Journal of Chromatography, 529 (1990) 145–154
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO 5283

Determination of megestrol acetate and cyproterone acetate in serum of patients with advanced breast cancer by high-performance liquid chromatography

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(First received November 27th, 1989; revised manuscript received February 14th, 1990)

ABSTRACT

A reversed-phase high-performance liquid chromatographic method with ultraviolet detection of megestrol acetate and cyproterone acetate in human sera is described. The proposed assay is linear up to 1400 ng/ml ($r=0.999$) and has a detection limit of 5 ng/ml. Recoveries of both compounds in spiked sera were ca. 95%; inter-assay coefficients of variation were 4 0 and 3.1% and intra-assay values were 1.3 and 1.4%, respectively. For validation of the method we also developed a gas chromatographic–mass spectrometric method for both steroids. The results obtained by the two methods showed good correlation: for megestrol acetate $r=0.98$, $n=31$, $p<0.0001$, and for cyproterone acetate $r=0.94$, $n=0$, $p<0.0001$. Large inter-individual differences in the serum concentrations of both substances were found in groups of patients with metastatic breast cancer receiving the same oral load of either steroid.

0378-4347/90/$03.50

INTRODUCTION

For more than a decade the anti-estrogen tamoxifen has been the first choice in the palliative treatment of metastatic breast cancer in postmenopausal women, leaving treatment with a synthetic progestin as a second option [1–5]. When tamoxifen has been shown to be ineffective, progestins may still be capable of remission induction. Since the first publications on the remission of breast cancer by progesterone therapy in the early 1950s [6], progestins in conventional doses have been used with moderate success [7,8], leading to a decreasing interest in this therapy. Progestin therapy became more effective when higher dosages were applied [9,10]. Medroxyprogesterone acetate (MPA) and megestrol acetate (MA) are the most commonly used synthetic steroids, and cyproterone acetate (CPA), an anti-androgen with progestative effects, is currently undergoing clinical trials [11].

If high dosages of MPA are administered, serum level monitoring is necessary, since large intra- and inter-individual differences in absorption and metabolism have been reported [12]. If the same kinetics apply to MA and CPA, drug monitoring during therapy may be desirable. Recently, radioimmunoassays for MPA, with a known extensive cross-reactivity for MA, were used to determine MA concentrations [13].

From an analytical point of view this method seems less appropriate, and therefore we developed a high-performance liquid chromatographic (HPLC) method for the determination of MA, using CPA as an internal standard. We also tested the method in reverse, making CPA determinations possible. The technique was used to determine MA and CPA in sera from patients with metastatic breast cancer, treated with either of the two steroids. To test the validity of the assay, the same samples were also determined by gas chromatography–mass spectrometry (GC–MS), which is considered to be a reference technique [14].

EXPERIMENTAL

Materials

The megestrol acetate reference substance was obtained from Sigma (St. Louis, MO, U.S.A.) and the cyproterone acetate reference was supplied by Schering Nederland (Weesp, The Netherlands). Cyproterone acetate tablets (Androcur) were obtained from Schering, and megestrol acetate tablets (Megace) from Bristol-Meyers (Weesp, The Netherlands). Sep-Pak C_{18} cartridges were from Waters Assoc. (Milford, MA, U.S.A.). All other reagents (analytical grade) were from Merck (Darmstadt, F.R.G.) and used as supplied.

Calibration curves

For the MA calibration curve, 70 mg of MA were dissolved in 50.0 ml of acetone and diluted with methanol to a final concentration of 14 μg/ml. Different volumes equivalent with amounts ranging from 0 to 600 ng were pipetted into six glass-stoppered 10-ml tubes. After the addition of 100 μl of the internal standard solution (CPA, 14 μg/ml) and mixing, the samples were evaporated to dryness under a gentle stream of nitrogen, at room temperature.

CPA (70.0 mg) was dissolved and diluted as described for MA, but the volumes pipetted into the 10-ml tubes ranged from 0 to 1400 ng. After the addition of the internal standard (100 μl of MA solution containing 14 μg/ml) the standards were treated as described above.

Extraction procedure

The Sep-Pak C_{18} extraction was essentially the same as described by Hofreiter et al. [15]. To 1.0 ml of serum, 100 μl internal standard and 100 μl of methanol were added. After mixing and equilibration (1 h at room temperature) the samples were applied to pre-washed Sep-Pak C_{18} cartridges. After washing with 5 ml of water, the steroids were eluted from the cartridges with 2 ml of methanol. The eluent, reduced to ca. 0.5 ml under a stream of nitrogen at room temperature, was diluted with 5 ml of water and extracted with 5 ml of dichloromethane. The dichloromethane layer was pipetted into an other tube and evaporated to dryness. The residue was dissolved in 100 μl of HPLC eluent.

HPLC procedure

A Waters M 6000 A pump equipped with a Brownlee analytical column (Spheri RP18, 220 mm × 4.6 mm I.D., particle size 5 μm) and a Kratos Spectroflow 773 UV detector set at a wavelength of 260 nm, was used to separate MA and CPA. A baseline separation was achieved with water–acetonitrile–methanol (35:30:35, v/v) as eluent at a flow-rate of 1.0 ml/min. After the injection of the redissolved samples and standards, peak-height ratios were determined and corresponding concentrations were derived from standard curves.

Preparative HPLC

The two peak fractions of each standard were collected in one tube and the solvent was evaporated under a stream of nitrogen at 40°C. The two peak fractions of each sample were also collected in one tube and treated likewise. The residues were redissolved in distilled hexane, and subjected without derivatization to GC–MS.

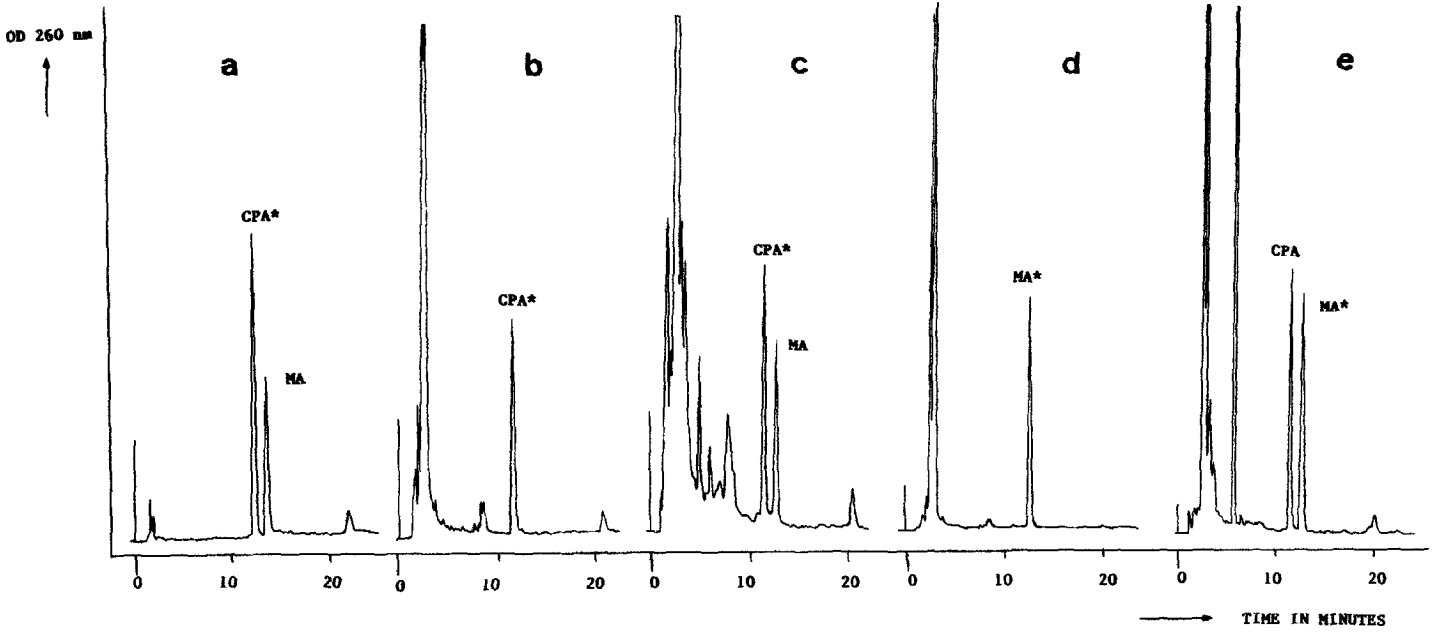

Fig. 1. Typical HPLC profiles of (a) a standard containing 426 ng/ml MA, (b) a serum containing no MA and (c) a serum of a patient receiving MA medication. To each sample ca. 740 ng/ml CPA was added as internal standard, indicated with an asterisk. The calculated MA concentration in the serum of the patient is 591 ng/ml. (d) Chromatogram of a serum containing no CPA. (e) Chromatogram of a serum of a patient receiving CPA. The internal standard in these samples (d and e) was ca. 1400 ng/ml MA, indicated with an asterisk. The calculated CPA concentration in the serum of the patient was 1550 ng/ml.

Gas chromatography–mass spectrometry

A Varian 3700 gas chromatograph, directly coupled to a Finnigan MAT 212 mass spectrometer and equipped with a SS 200 data system, was used. The temperature of the GC injection port was 250°C and the samples were introduced using a split ratio of 1:10. The initial oven temperature (200°C) was raised at 20°C/min to a final temperature of 300°C, and was maintained for another 5 min. The samples were separated on a CP-Sil-5 fused-silica column, (25 m×0.32 mm I.D., Chrompack, Middelburg, The Netherlands) using a carrier gas flow-rate of 1.9 ml/min. The mass spectrometer was used in the electron-impact mode (emission current, 0.5 mA; acceleration voltage, 3.0 kV; electron energy, 70 eV) at a resolution of 1000. The temperature of the ion source and the interface was set at 250°C. Mass spectra were recorded by scanning at 1.5 s/decade.

For the selected-ion monitoring of the HPLC peak fractions of the samples and the standards the detector of the mass spectrometer was set for the ions *m/z* 281 (MA) and *m/z* 313 (CPA). Peak-area ratios were calculated by means of the data system, and a linear calibration curve was constructed to determine the respective concentrations.

RESULTS

A typical chromatogram for an MA calibration standard is shown in Fig. 1a. After calculation of the peak-height ratios, the calibration curves obeyed the equation $y=0.56\cdot10^{-3}x-0.006$ ($r=0.9999$) for MA and $y=0.87\cdot10^{-3}x+0.013$ ($r=0.9997$) for CPA, where x is the steroid concentration in ng/ml and y is the peak-height ratio. When the same calibration standards, prepared in 1 ml of pool serum and extracted as described, were injected, the comparable equations were $y=0.54\cdot10^{-3}x-0.002$ ($r=0.9995$) for MA and $y=0.87\cdot10^{-3}x+0.008$ ($r=0.9998$) for CPA.

A sample containing MA was taken from a patient, and volumes ranging from 0.1 to 1.0 ml were pipetted into glass-stoppered tubes and diluted with blank human serum to a final volume of 1.0 ml. After the determination as patient samples, peak-area ratios (y) were plotted against the pipetted volume of patient serum (x) yielding a straight line ($y=1.712x+0.012$; $n=6$; $r=0.9991$). The same experiment for a CPA-containing serum from a patient again resulted in a straight-line equation: $y=1.847x-0.004$; $n=6$; $r=0.9999$.

Both experiments demonstrate that the proposed method is linear at least over the concentration range 0–626 ng/ml for MA and 0–2039 ng/ml for CPA. The lower level of detection is 5 ng/ml for both steroids.

The reproducibility of the assay was tested by spiking one pool serum with 70.9 ng of MA per ml and another with 736 ng of CPA per ml. Aliquots of 2 ml of the homogenated and equilibrated sera were stored at −20°C and, after

thawing just before use, determined on several occasions. The results are summarized in Table I.

We also analysed serum samples containing no MA and no CPA, and they demonstrated true blank values. Typical HPLC results, both of serum samples of patients receiving either MA or CPA, as well as the blank sera containing only MA or CPA as internal standard, are depicted in Fig. 1b–e.

To validate the HPLC assay and to exclude interference from metabolites, the patients' serum samples were also determined by GC–MS. In the preliminary experiments, mass spectra were recorded (Fig. 2) and the most abundant ion fragment ($M^+ - 103$), being the mass of the steroid molecule minus the C_{17}–α-acetate moiety and the C_{20-21} side-chain, was selected for each. Thus, for the selected-ion monitoring of the combined HPLC fractions from patient samples and from standards, the detector of the mass spectrometer was set at m/z 281 (MA) or at m/z 313 (CPA) (Fig. 3). After calculation of the peak-area ratios, concentrations were derived and compared with those determined by HPLC.

Fig. 4a indicates the correlation between the GC–MS (x) and the HPLC determinations (y) of MA in 31 serum samples from patients with metastatic breast cancer, receiving Megace ($y = 1.08x - 20$; $r = 0.98$; $p < 0.0001$). Likewise Fig. 4b shows the correlation between the two techniques when CPA was determined in sera from 60 patients receiving Androcur ($y = 0.96x + 30$; $r = 0.94$; $p < 0.0001$). These good correlations further demonstrate the reliability of the proposed HPLC assay.

The concentrations of MA in sera from 30 patients with metastatic breast cancer receiving 160, 400 or 800 mg of MA daily were determined by HPLC. The serum levels demonstrate the expected large inter-individual differences (Table II).

We also determined CPA in the serum samples from 60 patients with met-

TABLE I

REPRODUCIBILITY OF THE ASSAY

Compound	Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	n	Recovery (%)
Inter-assay					
MA	70.9	68.4 ± 2.7	4	17	96.5
CPA	736	696 ± 22	3.1	18	94.6
Intra-assay					
MA	70.9	68.4 ± 0.9	1.3	5	96.5
CPA	736	685 ± 11	1.6	4	93.1

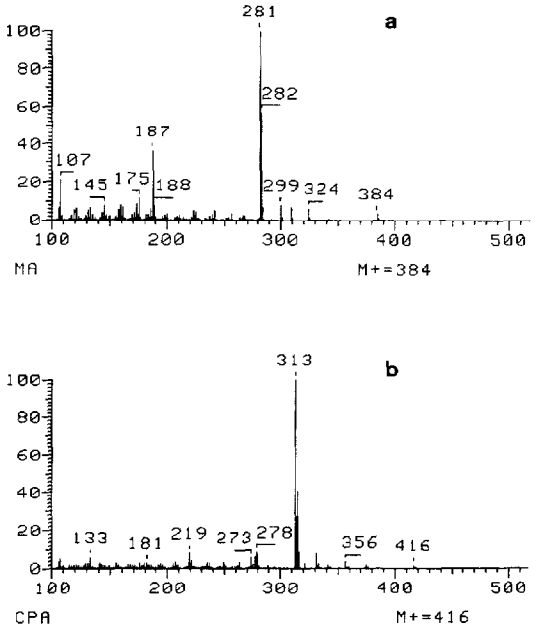

Fig. 2. Mass spectra of (a) megestrol acetate and (b) cyproterone acetate.

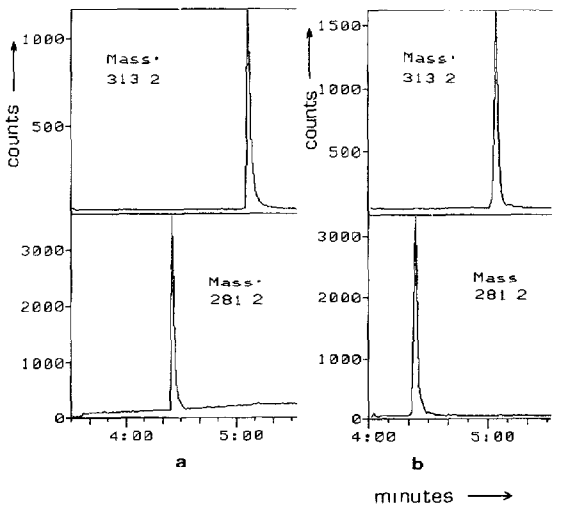

Fig. 3. Selected-ion monitoring of two patient sera. MA is monitored at *m/z* 281 and CPA at *m/z* 313. Examples are shown of (a) a serum sample of a patient receiving MA, to which ca. 740 ng/ml CPA was added as internal standard, and (b) a serum sample of a patient receiving CPA, to which MA was added as internal standard (ca. 1400 ng/ml). The corresponding calculated serum concentrations were 736 ng/ml for MA and 1512 ng/ml for CPA.

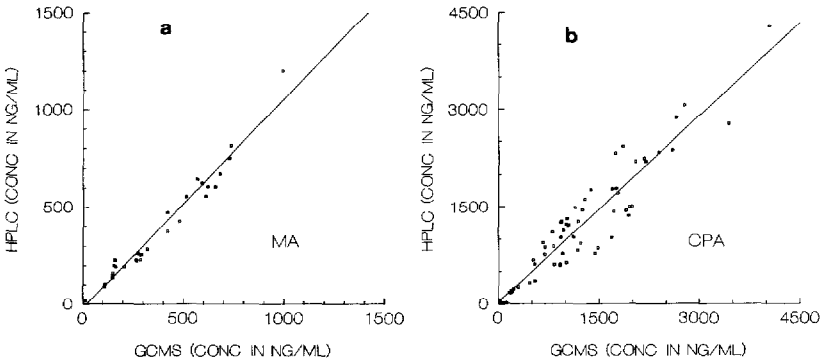

Fig. 4. Correlation graphs comparing serum concentrations of (a) MA and (b) CPA determined by the proposed HPLC method and by GC–MS. For MA $n=31$, $r=0.98$ and $p<0.0001$, and for CPA $n=60$, $r=0.94$ and $p<0.0001$.

TABLE II

DOSE–CONCENTRATION RELATION

Mean and median serum concentrations and the serum concentration range of groups of patients receiving the indicated oral dose of the steroid are presented.

Compound	Dose (mg)	Number of patients	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)
MA	160	17	202	227	91–285
	400	9	577	607	376–750
	800	4	778	721	471–1200
CPA	200	24	940	887	176–2373
	400	26	1700	1476	239–4293

astatic breast cancer receiving either 200 or 400 mg of Androcur daily. The results are also summarized in Table II.

DISCUSSION

Until recently MA concentrations were determined by radioimmunoassay, which was developed for MPA with its known cross-reactivity for MA. Since cross-reactivity with other steroids cannot be excluded we developed an HPLC method, which has the additional advantage of being devoid of radioactivity. Moreover, by simply replacing CPA with MA as internal standard, the method enables the determination of CPA.

An HPLC method for the determination of CPA using 17-α-hydroxypregna-4,5-diene-3,20-dione-17-butanoate as internal standard has been described

[16], as has an automated HPLC method for the determination of CPA and 15-hydroxy-CPA, its major metabolite, without using an internal standard [17]. The method described in this paper, however, makes use of basic routine HPLC equipment, does not require the synthesis of an internal standard and enables one to determine MA as well.

The linearity and precision in combination with the good correlation with the GC–MS results, establish the reliability of the assay. It is obvious that whenever MA and CPA are administered simultaneously, sera from these patients cannot be assayed with the proposed HPLC method. However, simultaneous administration is seldomly encountered in practice. When patient sera were analysed, large inter-individual differences in concentrations were found within groups of patients receiving the same oral load of either steroid (Table II). When the oral load of MA was increased from 160 to 400 mg per day, a concomitant raise in the mean concentration of MA in the serum from 202 to 577 ng/ml was observed. Although only four patients received a daily dose of 800 mg of MA, the mean serum concentration of 778 ng/ml suggests that a linear dose–concentration relation no longer exists when the dose is further increased.

Doubling the CPA dose from 200 to 400 mg per day results in a equivalent mean serum concentration rise from 940 to 1700 ng/ml.

These results indicate that the large differences in serum concentrations are individual- and steroid-dependent, and that, at least to a certain extent, a linear dose–concentration relation exists for MA as well as CPA. The differences in serum concentration and the deviation of the linear dose–concentration relation have been reported previously [18,19] and could be due to differences in absorption and metabolism. However, the true nature of the factors that cause these differences is still unclear and need further investigation.

ACKNOWLEDGEMENTS

The supply of cyproterone acetate by Schering is gratefully acknowledged. The authors also thank Mr. M. Volmer for his assistance and advice in the use of the computer facilities and Mr. W. de Jong for his technical support.

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