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Note

Clinical utility of adrenal steroid measurement by high-performance liquid chromatography in pediatric endocrinology

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We report a rapid analytical method for the measurement of 17-hydroxyprogesterone (17-OHP), cortisol (F), and 11-deoxycortisol (S) by high-performance liquid chromatography (HPLC) in serum. The method allows for the diagnostic measurement of these three steroids within 2 h in emergency situations of sexual ambiguity or glucocorticoid insufficiency in the neonate. Elevated levels of serum S and 17-OHP are diagnostic for congenital adrenal hyperplasia due to 21-hydroxylase deficiency or 11β-hydroxylase deficiency, the most common cause of female pseudohermaphroditism. This rapid screening greatly facilitates resolution of the medical emergency of the diagnosis of the intersex child.

Steroid analysis by HPLC offers the advantage of the simultaneous determination of several steroids with rapidity and specificity. This is in contrast to the more traditional method of radioimmunoassay (RIA). In fact, RIA is not entirely specific because antibodies may cross-react to varying degrees with other steroids [1, 2]. This is of particular importance in those circumstances where an elevated plasma concentration of steroids is present, such as in the newborn period, in pregnancy and in disorders of steroidogenesis [2]. Moreover, RIA is a time-consuming method, particularly when prior chromatographic purification is required.

We report the rapid and simultaneous measurement of F, 17-OHP and S in serum by HPLC with ultraviolet absorbance detection. We have measured these steroids and documented further hormonal abnormalities in two patients with Cushing's disease secondary to pituitary adenoma; one patient with a malignant, cortisol-producing, ovarian lipoid-cell tumor; two patients with

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11β-hydroxylase deficiency congenital adrenal hyperplasia (CAH); and twenty patients with 21-hydroxylase deficiency CAH. Results obtained by HPLC correlate with standard RIA measurements.

EXPERIMENTAL

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used consisting of a data module (Model 730), a system controller (Model 720), a WISP automatic injector (Model 710B), two pumps (Model M6000A) and a UV detector (Model 441) fitted with a mercury lamp at a fixed wavelength of 254 nm.

A Waters reversed-phase radial compression Novapak C_{18} column (10 cm × 8 mm I.D., 5-μm particles) with a Waters Bondapak C_{18} Corasil pre-column was used.

Blood samples

Blood was obtained by venipuncture and centrifuged; serum was stored at −20°C until assayed.

Serum sample extraction

A 0.5—1 ml volume of serum was extracted twice with diethyl ether; the aqueous phase was flash-frozen with acetone—dry ice and discarded; the supernatant was evaporated under air. The residue was reconstituted with 500 ml of a water—methanol (42:58, v/v) mixture; HPLC analysis was performed in duplicate runs.

Chromatographic procedure

Chromatographic separation was performed at room temperature using water—methanol (42:58, v/v) as the mobile phase, at a flow-rate of 1 ml/min for 12 min. Between 12 and 20 min the non-polar phase was increased to 61% and the run was continued for a total of 45 min. An external standard was used for sample quantitation; integration was performed by the Waters data module. The method of external standard quantitation with this instrumentation compares the response of each component directly to a calibration curve. Therefore, it provides the ability to calculate the absolute amounts of each component.

Radioimmunoassay

Cortisol and 17-OHP were determined by RIA after Celite chromatography according to methods previously described [3].

Clinical studies

In all patients serum samples were obtained under basal conditions or 1 h following an intravenous injection of 0.25 mg Cortrosyn (adrenocorticotropin, ACTH 1—24).

Statistical analysis

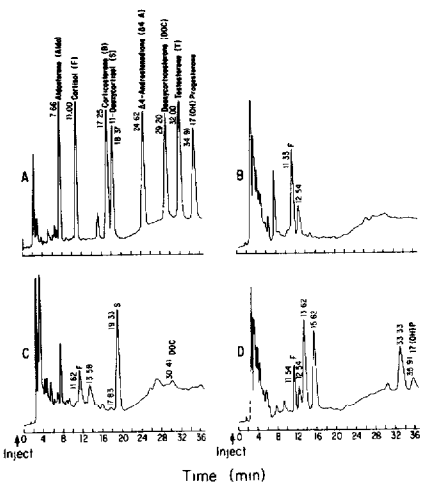
Selectivity was evaluated by comparing the retention times of serum samples

to purified F, 17-OHP and S. Fractions at 1-min intervals were collected and re-injected. In addition, purified steroids were added to serum samples in order to verify their retention times in serum. The addition experiments always yielded a single, sharp peak. Precision and reproducibility was evaluated by duplicate HPLC determination. Although the retention times varied plus or minus 1 min, depending on the age of the column and the temperature in the laboratory, standards were run twice consecutively at the beginning of each day. Accuracy was evaluated by comparing the HPLC assay with the chromatographed RIA of the same specimen for F and 17-OHP.

The differences between the two techniques were analyzed statistically by the paired t test and the one-way analysis of variance (ANOVA). Sensitivity was measured by assessing the lowest detectable limit of the three hormones. Under these experimental conditions, the signal-to-noise ratio is optimized. 11-Deoxycortisol values obtained by HPLC in patients with 11β-hydroxylase deficiency CAH were compared to values reported in the literature [4, 5].

RESULTS

Confirming previous studies [5—7], serum F, 17-OHP and S were separated by HPLC, identified by their chromatographic retention times (11.0, 18.37 and 34.91 min, respectively) and their concentration was determined. The chromatogram of a standard steroid mixture is shown in Fig. 1A, which demonstrates the resolution of the system. In Fig. 1, the chromatograms of a normal subject (Fig. 1B), a patient with 11β-hydroxylase deficiency (Fig. 1C) and a patient with 21-hydroxylase deficiency CAH (Fig. 1D) are also shown. The lowest detectable amount was found to be 6 ng/ml for each of the three hormones. Fig. 2 shows the corrrelation between 17-OHP as measured by HPLC and RIA (after column chromatography, $r = 0.9864$, $p < 0.001$). No significant



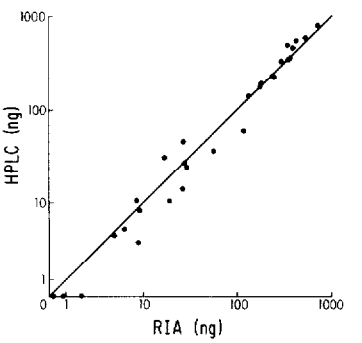


Fig. 1. HPLC profiles of (A) standard mixture of eight steroids; (B) normal control; (C) patient with 11β-hydroxylase deficiency, untreated; (D) patient with 21-hydroxylase deficiency, untreated.

Fig. 2. Comparison of 17-OHP values obtained by HPLC and RIA.

TABLE I

CORTISOL AND 17-OHP IN VARIOUS CLINICAL CONDITIONS BY HPLC AND RIA

Patient No.	Clinical condition	Time	Cortisol level (μg/dl)		17-OHP level (ng/dl)	
1	21-Hydroxylase deficiency	Pre-ACTH	3.4	2.7	176	177
		Post-ACTH	5.0	3.7	242	239
2	21-Hydroxylase deficiency	Pre-ACTH	3.7	3.3	133	142
		Post-ACTH	3.0	2.7	181	182
3	21-Hydroxylase deficiency	Pre-ACTH	1.3	1.1	137	149
		Post-ACTH	1.8	1.8	179	192
4	Cushing's disease	8 a.m. Preoperative	23.7	22.8	ND*	UD**
		4 p.m. Preoperative	25.3	24.4	ND	UD
		8 a.m. Postoperative	4.5	3.4	ND	UD
		4 p.m. Postoperative	2.5	2.0	ND	UD
5	Cushing's disease	8 a.m. Preoperative	23.3	24.0	ND	UD
		4 p.m. Preoperative	20.4	23.1	ND	UD
		8 a.m. Postoperative	11.2	10.7	ND	UD
		4 p.m. Postoperative	6.1	4.3	ND	UD
6	Ovarian tumor	Pre-ACTH	8.1	7.5	9.3	10.5
		Post-ACTH	32.8	26.4	8.6	8.3
7	Addison's disease	Pre-ACTH	3.4	2.2	ND	UD
		Post-ACTH	1.5	1.4	ND	UD

*ND = Not done.
**UD = Undetectable.

differences between the two methods were found ($p > 0.25$). The correlation coefficient between the two methods for cortisol has been previously reported [8]. Table I shows F and 17-OHP levels compared to RIA in the various clinical conditions studied to date. Serum S was undetectable in all subjects except for the two with 11β-hydroxylase deficiency.

DISCUSSION

Separation of a standard mixture of steroids by HPLC has been readily achieved [7, 9, 10]. However, the direct application of these methods to the quantitative analysis of biological fluids may not be possible due to interfering compounds of similar polarity. In Fig. 1 we demonstrate a standard mixture of eight steroids in the system described. This system optimizes the separation of corticosterone and 11-deoxycortisol, compounds which, in fact, share an identical chemical structure, but for the location of the hydroxy group. Using UV detection at 254 nm we have been able to simultaneously quantitate serum F, 17-OHP and S, three steroids of great importance in the diagnosis and management of adrenal disorders. States of glucocorticoid excess or insufficiency can be diagnosed in about 2 h from the time of sample collection, making

possible critical therapeutic decisions. For example, sex assignment in the neonate with ambiguous genitalia due to CAH is a medical emergency that can be rapidly assessed by the measurement of 17-OHP and S by HPLC. In 21-hydroxylase or 11β-hydroxylase deficiency CAH, the high levels of 17-OHP and S make HPLC sensitivity ideal for the precise diagnosis. In fact, we have measured deoxycorticosterone by HPLC in one untreated patient with 11β-hydroxylase deficiency (pre-ACTH levels: RIA 17.46 ng/ml, HPLC 20.5 ng/ml; post-ACTH: RIA 141.9 ng/ml, HPLC 156.1 ng/ml). The small amount of blood required makes the method readily suitable for pediatric patients.

HPLC determination of steroid hormones in amniotic fluid is a method which may be suitable for the rapid prenatal diagnosis of CAH.

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