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Detection of Exemestane and its metabolites using GC-MS and LC-MS-MS: a comparative study for screening purposes.

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Abstract

The detection of exemestane and its 17-dihydro derivative has been studied by gas chromatography-mass spectrometry (GC-MS) after trimethylsilylation with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and the trimethyliodosilane (TMIS) reagent MSTFA / NH_4I / ethanethiol 1000 / 2 / 3 (v/w/v). With the latter the formation of 6-methylene-estrone or 6-methylene-estradiol and other byproducts was observed in standard solutions as well as in urine samples from patients thereby drastically reducing the amount of detectable exemestane and 17-dihydroexemestane.

The two substances give a good response in positive atmospheric pressure chemical ionisation (APCI) and their evidence in urine is easily proven using LC-MS-MS techniques. So they have been incorporated into our screening procedure for glucocorticosteroids, beta-blockers, selected anabolic androgenic steroids and buprenorphine. Mass spectral data and a validation of the method for the analysis of exemestane and 17-dihydroexemestane are presented.

In addition, two other metabolites were detected using the GC-MS screening procedure for anabolic steroids: a dihydro- and a tetrahydro-exemestane. Their structures are not yet elucidated but due to the fact that they take two trimethylsilyl groups they are considered to be reduced in the A-ring.

Introduction

Exemestane (6-methyleneandrosta-1,4-diene-3,17-dione) is therapeutically used as steroidal aromatase inhibitor in treatment of metastatic breast cancer in postmenopausal women. Aromatase (estrogen synthetase) is an enzyme that catalyses the last step of the estrogen biosynthesis: the conversion of androgens to estrogens. Exemestane inhibits for example the conversion of adrenally generated androstenedione to estrone or testosterone to estradiol in ovaries and peripheral tissues, such as adipose tissue, as well as in some tumours [1]. It is orally active and structurally related to the natural substrate androstenedione [2].

The recommended therapeutical dose for exemestane is 25 mg per day [3,4]. Exemestane is extensively metabolised. Known is the reduction of the 17-keto group to give the 17ß-hydroxy steroid[5]. It was shown that exemestane boosts testosterone excretion [6]. In a cross-over study, 12 volunteers were randomly assigned to an oral administration of 25 and 50 mg exemestane/day for 10 days with a 14 days washout period. An increase in plasma testosterone concentrations by 56-60% was found.

Male athletes may feel encouraged to treat the adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) by using aromatase inhibitors. Therefore, since 2001, the use of aromatase inhibitors is prohibited by IOC and WADA for males. For female athletes the use of aromatase inhibitors is forbidden since January 2005 [7]. Methods for the analysis of exemestane and its 17-dihydro metabolite in human plasma using HPLC [8,9] and in urine as underivatised compounds using GC-MS analysis have been described [10]. Our intent was to incorporate these compounds into our screening method for anabolic steroids.

Experimental

Chemicals and reagents: Synthetic reference materials were exemestane (Thinker Chemical, Hangzhou, China), 17-dihydroexemestane (synthesised in-house by reduction of exemestane with $NaBH_4$) and Aromasin® containing 25 mg per tablet (Pharmacia & Upjohn GmbH, Erlangen, Germany). N-methyl-N-trimethylsilyl-trifluoroacetamide was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Other reagents and solvents were of analytical grade and provided by Merck (Darmstadt, Germany).

Excretion study urine samples: An excretion study was performed orally by a healthy male volunteer who had given his written consent to the excretion study which was approved by the local ethical committee. 25 mg of exemestane was orally administered. Aliquots of urine were collected for 8 days and thereafter morning urine samples over a time period of 10 days (in total over 18 days). Additionally spot urine samples from a woman suffering from metastatic breast cancer, treated with exemestane, were collected. Work-up and LC-MS or GC-MS conditions were as described elsewhere [11,12,13].

Degradation experiments: 25 µg and 2.5 µg of exemestane and 17-dihydroexemestane respectively were silylated with 100 µl of $MSTFA/NH_4I/ethanethiol$ (1000/2/3, v/w/v) at 60°C for 20 min. For studies in urinary matrix 2 ml of blank urine were worked up for silylation [12], 25 µg or 2.5 µg of the substances were added and silylated as above. The samples were placed into an automatic liquid sampler and measured repeatedly. An Agilent Technologies 6890 plus/5973N system was used. The GC conditions were: Injection port 300°C, oven: 0 min 180°C, +20°C/min, 3 min 320°C, carrier gas: He, flow: constant at 1 ml/min, interface: 300°C, column: 17 m Se54, I.D. 0.25 mm, film 0.25 µm. The MSD conditions were: Source temp 230°C, quad temp 150°C, scan 40 to 800 amu, threshold 100, sampling rate 2^2.

Validation:

* Calibration curves: Calibration curves were generated with the concentrations 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ng /ml of exemestane and 17-dihydroexemestane in urine. As internal standard 17α-methyltestosterone (MeT) was used (500 ng/ml). Each calibration point was prepared and analyzed once. For the evaluation the areas of the ion transitions *m/z* 297/121 (exemestane), 299/135 (17-dihydroexemestane) and 303/109 (MeT) were used.

* Lower limit of detection: The lower limit of detection was calculated from the signal to noise ratio of 10 blank urine samples and 10 specimens spiked with 5 ng of 17-dihydroexeme-stane/ml and 10 ng of exemestane/ml.

* Precision: Ten negative control urine samples spiked with 10 (low), 50 (medium) and 100 ng (high) of exemestane and 17-dihydroexemestane per ml were used.

* Specificity/robustness: Urine samples spiked with exemestane (10 ng/ml) and 17-dihydro-exemestane (5 ng/ml) were prepared.

* Recovery, Hydrolysis experiments, Extraction at different pH-values, Purification with n-pentane: Compare [11]

Results and Discussion

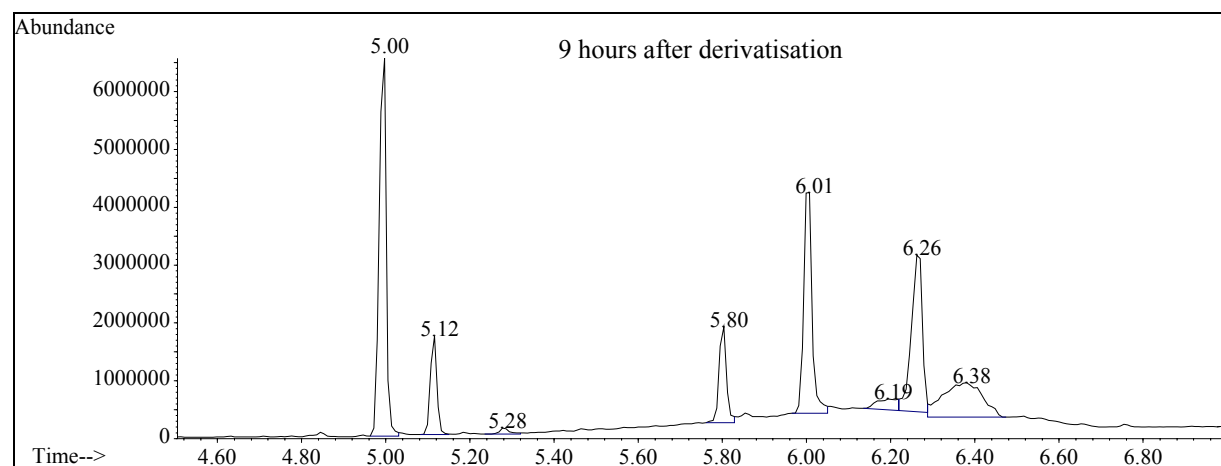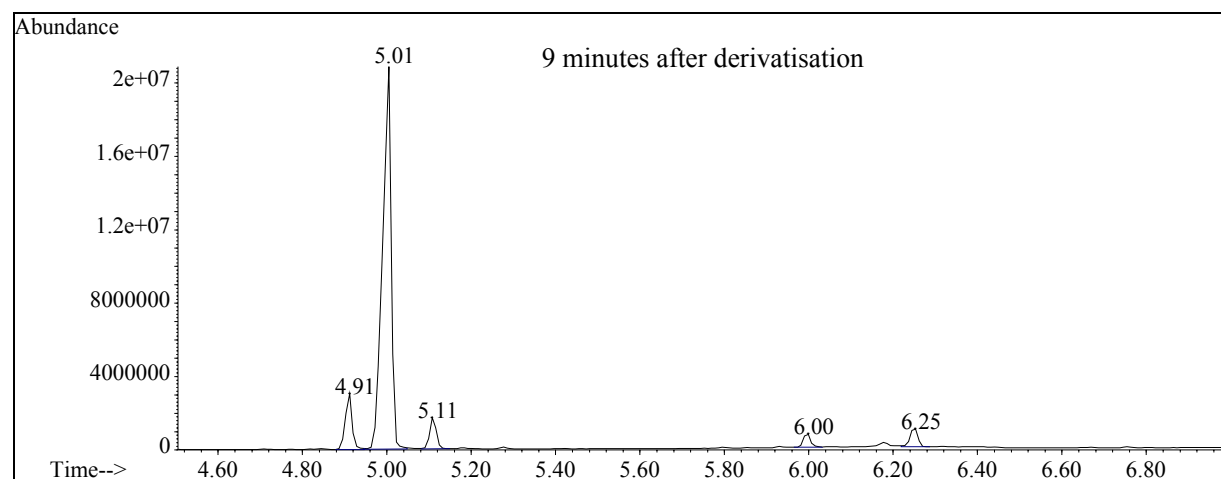The electron impact mass spectra of exemestane and 17-dihydro-exemestane and respective

Fig. 1: TIC's of exemestane 250 ppm derivatised with TMIS (Scan at 4.91 min exemestane, at 5.01 min exemestane 16-enol TMS ether).

trimethylsilylated derivatives have been published elsewhere [10, pages 217 and 220]. After silylation with MSTFA/NH$_4$I/ethanethiol (1000/2/3, v/w/v) and standing at ambient temperature the composition of the chromatograms changed dramatically (Fig. 1, Fig. 8): The amount of the reference substances decreased while at least 5 new peaks were detected.

Fig. 2 shows the mass spectrum of the substance at 5.12 min which indicates the formation of 6-methylene-estrone, bis-TMS. All ions greater than *m/z* 200 are increased by 12 and the relative intensities comply. As shown later this reaction occurs in the injection port and can be explained by the aggressiveness of the TMIS reagent and the reactivity of the oxo group of the 1,4-dien-3-one moiety: So the enolisation of the 3-oxo group is enforced under removal of the methyl group at carbon 10, most probably as CH$_3$I.
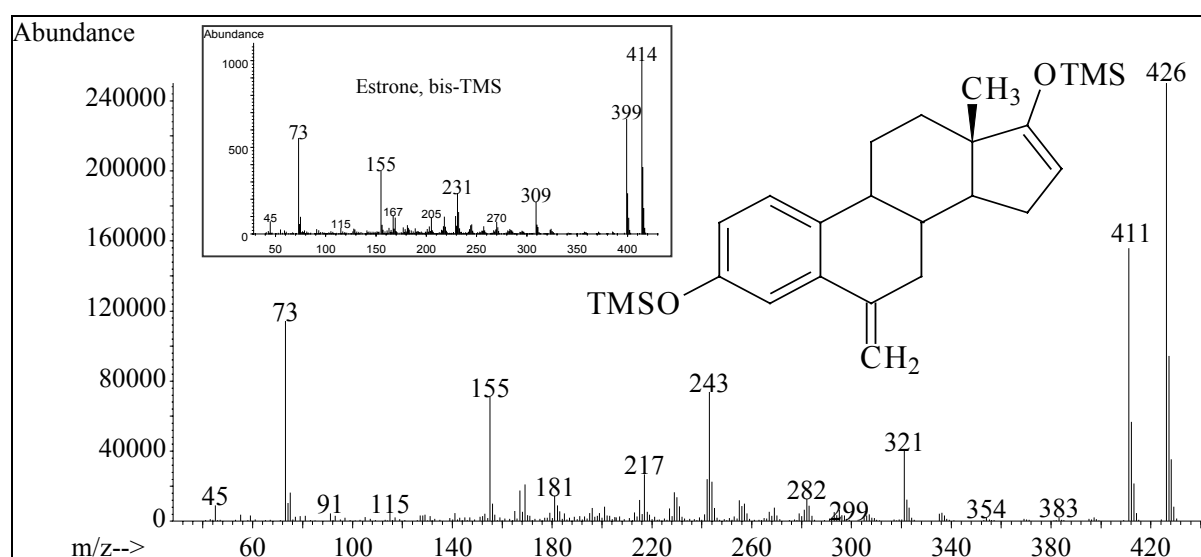

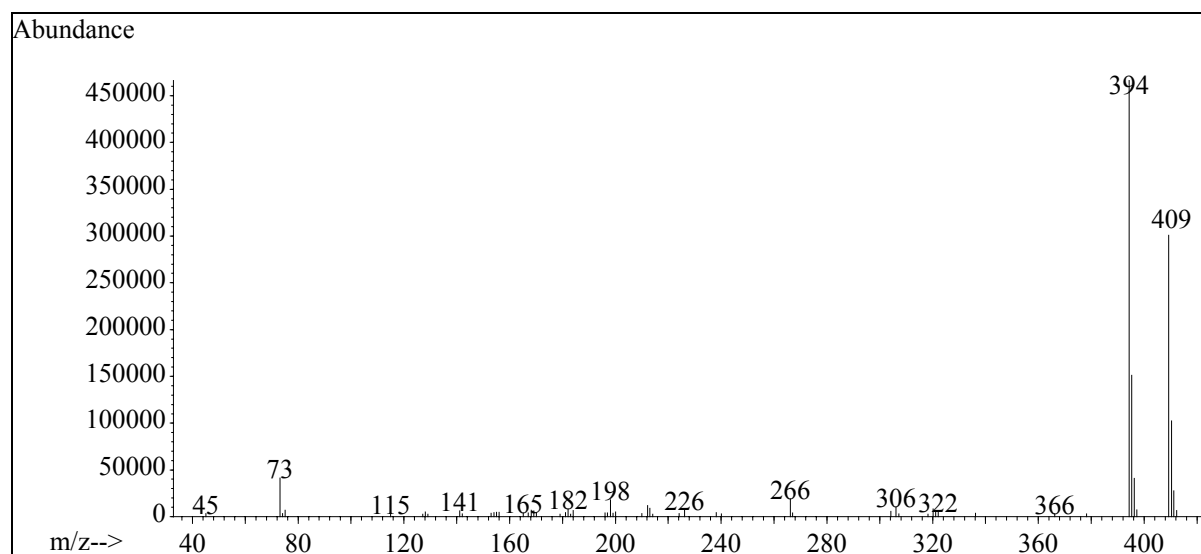*Fig 2: EI mass spectrum of the substance at 5.12 min: 6-methylene-estrone, bis-TMS.*

Fig. 3: EI mass spectrum of the substance at 5.80 min.

The mass spectrum of the substance at 5.80 min is shown in Fig. 3. The molecular ion of exemestane, TMS (368) is increased by 41 amu. This shift could not be explained yet.

Fig. 4 shows the mass spectrum of the substance at 6.01 min. The molecular ion at *m/z* 567 indicates an addition of MSTFA (M = 199). The fragment at *m/z* 440 can be explained by a neutral loss of N-methyl-trifluoroacetamide (M = 127) from the molecular ion and subsequent losses of a methyl radical and trimethylsilanol leading to the fragments at *m/z* 425 and 335. The most probable site for such an addition is the 3-oxo group (C-MTFA, O-TMS): the steric tension of the A-ring is reduced when the configuration of carbon 3 changes from sp^2 to sp^3.
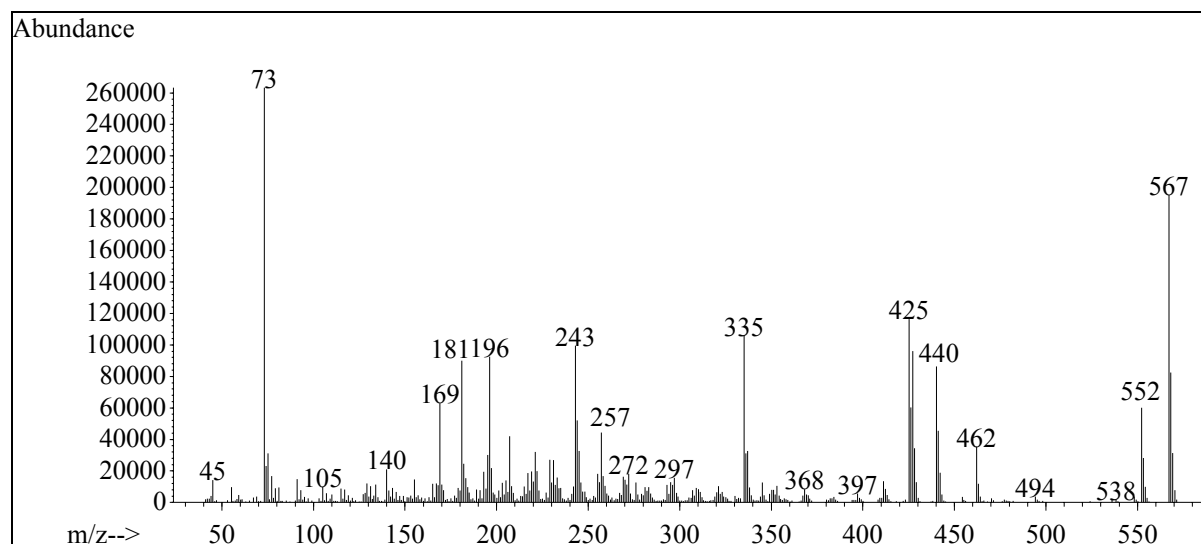

Fig. 4: EI mass spectrum of the substance at 6.01 min.
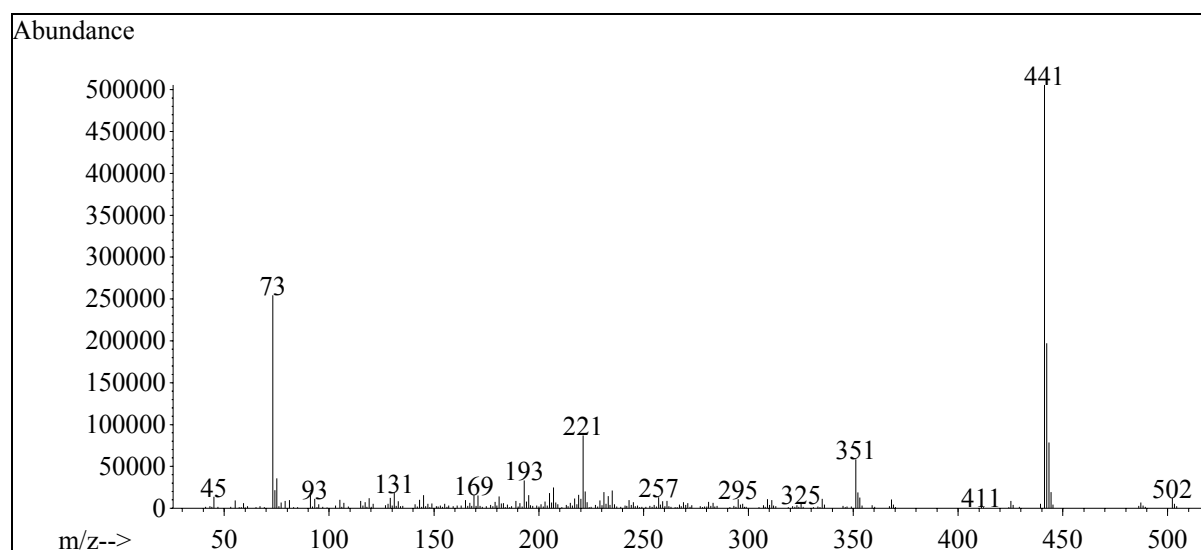

Fig. 5: EI mass spectrum of the substance at 6.26 min.

Fig. 5 shows the mass spectrum of the substance at 6.26 min. The molecular ion at *m/z* 502 indicates the addition of ethanethiol, TMS (M = 134) and the fragment ion at *m/z* 441 can be explained by the loss of an ethylmercapto radical. Again the 3-oxo group is in favour for such an addition (C-SC$_2$H$_5$, O-TMS).

Fig. 6 shows the selected ion profile of *m/z* 426 (molecular ion of 6-methylene-estrone, bis-TMS) and *m/z* 486. It reveals that the peak at 6.38 min does not correspond to a specific deri-
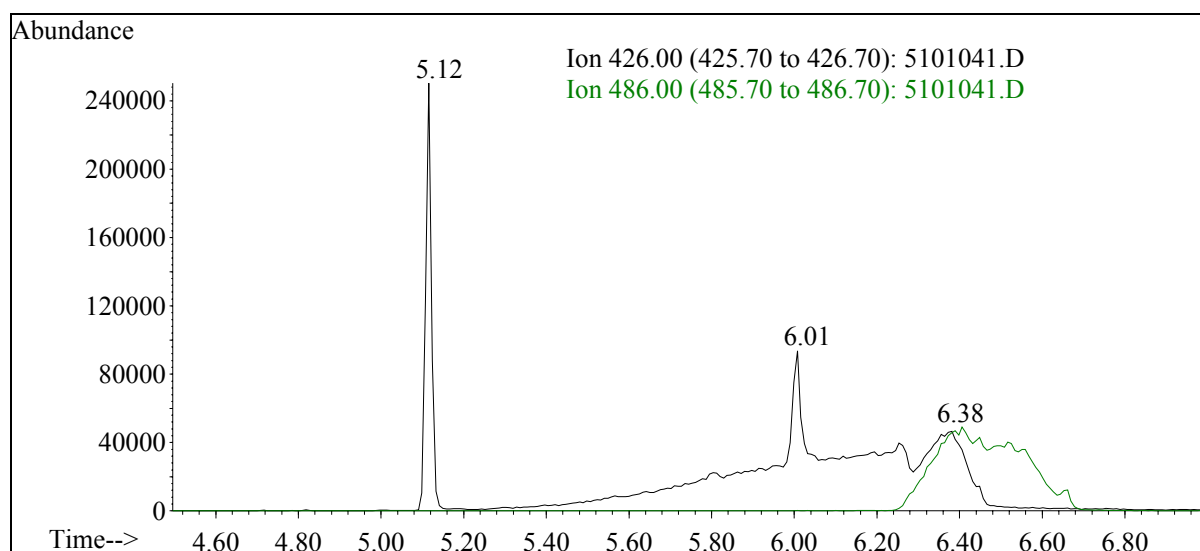
Fig. 6: Selected ion profile of m/z 426 and 486

vative but is generated by overlapping of two compounds. The mass spectrum of the latter (Fig. 7) contains too few informative fragment ions to elucidate its structure. The chromatographic behaviour of the 6-methylene-estrone, bis-TMS is reasonably explainable by the assumption that its formation not only occurs in the injection port but also in the column: the hotter the more (implicating that a part of the exemestane is irreversibly bound).
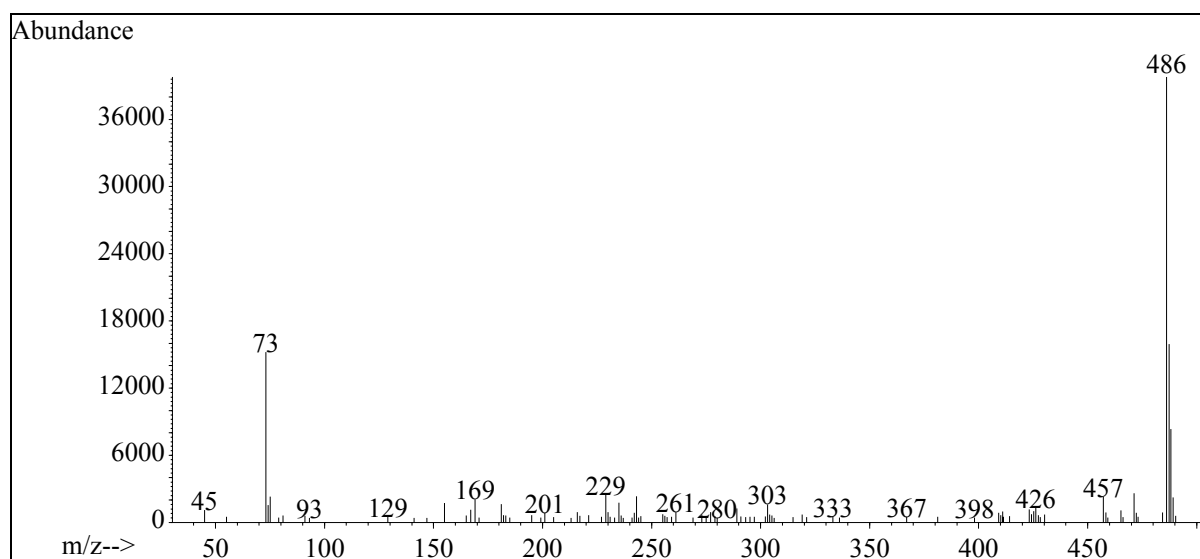

Fig. 7: EI mass spectrum taken at 6.52 min.

Dihydroexemestane shows a comparable behaviour to exemestane. In Fig. 8 the TIC of dihydroexemestane after silylation with TMIS reagent and standing at ambient temperature for 8.5 hours is presented. Comparison with Fig. 1 clearly demonstrates the analogy. The EI mass spectrum of the peak at 5.19 min (Fig. 9) is indicative for the formation of 6-methylene-estradiol, bis-TMS. All ions greater than *m/z* 200 are increased by 12 amu and the relative intensities comply with the mass spectrum of estradiol, bis-TMS. All other peaks correspond to the artifacts of exemestane: The molecular ions (and many of the fragments, if any) are increased
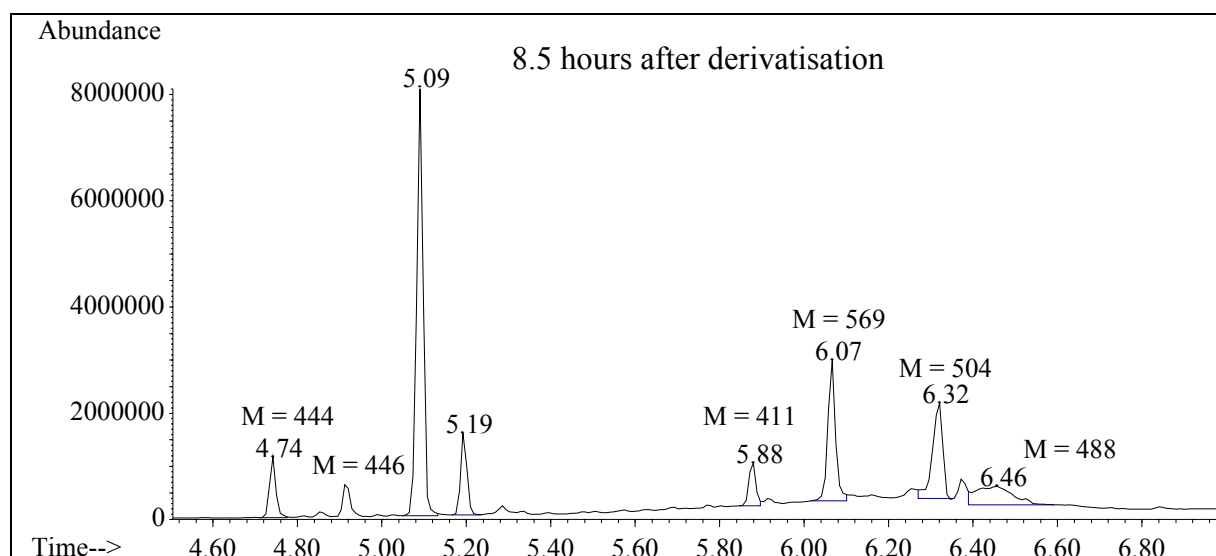
120

Fig. 8: TIC of dihydroexemestane derivatised with TMIS (Scan, peaks <5 min are impurities).

by 2 amu and the ion profiles of *m/z* 428 and 488 look very similar to the corresponding pro-
files of exemestane demonstrating that the formation of the artifacts is independent of the
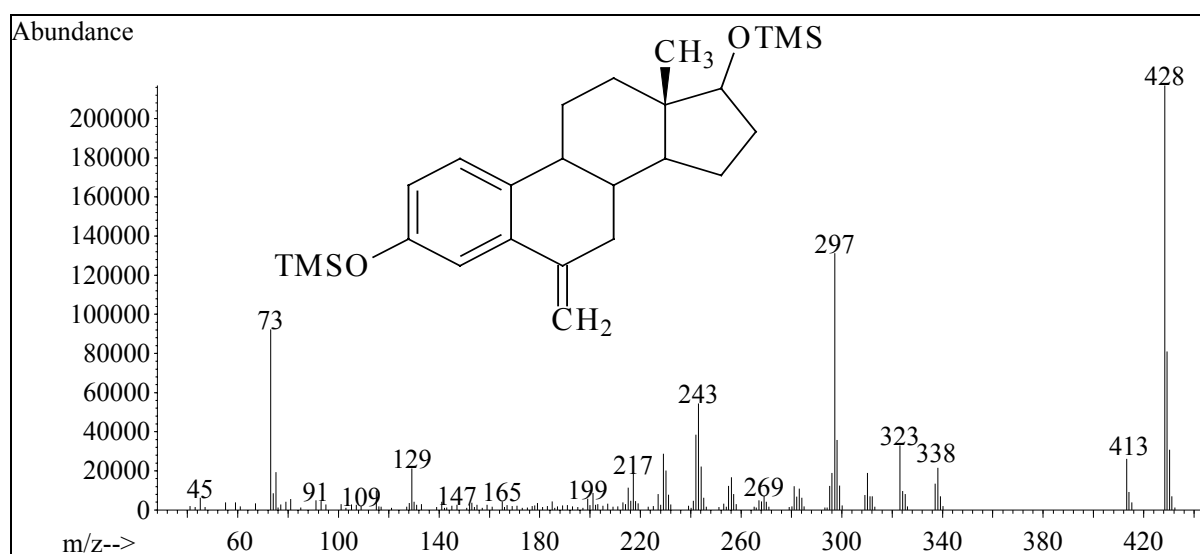structure of the D-ring and is based on the special structure of the A-ring of the steroid.

Fig. 9: EI mass spectrum of the substance at 5.19 min in fig. 8

The time course of the degradation of exemestane 25 ppm in a urinary matrix is displayed in
Fig. 10. While exemestane itself decreases very fast to 23 % within 4.7 h and the artifacts
increase mainly in this time, the formation of 6-methylene-estrone is nearly constant. From
this it is concluded that 6-methylene-estrone is generated mainly in the injection port. The
behaviour of 17-dihydroexemestane is nearly identical to that of exemestane itself, simply es-
trone is to be replaced by estradiol and the molecular weights and some fragments are in-
creased by 2 amu. Also the rates of degradation and formation of the byproducts are compa-
rable.The artifacts described above were found in standard solutions 250 ppm (Fig. 1, Fig. 8)
and 25 ppm, in blank urine samples spiked at 1.25 µg/ml (corresponding to a 250 ppm stan-
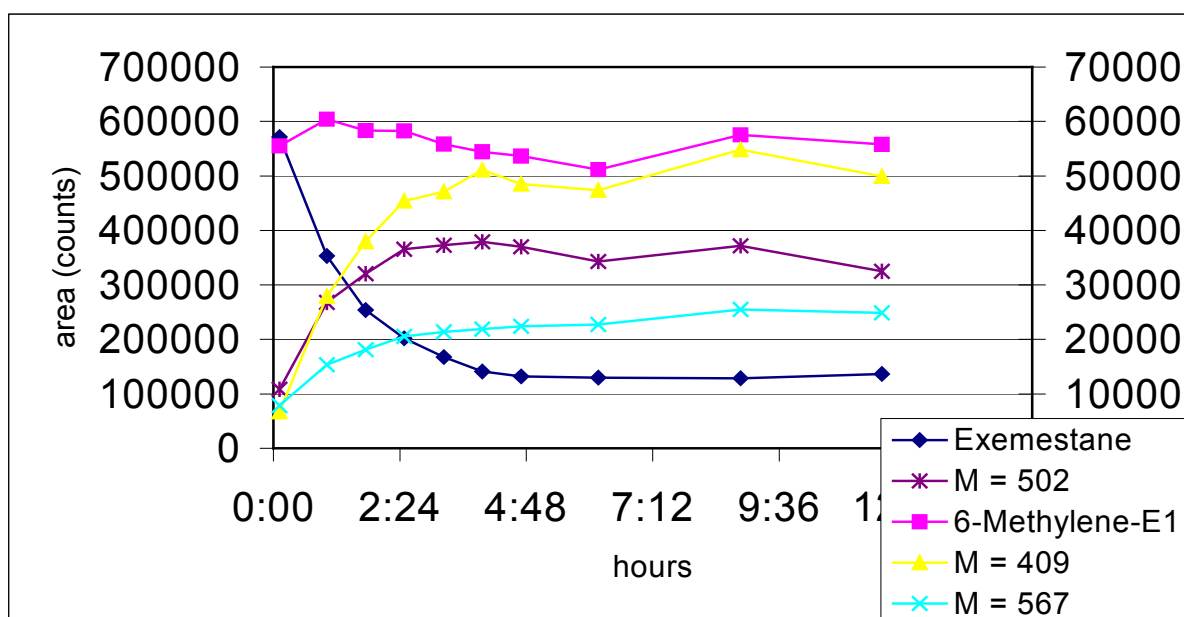
121

Fig. 10: Degradation of exemestane in a urine extract spiked at 125 ng/ml (corresponding to a 25 ppm standard) derivatised with TMIS reagent. Left axis: exemestane and m/z 502, right axis: 6-methylene-estrone, m/z 409 and m/z 567.

dard) and 125 ng/ml (Fig. 10) and in spot urine samples from excretion studies as well as in urine from a woman suffering from metastatic breast cancer and treated with exemestane. In a normal screening procedure the derivatised samples are put in an automatic liquid sampler and measured in sequence so that they remain at ambient temperature for 1 to 20 hours until analysis. After 1 hour up to 38 % of the exemestane is degraded and after 4.7 hours up to 77% which needs consideration in drug testing.
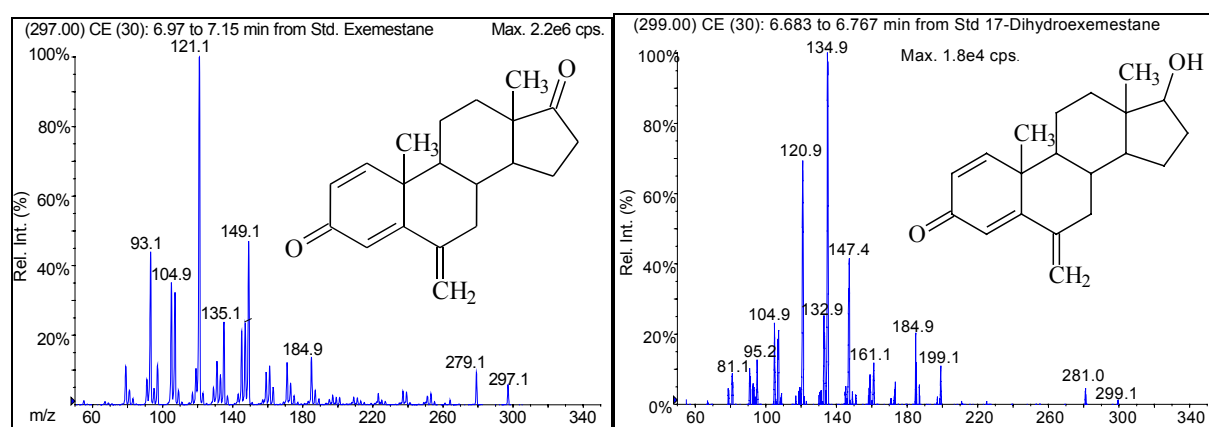

Fig 11: Left: Product ion spectrum of exemestane: $(M+H)^+ = 297$ (APCI, CE = 30 V), right: Product ion spectrum of 17-dihydroexemestane: $(M+H)^+ = 299$ (APCI, CE = 30 V).

Due to the difficulties using the TMIS reagent incorporation of exemestane and its 17-dihydro derivative into the screening procedure for glucocorticosteroids, beta-blockers, selected ana-bolic androgenic steroids and buprenorphine [11,12] was studied. Both substances give a good response in APCI. Fig. 11 shows a product ion spectrum of exemestane (collision offset

voltage of 30 V, the precursor ion *m/z* 297). Intensive fragment ions at *m/z* 149 and 121 are useful for monitoring exemestane in the multiple reaction mode (MRM) mode. Fig. 11 also shows the product ion spectrum of 17-dihydroexemestane (collision offset voltage of 30 V, precursor ion *m/z* 299 with intensive fragment ions at *m/z* 135 and 121, both useful for monitoring 17-dihydroexemestane in MRM).

Calibration curves for exemestane and 17-dihydroexemestane (10 –100 ng/ml)

Ten urine samples were spiked with exemestane and 17-dihydroexemestane from 10 ng/ml up to 100 ng/ml. The peak areas were obtained by integration of the ion transitions *m/z* 297/121 for exemestane, *m/z* 299/135 for 17-dihydroexemestane and *m/z* 303/109 for methyltestosterone. Subsequent calculation of their ratios yielded linear and homoskedastic calibration curves for exemestane (y = 0.0047x + 0.0188, residual standard deviation (RSD) 0.026) and 17-dihydroexemestane (y = 0.0215x + 0.0682, RSD 0.068)

Lower limit of detection [11]

The lower limit of detection was determined as 3.1 ng/ml for exemestane and 0.58 ng/ml for 17-dihydroexemestane at a signal-to-noise ratio ≥ 3.

Precision [11]

Exemestane: The intra-batch precision was 8.6, 8.7 and 4.4 % (n = 10 each) for the concentration levels at 10, 50 and 100 ng/ml, respectively. Inter-day precision ranged from 10.8 % (low) to 9.1 % (medium) and 8.3 % (high, n = 30 each).

17-Dihydroexemestane: The intra-batch precision was 5.5, 4.4 and 5.0 % (n = 10 each) for the concentration levels at 10, 50 and 100 ng/ml, respectively. Inter-day precision ranged from 6.6 % (low) to 6.7 % (medium) and 5.6 % (high, n = 30 each).

Recovery

The recovery was determined to be 85 % for exemestane and 89 % for 17-dihydroexemestane.

Specificity / Robustness

Successful screening for exemestane (*m/z* 297/121) and 17-dihydroexemestane (*m/z* 299/135) in each of the 10 urine sample was possible.

Hydrolysis experiments

Exemestane is excreted unconjugated. 17-Dihydroexemestane is excreted glucuronidated to a high extent. The hydrolysis with glucuronidase from E.coli was completed within 5 minutes.

Extraction at different pH-values

The extraction yields for exemestane and 17-dihydroexemestane at pH 7, 9.6 and 14 show similar results. Due to the fact that the extraction at pH 7 shows higher background an extraction at pH 9.6 is recommended.

Purification with n-pentane

The recoveries using n-pentane instead of TBME were determined as 80 % for exemestane and 72 % for 17-dihydroexemestane and were not significantly lower compared to TBME.

Screening for exemestane

Fig. 12 shows urine specimens spiked with exemestane and 17-dihydroexemestane. The response for 17-dihydroexemestane is greater than for exemestane. Therefore 17-dihydroexemestane should be included into the screening procedure.
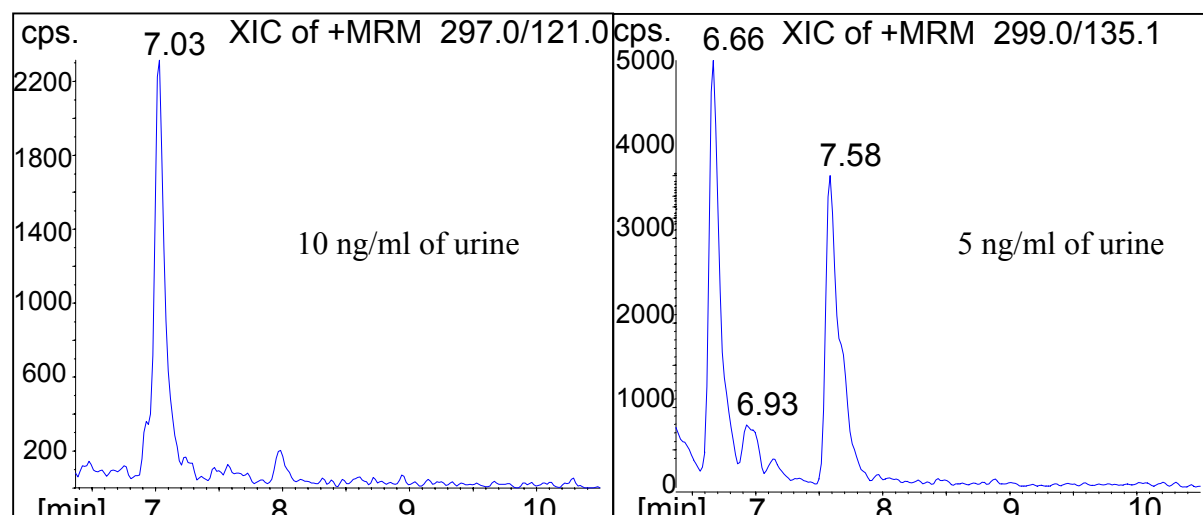

Fig. 12: Extracted ion chromatogram (297/121) for exemestane at 7.03 min (left)
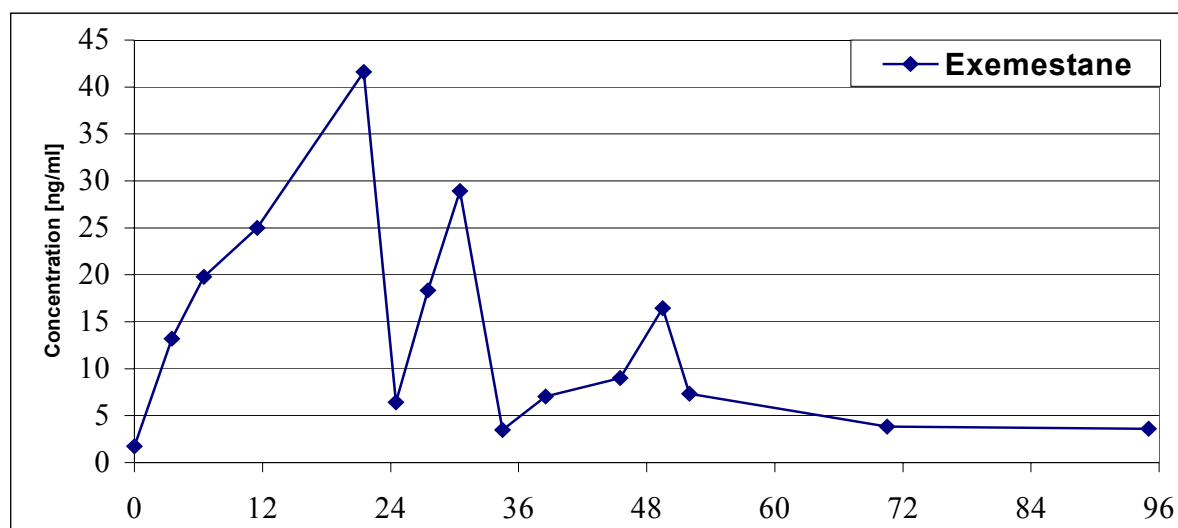and (299/135) for 17-dihydroexemestane at 6.66min (right).

Fig. 13: Urinary exemestane excretion after a single dose of 25 mg of exemestane.

The concentration-time curves of an excretion study with 25 mg of exemestane are shown in Fig. 13 and 14. While the concentration of exemestane arises and drops repeatedly the concentration of 17-dihydroexemestane reaches very rapidly a maximum and afterwards drops to small residual values.Values as low as 2.87 ng/ml and 0.14 ng/ml were obtained after 95 hours for exemestane and 17-dihydroexemestane respectively
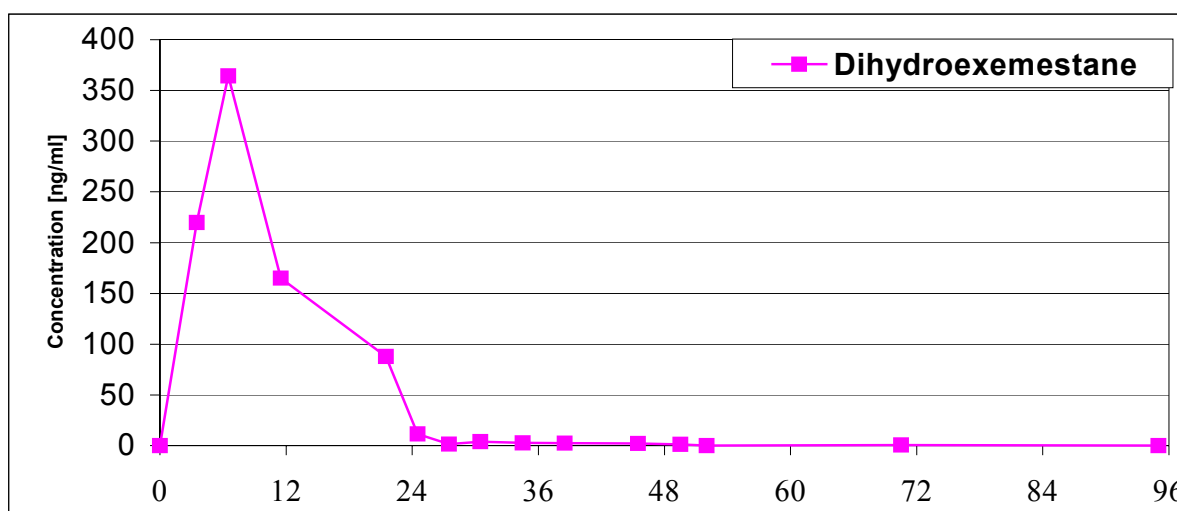
Fig. 14: Urinary 17-dihydroxemestane excretion after a single dose of 25 mg of exemestane.

Search for exemestane metabolites

Besides 17-dihydroexemestane hypothetical metabolites have been synthesised starting with the epoxidation of the 6-methylene group and subsequent reactions and their inhibitor properties against aromatase have been studied [14]. These metabolites were not detected here (scan and SIM).
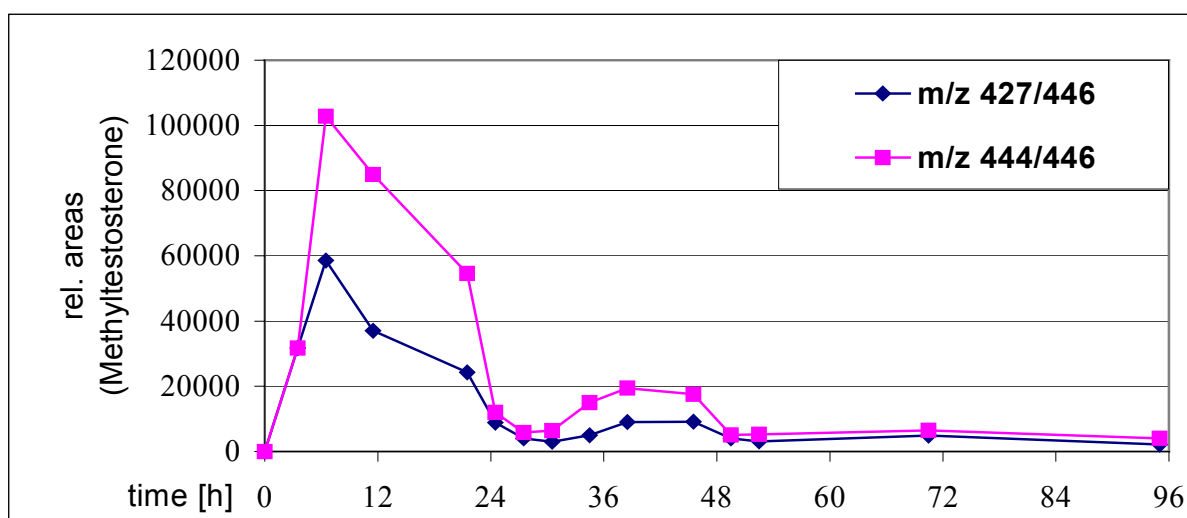

*Fig. 15: Excretion profile (relative areas * 100000) of dihydroexemestane II and tetrahydro-exemestane after a single dose of 25 mg exemestane.*

A search for other metabolites assuming a reduction in the A-ring was successful though full reliable spectra were not obtained. A substance with a molecular ion at *m/z* 442 and a fragment at *m/z* 427 (base peak) (dihydroexemestane II) and another substance with a molecular ion at *m/z* 444 (base peak) and a fragment at *m/z* 429 (tetrahydroexemestane) were found. Their excretion profiles are presented in Fig. 15.

Acknowledgements

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