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Determination of exemestane, a new aromatase inhibitor, in plasma by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic method for the determination of 6-methylen-androsta-1,4-diene-3,17-dione (exemestane) and its 17-dihydro metabolite in human plasma has been developed. The analytes and internal standard (Norgestrel) were extracted from plasma samples with a methylene chloride–iso-octane mixture; the organic phase was dried and the residue was reconstituted with an acetonitrile–water mixture, then analyzed by reversed-phase liquid chromatography. Quantification was achieved by ultraviolet detection of the eluate. The linearity, precision and accuracy of the method were evaluated. No interference from the constituents of human blank plasma was observed. The lower limit of quantification was 10 ng/ml plasma. The suitability of the method for *in vivo* samples was checked by analysis of plasma samples drawn from healthy male volunteers who had received a 200-mg single oral dose of the test compound.

INTRODUCTION

The aromatase system is an enzyme complex consisting of two components, a flavoprotein NADPH–cytochrome P-450 reductase, which transfers electrons from NADPH to the terminal enzyme, and a specific form of cytochrome P-450 known as aromatase cytochrome P-450. The latter protein is involved in the binding of the C_{19}-steroid substrates and catalyzes the multistep reaction leading to aromatization of the A ring of the steroid [1–3].

Inhibition of this enzyme, to reduce estrogen production by peripheral and ovarian tissue, could be a useful approach in treating hormone-dependent breast cancer [4,5]. Several irreversible aromatase inhibitors have been described including 4-hydroxyandrost-4-ene-3,17-dione [6] and 10-propargylestr-4-ene-3,17-dione [7] and 1-methyl-androsta-1,4-diene-3,17-dione [8]. These compounds offer significant advantages in comparison with the currently available drug aminoglutethimide, a non-specific reversible aromatase inhibitor [9].

Exemestane (6-methylen-androsta-1,4-diene-3,17 dione, FCE 24304, I, Fig. 1) is a new irreversible aromatase inhibitor which has been synthesized and studied in the Research and Development Laboratories of Farmitalia Carlo Erba (Milan, Italy). This type of inhibitor may be recognized as a substrate by the aromatase cytochrome P-450 and then be processed through the normal catalytic mechanism to a transformed

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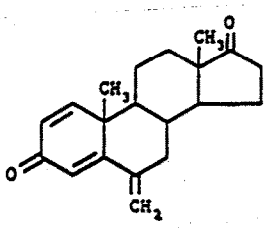

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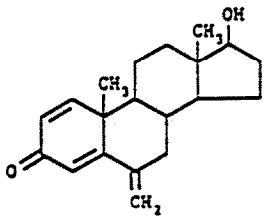

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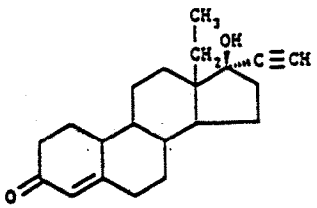

I.S.

Fig. 1. Structural formulae of FCE 24304 (I), FCE 25071 (II) and Norgestrel (I.S.).

compound, which binds covalently to, and thus inactivates, the active site of the enzyme.

Exemestane was shown to be a potent and selective inhibitor of aromatase *in vitro* and *in vivo*. In human-placental microsome preparations and rat-ovarian microsome preparations compound I has shown to have very great activity [10]; it was also reported to be effective on 7,12-dimethylbenzanthracene-induced mammary carcinoma in rats [11].

In the present study, optimal conditions were set up for the determination of the parent compound (I) as well as its 17-dihydro metabolite (6-methylen-androsta-1,4-diene-17-hydroxy-3-one, FCE 25071, II, Fig. 1) in plasma. The high-performance liquid chromatography (HPLC) method developed was fully validated and used in the determination of the two compounds in plasma samples obtained from the first preclinical and clinical studies [12].

EXPERIMENTAL

Chemicals and solutions

Compounds I and II were supplied by the Chemical Development Department of Farmitalia Carlo Erba (Milan, Italy). Norgestrel (internal standard, I.S.) was supplied by Sigma (St. Louis, MO, USA). All other chemicals and solvents were analytical grade from Farmitalia Carlo Erba (Milan, Italy). Stock solutions were prepared by dissolving a weighed amount of each compound in an acetonitrile–H_2O (4:6, v/v) solution. From these solutions working dilutions were prepared with distilled water.

Equipment

The HPLC system used in this study consisted of a Spectra Physics Model P 1000 isocratic pump (Santa Clara, CA, USA), a Spectra Physics Model AS 3000 temperature controlled autosampler equipped with a Rheodyne Model 7010-090 sampling valve with a 200-μl loop and a Knauer UV variable-wavelength detector. The peaks were recorded with a Spectra Physics Model SP 4270 integrator connected to an IBM computer equipped with Labnet software (Spectra Physics).

Chromatographic conditions

The chromatographic separation was performed with a 125 mm × 4.6 mm I.D. Lichrocart RP18 column, particle size 5 μm (Merck, Darmstadt, Germany) with a Survival pre-column packed with Pellicular ODS (particle size 37–53 μm; Whatman, Clifton, NY, USA). The mobile phase was acetonitrile–0.05 *M* KH_2PO_4, pH 4.5 (35:65, v/v). The flow-rate was set at 1.5 ml/min. The detector was set at 247 nm and a 100-mV output signal (0.08 absorbance units full scale, AUFS) was set to the integrator.

Sample extraction procedure

To 1 ml of human plasma, 0.1 ml of internal standard solution (*ca.* 100 ng) was added. The solution was placed in a 10-ml conical glass centrifuge tube and mixed with 0.6 ml of 0.5 *M* pH 7.4 KH_2PO_4. After the addition of 3 ml of methylene chloride–iso-octane mixture (2:3, v/v), the tube was capped and shaken on a mechanical stirrer for 10 min, then centrifuged at 1200 *g* for 15 min in order to clearly separate the two phases. The upper organic phase was transferred to another tube, and the extraction step was repeated. The combined organic phases were evaporated to dryness at 37°C under a stream of nitrogen. The residue was redissolved by addition of 200 μl of acetonitrile–H_2O mixture (1:1, v/v), and subsequently an aliquot (150 μl) was injected onto the chromatograph.

Determination of quality control and calibration samples

Analyses of blank human plasma spiked with known amounts of I, II and I.S. were carried out applying the above described procedure. Linearity was evaluated from six calibration curves prepared and run on six different days in the concentration range 10–1000 ng/ml plasma for both compounds. The precision and the accuracy were evaluated by repeated analyses of the two compounds in plasma at three concentrations (*ca.* 20, 250, 700 ng/ml) in 3 replicate samples analyzed on five different days.

All chromatograms obtained were evaluated by peak-height measurement. The quality controls and the unknown samples were calculated with the calibration curve generated on each day by least-square linear regression (weighting factor $1/y$) of the analyte/I.S. peak-height ratio against their concentration ratio in plasma. To evaluate the extraction recovery, the peak height of extracted plasma samples was compared to the peak height obtained with unextracted standard solution injected directly onto the chromatograph.

Suitability test of the chromatographic system

On each day, before the analysis of unknown and/or calibration samples the performance of the chromatographic system was checked in order to ensure that controlled conditions were used in the assay. Three parameters were used to define the suitability of the chromatographic system [13]. Analysis of the extracted samples was carried out only if the values of the three parameters checked were within the range of values described below.

Column efficiency. This was evaluated as the number of theoretical plates of the column calculated from the equation $N = 5.54\,(t_R/W)^2$, where t_R is the retention time (mm) of the compound tested and W is the peak width (mm) at half height. The value of N must be at least 5000 for each compound.

Peak symmetry. This was evaluated as symmetry factor *SF*, calculated from the equation $SF = W_{0.05}/2A$, where $W_{0.05}$ is the peak width (mm) measured at 1/20 of the peak heigth and A is the distance (mm) between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20 of the peak height. *SF* must be 1.3 or less.

Resolution factor. The resolution factors between the peaks of II *vs.* I and I *vs.* I.S. were calculated from the equation $R = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 refer to the respective retention times, w_1 and w_2 are their baseline band-widths. R must be greater than 2.0.

RESULTS AND DISCUSSION

Fast and reliable separation of analytes was achieved using a Lichrocart RP18 column eluted isocratically with acetonitrile–50 m*M* K_2HPO_4, pH 4.5 (35:65, v/v). The use of the phosphate buffer increased the sharpness of the chromatographic peaks, improving the performance of the column for the test compounds. Under the described conditions the retention times of the three analytes were *ca.* 10 (II), 14 (I) and 17 min (I.S.) (Fig. 2a). Several studies carried out for the determination of different steroids in biological fluids [14–16] employed a solid phase extraction (SPE) as pre-purification step before the HPLC analysis. Although SPE could also be employed

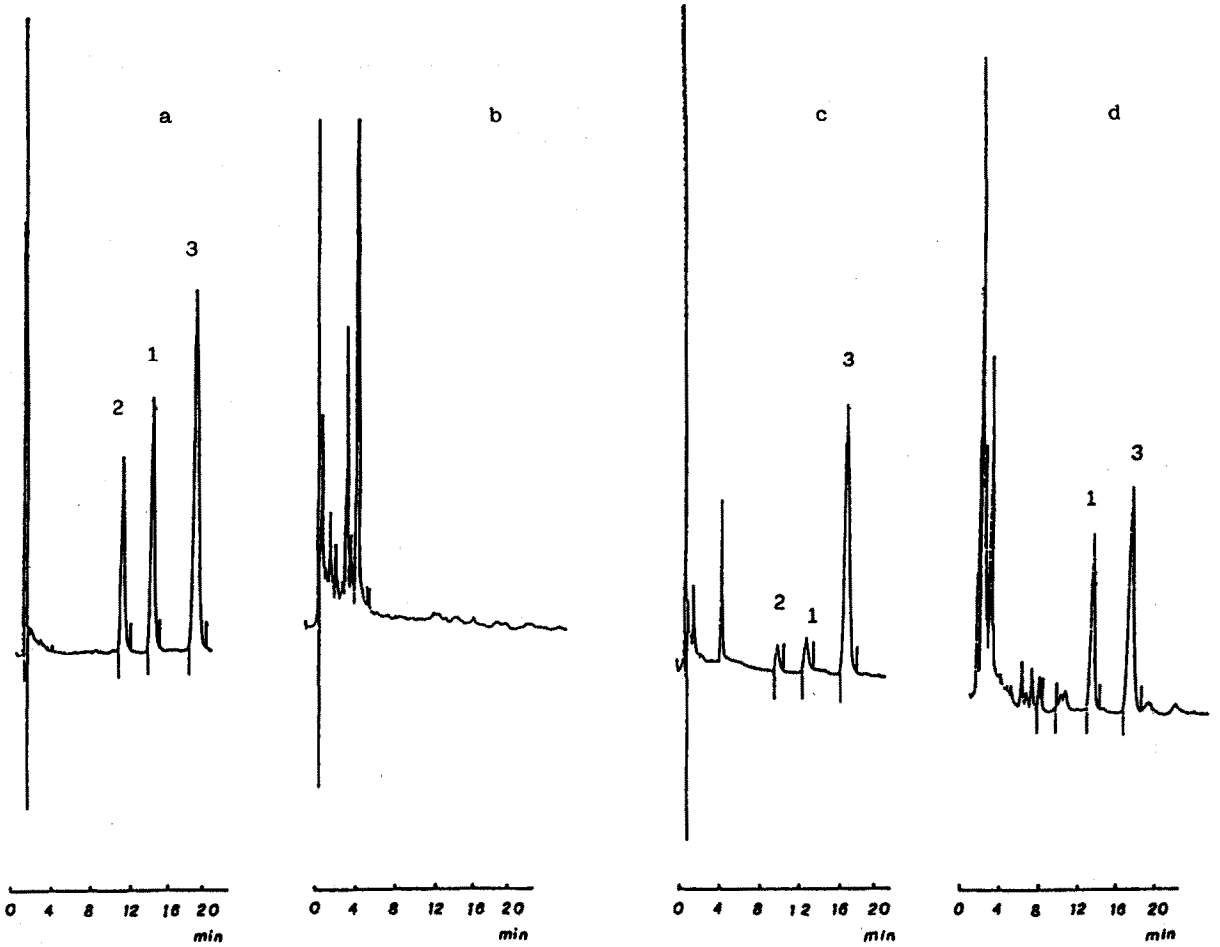

Fig. 2. (a) Chromatogram of unextracted standards, 50 ng of I (peak 1), 50 ng of II (peak 2) and 100 ng of Norgestrel (peak 3); (b) chromatogram of blank human-plasma (1 ml); (c) chromatogram of human plasma (1 ml) spiked with 10 ng of I (peak 1), 10 ng of II (peak 2) and 100 ng of Norgestrel (peak 3);(d) chromatogram of a plasma sample obtained from a healthy male volunteer 2 h after a single 200-mg oral dose of exemestane.

TABLE I

EXTRACTION RECOVERY OF THE THREE ANALYTES FROM PLASMA

$n = 5$.

Analyte	Label concentration in plasma (ng/ml)	Absolute recovery (%)	C.V. (%)
I	10	66.7	6.7
	200	71.5	3.7
	500	79.1	2.1
II	10	73.6	4.4
	200	68.8	5.6
	500	77.8	3.0
Norgestrel	100	76.0	6.1

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF FCE 24304 IN PLASMA

Control sample (ng/ml)	Day	No.	Accuracy			Precision	
			Mean found	Found/added (%)	S.D.	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day)
20.0	1	3	20.6	103.0	0.22	1.1	
	2	3	18.8	94.0	0.34	1.8	
	3	3	19.9	99.4	0.23	1.2	
	4	3	20.2	100.9	0.63	3.1	
	5	3	19.2	97.8	0.61	3.2	3.9
250.2	1	3	248.6	99.4	3.89	1.6	
	2	3	251.2	100.4	2.72	1.1	
	3	3	253.4	101.3	0.84	0.3	
	4	3	253.1	101.2	7.25	2.9	
	5	3	249.8	99.8	4.21	1.7	1.6
695.0	1	3	701.9	101.0	11.77	1.7	
	2	3	715.2	102.9	10.85	1.5	
	3	3	707.4	101.8	2.32	0.3	
	4	3	701.0	100.9	10.17	1.5	
	5	3	706.3	101.6	6.48	0.1	1.3

TABLE III

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF FCE 25071 IN PLASMA

Control sample (ng/ml)	Day	No.	Accuracy			Precision	
			Mean found	Found/added (%)	S.D.	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day)
20.0	1	3	18.7	98.8	1.61	8.2	
	2	3	16.9	84.5	1.38	8.1	
	3	3	18.7	93.5	0.17	0.9	
	4	3	19.9	99.5	2.86	14.3	
	5	3	18.9	94.2	3.18	16.8	11.2
250.6	1	3	219.9	87.7	3.44	1.6	
	2	3	221.2	88.3	2.21	1.0	
	3	3	223.7	89.3	0.67	0.3	
	4	3	244.2	97.5	40.05	16.4	
	5	3	223.4	89.1	1.87	0.8	8.0
716.0	1	3	643.8	89.9	11.77	1.8	
	2	3	647.1	90.4	4.69	0.7	
	3	3	653.5	91.3	4.30	0.7	
	4	3	698.9	97.6	103.36	14.8	
	5	3	654.9	91.5	3.62	0.6	6.8

for the extraction of exemestane, the use of liquid–liquid extraction gave less interferences from the plasma matrix and was therefore preferred. Blank human plasma assayed as described showed no significant peak at the retention time of the compounds of interest (Fig. 2b). The extraction recovery from plasma calculated for the two compounds at three concentrations (*ca.* 10, 200 and 500 ng/ml) ranged from 66.7 to 79.1% (I) and from 68.8 to 77.8 (II) (Table I).

Norgestrel showed good performances under the chromatographic conditions employed for the analysis of I and II. It did not interfere with the analytes nor with any component of the biomatrix since it was eluted in a free zone of the chromatogram. The mean extraction recovery of Norgestrel tested at a concentration of 100 ng/ml plasma was 76%: it was therefore considered suitable as I.S. in the assay.

The linearity of this HPLC assay was evaluated from six calibration curves run on different days in the concentration range 10–1000 ng/ml. A linear regression analysis of the peak-height ratio (I/I.S. and II/I.S.) *vs.* the concentration ratio (I/I.S. and II/I.S.) showed linearity over the whole range of concentrations tested. The mean slope was 1.17 (C.V. = 2.4%) for I and 1.16 (C.V. = 3.9%) for II. Back-calculated concentrations exhibited a C.V. of less than 4% for both compounds. Correlation coefficients (r) ranged from 0.9998 to 1 for I and from 0.9995 to 0.9999 for II.

The inter-day precision for concentrations between 20 and 700 ng/ml plasma expressed as C.V. ranged from 1.3 to 3.9% (I) (Table II) and from 6.8 to 11.2% (II) (Table III). At the same concentrations, the intra-day precision was better than 3.2% for I (Table II) and better than 16.8% for II (Table III).

The accuracy, evaluated on the same plasma samples and expressed as percentage ratio of the amount found to the amount added to plasma, ranged from 94 to 103% (I) (Table II) and from 84.5 to 99.5% (II) (Table III).

The instrumental limit of detection for both compounds (defined as the mass of the analyte on column able to produce a signal-to-noise ratio (S/N) of 3 in the absence of plasma) was 2 ng on column. The lower limit of quantification (LLOQ) for plasma samples was 10 ng/ml for both compounds ($S/N > 5$) (Fig. 2c).

The stability of I and II in plasma was also studied in spiked samples (at concentrations of *ca.* 30, 180 and 900 ng/ml) stored for 1, 3 and 6 months at −20°C and then assayed by the present HPLC method. Under these conditions both compounds proved to be stable. Compounds I and II were also stable when stored dissolved in acetonitrile–H_2O mixture for at least 24 h at room temperature, therefore allowing the use of an autosampler for automatic injection of the extracted samples onto the HPLC.

The present method was applied to the determination of plasma levels of the parent compound and its metabolite in several toxicity studies and in the first clinical studies. In one of these studies carried out at the Charing Cross & Westminster Hospital, London, after protocol approval by the institutional Ethics Committee, eight healthy male volunteers, who had given written informed consent, received a single oral dose of exemestane (200 mg, capsules). The subjects were between 19 and 31 years of age and between 58 and 91 kg body weight. The drug was administered 15 min after a high-fat breakfast, blood samples were drawn into heparinized tubes at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 24, 32 and 48 h after dosing. The samples were immediately centrifuged at 1200 *g* for 10 min. The plasma separated was stored at −20°C until assayed. A typical chromatogram obtained from these *in vivo* samples is shown in Fig. 2d. The only compound detected in plasma was I, the concentration of II being below the LLOQ of the method during this study, although it was detectable in some subjects in another study [12]. Fig. 3 shows the mean plasma levels ± S.E.M. of unchanged drug found after a single oral 200-mg dose of exemestane. Plasma concentrations peaked 4 h after dosing (C_{max} *ca.* 64 ng/ml), then declined to 18 ng/ml at 8 h and fell below the LLOQ 12 h after dosing.

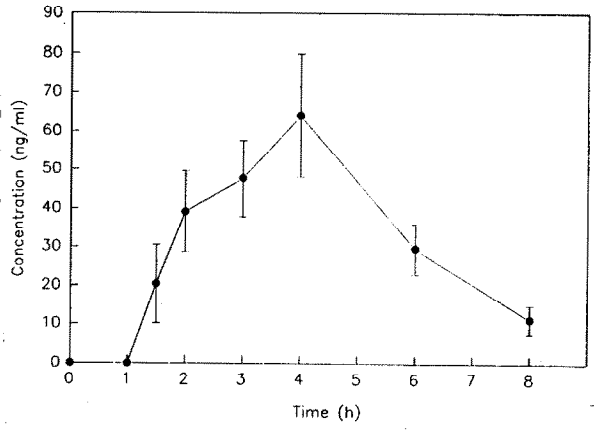

Fig. 3. Mean plasma levels ± S.E.M. of exemestane after a single 200-mg oral administration of exemestane to eight healthy male volunteers.

CONCLUSION

The method described here is selective for the determination of I and its metabolite, II, in plasma. It proved to be linear, precise and capable of accurately quantifying the two compounds in the concentration range 10–1000 ng/ml. The suitability of this method was demonstrated in a pharmacokinetic study of exemestane in humans. Therefore this method can be useful in further investigations on the pharmacokinetics of exemestane in preclinical and clinical studies with the test compound.

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