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Determination of cortisol and cortisone in urine using high-performance liquid chromatography with UV detection

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Abstract: The application of reversed-phase gradient high-performance liquid chromatography with UV detection to the determination of cortisol and cortisone in 24-h urine samples is described. The method employs Sep-pak C18 cartridges for the part-purification and concentration of the corticosteroids, with sample enrichment at the head of an HPLC pre-column and separation using water/acetonitrile gradient. The internal standard is 6α-methylprednisolone. Measurement of both cortisone and cortisol provides further information on adrenocortical function. 24-hour excretion rate data from normal subjects are reported.

Keywords: *Cortisol; cortisone; urine analysis; reversed-phase HPLC; UV detection.*

Introduction

Urinary free cortisol is a biochemical marker with proven clinical application, showing good correlation with states of adrenal hypo- and hyperfunction [1–3]. There is, however, a considerable amount of variation in the means and normal ranges reported by workers using different analytical techniques.

Radioimmunoassay (RIA) and competitive protein-binding assay (CPBA), often the methods of choice, have recently been criticized on the grounds that they are subject to interference [4–7]. The specificity and thus the accuracy of these techniques can be substantially improved by preliminary chromatographic procedures. Schoneshofer and Weber [8] have recommended high-performance liquid chromatography (HPLC) as a prelude to RIA quantitation of the purified cortisol fraction.

More direct HPLC methods, employing for example UV-absorbance detection, have been used in the analysis of plasma cortisol [9–13] but problems of sensitivity and interference have previously restricted the development of similar assays of urine. Recently, however, sample preparation methods and chromatography procedures have been devised which circumvent these problems and allow direct quantitation using fluorescence [14] or UV [15–17] detection.

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Urinary cortisone has received rather less attention despite the fact that it is interconverted with cortisol *in vivo* (primarily in the liver). Thus, although it is apparently the less active of the two compounds, it should not be disregarded. It provides a potentially useful further measure of adreno-cortical function. Simultaneous determination of cortisol and cortisone using HPLC has previously been reported for plasma [13] and for urine [15].

The present paper describes a new method in which an initial purification using Sep-pak C18 cartridges is used to prepare a fraction containing cortisol and cortisone. This can then be analysed directly using reversed-phase HPLC with UV-absorbance detection. Sensitivity and efficiency of separation are enhanced by enriching the sample at the head of a pre-column and using a water/acetonitrile gradient.

Sep-pak C18 cartridges have previously been used to prepare steroid extracts of biological fluids [13, 18, 19]. They contain a solid matrix with bonded C18 residues. By varying the composition of the solution passed through the cartridge, substances can be selectively retained or eluted. Manipulation of pH is used in the present method to separate a neutral steroid fraction (containing cortisol and cortisone) from steroid conjugates.

Materials and Methods

Urine collections

Twenty-three female nurses (mean age 23.8, range 20–34) involved in a study of the effects of shift changes on endocrine systems provided 24-h urine samples. Subjects collected three samples corresponding to three separate 24-h periods, incorporating either day-shifts or night-shifts. No subjects reported serious illness, but details of mild illnesses and medication were noted. Further samples were obtained from six male subjects (nurses and researchers).

Crystalline citric acid (5g) was added to each 2.5 l container before collection, primarily to preserve other hormones in the samples. Urines collected in the presence and absence of citric acid showed no discernible differences in corticosteroid content, even after several months of storage. After measuring total volume, aliquots were frozen and stored at −20°C prior to analysis.

Reagents

Cortisol, cortisone and internal standard, 1,4-pregnadiene-6α-methyl-11,17,21-triol-3,20-dione (6α-methylprednisolone) were obtained from Sigma (London, UK). Fisons "A.R." methanol and Fisons "HPLC" acetonitrile (Fisons Scientific Apparatus, Loughborough, UK) were used throughout. All other chemicals were of reagent grade and solutions were prepared using glass-distilled, deionized water.

Solutions (10 g/l) of the steroids in methanol were prepared and stored at −20°C. These showed no appreciable degradation over a period of several months. Working standards were prepared freshly as required by making suitable dilutions with methanol.

Sep-pak C18 cartridges were obtained from Waters Associates Ltd. (Cheshire, UK).

Sample preparation and chromatography

A Sep-pak cartridge was primed with 2 ml of methanol followed by 5 ml of water. A 20 ml sample of urine with 4 μg internal standard added was then passed through the cartridge followed by a wash solution of 5 ml of 0.1 mol/l ammonium carbonate. A

fraction containing a variety of unidentified UV-absorbing substances including steroid glucuronides was then eluted with 5 ml of 10 mmol/l ammonium carbonate in acetonitrile/water (18:82, v/v). Finally, a fraction containing cortisol, cortisone and internal standard was eluted with 1 ml of acetonitrile/water (90:10, v/v). After mixing, 0.5 ml of this fraction was diluted with water to give a total volume of 2.7 ml, of which 2.0 ml was injected into the chromatograph.

The efficiency of the extraction from urine and water was investigated. Recoveries (%) were calculated from extractions from urine and from water with 1 μg of each steroid (including the internal standard) in 10 ml of aqueous matrix.

The chromatograph comprised a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics Ltd., St Albans, UK), a Magnus M7110 autosampler (Magnus Scientific Instrumentation Ltd, Aylesbury, UK) with pneumatically operated Rheodyne injection valve and 2000 μl loop, an Altex Ultrasphere octadecyl column, 5 μm C18-bonded silica, 250 × 4.6 mm i.d. (Beckman-RIIC Ltd, High Wycombe, UK), a Hypersil 5μ-ODS precolumn, 50 × 5 mm i.d. (HPLC Technology Ltd, Macclesfield, UK), and a Pye–Unicam LC-UV detector (Pye–Unicam Ltd, Cambridge, UK) set at 260 nm. The chromatograms were analysed using a Spectra-Physics SP4100 computing integrator. The system was capable of continuous automatic handling of samples with a run-time of approximately 36 min for each sample. The water/acetonitrile gradient is shown diagrammatically in Fig. 1; a constant flow-rate of 0.9 ml/min was maintained. Solvents were degassed with helium.

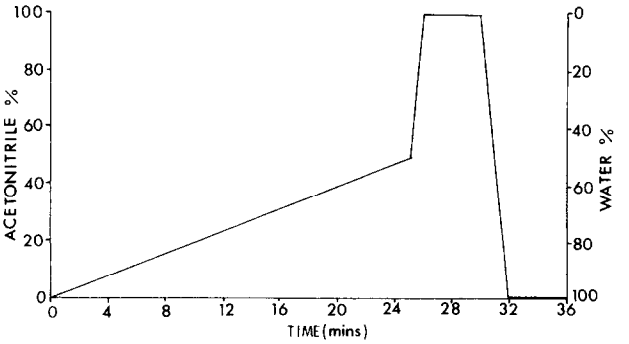

Figure 1
Variation in the composition of the solvent mixture with time after the injection of the sample.

Results

Figure 2 shows a chromatogram obtained from a 24-h urine sample, with peaks corresponding to cortisol, cortisone and the internal standard, 6α-methylprednisolone. Table 1 shows the mean percentage recoveries of cortisol, cortisone and 6α-methylprednisolone added to water and urine, and the mean ratios of percentage recoveries of the corticosteroids relative to the internal standard in individual extractions. Calculations of recoveries from urine are corrected for the endogenous steroids present. The results indicate some variability in the efficiency of recovery of cortisol and cortisone in the Sep-pak extraction stage. It is thus important to use an internal standard in the procedure: 6α-methylprednisolone is suitable in this respect, with percentage recoveries both from urine and water closely comparable with those of the endogenous steroids.

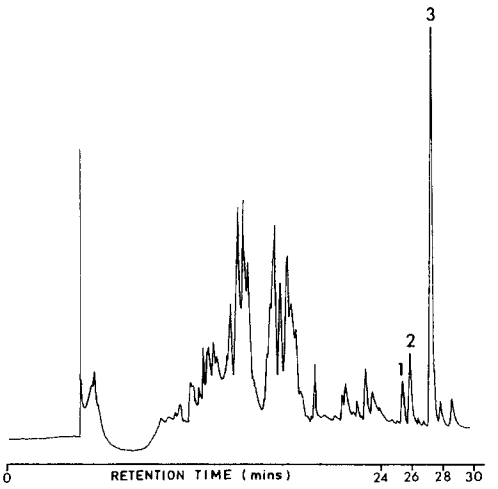

Figure 2
Chromatogram from a 24-h urine sample showing peaks corresponding to cortisol (1), cortisone (2) and 6-α-methylprednisolone (3). Determined cortisol concentration = 48.6 nmol/l, determined cortisone concentration = 109.9 nmol/l.

Table 1
Recoveries and recovery ratios (%) in extractions after addition of 1 μg of each steroid to 10 ml water (n = 4) and 10 ml urine (n = 4) I.S. = internal standard.

	% recoveries (mean + S.D.)						% recovery ratios (mean + S.D.)			
	Cortisol		Cortisone		I.S.		Cortisol/I.S.		Cortisone/I.S.	
Water	73.8	6.8	77.3	9.3	74.6	8.8	0.989	0.006	1.029	0.015
Urine	79.4	2.2	80.6	0.6	80.6	2.7	0.985	0.006	1.000	0.027

Interferences in the assays can be identified by abnormal peak shapes, deviation of absolute retention time or deviation of retention time relative to the internal standard. Relative retention times of standards in the system were highly constant, rarely deviating by more than 0.2%. The 75 samples assayed in the present study included two (from the same female subject) where problems of interference with the cortisol peak were apparent. The subject did not report any intake of drugs and the source of interference remains unclear. No problems of interference with the cortisone peaks were apparent.

The relatively large internal standard concentration (200 ng/ml) was chosen so that unidentified co-chromatographing substances would have minimal effect. In eight randomly selected samples, extracted without internal standard, the mean peak area of potentially interfering substances amounted to less than 0.5% of average 6-α-methylprednisolone peak areas.

Aliquots of urine spiked with cortisol and cortisone at five different levels in the range 0–1500 nmol/ml were analysed. The results showed that the expected and determined concentrations were closely correlated, both for cortisol (r = 0.999) and for cortisone (r = 0.999). Reported peak areas showed a linear relationship with added concentration.

Limits of detection are defined by the capacity of the computing integrator to discriminate between the baseline noise and peaks. Variation in detector voltage response can be automatically determined for a 'baseline' run with an injected blank sample. Peaks are thereafter identified by a suitably steep and characteristically constant increase in detector voltage response. Using these criteria, the limit of detection was approximately 2.5 nmol/l for both cortisol and cortisone. In the case of low-concentration urine samples, however, larger starting quantities can be used since the Sep-pak cartridge has a high capacity for adsorption [18]. Potential limits of detection are therefore somewhat lower than 2.5 nmol/l.

Repeated determinations of endogenous corticosteroids in aliquots of a urine sample gave a within-run coefficient of variation for cortisol of 5.2% at a mean determined concentration of 50.7 nmol/l, and for cortisone of 5.9% at a mean determined concentration of 127.3 nmol/l ($n = 5$). Between-run coefficients of variation were 7.6% for cortisol at a mean concentration of 77.8 nmol/l, and 7.0% for cortisone at a mean concentration of 153.3 nmol/l ($n = 4$).

Table 2 shows corticosteroid excretion data from female nurses determined from urine collected over 24-h periods incorporating a day-shift. Results from six male subjects are also presented. Mean cortisol excretion in the same group of female nurses was lower when determined over two 24-h periods incorporating night-shifts (54.26 and 63.34 nmol/24 h): mean cortisone excretion, on the other hand, was slightly higher (179.94 and 170.43 nmol/24 h). None of these differences, however, were statistically significant.

Table 2
Results from female and male subjects: mean urinary corticosteroid concentrations and 24-h excretion rates

	Females				Males			
	Mean	S.D.	*n*	Range	Mean	S.D.	*n*	Range
Cortisol (nmol/l)	55.28	22.32	22	18.32–111.59	49.35	15.01	6	25.10–66.21
Cortisone (nmol/l)	134.51	42.39	23	75.15–208.07	135.37	38.83	6	89.88–190.79
Cortisol (nmol/24 h)	66.84	29.93	22	27.61–140.30	77.30	30.23	6	41.16–124.47
Cortisone (nmol/24 h)	165.02	72.98	23	47.63–326.13	204.33	40.00	6	144.69–251.37

Discussion

Values for 24-h cortisol excretion in normal male and female subjects in the present study are similar to those obtained by workers employing other HPLC-based methods or immunoassay quantitation in combination with chromatographic separation techniques (Table 3). They are, however, substantially lower than those obtained by other workers using fluorescence-based methods or immunoassay methods without initial chromatographic steps, giving support to the proposition that these earlier methods tend to overestimate true cortisol concentrations.

Urinary cortisone excretion has so far received little attention, although Lewbart and Elverson report a closely comparable mean value of 149.8 nmol/24 h/g creatinine in a mixed group of 51 male and female subjects [15]. It should now be possible to investigate associations of cortisone excretion with states of health and disease and to assess its value as an additional marker of adrenocortical activity.

Table 3
Mean 24-h cortisol excretion rates (nmol/24 h) determined in normal subjects using different analytical techniques

Ref.	Technique	Males Mean	*n*	Females Mean	*n*	Sex unspecified Mean	*n*
20	Fluorescence	—	—	—	—	195	38
21	CPBA	128	7	—	—	—	—
22	RIA	—	—	—	—	119	8
23	Column chrom./RIA	155	11	166	9	—	—
4	Column chrom./CPBA	56.6	6	38.6	6	—	—
24	TLC/RIA	—	—	51.0	9	—	—
6	I.D./Mass Spec.	122	20	51	25	—	—
15	TLC/HPLC	—	—	—	—	83	51
8	HPLC/RIA	68.3	32	—	—	—	—
16	HPLC (2-cycle)/UV	—	—	—	—	112.8	32
17	HPLC/UV	—	—	—	—	55.5	45
—	HPLC (present method)	77.3	6	66.8	22	—	—

CPBA = competitive protein binding assay, RIA = radioimmunoassay, I.D. = isotope dilution.

It has been demonstrated that the pituitary–adrenocortical axis is responsive to numerous environmental, social and psychological stimuli [25]. Substantial variations both within and between populations might thus be expected. Assuming specificity and accuracy of technique, attention should be paid to defining population parameters and the circumstances of collection before means and ranges are compared.

Of the currently available methods, the immunoassays do have a history of successful clinical application and have been shown to correlate well with more specific methods [6]. For more demanding applications, however, HPLC is a useful adjunct or alternative. The present method dispenses with RIA quantitation, thus reducing costs and eliminating the use of radioactive materials. The extraction procedure is straightforward and rapid and the use of automatic equipment facilitates unattended operation.

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