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SELECTIVE DETERMINATION OF URINARY FREE CORTISOL BY LIQUID CHROMATOGRAPHY AFTER SOLID-STATE EXTRACTION

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SUMMARY

We have developed a selective and precise high-performance liquid chromatographic method for urinary free cortisol with an improved and efficient sample clean-up using C_{18} Sep-Pak cartridges. The urine sample (2 ml), with 11-deoxycortisol as internal standard, is applied to the Sep-Pak, which is then sequentially washed with acetone–water (1·4, v/v), water and hexane. Cortisol is eluted with diethyl ether, evaporated to dryness and redissolved in 2 ml of water. The wash cycle is repeated once using the same Sep-Pak cartridge. This double extraction greatly improves sample clean-up and allows modification of the mobile phase (tetrahydrofuran–methanol–water) so that cortisol is rapidly eluted as a single well resolved peak at 13 min. Chromatography is performed isocratically on a reversed-phase column with detection at 254 nm. Detection limits for urinary free cortisol by this procedure were two or three times lower than those obtained with two commercial radioimmunoassay kits. The chromatographic method was used successfully in the diagnosis of patients with hypercortisolism and Cushing's syndrome.

INTRODUCTION

Urinary free cortisol (UFC) is considered to be a specific and sensitive test for the diagnosis of Cushing's syndrome [1]. Methods for determining UFC include competitive protein binding assays, radioimmunoassays (RIA) and high-performance liquid chromatography (HPLC). RIA procedures are designed primarily for the assay of serum cortisol. These assays are applied either directly to urine samples or after extraction of urine with organic solvents. Although the specificity of antisera employed in most RIA systems is generally acceptable for serum

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cortisol measurements, it is clear that urine contains many cross-reacting substances that make RIA of urine cortisol generally non-specific [2,3]. Efforts to improve the specificity by extraction of cortisol with methylene chloride and washing the solvent extract with aqueous alkaline and acidic solutions were only partially successful in removing all of the interferences [4]. Some interfering compounds are known but most of them, presumably corticosteroid metabolites, remain unidentified [2,5].

Chromatographic separation of cortisol from interfering compounds is an efficient way of improving UFC assay specificity. Liquid chromatographic separations of steroids have been reviewed [6]. Schoneshofer and co-workers [7,8] quantified cortisol and other urinary steroids after collecting fractions separated by HPLC and assaying these fractions by RIA. Recently, Canalis et al. [9] used a direct quantitation technique by monitoring UV absorbance at 254 nm. These methods are considered selective for UFC. The differences in selectivities between various methods for UFC determination can be appreciated by comparing the reference ranges recommended for these methods. Ranges obtained by RIA are generally two to three times higher than those obtained using HPLC alone [9] or coupled HPLC–RIA methods [7,8]. One commercial kit [10] cites a reference range for the direct assay protocol that is three times greater than for the methylene chloride extraction procedure using the same reagents. In this paper we describe an improved liquid chromatographic procedure for UFC. This method was successfully applied in the diagnosis of patients with hypercortisolism and Cushing's syndrome.

EXPERIMENTAL

Materials and reagents

Urine was collected over a 24-h period without preservative. The volume was recorded and creatinine measured by a routine method (Beckman Astra analyzer). Aliquots for UFC were stored at $-20°C$ until assayed. For the normal range determination, urine specimens were selected from individuals without apparent endocrine problems. All solutions were prepared and stored in all-glass or PTFE-lined glass apparatus. UV-grade tetrahydrofuran (THF), methanol, hexane and acetone were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) Diethyl ether was of the stabilized form (Aristar grade from BDH Chemicals, containing 1 ppm pyrogallol as stabilizer and butylated hydroxytoluene as preservative). Steroids were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of steroids (500 mg/l) were prepared in methanol. Dilutions were made in mobile phase. Working solutions (5 mg/l) consisting of a mixture of cortisol and 11-deoxycortisol (internal standard) and an 11-deoxycortisol solution were prepared each week in mobile phase. The mobile phase was prepared as follows: 265 ml of THF and 30 ml of methanol were mixed in a 1-l glass volumetric flask and made up to 1 l with water. The mobile phase solution was degassed and filtered through a 0.45-μm PTFE filter under vacuum before use. Acetone (200 ml) was diluted to 1 l with water to make the Sep-Pak washing solution. Solvents and washing solutions were stored at room temperature in PTFE-lined glass dispens-

ers for convenient dispensing of contaminant-free solutions. RIA measurements of UFC were carried out with Gammacoat (Clinical Assays) and Amerlex (Amersham) kits.

Extraction procedure

To 2-ml samples of urine in 10-ml glass tubes were added 150 ng (30 μl) of the internal standard. The tubes were vortexed. The Sep-Pak cartridges were washed with 3 ml of methanol, followed by 6 ml of water. The flow-rate through the cartridge was adjusted to 1–2 drops/s for all steps in the procedure by adjusting the vacuum suction. The entire 2-ml urine sample (with added internal standard) was quantitatively transferred to the cartridges and allowed to flow through. This was followed by successive washing with 2 ml of acetone–water (1 : 4, v/v), 2 ml of water and 3 ml of hexane. The washings were discarded. Cortisol was then eluted from the cartridges with 3 ml of diethyl ether into 10-ml glass tubes. The diethyl ether was evaporated at 37°C under a stream of air. The eluate should be clear and no suspension seen. The residue obtained after evaporation of the diethyl ether was reconstituted with 2 ml of water with vortexing. The previously used Sep-Pak was washed with 3 ml of methanol, followed by 6 ml of water. The aqueous cortisol solution was passed through the Sep-Pak with repeated washings of acetone–water (1 : 4, v/v), water and hexane, as described above. Cortisol was eluted a second time with 3 ml of diethyl ether into glass tubes, and the eluate was evaporated to dryness. The final residue was reconstituted with 100 μl of mobile phase, of which 80 μl were routinely injected onto the column. With this procedure urine samples are extracted twice with one Sep-Pak cartridge. Some cortisol is lost in the second cycle but the double extraction greatly improves sample clean-up and permits modification of the mobile phase to elute cortisol in 13 min without compromising on the resolution.

Chromatography

The equipment consisted of a Waters 6000A pump with U6K injector, a Perkin-Elmer LC-75 variable-wavelength UV detector, a Hewlett-Packard 3390A integrator and a recorder. Detection was at 254 nm with a sensitivity of 0.02 a.u.f.s. The column was a μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D., Waters Assoc.) with a guard column (3 cm $\times$ 2 mm I.D.) packed with C_{18} material. Chromatography was carried out at ambient temperature and a flow-rate of 1.0 ml/min.

Calculations

UFC concentrations were calculated from peak heights of internal standard (I.S.) and cortisol (C) with the following formula:

$$\frac{\text{UFC}}{(\text{ng/ml})} = \frac{\text{peak height of C}}{\text{peak height of I.S.}} \times \frac{\text{amount of I.S.}}{\text{per ml urine}} \times \text{factor}$$

Amount of I.S. per ml = 75 ng, factor = relative response (I.S./C) $\times$ relative recovery (I.S./C).

The relative response was obtained from the ratio of I.S. to C peak heights (see Table I) and equalled an average of 0.72; the recovery factor, which may vary

with different Sep-Pak cartridges, is equal to the ratio of absolute recoveries of I.S. and C added to urine (see Results section).

On rare occasions, interfering peaks may distort the I.S. peak, and the UFC is then calculated without I.S. by the following formula:

$$\frac{\text{UFC}}{(\text{ng/2 ml})} = \frac{\text{peak height of C}}{\text{peak height of daily standard C}} \times \text{factor}$$

In this case, the factor is derived from the product of: (a) the amount of C standard (150 ng) injected at the beginning of each working day; (b) correction factor (1.25) to correct for the 80 μl injected out of a total extract volume of 100 μl; (c) the absolute recovery factor for cortisol after Sep-Pak treatment (see Results below).

The above formula applies to 2 ml of urine extracted; all results are finally expressed as nmol UFC per day, using standard SI conversion and the 24-h urine volume.

RESULTS

Chromatography

Typical chromatograms of steroid standards and a urine sample are shown in Figs. 1 and 2, respectively. The coefficient of variation (C.V.) of the retention times of cortisol and 11-deoxycortisol was less than 3% on a day-to-day basis. The majority of urine samples in our experience gave chromatograms similar to Fig. 2. Occasionally, samples contained high amounts of early eluting substances, which may cause deflection of the recorder pen outside the measuring scale, but this did not affect either the location or the size of the cortisol peak. Rarely, a sample demonstrated high background absorbance even after double Sep-Pak extraction. These samples were successfully analysed following a 1:1 dilution of the sample with water.

Calibration

The calibration curves for cortisol and 11-deoxycortisol, injected separately or as a mixture, were linear up to 300 ng. A linear relationship was also obtained when the ratio of peak heights of various concentrations of cortisol was plotted against a fixed amount of 11-deoxycortisol (150 ng). A relative response factor of 11-deoxycortisol compared with that of cortisol was calculated and found to be 0.72 ± 0.02 (S.D.) for the entire range. This factor was incorporated into the formula for internal standard quantitation as described in Calculations.

Recovery

Absolute recoveries of cortisol and 11-deoxycortisol are different (ca. 80% for cortisol and 75% for 11-deoxycortisol), so a relative recovery factor must be incorporated in the equations for the calculation of UFC concentrations (see Calculations). In our experience of over a year with this method it became clear that these recovery rates may vary between lots of Sep-Pak cartridges. We therefore recommend obtaining sufficiently large supplies of cartridges with the same lot
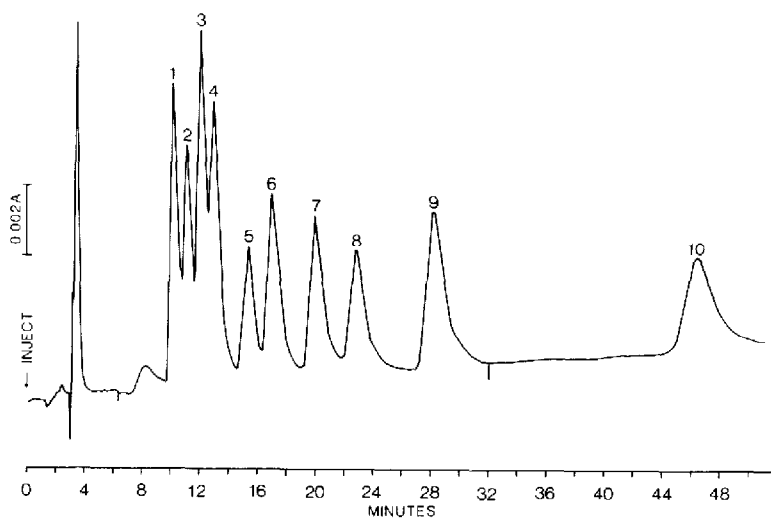
Fig. 1. Chromatographic separation of steroids by HPLC. Peaks: 1=prednisone; 2=cortisone; 3=prednisolone; 4=cortisol; 5=fludrocortisone; 6=corticosterone; 7=11-deoxycortisol; 8=dexamethasone; 9=testosterone; 10=17-hydroxyprogesterone. The amount injected was ca. 150 ng for each steroid.
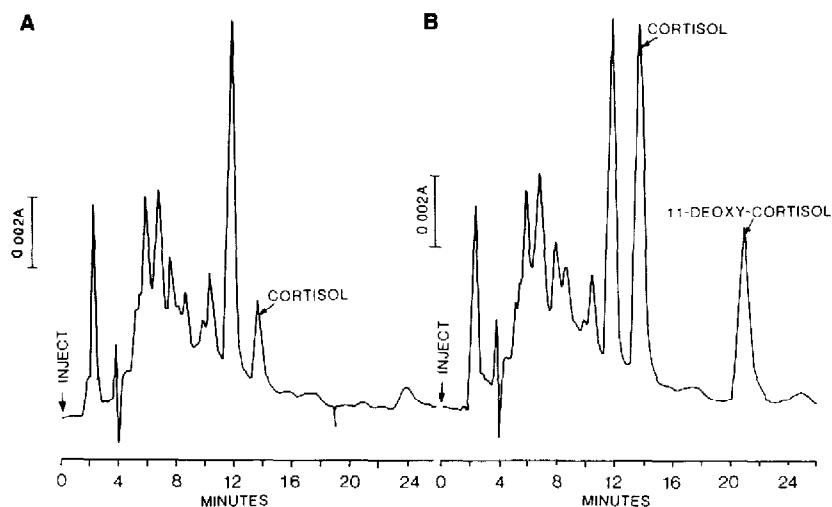

Fig. 2. Typical chromatogram of a urine sample (A) before and (B) after the addition of 250 ng of cortisol and 250 ng of 11-deoxycortisol.

and determining the absolute recoveries by adding various amounts of pure standards of both compounds to preanalysed urines, performing the entire analysis and correcting for baseline levels. The variation within lot was minimal.

Precision

The within-day and between-day precision for the method are shown in Table I. These determinations reflect the entire process, including extraction, and were conducted on urine pools stored in frozen aliquots.

TABLE I

PRECISION OF URINARY FREE CORTISOL METHOD

Mean UFC (nmol/day)	n	Coefficient of variation ($\%$)
Within-day		
89	13	3.5
181	20	2.2
Between-day		
89	15	4.9
181	20	3.9

Reference ranges

We analysed 33 urine samples (24-h collection) from individuals who, to the best of our knowledge, did not have any adrenal-related disease. The results ranged from undetectable (three samples) to 120 nmol/day. The overall mean and S.D. values were 45 nmol/day and 34 nmol/day. An upper limit of normal of 110 nmol/day was set for this method, which is in agreement with previously published data using other specific techniques [7–9].

Comparison with RIA

The results of the HPLC method were compared with those obtained with the Amerlex (Amersham) RIA kit (direct method without extraction). Thirty-three urine samples from individuals with normal adrenal function and ten specimens from a patient diagnosed with Cushing's disease due to a pituitary adenoma were analysed. All ten specimens from this patient had elevated UFC presurgically by HPLC (range 386–2550 nmol/day) and by RIA (range 1456–2834 nmol/day). The regression equation obtained with all 43 specimens was $y(\text{HPLC}) = 0.579x(\text{RIA}) - 535$, $S_{y/x} = 320$, $r = 0.778$. For seventeen of these samples, including seven specimens from the patient with Cushing's disease, cortisol

TABLE II

URINARY FREE CORTISOL LEVELS AFTER DEXAMETHASONE ADMINISTRATION: HPLC COMPARED TO RIA

Dexamethasone		UFC (nmol/day)	
Dose	Day	HPLC	RIA
Baseline	0	1250	1912
0.5 mg q.6.h*	1	340	795
0.5 mg q.6.h	2	235	500
2 mg q.6.h	3	105	500
2 mg q.6.h	4	N.D.**	435

*q.6.h = every 6 h.
**N.D. = not detected.

TABLE III

URINARY FREE CORTISOL IN PATIENTS WITH CUSHING's SYNDROME

Patient	Diagnosis*	UFC (nmol/day)	
		HPLC	RIA
1	⎰ Bilateral multinodular	204	543
2	⎱ Adrenal hyperplasia	540	1151
3	Adrenocortical carcinoma	734	2054
4	Islet cell carcinoma (ectopic ACTH)	198	800
5	Pituitary-dependent Cushing's syndrome	455	1417
6	Adrenal hyperplasia	1090	2888

*Diagnosis was based on surgical or autopsy reports. RIA was carried out with the Amersham kit.

was also measured by the Gammacoat (Clinical Assays) RIA kit after urine extraction with methylene chloride. The regression equation for the seventeen specimens was $y(HPLC) = 0.609x(RIA) - 31$, $S_{y/x} = 319$, $r = 0.802$. Table II compares the results obtained with the Amerlex direct RIA assay in another patient on low and high dose of dexamethasone. Suppression was complete to undetectable levels with the HPLC method, whereas the RIA procedure (reference range 100–400 nmol/day) measured a significant level of apparent cortisol following the high dose of dexamethasone. This patient subsequently underwent hypophysectomy and no pituitary adenoma was found.

Table III shows a comparison of the two methods on six patients who were confirmed surgically as suffering from Cushing's syndrome due to different etiologies. The HPLC results correlated with the RIA but were lower, suggesting better specificity.

DISCUSSION

Steroids have been traditionally isolated from urine by organic solvent extraction procedures. There are many reports in the literature dealing with the use of Sep-Pak or similar cartridges for steroid extraction from biological specimens [11–15]. These devices contain octadecylsilane-bonded phase packing and offer a rapid and quantitative means of steroid extraction. Under optimized conditions these cartridges can yield clean solutions with a low background absorbance suitable for analytical liquid chromatography. In our studies, we found that the washing step with acetone–water (1:4, v/v) removed much of the interfering material present in urine, which is initially retained by the Sep-Pak. Additionally, the use of diethyl ether instead of methanol led to the elution of cortisol, while a second group of interfering materials, which had not eluted in acetone, was left behind on the Sep-Pak. The incorporation of a second extraction step further improves sample clean-up and yields a final extract suitable for the injection. Solvent extraction alone for urinary cortisol clean-up before liquid chromatography was found inadequate in one study [16].

It is important to avoid contact of plastic materials with the solvents used in this study. We observed "ghost" peaks in the chromatograms from use of plastic

material. We therefore recommend that all-glass or PTFE-lined glassware be used throughout. Urine is a complex matrix containing variable amounts of steroids and their metabolites. Rarely, unknown peaks may elute close to the I.S. peak and may distort it; in that case UFC can be calculated without the use of I.S., as described in *Calculations*.

Both the HPLC and the RIA methods used were able to differentiate the pathological samples from the normal population. A notable exception was one urine sample that was repeatedly found to contain undetectable levels of UFC by HPLC and 650 nmol/day by the Amersham procedure (normal range 100–400 nmol/day) and 722 nmol/day by the Clinical Assays extraction procedure (normal range 55–250 nmol/day). It was later found that the patient's sample contained prednisolone, which was easily seen on the chromatogram as a peak eluting 1 min before the expected cortisol peak. Prednisolone is the major steroid known to cross-react with the cortisol antibody used in most RIA kits.

Although RIA procedures for UFC give clinically useful results in most cases, it is clear that these methods are not specific even after extraction of urine with organic solvents. Antisera to cortisol cross-react with many urine constituents, including drugs, and falsely elevated results may occur with some specimens. A selective and precise HPLC method such as the one described in this paper would be preferred. The simple and convenient extraction procedure effectively removes many interferences. This allows the mobile phase to be modified in order to achieve early elution of cortisol in a well resolved peak.

REFERENCES

1 L. Crapo, Metabolism, 28 (1979) 955.
2 B.E.P. Murphy, L.M. Okouneff, G.P. Klein and S.C. Ngo, J. Clin. Endocrin. Metab., 53 (1981) 91.
3 M. Schoneshofer, A. Fenner and H.J. Dulce, Clin. Chim. Acta, 101 (1980) 125.
4 R.M. Gough and G. Ellis, Clin. Biochem., 14 (1981) 74.
5 C. Monder and H.L. Bradlow, Recent Prog. Horm. Res., 36 (1980) 345.
6 E. Heftmann and I.R. Hunter, J. Chromatogr., 165 (1979) 283.
7 M. Schoneshofer, A. Fenner, G. Altinok and H.J. Dulce, Clin. Chim. Acta, 106 (1980) 63.
8 M. Schoneshofer and B. Weber, J. Steroid Biochem., 18 (1983) 65.
9 E. Canalis, G.E. Reardon and A.M. Caldarella, Clin. Chem., 28 (1982) 2418.
10 Gammacoat [125]I Cortisol Radioimmunoassay Kit, Clinical Assays, Cambridge, MA.
11 C.H.L. Shackleton and J.O. Whitney, Clin. Chim. Acta, 107 (1980) 231.
12 G.R. Cannell, J.P. Galligan, R.H. Mortimer and M.J. Thomas, Clin. Chim. Acta, 122 (1982) 419.
13 M. Zief, L.J. Crane and J. Horvath, Am. Lab., 14 (1982) 120.
14 P.G. Stoks and T.J. Benraad, J. Chromatogr., 276 (1983) 408.
15 B.T. Hofreiter, A.C. Mizera, J.P. Allen, A.M. Masi and W.C. Hicok, Clin. Chem , 29 (1983) 1808.
16 J Nakamura and M. Yakata, Clin. Chem., 28 (1982) 1497.