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Note

Simultaneous analysis of cortexolone and cortisol by high-performance liquid chromatography for use in the metyrapone test

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Analysis of cortexolone, cortisol, and other endogenous steroids has become important in assessing the integrity of the hypothalamic-pituitary-adrenal (HPA) axis in Addison's disease, Cushing's syndrome and depression [1—3]. Under physiological conditions, low concentrations of most endogenous steroids have necessitated the use of sensitive assay methods such as the radioimmunoassay (RIA). The higher physiological concentrations of cortisol have allowed its analysis by fluorometry [4], competitive protein binding [5], liquid chromatography [6], as well as RIA techniques [7]. Many of these methods lack specificity in separation or specific antibodies to distinguish various endogenous steroids. This may be particularly important with the metyrapone test. The administration of metyrapone results in elevated cortexolone concentrations to a range which can be determined by current high-performance liquid chromatography (HPLC) techniques. The concomitant analysis of cortisol has been suggested as a measure of sufficient adrenal hydroxylase inhibition [3]; however, the supra-physiological cortexolone concentrations may cause falsely elevated cortisol values due to lack of antibody specificity with the RIA technique [6, 8]. Previous liquid chromatography methods for cortexolone and cortisol lack the ability to assay both steroids simultaneously and therefore require two separate internal standards and chromatographic conditions [9]. Other techniques do not utilize an internal standard and fail to selectively extract the steroids without extracting metyrapone [6]. In the single dose version of the test, metyrapone is present in μg/ml quantities and may interfere with the quantitation of ng/ml quantities of the steroids especially under reversed-phase chromatographic conditions.

This report describes an HPLC method which was modified from our previ-

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ous exogenous steroid assays [10—12] to allow selective and simultaneous quantitation of cortexolone and cortisol with a single internal standard in human plasma for use in the metyrapone test.

EXPERIMENTAL

Materials

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system and a Model 441 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 universal loop injector (Rheodyne, Berkeley, CA, U.S.A.), and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). A Zorbax Sil (DuPont, Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5—6 μm particle size) and a 70 × 6 mm stainless-steel precolumn packed with HC-Pellocil (37—53 μm particles) (Whatman, Clifton, NJ, U.S.A.) were used to separate the compounds.

Methylene chloride, used in the extraction procedure, and hexane, used in the mobile phase, were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Pure ethanol (U.S.P.), obtained from U.S. International Chemicals (New York, NY, U.S.A.) and glacial acetic acid, purchased from J.T. Baker (Philipsburg, NJ, U.S.A.), were employed in the mobile phase. The cortisol, dexamethasone, and cortexolone analytical standards were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmaceutical grade decolorizing carbon, neutral, was purchased from Amend Drug and Chemical Co. (Irving, NJ, U.S.A.). Anhydrous sodium sulfate, sodium hydroxide solution (1 *M*) and hydrochloric acid solution (1 *M*) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Standard preparation

Decolorizing carbon (12.5 g) was added to 240 ml of pooled human plasma and stirred for 2 h at room temperature. Carbon was then removed by centrifugation at 17,000 *g* for 6 h at 4°C and by filtration through 5-μm and 0.45-μm Millipore filters (Millipore, Bedford, MA, U.S.A.). To this cortisol-stripped plasma, standards of cortisol and cortexolone in acetonitrile—methanol (1:1) were added to provide concentrations of 5 to 500 ng/ml.

Extraction procedure

Plasma samples (1 ml) were placed into acid-washed glass extraction tubes with PTFE-lined screw caps (20 × 150 mm) and 30 μl of a 5 μg/ml solution of dexamethasone in acetonitrile—methanol (1:1) were added as the internal standard. Then 0.3 ml of 1 *M* hydrochloric acid and 15 ml of methylene chloride were added. The tubes were capped, shaken for 15 min, and then centrifuged for 10 min. The aqueous layer and creamy interface were aspirated. The remaining organic phase was then washed with 1 ml of 0.1 *M* sodium hydroxide, with 1 ml of distilled water, and dried by adding 1 g of anhydrous sodium sulfate to each tube. After decanting, the organic phase was evaporated to dryness at 45°C under a nitrogen gas stream.

Chromatography

The residue was reconstituted with approximately 250 μl of mobile phase for injection. All chromatography was carried out at ambient temperature. The mobile phase, composed of a hexane—methylene chloride—ethanol—acetic acid (26:69:3.4:1) mixture, was pumped through the column at a constant flow-rate of 2 ml/min (85 bars). Concentrations of cortexolone and cortisol were determined by comparison of the peak height ratio of drug to internal standard with the peak height ratio of known standard concentrations of the drugs.

Steroid recovery

The assay recovery of each steroid was assessed at 50 ng/ml and 300 ng/ml. The peak heights from ten extracted plasma samples (1 ml) and from ten direct injections of the same amount of steroid (e.g. 50 and 300 ng) in mobile phase were compared. The assay recovery of each steroid was computed using the following equation:

$$\text{Percent recovery} = \frac{\text{peak height, extracted drug}}{\text{mean peak height, direct injection}} \times 100$$

Assay comparison

Plasma samples from depressed-psychiatric patients receiving the metyrapone test to assess the hypo-activity of the HPA axis were measured by the HPLC method and a radioimmunoassay (RIA) method. The commercial RIA kits for cortexolone and cortisol were purchased from Radioassay Systems Labs. (Carson, CA, U.S.A.).

RESULTS

The chromatogram shown in Fig. 1a illustrates the response to steroid concentrations of approximately 25 ng/ml in plasma from which endogenous steroids were removed by charcoal. Each steroid eluted with a sharp peak and distinct separation at baseline. Cortexolone eluted before cortisol, and also showed a relatively greater detector response. Good separation is maintained in chromatograms of plasma samples taken with and without elevated cortexolone from the metyrapone test (Fig. 1b and c). The mean assay recoveries were 59% for cortexolone and 50% for cortisol. Similar recoveries were seen with the internal standard. The apparent minimum quantification limit for both cortexolone and cortisol is 5 ng/ml. Calibration plots of peak height ratio versus steroid concentration were linear over the range of 5 to 1000 ng/ml.

The within-day and between-day precision of the assay were determined by analysis of ten plasma samples containing high (300 ng/ml) and low (50 ng/ml) concentrations. These data are presented in Table I. Assay specificity was determined by comparing retention times of standards to those of samples (Table II) and by analysis of samples from patients receiving other drugs. These drugs and their metabolites which had no apparent assay interference included: imipramine, amitriptyline, doxepin, oxazepam, flurazepam, chlorpromazine,

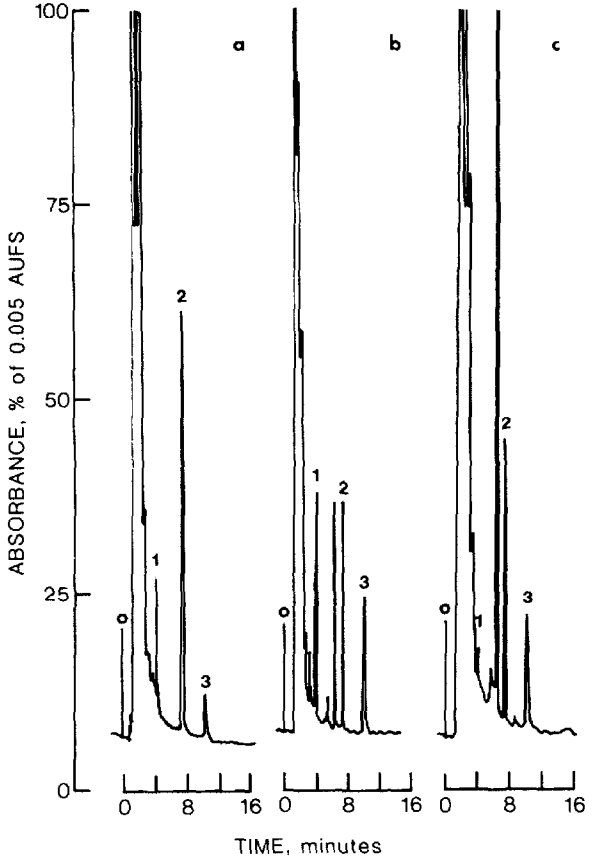

Fig. 1. Chromatograms of (a) charcoal-stripped plasma extract spiked with 25 ng/ml of cortexolone (1) and cortisol (3); (b) plasma extract from a patient with metyrapone and, (c) plasma extract from a patient without metyrapone. Dexamethasone, 150 ng (2) is the internal standard. The symbol (o) designates the injection point.

TABLE I

WITHIN-DAY AND BETWEEN-DAY VARIABILITY OF ASSAYS

Steroid	Within-day				Between-day			
	Mean concn. (μg/l)	S.D.	C.V. (%)	*n*	Mean concn. (μg/l)	S.D.	C.V. (%)	*n*
Cortexolone	50.9	6.04	11.9	10	47.2	6.19	13.1	11
	276	14.2	5.16	10	290	31.7	10.9	10
Cortisol	48.8	2.47	5.06	10	48.6	7.83	16.1	11
	277	5.33	1.92	10	291	22.6	7.76	11

haloperidol, fluphenazine, thiothixene, acetaminophen, various bronchodilators, anticonvulsants, and antibiotics as has been previously reported [12].

Twelve plasma samples were measured by the HPLC method and the commercial RIA method. The two assays showed excellent correlation for cortexolone with a regression line slope of 1.05 (Fig. 2a). Cortisol was also assayed in five of these samples as well as in eight other samples from patients who had not received metyrapone. The RIA method yielded similar results as

TABLE II

RELATIVE CHROMATOGRAPHIC DRUG RETENTION TIMES

Drug	Relative retention time
Deoxycorticosterone	0.31
Cortexolone	0.45
Fluocinonide	0.51
Cortisone	0.56
Corticosterone	0.57
Methylprednisone	0.58
Prednisone	0.66
Beclomethasone	0.73
Aldosterone	0.84
Betamethasone	0.86
Dexamethasone	0.93
Cortisol	1.00
Methylprednisolone	1.2
Prednisolone	1.3

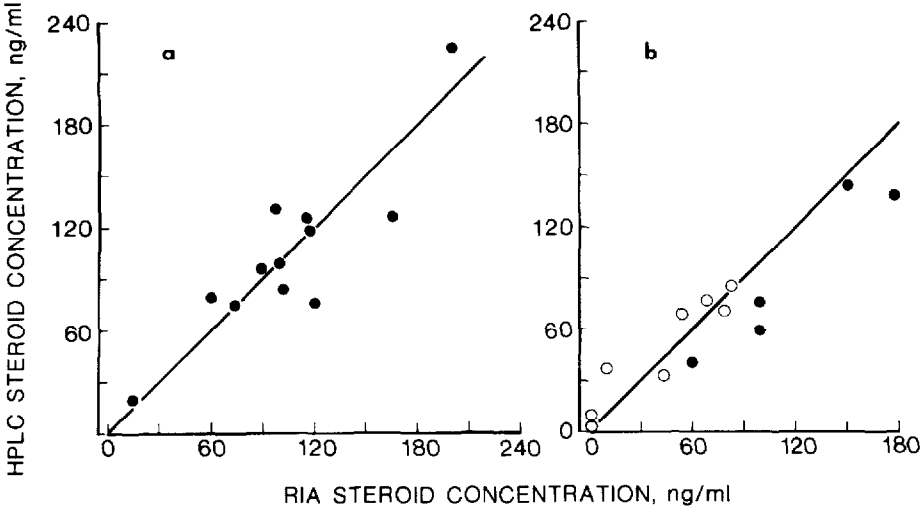

Fig. 2. Correlation between plasma cortexolone (a) and cortisol (b) concentrations, as measured by HPLC and RIA methods. Plotted lines denote slopes of unity. For cortexolone, the calculated slope = 1.05, the intercept = —4.65, and the correlation coefficient = 0.881. For cortisol without metyrapone testing (○), the slope = 1.01, the intercept = 9.77, and the correlation coefficient = 0.935. For cortisol with metyrapone testing (●), the slope = 1.00, the intercept = —25.6, and the correlation coefficient = 0.960.

the HPLC method for cortisol in the absence of the metyrapone test; however, in the presence of metyrapone and elevated cortexolone concentration, the RIA assay yielded higher values than the HPLC method (Fig. 2b). The mean cortisol concentration (117 ng/ml) measured by RIA in the presence of metyrapone was significantly higher ($p = 0.017$, non-paired Student's t test) than the mean cortisol concentration (92 ng/ml) without metyrapone.

DISCUSSION

The simultaneous measurement of cortexolone and cortisol by this HPLC

method is efficient, precise, sensitive and selective. To date, over 150 cortexolone and over 2000 cortisol and exogenous steroid samples have been analyzed by this chromatographic procedure in studies of the disposition kinetics of exogenous steroids, cortisol, and in psychoendocrinologic studies.

Variability between assay methods in population mean cortisol concentrations has been described following the single-dose metyrapone test [6, 8, 9]. This may be a function of variability in both the assay method and in adrenal hydroxylase inhibition. The latter may be caused by interpatient differences in metyrapone pharmacokinetics or perhaps variability in sampling and handling methods. To further assess this discrepancy and to avoid interpatient variability, the same serum samples were analyzed by both HPLC and RIA methods. Therefore, the increased variability of cortisol measurements following the metyrapone test can be attributed solely to differences in the assays. In our small patient sample, the RIA values were 4 to 66% higher than the HPLC values. This is similar to the 50 to 90% difference between HPLC and RIA results reported by Reardon et al. [6] between reversed-phase HPLC with UV detection and a commonly available RIA method (Beckman Instruments, Irvine, CA, U.S.A.). The more pronounced difference reported by Reardon et al. [6] may be due to differences in antibody specificity between our two RIA assays. On the contrary, Schoneshofer et al. [13] describe a gradient HPLC method with UV detection following a solid phase extraction method which produced interferences in 21% of 195 serum samples analyzed for cortisol in comparison with a commonly available RIA method. The intermediary washing steps usually exclude such interferences in our liquid—liquid extraction method.

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REFERENCES

1 W. Jubiz, A.W. Meikle, C.D. West and F.H. Tylor, Arch. Int. Med., 125 (1970) 472.
2 B.J. Carroll, M. Feinberg, J.F. Greden, J. Tarika, A.A. Albala, R.F. Harkett, N. McI. James, Z. Kranfol, N. Lohr, M. Steiner, J.P. deVigne and E. Young, Arch. Gen. Psychiat., 38 (1981) 15.
3 M. Spiger, W. Jubiz, A.W. Meikle, C.D. West and F.H. Tylor, Arch. Int. Med., 135 (1975) 698.
4 J.W. Goldzieher and P.K. Besch, Anal. Chem., 30 (1958) 962.
5 B.E.P. Murphy, J. Clin. Endocrinol. Metab., 27 (1967) 973.
6 G.E. Reardon, A.M. Caldarella and E. Canalis, Clin. Chem., 25 (1979) 122.
7 J. Seth and L.M. Brown, Clin. Chim. Acta, 86 (1978) 109.
8 E. Canalis, A.M. Caldarella and G.E. Reardon, Clin. Chem., 25 (1979) 1700.
9 C.P. de Vries, M. Lomecky-Janousek and C. Popp-Snijders, J. Chromatogr., 183 (1980) 87.
10 J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273.
11 M.L. Rocci, Jr. and W.J. Jusko, J. Chromatogr., 224 (1981) 221.
12 W.F. Ebling, S.J. Szefler and W.J. Jusko, J. Chromatogr., 305 (1984) 271.
13 M. Schoneshofer, R. Skobolo and H.J. Dulce, J. Chromatogr., 222 (1981) 478.