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High-performance liquid chromatographic determination of FCE 24928, a new aromatase inhibitor, in human plasma

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic method for the determination of FCE 24928 (4-aminoandrosta-1,4,6-triene-3,17-dione) in human plasma is reported. The drug was extracted from buffered (pH = 8) plasma samples with methylene chloride–isooctane, then analysed by reversed-phase liquid chromatography. Quantitation was achieved by ultraviolet detection of the eluate at 238 nm. Blank plasma samples from humans, dog and rat assayed as described showed no significant peak at the retention time of the compound of interest. The suitability of the method for *in vivo* samples was tested by measuring the plasma levels of FCE 24928 in rats that received oral doses of the test compound.

INTRODUCTION

Increasing attention is being focused upon the possibility of treating estrogen-dependent diseases [1], such as breast cancer, by inhibition of aromatase, the enzyme catalysing the conversion of androgen to estrogen [2].

Some promising irreversible aromatase inhibitors, including exemestane [3], a molecule synthesized and studied in the Laboratories of Farmitalia Carlo Erba, are at various stages of clinical development, and one of them, formestane [4], has been recently introduced onto the market for the treatment of post-menopausal breast cancer.

In a programme aimed at finding new irreversible aromatase inhibitors, a series of 4-aminoandrostenedione derivatives were synthesized and tested for their aromatase inhibitory properties [5,6]. Among them, FCE 24928 (Fig. 1) was found to be active both *in vitro* and *in vivo* [7]. The compound was orally active, showed no affinity for the rat androgen receptor and in immature castrated rats did not show androgenic activity [8].

In addition, FCE 24928 was effective against 7,12-dimethylbenzoanthracene-induced mammary tumours in ovariectomized rats treated with testosterone propionate, a post-menopausal mammary tumour model [9].

In the present paper a sensitive and selective HPLC assay for the determination of FCE 24928 in human plasma and its validation are described. The method has been developed for the quantitation of the compound in rat, dog and human plasma during toxicokinetic and pharmacokinetic studies.

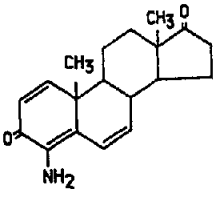

Fig. 1. Structural formula of FCE 24928 (4-aminoandrosta-1,4,6-triene-3,17-dione).

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EXPERIMENTAL

Chemicals and solutions

FCE 24928 was supplied by the Chemical Development Department of Farmitalia Carlo Erba. All other chemicals and solvents were analytical grade from Farmitalia Carlo Erba (Milan, Italy). Buffer solutions were filtered before use through a Millipore 0.45-μm filter. The stock standard solution was prepared by dissolving a weighed amount of FCE 24928 in 0.1 *M* H_3PO_4. From this solution (stable for at least 2 weeks if stored at 0–4°C in the dark) working solutions were prepared daily by dilution with 0.1 *M* H_3PO_4.

Equipment

The chromatographic system consisted of a Milton Roy (Rochester, NY, USA) Model CM III pump equipped with a Rheodyne Model 7125 sampling valve with a 100-μl loop, a Fisons Instruments Model MICRO UVIS20 variable-wavelength detector (Milan, Italy) and a Spectra-Physics (San José, CA, USA) Model SP4100 integrator.

Chromatographic conditions

The chromatographic separation was performed on a Lichrocart, Superspher 100 RP-18 analytical column (25 cm × 4 mm I.D.; particle size 4 μm) purchased from Merck (Darmstadt, Germany), at ambient with a flow-rate of 0.4 ml/min. Isocratic elution with a mobile phase consisting of acetonitrile–0.05 *M* KH_2PO_4 adjusted to pH 4.3 with 1 *M* H_3PO_4 (1:1, v/v) was used. The detection wavelength chosen was 238 nm.

Sample extraction

Human plasma (1 ml) was placed into a 10-ml conical glass centrifuge tube, the working FCE 24928 solution (0.1 ml) added and mixed with 1 *M* pH 8 borate buffer (0.5 ml). The mixture was extracted with 3 ml of a methylene chloride–isooctane mixture (2:3, v/v) on a rotating tube holder (10 min) then centrifuged (10 min, 1200 *g*). The organic layer was collected and the extraction step was repeated as above. The organic phases were then combined and evaporated to dryness at room temperature in the dark under a stream of nitrogen. After reconstitution of the residue with the mobile phase (150 μl), an aliquot (100 μl) was injected onto the column.

Assay validation

Blank human plasma samples spiked with a known amount of the FCE 24928 working solution were processed as described above. All chromatograms obtained were evaluated by peak-height measurement. Three calibration curves were prepared and run on three different days in the concentration range 2.16–540 ng/ml in order to evaluate the linearity of the method. The precision was assessed by analysing three replicate samples of five concentrations (2.16, 10.8, 54, 108 and 540 ng/ml) prepared on three different days. The accuracy, expressed as mean percentage ratio of found to added amounts, was evaluated by repeated analyses carried out at three concentrations (13.5, 216 and 432 ng/ml).

The percentage of drug recovered was calculated by comparison of the peak height of the extracted plasma samples with the peak height obtained from direct injection of the unextracted standard solutions at four different concentrations.

Chromatographic system suitability test

The suitability [10] of the chromatographic system was checked daily before analysis by evaluating the following three parameters.

Column efficiency. This was evaluated as the number of theoretical plates of column calculated by the equation $N = 5.54(t_R/W)^2$, where t_R is the retention time (mm) of FCE 24928 and W (mm) is the peak width at half-height. N must be $\geq$9000.

Peak symmetry. This was evaluated as symmetry factor S_F calculated from the equation $S_F = W_{0.05}/2A$ where $W_{0.05}$ is the peak width (mm) measured at 1/20 of the peak height 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. S_F must be in the range 1–1.2.

System reproducibility. The detection response of four replicate injections of a standard

solution (50–100 ng/ml) must show a relative standard deviation (R.S.D.) not exceeding 2%.

RESULTS AND DISCUSSION

A range of pH values (4.5–8.8) was explored in order to define the best extraction yield of the product from plasma, and the maximum recovery was obtained at pH 8.

From several solvents (*e.g.* methylene chloride, ethyl acetate, diethyl ether, isooctane) tested as extractants, a mixture of methylene chloride–isooctane (2:3, v/v) provided the best compromise between high recovery of FCE 24928 and an acceptable degree of coextraction of interfering plasma components. The mean recovery of FCE 24928, using the liquid–liquid extraction conditions described above, was 72% with 6.9% R.S.D. (Table I).

A satisfactory and reliable separation of FCE 24928 from endogenous substances coextracted from human plasma samples was obtained with the Superspher C_{18} reversed-phase analytical column and the mobile phase system reported in the Experimental section (Fig. 2). Blank plasma samples from rat and dog assayed as described showed no significant peak at the retention time of the compound of interest (Fig. 3). The UV spectrum of FCE 24928 recorded in the mobile phase in the range 220–600 nm showed the highest absorption maximum at 238 nm; therefore this wavelength was chosen to quantify the product.

TABLE I

EXTRACTION RECOVERY OF FCE 24928 FROM HUMAN PLASMA ($n = 3$)

Concentration added (ng/ml)	Absolute recovery (%)	R.S.D. (%)
10.8	72.8	1.9
54.0	68.6	3.9
108.0	67.9	3.7
540.0	79.0	1.7
Overall average ($n = 12$)	72.1	6.9

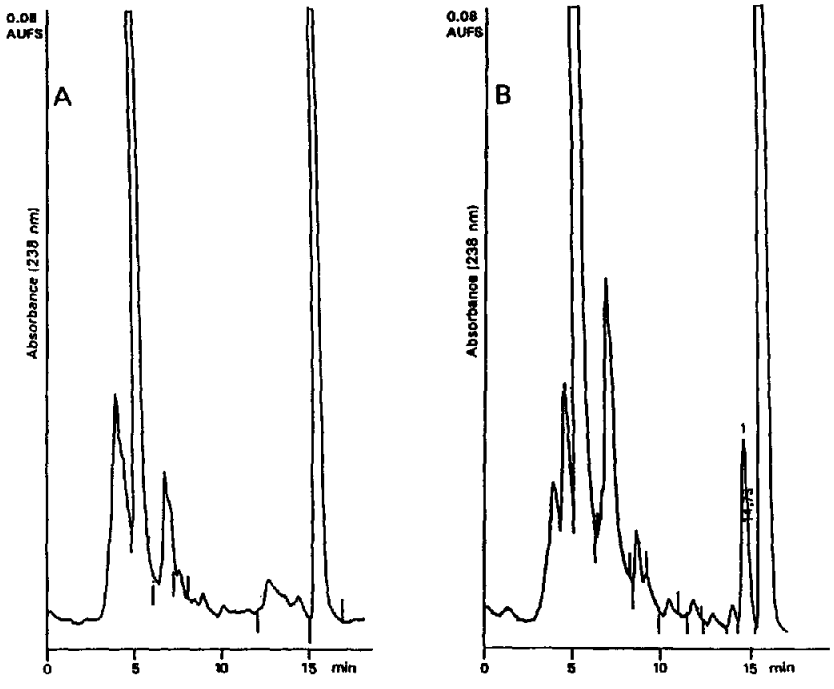

Fig. 2. Chromatograms obtained by assaying (A) 1 ml of human blank plasma and (B) 1 ml of human plasma spiked with 10.8 ng of FCE 24928 (1).

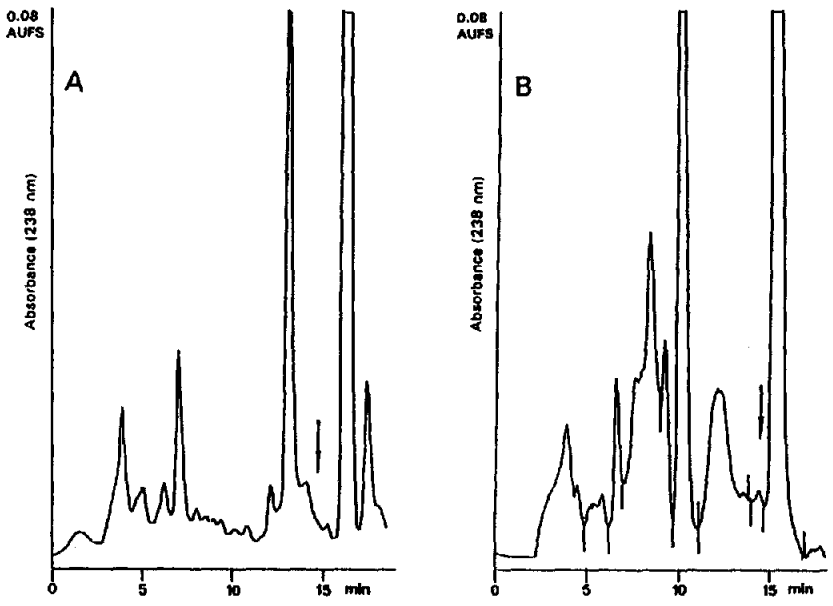

Fig. 3. Chromatograms obtained by assaying (A) 1 ml of blank dog plasma and (B) 1 ml of blank rat plasma. Arrows indicate the peak position of FCE 24928.

TABLE II

INTRA- AND INTER-DAY PRECISION OF THE METHOD FOR THE DETERMINATION OF FCE 24928 IN HUMAN PLASMA

Control sample (ng/ml)	Day	n	Mean found (ng/ml)	S.D.	R.S.D. (%)	
					Intra-day	Inter-day ($n = 9$)
2.16	1	3	2.15	0.17	7.90	
	2	3	2.40	0.17	7.08	
	3	3	2.23	0.04	1.79	7.38
10.8	1	3	11.25	0.23	2.04	
	2	3	11.25	0.77	6.84	
	3	3	11.18	0.74	6.62	4.87
54.0	1	3	52.49	2.30	4.38	
	2	3	50.01	3.67	7.34	
	3	3	48.61	2.22	4.57	5.89
108.0	1	3	100.6	1.65	1.64	
	2	3	98.0	3.61	3.68	
	3	3	102.5	5.76	5.62	3.98
540.0	1	3	577.0	35.18	6.09	
	2	3	549.0	26.49	4.82	
	3	3	582.6	2.77	0.48	4.74

TABLE III

ACCURACY OF THE METHOD FOR THE DETERMINATION OF FCE 24928 IN HUMAN PLASMA

Control sample (ng/ml)	n	Mean found (ng/ml)	Found/ added (%)	S.D. (%)	R.S.D. (%)
13.5	4	12.78	94.6	2.5	2.6
216.0	4	204.40	94.7	6.5	6.8
432.0	4	413.07	95.6	5.5	5.8
Overall average ($n = 12$)			95.0	4.7	4.9

Three separate calibration curves obtained on three different days by plotting the peak height *versus* concentration were found to be linear when evaluated by linear regression analysis in the concentration range 2.16–540 ng per ml of human plasma. The correlation coefficients were > 0.999 and the mean slope was 228 (R.S.D. = 3.8%). Intercept values, when submitted to Student's t test were not significantly different from zero ($p > 0.05$).

The inter-day precision ranged from 3.98 to 7.38% R.S.D. for concentrations between 2.16 and 540 ng/ml, and the intra-day precision was better than 7.9% R.S.D. (Table II).

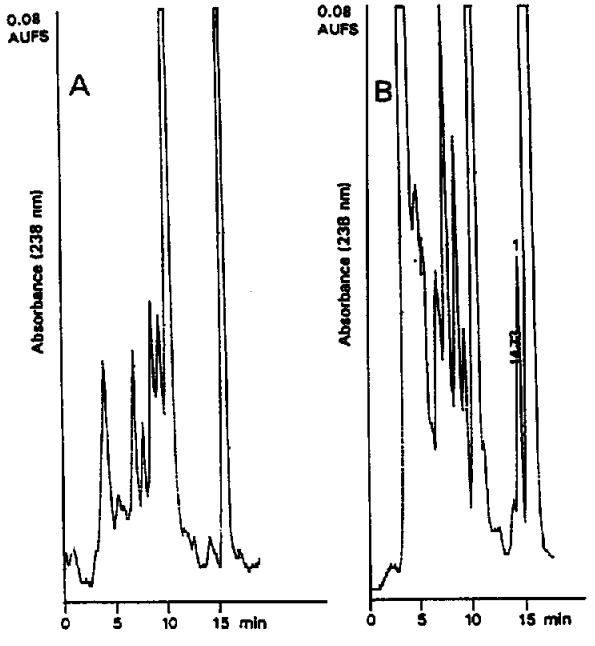

Fig. 4. Chromatograms obtained by assaying (A) 1 ml of rat plasma from a control animal and (B) 1 ml of rat plasma 4 h after oral administration of 40 mg/kg FCE 24928 (1).

The accuracy, expressed as mean percentage of the found to added amounts calculated at three concentrations (13.5, 216 and 432 ng/ml) with samples analysed on different days, was 95% with 4.9% R.S.D. (Table III).

The limit of quantitation, defined here as the lowest plasma concentration that can be measured routinely with acceptable precision (R.S.D. $< 10\%$) and accuracy (bias $\pm$ 10%), was 2.16 ng/ml (see Tables II and III).

The developed method has been used in a preliminary study to monitor the plasma levels of FCE 24928 after administration of a single oral dose of 40 mg/kg to three rats.

Blood samples were drawn from the animals kept under ether anaesthesia, from sublingual vein at 1 and 4 h and from abdominal aorta at 24 h after administration, and collected into heparinized tubes. Plasma was separated after centrifugation at 1200 *g* for 10 min at room temperature, then stored at −20°C until assayed. A typical chromatogram obtained from these *in vivo* samples is shown in Fig. 4B. Blank plasma samples of control animals showed no detectable peak with the same retention time as FCE 24928 (Fig. 4A). Mean plasma concentrations of 407 $\pm$ 85 ng/ml (mean $\pm$ standard error of the mean) occurred at 1 h after administration, then a relatively rapid disappearance of the compound was observed with mean levels of 75 $\pm$ 20 ng/ml and of 12 $\pm$ 4 ng/ml at 4 and 24 h, respectively. It may be interesting to express in molarity the

mean plasma concentrations obtained at 1, 4 and 24 h after administration of 40 mg/kg, a dose which is about three times the ED_{50} for reduction of ovarian aromatase activity in the rat [8]. These values (1368, 252 and 40 nM) are higher than or similar to the K_i value for inhibition of human placental aromatase [8].

CONCLUSIONS

The isocratic reversed-phase HPLC method described here is the first analytical method for the selective determination of FCE 24928 in plasma. It proved to be reproducible, linear, precise and accurate in the concentration range 2.16–540 ng/ml; therefore it is suitable for monitoring the unchanged drug plasma levels in animals and in humans.

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