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THE EXCRETION STUDY OF EXEMESTANE IN MAN

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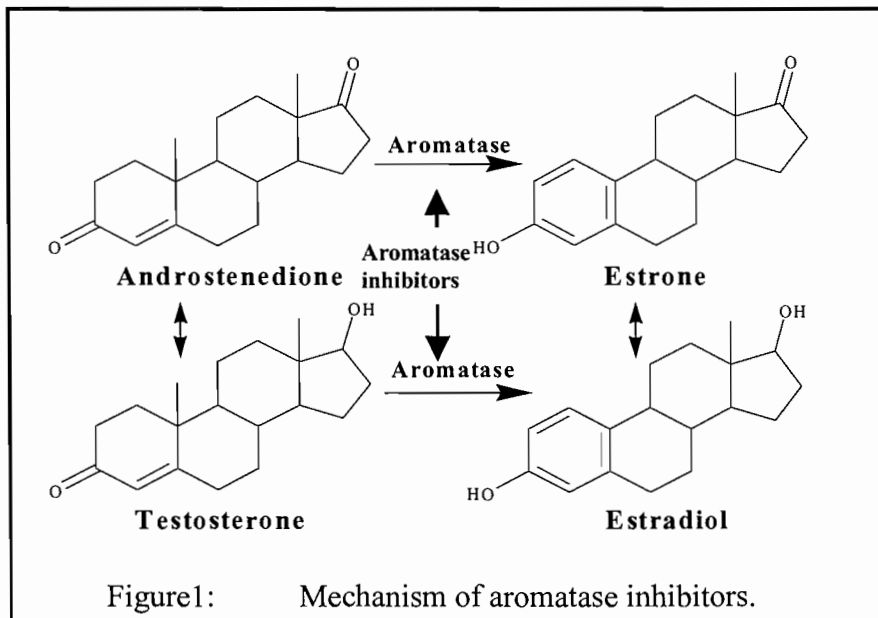
Abstract

Exemestane (Aromasin®) is a potent steroidal aromatase inhibitor strongly blocking the conversion of androgens to estrogens. The drug has been abused in doping to elevate androgens. Recently, aromatase inhibitors have been in the prohibited list of both the International Olympic Committee (IOC) and the World-Antidoping Agents (WADA).

The aims of this study are to investigate exemestane in 3 parts; first the urinary excretion profile, second the effect of exemestane on endogenous steroid level and third an attempt to develop a convenient screening method for urinary exemestane for doping analysis. Urine collection was performed at intervals up to 6 days from 20 healthy male volunteers after a single 200 mg oral administration. The urinary excretion profile of exemestane was analyzed by HPLC/DAD. The maximum excretion rate was observed at 2 hours after drug administration and exemestane was detectable in urine up to 48 hours after drug administration. The effect of exemestane on steroid levels revealed a remarkable increase in androstenedione and testosterone to estrone ratio. (T/E₁) was increased continuously in the last 3 days. A screening detection of exemestane was performed in GC/MS to explore a suitable method for doping analysis. Exemestane parent compound was observed in procedure 1 (detection of nitrogen containing compounds excreted free in urine) and 4A (detection of steroids excreted free in urine) with the characteristic ions 148 and 296 (molecular ion). A suspected exemestane metabolite was found in procedure 4A with the characteristic ions 355 and 370 (molecular ion)..

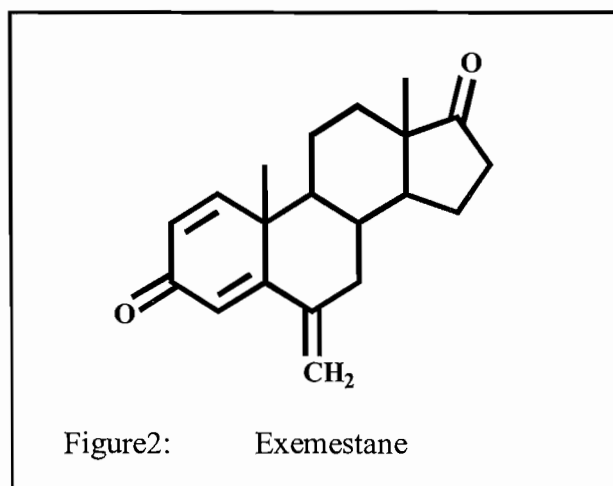
Introduction

Aromatase inhibitors have been in the prohibited list of both the International Olympic Committee (IOC) and the World-Antidoping Agents (WADA) since 2001[1]. Aromatase



inhibitors inhibit the conversion of androstenedione to estrone and testosterone to estradiol so possibly leading to the accumulation of androstenedione and testosterone [2].

These drugs can be divided into 2 main groups; steroidal and non-steroidal [3]. Exemestane is an example of an aromatase inhibitor of steroidal type; its trade name is Aromasin® [4]. It is an analogue of androstenedione and acts as a competitive inhibitor to aromatase. The differences from androstenedione are the methylene group at position 6 and the additional double bond at position 1 (Figure2). Half life in blood circulation is 24 hours. These drugs are widely used for treatment of estrogen-dependent breast cancer with the purpose to lower estrogen concentration [5].



Most studies of the exemestane metabolism in man was performed in blood. Increased serum levels of androstenedione after exemestane administration have been previously reported [6,7]. However, studies of exemestane in urine, particularly in relation to doping control analysis, are not well established. It is therefore of interest to investigate urinary excretion of the drug and its effect on urinary endogenous steroid profiles in man. Moreover, identification of the drug excreted in urine, or other possible related parameters, would also be explored with potential application in doping control.

Experiments and results

Experiments were performed with 20 healthy male volunteers (ages 20 – 42 years, mean 29.8 ± 5.9 years). Ethical clearance for studying in human was obtained from the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. All volunteers signed the informed consent and blood examinations were performed before drug administration.

Blank urine was collected the day before drug administration. One day after, the volunteers took 200 mg of exemestane (n=15) or placebo (n=5), and urines were subsequently collected at intervals up to 6 days. All samples were stored at 2-8 °C without preservative.

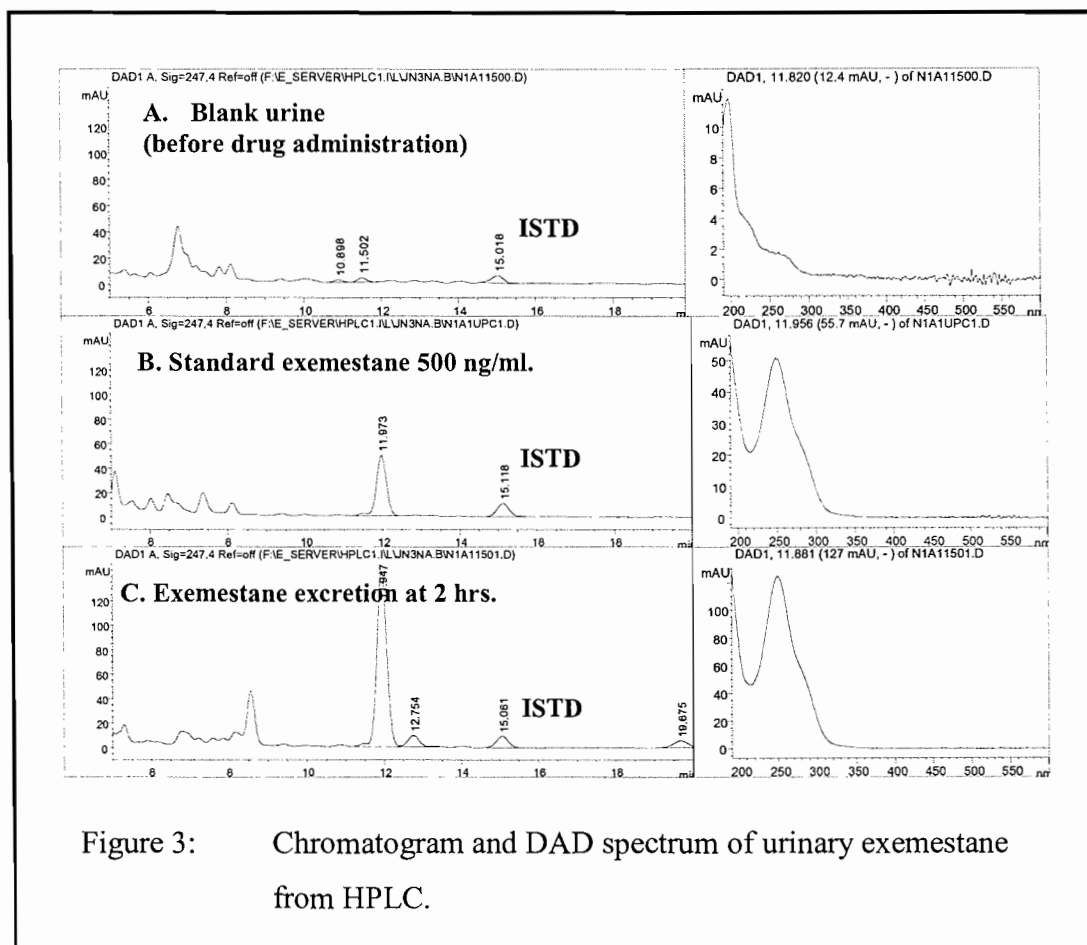
Urinary excretion of exemestane

The extraction and analysis of exemestane were performed according to the method previously reported [8]. Briefly, 5 ml of urine were extracted with ethylacetate after addition of solid carbonate buffer ($\text{NaCO}_3/\text{NaHCO}_3$ 2:1) and saturation with sodium chloride. The organic layer was evaporated to dryness and dissolved in 100 μl acetonitrile: water (70:30,v/v) from which 25 μl were injected into the HPLC (Hewlett Packard 1090 Series II) using a Symmetry® C18 column (15m x 4.6 mm i.d. x 3.5 μm). Mobile phase was acetonitrile/water, 40:60(v/v), flow rate 1.3 ml/min and the absorbance was monitored by a diode array detector (Agilent 1100 series DAD) between 210 - 600 nm.

The improved method gave limit of detection of 9 ng/ml urine. The recovery of exemestane and internal standard (norgestrel) were 78 and 89 % respectively. Linear calibration curves were obtained in the concentration range of 10 – 2000 ng/ml urine ($R^2 = 0.9993$).

An Exemestane standard (200 ng/ml urine) was used as one point-calibrator to quantify excreted exemestane calculated from the area ratio of exemestane and norgestrel (Figure3). Exemestane was observed at 11.90-12.07 minutes for excretion urine and the spectrum was identical to that observed from positive control. In blank urine, exemestane peak was observed at the expected retention time.

The maximal mean excretion rate of exemestane in urine observed at hour 2 after drug administration is approximately $160 \pm 37 \mu\text{g/hr}$ and declined to approximately $1 \mu\text{g/hr}$ after 2 days (Figure 4).

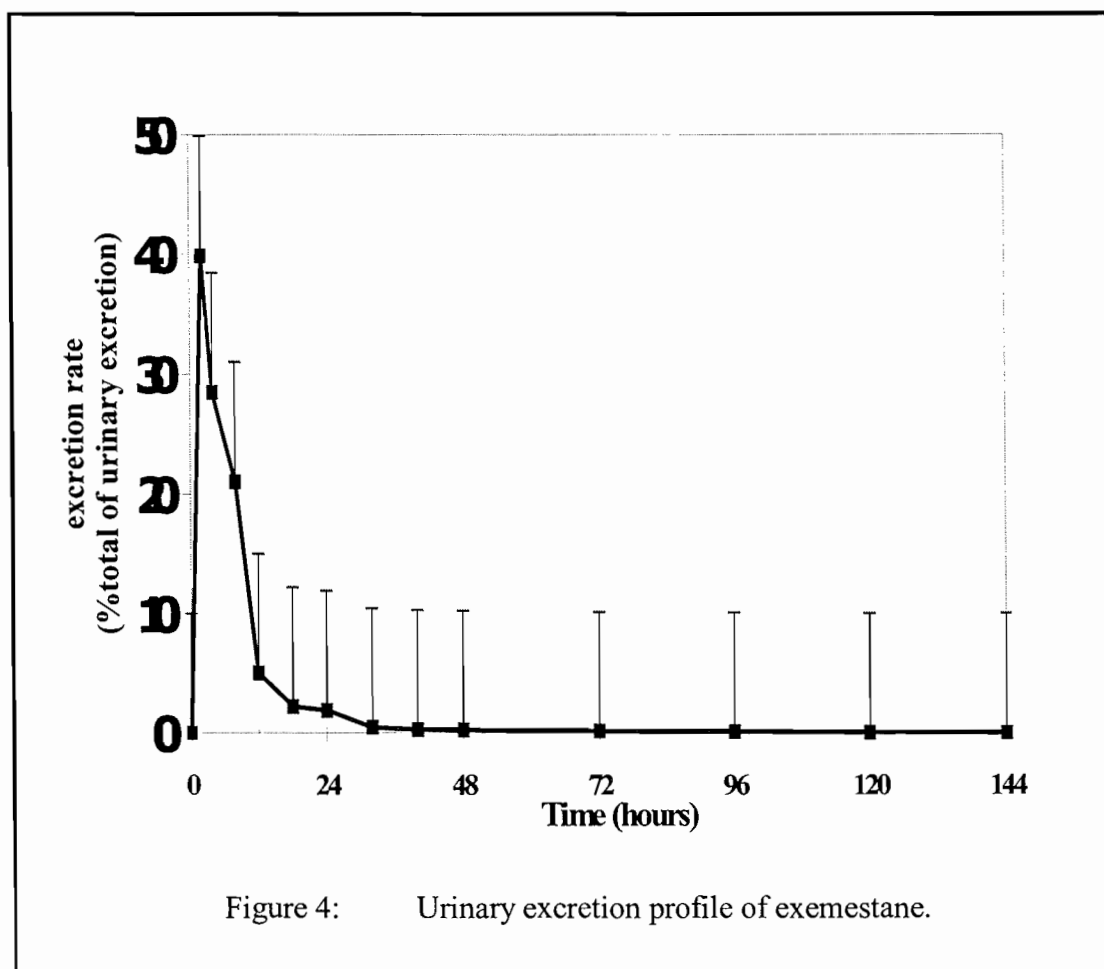


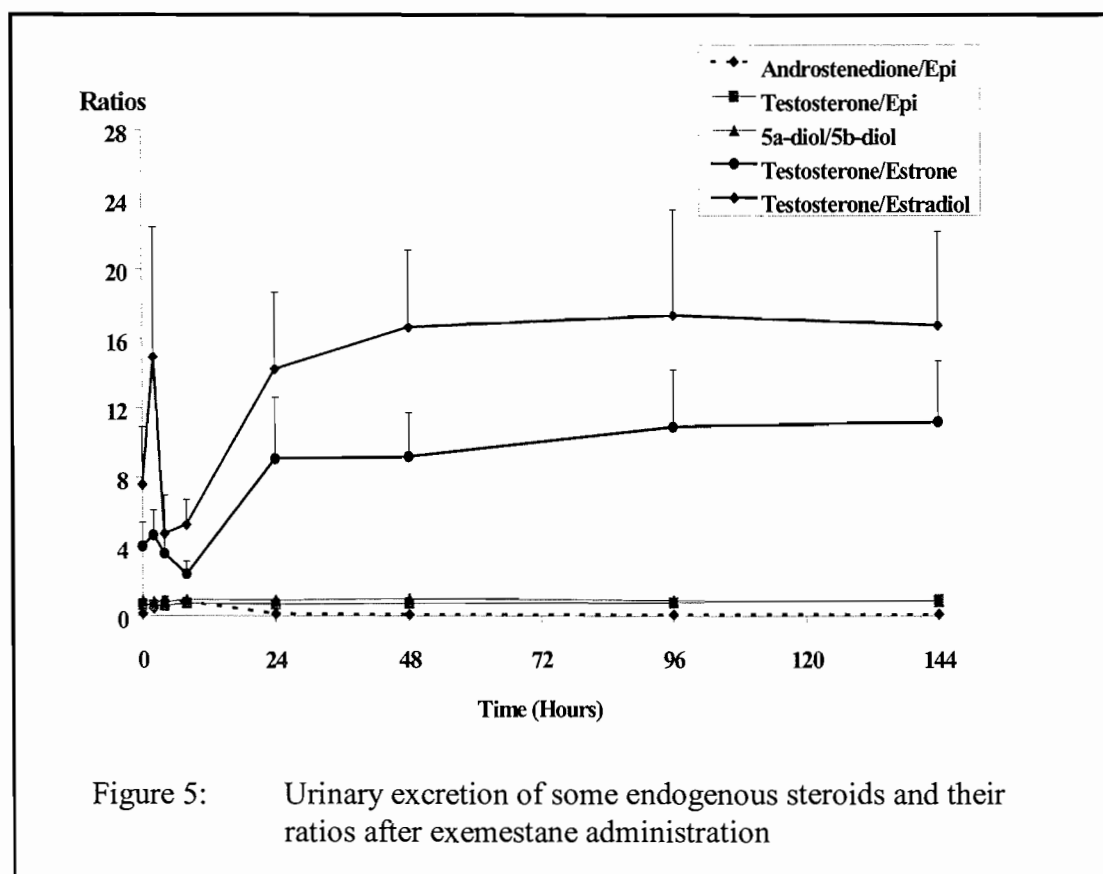
Effect of exemestane administration on endogenous steroids .

Endogenous steroid levels in urine samples were measured by GC/MS. The extraction method and analytical condition performed as described for procedure 4B (detection of endogenous steroids in the combined fraction). Briefly, solid-phase extraction was performed using C18 Sep-pak followed by enzymatic hydrolysis with β -glucuronidase (*E.coli*) in 0.2 M sodium phosphate buffer at pH 7.0. Liquid-phase extraction was performed using t-butyl

methyl ether and derivatization with MSTFA/NH₄I/2-mercaptoethanol (1000:2:6,v/w/v). Instrumental analysis was performed by HP6890 Series 2 GC with MSD 5973 using the HP Ultra1 column at a constant pressure of 17 psi. GC/MS analysis was run in SIM mode.

Results showed significant increase of androstenedione ~500% higher than baseline at 2-8 hours after drug administration. Urinary testosterone was slightly elevated at approximately 55% from baseline at 2 hours. The testosterone to estrone (T/E₁) and testosterone to estradiol (T/E₂) ratios increased significantly 1 day after the drug administration and were maintained at this increased level for the period of drug monitoring (Figure5). However the testosterone to epitestosterone ratio (T/E) showed no significant change.



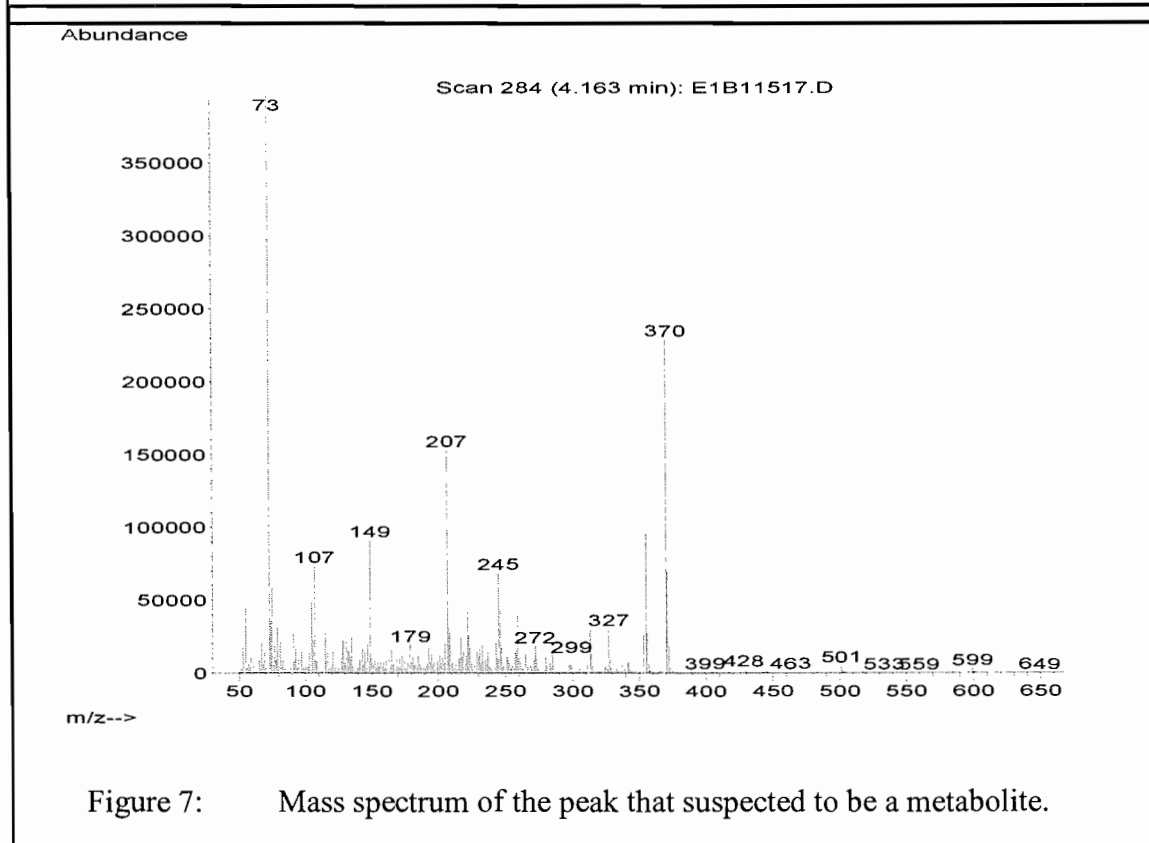
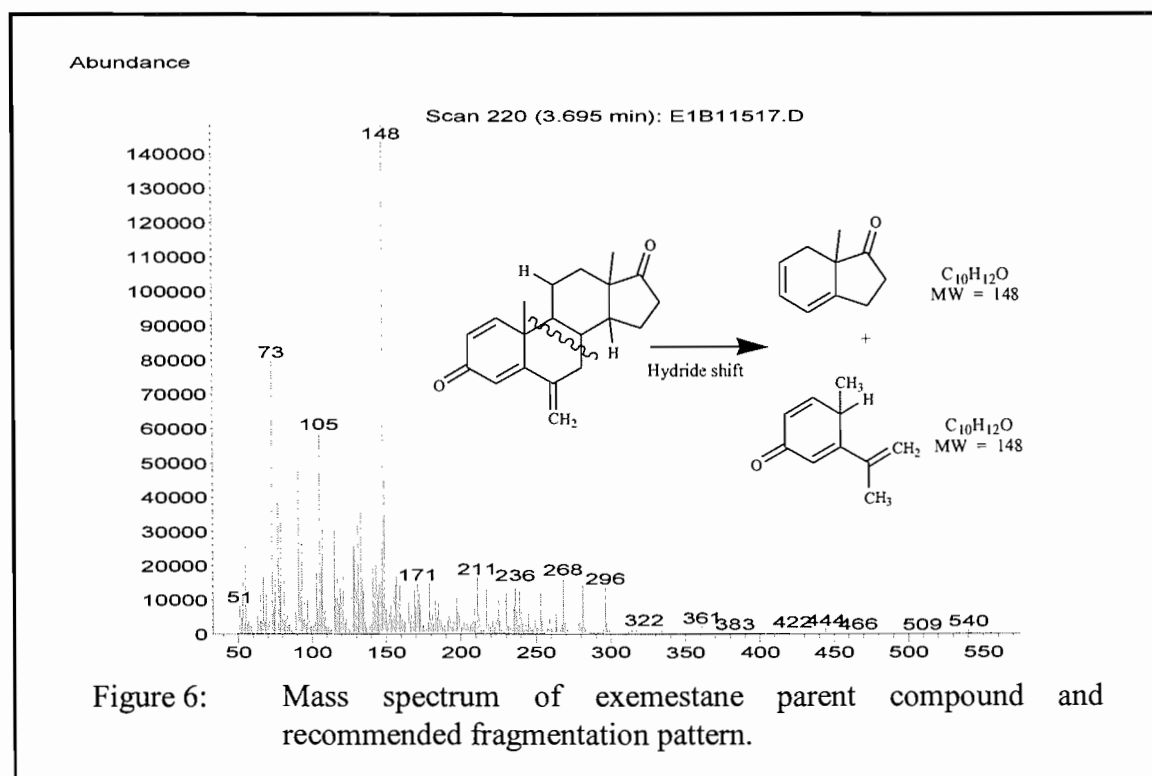


Identification of excreted exemestane in doping control analysis.

The determination of exemestane was carried out by routine analytical procedures in doping control analysis. Six samples from 3 volunteers that had been collected before and after drug administration were analyzed. Blank urine of each volunteer was analyzed in parallel to the excreted urine. The exemestane concentrations in these samples were calculated. The concentrations from 2 volunteers were approximately 1500 ng/ml and from the third volunteer was approximately 3600 ng/ml.

Exemestane parent compound was observed in the procedure 1 at 3.69-3.71 min and in the procedure 4A at 7.89-7.92 min. The mass spectra showed identical pattern to exemestane standard with the characteristic ions 148 and 296 (molecular ion) (Figure 6). The proposed fragmentation scheme is a scission through the B ring to 2 fragments of mass 148.

A peak, presumably an exemestane metabolite, was detected in the procedure 4A with the characteristic ions 355 and 370 (molecular ion) (Figure7). In addition, the peak areas of these suspected peak corresponded to the concentration of the parent compound in each sample (data not shown).



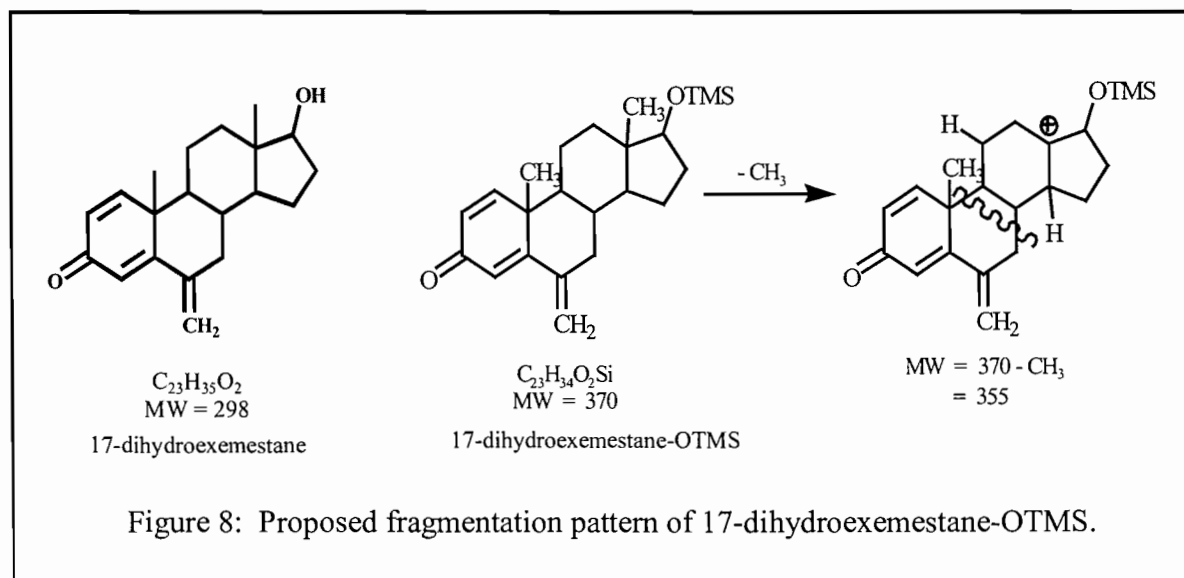
EI mass spectra of 17-dihydroexemestane (free and as TMS derivative) and exemestane, 16-enol TMS, are attached hereto as annex and were provided by Prof.Dr.W.Schänzer, German Sport University Cologne, Institute of Biochemistry, Carl-Diem-Weg 6, D-50933 Köln.

Conclusion and discussion

The function of aromatase is to mediate the conversion of either androstenedione to estrone or testosterone to estradiol. There was approximately 500% and 50% increase of androstenedione and testosterone at 2 hrs coupled with 60% and 50% decrease in estrone and estradiol at 7 days after administration. The rise in aromatase substrate is probably due to substrate accumulation caused by the aromatase inhibition. The higher degree of androstenedione excretion compared to testosterone shows that the effect of aromatase inhibition in male is predominantly an estrone suppression. Also the conversion of testosterone to androstenedione by steroidogenase may be involved.

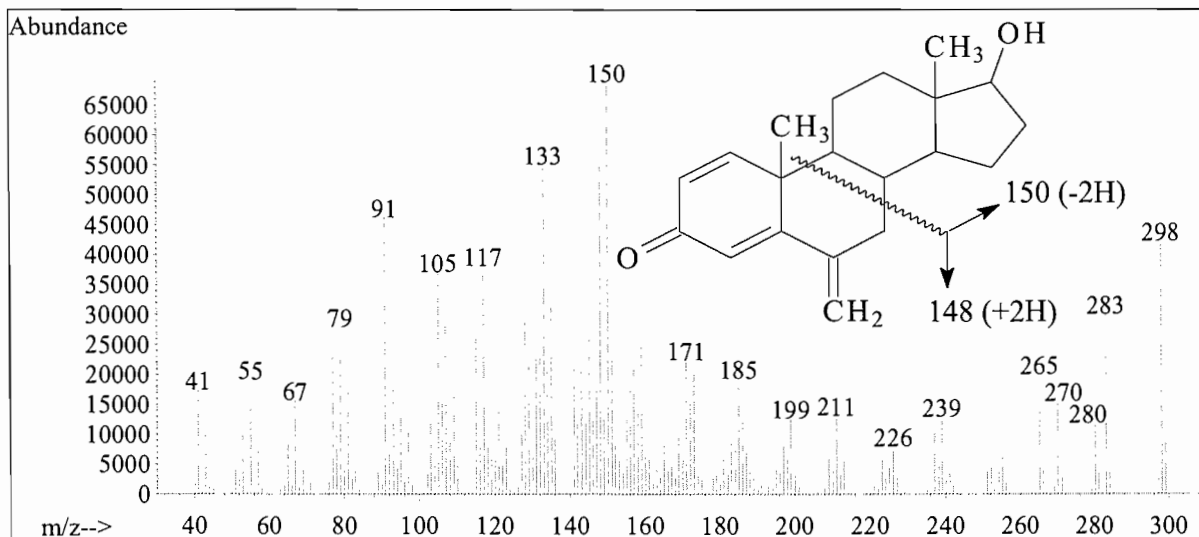
Sustained increase of T/E₁ and T/E₂ at 5-7 days after a single oral dose of exemestane was observed. These results are due to the suppression of estrone and estradiol and this persisted along a period when the parent compound was no longer detectable. This criterion may perhaps be used as supportive evidence of aromatase inhibition.

The detection of exemestane parent compound as free drug can be carried out using both procedures 1 and 4A with the characteristic ions 148 and 296. In addition, a suspected metabolite was also detected in procedure 4A at retention time 4.163 min. with the characteristic ions 355 and 370. This metabolite is very likely to be 17-dihydroexemestane (Figure 8), as suggested previously [8]. Conclusive information could be obtained when the reference compound of the proposed metabolite is available for further confirmation.

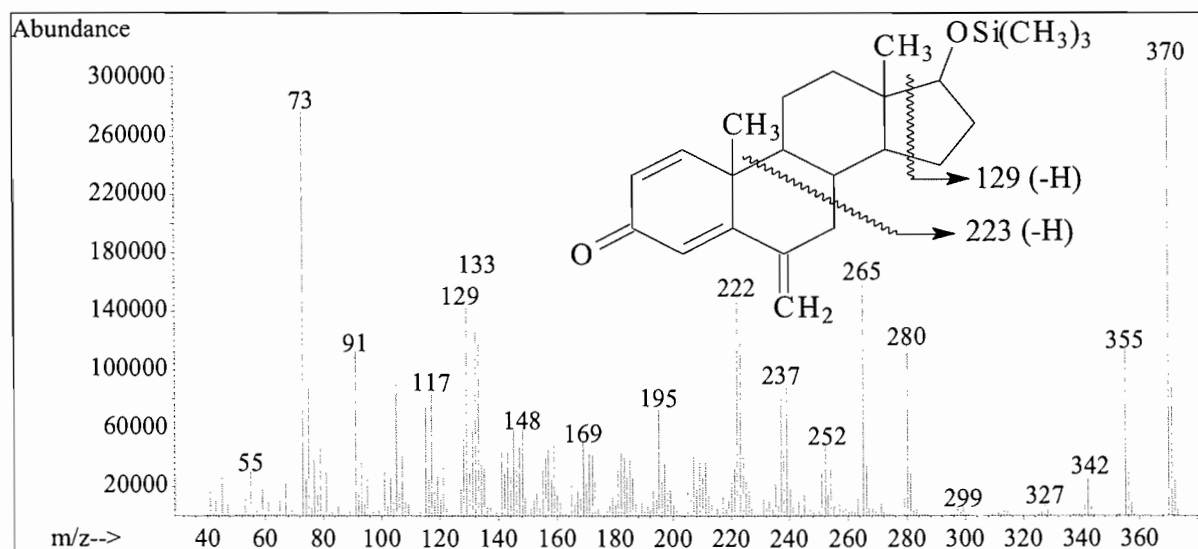


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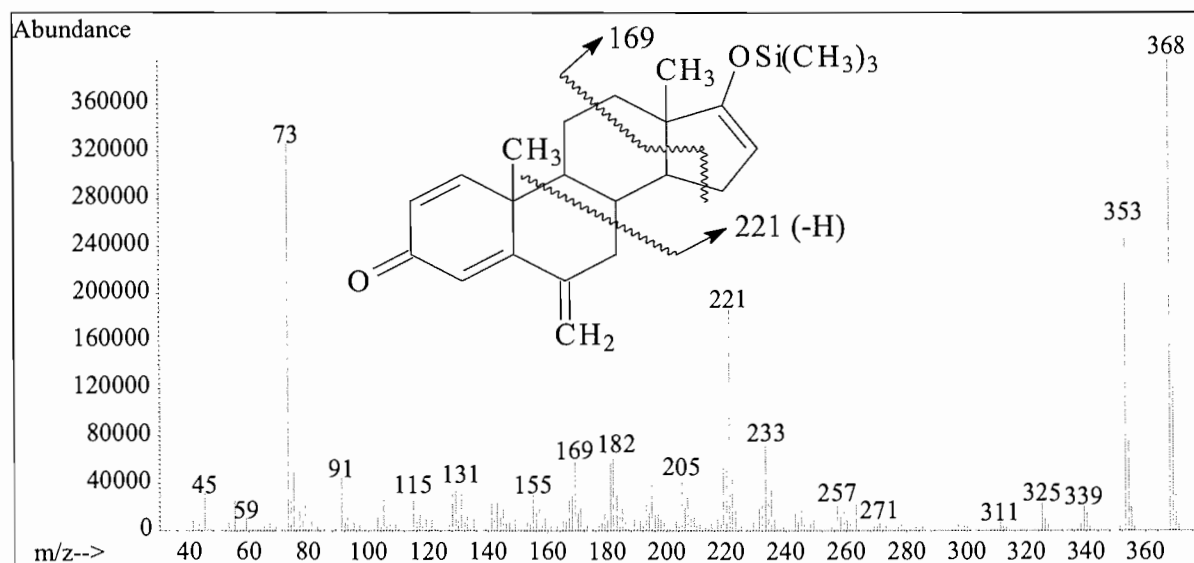
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EI mass spectrum of 17-dihydro-exemestane



EI mass spectrum of 17-dihydro-exemestane, O-trimethylsilyl



Ei mass spectrum of exemestane, 16-enol trimethylsilyl