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Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 451–460

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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LC–MS–MS determination of exemestane in human plasma with heated nebulizer interface following solid-phase extraction in the 96 well plate format

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Received 10 May 1999; received in revised form 21 September 1999; accepted 26 September 1999

Abstract

A sensitive, specific and rapid analytical method for the quantitation of exemestane (EXE) in human plasma has been developed. EXE, 6-methylen-androsta-1,4-diene-3,17-dione, is an orally active irreversible steroidal aromatase inhibitor used for the therapy of metastatic postmenopausal breast cancer, with estrogen-dependent pathological conditions. The method involves extraction of EXE from human plasma by solid phase extraction using C2 endcapped sorbent in the 96 well plate format (50 mg/2 ml). After conditioning of the sorbent with 1 ml of acetonitrile ($x2$) the plates were rinsed with 1 ml of water ($x2$). The prepared samples (0.5 ml plasma, spiked with [$^{13}C_3$] EXE as internal standard (IS) and diluted with 0.5 ml water) were loaded and drawn through the plate with a minimum of vacuum. The plates were then washed with 1 ml acetonitrile:water (10:90) followed by a drying step for 30 min at full vacuum. Elution was by 0.15 ml of 0.1% trifluoracetic acid in acetonitrile ($x2$) under a minimum of vacuum. Aliquots of 80 μl were finally injected into the LC–MS–MS system. A Zorbax SB C8 column (4.6 × 150 mm, 5 μm) was used to perform the chromatographic separation; the mobile phase was 100% acetonitrile. MS detection used the heated nebulizer interface, with multiple reaction monitoring (MRM) (297 → 121 m/z for EXE and 300 → 123 m/z for IS) operated in positive ion mode. A weighed linear regression analysis (weighing factor $1/x^2$) was used to calculate EXE concentration in standard and unknown samples. The method was fully validated in the concentration range 0.05–25 ng ml^{-1}.

Keywords: Exemestane; Human plasma; LC–MS–MS system; 96 Well plate format

1. Introduction

Estrogens are the most important hormones involved in human breast hormone-dependent cancer [1]. In post-menopausal women, estrogens are produced essentially by the conversion of androstenedione to estrone, via the aromatase enzyme in peripheral tissues [2].

Aromatase is a complex enzyme consisting of two proteins: the aromatase cytochrome P450, a hemoprotein, and reduced nicotinamide adenine dinucleotide diphosphate (NADPH) cytochrome

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PII: S0731-7085(00)00235-1

P450 reductase, that donates electrons to the P450 aromatase [3].

Exemestane (EXE, Fig. 1A), 6-methylen-androsta-1,4-diene-3,17-dione, is a highly specific and irreversible steroidal aromatase inhibitor. EXE binds covalently to the active site cytochrome P450, making it inactive [4].

At receptor level EXE shows good selectivity (both in vitro and in vivo), since it does not interact with other steroidogenic enzymes and has very low or no affinity for the steroid receptors [5].

In the past, three high performance liquid chromatographic (HPLC) methods for plasma determination of EXE were developed; one with UV detection (with limit of quantitation 10 ng ml^{-1}) [6], and the others with mass spectrometric detection (with limit of quantitation 1 ng ml^{-1} [7] and 0.25 ng ml^{-1} [8]. The sample preparation of the last two methods consisted of a solid phase extraction (SPE) of plasma using C18 cartridges and a single step liquid–liquid extraction of plasma with ethylacetate, respectively. As previous methods had insufficient sensitivity to determine the drug in plasma obtained from clinical trials where the doses administered ranged from 10 to 25 mg/day, a high performance liquid chromatographic method combined with radioimmunoassay (RIA) technique, that decreased the limit of quantitation to 0.012 ng ml^{-1}, was developed [9].

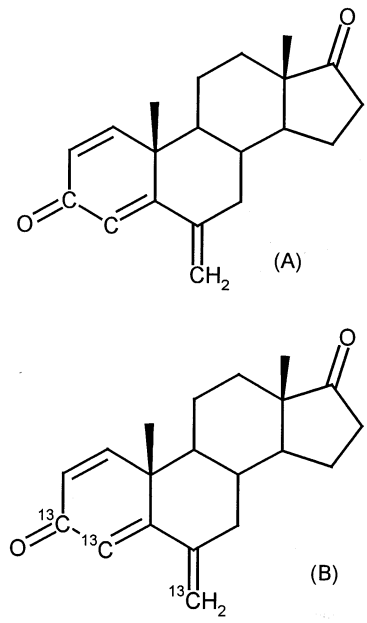

Fig. 1. Structural formulae of exemestane (A) and internal standard (B).

However, the throughput of this method was very poor, as only about 70 samples/week could be analysed. For pharmacokinetic studies where a limit of quantitation of about 0.05 ng ml^{-1} is needed, the analysis time of the method described by Allievi et al., had to be reduced. A new LC–MS–MS method for the determination of EXE in human plasma, using SPE with C2 endcapped normal cartridges, was therefore developed and validated.

In order to further increase the throughput of the method, it was then transferred to the 96 well plate format and revalidated with inclusion of a stable label EXE as internal standard (IS).

2. Experimental

2.1. Chemicals and solutions

EXE and [$^{13}C_3$] EXE, as IS (Fig. 1B) were supplied by the Chemistry Department and by the Radio-Labelled Compound Synthesis Laboratory (DMR Department) of Pharmacia and Upjohn (Nerviano, Milan, Italy), respectively.

Acetonitrile, methanol and trifluoracetic (TFA) acid were analytical grade from Carlo Erba Reagents (Milan, Italy).

Stock solutions of EXE were prepared by dissolving a weighed amount of the compound in methanol and consequently working solutions were prepared by dilution with methanol and distilled water (50:50, v/v) mixture.

Distilled water was produced in house by the Milli-Q Plus 185 instrument (Millipore, Bedford, MA, USA).

2.2. LC equipment and conditions

The HPLC system used in this study included HP 1100 modules (Hewlett Packard, Waldbronn, Germany) in terms of pump, oven and degaser,

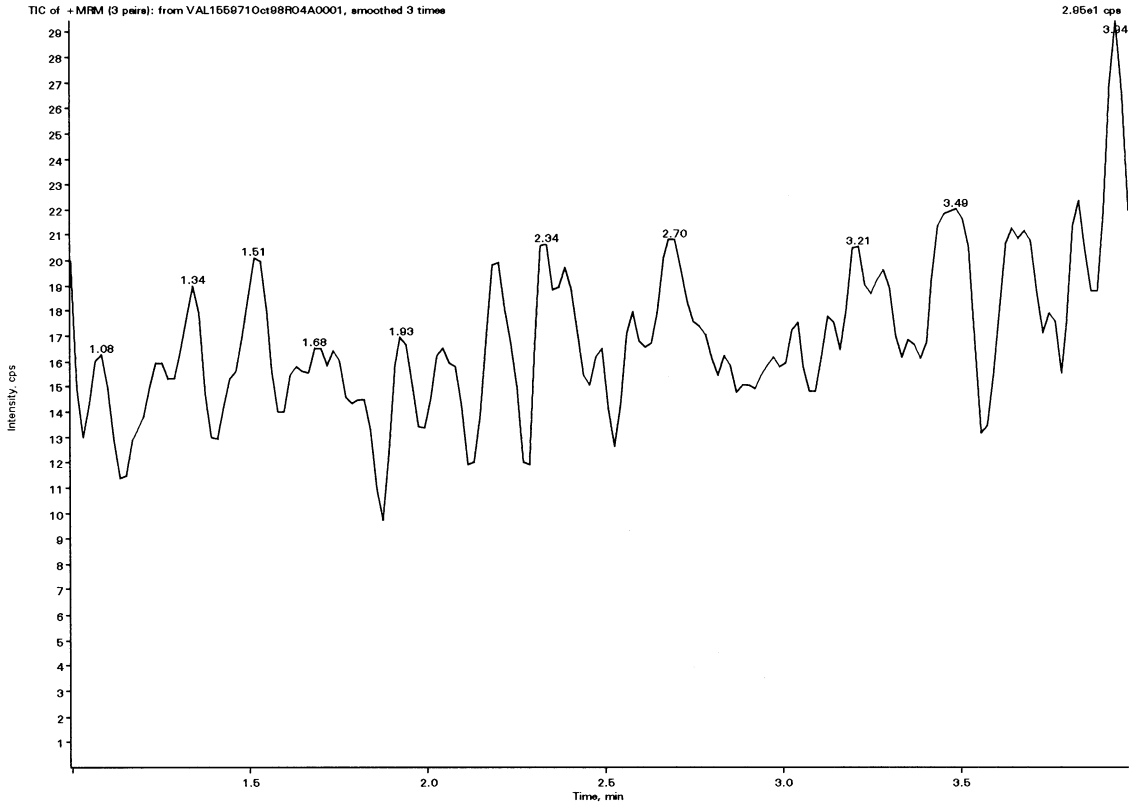

Fig. 2. Chromatogram of a blank human plasma spiked with internal standard (MRM transition 297→121 m/z).

and Gilson 233 XL autosampler (Gilson, Villiers-le-Bel, France).

The chromatographic separation was performed on a Zorbax SB C8 (4.6 × 150 mm, 5 μm, Hewlett Packard) column equipped with a New Guard RP 8, Aquapore Octyl, (15 × 3.2 mm, 7 μm, PE Brownlee, Norwalk, CT) spherical guard column, both maintained at 45°C. The mobile phase was 100% acetonitrile at a flow rate of 1 ml min^{-1}. The total cycle time was 6.0 min.

2.3. MS equipment and conditions

The LC–MS–MS analysis was performed using a Perkin Elmer Sciex API 300 triple quadruple mass spectrometer (PE SCIEX, Concord, Ont., Canada).

The instrument was operated in atmospheric pressure positive-ion chemical ionisation (APCI) mode utilising the heated nebulizer interface. The temperature of the nebulizer probe was 375°C. Multiple reaction monitoring (MRM) was employed with a collision energy of 30 eV. Molecular-to-product transitions of 297–121 m/z for EXE and 300–123 m/z for IS were used. The dwell time for each transition was 500 ms. Peak-area for the selected ions were determined using the PE Sciex software package MACQUAN, version 1.5.

2.4. Sample preparation

To 0.50 ml of plasma aliquots in 1.5 ml polypropylene microcentrifuge tubes, were added 50 μl of IS solution (1.11 μg ml^{-1}), 0.50 ml of water and each tube was vortex mixed. The 96-extraction plates were primed with 1 ml of acetonitrile ($x2$) and with 1 ml of water ($x2$). The prepared samples were loaded and drawn through the plates with a minimum of vacuum. The plates

were washed with 1 ml of acetonitrile:water (10:90, v/v) mixture, followed by a drying step for 30 min at full vacuum. Elution was achieved with 0.15 ml of 0.1% TFA in acetonitrile ($x2$) under a minimum of vacuum. The eluates were centrifuged at 1500 rpm for 2 min to remove any precipitated material and aliquots of 80 μl were injected onto the LC–MS–MS system. All these steps were performed manually. The manifold used (mod. M005.DW) was supplied by Porvair Sciences Ltd (Shepperton Business Park, UK).

2.5. Assay validation experiments

The linearity was evaluated using results of three calibration curves which were run on 3 different days over the concentration range 0.05–25 ng ml^{-1} of human plasma. Calibration curves were constructed by plotting the peak area ratio of the compound and IS (y) against the concentration of EXE (x) in plasma. A weighed linear regression analysis (weighing factor $1/x^2$) was used to calculate the drug concentrations in quality control and unknown samples.

The lower limit of quantitation (LLOQ), at 0.05 ng ml^{-1} was assessed by analysis of six spiked plasma samples in one analytical run.

Intra-day accuracy and precision at 0.21, 2.06 and 20.60 ng ml^{-1} concentrations (quality control, QC) were determined by analysis of six spiked plasma samples in one analytical run and three spiked plasma samples in two analytical runs. Inter-day accuracy and precision at the same concentrations as above were assessed from the analysis of three calibration curves with QC samples, run on 3 different days. The extraction recovery was evaluated comparing the peak area of extracted plasma samples to the peak area obtained with unextracted standard solutions dissolved in mobile phase, directly injected onto the LC–MS–MS system.

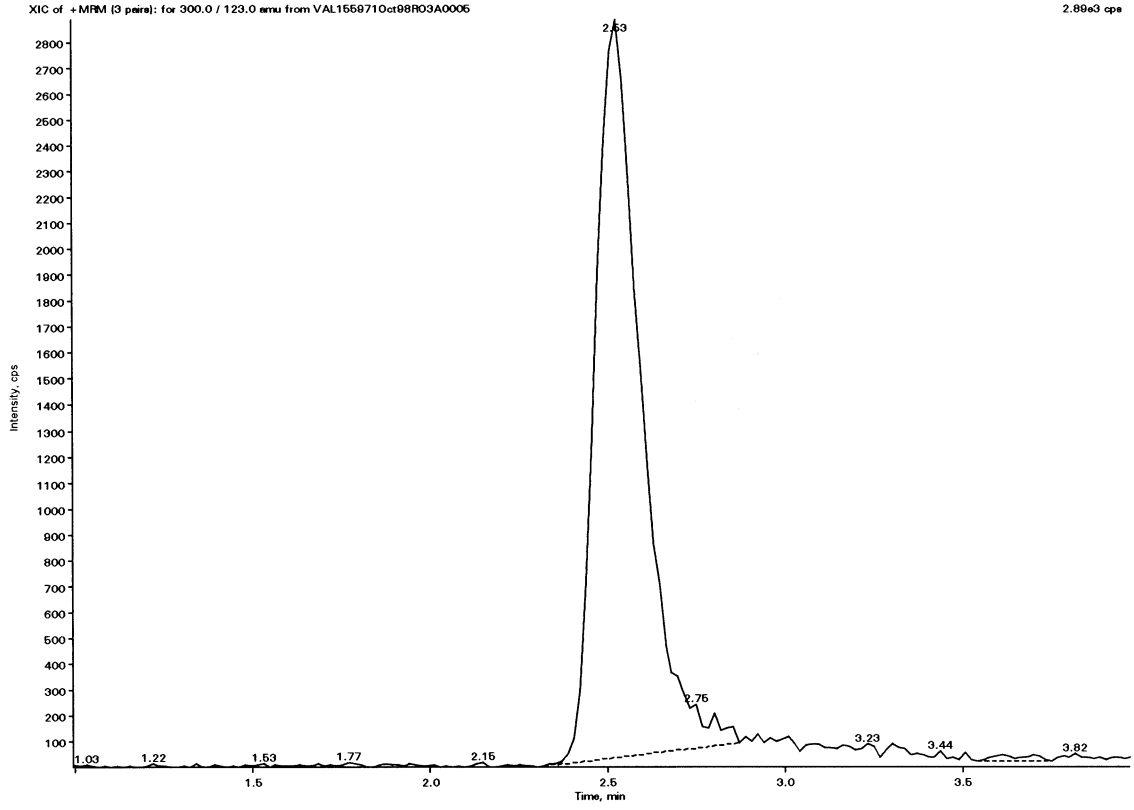

Fig. 3. Chromatogram of a blank human plasma spiked with internal standard (MRM transition 300 → 123 m/z).

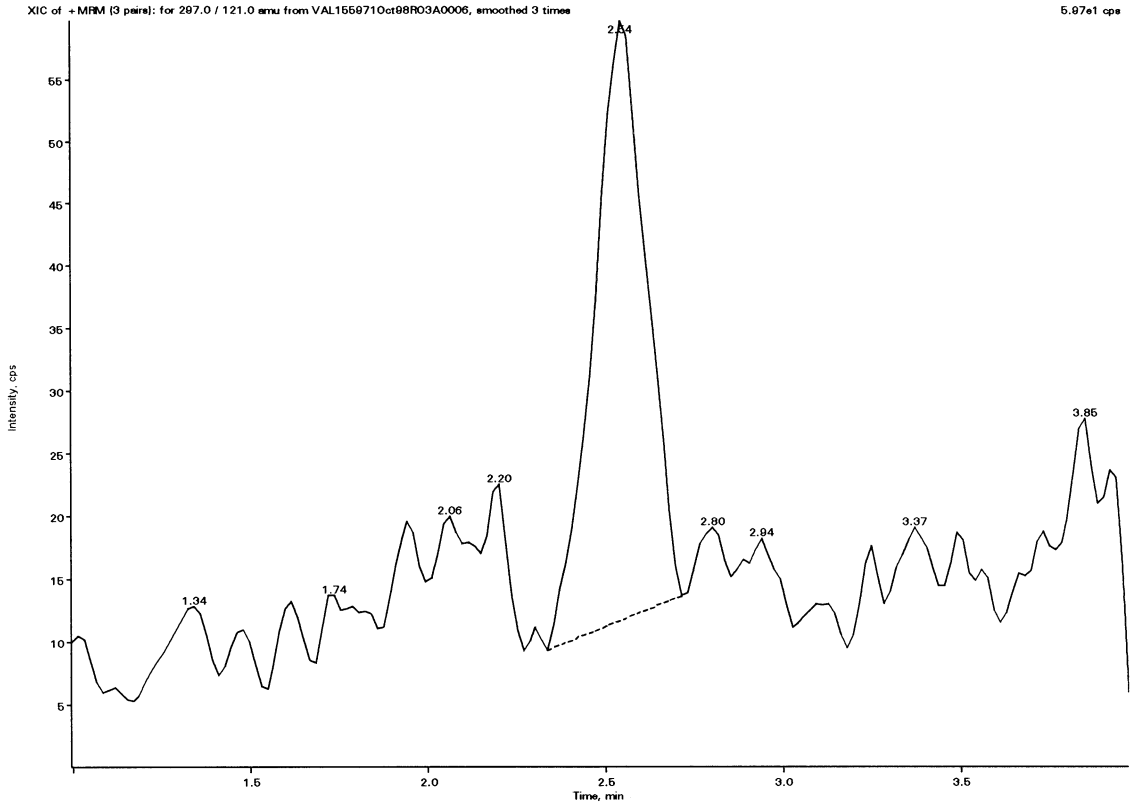

Fig. 4. Chromatogram of lower calibration standard (0.05 ng ml^{-1}).

Stability of EXE was examined after storage in plasma at room temperature for 24 h, after storage of final extracts at room temperature for 24 h and after three freeze/thaw cycles.

For each stability experiment, QC concentrations at 0.21, 2.06, 20.6 ng ml^{-1} were analysed in triplicate.

3. Results

The retention time for EXE was 2.3 min and the total cycle time was 6 min to allow endogenous peaks to elute without interfering with the next chromatographic run.

Figs. 2 and 3 show the chromatograms of a blank human plasma spiked with internal standard at two different transition ($297 \rightarrow 121$ m/z and $300 \rightarrow 123$ m/z, respectively). Figs. 4 and 5 show the chromatograms of the lowest standard (0.05 ng ml^{-1}) and of the highest standard (25 ng ml^{-1}), respectively.

The coefficient of linear regression (r), using a weighing factor of $1/x^2$ was always better than 0.992. The mean calibration curve obtained was described by the following equation: $y = 0.195x + 0.006$ ($n = 3$). The precision (expressed as % relative standard deviation, RSD) of the slope of the calibration line was 5.7%. The back calculated calibration standard values for the compound showed a RSD ranging from 3.3 to 10.5%. The lower limit of quantitation, chosen as the lowest point on the calibration curve having a back-calculated concentration within 20% of the nominal value (LLOQ), had a RSD of 6.1%.

The intra-day precision evaluated from QC samples, ranged from 1.6 to 9.5%. The inter-day precision evaluated at the same concentrations ranged from 3.2 to 9.1%. The intra-day accuracy (expressed as percentage ratio of the mean

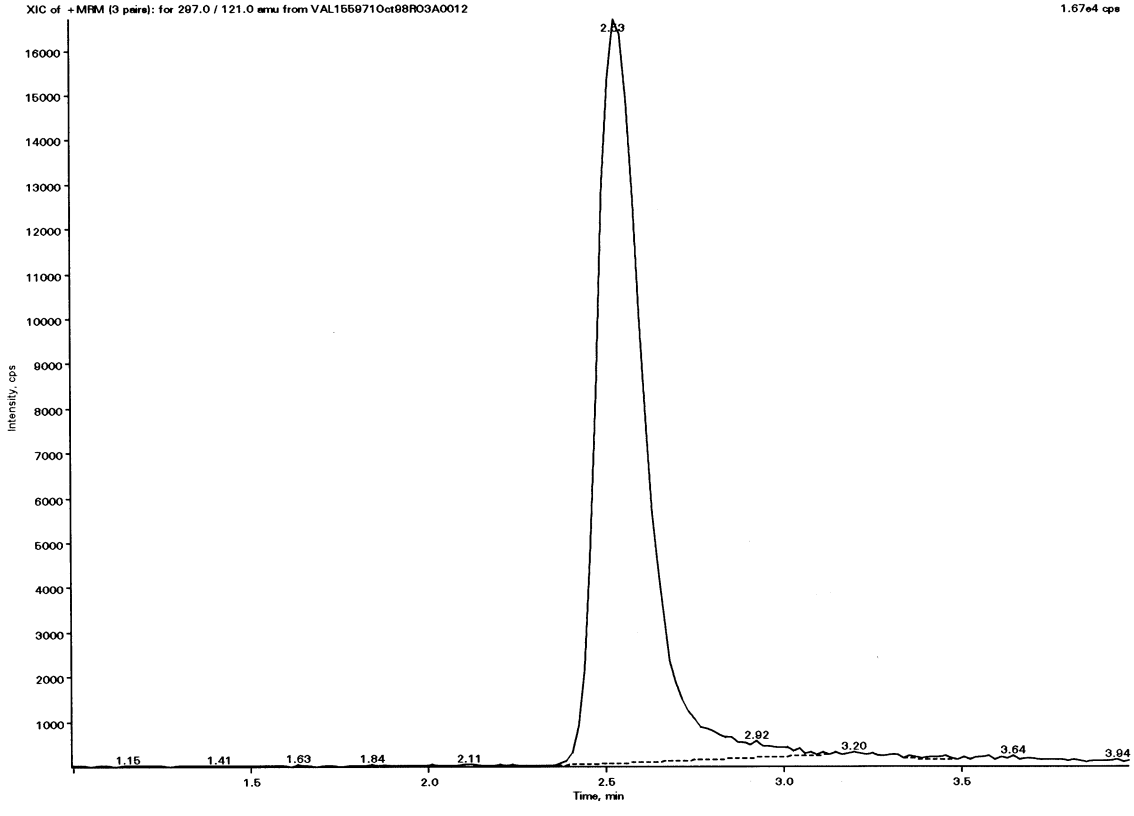

Fig. 5. Chromatogram of higher calibration standard (25 ng ml^{-1}).

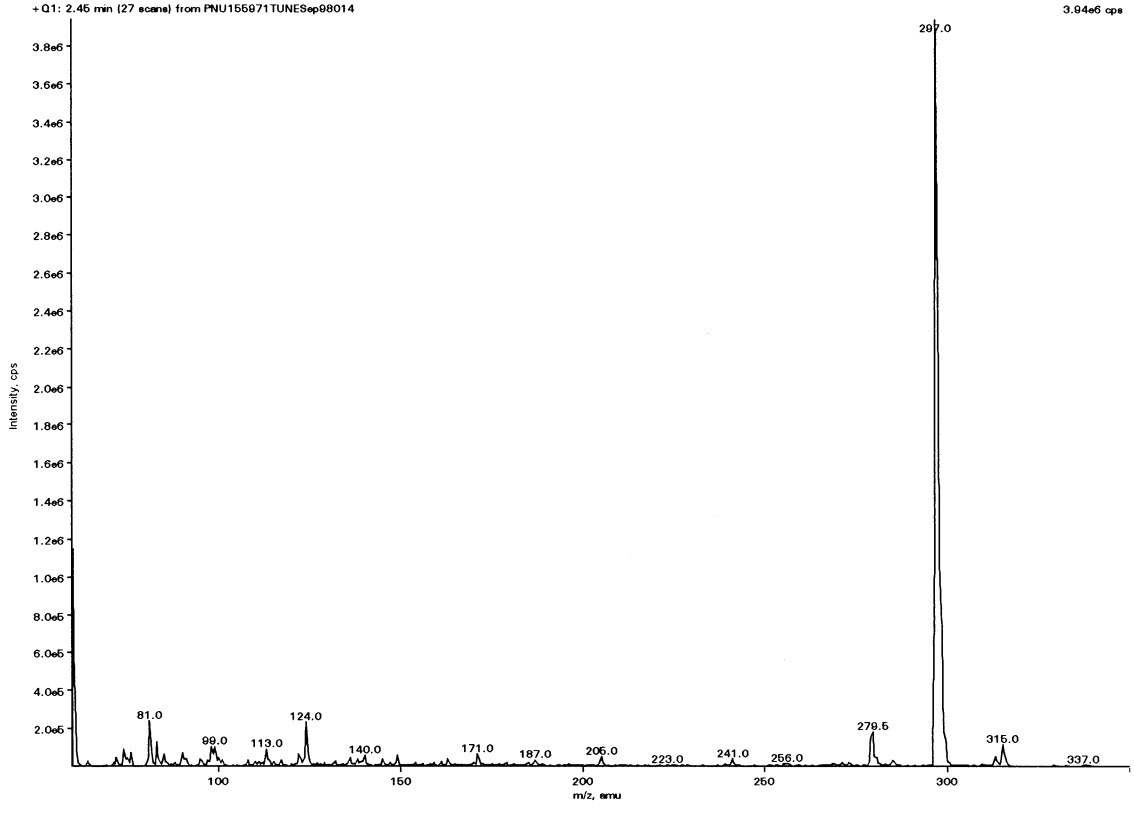

Fig. 6. Full scan spectrum of exemestane.

Table 1
Accuracy and precision of the method for the determination of EXE in human plasma

Control sample (ng ml^{-1})	Day	n	Mean found (ng ml^{-1})	Accuracy		S.D.	Precision	
				Mean (%) nominal (intra-day)	Pooled (%) nominal (inter-day)		% RSD (inter-day)	Pooled % RSD (inter-day)
0.21	1	6	0.21	100		0.02	9.5	
	2	3	0.23	109.5		0.01	4.3	
	3	3	0.23	109.5	104.8	0.01	4.3	9.1
2.06	1	6	1.90	92.2		0.07	3.7	
	2	3	1.85	89.8		0.04	2.2	
	3	3	1.87	90.8	91.3	0.03	1.6	3.2
20.6	1	6	21.28	103.3		0.80	3.8	
	2	3	20.66	100.3		0.63	3.0	
	3	3	20.58	99.9	101.7	0.37	1.8	3.4

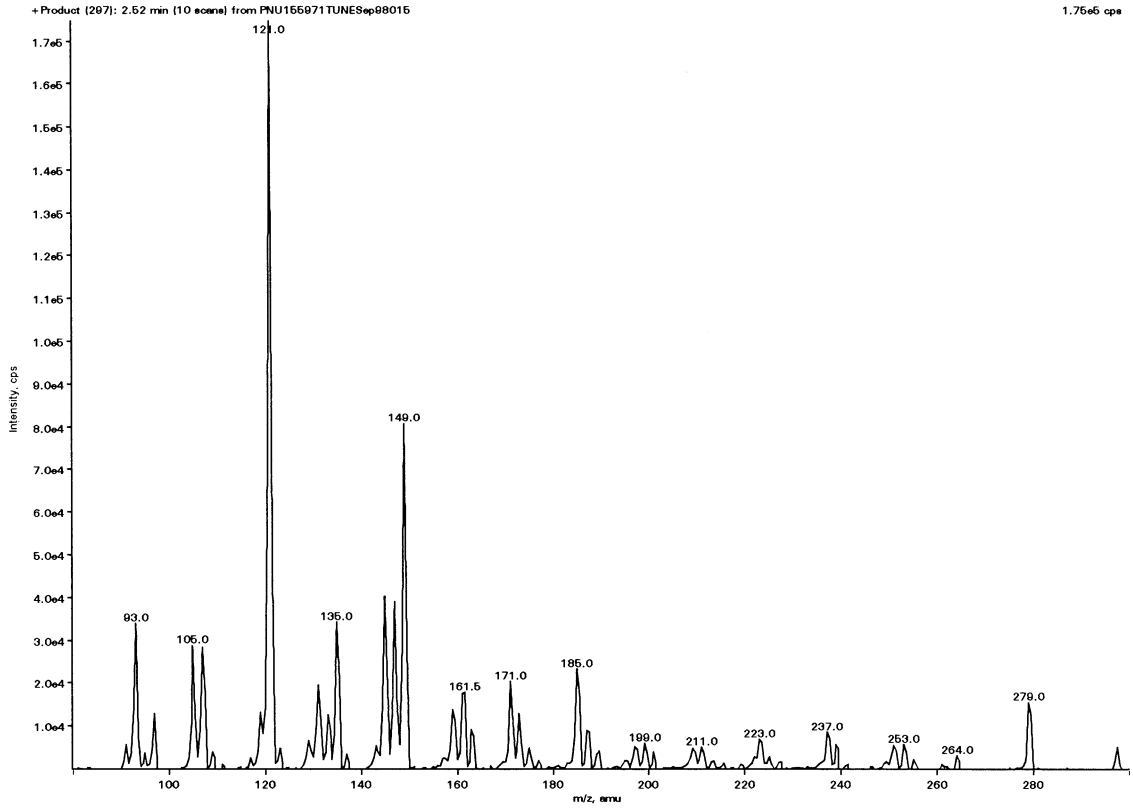

Fig. 7. Product ion spectrum of exemestane.

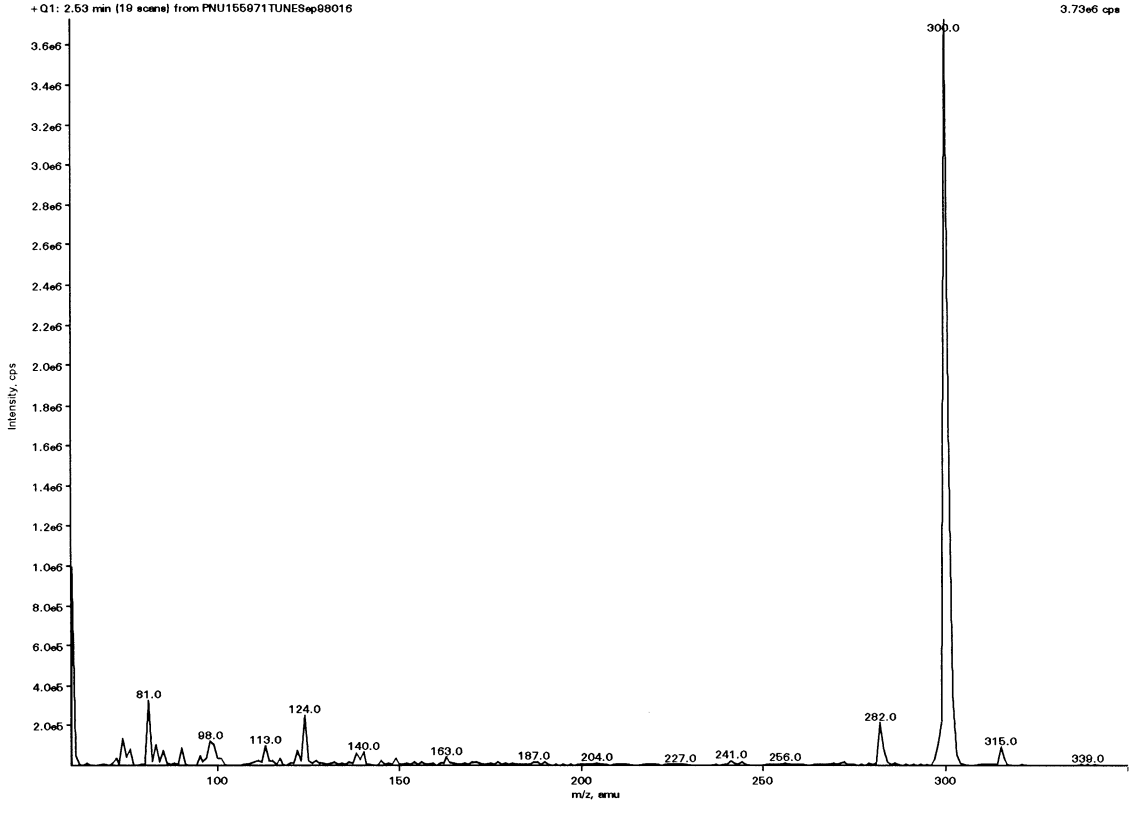

Fig. 8. Full scan spectrum of internal standard.

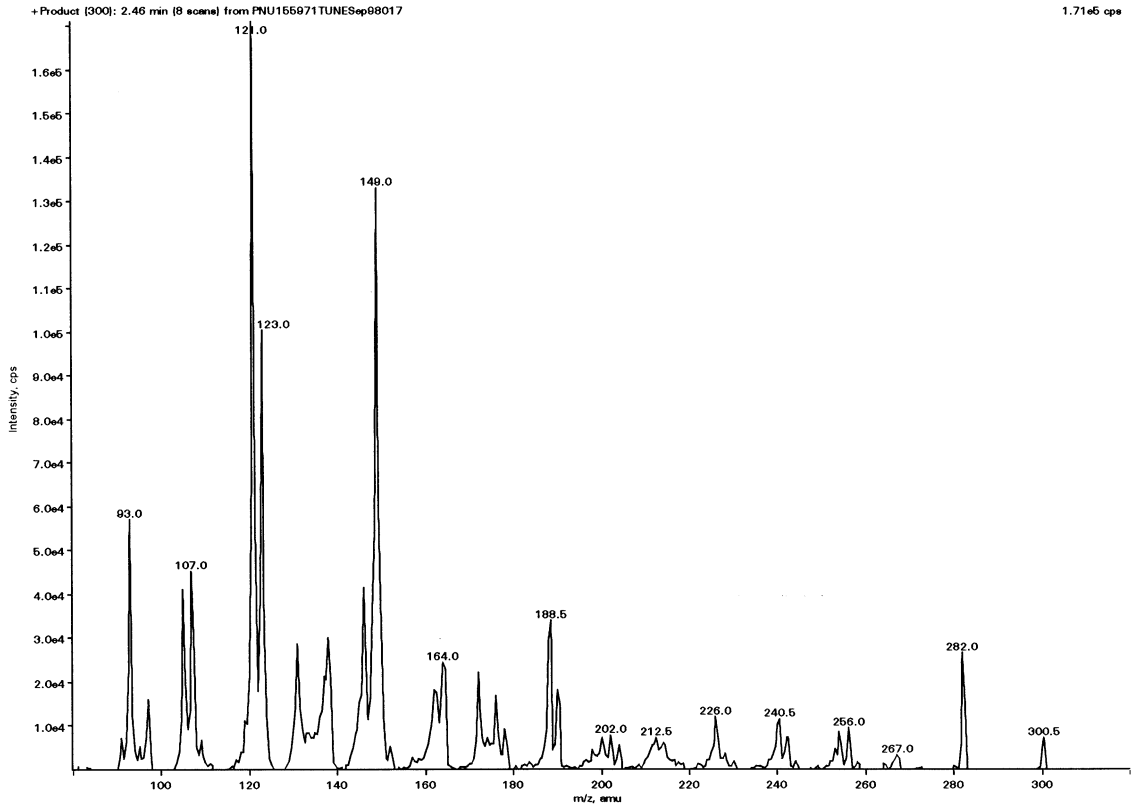

Fig. 9. Product ion spectrum of internal standard.

amount found to the amount added to plasma, mean % nominal), evaluated on the same plasma samples, ranged from 89.8 to 109.5%. The inter-day pooled accuracy (expressed as pooled % nominal) ranged from 91.3 to 104.8% (Table 1).

The extraction recovery of the method for the determination of EXE following SPE in the 96 well plate format evaluated at three concentrations ranged from 94.5 to 106.3%.

There was no evidence of degradation of EXE after storage in plasma at room temperature for 24 h, during storage of final extracts at room temperature for 24 h and after three freeze/thaw cycles using spiked QC samples.

Study plasma samples, however, may contain a substance thought to be a metabolite, that is unstable at room temperature and which consequently resulted in increased EXE levels. Therefore, plasma must be kept on ice during the sample preparation, but extracted samples appear to be stable.

The full scan spectrum of EXE (Fig. 6) revealed the protonated parent molecule $(M+H)^+$ to be in abundance with a mass to charge ratio (m/z) of 297 and the product ion spectrum of EXE (Fig. 7), using a collision energy of 30 eV, resulted in a major fragment at m/z 121.

For the IS, a full scan spectrum revealed the protonated parent molecule to be in abundance with a mass to charge ratio (m/z) of 300 (Fig. 8), as expected, and the product ion spectrum, using the same collision energy as above, showed the presence of two fragments at 121 and 123 m/z (Fig. 9). The transition ($300 \rightarrow 123$ m/z) was chosen to monitor IS and, in order to minimise the cross talk (less than 0.1%), a dummy transition of ($600 \rightarrow 30$ m/z) was also inserted.

4. Conclusions

This LC–MS–MS method proved to be linear, precise and capable of accurately quantifying EXE in the concentration range 0.05–25 ng ml^{-1}.

The procedure described above is substantially more sensitive compared to previously published HPLC–UV and LC–MS–MS methods (0.05 ng ml^{-1} instead of 10, 1 and 0.25 ng ml^{-1}, respectively), but is less sensitive than the HPLC–RIA method (0.012 ng ml^{-1}).

Neverthless, at first using SPE with traditional cartridges and then using SPE with 96 well plate, the throughput of the method was considerably improved with up to 170 samples/day, compared to HLPC–RIA procedure.

The introduction of 96 well plate system greatly improved the efficiency of extracting samples.

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