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Determination of Plasma Levels of Exemestane (FCE 24304), a New Irreversible Aromatase Inhibitor, Using Liquid Chromatography/ Thermospray Mass Spectrometry

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A high performance liquid chromatographic mass spectrometric method with thermospray ionization for the determination of concentrations of exemestane (6-methylenandrosta-1,4-diene-3,17-dione, FCE 24304) in human plasma has been developed. The sensitivity of the method allowed the determination of exemestane concentrations as low as 1 ng ml^{-1}. A suitable internal standard was used for quantification. The intra-day precision (relative standard deviation, R.S.D.) ranged from 16.3% near to the lower limit of quantification to 0.3% close to the upper end of the calibration graph. The mean accuracy of the method was found to be 97.8% (R.S.D. = 7%). Unlike a previously developed gas chromatographic/mass spectrometric method, the new method proved to be suitable for the determination of unchanged drug plasma levels in exemestane-treated subjects.

INTRODUCTION

Exemestane (6-methylenandrosta-1,4-diene-3,17-dione, FCE 24304; Fig. 1) is an irreversible aromatase inhibitor currently under investigation for the treatment of breast cancer.[1–3] Inhibitors of aromatase, the enzyme responsible for the conversion of androgens to estrogens, are potentially useful therapeutic agents for estrogen-dependent tumours such as breast cancer.[4]

The pharmacokinetics of single doses of exemestane have been investigated in 12 healthy post-menopausal volunteers after administration of single doses of 50, 200, 400 and 800 mg of exemestane.[5] Plasma levels were determined by HPLC with UV detection[6] and were found to decline rapidly, falling below the limit of quantification (10 ng ml^{-1}) at 4 h (50 mg) and 24 h (200 and 400 mg) post-dosing. However, there is evidence that exemestane plasma levels lower than the detection limit of the HPLC assay may still be pharmacologically active[5]. Therefore, it was deemed necessary to develop a more sensitive method in order to detect plasma levels lower than 10 ng ml^{-1}.

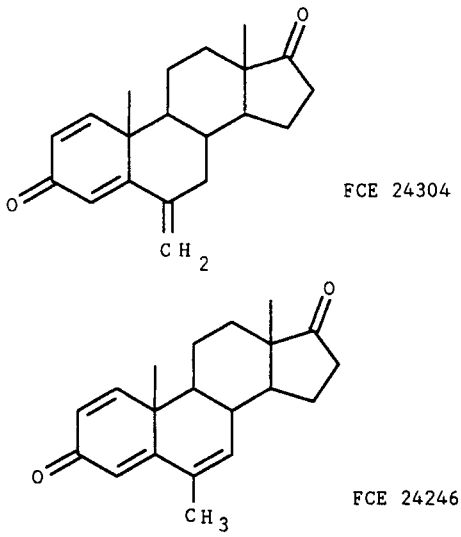

Figure 1. Structures of exemestane (FCE 24304) and FCE 24246 (internal standard).

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The combination of high-resolution gas chromatography with mass spectrometry (GC/MS) was the first approach investigated for the determination of low plasma levels of exemestane. A GC/MS method was developed with a quantification limit of 0.25 ng ml^{-1} of exemestane in plasma. Briefly, the method entailed a solid–liquid extraction with methanol as eluent followed by GC/MS with chemical ionization. Despite the good results obtained during the validation of the method using blank human plasma (accuracy 99.2 ± 7.4%, relative standard deviation (R.S.D.) 7.4%; $n = 54$), this method proved unsuitable for the analysis of plasma from treated subjects, most likely because of the presence of thermolabile exemestane-producing metabolite(s) in plasma from 8 h onwards.

As an alternative, the combination of HPLC with mass spectrometry with thermospray (TSP) ionisation, together with a more selective extraction procedure, was then envisaged. TSP ionization has often been found to be a suitable method for scarcely volatile and thermolabile compounds. In addition, free and conjugated[7–11] steroids were successfully analysed by HPLC/TSP-MS. In this paper we report a procedure that has been developed for the determination of unchanged drug concentrations in plasma of subjects administered exemestane. A fast and simple extraction procedure was used, followed by quantitative analysis using HPLC/TSP-MS

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and an appropriate internal standard (I.S.) (6-methylandrosta-1,4,6-triene-3,17-dione, FCE 24246; Fig. 1). The method allowed the determination of exemestane plasma levels as low as 1 ng ml^{-1}. The suitability of the method was established by analyses of plasma samples obtained from subjects treated with exemestane. The plasma concentration versus time profile after administration of a single oral dose of 50 mg of exemestane to a post-menopausal healthy volunteer is also presented.

EXPERIMENTAL

Chemicals and reagents

FCE 24304 and FCE 24246 (I.S.) were synthesized at Farmitalia Carlo Erba (Milan, Italy). C_{18} cartridges (200 mg) were obtained from Lida (Kenosha, WI, USA). Ammonium acetate (purity 98%) and all analytical-grade reagents were supplied by Farmitalia Carlo Erba (Milan, Italy).

HPLC/MS equipment and instrumental conditions

A Finnigan Mat (San Jose, CA, USA) Model 4600 quadrupole mass spectrometer equipped with a Super Incos data system and a TSP1 thermospray source modified as follows was used. The original vaporizer pulsed heater controller (18 V a.c.) was replaced with a new constant heater controller (24 V d.c.). This system maintains the stability of the vaporizer temperature within ± 1 °C. The source temperature controller was replaced with a more stable one (± 2 °C) and the original repeller and lens controllers were replaced with a new power supply with a better voltage stability (± 1 V). The main advantage of these modifications was a definite reduction in the flickering of the signals, providing a more precise calculation of the peak areas.

A Waters 600-MS HPLC pump (Millipore, Milford, MA, USA) equipped with an ODS Ultrasphere column, 250 × 4.6 mm i.d., particle size 5 μm (Beckman, San Ramon, CA, USA) and a C_{18} 37–53 μm particle size guard column (Whatman, Maidstone, UK) was coupled to the mass spectrometer. The eluent composition was 0.1 M ammonium acetate buffer (pH 4.5)–acetonitrile (60:40, v/v) at a flow-rate of 1.3 ml min^{-1}. The presence of unknown and non-polar compounds in the plasma extracts made it necessary to wash the chromatographic column with ammonium acetate–acetonitrile (40:60, v/v) for 15 min every two injections of plasma extracts and to recondition the system with the eluent for 5 min.

The TSP parameters at the flow-rate and solvent composition chosen were determined in order to obtain the highest molecular ion abundance and were: vaporizer temperature 80 °C, source block temperature 270 °C and repeller voltage 40 V.

Quantitative data were obtained using selected-ion monitoring (SIM). The ion selected to run was *m/z* 297 (protonated molecular ion MH^+) for both FCE 24304 and FCE 24246 (I.S.) with a total scan time of 0.95 s and a peak width of 0.5 u.

Standard solutions and sample preparation

Stock standard solutions of exemestane and of the I.S. were prepared separately at a concentration of 1 mg ml^{-1} dissolved in the minimum amount of acetonitrile and brought to volume with water. Two working standard solutions were prepared from the stock standard solution of exemestane at concentrations of 1 and 0.1 ng μl^{-1}. One working standard solution containing 0.1 ng μl^{-1} was prepared from the stock standard solution of the internal standard.

Five test-tubes with 1 ml of human plasma each containing 5 ng of internal standard and 1, 5, 10, 25 and 50 ng of exemestane, respectively, and one test-tube containing 1 ml of drug-free human plasma were prepared.

Extraction procedure

A 1 ml volume of plasma was loaded onto the C_{18} cartridge previously conditioned with 10 ml of methanol followed by 20 ml of water. A light vacuum was applied to the manifold of the Vac-Elut chamber (Analytichem International) at each stage of the extraction procedure. The cartridge was washed with 20 ml of water, then exemestane and internal standard were eluted with 5 ml of ethyl acetate. The organic phase was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in 120 μl of HPLC eluent and injected into the HPLC/MS system through a 100 μl fixed-loop sample injector (Reodyne model 7125).

Recovery

The extraction recovery of exemestane from human plasma was assessed with spiked samples at three concentrations (1.5, 8.2 and 40.8 ng ml^{-1}) within the range of interest. The recovery was calculated from the ratio between the peak area of extracted exemestane and the peak area of internal standard added after the extraction process, compared with the ratio of the peak areas of the same amount of the analytes added directly to a drug-free plasma extract. The recovery of the internal standard was calculated by comparing the area counts of the internal standard compound before extraction and the area counts after the extraction procedure.

Calibration graphs

Seven calibration graphs were established using blank human plasma spiked with amounts of exemestane ranging from 1 to 50 ng ml^{-1}. Each sample containing 5 ng ml^{-1} of I.S., was submitted to the whole procedure of extraction and analysis.

Linearity and limit of quantification

Linear calibration graphs were generated by least-squares linear regression of the values $(A_{FCE\ 24304}/A_{I.S.}) \times Q_{I.S.}$ (peak area of FCE 24304/peak area I.S.) × amount of I.S.) versus the concentration of FCE 24304 in plasma (1–50 ng ml^{-1}).

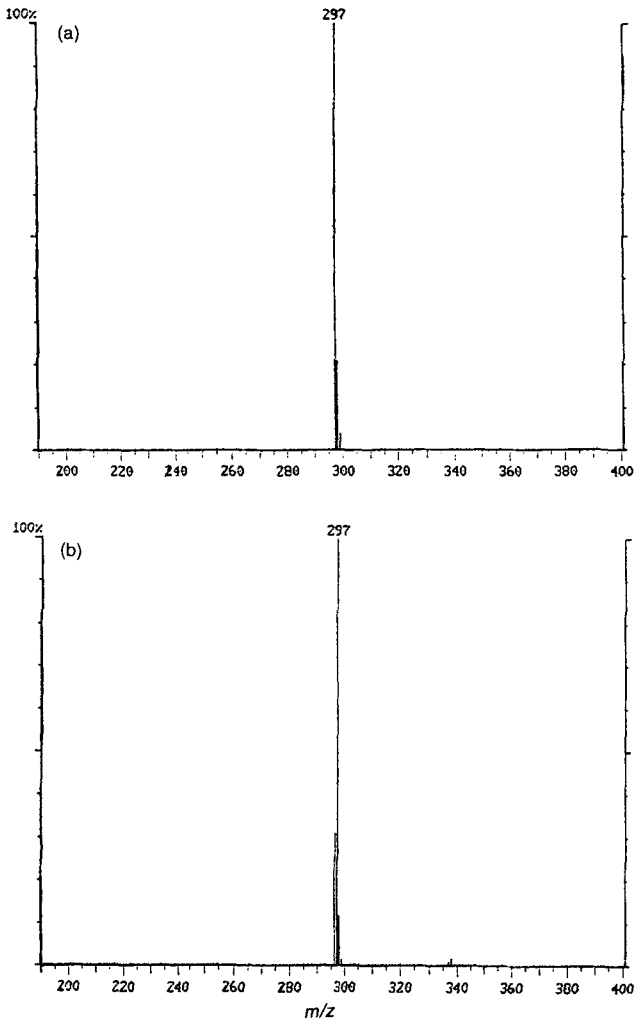

Figure 2. Thermospray mass spectra of (a) FCE 24304 and (b) FCE 24246.

The minimum quantifiable level was defined as the minimum concentration of the analyte in plasma giving a signal-to-noise ratio of at least 4:1 under the assay conditions measured with a good level of confidence.

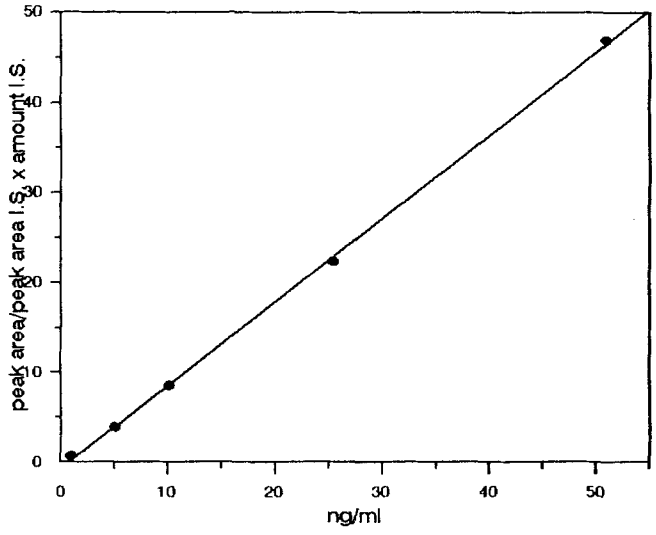

Figure 3. Typical calibration graph for FCE 24304 in plasma in the range 1–50 ng ml^{-1}.

Table 1. Intra-day and inter-day precision of the HPLC/TSP-MS method for the determination of FCE 24304 in human plasma

Spiked amount (ng ml^{-1})	Day	No. of analyses	Precision (R.S.D. (%)) Intra-day	Inter-day
1.53	1	3	7.6	
	2	3	2.9	
	3	3	16.3	
	4	3	3.2	
	5	3	6.3	
	6	3	3.3	9.2 ($n = 18$)
8.16	1	3	2.8	
	2	3	4.5	
	3	3	2.6	
	4	3	3.0	
	5	3	5.4	
	6	3	2.3	5.1 ($n = 18$)
4.08	1	3	4.9	
	2	3	2.4	
	3	3	0.3	
	4	3	6.1	
	5	3	1.6	
	6	3	4.8	5.6 ($n = 18$)

Precision and accuracy

Intra-day and inter-day precisions were assessed by replicate determinations of exemestane concentrations in plasma on the same day and on different days, respectively.

The same samples analysed for the intra-day precision were used to calculate the accuracy of the method.

Determination of exemestane concentration in plasma samples from exemestane-treated subjects[12]

Twelve healthy post-menopausal volunteers were served a light continental breakfast. Within 30 min after completing the meal, they were administered 50 mg of exemestane, swallowed with tap water. A standard lunch was served after the fourth post-dosing blood sampling. Blood samples were collected into heparinized tubes immediately before dosing and at 15 min, 30 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 32 and 48 h post-dosing, and stored at −20 °C pending analysis. Before assay, 5 ng ml^{-1} of I.S, were added to the plasma samples.

RESULTS

FCE 24246 (Fig. 1), chosen as the I.S. was the best alternative to a deuterated compound, which was not available. In fact, FCE 24246, which differs from exemestane only in the position of the double bond in ring B, is likely to have very similar physico-chemical properties and analytical behaviour to those of exemestane. Both compounds generate an abundant protonated molecular ion and show the best sensitivity under the thermospray conditions used (Fig. 2) The internal standard chosen was found not to interfere with the analyte or with endogenous substances present in blank or exemestane-treated human plasma.

Table 2. Accuracy of the assay for exemestane in human plasma

Spiked amount (ng ml^{-1})	*n*	Found/added (%) ±S.D.	R.S.D.
1.53	18	100.0 ± 9.2	9.2
8.16	18	95.8 ± 4.9	5.1
40.8	18	97.6 ± 5.5	5.6
Overall mean	54	97.8 ± 6.9	7.0

The mean recovery of the method, calculated for concentrations of 1.53, 8.16 and 40. 8 ng ml^{-1} of exemestane in plasma, was 90.8 ± 7.0% (R.S.D. 7.7%, $n = 6$). No significant difference in the extraction efficiency was found at the lowest concentration. The recovery of the internal standard was 90%.

The linearity of the response was evaluated on seven different days. The calibration graphs obtained showed excellent linearity and good reproducibility of the slope. The mean calibration graph was defined by the equation $y = 0.88x - 0.672$ (slope R.S.D. = 6.8%; correlation coefficient $r = 0.999$); y-intercept values submitted to Student's t-test were not significantly different from zero ($p > 0.05$). A typical calibration graph is shown in Fig. 3.

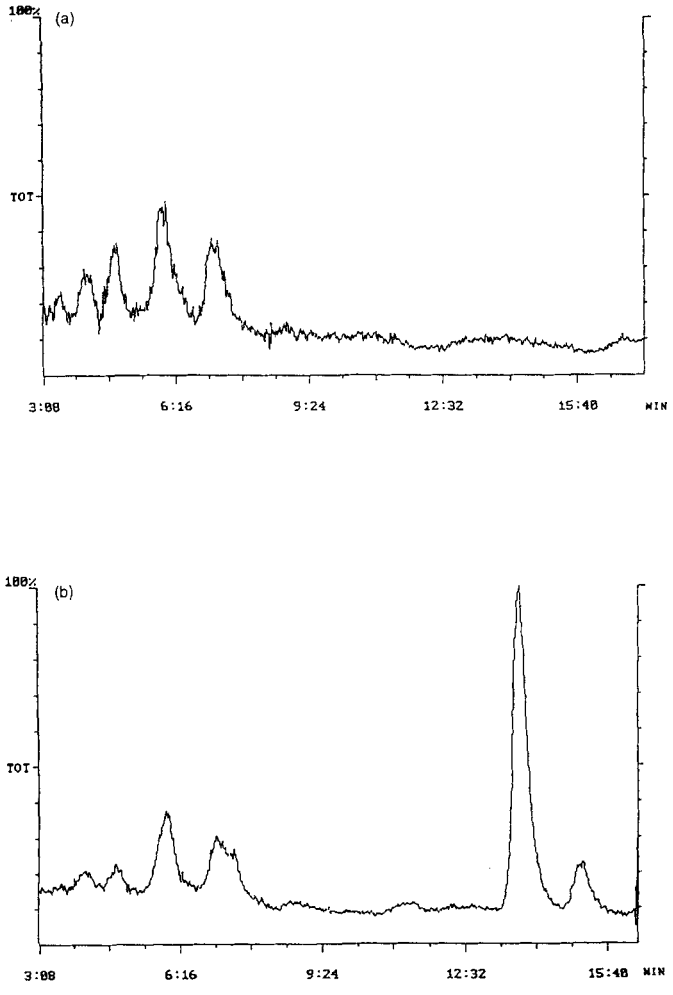

Figure 4. Thermospray selected—ion chromatograms obtained after processing samples. (*a*) 1 ml of blank human plasma. This chromatogram shows the absence of peaks at the retention time of FCE 24304 and I.S. (15.2 and 13.5 min, respectively. (b) 1 ml of plasma spiked with 1 ng of FCE 24304 retention time 15.2 min) and 5 ng of FCE 24246 (I.S.) (retention time 13.5 min).

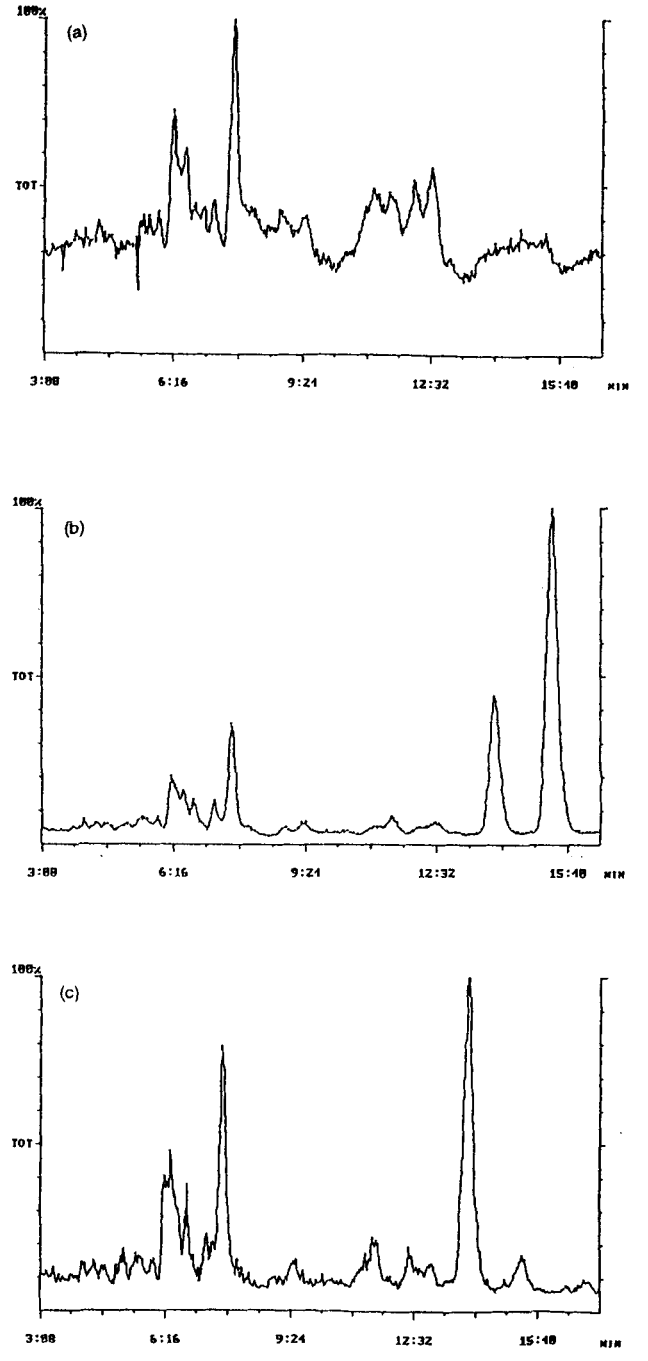

Figure 5. Thermospray selected-ion chromatograms of 1 ml of plasma extract from a healthy subject (B.I.W.) (a) before adminstration of FCE 24304, (b) 1 h after adminstration of a 50 mg dose of the drug and (c) 24 h after adminstration of the drug. FCE 24246 as I.S. (5 ng) was added to plasma in (b) and (c). The amount of FCE 24304 calculated was 13.8 ng at 1 h and <1 ng at 24 h.

The intra-day precision, determined by assaying plasma samples containing three different concentrations of exemestane (1.53, 8.16 and 40.8 ng ml^{-1}) processed in triplicate, was found to range from 0.3 to 16.3% (R.S.D.). The inter-day precision was assessed by analysing plasma samples used for the intra-day precision on six different days (Table 1). The same samples

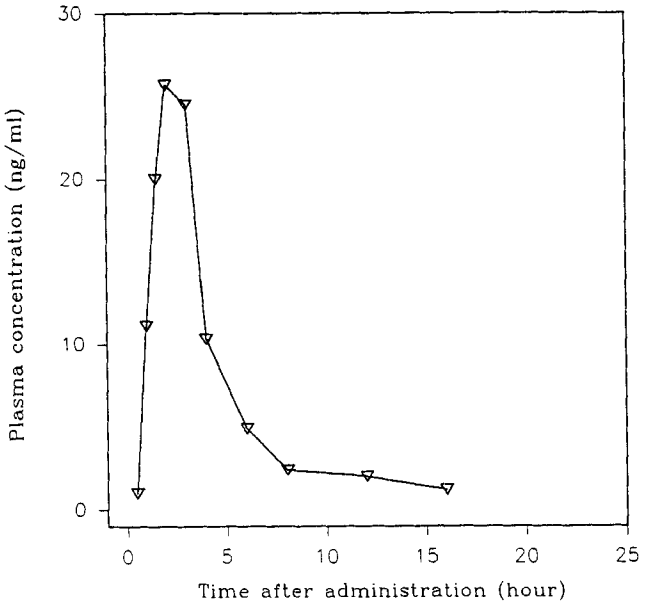

Figure 6. Time course of FCE 24304 plasma concentrations after adminstration of a 50 mg dose to a healthy post-menopausal volunteer (B.I.W.).

were used to calculate the accuracy of the method. The accuracy, defined as the percentage ratio of found to added amounts, was on average 97.8 ± 6.9% (R.S.D. 7.0%, $n = 54$) (Table 2).

Figure 4(a) shows the reconstructed selected-ion chromatogram obtained from 1 ml of blank human plasma processed following the analytical procedure described. No interfering signal was present in the range of retention times of the compounds of interest. The chromatogram obtained from 1 ml of plasma spiked with 1 ng of exemestane and 5 ng of I.S. and submitted to the extraction procedure is shown in Fig. 4(b). The retention times were 15.2 and 13.5 min for exemestane and the I.S., respectively. The limit of quantification was 1 ng ml^{-1}, corresponding to a mean signal-to-noise ratio of 7.7 ± 2.6 (R.S.D. = 33.8%, $n = 7$).

Figure 5(a) shows a chromatogram for a pre-dosing plasma sample from one subject (B.I.W.) who participated in the study. The chromatogram shows the absence of an interference peak at the retention times of exemestane and the I.S. Figure 5(b) and (c) show chromatograms of plasma from blood samples taken 1 and 24 h post-dosing, respectively. The plasma concentration–time profile after administration of a single oral dose of 50 mg exemestane to subject B.I.W. is shown in Fig. 6.

DISCUSSION

The combination of liquid chromatography and mass spectrometry is making a growing contribution to the analysis of biological samples when gas-phase analysis is either not possible or inadequate. In the case of exemestane, the limit of quantification of the HPLC/MS method (1 ng ml^{-1}) was not as low as that obtained with the previously developed GC/MS method (0.25 ng ml^{-1}). However, the mild conditions typical of thermospray ionization, together with the higher selectivity of the extraction procedure compared with that developed previously in the GC/MS method, allowed the determination of exemestane plasma levels in treated subjects. No abnormal course of plasma concentrations was observed, as was the case when the GC/MS method was used. In contrast, a normal plasma concentration–time course was observed, with plasma levels falling below the detection limit 16 h after administration of the drug (Fig. 6).

The modification of the vaporizer heater control module improved the reproducibility of the ionization yield. In fact, both the stability of the signal and the sensitivity of detection were found to depend greatly on the maintenance of a constant temperature in the spray. In addition, the performance of the instrument and the quality of the results obtained are also strictly dependent on the mechanical characteristics of the vaporizer.

The suitability of this HPLC/MS method was established in a pharmacokinetic study where the relative bioavailability of two different formulations of FCE 24304 was determined.[12]

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