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Note

Simultaneous quantification of natural glucocorticoids and progestins in serum

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Both qualitative and quantitative analyses of glucocorticoids in human serum have been reported using a variety of analytical techniques such as gas chromatography–mass spectrometry (GC–MS) [1], liquid chromatography–mass spectrometry (LC–MS) [2], gas chromatography (GC) [3], as well as radioimmunoassay (RIA) [4] and high-performance liquid chromatography (HPLC) [5,6], separately and in tandem [7]. While analyses based on RIA procedures are characterized by high sensitivity, these benefits can be offset by potential lack of specificity due to cross-reactivity of related compounds. Analyses of these compounds by GC, while both highly sensitive and specific, require derivatization of samples prior to analyses. HPLC, however, provides a rapid, simple and sufficiently sensitive analytic tool for the quantification of glucocorticoid levels in biological material.

The method reported here was developed in response to our need for a quantitative analytical procedure that could reveal more information than standard RIA procedures about glucocorticoids, progestins, and their metabolites in single serum samples from pregnant women. What follows is the description of an analytical procedure which allows the simultaneous quantification of ten glucocorticoids, progestins, and metabolites in human serum.

EXPERIMENTAL

Materials

Steroids used as standards was obtained from Steraloids (Wilton, NH, U.S.A.). These included: aldosterone (11β,21-dihydroxy-4-pregnene-3,18,20-trione); cor-

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tisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione); cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione); 21-desoxycortisone (17α-hydroxy-4-pregnene-3,11,20-trione); 21-desoxycortisol (11β,17α-dihydroxy-4-pregnene-3,20-dione); corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione); 11-desoxycortisol (17,21-dihydroxy-4-pregnene-3,20-dione); desoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione); 17α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione); and progesterone (4-pregnene-3,20-dione). The HPLC apparatus (all from Waters Assoc., Milford, MA, U.S.A.) consisted of a Model U6K injector fitted with a 2-ml sample loop, a Model 440 fixed-wavelength absorption detector, a Model 680 gradient controller, and two pumps (a Model 6000A and a Model M45). A Vydac HS Silica C_{18} reversed-phase 10-μm column (250$\times$4.6 mm) was used along with an Uptight guard column packed with HS C_{18} (Upchurch Scientific, Sunnyvale, CA, U.S.A.). Chromatograms were recorded on a two-channel variable millivolt setting recorder from LKB (Gaithersburg, MD, U.S.A.). All solvents were HPLC grade from Fisher Scientific (Santa Clara, CA, U.S.A.). All glassware used in the study was deactivated by treatment with a 10% solution of dimethyldichlorosilane (Pierce, Rockford, IL, U.S.A.) in toluene prior to use. Individual stock standards containing 1 mg/ml in methanol of each compound were diluted to make working standards of 10 and 2 ng/μl. A 20 ng/μl methanolic standard of 6β-hydroxyprogesterone (4-pregnen-6β-ol-3,20-dione) was used as an internal standard.

Standard curve

Serum from female rats (ovariectomized and adrenalectomized ten days prior to decapitation) was the biological matrix for generating the standard curve. A standard curve was produced by placing 1 ml of the steroid-free rat serum into 125$\times$16 mm screw-cap tubes. To each tube were added various volumes of the working standards to generate a standard curve ranging from 12.5 to 250 ng/ml. Each sample also received 200 ng 6β-hydroxyprogesterone to serve as an internal standard. In addition, one tube received no added steroid and served as a blank. Quantitation was achieved by comparing the peak-height ratios found in case samples to those generated in the standard curves. The signal from the detector was monitored at two different millivolt settings to ensure that peaks of variable size would be on scale and measurable. Retention times and relative retention times were used to identify peaks in the patient samples.

Extraction

As each sample was being vortex-mixed, 2 ml of acetonitrile were added to aid in the deproteinization of the serum. Methylene chloride (10 ml) was added to each tube and the samples were then extracted for 30 min on a shaker. After centrifugation for 15 min at 2000 *g*, the organic layer was transferred to a clean 125$\times$16 mm screw-cap tube, 2 ml of saturated sodium borate buffer (pH 9.4) were added, and the tubes were shaken for 10 min. After centrifugation for 10 min, the borate buffer was discarded and replaced with 2 ml of water. The sample was extracted for an additional 10 min, centrifuged to separate the layers, and the organic layer transferred to a clean 100$\times$16 mm centrifuge tube. The organic

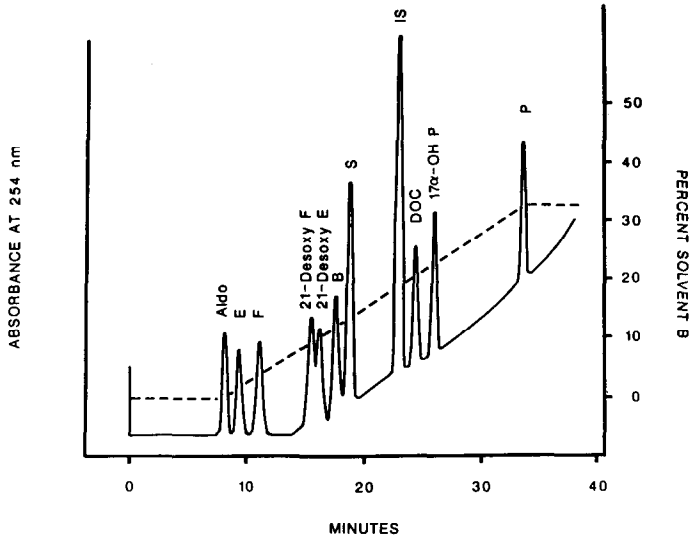

Fig. 1. HPLC separation of some glucocorticoids and progestins. A mixture of 50 ng each of ten compounds and 200 ng of 6β-hydroxyprogesterone, internal standard, were separated on a C_{18} column as described in Experimental. The compounds were detected by their absorbance at 254 nm (solid line) with a sensitivity of 0.01 a.u.f.s. The compounds separated were: aldosterone (Aldo); cortisone (E); cortisol, (F); 21-desoxycortisol (21-desoxy F); 21-desoxycortisone (21-desoxy E); corticosterone (B); 11-desoxycortisol (S); 6β-hydroxyprogesterone (IS); desoxycorticosterone (DOC); 17α-hydroxyprogesterone (17α-OH P); progesterone (P). The gradient profile is shown by the dashed line.

solvent was evaporated under a stream of nitrogen in a 50°C water bath. (Samples may be stored for as long as 48 h at 4°C prior to evaporation without adverse effect.) The dried residue was reconstituted in 400 μl of 0.001 *M* potassium dihydrogen phosphate–methanol (48:52, v/v) and vortex-mixed. If the reconstituted sample was cloudy, it was centrifuged to produce a clear supernatant suitable for injecting into the separation system.

Chromatography

The HPLC conditions and gradient program used are as follows. The mobile phases utilized were: (A) 1 m*M* potassium phosphate (monobasic)–methanol (48:52, v/v) and (B) methanol–isopropanol (50:50, v/v). The flow-rate was set at 1.5 ml/min. Mobile phase A was held at 100% for the first 8 min of analysis. At this point mobile phase B was introduced into the system and was increased linearly over the next 26 min to a final proportion of 34%. Compounds were detected by their absorbance at 254 nm. Total analysis time for each sample including regeneration time is 47 min. A typical chromatogram is shown in Fig. 1. A list of naturally occurring and synthetic steroids detected at 254 nm and their retention characteristics are shown in Table I.

TABLE I

RETENTION TIMES OF SOME NATURAL AND SYNTHETIC STEROIDS

Steroid	Retention time (min)	Retention time relative to 6β-hydroxyprogesterone
Aldosterone	8.3	0.36
Cortisone	9.7	0.42
Cortisol	11.4	0.49
Prednisolone	11.4	0.49
21-Desoxycortisol	16.2	0.70
21-Desoxycortisone	17.0	0.74
Betamethasone	17.2	0.74
Dexamethasone	17.4	0.75
Corticosterone	18.6	0.81
11-Desoxycortisol	19.8	0.86
16α-Hydroxyprogesterone	20.8	0.90
11α-Hydroxyprogesterone	21.0	0.91
6β-Hydroxyprogesterone	23.1	1.0
Deoxycorticosterone	25.4	1.1
11β-Hydroxyprogesterone	25.7	1.11
Testosterone	25.8	1.12
17α-Hydroxyprogesterone	27.0	1.17
Deoxycorticosterone-hemisuccinate	29.8	1.29
Depo-Provera	31.2	1.35
Progesterone	34.8	1.51
20β-Hydroxyprogesterone	36.0	1.56

RESULTS

Recovery data

Four human serum samples containing added levels of steroid (100 ng/ml) were extracted as described. A 6-ml aliquot of the 10 ml of methylene chloride used in the extraction was evaporated. Two aliquots of a methanolic standard containing 60 ng of the ten glucocorticoids were injected onto the column to simulate 100% extraction and recovery. Peak heights were compared between standards and extracted samples. The recovery was greater than 90% for all of the compounds examined.

Reproducibility

Intra-assay coefficients of variation (C.V.) were established at 60 ng/ml concentration with $n = 11$; these samples were analyzed on five different occasions to determine the inter-assay variation. For all ten compounds, the C.V. for within-day and for day-to-day determinations were less than 5.5 and 12%, respectively. The actual values obtained are shown in Table II.

Linearity and accuracy

Standard curves were linear from 12.5 to 250 ng/ml with correlation coefficient (r) values of 0.995 or greater for each of the compounds of interest.

TABLE II

PRECISION OF HPLC DETERMINATION OF STEROID LEVELS AT A CONCENTRATION OF 60 ng/ml

Compound	Within-day		Day-to-day	
	n	C.V. (%)	*n*	C.V. (%)
Aldosterone	11	5.3	5	6.0
Cortisone	11	5.3	5	12.0
Cortisol	11	4.5	8	10.3
21-Desoxycortisol	11	4.0	8	7.1
21-Desoxycortisone	11	3.7	8	11.2
Corticosterone	11	2.2	7	7.8
11-Desoxycortisol	11	1.6	8	8.1
Deoxycorticosterone	11	3.6	8	7.5
17α-Hydroxyprogesterone	11	3.0	8	4.2
Progesterone	11	2.9	8	6.3

TABLE III

COMPARISON OF STEROID VALUES OBTAINED BY RIA AND HPLC

Steroid (110 μg/ml) was added to control human serum. The levels of each were then determined repeatedly by both RIA and HPLC as described. In all cases, $n=6$.

Steroid	RIA			HPLC		
	Mean (pg/ml)	S.D. (pg/ml)	C.V. (%)	Mean (pg/ml)	S.D. (pg/ml)	C.V. (%)
Cortisol	161.2	8.84	5.5	175.3	5.0	2.86
Progesterone	118.5	7.7	6.9	105.2	3.7	3.5

Stability

Human serum samples with added concentrations of 100 ng/ml of the ten compounds were stored in either deactivated glass or polypropylene vials in a $-20°C$ freezer. Over a six-month period, these samples were periodically analyzed under our standard conditions. In both deactivated glass and polypropylene-stored samples there were no observable losses in concentration levels of any of the compounds. If, however, samples were stored under similar conditions in non-deactivated glass vials, significant losses of aldosterone and progesterone were observed.

Comparison of values with those obtained RIA

The quantification of cortisol and progesterone by HPLC analyses was compared with that achieved by RIA. Each of the RIAs utilized was highly specific and did not detect any compounds in the serum other than cortisol or progesterone (unpublished results). When sera containing different levels of steroid were analyzed by each technique, there was good agreement between the values obtained by HPLC and RIA ($r=0.96$, data not shown). To compare the precision of the

two methods, 100 μg/ml cortisol or progesterone was added to control human serum samples. Steroid values were determined six separate times by both RIA and HPLC techniques and the coefficient of variation was determined for each method. As shown in Table III, comparable steroid values were obtained. The precision of the HPLC method, however, was about two times better than that observed with RIA.

DISCUSSION

We described here a novel method for resolving and quantitating glucocorticoids and progestins by reversed-phase HPLC. While not as sensitive as most RIAs, this method allows the simultaneous determination of at least ten compounds and is free from problems of cross-reactivity which frequently plague RIAs. In addition, we have found it to have greater reproducibility than most RIAs. Although extraction of the compounds of interest is required, the method as described is relatively simple to use and a sufficient number of samples can be analyzed at one time to make it of practical use.

Steroids exist in plasma both in free form and bound to albumin and specific steroid-binding proteins such as corticosteroid-binding globulin (CBG) and sex hormone-binding globulin (SHBG) [8]. Disruption of the interaction of steroid and binding proteins during the extraction procedure must be achieved for maximum extraction efficiency of total steroids. The method described, which includes the efficient denaturation of specific binding proteins during the extraction procedure, meets this criteria. When a simple liquid–liquid extraction of physiologic pH was used, the extraction efficiency was low; it increased for some compounds (e.g. cortisol and corticosterone) if the samples were heated at 60°C for 35 min prior to extraction but reached maximum values only after treatment with acetonitrile. The greater than 90% recovery of progesterone in particular is dependent upon this step since progesterone is bound with high affinity to the specific steroid-binding protein, CBG [8]. The extraction procedure is readily adaptable to the handling of multiple samples. Routinely, a batch containing ten samples, five concentrations to establish a standard curve and five patient samples can be extracted in 2–3 h. The final extracts may then be stored overnight, dried down the next day and injected over an 8–9 h period.

Although HPLC separation procedures for glucocorticoids, progestins, and androgens have been described [5,6], we feel that this method has several advantages. The extraction system utilized is both highly efficient and convenient. It results in excellent recoveries of all steroids examined. The solvent system is a relatively simple mixture of frequently used inexpensive HPLC chemicals. Isopropanol was found to be particularly useful for decreasing the elution time of some compounds, thus improving peak shape and allowing reproducible quantification of even late eluting less polar compounds. While the use of gradient elution methods is not ideal, the one employed here is simple and fast as well as readily adaptable to isocratic conditions for the analysis of individual steroids of interest using one of the synthetic steroids as an internal standard. For example,

dexamethasone may be used as an internal standard for the quantification of corticosterone.

With a correlation coefficient of greater than 0.995 and coefficients of variation of less than 7%, we have developed a fast, reliable, reproducible and accurate analytical procedure for the simultaneous quantification of some glucocorticoids and progestins.

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