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Note

High-performance liquid chromatographic estimation of cyproterone acetate in human plasma

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Cyproterone acetate (17α-hydroxy-6-chloro-1,2α-methylenepregn-4,6-diene-3,20-dione acetate, CPA) possesses antiandrogen and progestational properties [1]. CPA is used in the treatment of hirsutism [2], severe acne [3] and precocious puberty [4]. Although the pharmacokinetic [5] and pharmacological [6] aspects have been studied, there is a dearth of information concerning peripheral blood levels and clinical effects in patients treated with CPA. For this reason a selective, rapid assay was developed utilising high-performance liquid chromatography (HPLC). HPLC allows potentially clinically important metabolites to be measured, offering an advantage over a previously reported radioimmunoassay method [7].

MATERIALS AND METHODS

Chemicals and reagents

Cyproterone acetate (Schering, Sydney, Australia) was added to pooled human plasma to prepare plasma samples and standards. Ethyl acetate and hexane were of analytical grade and distilled before use. Methanol was HPLC grade (Waters Assoc., Milford, MA, U.S.A.) and chromatographic grade alumina was obtained from Merck (Darmstadt, G.F.R.). The internal standard starting material, 17α-hydroxyprogesterone, was obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The chromatographic equipment consisted of a high-performance liquid chromatograph (Tracor, Austin, TX, U.S.A., Model 985 LC master, Model 951

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pump) with a 100-μl valve injection loop and a variable-wavelength detector (Model 970A).

The column was a 300 × 3.9 mm octadecylsilane reversed-phase (μBondapak, 10 μm Waters Assoc.). Retention times and peak heights were measured with a recording integrator (Model SP4100, Spectra Physics, Santa Clara, CA, U.S.A.).

Chromatographic operating conditions

The detector was set at 282 nm, determined as λ_{max} by a stopped flow scan of cyproterone acetate standard. The molar extinction coefficients of cyproterone acetate and the internal standard in the eluting solvent measured at 282 nm are 17,700 and 16,600 respectively. The sensitivity was 0.030 a.u.f.s. The flow-rate was held constant at 2 ml/min and all measurements were at ambient temperatures. The eluting solvents, water and methanol, were filtered through a glass filter (pore size, 0.5 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum. The LC master was set to deliver a 70:30 ratio (by volume) of methanol to water.

Internal standard

The internal standard, 17α-hydroxypregn-4,6-diene-3,20-dione 17-butanoate, was synthesized from 17α-hydroxyprogesterone using conventional methods [8, 9].

17α-Hydroxyprogesterone was esterified with butanoic anhydride and pyridine and the reaction monitored by high-performance liquid chromatography (HPLC) for completion. The product, after purification on silica gel with hexane—diethyl ether mixtures as eluent, was treated with chloranil in *tert.*-butanol to give the internal standard. The overall yield following purification on neutral alumina (hexane—diethyl ether mixtures as eluent) was 37%.

A stock internal standard solution of 250 mg/l was prepared in methanol and diluted to 12.5 mg/l.

Extraction procedure

Spiked plasma or patient sample (0.5 ml) was mixed with 0.5 ml sodium hydroxide (0.25 *M*), 100 μl of internal standard and 10 ml of ethyl acetate in an assay tube (Pyrex tube, 150 × 20 mm with PTFE-lined screw cap). After shaking (5 min) and centrifuging (5 min at 900 *g*), the organic layer was transferred to a 100 × 24 mm pyrex tube and the solvent removed in vacuo at 40°C. The residue was chromatographed on 0.5 g silica gel with 4 ml of 5% (v/v) ethyl acetate—hexane followed by 5 ml of ethyl acetate. The ethyl acetate fraction was evaporated in vacuo at 40°C, the residue dissolved in 100 μl of methanol and injected onto the column.

Stability

The effect of anticoagulants on the assay is shown in Table I. Each assay contained the same amount of CPA and each tube was assayed at the same time. Lithium heparin results agree closely with the non-extracted value and this anticoagulant was used for all patient samples.

The long-term stability of CPA with lithium heparin as an anticoagulant is

shown in Table II. Aliquots of a blood sample containing lithium heparin and spiked with CPA were centrifuged and plasma stored at −20°C at the times shown. The samples were assayed concurrently and showed no decomposition of CPA over a 24-h period.

TABLE I

EFFECT OF VARYING ANTICOAGULANT

Anticoagulant	Peak height ratio*
Theoretical	1.01
Plain tube	1.04
Lithium heparin	1.01
Oxalate	1.08
Oxalate/fluoride	1.06
EDTA	1.05

*Peak height of drug:peak height of internal standard.

TABLE II

LONG TERM EFFECT OF LITHIUM HEPARIN

Time (h)	Peak height ratio*
0	1.38
0.5	1.44
1	1.47
2	1.42
4	1.50
6	1.38
8	1.49
24	1.41

*Peak height of drug:peak height of internal standard.

RESULTS

Linearity of response and sensitivity

Pooled plasma was spiked with CPA from 0.1 μg/ml and assayed. Peak height ratio (peak height of CPA divided by peak height of internal standard) was used as the response. A least squares linear regression analysis was used to determine the slope, y-intercept, and correlation coefficient. The regression line obtained was $y = 1.735x - 0.012$ ($r = 0.999$). Concentrations as low as 0.02 μg/ml could be measured accurately. The lower limit of detection was 4 ng.

Precision and accuracy

The reproducibility of the assay was determined using spiked plasma pools. These results are given in Table III. Recoveries were calculated using plasma

pools containing 2 μg/ml and 0.5 μg/ml CPA (n = 6 for each). Recovery from the 2 μg/ml sample averaged 95.6% and 88.3% from the 0.5 μg/ml sample.

Selectivity

Interference in the assay by other drugs was studied by using either solutions of the drug or plasma samples from patients ingesting the drug (Table IV). Medroxyprogesterone acetate and 17α-hydroxypregnenolone have similar retention volumes to CPA but absorption of these compounds at 282 nm is negligible.

A typical chromatogram of extracted human plasma spiked with CPA and internal standard is shown in Fig. 1. The retention times for the assay conditions are 5.3 min and 7.5 min with baseline separation. A pooled plasma sample without CPA or internal standard shows no interfering compounds.

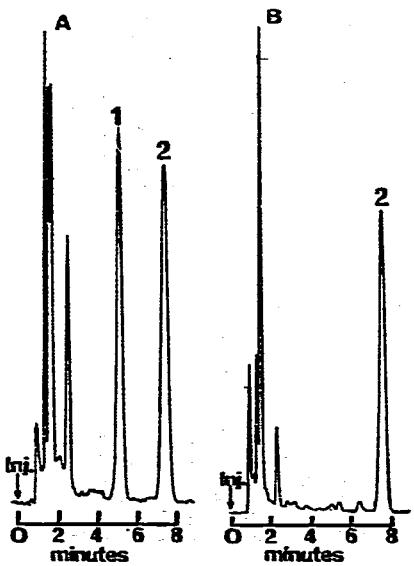

Fig. 1. (A) Chromatogram of extracted pooled plasma spiked with CPA 2.1 μg/ml (1), and internal standard (2). (B) Chromatogram of extracted blank plasma containing only internal standard (2); 0.03 a.u.f.s.

DISCUSSION

An internal standard with a larger k' value than CPA was synthesized to achieve baseline separation and an absorption maximum with close correspondence to that of CPA. The butanoate ester of 17α-hydroxypregn-4,6-diene-3,20-dione used as internal standard was separated from all endogenous plasma components and drugs tested for specificity of the assay.

CPA levels have been measured by radioimmunoassay in plasma of patients following administration of a contraceptive containing 2 mg of CPA [10]. In the latter study the maximum CPA concentration of 11.0 ± 3.4 ng/ml plasma was observed 1.6 ± 0.6 h after a single administration. We have used the HPLC

TABLE III

PRECISION OF ASSAY FOR CPA

	Intra-assay* (n = 10)			Inter-assay** (n = 10)		
Theoretical	0.55	1.11	2.21	0.55	1.11	2.21
Mean ± S.D. (μg/ml)	0.53 ± 0.015	1.06 ± 0.028	2.19 ± 0.05	0.54 ± 0.021	1.11 ± 0.039	2.14 ± 0.074
C.V. (%)	2.74	2.65	2.30	3.80	3.50	3.50

*Within-assay precision.
**Between-assay precision.

TABLE IV

INTERFERENCE IN THE ASSAY BY OTHER DRUGS

Tablet preparations and pure drugs tested		Plasma from patients ingesting these drugs tested	
Androstenedione	17α-Hydroxyprogesterone	Acetylsalicylic acid	Oxazepam
Cortisol	Medroxyprogesterone acetate	Chlorothiazide	Paracetamol
Cortisone	Methylprednisolone	Chloral hydrate	Phenytoin
Dehydroisoandrosterone	Prednisolone	Digoxin	Propranolol
Dexamethasone	Prednisone	Erythromycin	Pseudoephedrine
17β-Estradiol	Progesterone	Hydralazine hydrochloride	Salbutamol
Estriol	Spironolactone	Isoniazid	Theophylline
Ethinyl estradiol	Testosterone	Nitrazepam	Thiamine
17α-Hydroxypregnenolone			

assay to measure CPA in patients with precocious puberty treated with 75—200 mg daily (Table V).

The HPLC assay is rapid and selective. Each assay requires about 30 min from receipt of the plasma sample. It is linear to 4 μg/ml and recoveries average between 88—96%. The assay is being adapted to measure metabolites of CPA in plasma.

TABLE V

ASSAYS OF PATIENT SAMPLES

Random samples were used.

Patient	CPA (μg/ml)	Daily dose (mg)
A	0.59	75
B	0.52	125
B	0.74	125
B	0.63	125
B	0.66	200
B	0.62	200
B	0.97	200

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