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15-HYDROXYCYPROTERONE ACETATE AND CYPROTERONE ACETATE LEVELS IN PLASMA AND URINE

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SUMMARY

15β-Hydroxycyproterone acetate (15HOCPA) and cyproterone acetate (CPA) have been quantitated in human plasma and urine by a selective high-performance liquid chromatographic assay. The levels of 15HOCPA in plasma are generally twice those of its precursor CPA, although both compounds appear to have similar clearance rates. Approximately 6% of the dose is excreted into the urine, predominantly (> 90%) as the free form of 15HOCPA and CPA.

INTRODUCTION

Cyproterone acetate (CPA) is a synthetic steroid, whose efficacy as an antiandrogen in the treatment of acne and hirsutism is well documented [1]. Some controversy exists, however, whether CPA or one of its metabolites is the active compound [2—4]. The main evidence for the latter inference is based either on the lack of CPA activity when applied topically [2, 3] or the increase in patient responders when CPA is administered intramuscularly [4].

The major metabolite of CPA, in both plasma and urine, has been identified as the 15β-hydroxy analogue (15HOCPA) [5]. While assays for plasma CPA based on either radioimmunoassay (RIA) or high-performance liquid chromatography (HPLC) have been reported [6, 7], no assays for 15HOCPA have been published.

The significance of 15HOCPA is that while its antiandrogen properties are comparable to CPA, its progestational effect is considerably less [3, 8]. These results infer that 15HOCPA is potentially better suited than CPA for the treatment of androgen-induced problems in both women and men.

In this paper we describe a HPLC assay which allows quantitation of both

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CPA and HOCPA in either plasma or urine. Application of this assay allows the accurate simultaneous assessment of CPA and 15HOCPA levels.

EXPERIMENTAL

Chemicals

Cyproterone acetate and cyproterone (CP) were donated by Professor F. Neuman and 15β-hydroxycyproterone acetate by Professor I.D. Cooke.

All solvents were of the highest analytical grade and were redistilled prior to use. Organic extracts were dried using anhydrous sodium sulphate.

Thin-layer chromatography (TLC) was performed on Eastman precoated silica-gel sheets, in chloroform as solvent.

Synthesis of cyproterone propionate

A heterogenous mixture of cyproterone (1 g, 2.6 mmol) and Amberlite IR 120 resin (2 g) was stirred in redistilled propionic anhydride (5 ml) at 50°C for three days. The mixture was then poured into water, stirred for a further 1 h and then extracted with diethyl ether (20 ml three times). The ethereal extract was then washed with saturated sodium bicarbonate solution, water, brine and then dried. The solvent was removed using a rotary evaporator to leave a solid which was then chromatographed on a silica column.

Elution with diethyl ether—hexane (3:5, v/v) and recrystallization of the solid from diethyl ether gave cypropterone propionate, CPP (513 mg, 47%) as a pale yellow solid, m.p. 193—194°C. The product was homogenous by HPLC, TLC and had expected spectroscopic properties i.e. UV, IR and mass spectrometric (MS).

High-performance liquid chromatography

A Waters instrument was used (Waters Assoc., Milford, MA, U.S.A.) fitted with a univeral liquid injector and a variable-wavelength UV detector, set at 282 nm.

The column (300 × 3.9 mm) was an octadecylsilane reversed-phase (μBondapak, 10 μm), operated at ambient temperature, using acetonitrile—water (65:35, v/v) as the eluting solvent mixture.

Extraction procedure

Patient plasma (0.25—0.5 ml) or urine (0.5 ml) was added to the internal standard, CPP, (100 ng for plasma, 300 ng for urine) and the mixture sonicated for 30 min. Diethyl ether was then added (5 ml) and the mixture was shaken for 30 min, the ether extract was then separated and washed with aqueous sodium hydroxide (1 ml, 0.25 *M*), water (1 ml) and then dried. The ether extract was then transferred to another tube and the sodium sulphate washed with a further aliquot of diethyl ether (2.5 ml).

The combined ether extracts were evaporated to dryness at ambient temperature and then reconstituted in diethyl ether (1 ml) prior to Florisil column chromatography (300 mg). The column was developed with *n*-heptane (5 ml), diethyl ether (5 ml) and the required fraction then eluted with methanol—diethyl ether (1:10, v/v, 5 ml). The latter fraction was evaporated

to dryness, reconstituted in the HPLC solvent (0.3 ml) and a portion (0.015 ml) injected onto the column.

Quantitation was based on peak height measurements with respect to the standard curves. The relative retention times of the compounds are for 15HOCPA 0.41, CP 0.56, CPA 0.72 and CPP 1.00.

Standard curves

For each assay blank plasma or urine (i.e. no CPA therapy) was used to establish the response between CPA, 15HOCPA and CPP. Amounts of CPA and 15HOCPA added comprised of two ranges: (a) 10, 20, 30, 40, 50, 60, 70 and 80 ng; and (b) 30, 50, 100, 150 and 200 ng. Work-up was identical to that specified above for patient samples.

Radioimmunoassay of CPA

RIA was performed at the Institute of Medical and Veterinary Science (Adelaide, Australia) using an antibody to CPA supplied by Schering. Full details of the methodology have been previously published by Nieuweboer and Lubke [6].

Urinary conjugates of CPA and 15HOCPA

The urine sample was first extracted to quantitate the free fraction. The urine, which then only contained conjugates, was split into two equal fractions. One fraction was subjected to sulphate hydrolysis, while the other was treated with a glucuronidase enzyme [9]. Each sample was then processed in the usual manner.

Verification of 15HOCPA in patient samples

15HOCPA was converted to the respective 15-β-acetate (15ACCPA) by the standard acetic anhydride—pyridine procedure. The synthesised 15ACCPA had R_F 0.38 compared to 0.27 for 15HOCPA on TLC, and a retention time 0.75 min longer on HPLC.

Several plasma samples were processed in the normal manner and the final extracts subjected to preparative HPLC with respect to the peak assigned as 15HOCPA. Acetylation of the isolated compound gave a single major component with identical properties to 15ACCPA on both TLC and HPLC.

Other plasma samples were extracted and then the final solution prior to HPLC partitioned. One aliquot was chromatographed as the control. The other aliquot was acetylated and then subjected to HPLC. The retention time of the peak attributed to 15HOCPA in the control was absent after acetylation, with a new peak appearing at the retention time associated with 15ACCPA.

Patient samples

In this institution plasma and urine samples were collected from women undergoing CPA therapy on the reverse sequential regimen of CPA (100 mg, days 5—15) and ethinyl estradiol (0.05 mg, days 5—25). Samples were taken on different days of the cycle, however, the length of time each individual patient had been on treatment was not constant.

The samples used for the RIA versus HPLC comparison were supplied by

IMVS. These represented a number of varied regimens, including both continuous and intermittent treatment, as well as the standard reverse sequential course.

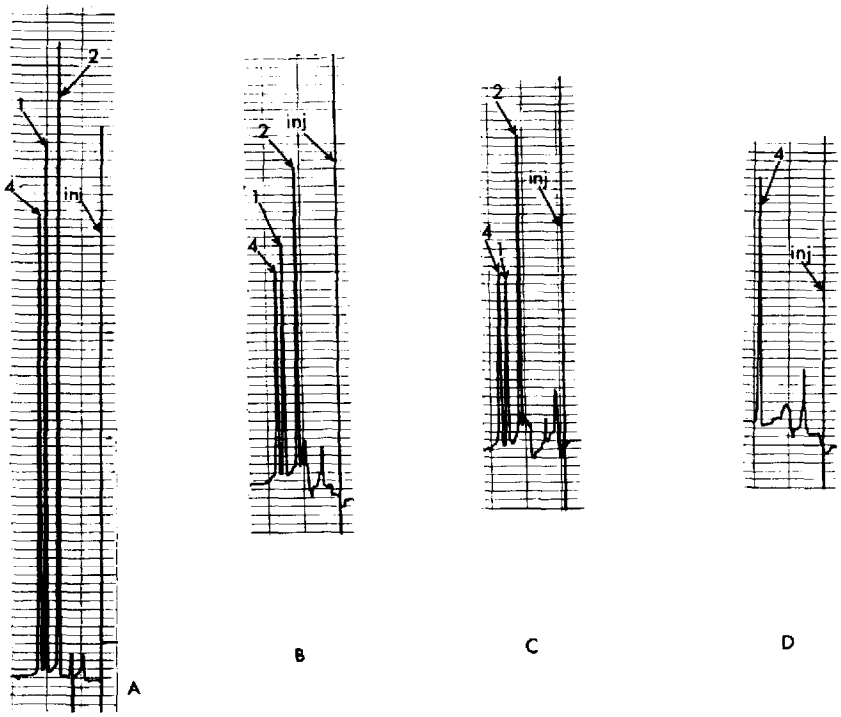

Fig. 1. HPLC profiles of (A) standards; (B) actual plasma sample; (C) previous sample spiked with authentic 15HOCPA; (D) plasma sample from subject not on CPA therapy. Peaks: 1 = CPA; 2 = 15HOCPA; and 4 = CPP.

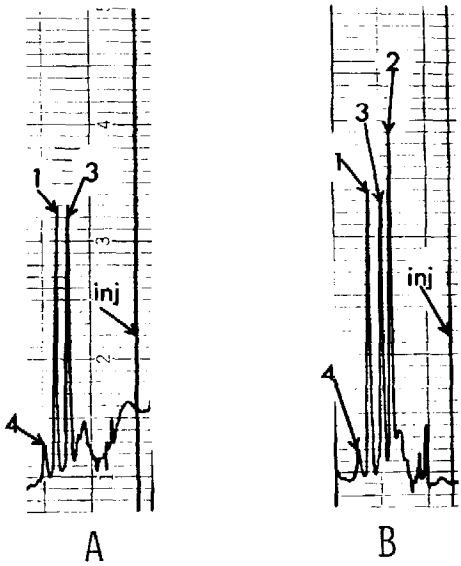

Fig. 2. HPLC profiles of (A) CPA plasma sample after acetylation; and (B) same sample spiked with 15HOCPA. Peaks: 1 = CPA; 2 = 15HOCPA; 3 = 15ACCPA; and 4 = CPP.

RESULTS

Initial experiments showed that simple extraction of CPA and 15HOCPA from plasma resulted in many interfering peaks in the HPLC trace. Consequently, a step involving Florisil chromatography was added. Fig. 1 shows typical

TABLE I

INTER-ASSAY PRECISION OF HPLC METHOD

Units are nmol/l and nmol/ml for plasma and urine, respectively.

Sample	*n*	CPA			15HOCPA		
		Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Plasma	6	111	11.9	11	241	45.5	19
Plasma	6	290	16.9	6	771	169.0	21
Plasma	3	472	38.0	8	349	40.0	11
Plasma	8	900	158.0	17	2695	515.0	19
Urine	3	1.71	0.28	16	5.99	0.84	14

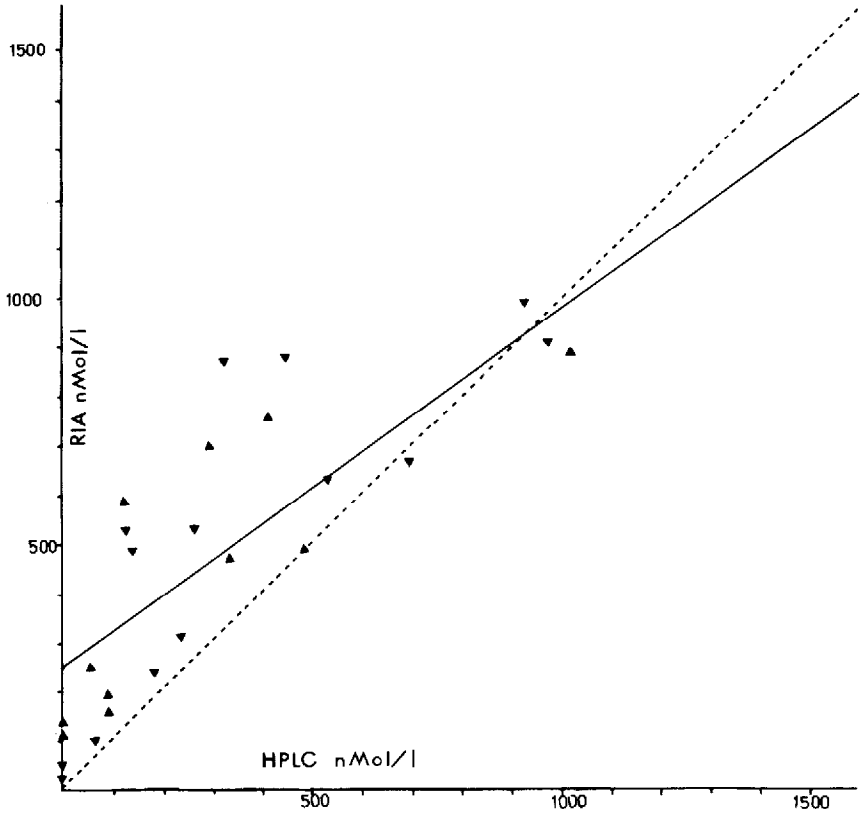

Fig. 3. Comparison of CPA values measured on the same plasma sample by RIA and HPLC. The dashed line represents the optimum 1:1 response slope. Intercept = 252.8; slope = 0.775; $r = 0.81$; and $S_{yx} = 177.2$.

HPLC traces for the procedure described in Experimental. Identity of the two analytes CPA and 15HOCPA was not only confirmed by spiking with authentic samples, but also by collection and chemical derivatisation for 15HOCPA (see Experimental and Fig. 2).

To obviate any problems associated with recovery from plasma or urine a homologous analogue of CPA was synthesised as the internal standard. Other experiments established that the absolute recoveries for CPA and 15HOCPA through the whole procedure were 78% and 84%, respectively. Standard curves of CPA versus CPP and 15HOCPA versus CPP were linear with correlation coefficients > 0.99 in all cases. The minimum detectable concentration was approximately 20—40 nmol/l for both CPA and 15HOCPA.

The precision of the HPLC method was assessed by repeated measurements on different patient samples (Table I). Comparison of CPA values by RIA and HPLC are shown in Fig. 3. The results indicate the existence of both proportional (slope) and constant (intercept) errors between the assays.

The levels of 15HOCPA and CPA in the same plasma samples are shown in

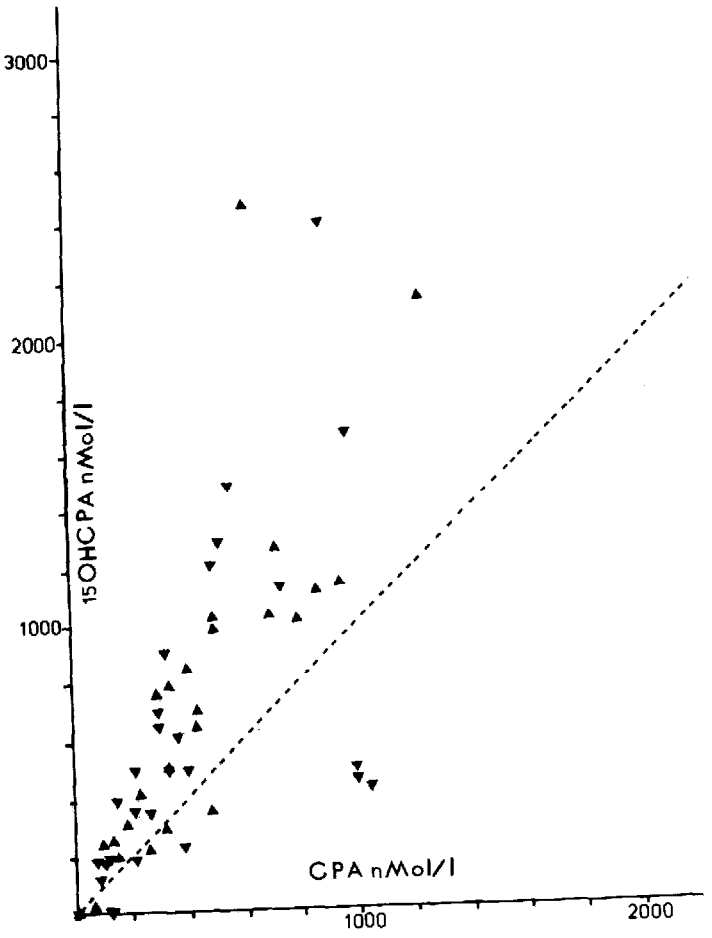

Fig. 4. Comparison of CPA and 15HOCPA values in the same plasma sample as measured by HPLC. The dashed line represents equal molar values.

Fig. 4. Ratios of 15HOCPA/CPA ranged from 0.38 to 3.2, with a median value of 1.85. No relationship was apparent between the 15HOCPA/CPA ratio and the day of the cycle (5—15) on which the medication was taken. Similarly, absolute values of CPA and 15HOCPA were not clearly related to the number of times the drug regimen had been completed. Analysis of plasma samples taken on day 5, prior to the commencement of a new course, showed negligible levels of CPA and 15HOCPA.

Analysis of the urinary results (Table II) shows that CPA and 15HOCPA are excreted almost exclusively in the free form. The relative amount of 15HOCPA to CPA (7.3, 4.3 and 3.9), however, appears more elevated than in plasma. This high ratio of 15HOCPA/CPA is diminished for the urinary conjugates. These results imply that the higher excretion of free 15HOCPA may be related to a greater water solubility than CPA.

TABLE II

URINARY EXCRETION OF CYPROTERONE ACETATE AND 15β-HYDROXYCYPROTERONE ACETATE

	Subject I	Subject II	Subject III
Free			
CPA (nmol/ml)	2.31	3.13	1.71
15HOCPA (nmol/ml)	17.0	13.4	6.63
Glucuronides			
CPA (nmol/ml)	0.48	0.62	n.m.*
15HOCPA (nmol/ml)	1.51	1.12	n.m.
Total (nmol/ml)	21.30	18.27	
Percentage free	90.7	90.5	
Volume (ml)	660	770	S**
24 h Total (μmol)	14.06	14.06	
Percentage 100 mg dose	5.92	5.92	

*n.m. = value not measured.
**S = sample not a 24-h sample.

DISCUSSION

The HPLC assay described herein is shown to allow reliable quantitation of CPA and 15HOCPA in both plasma and urine. No assay has previously been published for 15HOCPA (see ref. 4). The advantages of HPLC assay, in comparison to RIA techniques, centre on the increased specificity and the ability to simultaneously measure more than one analyte.

The results of the present investigation, with respect to CPA plasma levels, are similar to those previously published [4]. Plasma levels of 15HOCPA were found to be approximately twice those of CPA (Fig. 4). The plasma clearance rate of both CPA and 15HOCPA, however, appears to be similar based on the following observations. First, negligible amounts of either CPA or 15HOCPA were present, prior to initiation of a new drug course. Secondly, the 15HOCPA/CPA ratio did not show any marked change between days 5 and 15 of the drug regimen.

Approximately 6% of the typical daily CPA dose (100 mg), was found to be excreted in the urine (Table II). Only a minor fraction (10%) of the urinary products, however, were conjugated. The conjugates were glucuronides, with no evidence for sulphates. Based on previous radioactive studies, 5% and 30%, respectively, of the CPA dose had been reported as excreted in the urine [10, 11]. Our finding of substantial amounts of free urinary 15HOCPA and CPA agrees with the earlier work cited for males [5].

The present study shows that a major part of the administered CPA dose is rapidly metabolised to 15HOCPA, and that 15HOCPA has a similar plasma clearance to CPA. This finding would add support to the suggestion that 15HOCPA replaces CPA in the reverse sequential therapy, because of its reduced progestational effect relative to its antiandrogen properties [4]. Practical questions of 15HOCPA bioavailability and cost of synthesis however, would also need to be addressed.

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