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Note

Semi-automated high-performance liquid chromatographic method for the simultaneous assay of plasma cortisol and 11-deoxycortisol in the metyrapone test

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The metyrapone test is a well established diagnostic method for assessing pituitary adrenal function. Metyrapone selectively inhibits 11-hydroxylation in steroid biosynthesis. In patients with normal pituitary adrenal function, administration of metyrapone causes a decrease in the secretion rate of cortisol and corticosterone, which results in a compensatory increase in pituitary ACTH production to which the adrenal responds by secreting 11-deoxycortisol. The increase and decrease in secretion rates is reflected in a respective increase and decrease of blood levels of these steroids. Plasma levels of these steroids (cortisol and 11-deoxycortisol) can be determined by radioimmunoassay (RIA), and several methods have been published using this procedure [1–4]. The advent of high-performance liquid chromatography (HPLC) has offered a method whereby compounds can be isolated and purified and then assayed through their UV-absorbing and fluorescence properties. However, the sensitivity of HPLC detectors, particularly UV spectrophotometers, limits the applicability of HPLC methodology to compounds present at nanogram levels in biological fluids. Cortisol and 11-deoxycortisol (after metyrapone administration) have high enough plasma levels to theoretically be measurable by HPLC.

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EXPERIMENTAL

Materials

Acetonitrile, water, methylene chloride, HPLC grade, were from Baker (Phillipsburg, NJ, U.S.A.). Steroids were purchased from Steraloids (Wilton, NH, U.S.A.).

Instrumentation

The HPLC system contained the following components: Model 5040 ternary liquid chromatograph, Vista 401 chromatography data system, all from Varian Instruments (Sunnyvale, CA, U.S.A.); Spectroflow 757 UV–VIS detector equipped with a 12-μl flow-cell was from Kratos (Ramsey, NJ, U.S.A.). Filtertips (2 μm), Supelcosil LC-18-DB column (25 cm$\times$0.46 cm I.D.), and guard column containing LC-18 pellicular packing were purchased from Supelco (Bellefonte, PA, U.S.A.) and WISP automatic sample injector was from Waters Assoc. (Milford, MA, U.S.A.). Commercial coated-tube RIA kits were purchased from Dade Baxter Travenol Diagnostic (Cambridge, MA, U.S.A.) (cortisol) and ICN Biomedicals (Carson, CA, U.S.A.) (11-deoxycortisol) and limited-volume insert vials from Sunbrokers (Wilmington, NC, U.S.A.).

Metyrapone testing

Subjects were fifteen normal volunteers, aged 24–41 years. All were healthy and were taking no medications. Metyrapone was administered as a single, oral, bedtime dose according to body weight as described by Jubiz et al. [5]. Pre-metyrapone blood samples were drawn the morning before metyrapone was given; post-metyrapone samples were drawn between 8:00 a.m. and 9:00 a.m. following the metyrapone dose. There were no side-effects from the metyrapone. This protocol was reviewed and approved by the Human Subjects' Committee of our institution, and subjects were studied only after giving their informed consent.

Extraction of plasma

A 100-ng amount of 11α-hydrocortisone as an internal indicator in 100 μl of ethanol was added to 0.5 ml of plasma, the total volume was made up to 1 ml with water and then extracted with 10 ml of cold methylene chloride. The methylene chloride extract was washed twice with 1 ml of water and then evaporated to dryness in a 20-ml glass vial (vacuum, oven, 55°C).

Chromatography

A 250-μl volume of a 1:4 (v/v) mixture of acetonitrile–water was added to the dried extract in the vial, and the solution was filtered by drawing it up into a 250-μl Eppendorf tip, then fitting a 2-μm filter to the end of the pipette and

pushing the solution through it into a limited-volume insert vial. The vials were placed into the WISP and 200 μl of the solution were injected directly onto the HPLC column. The column was eluted using a gradient system consisting of water (A) and acetonitrile (B), flowing at 2.0 ml/min; 0 min, A–B, 75:25; 10 min, A–B, 75:25; 20 min, A–B, 50:50; 23 min, A–B, 50:50; 30 min, A–B, 75:25. The eluate from the column is scanned in the UV detector and the cortisol, 11-deoxycortisol and 11α-hydrocortisone were detected by absorption at 242 nm.

RESULTS

Chromatography

Figs. 1 and 2 show the 242-nm UV absorption chromatograms of an extract of plasma drawn before (Fig. 1) and after metyrapone administration (Fig. 2). The VISTA is programmed to compute the concentration of steroid in μg per 100 ml plasma from the area of the UV absorption peaks of cortisol and 11-deoxycortisol and the area of the 11α-hydrocortisone added to the plasma as an internal standard. In this system metyrapone has an elution time of 7.1 min. At 242 nm it is 250 times less sensitive to UV absorption than cortisol, and no peak for metyrapone was detected on the chromatograms for any of the plasma samples assayed. Elution times for other steroids are: prednisolone, 7.8 min; prednisone, 7.9 min; cortisone, 9.7 min; corticosterone, 16.2 min.

Accuracy, precision and recovery

Accuracy studies were performed by adding 25, 50, 100 and 200 ng of cortisol and 11-deoxycortisol to 0.5-ml aliquots of a plasma pool and processing them through the method in duplicate. A 100-ng amount of 11α-hydrocortisone was added as an internal standard. The duplicate values were averaged and the values were corrected for the endogenous levels of cortisol and 11-deoxycortisol in the plasma pool (0 ng added). The results are shown in Table I. The overall mean value ($\pm$S.D.) for the recovery of added amounts of cortisol and 11-deoxycortisol recovered above the endogenous values were: cortisol, $111 \pm 7.4\%$; 11-deoxycortisol, $101 \pm 6.5\%$ ($n=8$). The intra-assay precision on 0.5-ml aliquots of a post-metyrapone plasma pool was 8.6 ± 0.14 μg per 100 ml of plasma with a coefficient of variation (C.V.) of 5.2% ($n=10$) for cortisol and 21.2 ± 0.52 μg per 100 ml of plasma with a C.V. of 7.8% for 11-deoxycortisol. The average ($\pm$S.D.) recovery of 11α-hydrocortisone added as the internal standard was $59.6 \pm 5.2\%$ ($n=24$).

Comparison of HPLC and RIA methods

Cortisol and 11-deoxycortisol were assayed in fifteen plasma samples by both HPLC and RIA. A least-squares regression analysis of the results for the two methods gave a significant correlation of the values for cortisol both pre- and

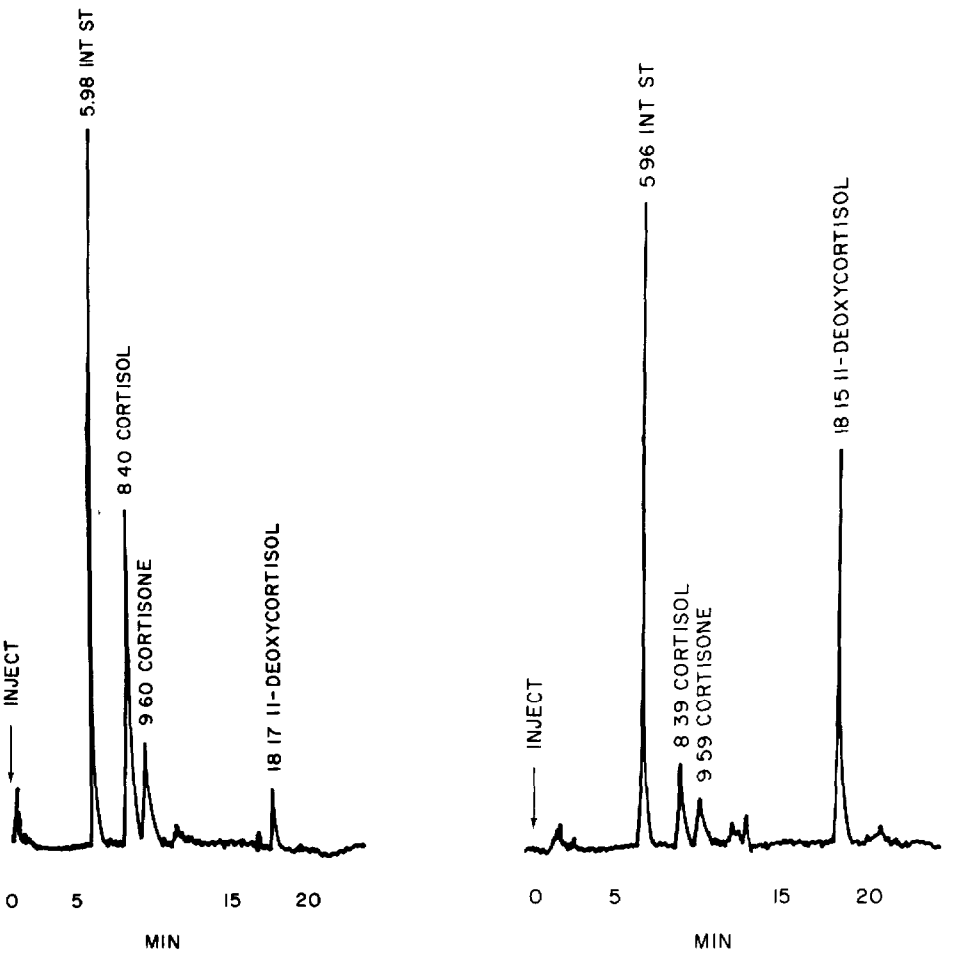

Fig. 1. Chromatogram of an extract of 0.5 ml plasma drawn before metyrapone administration.

Fig. 2. Chromatogram of an extract of 0.5 ml plasma drawn after metyrapone administration.

post-metyrapone administration and for 11-deoxycortisol post-metyrapone administration (i.e. $r=0.98, P<0.004$; $r=0.96, P<0.001$; $r=0.94, P<0.02$, respectively). However, there was a significant difference between the pre-metyrapone 11-deoxycortisol values, most probably due to the lack of precision of the HPLC assay at these low pre-metyrapone levels. The results are shown in Table II. The sensitivity of the HPLC method is approximately 2 ng cortisol and 11-deoxycortisol, or 0.4 μg per 100 ml of plasma.

DISCUSSION

The major advantages of HPLC over RIA for assaying steroids is the shorter length of assay time, the ability to automate the procedure and the simulta-

TABLE I

RECOVERY OF STANDARD AMOUNTS OF CORTISOL AND 11-DEOXYCORTISOL ADDED TO 0.5-ml ALIQUOTS OF A PLASMA POOL AND PROCESSED THROUGH THE METHOD IN DUPLICATE

Compound	Added (ng)	Recovered (ng)		Recovery (%)
Cortisol	20	21.5		107
	50	52.5		105
	100	112		112
	200	243		121
			Mean ± S.D.	111 ± 7.4
11-Deoxycortisol	20	19.5		97.5
	50	54.5		109
	100	94.5		95
	200	207		104
			Mean ± S.D.	101 ± 6.5

TABLE II

COMPARISON OF CORTISOL AND 11-DEOXYCORTISOL LEVELS (μg PER 100 ml OF PLASMA) IN THE METYRAPONE TEST DETERMINED BY HPLC AND RIA

Sample No.	Cortisol				11-Deoxycortisol			
	Pre-metyrapone		Post-metyrapone		Pre-metyrapone		Post-metyrapone	
	HPLC	RIA	HPLC	RIA	HPLC	RIA	HPLC	RIA
1	24.3	18.0	3.5	5.8	0.7	0.6	12.7	15.0
2	10.5	11.0	5.0	6.5	<0.4	0.2	12.4	13.0
3	11.3	9.8	0.6	1.2	2.4	0.4	23.1	21.0
4	14.0	13.0	1.0	1.8	1.5	0.4	10.0	10.0
5	23.4	18.0	14.6	14.0	1.3	0.5	7.8	7.0
6	33.4	25.0	4.4	5.4	2.5	0.5	14.7	12.0
7	12.2	11.0	2.7	3.8	3.6	0.5	22.2	18.0
8	9.6	9.9	3.8	5.4	<0.4	0.2	11.3	10.0
9	23.9	19.0	4.2	5.8	<0.4	0.5	11.0	12.0
10	19.9	16.0	8.6	11.0	3.6	0.3	16.8	16.0
11	12.0	9.7	5.6	6.0	<0.4	0.4	12.9	14.0
12	9.8	8.2	4.5	5.7	1.6	0.3	17.2	17 0
13	21.5	19.0	10.3	9.2	2.3	0.5	5.5	4.0
14	9.9	10.0	3.6	4 3	<0.4	0.2	14.4	13.0
15	15.1	14.0	4.1	6.1	<0.4	0.4	15.6	13.0
Mean	16.7	14.1	5.1	6.1	–	0.4	13.8	13.0
r	0.98		0.96		–		0.94	
P	<0.004		<0.001		–		<0.02	

neous assay of several steroids in a single sample of plasma. Also, particularly when only one or two samples have to be assayed, as is the case when the metyrapone test is used, the HPLC method is far less expensive than RIA. However, HPLC is less sensitive than RIA and only steroids generally at the μg per 100 ml level in plasma, such as cortisol, corticosterone and also 11-deoxycortisol after metyrapone administration, can be assayed by this technique using UV absorption.

A significant difference in cortisol values after the metyrapone test when measured by HPLC and a RIA method has been reported by several workers [6–8]. Carson and Jusko [6] found RIA values 4–66% higher than HPLC values and Reardon et al. [7] reported 50–90% lower HPLC than RIA values. Bouquet et al. [8] found levels 20–30% lower than those assayed by a fluorimetric method. In contrast we found very good correlation between the HPLC and RIA methods for cortisol both before and after metyrapone administration (Table II) with correlation coefficients of 0.98 and 0.96, respectively. Our results may differ because we had more specific antibodies than those used by the other investigators, and their HPLC system removed a compound which cross-reacts with the antibody, thus giving a value by HPLC which is lower than by RIA.

In summary, the present automated HPLC procedure allows samples to be assayed overnight with simultaneous measurement of cortisol and 11-deoxycortisol and should prove useful in the clinical assay laboratory for application to the metyrapone test.

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