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AUTOMATED LIQUID CHROMATOGRAPHIC DETERMINATION OF THE 20-DIHYDRO ISOMERS OF CORTISOL AND CORTISONE IN HUMAN URINE

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SUMMARY

A fully automated method for the simultaneous assessment of cortisol, cortisone and their 20-dihydro isomers in human urine is described. On-line sample enrichment, prepurification, focusing and injection are combined with automated high-performance liquid chromatographic separation and quantification. Losses of steroids throughout the total procedure are negligible. Thus, external calibration is feasible for quantification. Coefficients of variation range between 8.7 and 17.0% for inter-assay variability and between 1.3 and 5.2% for intra-assay variability. Assay sensitivity is 15 nmol/l. In normal students, the medians of the relative excretion rates of free 20α-dihydrocortisol, 20α-dihydrocortisone, 20β-dihydrocortisol and 20β-dihydrocortisone were 10.9, 6.1, 7.7 and 4.4 μmol/mol creatinine. The fully automated feature renders the present method well suited for routine diagnosis of hypercorticoidism.

INTRODUCTION

In a hypercortisolemic but hypocortisoluric patient with Cushing's disease, we recently found high amounts of two urinary cortisol-immunoreactive compounds*, which were identified as 20α- and 20β-dihydrocortisol by mass spectrometry [1]. It is conceivable that such a shift of cortisol metabolism to C-20 reduction under chronic hypercorticoid conditions is dictated by mechanisms similar to those inducing 6β-hydroxylation of cortisol [2]. Thus, the simultaneous monitoring of urinary cortisol and its 20-dihydro isomers may

*Compounds: 20α-dihydrocortisol = 4-pregnene-11β,17α,20α,21-tetrol-3-one; 20β-dihydrocortisol = 4-pregnene-11β,17α,20β,21-tetrol-3-one; 20α-dihydrocortisone = 4-pregnene-17α,20α,21-triol-3,11-dione; 20β-dihydrocortisone = 4-pregnene-17α,20β,21-triol,3-11-dione.

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provide a valuable tool for the definitive biochemical diagnosis of chronic hypercortisolism.

We therefore developed a liquid chromatographic method, which allows the simultaneous estimation of urinary cortisol and cortisone as well as of their 20-dihydro isomers. In this method, an "on-line" sample pretreatment procedure preceding the liquid chromatographic determination is used [3, 4]. It provides fully automated operation of the complete assay and facilitates its use in a clinical routine laboratory.

EXPERIMENTAL

Chemicals and solvents

Cortisol and cortisone were obtained from Sigma (Munich, F.R.G.); 20α- and 20β-cortisol as well as 20α- and 20β-cortisone were from Makor Chemicals

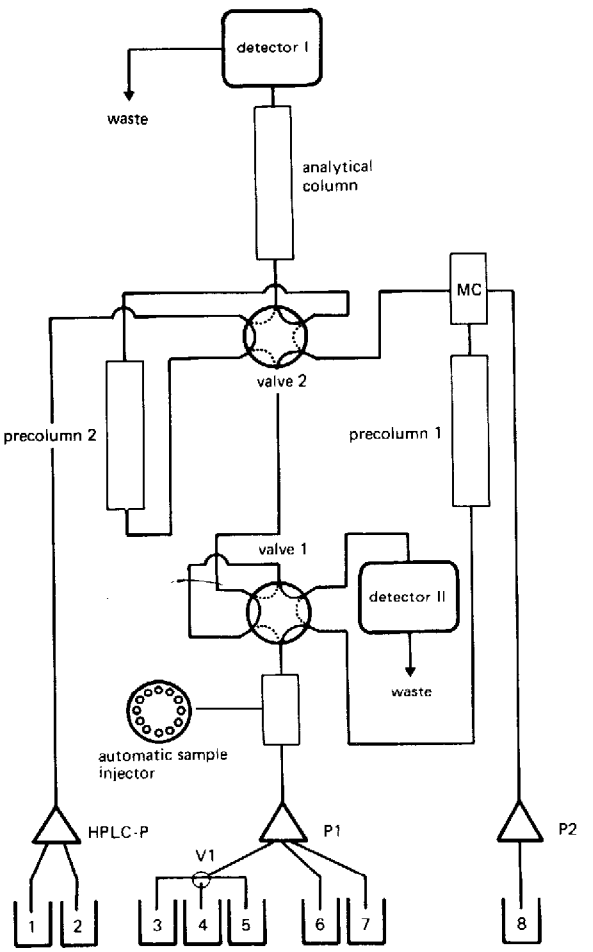

Fig. 1. Flow diagram of the automated liquid chromatographic assay of urinary free cortisol. Valves 1 and 2 are shown in position I; the broken lines indicate position II. The pumps (P1, P2, HPLC-P), sample injector, precolumns, analytical column, mixing chamber (MC) and other valves are described in the text.

(Jerusalem, Israel); Synacthen® was from Ciba-Geigy (Wehr, F.R.G.). Other reagents were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

The following solvents were used for sample clean-up and chromatography: methanol, acetonitrile, deionized water, 20 mmol/l sodium hydroxide, and 20 mmol/l hydrochloric acid. After being degassed by purging with helium, they were freshly purified "on-line": water and the hydrochloric acid solution by "on-line" passage through Sep-Pak C_{18} cartridges (Waters Assoc., Königstein/Taunus, F.R.G.), sodium hydroxide by passage through cartridges packed with 10-μm PRP-I® particles (Hamilton, Reno, NV, U.S.A.), methanol and acetonitrile by passage through cartridges packed with 10-μm alumina particles (Machery-Nagel, Düren, F.R.G.). Mixtures of aqueous and organic solvents were prepared "on-line" by the corresponding pumps (Fig. 1).

Instrumentation

The principle of the analyser system has been outlined previously [3]. It includes the following units (Fig. 1): the automated sample injector, the sample clean-up unit, the chemical modulator, the chromatographic and quantification unit, and a microprocessor control unit.

The sample injector consists of a sampler and a peristaltic pump from an AutoAnalyzer I (Technicon Instruments, Tarrytown, NY, U.S.A.). Samples were loaded by switching a 1-ml loop of a six-way valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.) into the line of the analyser.

The sample clean-up unit consists of a motor-driven six-way valve (V1) (Latek, Heidelberg, F.R.G.), a high-pressure pump (P1) with a ternary mixing device (Model LC-4A; Shimadzu, Kyoto, Japan), a six-way valve (valve 1) (Model 7010; Rheodyne) and a 60 × 4 mm I.D. precolumn (precolumn 1) (Knauer, Berlin, F.R.G.) packed with PRP-I (Hamilton).

The chemical modulator consists of a mixing chamber (Kontron, Eching, F.R.G.) with an internal volume of 1.1 ml; a double-plunger pump (P2) (Milton Roy, Riviera Beach, FL, U.S.A.), a six-way valve (valve 2) (Model 7010; Rheodyne) and a 30 × 4 mm I.D. precolumn (precolumn 2) packed with silica ODS (Shandon Southern Products, Cheshire, U.K.).

The quantification unit consists of a high-pressure pump (HPLC-P) (Model 1084 B; Hewlett-Packard, Böblingen, F.R.G.), a 250 × 4 mm I.D. analytical column, prepacked with 5-μm Hypersil ODS (Shandon), a fixed-wavelength detector (detector I) set at 254 nm, and a Hewlett-Packard integrator.

All modules of the system were operated by the microprocessor control unit, a time-relay electronic controller (Izumi Denki, Osaka, Japan).

The degassed solvents were positioned as followed: water in bottles, 1, 3 and 8; acetonitrile in bottles 2 and 6; 0.01 *M* sodium hydroxide in bottle 4; 0.01 *M* hydrochloric acid in bottle 5; methanol in bottle 7.

Methods

The following analytical steps were time-controlled by the electronic controller in such a manner that steps 1—7 as well as 9 and 10 were run concomitantly with the chromatographic step 8. All UV-absorbing material that was not loaded onto the analytical column was monitored by detector II, also set at 254 nm.

Step 1: A 1-ml volume of urine is transferred from the sampler into the sampling loop of the sampling unit.

Step 2: The sampling loop is switched into the analytical line; pump P1 is activated to deliver water from bottle 3 (flow-rate 1 ml/min); valves 1 and 2 are in position I. By this step, all lipophilic compounds are adsorbed on precolumn 1; polar compounds are eluted.

Step 3: A mixture of sodium hydroxide and acetonitrile (85:15) is delivered by pump P1. Anionic forms of acids or phenolic compounds are eluted from precolumn 1.

Step 4: A mixture of hydrochloric acid and acetonitrile (85:15) is delivered by pump P1. Cationic forms of basic compounds are eluted from precolumn 1.

Step 5: A water—methanol (70:30) mixture is delivered by pump P1; lipophilic compounds, more polar than cortisol/cortisone and their 20-dihydro isomers, are eluted from precolumn 1.

Step 6: A water—methanol (30:70) mixture is delivered by pump P1. The steroid-containing fraction elutes into the chemical modulator.

Step 7: Water (flow-rate 2.5 ml/min) is delivered from bottle 8 by pump P2 into the mixing chamber and valve 2 is switched into position II. Thus, the eluent of the steroid-containing fraction is polarized in the mixing chamber and then focused onto the top of precolumn 2.

Step 8: Valve 2 is switched into position I; a gradient is run from 20 to 35% of acetonitrile in water by pump HPLC-P. The prepurified, focused fraction is chromatographed on the analytical column and separated steroids are quantified in detector I.

Step 9: Valve 1 is switched into position II; an acetonitrile—water (80:20) mixture is delivered by pump P1. All residual material, more lipophilic than the steroid-containing fraction, is eluted from precolumn 1 in "back-flush" mode into the waste.

Step 10: Valve 1 is switched into position I and pure water is delivered by pump P1. Precolumn 1 is equilibrated and ready for adsorption of the next sample.

RESULTS

Procedural variables

Efficiency of focusing and separating the steroid fraction

Fig. 2a illustrates the chromatogram of the steroid standards under study obtained by injection of 20 μl of water containing ca. 100 ng of each steroid directly onto the analytical column. To study the efficiency of the present technique for focusing the steroid-containing fraction onto the top of precolumn 2 prior to chromatography (step 7), the same amounts of steroid were dissolved in 1 ml of water and assayed by the present method. Compared with direct injection, there was virtually no difference in peak width, height and resolution. Chromatographic data are listed in Table I.

Efficiency of sample clean-up

The efficiency of the different sample clean-up steps for elimination of non-

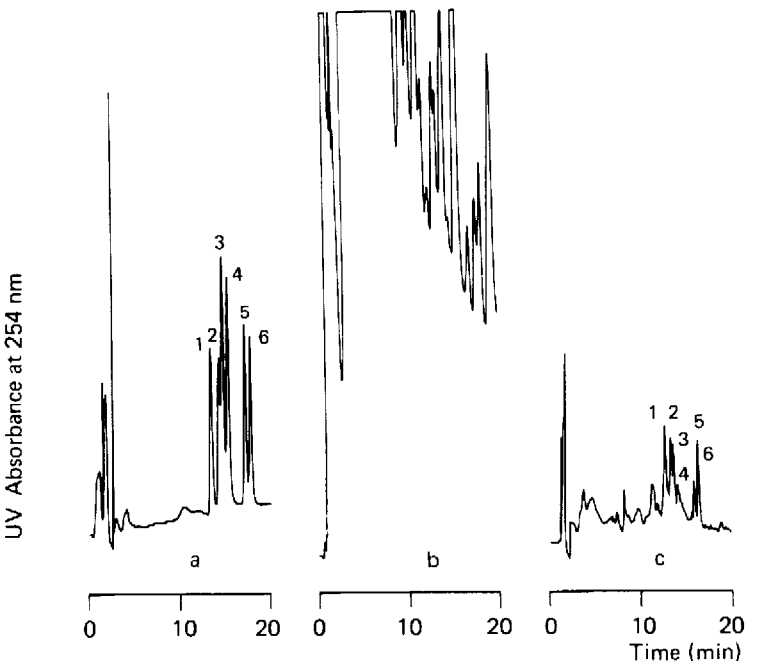

Fig. 2. UV chromatograms of pure steroid standards. (a) A normal urine sample cleaned up without (b) and with (c) the different purification steps of the on-line pretreatment device. Peaks: 1 = 20α-dihydrocortisol, 2 = 20α-dihydrocortisone, 3 = 20β-dihydrocortisol, 4 = 20β-dihydrocortisone, 5 = cortisol, 6 = cortisone.

TABLE I

PARAMETERS OF LIQUID CHROMATOGRAPHIC EFFICIENCY

Steroid	Retention time (min)	Coefficient of variation (%)	Resolution of adjacent steroids
20α-Dihydrocortisol	13.16	0.25	
			0.93
20α-Dihydrocortisone	13.72	0.38	
			0.56
20β-Dihydrocortisol	14.06	0.23	
			0.61
20β-Dihydrocortisone	14.43	0.39	
			1.44
Cortisol	16.16	0.22	
			0.75
Cortisone	16.61	0.37	

specific, UV-absorbing chromogens was studied in a normal urine sample. Fig. 2b depicts the chromatogram monitored by detector I when the crude urine sample is loaded directly onto precolumn 2, thereby replacing precolumn 1 by a zero dead-volume conjunction and limiting the washing procedure to 3 ml of water only. No discrete steroid peaks were discernible against the large, non-specific UV background of urinary chromogens. When running the complete analytical procedure, the non-specific background was almost completely eliminated and the steroids under study almost completely resolved (Fig. 2c).

Procedural losses

To investigate the potential loss of steroids during the complete multi-step analytical procedure, a 1-ml water sample containing 10 mg/l of both 20α-dihydrocortisol (the most polar steroid) and cortisone (the most lipophilic steroid studied here) was assayed. UV absorbance was monitored at the same time with both detectors. Detector II (limit of detection ca. 60 ng) detected no UV absorbance, thus indicating that the steroids were almost quantitatively transferred to the analytical column. During routine analyses, we checked for potential analytical losses such as these every 20th sample.

Ruggedness test of the chromatographic system

For 200 urine samples, which had been analysed with the same instrument settings, the chromatographic resolution and peak shape were of consistent quality, as was the recovery of 100 ng of 20α-dihydrocortisol and cortisone in an aqueous sample. There was no significant alteration of retention time.

Memory effect

There was no measurable memory effect when pure water was analysed immediately after a sample containing 500 μg/l of each steroid.

Analytical variables

Standard curve and sensitivity

A standard curve for external calibration was obtained using aqueous standard solutions of cortisol in the concentration range of 40—1360 nmol/l. Since all steroids under study have almost the same absorptivities, they were all evaluated from this cortisol curve. Values below 500 nmol/l were evaluated by comparison of peak heights, values higher than 500 nmol/l by peak integral interpolation [4]. The detection limit (a signal three-fold the height of the noise level) was ca. 15 nmol/l.

Precision

Intra-assay variability was assessed by replicate analyses (n=10) of a normal urine sample. Coefficients of variation ranged between 1.3% for cortisone and 5.2% for 20α-dihydrocortisol.

Inter-assay variability, checked in the same urine sample, ranged between 8.7% for 20α-dihydrocortisol and 17.0% for 20α-dihydrocortisone (n=12).

Recovery

Analytical recovery was determined from replicate analyses of a normal urine sample spiked with 100 ng/ml of each steroid. Recovery ranged from 94.8% for 20α-dihydrocortisol to 105.9% for cortisol.

Specificity

The intensive prepurification almost completely eliminated the unspecific background due to urinary chromogens leaving only neutral compounds closely related to the steroids under study for quantitative analysis on the analytical column (Fig. 2). The chromatographic separation obtained using the reversed-

phase analytical column provided sufficient resolution of 20α-dihydrocortisol, cortisol and cortisone. The peaks of 20α-dihydrocortisone, 20β-dihydrocortisol and 20β-dihydrocortisone overlapped slightly (Table I). Relevant adrenal or gonadal steroids were already eliminated by the prepurification steps. No interferences were observed in the samples from 25 normal students (Fig. 2c). However, interferences were encountered in some patients under intensive care. The less-excreted steroids, 20α- and 20β-dihydrocortisone, were more involved in unspecific effects than the other steroids. In most cases, these interferences were caused by high dosage therapy of synthetic corticoids or related substances.

Practicability

The practicability of the present automated system is identical to that for estimating urinary free cortisol and has been detailed previously [4]. Thus, for the complete assay as described here, 48 samples may be handled during one day, if overnight operation is included.

Excretion rates in normal humans

Results of excretion rates of urinary free steroids in healthy students are listed in Table II. The excretion rates are expressed relative to creatinine. A logarithmic distribution of values was assumed. In five of the 25 samples no 20α-dihydrocortisone was detectable, and in 14 samples no 20β-dihydrocortisone was detectable.

TABLE II

RELATIVE URINARY EXCRETION RATES OF CORTISOL AND CORTISONE AS WELL AS OF THEIR C-20 REDUCED ISOMERS FROM NORMAL SUBJECTS.

Results expressed as lower limit, median and upper limit of measured data.

Steroid	Steroid (μmol)/creatinine (mol)		
	Lower	Median	Upper
20α-Dihydrocortisol	6.2	10.9	19.1
20α-Dihydrocortisone	3.6	6.1	10.5
20β-Dihydrocortisol	4.1	7.7	14.5
20β-Dihydrocortisone	2.4	4.4	8.3
Cortisol	5.9	8.8	12.9
Cortisone	5.3	10.3	19.9

DISCUSSION

The recent finding of an elevated urinary excretion of 20α-dihydrocortisol in a hypercortisolemic but hypocortisoluric patient with Cushing's disease [1] indicates that the estimation of the C-20 reduced corticoids in urine may improve the biochemical diagnosis of hypercorticoid states. Thus, its diagnostic usefulness seems to be comparable to that of 6β-hydroxycortisol [2]. However, 6β-hydroxycortisol cannot be assayed simultaneously with cortisol from one sample by immunological [5] as well as by simple chromatographic techniques [6]. Due to the chemical similarities of cortisol/cortisone and their C-20 reduced derivatives, a simultaneous estimation is possible using the described

method of sample clean-up [3]. Therefore, the development of a fully automated method is justified in view of its need in a routine laboratoty.

The analytical variables of the present method, such as precision, accuracy and practicability, were comparable with those of the urinary free cortisol assay [4].

The present normal values for the excretion rates of urinary free cortisol and cortisone are in agreement with the reported data [4, 7]. Reference intervals of the excretion rates of 20α- and 20β-cortisone have not been described so far. Those of 20α- and 20β-cortisol reported in older studies [8—10] are only of approximative nature. Thus, Fukushima et al. [8] roughly estimated excretion of 20α-dihydrocortisol to correspond to ca. 0.9% of the total administered dose of cortisol. 20β-Dihydrocortisol reportedly [9] is excreted at the rate of 490 nmol/24 h (mean of five normals). From cross-reactivity studies at 50% displacement, Murphy et al. [10] estimated that the amounts of the 20-dihydro isomers of cortisol are approximately of the same order of magnitude as those reported in earlier studies [8, 9]. In a previous approach, we immunologically estimated 20α- and 20β-dihydrocortisol using a cross-reacting cortisol-antiserum and liquid chromatographic prepurification [1]. Apart from the fact that this method was unpracticable, the measured values could not be accurate since chromatographic fractionation prior to immunoassay was insufficient (see Fig. 2). Accordingly, the reference values monitored with the present technique are lower than all those reported previously. As to the excretion rates of 20α- and 20β-cortisone in normals, it must be stated that assay sensitivity is too low for precise estimation of all normal levels.

Since urinary 20α-dihydrocortisol represents the most abundant steroid of all four 20-dihydro isomers studied, its estimation is preferable to that of other 20-dihydro steroids for routine purposes. Therefore, the measurement of urinary 20α-dihydrocortisol or its ratio to urinary cortisol may be a better screening test for hypercortisolism than the estimation of free cortisol itself, if no physiological or pharmacological influences alter the cortisol metabolism.

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