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Note

Automated high-performance liquid chromatographic assay for cyproterone acetate and 15β-hydroxycyproterone acetate in plasma

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Cyproterone acetate (CPA) is an antiandrogen that is widely used in the treatment of hirsutism and acne [1]. 15β-Hydroxycyproterone acetate (OH-CPA) is the major metabolite of CPA found in mammals after oral or parenteral administration [2]. This metabolite still exerts some pharmacological activity, and it is known from other studies that its concentration in plasma can exceed that of CPA [1,3]. Therefore, it would be of interest to know the concentrations of both CPA and OH-CPA in plasma during and after CPA treatment in the course of animal experiments and clinical studies. However, conventional radioimmunoassay (RIA) for CPA determination does not provide this additional information. In fact, owing to cross-reactivity of OH-CPA (15–20%) [4], high metabolite concentrations could even interfere with CPA measurement and lead to an overestimation of CPA plasma levels.

Although high-performance liquid chromatographic (HPLC) procedures for the determination of either CPA alone [5] or CPA and its metabolite OH-CPA [3] in plasma have been described, they required time-consuming sample clean-up. The present investigation describes an automated HPLC procedure that allows quantitative determination of CPA and OH-CPA by direct injection of plasma samples. This technique was applied to plasma samples from patients under CPA therapy.

EXPERIMENTAL

Materials

The hardware consisted of two pumps (5200, Knauer, Berlin, F.R.G.), an autosampler (ISS-100, Perkin-Elmer, Überlingen, F.R.G.), a column-switching mod-

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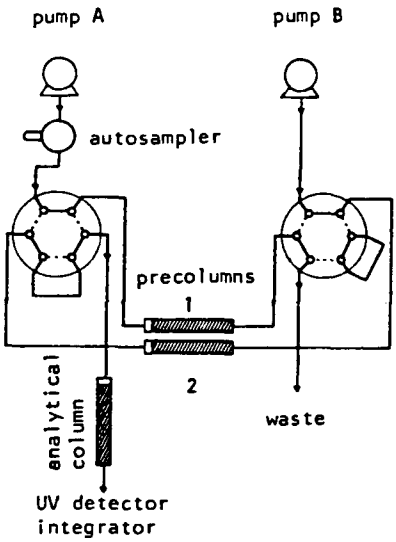

Fig. 1. Schematic representation of the automated HPLC configuration with guard column switching facility.

ule (SE-2, Gynkotek, Munich, F.R.G.), a thermostat-regulated column oven (Gynkotek) and a variable-wavelength UV detector (Spectroflow 773, Kratos, Karlsruhe, F.R.G.). The UV detector was connected via an interface with a CHB computer system for data acquisition and evaluation.

Analytical-grade acetonitrile was obtained from Merck (Darmstadt, F.R.G.) and water was freshly distilled in an all-glass still. Solvents were degassed separately under water-jet vacuum, and the mobile phase was freshly prepared each day.

Chromatographic procedure

A schematic representation of the HPLC system, which is similar to one already described by Roth et al. [6], is given in Fig. 1. Plasma samples were injected by the autosampler and transported by a flow of water (pump A) onto a guard column (1). At the same time, a clock in the column-switching module was triggered, which defined a preset washing period. Constituents of the biological matrix (e.g., plasma proteins, salts) were eluted (waste), while CPA and OH-CPA were retained on the guard column. Meanwhile, another guard column (2) and the analytical column were in the solvent stream (pump B), which was permanently monitored by the UV detector.

When the washing period ended, two pneumatically driven valves (SE-2) were switched to such a position that guard column 1 was now in the solvent stream and in line with the analytical column, while guard column 2 was connected to pump A and equilibrated with water, ready to be loaded by the following injection. Compounds retained on guard column 1 were now back-flushed onto the analytical column, separated and recorded. After a total of 15 min one chromatographic analysis was terminated. The use of two guard columns allowed one sample to be

loaded on the second guard column while the previously injected sample was still being separated on the analytical column. This arrangement allowed straightforward automation of the whole procedure.

Chromatographic conditions

Guard columns (40×4.6 mm I.D.) packed with LiChroprep RP-18 (25–40 μm, Merck) were equilibrated with distilled water before the plasma samples (200 μl) were injected. After injection, the loaded guard column was washed for 3 min with distilled water at a flow-rate of 2 ml/min.

The analytical column (125×4.6 mm I.D.) was packed with ODS-Hypersil, 5 μm (Shandon) and the mobile phase consisted of acetonitrile–water (45:55). Both guard columns and the analytical column were thermostatted at 40°C, and at a flow-rate of the mobile phase of 1.6 ml/min the overall back-pressure was ca. 70 bar. The compounds were detected by their UV absorption at 282 nm, and the retention times of OH-CPA and CPA on the analytical column were 3.4 and 8.9 min, respectively (Fig. 2).

Calibration curves

For calibration, blank plasma samples were spiked with CPA and HO-CPA to obtain the following concentrations: 50, 100, 250, 500 and 1000 ng/ml. A 200-μl volume of each standard plasma was injected, and peak areas of the signals corresponding to CPA and OH-CPA were determined. Each standard was analysed in duplicate.

Patient samples

Plasma samples were collected from women undergoing long-term CPA therapy of severe acne and hirsutism. According to the reverse sequential regimen they received CPA (100 mg, days 5–15) and ethinyl estradiol (0.05 mg, days 5–25) (group I, five patients) or the same regimen but 50 mg of CPA instead of 100 mg (group II, seven patients). Patients in group III ($n=3$) received daily doses of 100 or 50 mg of CPA alone, throughout the cycle. The other plasma samples used in this study were collected from three male volunteers (group IV), who received during a pharmacokinetic study a daily oral dose of 150 mg of CPA in combination with 0.5 mg Suprefact (LHRH-agonist) over a period of 7 days.

RESULTS

Recovery

Analytical recovery of CPA and OH-CPA from plasma was determined by a comparison of peak areas obtained from processed plasma samples with known drug concentrations with those of pure drug standards in diluted methanol. As shown in Table I, recoveries were between 83 and 101%.

Precision

The precision of the method was assessed by repeated analysis of plasma samples that contained OH-CPA and CPA at low, medium and high concentrations.

TABLE I

ANALYTICAL RECOVERY OF CPA AND OH-CPA FROM SUPPLEMENTED BLANK PLASMA SAMPLES

Compound	Concentration (ng/ml)	Recovery (mean ± S.D., $n=4$) (%)
CPA	100	94±3
	500	88±7
	1000	83±3
OH-CPA	100	94±2
	500	101±7
	1000	96±2

Coefficients of variation (C.V.) of within-day and day-to-day precision were in the ranges 1–4% and 2–11%, respectively (Table II).

Accuracy

Each set of samples analysed included calibration standards and test-plasma samples, which contained known concentrations of CPA and OH-CPA. The drug concentrations of both actual and test-plasma samples were evaluated with the same calibration. The deviation of calculated values from real concentration values in the test samples gave a measure of accuracy. Day-to-day variation ranged from 1.3 to 6.6% (Table II).

Calibration curve, limit of detection

In the range 50–1000 ng/ml, a linear regression line was obtained for OH-CPA and CPA ($r=0.9999$). Injection of a drug-free plasma sample revealed no interfering background signals, and the limit of detection was 50 ng/ml for both compounds.

TABLE II

PRECISION AND ACCURACY OF THE AUTOMATED DETERMINATION OF CPA AND OH-CPA IN PLASMA SAMPLES

Compound	Concentration (ng/ml)	Within-day C.V. (%)	Day-to-day C.V. (%)	Accuracy (% deviation)
CPA	100	4.3 ($n=5$)	10.8 ($n=8$)	+6.6
	500	1.9 ($n=5$)	4.0 ($n=12$)	−2.1
	1000	3.0 ($n=5$)	8.2 ($n=12$)	−1.3
OH-CPA	100	2.9 ($n=5$)	9.9 ($n=9$)	+2.8
	500	1.1 ($n=5$)	2.1 ($n=12$)	−2.5
	1000	1.8 ($n=5$)	7.0 ($n=12$)	−1.8

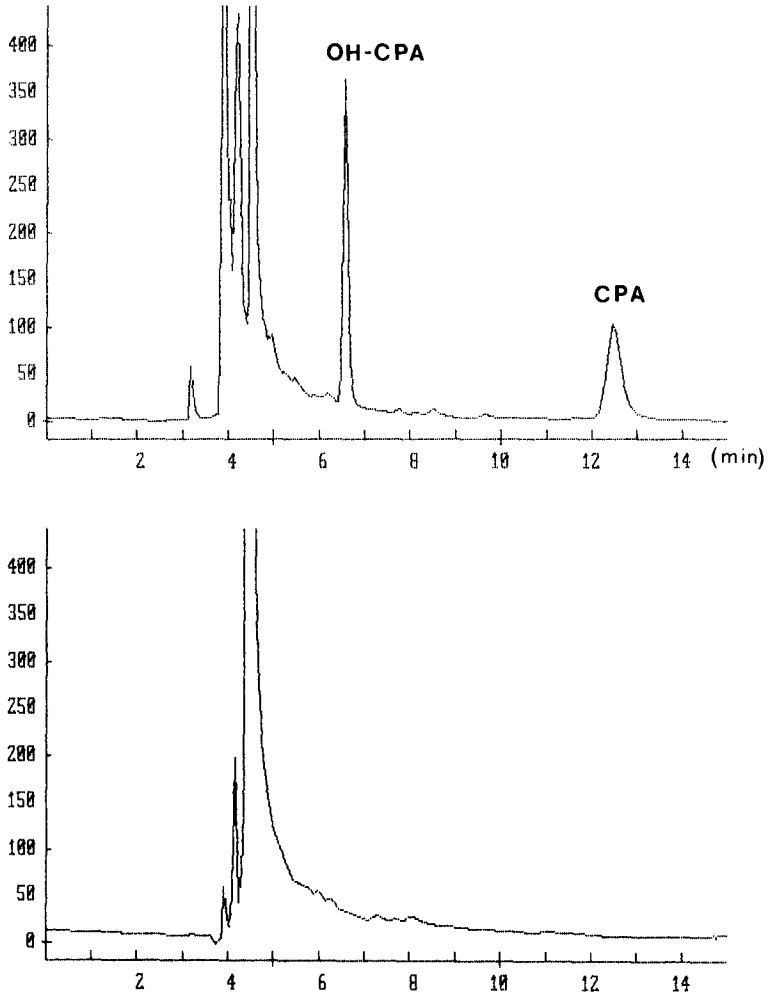

Fig. 2. (Top) Chromatogram of a 200-μl plasma sample that contained both CPA and OH-CPA at concentrations of 500 ng/ml. At time zero, the sample was injected onto the guard column. (Bottom) Chromatogram of a 200-μl blank plasma sample.

OH-CPA/CPA concentration ratios in human plasma samples

The concentrations of CPA and OH-CPA were determined in 77 plasma samples obtained from seventeen female patients and three male volunteers. The OH-CPA/CPA concentration ratios were in the range 0.4–4.2, with a mean value of 1.8 ± 0.9. CPA concentrations in plasma were in the range 86–780 ng/ml (Table III).

Stability of the compounds

In order to assess the influence of storage at room temperature over a prolonged time on the stability of OH-CPA and CPA in plasma, repeated analyses were performed on the same samples. When the samples were kept at room tempera-

TABLE III

CONCENTRATION OF CPA AND CONCENTRATION RATIO OF OH-CPA TO CPA

Values determined in plasma samples from female patients (group I–III) and male volunteers (group IV), who were treated with CPA (for details refer to Experimental).

Group	*n*	OH-CPA/CPA concentration ratio		CPA concentration range (ng/ml)
		Mean	Range	
I	5	1.8	1.6–2.4	227–521
II	7	2.1	1.0–4.1	86–344
III	5	1.8	0.9–2.5	110–631
IV	60	1.7	0.4–4.2	108–780
I–IV (mean ± S.D.)		1.8 ± 0.9		

ture for 24 h, no change in the peak areas of OH-CPA and CPA was observed when the chromatograms obtained at different days were compared.

DISCUSSION

HPLC with precolumn-switching facility allows direct injection of plasma samples without additional off-line purification steps. This minimizes sample losses, and owing to the high reproducibility of the procedure, internal or external standardization is optional. The whole procedure can easily be automated, which makes it especially suited for unattended operation (e.g., overnight). The lower limit of detection is mainly determined by the sample volume injected. In the particular case of CPA and OH-CPA, injection of 400 μl of plasma would allow the quantification of 25 ng/ml. Column efficiency was slightly impaired after the injection of ca. 25 plasma samples (injection volume 200 μl), which was indicated by an increased peak-width and decreased peak-height. This, however, had no influence on the accuracy of the determination. Repeated analyses of test-plasma samples of known drug concentration, which were injected at the end of a sample set, revealed a maximum deviation of 7% of the true value. Nevertheless, guard columns should not be used for more than 100 injections. When a large number of samples is to be analysed, the following approach would be recommended to compensate for possible changes in column performance: first, the calibration standards (3–5) should be injected, followed by actual samples (ca. 25); then, another set of calibration standards plus actual samples could be injected, etc. This procedure would provide an indication of the changes in column performance, and also compensate for possible changes by offering a calibration curve corresponding to actual column performance.

In the present investigation, the concentration ratios of OH-CPA to CPA were determined in a number of plasma samples obtained from patients and volunteers who were treated repeatedly with CPA. A mean value of 1.8 ± 0.9 indicated that the metabolite concentrations usually exceeded those of the parent compound.

This result is in agreement with previous data [1,3]. The relatively wide range of OH-CPA/CPA concentration ratios we found in our study (Table III) is mainly due to interindividual differences within one group, whereas repeated sampling from the same group of individuals over a period of 7 days showed only small intra-individual variations.

It is well known that OH-CPA interferes with the radioimmunological analysis of CPA owing to a cross-reactivity of 15–20% [4]. Taking into account a mean value of 1.8 for the OH-CPA/CPA concentration ratio, a mean overestimation of ca. 30% can be expected for the CPA determination. However, ongoing studies show that there are no differences in basic pharmacokinetic parameters when calculated from plasma concentration–time curves determined by either HPLC or RIA, although the accuracy of CPA determination is better in the former method. Conversely, the HPLC method described here cannot be used in pharmacokinetic studies in which low-dosage CPA preparations, such as Diane® (2 mg CPA plus 0.05 mg ethinylestradiol per day), are investigated.

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