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Identification of non-steroidal Aromatase Inhibitors in Doping Analysis

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Abstract

Anastrozole, letrozole and vorozole are therapeutically used as non-steroidal aromatase inhibitors in treatment of metastatic breast cancer in postmenopausal women.
For doping purposes these substances may be used for treatment of adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) and to increase the testosterone concentration and stimulation of the testosterone biosynthesis.
The use of aromatase inhibitors is prohibited for male athletes since September 1, 2001.
Purpose of this study was the identification and enclosure of the parent compound or the main metabolite(s) of the above mentioned non-steroidal aromatase inhibitors into established, running screening procedures in doping analysis.
Excretion studies were carried out by oral application of one single dose Arimidex® (2 mg) and Femara® (5 mg) respectively.
The analysis was performed by gas chromatography-mass spectrometry (GC-MS).
The unchanged drug of anastrozole is detectable in different screening procedures for up to 152 hours; letrozole is detectable only in the screening procedure for heavy volatile nitrogen containing drugs for up to 43 hours.
Full scan spectra and diagnostic ions for the analysis of anastrozole and letrozole are presented.

Introduction

The aromatase pathway transforms testosterone and its precursor androstenedione into estrogens.
Aromatase inhibitors are therapeutically used in treatment of metastatic breast cancer in post-menopausal women because the inhibition of the biosynthesis of estrogens may lead to the degeneration of tumor tissue.
Male athletes may be encouraged to treat the adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) by using aromatase inhibitors.

Aromatase inhibitors have been divided into three classes:

1. Aminoglutethimide
2. Non steroidal aromatase inhibitors (anastrozole, letrozole, vorozole)
3. Steroidal aromatase inhibitors (exemestane, formestane)

As notified by the IOC and WADA on June 1, 2001 the use of aromatase inhibitors is prohibited for males beginning on September 1, 2001.

Aromatase inhibitors are listed (since 1.01.2003) under prohibited classes of substances in the class of agents with anti-estrogenic activity where the anti-estrogenic drugs clomiphene, cyclofenil and tamoxifen are specifically mentioned.

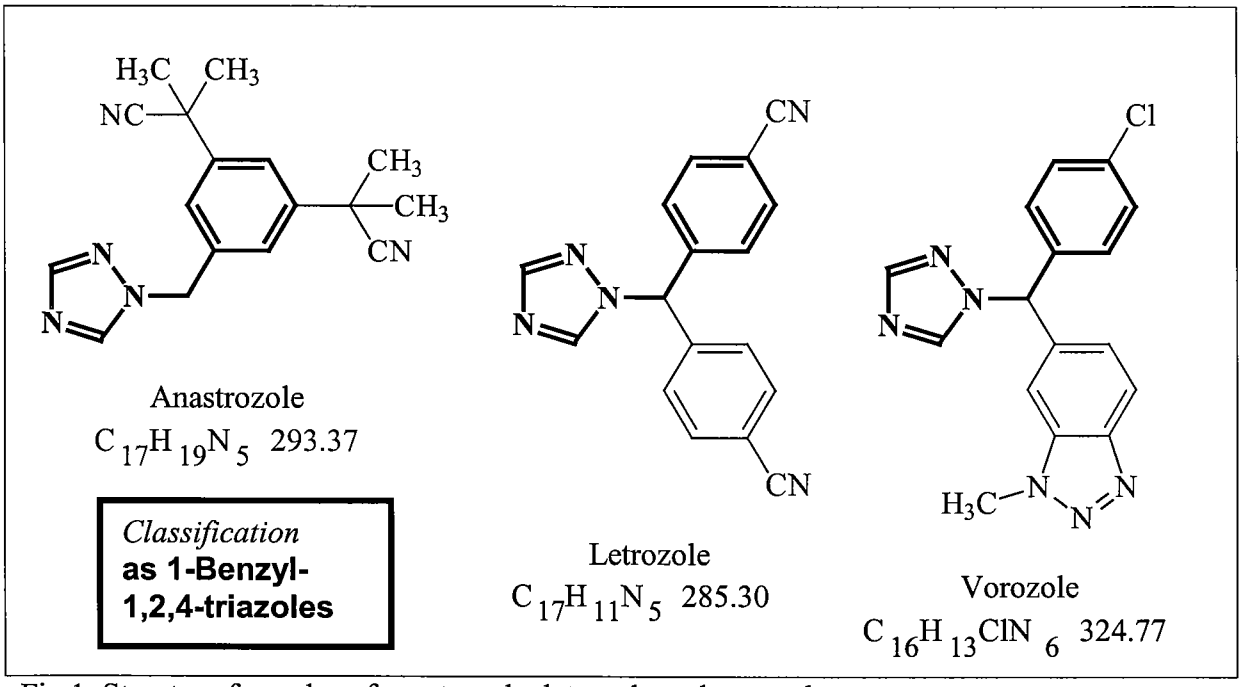

Fig 1: Structure formulas of anastrozole, letrozole and vorozole.

Non steroidal aromatase inhibitors belong to a new generation in aromatase inhibition. The use of the aromatase inhibitor aminoglutethimide is limited by its lack of selectivity for aromatase and its toxicity. The new agents are more selective and their use does not lead to an inhibition of the cortisone synthesis. Also the recommended therapeutical dose is different: for aminoglutethimide a daily dose of 250 mg is recommended, for anastrozole 1 mg/d and for letrozole 2.5 mg/d. [1]

It was shown that Arimidex® boosts testosterone excretion [2]. After a 10 weeks treatment with 1 mg of Arimidex per day an increase of testosterone by 58% and also of LH and FSH concentrations in serum was detected.

The effect on male infertility was investigated. If the infertility results from a low serum testosterone to estradiol ratio treatment with anastrozole is recommended. [3]

In the underground literature anastrozole is freely offered as "the aromatase inhibitor of choice". The drug "should be appropriately used when using substantial amounts of

aromatizing steroids or when the user is prone to gynecomastia while using moderate amounts of such steroids". [4]

Anastrozole has a mean terminal half-life of approximately 30 to 60 hours [5,6].

It is extensively metabolized with about 10% of the dose excreted in the urine as unchanged drug within 72 hours of dosing, and the remainder (about 60% of the dose) excreted in urine as metabolites. Anastrozole and letrozole are metabolized by N-dealkylation, hydroxylation and glucuronidation. Three metabolites of anastrozole have been identified in human plasma and urine. The known metabolites are triazole, a glucuronide conjugate of hydroxy-anastrozole and a glucuronide of anastrozole itself. [7]

For letrozole bis-4-cyanophenyl-methanol is described as metabolite [8,9].

Studies of vorozole are also published [10] but it was not possible to get this drug as reference substance. According to informations of Janssen Cilag vorozole (R83842) failed in 1997 the third clinical study and thereafter the development was terminated due to economical reasons.

Experimental

Sample preparation

The urine samples were prepared and analyzed according to the standard operating procedures for volatile nitrogen containing drugs [11], heavy volatile nitrogen containing drugs [11,12,13] and anabolic steroids [11,14].

GC-MS parameters

GC-MS: HP 6890-HP 5973 (Hewlett Packard)

electron impact: 70 eV

Screen1 (volatile nitrogen containing drugs):

column: HP 5MS, 24m, 0.25mm i.d., 0.25 µm film thickness
carrier gas: helium 12 psi, split 1:8
temperature program: 0 min100°C, + 22°C / min, 2 min 330°C
injection volume: 5 µl

Screen 2 (heavy volatile nitrogen containing drugs):

column: HP 5MS, 24m, 0.25mm i.d., 0.25 µm film thickness
carrier gas: helium 12 psi, split 1:8
temperature program: 0 min 95°C, + 22°C / min, 2 min 330°C
injection volume: 3 µl
A modified standard operating procedure was performed: After the extraction with t-butylmethyl ether / t-butanol and evaporation to dryness in vacuo the residue is reconstituted in methanol. A part is injected into GC-MS without derivatisation. [12,13]

Screen 4 (anabolic steroids):

column: HP Ultra I (OV-1), 17m, 0.2mm i.d., 0.11 µm film thickness
carrier gas: helium 12 psi, split 1:10
temperature program: 0 min 180°C, + 3°C / min, 0 min 229°C, + 40°C / min, 2 min 320°C
After extraction with t-butylmethyl ether [11, 14] the dry residue was derivatized with 100 µl of MSTFA/NH_4I/ethanethiol 1000:2:3 (v:w:v) and heated for 20 min at 60°C.
injection volume: 3 µl

Urine samples
Excretion studies were performed by healthy male volunteers. One single dose of anastrozole (2 mg) and of letrozole (5 mg) was administered. For 30 hours all urine samples were collected and thereafter only morning urine samples for a total of 7 days.

Metabolites
Anastrozole and letrozole in the urinary matrix were identified by comparison of their mass spectra and retention times with those of a anastrozole reference (Arimidex®) or letrozole reference (Femara®), respectively.

Results and Discussion

Anastrozole
Anastrozole was detected as unchanged drug in screen 1, 2 and 4 over a time period of 152 hours after administration of 2 mg Arimidex®.
The mass spectrum of anastrozole shows as molecular ion *m/z* 293 (Fig 2). Loss of the triazolyl radical (α-fission from the aromatic system) leads to *m/z* 225 and neutral loss of triazole to *m/z* 224. The basepeak *m/z* 209 is generated by the subsequent loss of a methyl radical. Additional neutral loss of HCN leads to *m/z* 182.
On the other hand cleavage of HCN from the molecular ion leads to *m/z* 266, following neutral loss of triazole to *m/z* 197 and additional loss of a methyl radical again to *m/z* 182.
The ions at *m/z* 209 and 224 are monitored in screen 4 (SIM mode), and the same ions are extracted from full scan in screen 2 (Fig 3).

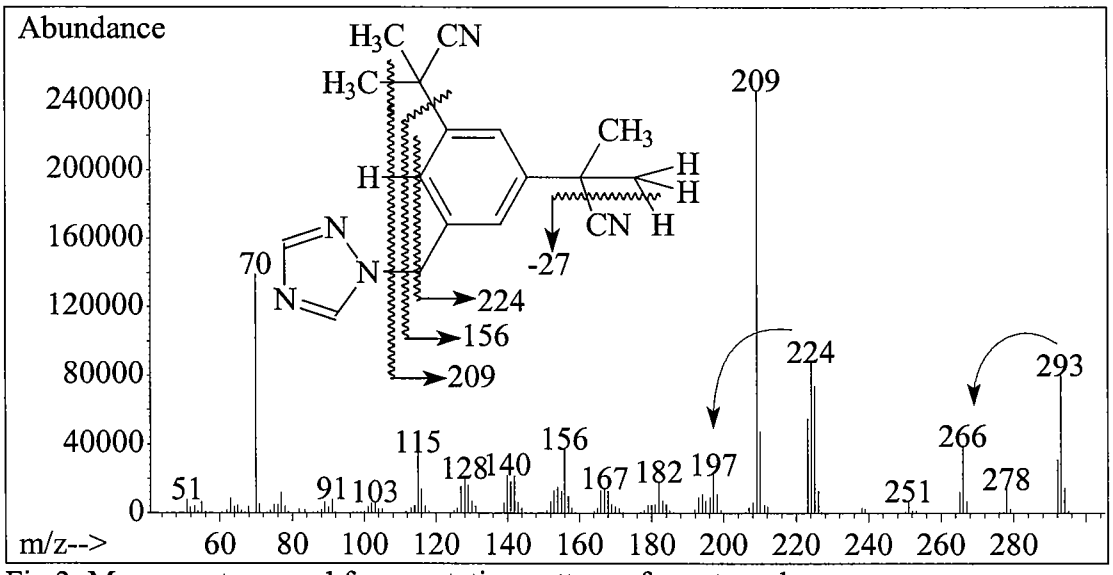

Fig 2: Mass spectrum and fragmentation pattern of anastrozole.

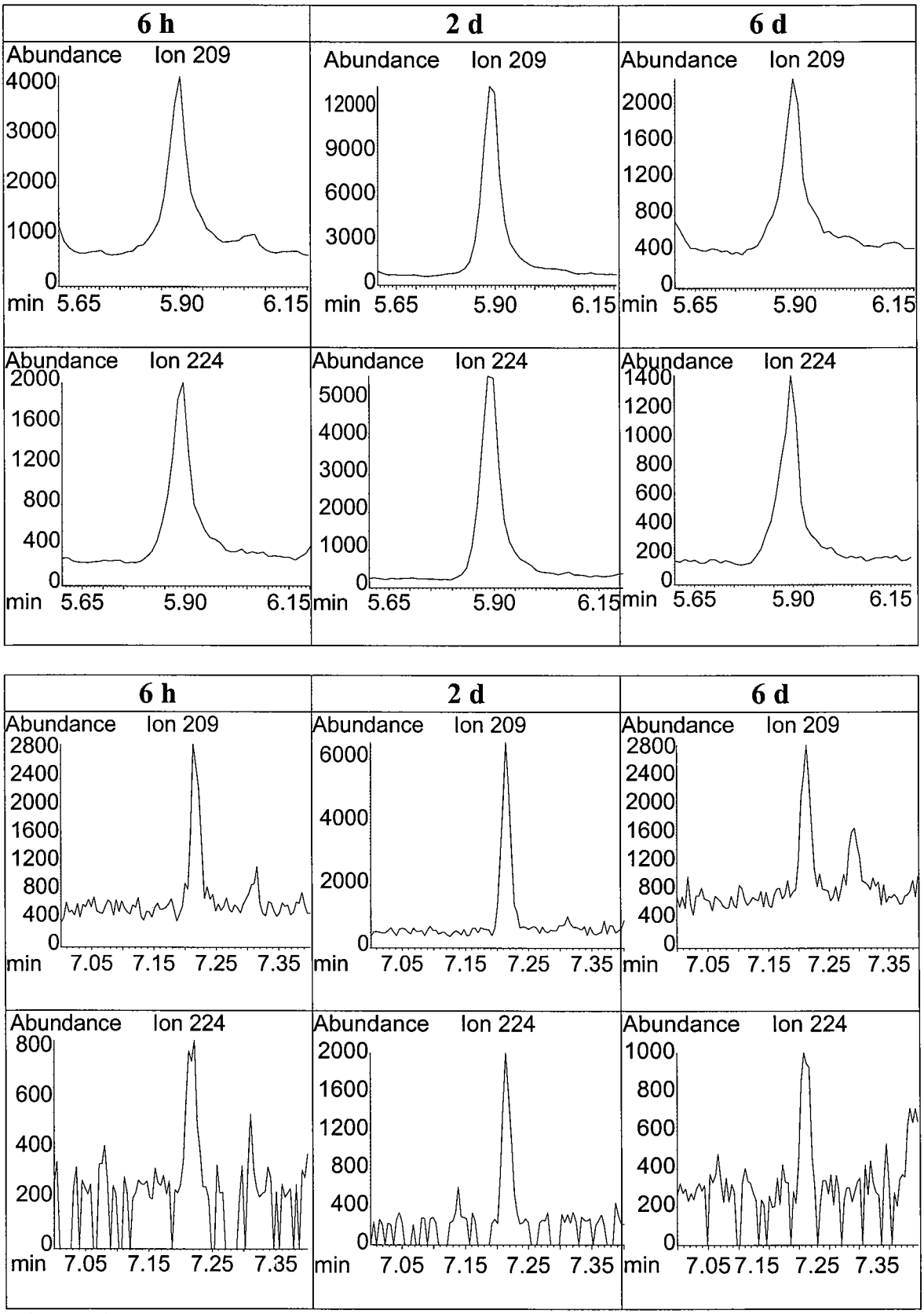

Fig 3: Three samples, positive for anastrozole, taken at different times after administration. upper: screen 4 (SIM), lower: screen 2 (scan)

Letrozole

Letrozole was detected as unchanged drug only in screen 2 over a time period of 43 hours after administration of 5 mg Femara®. In screen 4 the detection of letrozole parent compound is not possible resulting from its bad chromatographical behaviour during analysis with a OV 1 column.

The mass spectrum of letrozole shows a molecular ion *m/z* 285 (Fig 4). The basepeak *m/z* 217 is generated by loss of the triazolyl radical (α-fission from the aromatic system) and neutral loss of HCN leads to *m/z* 190. The loss of a hydrogen radical from the molecular ion leads to m/z 284, and following losses of HCN lead to m/z 257 and 230. The loss of a cyanophenyl radical leads to *m/z* 183 and following losses of HCN lead to *m/z* 156, 129, 102 and 75.
The ions *m/z* 217 and 190 are extracted from full scans in screen 2 (Fig 5).

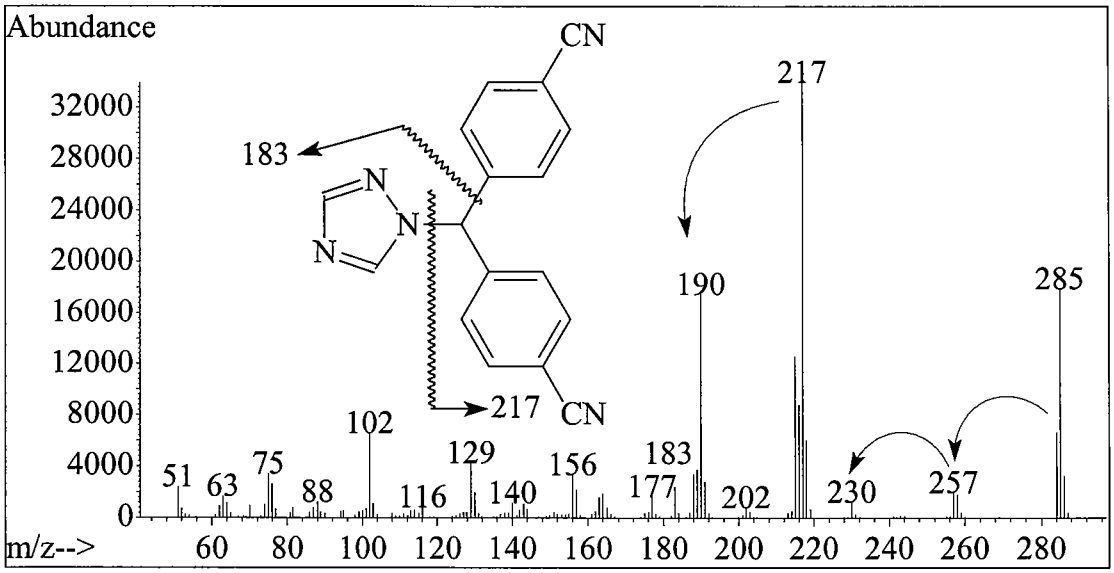

Fig 4: Mass spectrum and fragmentation pattern of letrozole.

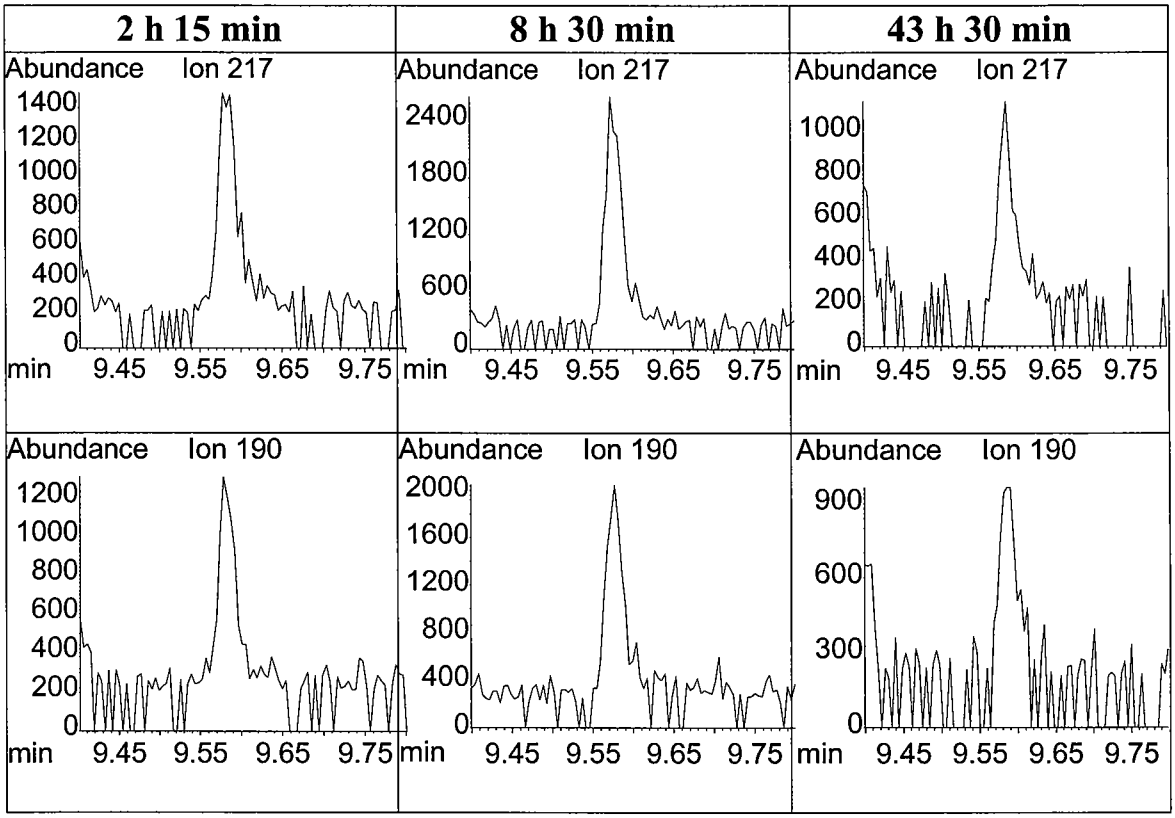

Fig 5: Results of screen 2 analysis (underivatised) for three positive letrozole samples taken at different times after administration.

Steroid profiling

The volunteer undertaking the excretion study with letrozole also performed the excretion studies with tamoxifen, clomiphene and cyclofenil [15]. He has also participated in several studies concerning stability of steroid profiles [16,17,18]. For these studies morning urine samples were collected during one month every day (30d) and during one year one time a month (12 per year). Over a time period of 24 hours urine samples were collected every two hours even during nighttime (12 samples).

For the excretion study with letrozole one single dose of 5 mg was administered. For 30 hours all urine samples were collected and thereafter only morning urine samples for in total 7 days. One of the described side effects of aromatase inhibitors is the increase of serum testosterone. The most stable steroid ratios androsterone/etiocholanolone (A/E), testosterone/epitestosterone (T/epiT) and 5α-androstane-3α,17ß-diol (Adiol)/5ß-androstane-3α,17ß-diol (Adiol/Bdiol) and the excretion rates of androsterone (A), etiocholanolone (E), testosterone (T), epitestosterone (epiT), 5α-androstane-3α,17ß-diol (Adiol) and 5ß-androstane-3α,17ß-diol (Bdiol) from the excretion study with letrozole and previous longitudinal studies were compared (Tab 1, 2).

The steroid ratios show generally low variations. An exception is a slight increase in Bdiol excretion. A similar reaction was shown in the excretion study with cyclofenil [15].

The excretion study with the aromatase inhibitor letrozole was performed with a single dose, in order to get more information about the effects from aromatase inhibitors on steroid profile parameters. Other excretion studies (with more volunteers and multiple application) have to be performed.

ratio	letrozole (20 samples)	30d (30 samples)	24h (12 samples)	12p/y (12 samples)
A/E	1,02 *(0,12)*	1,04 *(0,07)*	1,12 *(0,09)*	1,02 *(0,05)*
T/epiT	1,12 *(0,27)*	0,91 *(0,10)*	0,94 *(0,23)*	1,01 *(0,14)*
Adiol/Bdiol	0,23 *(0,03)*	0,22 *(0,02)*	0,24 *(0,02)*	0,25 *(0,02)*

Tab 1 : Stability of steroid profile : mean values of ratios *(StDev.)*.

ratio	letrozole [µg/h]	30d [µg/h]	24h [µg/h]	12p/y [µg/h]
A	98 *(28)*	84 *(12)*	97 *(34)*	91 *(55)*
E	88 *(28)*	82 *(12)*	87 *(34)*	91 *(57)*
epiT	1,5 *(0,5)*	1,5 *(0,2)*	1,6 *(0,7)*	1,8 *(1,1)*
T	1,7 *(0,6)*	1,4 *(0,2)*	1,4 *(0,5)*	1,7 *(1,0)*
Adiol	3,1 *(1,1)*	2,0 *(0,3)*	2,4 *(0,9)*	2,5 *(1,4)*
Bdiol	14 *(5,1)*	9 *(1,0)*	10 *(3,0)*	10 *(6,0)*

Tab 2: Stability of steroid profile: mean values of excretion rates [µg/h] *(StDev.)*.

Conclusion

In principle all tested screening procedures are suitable for the analysis of anastrozole.
In full scan mode (screen 2) and in SIM-mode (screen 4) monitoring of the ions *m/z* 209, 224 and 293 are suitable.
However, since screen 1 and 2 are only performed for competition samples, all screening procedures should be used together to cover all kind of urine samples.
The detection of letrozole is only possible in screen 2 in full scan mode monitoring the ions *m/z* 217 and 190. For the detection in screen 4 the analysis of the hydroxy metabolite can be taken into consideration.
The analysis with LC-MS should be an appropriate method for the analysis of anti-estrogenic substances and aromatase inhibitors. At present time the mentioned analytical method is still in preparation.

Acknowledgements
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