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DETERMINATION OF CORTISOL IN HUMAN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the measurement of cortisol in human plasma using 45% aqueous methanol eluent on a 120 mm × 4.5 mm I.D. Hypersil octadecylsilane column with UV detection at 239 nm after a simple dichloromethane extraction and evaporation with a prednisone internal standard.

The sample preparation time and chromatography time are each about 15 min and linear correlations have been obtained with plasma samples assayed by the Mattingly fluorimetric technique and a commercial-kit competitive protein binding method. Concentrations down to 30 nmol/l may be measured and the method can be used when fluorimetry is invalidated by interference, particularly from spironolactone.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) for the determination of plasma cortisol concentrations using normal-phase systems has been described by Trefz et al. [1] using silica columns and by Van den Berg et al. [2] with Nucleosil-NO_2 columns. Schwedt et al. [3] studied both normal- and reversed-phase systems for determining urinary free cortisol but favoured normal-phase adsorption chromatography. The simplicity and versatility of the reversed-phase system aqueous methanol with octadecylsilane (ODS)-silica makes this the most likely system to be available in clinical chemistry laboratories at present, and this paper describes its application to the measurement of plasma cortisol when the commonly used fluorimetric technique described by Mattingly [4] is invalidated by interfering substances, particularly spironolactone.

MATERIALS AND METHODS

Equipment

An Applied Chromatography Systems (Model 750/03) reciprocating pump was used with detection at 239 nm by a Cecil CE 212 variable wavelength

UV monitor fitted with an 8-μl chromatography cell. A 120 mm × 4.5 mm I.D. stainless-steel column was packed by a slurry technique with Hypersil ODS (Shandon, London, Great Britain). Injection was performed with a "Specac" loop injector fitted with a 72-μl loop.

Reagents

Dichloromethane and methanol, both HPLC grade, were obtained from Rathburn Chemicals, Walkerburn, Great Britain. The sodium hydroxide used was 0.25 *M* in water.

Standard cortisol was obtained from Sigma (St. Louis, Mo., U.S.A.). A stock solution of 18.1 mg/l (50 μmol/l) in ethanol was diluted 1:100 to a working standard of 181 μg/l (500 nmol/l) with 3% bovine serum albumin in water. Prednisone (Koch-Light, Colnbrook, Great Britain) was used as internal standard at a concentration of 2 mg/l (5.89 μmol/l) in 10% aqueous ethanol. The competitive protein binding (CPB) "Cortipac" Kits (Radiochemical Centre, Amersham, Great Britain) were used.

Procedure

1 ml plasma, 100 μl of internal standard (corresponding to 200 ng prednisone), 100 μl 0.25 *M* sodium hydroxide and 7 ml dichloromethane were vortexed for 30 sec in a stoppered tube and then centrifuged for approximately 5 min at 2000 r.p.m. (approximately 1300 *g*). A 5-ml aliquot of the organic layer was evaporated to dryness with a stream of air in a water bath at 40°. The residue was dissolved in 100 μl eluent, 72 μl of which was injected. The eluent system was 45% methanol in water at a flow-rate of 1 ml/min corresponding to a pressure drop of 100 bars. The ratios of peak heights of cortisol and prednisone in chromatograms from serum samples were compared with that obtained when 1 ml working standard (equivalent to 500 nmol/l cortisol) was similarly processed.

RESULTS

Fig. 1 shows a separation of six steroids chromatographed in this system. Cortisol is well separated from prednisone, corticosterone, dexamethasone, 11-deoxycortisol, and 17-hydroxyprogesterone but not from prednisolone. Spironolactone eluted more than 30 min after cortisol (Fig. 2). The steroid retention data are shown in Table I. A chromatogram of a normal plasma is shown in Fig. 3.

The recovery of prednisone and cortisol from a pooled plasma sample was calculated by chromatographing extracts before and after adding known amounts of prednisone and cortisol. There was a constant recovery of 86.1% for cortisol and 80.0% for prednisone. Recovery from water was found to be about 10% greater and therefore it was necessary to prepare the standard cortisol solution in a protein matrix; 3% bovine serum albumin (BSA) was sufficient and convenient. Vortex mixing for 30 sec did not produce emulsions; it was compared with extraction by gentle rotation for 15 min and found to be equally efficient.

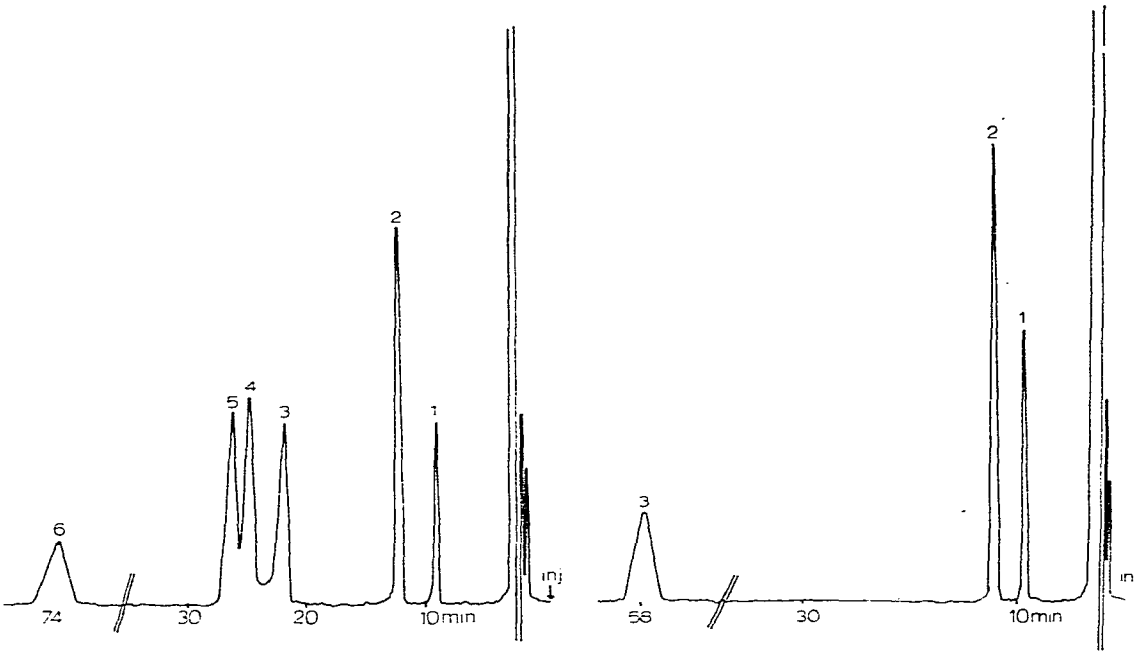

Fig. 1. Separation of a standard steroid mixture. Peaks: 1 = prednisone; 2 = cortisol; 3 = corticosterone; 4 = dexamethasone; 5 = 11-deoxycortisol; 6 = 17-hydroxyprogesterone. Column, Hypersil ODS 120 × 4.5 mm I.D.; eluent, methanol—water (45:55); flow-rate, 1 ml/min; sample injection, 72-µl sample loop. Detection at 239 nm.

Fig. 2. Separation of spironolactone from cortisol. Peaks: 1 = prednisone; 2 = cortisol; 3 = spironolactone. Chromatography as in Fig. 1.

Standard solutions in 3% BSA were assayed in duplicate over the range 62—2000 nmol/l; the response was linear; the average standard deviation of the duplicates over the whole range was 6.76 nmol/l (CV 4.4%). A blank extract of the 3% BSA showed no significant interference (Fig. 4). Six replicate analyses of the same sample in a single run gave values 511 ± 10.6 (S.D.) nmol/l

TABLE I

STEROID RETENTION DATA FOR FIG. 1

Compound	Capacity factor (k')	Retention relative to cortisol
Prednisone	7.7	0.72
Cortisol	10.7	1.00
Prednisolone	11.0	1.03
Corticosterone	19.7	1.85
Dexamethasone	22.0	2.06
11-Deoxycortisol	23.7	2.23
Spironolactone	46.0	4.35
17-Hydroxyprogesterone	68.0	6.10

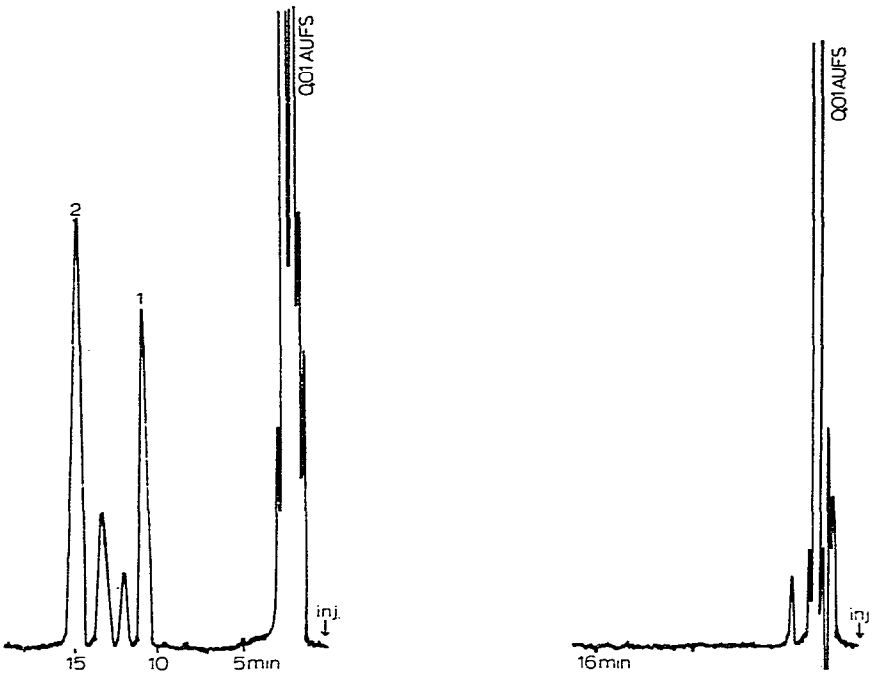

Fig. 3. Chromatography of a normal plasma extract. Peaks: 1 = prednisone; 2 = cortisol. Chromatography as in Fig. 1.

Fig. 4. Blank extraction of 3% bovine serum albumin.

(CV 2.1%). The same sample analysed on five different days gave a between batch CV of 3.6%.

In 41 random laboratory samples the results obtained by HPLC were compared with those obtained by the Mattingly method; the correlation is shown in Fig. 5 (r = 0.8792). A competitive protein binding method was compared for 17 samples and this correlation is shown in Fig. 6 (r = 0.8918).

A sample can be prepared for chromatography in 20 min; uncontaminated samples can be injected at 20-min intervals.

DISCUSSION

Fluorimetric procedures are commonly employed for routine cortisol analysis but they lack specificity and the administration of fluorogenic drugs, particularly spironolactone, invalidates the results. Competitive protein binding has improved specificity but several naturally occurring steroids possess a high affinity for the corticosteroid binding globulin and may disturb the determination in newborns, pregnancy and the adrenogenital syndrome. Radioimmunoassay procedures have similar problems.

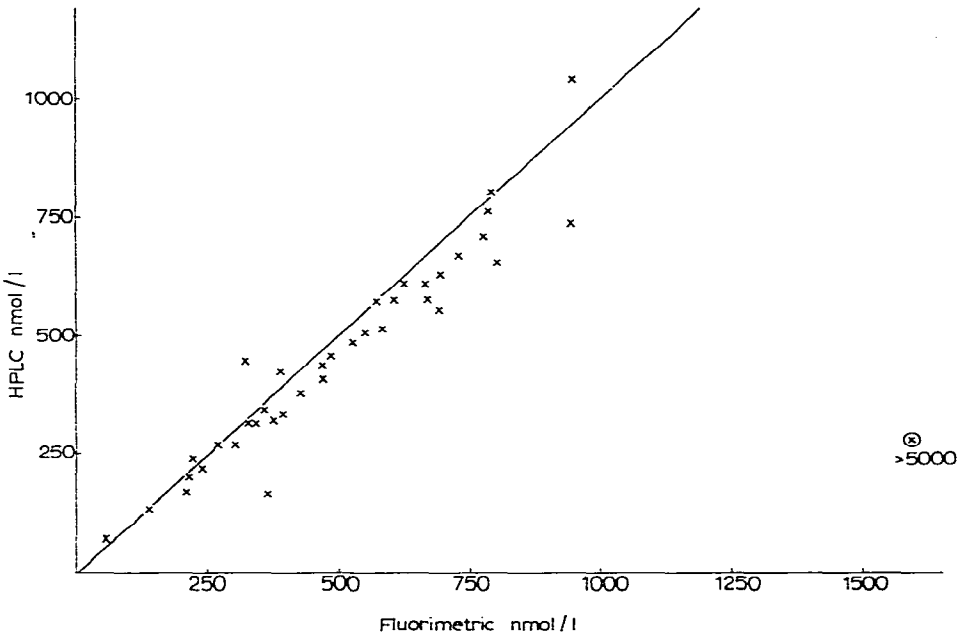

Fig. 5. Scattergraph of HPLC method vs. fluorimetry (Mattingly). The line shown is $x = y$. $r = 0.8792$; $y = 0.88\ x + 18$ (where y = HPLC). ⊛ = plasma from patient receiving spironolactone.

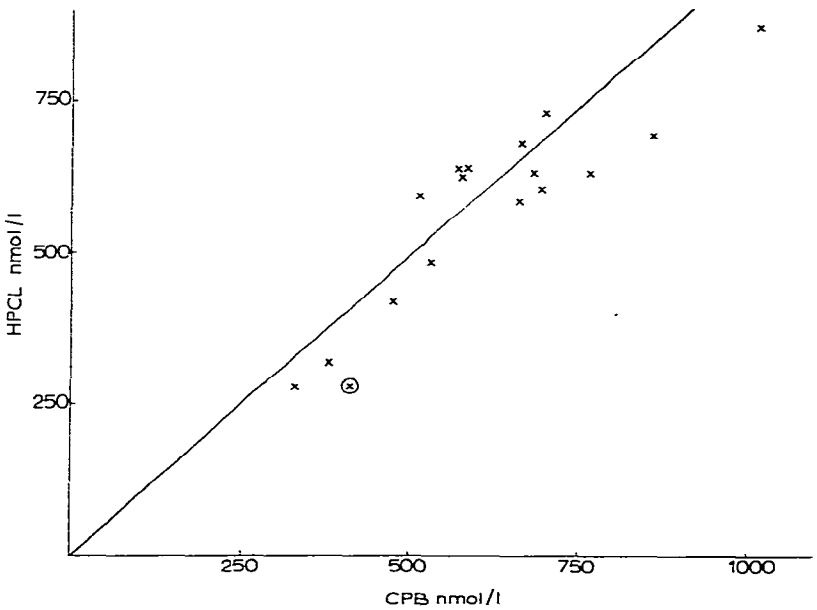

Fig. 6. Scattergraph of HPLC method vs. CPB (Cortipac). The line shown is $x = y$. $r = 0.8918$; $y = 0.835\ x + 57.6$ (where y = HPLC). ⊛ = plasma from patient receiving spironolactone.

The method described in this paper allows the specific determination of cortisol in plasma when other methods are invalidated. An example of a patient on spironolactone is shown in Figs. 5 and 6 and HPLC has been used in this laboratory for the determination of cortisol on several patients receiving this drug. The good correlations shown in Figs. 5 and 6 on patients receiving a variety of hospital prescriptions indicates that other commonly used drugs do not interfere with the HPLC procedure.

The simple reversed-phase procedure described is one likely to be available in any laboratory practising HPLC and is cheap and easy to run and maintain. In particular, it avoids the commonly encountered frustrations of normal-phase systems which use fully or partly water saturated solvents. It has proved robust in use with fast analysis time and no significant problems of late eluting peaks. It is possible to perform at least 20 analyses by this method in one day and so it may be used for a small routine workload, except for patient receiving large doses of prednisone or prednisolone, when the steroid therapy would require to be substituted. In patients receiving small doses the usual request is for the short-term synacthen response which could be readily measured.

ACKNOWLEDGEMENT

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