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Simultaneous determination of serum cortisol and cortisone by reversed-phase liquid chromatography with ultraviolet detection

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ABSTRACT

A rapid high-performance liquid chromatographic (HPLC) method for the simultaneous determination of cortisol and cortisone in a single extract of 1 ml of serum is described. The method employs meprednisone as the internal standard. The steroids were analysed isocratically by reversed-phase HPLC with an octadecylsilane-bonded (ODS) column using ultraviolet detection. The matrix effect was reduced by lowering the sample pH by adding glacial acetic acid to the sera. The samples were then filtered through regenerated cellulose membranes at 4°C and extracted with diethyl ether. The dried eluates were redissolved in the mobile phase and injected into the column. The detection limit of the assay for both steroids was 500 ng/l. Cortisol was determined in twenty serum samples by both HPLC and radioimmunoassay (RIA). The results were similar. Interference by other steroids and certain steroid analogue drugs was also studied. The HPLC method yielded no cross-reactivity between the different steroids as may occur with the RIA technique. The HPLC method was technically easy to perform and it allowed us to quantify both cortisol and cortisone in a single serum extract with high specificity.

INTRODUCTION

Cortisol (11β,17α,21-trihydropregn-4-ene-3,20-dione) is an important product of the adrenal gland needed in several metabolic processes and in adaptation to stress. The main inactivation route of cortisol is interconversion to cortisone (17,21-dihydroxy-4-pregnene-3,11,20-trione) [1]. This reaction is catalysed by 11β-hydroxysteroid dehydrogenase (11β-HSD) in many peripheral tissues [2,3]. In normal adult serum the concentration of physiologically active cortisol is five to ten times that of inactive cortisone [4].

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Defective inactivation of cortisol because of either a congenital [5–8] or an acquired [9–12] deficiency of 11β-HSD has been shown to result in clinical symptoms of low-renin hypertension, hypokalaemia, apparent mineralocorticoid excess and virilization. Suggestive of 11β-HSD deficiency is the finding of normal serum cortisol concentration with the virtual absence of serum cortisone [8,13,14]. The accurate and simultaneous measurement of serum cortisol and other glucocorticoids may also be used in the diagnosis and management of various clinical disorders of the adrenal and pituitary glands.

During the past decades several clinical methods involving fluorimetry [15,16], enzyme immunoassay (EIA) [17,18], radioimmunoassay (RIA) [19], high-performance liquid chromatography

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(HPLC) [20–25], gas chromatography–mass spectrometry (GC–MS) [26] and HPLC–MS [27] have been developed for the measurement of serum cortisol. The specificity of the methods used has not always been adequate, as reviewed recently [24]. Both normal-phase and reversed-phase HPLC with either liquid–liquid or solid-phase extraction procedures have been used for the analysis of these steroids. There are quite a few reports on the simultaneous determination of cortisol and cortisone in serum with the HPLC technique [26,28–33]. Unfortunately, many of them require tedious sample pretreatment procedures. Cortisone has been measured with a sensitive RIA [4]. In earlier reports HPLC has been used for separating the steroids before quantification by RIA [34]. However, many RIAs used in clinical laboratories are not specific for cortisol, and the result can be misleading when the patient has abnormalities of cortisol metabolism, renal failure [35] or is on corticoid therapy [36]. More specific HPLC assays for cortisol have not gained wide acceptance in clinical laboratories, partly because the current HPLC detectors have not been sensitive enough as compared with RIA. Noma *et al.* [33] recently described an automated direct assay for measuring estetrol, estriol, cortisone and cortisol in serum and amniotic fluid (0.5–1.0 ml) using column-switching techniques and compared the results with RIA. The correlations between the two techniques were found to be significant for all the compounds measured, and the reproducibility with low coefficients of variation (C.V.s) was excellent. Wade and Haegele [37] used an extraction column mounted in place of the sample loop of a conventional injection valve to concentrate a sample with special emphasis upon the glucocorticoid hormones cortisol and corticosterone and were able to measure routinely as little as 300 pg of corticosterone per rabbit serum sample with 5% C.V. Recently, a thermospray LC–MS assay has been developed for glucocorticoids including cortisol and cortisone [38]. The structural confirmation of compounds can be established with this technique and without the derivatization of steroids.

With the method presented in this paper, total serum cortisol and cortisone can be analysed simultaneously with specificity using a sensitive diode-array spectrophotometric analysis. The present simple and low-cost reversed-phase procedure is easily maintained in any laboratory provided with the HPLC equipment.

EXPERIMENTAL

Apparatus

The HPLC system consisted of an M600 E pump, a 991 photodiode-array detector and a Rheodyne manual injector from Waters (Milford, MA, USA) equipped with a Beckman Ultrasphere ODS column (150 mm × 4.6 I.D., particle size 5 μm) from Beckman (San Ramon, CA, USA) protected by a precolumn filter with a 2-μm frit (Upchurch Scientific, Oak Harbor, WA, USA).

Chromatography

The steroids were eluted with two solvent systems. Mobile phase 1 was methanol–tetrahydrofuran–water (25.5:9.0:65.5, v/v/v) and mobile phase 2 was methanol–acetonitrile–58 m*M* sodium dihydrogenphosphate containing 6 m*M* heptanesulphonic acid (4:22:74, v/v/v). The flow-rate of mobile phase 1 was 1.0 ml/min and of mobile phase 2 was 1.5 ml/min at ambient temperature. The UV–VIS detection was in the range 190–800 nm. An aliquot of 40 μl of the total sample volume (80 μl) was injected. The steroids were quantified in the biological samples by comparing the results with a standard curve based on the ratios of the cortisol and cortisone peak heights to the peak height of the internal standard.

Chemicals and reagents

Aldosterone [(11β)-11,21-dihydroxy-3,20-dioxopregn-4-en-18-al], 1-dehydroaldosterone (1,4-pregnadien-18-al-11β,21-diol-3,20-dione), 17-isoaldosterone (17β-pregn-4-en-18-al-11β,21-diol-3,20-dione), 18-hydroxycorticosterone (11β, 18,21-trihydroxy-4-pregnene-3,20-dione), prednisone (17α,21-dihydroxy-1,4-pregnadiene-3,11, 20-trione), androsten-16α-ol-3,17-dione, cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-

trione), prednisolone (1,4-pregnadiene-11β,17α, 21-triol-3,20-dione), cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), 4-androstene-11β-ol-3,17-dione, 18-hydroxy-11-deoxycorticosterone (18,21-dihydroxypregnene-3,20-dione), fluprednisolone (6-fluoro-11,17,21-trihydroxypregna-1,4-diene-3,20-dione), meprednisone (17,21-dihydroxy-16β-methylpregna-1,4-diene-3,11,20-trione), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), 21-deoxycortisol (4-pregnene-11β,17α-diol-3,20-dione), betamethasone (9α-fluoro-16β-methyl-11β,17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione), 11-deoxycortisol (17α,21-dihydroxy-4-pregnene-3,20-dione), methylprednisolone (11,17,21-trihydroxy-6-methyl-1,4-pregnadiene-3,20-dione), dexamethasone (9α-fluoro-16α-methyl-11β,17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione), 4-androsten-4-ol-3,17-dione 4-methyl ether, 4-androsten-4-ol-3,17-dione acetate, androstenedione (4-androstene-3,17-dione), paramethasone (6α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione), fluoromethalone (6α,11β)-9-fluoro-11,17-dihydroxy-6-methylpregna-1,4-diene-3,20-dione), fluandrenolide, 4-androsten-4-ol-3,17-dione, 5α-androst-1-ene-3,17-dione, pregnenolone (3-hydroxypregn-5-en-20-one), fluocinolone acetonide {6,9-difluoro-11,21-dihydroxy-16,17-[(1-methylethylidene)bis(oxy)]-pregna-1,4-diene-3,20-dione}, medrysone (11-hydroxy-6-methyl-pregn-4-ene-3,20-dione), nivazolol and tralonide were purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) was from Orion (Helsinki, Finland). Tetrahydrofuran and diethyl ether (p.a.) were from Merck (Darmstadt, Germany). Water was taken from a Milli-Q water purification system (Millipore, MA, USA). Sodium phosphate monobasic was from Mallinckrodt (St. Louis, MO, USA) and heptanesulphonic acid sodium salt monohydrate from Fluka Chemie (Buchs, Switzerland). For the cortisol determinaton by RIA the Farmos Diagnostica cortisol radioimmunoassay kit (Farmos RIA) was used (Farmos Group, Turku, Finland).

Steroid stock solutions were prepared separately at concentrations of about 400 mg/l in absolute ethanol. All stock calibration standards were stable at 4°C over a year. A working standard solution, containing 1–3 mg/l, was diluted from a stock solution with water and was found to be stable for at least two weeks at 4°C.

Sample preparation

A 50-μl volume of glacial acetic acid and 250 ng of the internal standard were added to 1.0 ml of the serum sample in the Ultrafree-PF filter unit (10000 M_r cut-off, Nihon Millipore Kogyo K.K). The filter unit was capped, shaken carefully, and air was applied with a 20-ml syringe to get pressure (about 2 bar). The sample was left to filtrate overnight at 4°C in a glass conical tube. After filtration 4 ml of diethyl ether were added. The sample tube was mixed for 5 min. The organic layer was removed with a 5-ml Finnpipet. The diethyl ether extraction was repeated, and the combined organic extract was evaporated to dryness under nitrogen at 40–50°C. The residue was dissolved in 80 μl of the mobile phase, of which 40 μl were injected into the HPLC system.

RESULTS

Chromatographic separation of cortisol and cortisone by HPLC

Cortisol, cortisone and the internal standard (meprednisone) were separated from the endogenous serum components. In Fig. 1, a standard mixture of nine steroids is demonstrated in the systems described. With mobile phase 1, methanol–tetrahydrofuran–water (25.5:9.0:65.5, v/v/v) (Fig. 1a), cortisone and cortisol were eluted with retention times of 9.7 and 13.2 min, respectively, while with mobile phase 2, methanol–acetonitrile–58 m*M* sodium dihydrogenphosphate containing 6 m*M* heptanesulphonic acid (4:22:74, v/v/v) (Fig. 1b), the retention times were 9.7 and 9.1 min, respectively. In Fig. 2, the patient sample containing 500 nmol/l cortisol is analysed for peak purity at three wavelengths (221, 245 and 260 nm).

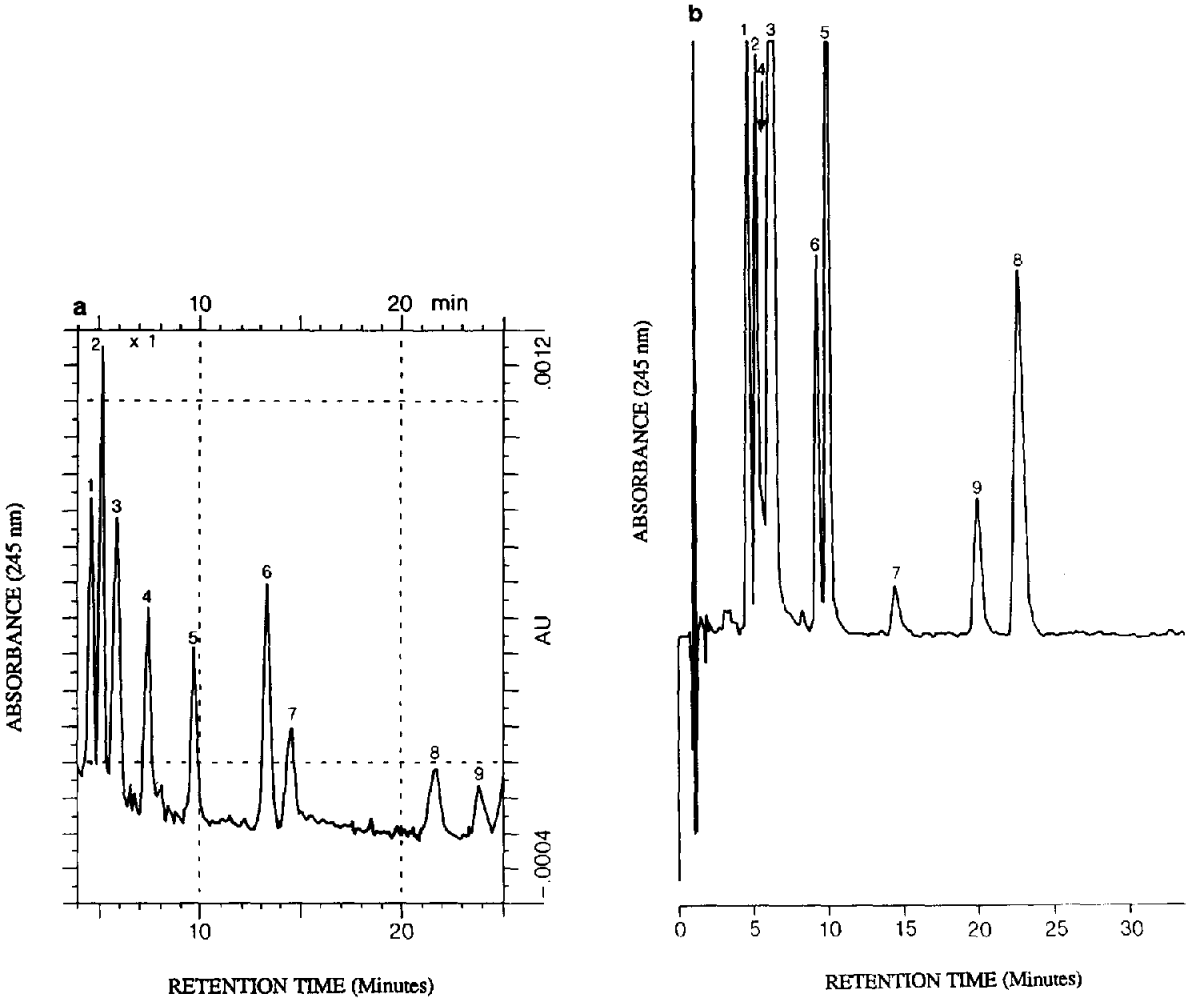

Fig. 1. (a) Chromatogram of a standard solution containing 1-dehydroaldosterone (1), 17-isoaldosterone (2), aldosterone (3), 18-hydroxycorticosterone (4), cortisone (5), cortisol (6), 18-hydroxy-11-deoxycorticosterone (7), corticosterone (8), and 21-deoxycortisol (9) using mobile phase 1 (methanol–tetrahydrofuran–water, 25.5:9.0:65.5, v/v/v), flow-rate 1.0 ml/min, detection at 245 nm. Amounts injected: 46 ng (1), 50 ng (2), 40 ng (3), 40 ng (4), 21 ng (5) 48 ng (6), 20 ng (7), 12 ng (8), and 19 ng (9). (b) Chromatogram of a standard solution containing the same steroids as in (a) with mobile phase 2 (methanol–acetonitrile–58 m*M* sodium dihydrogenphosphate containing 6 m*M* heptanesulphonic acid, 4:22:74, v/v/v), flow-rate 1.5 ml/min, detection at 245 nm with a Spectroflow 757 absorbance detector. Amounts injected: 10 ng (1), 11 ng (2), 50 ng (3), 25 ng (4), 26 ng (5), 13 ng (6), 5 ng (7), 15 ng (8), and 8 ng (9).

Precision

The intra-assay precision of the method (with mobile phase 1) was estimated by assaying twenty samples of spiked human serum at four cortisol levels over a concentration range of 215–861 nmol/l and over a range of 14–721 nmol/l for cortisone. The C.V.s were less than 5.1 and 5.7%, respectively. The inter-assay precision of the HPLC method was evaluated using Lyphochek immunoassay control serum of medium level in the analytical range (level 2) for cortisol and a pooled serum sample for cortisone (70 nmol/l) during a period of one month (n = 10). The C.V.s were 4.2 and 5.4%, respectively.

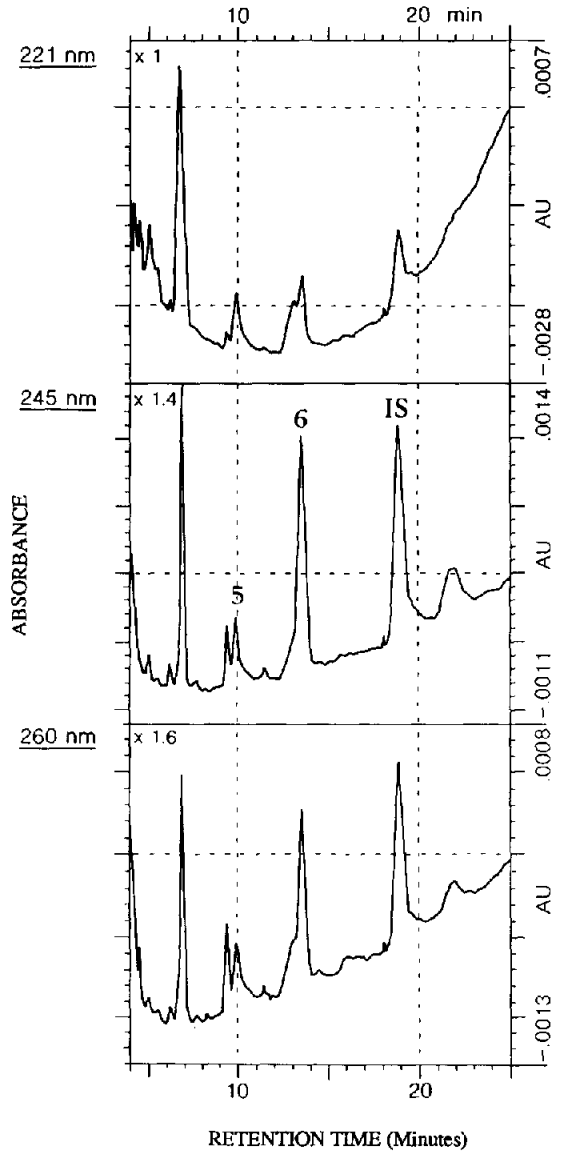

Fig. 2. Patient sample containing 500 nmol/l cortisol analysed simultaneously at three wavelengths (221, 245 and 260 nm). Internal standard (IS) = meprednisone (250 ng/ml).

Recovery

Known amounts of cortisol in the concentration range 10–500 ng/ml were added to a serum specimen containing 12 ng/ml cortisol. A constant amount of the internal standard (250 ng) was added to each sample and processed as described under *Sample preparation*. The mean recoveries at different concentrations ranged from 90 to 95% in double determinations. All calculations were carried out on the basis of the peak-height ratios of the drug and the internal standard. Likewise, cortisone was added in the concentration range of 5.2–260 ng/ml to a serum specimen containing 10 ng/ml cortisone. The recoveries were more than 90% in double determinations.

Limit of detection and linearity

Minimal detectable amounts (signal-to-noise ratio = 3) for cortisol and cortisone were 500 ng/l if monitored at 0.001 a.u.f.s. at 245 nm. The standard curves were shown to be linear up to 2000 nmol/l for cortisol and up to 1000 nmol/l for cortisone.

Interference studies

Potential interference with other drugs and steroids was investigated by chromatographing each drug and steroid individually using mobile phase 1. Table I lists the relative retention times for the various steroids studied. Prednisolone, a commonly used synthetic glucocorticoid, was found to have the same retention time as cortisol when mobile phase 1 was used and could thus give falsely high results if present in the same sample.

Comparison of the HPLC method with Farmos RIA–cortisol

The standard calibration curve of the Farmos RIA standards measured by HPLC is shown in Fig. 3. Twenty serum samples were analysed by both HPLC and RIA. Fig. 4 shows the values obtained by the present method using mobile phase 1 and RIA. The correlation coefficient between the described liquid chromatographic and RIA method was 0.938 with a slope of 0.861 and a y-intercept of 31.2 nmol/l.

Lyphochek immunoassay control serum (Human) from Bio-Rad, levels 1, 2, and 3, were analysed by HPLC. The cortisol contents measured were in agreement with the values given for the Farmos RIA kit. The acceptable ranges for levels 1, 2 and 3 were 69.80–94.36, 382.9–519.0 and

TABLE I

INTERFERENCE STUDIES WITH MOBILE PHASE 1

Compound tested	Concentration (mg/l)	Relative retention time[a]
1-Dehydroaldosterone	2.28	0.25
17-Isoaldosterone	2.52	0.28
Aldosterone	2.00	0.32
18-Hydroxycorticosterone	2.00	0.40
Prednisone	10.00	0.48
Androsten-16α-ol-3,17-dione	2.00	0.49
Cortisone	1.04	0.53
Prednisolone	10.00	0.71
Cortisol	3.12	0.72
4-Androsten-11β-ol-3,17-dione	2.80	0.74
18-Hydroxy-11-deoxycorticosterone	1.00	0.78
Fluprednisolone	10.00	0.91
Meprednisone	10.00	1.00
Corticosterone	0.58	1.16
21-Deoxycortisol	0.96	1.29
Betamethasone	10.00	1.34
11-Deoxycortisol	1.52	1.36
Methylprednisolone	10.00	1.37
Dexamethasone	10.00	1.48
4-Androsten-4-ol-3,17-dione 4-methyl ether	10.00	1.50
4-Androsten-4-ol-3,17-dione acetate	10.00	1.52
4-Androstene-3,17-dione	1.50	1.69
Paramethasone	10.00	2.00
Deoxycorticosterone	0.68	2.35
Fluoromethalone	10.00	2.43
Fluandrenolide	10.00	2.49
4-Androsten-4-ol-3,17-dione	3.00	2.59
5α-Androst-1-ene-3,17-dione	5.20	2.90
Pregnenolone	10.00	>3.0
Fluocinolone acetonide	10.00	>3.0
Medrysone	10.00	>3.0
Nivazol	10.00	>3.0
Tralonide	10.00	>3.0

[a] Relative to the internal standard (meprednisone).

615.0–832.9 nmol/l, respectively. The corresponding values with the described method were 90.10, 473.1 and 792.0.

DISCUSSION

With the present assay it is possible to measure simultaneously serum cortisol and cortisone using reversed-phase HPLC with ultraviolet detection. The retention times of cortisone, cortisol and meprednisone (used as the internal standard) were 9.8, 13.2 and 18.9 min, respectively. In addition, pure standards of 1-dehydroaldosterone, 17-isoaldosterone, aldosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone, corticosterone and 21-deoxycortisol could also be detected spectrophotometrically. Cortisol and cortisone did not interfere with other steroids studied for interference except with prednisolone, which was co-eluted with cortisol when mobile

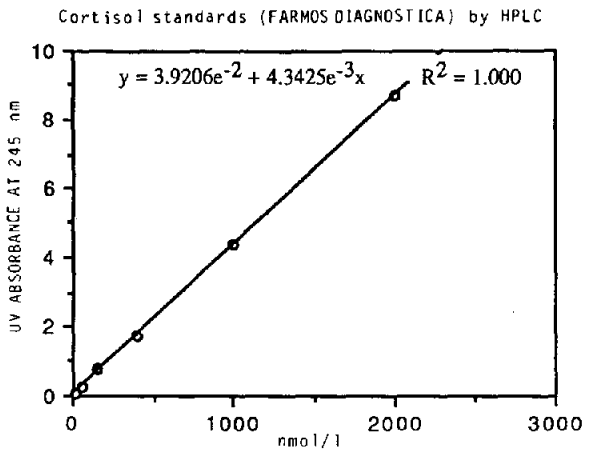

Fig. 3. Farmos RIA standards measured by the HPLC method.

phase 1 was used. By using mobile phase 2 it was possible to separate prednisolone and cortisol successfully. When using precolumn sulphuric acid–ethanol derivatization for serum cortisol, Nozaki *et al.* [39] found that synthetic steroidal drug (*e.g.* prednisolone) derivatives did not emit any fluorescence and did not interfere with the measurement of cortisol, but this method allowed no simultaneous determination of cortisol and cortisone. A typical chromatogram of a profile of the present 1-ml serum sample shows that the accurate and specific estimation of cortisol and cortisone can be difficult in the presence of closely related steroids (Fig. 1). Currently, RIA is the technique most widely used to determine individual glucocorticoids in human biofluids. However, cross-reactivity may be a serious problem in RIA methods depending on the antiserum used. For comparison, twenty serum samples assayed for cortisol by the Farmos RIA kit were analysed. The values obtained by HPLC were in good agreement with those by RIA (Fig. 4). However, when low concentrations of glucocorticoids are measured, the values obtained by HPLC were found to be more accurate than those obtained by RIA [40].

More than 90% of circulating cortisol is bound to proteins, of which about 85% is bound to corticosteroid-binding globulin (CBG) and 10% to albumin. CBG is also the high-affinity binding protein for cortisone, but with an association constant about ten times lower than that for cor-

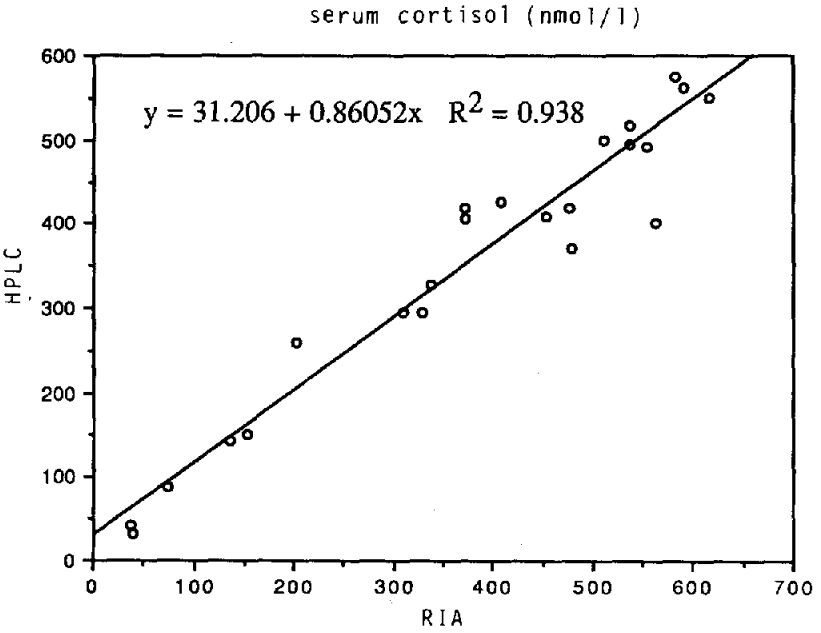

Fig. 4. Correlation between the values obtained by HPLC and RIA.

tisol. In the present method Ultrafree-PF filter units were used to remove macromolecular contaminants from the acidified 1-ml sample followed by an extraction and concentration with diethyl ether. The pH of the serum sample was reduced to around 4 with a minimal volume (50 μl) of glacial acid to minimize the interaction of cortisol and cortisone with CBG before the filtration [41].

In the present study the HPLC conditions were optimized by using two mobile phases to resolve cortisol (or cortisone) from endogenous compounds and synthetic drugs. Mobile phase 1 was chosen and modified according to previous studies by others [42]. By using mobile phase 2 the retention times of cortisone and the internal standard were almost unaffected, but cortisol was eluted more quickly, thus allowing successful separation of cortisol and cortisone from interfering compounds.

The limit of a determination of cortisol with UV detectors in the HPLC methods reported so far has generally been around 0.5 ng. The sensitivity of the HPLC method is dependent on the type of UV detector used and the performance of the column. Under the experimental conditions in the present assay, serum cortisol and cortisone concentrations as low as 500 ng/l could be measured. When a small amount of serum is used, most of the steroids at low concentrations cannot be detected with the monitoring systems commonly used. Hariharan *et al.* [43] recently reported a detection limit of 300 ng/l for both cortisol and corticosterone in 1 ml of plasma by a solid-phase extraction method, using a C_{18} minibore (100 mm × 2 mm I.D.) analytical column and UV detector with a 10-mm-pathlength flow cell (the same cell as used in the present study). They heated the plasma with the internal standard at 50°C for 10 min to free the CBG-bound cortisol. Similar low detection limits have been reported by others [40]. Wade and Haegele [44] used cyclodextrin media for solid phase extraction and a concentrating reversed-phase extraction column connected directly to an injector to analyse dilute samples. In the present study the serum filtrate (700–800 μl) was extracted with diethyl ether, which is easily evaporated. An on-line processing of the filtrate with a concentrating column could also improve the sensitivity of the present method. Underwood *et al.* [45] used only 0.5 ml of plasma for extraction with cold methylene chloride and could measure cortisone reliably. They obtained a recovery of more than 103%, while it was above 90% in the present study. The use of diode-array UV spectrometry in combination with thermospray LC–MS or atmospheric pressure ionization LC–MS has recently been suggested for the determination and structural elucidation of glucocorticoid steroids in plasma or serum [38,46]. These procedures have greatly extended the ease with which the LC–MS data can be aquired without the need for individual compound isolation.

To conclude, the described rapid HPLC method is a simple, reliable and reproducible method for separating and measuring cortisol and cortisone in a single serum sample with high specificity.

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