Reversed-Phase High-Performance Liquid Chromatography Separation of Adrenal Steroids Prior to Radioimmunoassay: Application in Congenital Adrenal Hyperplasia

Vânia Tonetto Fernandes[1,2], Luciane M. Ribeiro-Neto[1], Samira Barbosa Lima[3], José G.H. Vieira[1], Ieda T.N. Verreschi[1], and **Claudio E. Kater[1,*]**

[1]Division of Endocrinology, Department of Medicine, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil; [2]Pediatric Endocrinology Service, Hospital Infantil Darcy Vargas, São Paulo, SP, Brazil; and [3]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo–USP, Brazil

Abstract

21-Hydroxylase deficiency (21-OHD) is the most common form of congenital adrenal hyperplasia (CAH), followed by 11β-hydroxylase deficiency (11β-OHD). Diagnostic serum markers for these conditions are 17-hydroxyprogesterone (17-OHP) and 11-desoxycortisol (S), respectively. In 21-OHD, the large amounts of 17-OHP are further 11β-hydroxylated to form 21-deoxycortisol (21-DF), making it also an excellent marker of this disease. These steroids can be measured in blood by radioimmunoassay (RIA). In this paper, we report the use of high-performance liquid chromatography (HPLC) for steroid purification, prior to RIA determinations of 21-DF, S, 17-OHP, and testosterone (T) in ether-extracted serum. The chromatographic separation is developed in a BDS-Hypersil column using water–methanol (53:47, v/v) as the mobile phase. The method is applied to 35 patients with the classic form of 21-OHD (18 females, 17 males, 5.1–14.2 years old) and 2 with 11β-OHD (1 female, 1 male, 9.5 and 12.6 years old). Thirteen control children (5 females and 8 males, 5.2–15.2 years) are also studied. The results obtained for all measured steroids are compatible with those reported in the literature. The method is precise, and recovery is adequate. The HPLC technique proved to be of value for the purification of several steroids from single serum samples prior to RIA in patients with CAH.

Introduction

Congenital adrenal hyperplasia (CAH) is composed of a group of adrenal enzymatic defects in steroid biosynthesis, from which the most common is 21-hydroxylase deficiency (21-OHD) followed by 11β-hydroxylase deficiency (11β-OHD), which are responsible for 90% and 5% of all cases, respectively (1,2). Reduced or absent activity of these enzymes impairs cortisol synthesis. This leads to excessive adrenocorticotrophic hormone secretion and overproduction of testosterone (T) and the cortisol precursors [17α-hydroxyprogesterone (17-OHP) and 21-deoxycortisol (21-DF)] in 21-OHD, 17-OHP, and 11-deoxycortisol (S) in 11β-OHD. In both deficiencies, the clinical picture can be similar because of androgen excess: ambiguous genitalia (in girls) and precocious puberty. Salt loss is present in approximately 75% of patients with 21-OHD (1), whereas hypertension and hypokalemia are common in those with 11β-OHD.

In 21-OHD, substantially elevated serum levels of 17-OHP, the immediate precursor to the enzymatic block, make it the major steroid marker for diagnosis. Deficiency in the next steroid biosynthetic step downstream (11β-OHD) causes S to accumulate, making it the specific marker for this rarer disease. Because 17-OHP is also elevated to some extent in 11β-OHD, both conditions may be biochemically indistinguishable when diagnosis is based solely on measurements of 17-OHP, as is the case in most centers. However, in 21-OHD the large amounts of 17-OHP that would skip 21-hydroxylation will be further 11β-hydroxylated to form 21-DF. This alternate pathway is negligible in normal conditions and virtually nonfunctional in 11β-OHD, but this is greatly activated in 21-OHD, making 21-DF also an excellent marker of this disease (3,4).

All mentioned steroids can be routinely determined in biological fluids by radioimmunoassays (RIA). However, because of the difficulty in obtaining highly specific steroid antibodies for use in this technique, previous chromatographic separation of steroids is often required. Although laborious, the usual chromatographic method for individual steroid isolation is column chromatography with sephadex (5–8) or celite (4,10–12).

In this paper, we report the use of high-performance liquid chromatography (HPLC) for steroid purification prior to RIA determinations of 21-DF, 17-OHP, S, and T in serum samples from children with CAH caused by 21-OHD and 11β-OHD.

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* Author to whom correspondence should be addressed: email kater@endocrino.epm.br.

Experimental

Chemicals and materials

All standard steroids were purchased from Steraloids (Wilton, NH). Methanol (EM Science, Gibbstown, NJ) and ether (Sinth, São Paulo, Brazil) were HPLC and analytical-reagent grade, respectively. Water was freshly distilled, deionized, and purified with a Milli-Q plus equipment (Millipore, Bedford, MA).

Preparation of standards

The standard solutions (10 μg/mL) for 21-DF, 17-OHP, S, T, and dexamethasone (Dex) were prepared in methanol. Dex was used as a reference standard for chromatographic analysis.

Sample extraction

Aliquots (1 mL) of serum samples were extracted with 3 mL ether under vigorous shaking for 20 s, followed by centrifugation at 2000 rpm for 10 min. The organic layer was evaporated to dryness under a nitrogen flow, and the residues were reconstituted in 200 μL of mobile phase, filtered through a reduced cellulose syringe filter (0.45-μm pore size, 13-mm i.d.) from Hewlett-Packard (HP, Weiterstadt, Germany), and injected into the chromatographic column.

HPLC method

An HP-1100 (Hewlett-Packard, Palo Alto, CA) liquid chromatograph equipped with a UV detector set at 246 nm was employed. A BDS-Hypersil column (125 × 4 mm, 5-μm particle size) and column guard equivalent (4 × 4 mm, 5 μm), both from HP, were kept at 40°C throughout the analysis. The isocratic water–methanol (53:47, v/v) mobile phase was used at a flow rate of 1.0 mL/min for 22 min. At the 23rd min, the mobile phase was changed to 100% methanol and maintained for 1 min at a flow rate of 1.5 mL/min. At the 24th min, the starting mixture was reinstituted and maintained until the 30th min. The injection volume was 50 μL. The eluent was fractionated using a fraction collector FRAC-100 (Pharmacia Biotech, Uppsala, Sweden). Each fraction corresponded to a 2-min running time. Fraction numbers 4, 5, and 8 correspond respectively to 21-DF, S, and T, and fractions 10 and 11 were grouped and correspond to 17-OHP. All other fractions were discarded. The collected fractions were evaporated under a stream of nitrogen and stored at –20°C until analysis.

RIA

Serum concentrations of 17-OHP, T, S, and 21-DF were determined by routine in-house RIAs: 17-OHP and T used RIA methods adapted from Vieira et al. (12,13), S used an RIA method adapted from Araújo et al. (15) using an antibody with greater specificity, and 21-DF used an RIA method recently described by Fernandes et al. (14). Table I shows the characteristics of these assays. Final serum concentrations were calculated by RIACALC software (Wallac Oy, Turku, Finland), and the results obtained were corrected for the initial volume and correspondent dilutions.

Sensitivity, precision, and recovery

The sensitivity of the assay, defined as the smallest amount of steroid that can be measured in a serum sample, was established as the mean minus 2 standard deviations (SDs) of the mean of free-steroid serum samples ($n = 6$). Precision and recovery were studied by concomitant evaluation throughout the procedure of prepared steroid-free serum samples with added concentrations of the steroids. Intra- and interassay coefficients of the variations were determined, respectively, by analyzing 5–6 replicate samples in the same assay and on 2-day repeated determinations.

Subjects

Venous blood samples were obtained from 35 outpatients with the classic form of 21-OHD (18 females and 17 males, 5.1–14.2 years of age, 17 being salt losers), and 2 with 11β-OHD (1 female and 1 male, 9.5–12.6 years). All patients had their replacement glucocorticoid and mineralocorticoid therapy discontinued for 48 h prior to sampling. Diagnosis of CAH caused by 21-OHD and 11β-OHD was based of elevated basal serum levels of 17-OHP and S, respectively. Thirteen control children (5 females and 8 males, 5.2–15.2 years) were also studied. Informed written consent was obtained from all parents or legal guardians according to a protocol previously approved by the Committee on Human Research of the institutions involved.

All samples were centrifuged at room temperature within 2 h from collection, and serum was separated and stored at –20°C until processing.

Results and Discussion

RIA is still widely used for the measurement of steroids, although to date problems with specificity have been only partially solved. The major difficulties are still the production of specific steroid antibodies caused by a high percentage of

Table I. Percentage of Steroid Cross Reactivity and Antibody Titers from RIAs Used to Determine Serum Levels of 21-DF, 17-OHP, S, and T

RIA	Antibody titer	Cross reactivity (%)				
		21-DF	17-OHP	S	T	F
21-DF	1/8000	100.00	9.50	9.50	ND	100.00
17-OHP	1/100,000	ND	100.00	0.80	< 0.05	< 0.06
S	1/7000	ND	100.00	100.00	8.00	6.00
T	1/700,000	ND	0.30	ND	100.00	0.30

* F, cortisol. ND, not detected.
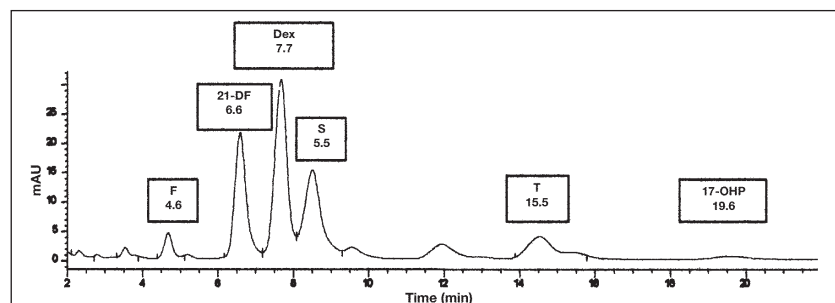
Figure 1. Chromatogram obtained from sample serum with known concentrations of the cortisol (F), 21-DF, Dex, S, T, and 17-OHP.

cross reactivity with structurally similar substances and the wide differences in serum concentrations. Thus, it is often necessary to apply additional procedures in order to improve analytical sensitivity and specificity. Solid-phase column chromatography using Sephadex (5–8) or Celite (4,10–12) is still widely used worldwide. However, this technique is time consuming, less robust, and technician-dependent, whereas HPLC is highly selective and efficient, allows for automation, and in some cases can also be used for direct determination (6,16).

In this study, the chromatographic separation proved to be selective. Dex, used as a reference standard, showed a retention time (t_R) of 7.7 min, with a relative t_R of 0.86, 1.10, 2.01, and 2.55 min, respectively, for 21-DF, S, T, and 17-OHP (Figure 1). The obtained chromatographic profile separation was similar to those reported by others using reversed-phase HPLC (6,16–19), whereas a longer time interval for separation (30 min) is usually needed when normal phase is employed (11,20).

Ether extraction of serum steroids (8,11,18) is a simple and useful procedure that permits efficient sample purification when coupled to HPLC. Because of automation, the HPLC technique is easier and advantageous, reducing the time-consuming step of column chromatography.

Data on sensitivity, recovery, and precision are summarized in Table II. The method sensitivity for determining 21-DF permitted evaluation in the range seen in patients with 21-OHD; however, in patients with 11β-OHD and those in the control group, serum levels of 21-DF were all below the limit of sensitivity. Recovery was similar to those previously reported for 21-DF (7,21), 17-OHP (20), S (20), and T (13), although protocols were not exactly the same. Intra- and interassay coefficients of variation were appropriate and in accordance with those published (3,7,9,21).

Results obtained from patients with 21-OHD and 11β-OHD and control subjects are presented in Table III. In patients with 21-OHD, serum levels of 17-OHP and 21-DF were elevated and comparable with those previously reported (5,7,9,10,21); Franks et al. (5) found higher levels of 21-DF, although his patients were mostly untreated. Serum levels of S were also in the same range as those found by Fukushima et al. (10). Because pubertal patients were included in our study, serum T levels were somewhat higher than those found by Schnakenburg et al. (8). Both patients with 11β-OHD had significantly elevated S levels in the presence of only moderately elevated 17-OHP; the S levels were actually even higher than those reported by Fukushima et al. (10) and Weisman et al. (16).

In the control group, serum levels of 17-OHP (21,22), S (22), and T (8,22) were all in the range previously reported.

Conclusion

This reversed-phase HPLC technique proved to be of value for the purification of several useful diagnostic steroids prior to RIA in single serum samples from patients with CAH.

Acknowledgments

Table II. Summary Data Obtained from the Study of Sensitivity, Recovery, and Precision for the Method Using HPLC Prior to RIA

Steroids	Sensitivity (ng/dL)	Concentration (ng/dL)	Recovery (%)	Intra-assay CV* (%)	Interassay CV (%)
21-DF	156[†]	750	93.0[†]	4.8[†]	15.0
S	24[†]	1000	69.0[†]	7.1[†]	22.0
T	13[†]	200	74.0[‡]	7.6[†]	9.0
17-OHP	11[†]	5000	54.0[†]	3.1[§]	8.6

* CV, coefficient variation.
[†] $n = 6$.
[‡] $n = 3$.
[§] $n = 5$.

Table III. Serum Steroid Levels from 35 Patients* with the 21-OHD, 2[†] with the 11β-OHD Forms of Congenital Adrenal Hyperplasia, and 13[‡] Age-Control Children

21-OHD		21-DF (ng/dL)		17-OHP (ng/dL)		S (ng/dL)		T (ng/dL)	
		F	M	F	M	F	M	F	M
	Mean	1423	1244	8761	9186	73	91	165	214
	SD	1299	1043	5232	7864	38	41	155	149
	Median	811	940	8451	5852	58	93	87	172
	Range	254–4642	218–3935	1019–19,927	774–25,049	28–148	24–170	28–524	21–552
11β-OHD		ND	ND	344	466	6357	6088	39	73
Controls	Mean	ND	ND	104	88	93	52	25	118
	SD			86	58	59	26	6	114
	Median	ND	ND	72	81	93	45	23	72
	Range			12–221	12–188	24–179	24–83	19–36	21–330

* Patients: 18 females, 17 males.
[†] Patients: 1 female, 1 male.
[‡] Patients: 5 females, 8 males.
[§] Abbreviations: F, female; M, male; and ND, not detectable (< 156 ng/dL).

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