A Reversed-Phase High-Performance Liquid Chromatographic Method for the Simultaneous Determination of Serum Concentrations of Cyproterone Acetate and 15β -Hydroxycyproterone Acetate

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Abstract

A reversed-phase high-performance liquid chromatographic method for the simultaneous determination of cyproterone acetate (CPA), 15β -hydroxycyproterone acetate (15β -OH-CPA) and cyproterone (CP) was reported. This method was specific, sensitive, precise, easy and rapid for determination of the serum concentrations of these steroids in patients receiving CPA. Although no peak corresponding to CP was observed for serum, peaks corresponding to CPA and 15β -OH-CPA were detected and well separated in all subjects undergoing long-term CPA therapy. In these patients, there seemed to be a dose-dependent relationship between the amount of CPA administered and the serum concentrations of these steroids, and the serum concentrations of CPA were either similar or low compared with those of 15β -OH-CPA.

In conclusion, this simplified method is thought to be very valuable for studies on the pharmacokinetics of CPA and 15β -OH-CPA, and on the relationship between the CPA dosage and the therapeutic or side effects on adrenal and gonadal steroid production.

Cyproterone acetate (17-acetoxy-6-chloro- 1α , 2 α -methylene-4, 6-pregnadiene-3, 20-dione: CPA) is one of the agents which are generally used for the treatment of sexual deviations, especially of true precocious puberty in childhood. Although several papers dealing with methods for the determination of CPA, involving radioimmunoassaying (RIA) (Hümpel *et al.*, 1978; Nieuweboer and

Lübke, 1977) or high-performance liquid chromatography (HPLC) (Cannell *et al.*, 1981; Frith and Phillipou, 1985), have appeared the pharmacokinetics of CPA and its metabolites in patients undergoing long-term CPA therapy have not been sufficiently examined. In addition to this, not only an appropriate dosage and CPA therapy schedule but also the relationship between the dosage of CPA and the side effects on adrenal intrinsic steroid production has not yet been determined. In this paper, therefore, as one means of resolving these points, we report a modified reversed-phase HPLC

Received September 16, 1987

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method for the simultaneous determination of serum CPA, cyproterone (CP) and 15β -hydroxycyproterone acetate (15β -OH-CPA), which is known to be the main and potential metabolite of CPA in serum (Schleusener *et al.*, 1980).

Subjects and Methods

Subjects

Twenty-three serum samples for 11 patients with true precocious puberty and one patient with idiopathic hirsutism were examined. Two of them were patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency who developed true precocious puberty after the initiation of hydrocortisone therapy. The doses of CPA orally administered ranged from 41.6 to 208 mg/m²/day. Most of the samples were obtained two hours after the oral administration of CPA.

Methods

The methanol and acetonitrile used for the mobile phase and the diethyl ether for sample extraction were all of HPLC-grade. Cyproterone (CP), cyproterone acetate (CPA) and 15β -hydroxycyproterone acetate (15β -OH-CPA) were kindly donated by Schering AG (Berlin, West Germany), and 19-nortestosterone (19-NT) and other steroids were all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

The HPLC pump and UV spectrometer used were a TWINCLE and a UVIDEC 100-V obtained from JASCO (Tokyo, Japan). The UV spectrometer was operated at a wavelength of 284 nm, since CP, CPA and 15β -OH-CPA showed maximum absorbance at around this wavelength under our assay conditions, though the maximum absorbance of the internal standard, 19-NT was at around 246 nm. We used ERC-ODS-1161 (ϕ 6 mm $\times$ 20 cm; particle size, 3 μ) and ERC-ODS-1652 (ϕ 6 mm $\times$ 3 cm; particle size, 5 μ) (both from ERMA, Tokyo, Japan) for the main column and the pre-column, respectively. A mixed solvent containing methanol, acetonitrile and water, 55/6/39 by volume, was used as the mobile phase, which was pumped at a constant flow rate of 1.8 ml/min. The HPLC was conducted at room temperature.

One half milliliter of serum and 50 μ l (5 μ g)

of the internal standard, 19-NT, was extracted with 5.0 ml of diethyl ether by vortex mixing in a test tube. The organic phase was washed with 0.5 ml of distilled water and then separated by centrifugation at 3,000 rpm for 2 min. After evaporation of the solvent, the residue was dissolved in 50 μ l of methanol and then 20 μ l of the resultant solution was injected into the HPLC system.

We examined the retention time and recovery rate for each steroid, and the ratio of peak height for each steroid to that of the internal standard, 19-NT. Each steroid was identified on the basis of its retention time relative to that of 19-NT. We used the peak height ratio as the basis for quantitation.

The estimated values were expressed as mean $\pm$ SD. Statistical analyses were performed with Student's unpaired *t*-test, and differences with *P* values of less than 0.05 were considered to be statistically significant.

Results

Tables 1 and 2 show the retention time and recovery rate for each steroid. Fig. 1

Table 1. Retention times and retention time ratios to the internal standard (19-nortestosterone).

	Retention time (min)	CV (%)	Retention time ratio	CV (%)
15β -OH-CPA	5.5 $\pm$ 0.1	0.9	0.72 $\pm$ 0.01	1.6
19-NT	7.6 $\pm$ 0.1	1.3	1	—
CP	12.9 $\pm$ 0.1	0.5	1.58 $\pm$ 0.01	0.9
CPA	18.1 $\pm$ 0.3	1.5	2.39 $\pm$ 0.02	0.9

Mean $\pm$ SD (n=4)

Table 2. Recovery rates and peak height ratios to the internal standard (19-nortestosterone).

	Recovery (%)	CV (%)	Peak height ratio	CV (%)
15β -OH-CPA	70.5 $\pm$ 3.1	4.4	57.7 $\pm$ 1.2	2.2
19-NT	74.6 $\pm$ 4.0	5.4	1	—
CP	79.6 $\pm$ 4.7	5.8	43.3 $\pm$ 1.4	3.3
CPA	81.4 $\pm$ 2.9	3.6	27.2 $\pm$ 0.7	2.7

Mean $\pm$ SD (n=4)

shows a chromatogram for a normal adult serum sample supplemented with 125 ng each of CP, CPA and 15 β -OH-CPA, and 2.5 μ g of 19-NT, per 0.5 ml of serum. The steroids showed good separation and the recovery rates were all sufficient. The coefficients of variation for the retention time and recovery rate were both satisfactorily low. The minimum detectable levels for CPA and 15 β -OH-CPA were considered to be 15 and 5 ng/ml, respectively, when 0.5 ml of serum was used.

Fig. 2 shows a typical profile for a

serum sample obtained from a patient with idiopathic true precocious puberty undergoing long-term CPA therapy (62.5 mg/m²/day), for which peaks of CPA and 15 β -OH-CPA were detected and the concentrations of CPA and 15 β -OH-CPA were calculated to be 318 and 466 ng/ml, respectively. Peaks of CP and other metabolites were not detected for any serum sample obtained from patients undergoing CPA therapy.

Intra-assay variances ranged from 0.9 to 6.2% for CPA and from 2.1 to 5.7% for 15 β -OH-CPA, and the inter-assay variances

Fig. 1. Chromatogram of a normal adult serum sample supplemented with 125 ng each of CP, CPA and 15 β -OH-CPA, and 2.5 μ g of 19-NT, per 0.5 ml serum. 1: 15 β -OH-CPA, 2: 19-NT, 3: CP, 4: CPA.

Fig. 2. A typical chromatogram for a patient with true precocious puberty undergoing long-term oral CPA administration. 1: 15 β -OH-CPA (446 ng/ml), 2: 19-NT, 3: CPA (318 ng/ml).

were 1.6 to 5.5% for CPA and 6.1 to 7.3% for 15β -OH-CPA. These values were all considered to be sufficiently low for the determination of the serum concentrations of the two steroids (Table 3).

Table 4 shows the serum concentrations of CPA and 15β -OH-CPA in patients undergoing long-term oral CPA administration with various doses. The mean 15β -OH-CPA concentrations were higher than those of CPA in all groups divided as to the doses administered. There were positive relationship between the serum concentrations of these steroids and the CPA dosage (up to 150 mg/m²/day) administered, but we did not observe a significant differences in these serum concentrations between the patients receiving high dose of CPA (100–150 mg/m²/day), and those receiving very high doses of CPA (above 150 mg/m²/day).

Table 3. Precision of the assay

Intra-assay variance

	CPA (ng/ml)	CV (%)	15β -OH-CPA (ng/ml)	CV (%)
Sample A	570±11	1.9	1264±27	2.1
B	420±26	6.2	492±28	5.7
C	377±4	0.9	1394±39	2.7
D	64±4	5.4	41±2	4.9

Inter-assay variance

	CPA (ng/ml)	CV (%)	15β -OH-CPA (ng/ml)	CV (%)
Sample E	113±2	1.6	278±17	6.1
F	337±18	5.5	478±35	7.3

Mean±SD (n=3)

Table 4. Serum concentrations of CPA and 15β -OH-CPA in patients undergoing oral CPA administration.

Dose (mg/m ² /day) (mean)	n	CPA (ng/ml)	15β -OH-CPA (ng/ml)
<50 (30.5)	4	92.0±42.7	253±34.5
50~100 (73.5)	9	134±50.0	384±158
100<150(127.9)	14	400±195	1176±495
150< (175.3)	7	428±118	1215±585

Mean±SD

Discussion

CPA has been used as an antiandrogen in the treatment of adult women with acne vulgaris and/or hirsutism (Hammerstein *et al.*, 1975; Moltz *et al.*, 1982), and of adult men with advanced prostatic carcinoma (Jacobi, 1983) or hyper-sexuality (Laschet and Laschet, 1975), and the pharmacokinetics of CPA in humans have only been investigated in these adult subjects. Apart from these diseases, the most important use of CPA today is in the treatment of precocious puberty in children. However, not only the pharmacokinetics of CPA and its main and potential metabolite, 15β -OH-CPA, but also the relationship between the serum concentrations of these steroids and the clinical or endocrinological effects has not been sufficiently examined in children undergoing long-term oral CPA administration for the treatment of precocious puberty. Moreover, although it is well known that CPA has a suppressive effect on the adrenocortical function (Heinze *et al.*, 1978), the relationship between the serum CPA and 15β -OH-CPA levels and the extent of adrenocortical suppression is less well defined. We therefore attempted to resolve these points through more accurate determination of serum CPA and 15β -OH-CPA concentrations.

As to HPLC methods for the determination of CPA and its metabolites, Frith and Phillipou (1985) reported a reversed-phase HPLC method which was applicable for the simultaneous determination of the CPA and 15β -OH-CPA levels in plasma and urine. The equipment and the wavelength they used were basically the same as ours, but their extraction procedure was much more complex than ours. Instead of cyproterone propionate, which they originally synthesized and used as an internal standard, commercially available 19-NT was used for our method. Because the maximum

absorbance of 19-NT was at around 246 nm and is different from the wavelength used (284 nm), it is necessary to use a large amount of 19-NT for the internal standard in this study. However, we have demonstrated that this large amount of 19-NT used does not have any undesirable effect on this analysis. With our modified HPLC method, the specific detection of the peaks of CPA and 15 β -OH-CPA in serum is considered to be much easier for the following reasons. (1) The appropriate wavelength for CPA and 15 β -OH-CPA determination was 284 nm with our method. Since many kinds of unconjugated intrinsic adrenal and gonadal steroids are not detectable at this wavelength (Cannell *et al.*, 1981), and our extraction procedure involving diethyl ether excludes the sulfoconjugated intrinsic steroids which may exist in sera in great quantities, our method is considered to be specific for the determination of CPA and 15 β -OH-CPA. As a matter of fact, no peaks other than those of CPA, 15 β -OH-CPA and 19-NT (internal standard) were seen in the chromatogram. With the RIA method, the peak concentration of serum CPA was reported to be 300 ± 30 (mean $\pm$ SD) ng/ml in normal adults after single oral administration of a 50 mg tablet of CPA (Humpel *et al.*, 1978). This value was approximately three times higher than that obtained with our method, i.e., 76.1 ng/ml. This difference is thought to be due to the cross-reactivities of other related steroids including 15 β -OH-CPA, which inevitably interfere with the radioimmunoassay method. (2) The minimum detectable levels with this method were 15 ng/ml for CPA and 5 ng/ml for 15 β -OH-CPA, respectively. These values are considered to be sufficiently low for determination of the serum concentrations of these steroids in patients undergoing routine oral CPA administration. (3) The precision of this method was satisfactory, and the recovery rate on extraction was also sufficient and constant.

Although the serum concentration of CP can be simultaneously measured with our method, if it is present in serum, it was, as reported previously (Frith and Phillipou, 1985), not detected in any serum samples from the patients receiving CPA. In humans, CP is known to be present in urine and bile juice chiefly as a glucuronide conjugate (Kopp *et al.*, 1976), however, we ourselves have not yet studied the CP contents in such specimens. They also reported that highly polar unconjugated steroid was detected in human serum by a thin layer chromatographic procedure after ^{14}C -labelled CPA administration, but we could not find any peak representing this metabolite by our method.

In conclusion, using our new reversed-phase HPLC method, we could measure CPA and 15 β -OH-CPA in serum easily and simultaneously without any interference due to other intrinsic adrenal or gonadal steroids. By means of this method it will be possible to study not only the pharmacokinetics of CPA and 15 β -OH-CPA but also the relationship between CPA dosage and the effects on adrenal and gonadal steroid production in children suffering from precocious puberty who are undergoing long-term CPA therapy.

Acknowledgements

The authors wish to acknowledge the donation of cyproterone acetate, cyproterone and 15 β -hydroxycyproterone acetate by Schering AG.

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