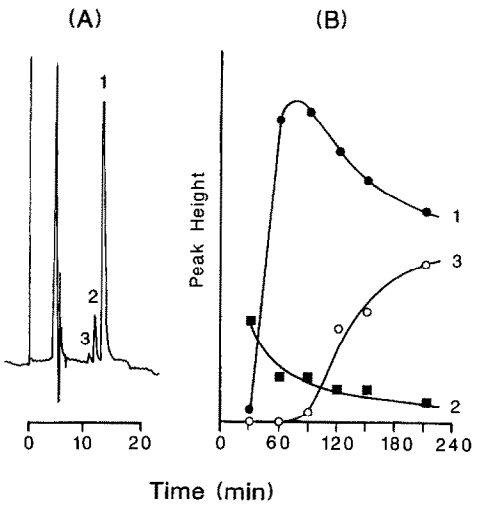


Fig. 2. Dehydration of I with *p*-toluenesulphonic acid. (A) Elution profiles of reaction products sampled at 90 min. Peaks: 1 = II; 2 = a possible direct precursor; 3 = degradation product. (B) Time-course of dehydration: (●) peak 1; (■) peak 2; (○) peak 3.

steronism had plasma aldosterone concentrations higher than 250 pg/ml (control, less than 150 pg/ml). The patient with renal tubular acidosis exhibited the most severe aldosteronism: 703 pg/ml plasma aldosterone concentration. Drugs were withdrawn for two weeks prior to this study. Urine samples were stored at −70°C until use without preservatives.

*Extraction*

Compound III (internal standard) was added at a concentration of 0.5 mg/l to a 5-ml sample of urine. Urine was then applied to a Bond Elut column (Analytichem International, Harbor City, CA, USA) and was eluted in 5 ml of methanol after a wash with 5 ml of water. The extraction recoveries for I and III ranged from 75 to 85%.

*Dehydration*

The extract, dried in a centrifugal evaporator, was dehydrated in 2 ml of chloroform containing 1% (w/v) *p*-toluenesulphonic acid at 25°C for 75 min in a screw-capped glass tube. After a wash with water saturated with $NaHCO_3$, 1 ml of the chloroform phase was sampled and evaporated under a nitrogen stream. The dehydrated materials were dissolved in 50 μl of the HPLC mobile phase solvent, and 4 μl of the solution were applied to the HPLC column.

To establish the above procedures, the time-course of dehydration for I was studied. During 30–210 min of dehydration under the above conditions, the reaction products were analysed by HPLC, under the conditions described. Fig. 2A shows the chromatogram for the reaction product at 90 min. Peaks 1, 2 and 3 correspond, respectively, to II, a possible direct precursor of II and a degradation product of II. As shown in Fig. 2B, the formation of II reached a maximum at 60–90 min and, subsequently, degradation of the epoxide proceeded. Thus, dehydration of I was terminated at 75 min in the following studies. The conversion yields from I to II were the same as those for III to IV in seven assays, ranging from 82 to 88%.

*Concentration*

Since the extraction recovery and epoxidation yield for the internal standard (III) were equal to

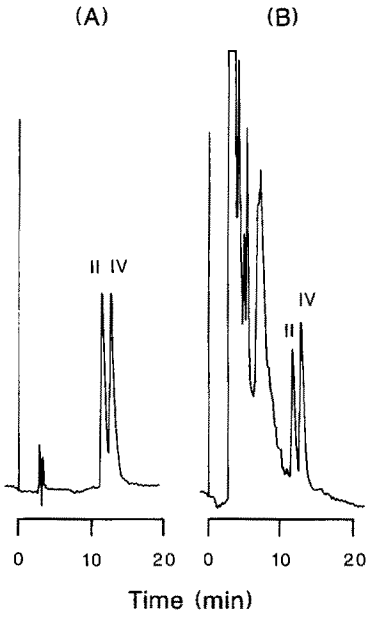


Fig. 3. (A) HPLC elution profile of authentic II and IV. (B) Typical chromatogram for a urine sample from a patient with primary aldosteronism after the dehydration procedure.

TABLE I

RECOVERIES AND PRECISION OF THE HPLC ASSAY

| Concentration added (mg/ml) | Recovery (mean ± S.D.; $n$ = 15) (%) | Coefficient of variation (%) | |
|---|---|---|---|
| | | Intra-day ($n$ = 10) | Inter-day ($n$ = 6) |
| 0.5 | 54.5 ± 6.7 | 4.4 | 5.2 |
| 2.0 | 66.8 ± 6.3 | 6.3 | 5.6 |

those for I, the concentration of I in urine was calculated from the peak-height ratio of II to IV and from the concentration of added internal standard in urine (0.5 mg/l).

*Assay validation*

The recovery and precision of the assay were determined by adding I into control urine at two concentrations: 0.5 and 2.0 mg/l. The urine was stored at −50°C in aliquots until use.

## RESULTS AND DISCUSSION

Compounds II and IV gave two distinct HPLC peaks with retention times of 11.5 and 13.0 min for II and IV, respectively (Fig. 3A). Fig. 3B shows a chromatogram of a urine, sampled from a patient with primary aldosteronism, extracted and dehydrated as described above. Although several unidentified peaks were observed, no interference was encountered.

The calibration curve for II, constructed by plotting the peak-height ratio of II to IV, added at a concentration of 25 ng/μl, against the amount of II was linear over the range 12.5–300 ng per 4-μl injection: $y = 0.0098x + 0.00096$ ($r = 1.000$); $y$ = peak-height ratio; $x$ = amount of II (ng). The recoveries for I, and the intra-day and inter-day coefficient of variation (C.V.) are listed in Table I. Although the recoveries were relatively low, the use of III as the internal standard resulted in high reproducibility.

The urinary concentration of I was undetectable in seven patients with secondary aldosteronism and in ten normal subjects (Table II). Elevated urinary excretion of I was found in patients with primary aldosteronism, as previously described [1,2,4]. After removal of adrenocortical

TABLE II

18-HYDROXYCORTISOL (I) CONCENTRATIONS IN URINE AS MEASURED BY THE HPLC ASSAY

| | $n$ | Concentration (mg/l) | | | Excretion rate (mg/day) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | S.D. | Range | Mean | S.D. | Range |
| Primary aldosteronism | | | | | | | |
| Before adrenalectomy | 7 | 0.64 | 0.39 | 0.24–1.5 | 1.1 | 0.49 | 0.56–2.0 |
| After adrenalectomy | 6 | <0.1 | | | | | |
| Secondary aldosteronism | | | | | | | |
| Renal tubular acidosis | 1 | <0.1 | | | | | |
| Renovascular hypertension | 1 | <0.1 | | | | | |
| Diuretics-induced | 5 | <0.1 | | | | | |
| Normal subjects | 10 | <0.1 | | | | | |

adenoma, urinary concentrations of I decreased to the control level in each patient.

## CONCLUSION

Epoxidation of I, coupled with the use of III as the internal standard, enabled a sensitive and reproducible measurement of I in urine by HPLC. Since compound I is highly specific of primary aldosteronism, our method is useful for differential diagnosis of this disease from other causes of hypertension.

## ACKNOWLEDGEMENTS

This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, 01 771 198, 02 770 990 and 04 670 946.

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