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## **Screening on Letrozole and its Metabolite in Doping Analysis**

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### **Abstract**

Letrozole (1-(bis-(4-cyanophenyl)methyl)-1,2,4-triazole) is therapeutically used as non-steroidal aromatase inhibitor in treatment of metastatic breast cancer in postmenopausal women. For doping purposes it may be used for treatment of adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) and to increase the testosterone concentration by stimulation of the testosterone biosynthesis.

The use of aromatase inhibitors is prohibited for male athletes since September 1, 2001.

An excretion study was carried out by oral application of one single dose Femara® (5 mg).

The analysis was performed by gas chromatography-mass spectrometry (GC-MS).

The main metabolite of letrozole (bis-4-cyanophenyl-methanol) is detectable in the screening procedure of anabolic steroids for more than 8 days, whereas letrozole itself is only detectable for about two days.

Bis-4-cyanophenyl-methanol is enclosed into the screening procedure of anabolic steroids.

The full scan spectrum, diagnostic ions and a characterisation of the method for the analysis of the letrozole metabolite are presented.

### **Introduction**

Aromatase (estrogen synthetase) is an enzyme that catalyses various steps in the conversion of androgens to estrogens.

Letrozole is a potent and highly specific non-steroidal aromatase inhibitor. It inhibits the aromatase enzyme by competitively binding to the heme of the cytochrome P450 subunit of the enzyme, resulting in a reduction of estrogen biosynthesis in all tissues. Letrozole exerts its antitumour effect by depriving estrogen-dependent breast cancer cells of one of their growth stimuli. In postmenopausal women, estrogens are derived mainly from the action of the aromatase enzyme, which converts adrenal androgens – primarily androstenedione and testosterone – to estrone and estradiol. The suppression of estrogen biosynthesis in peripheral

tissues and the malignant tissue can be achieved by specifically inhibiting the aromatase enzyme.

The recommended therapeutical dose for letrozole is 2.5 mg per day. The major route of elimination of letrozole is via metabolism to bis-4-cyanophenyl-methanol. Letrozole and its metabolites are excreted mainly via the kidneys. 6% of the drug is excreted unchanged. Its terminal plasma half-life time is 2 days. [1,2]

There is no medical indication for letrozole in males, but 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.

Previous investigations have shown that letrozole itself was detected only in the screening for heavy volatile nitrogen containing drugs over a time period of 43 hours after administration of 5 mg Femara® [3]. Further characterisation of this screening method shows recovery yields of 10% for letrozole, resulting from its decomposition during hydrolysis with 1 M hydrochloric acid. In a routine screening method for anabolic steroids the detection of letrozole was not possible resulting from its poor chromatographical behaviour during analysis on the applied OV-1 column.

Regarding this insufficient detection of letrozole itself, a possible screening analysis of the letrozole metabolite bis-4-cyanophenyl-methanol [4] was investigated.

## **Experimental**

### *Sample preparation [5,6]*

Conjugated and unconjugated anabolic steroids were extracted from 2 ml of urine at pH 9.6 with t-butyl methyl ether (TBME) following enzymatic hydrolysis at pH 7 with  $\beta$ -glucuronidase from E.coli. The ethereal layer was evaporated to dryness in vacuo. The dry residue was derivatised with 100  $\mu$ l of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA)/NH<sub>4</sub>I/ethanethiol 1000:2:3 (v:w:v) for 20 min at 60°C.

### *GC-MS parameter*

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

Column: J&W Scientific Ultra I (OV-1), 17m, 0.2mm i.d., 0.11  $\mu$ m film thickness

Carrier gas: helium 12 psi, split 1:10

Injection volume: 3  $\mu$ l

Injector temperature: 300°C

Temperature program: 0 min 181°C, + 3°C / min, 0 min 230°C, + 40°C / min, 2 min 310°C

Transfer line temperature: 300°C

Ionisation mode: Electron impact at 70 eV

Acquisition mode: SIM  
Resolution: Low  
Source temperature: 230°C  
Quadrupole temperature: 150°C  
Electron multiplier voltage (EMV): +294V relative to the value established by tuning  
For experiments in scan mode the following MS parameter were applied:  
Scan range: 50-650 amu  
Scans/sec: 1.27  
Scan threshold: 20  
EI source temperature : 230°C  
Quadrupole temperature: 150°C  
EMV: +294V relative to the value established by tuning

#### *Excretion urine samples*

An excretion study was performed orally by a healthy male volunteer who has given his written consent to the excretion study. The excretion study was approved by the local ethical committee. One single dose of letrozole (5 mg) was administered. For 30 hours all urine samples were collected and thereafter only morning urine samples for a total period of 188 hours.

Additionally spot urine samples from women suffering from metastatic breast cancer, treated with letrozole, were collected. All urine samples were made available from Dr. Wolfgang Kauffels (Gynaecology and Obstetrics, Hannover Medical School).

#### *Letrozole metabolite*

The letrozole metabolite bis-4-cyanophenyl-methanol described in the literature [4] was identified by its mass spectrum.

#### *Hydrolysis*

The letrozole metabolite is excreted renally almost totally as its glucuronide [1, 2]. The kinetic of the hydrolysis of the conjugated letrozole metabolite was investigated. Aliquots of 2 ml of a clinical urine sample were spiked with 25 µl of E.Coli enzyme solution and incubated at 50°C. The hydrolysis was stopped after different time periods (5, 15, 30, 120 and 240 minutes). The sample preparation was continued according to the standard operating procedure (SOP) for the screening of anabolic steroids.

#### *Extraction at different pH-values*

Aliquots of 2 ml of a clinical urine were prepared according to the SOP for the screening procedure of anabolic steroids [5, 6]. After hydrolysis the urine samples were adjusted to different pH-values: 7, 9.6 and 14. The remaining sample preparation followed the SOP for the screening procedure of anabolic steroids.

#### *Purification with n-pentane*

Extraction with n-pentane instead of tert.-butyl methyl ether is often used for confirmation methods of anabolic steroids (e.g. T/epiT, 19-norandrosterone) to exclude polar coeluting substances.

Purification tests with clinical urine samples were conducted.

## Results and Discussion

### *The letrozole metabolite bis-4-cyanophenyl-methanol*

The major route of elimination of letrozole is via metabolism to a pharmacological inactive carbinol metabolite bis-4-cyanophenyl-methanol [1,2,9]. Triazole, another of its metabolites, is not suitable for routine GC-MS screening in urine due to its low boiling point.

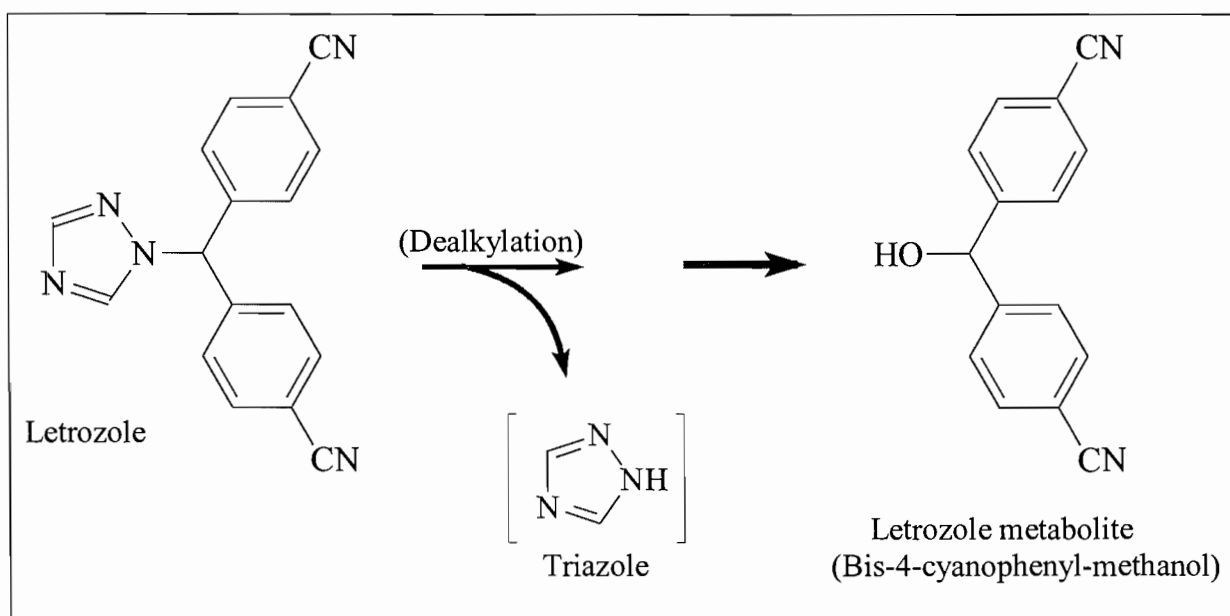


Fig 1: Metabolism of letrozole

For improvement of analytical detection by GC/MS a modification of the metabolite is necessary [7,8]. The derivatisation with MSTFA/ $\text{NH}_4\text{I}$ /ethanethiol converts bis-4-cyanophenyl-methanol to its trimethylsilyl (TMS) ether.

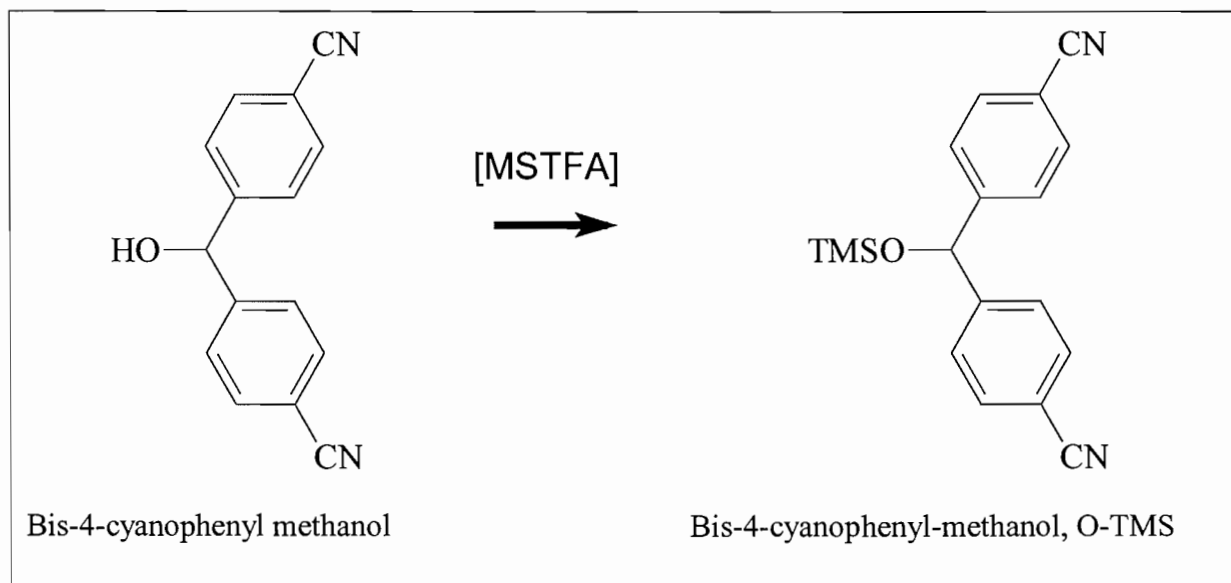


Fig 2: Derivatisation of the letrozole metabolite bis-4-cyanophenyl-methanol with MSTFA.

The mass spectrum of bis-4-cyanophenyl-methanol, O-TMS shows a molecular ion at  $m/z$  306 (Fig 3). The loss of a methyl radical leads to  $m/z$  291. The base peak  $m/z$  217 is generated by loss of the trimethylsiloxy group and subsequent loss of HCN leads to  $m/z$  190. Neutral loss of benzonitrile and rearrangement of hydrogen from the molecular ion leads to the fragment  $m/z$  203 and subsequent loss of HCN leads to  $m/z$  176.

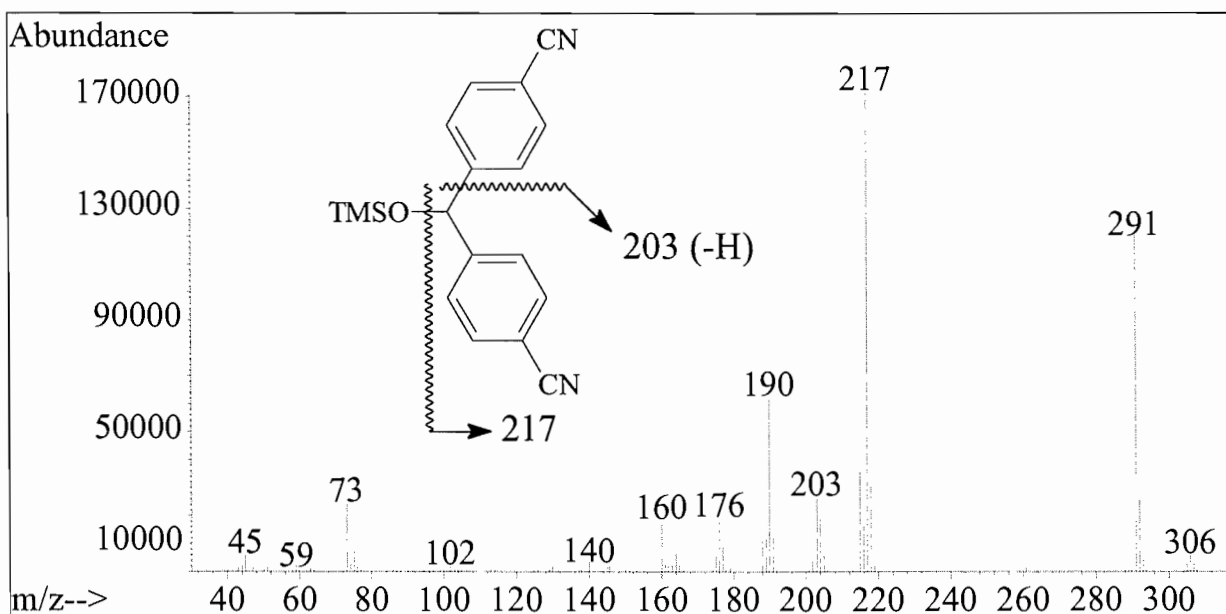


Fig 3: Mass spectrum and fragmentation pattern of the letrozole metabolite bis-4-cyanophenyl-methyl TMS ether.

#### *Hydrolysis*

The hydrolysis with E.coli of bis-4-cyanophenyl-methanol glucuronide is completed in less than 5 minutes.

#### *Extraction at different pH-values*

The extraction yields for the letrozole metabolite in the urine samples extracted at pH 7 and pH 9.6 show similar results. The extraction yield at pH 14 is reduced (perhaps because of hydrolysis of the nitrile function)

Due to the fact that the extraction at pH 7 show higher extraction of substances leading to disturbing background an extraction at pH 9.6 is recommended.

#### *Purification with n-pentane*

Extraction yields for n-pentane extraction of 4-cyanophenyl-methanol were determined with 10%.

For confirmatory analysis of 4-cyanophenyl-methanol the extraction with n-pentane may be used as an additional cleaning step (pre extraction).

#### Screening on bis-4-cyanophenyl-methanol

For the detection of letrozole misuse analysis of the letrozole metabolite is recommended.

Suitable ions for screening are  $m/z$  217 (base peak) and  $m/z$  291 ( $M^+ - 15$ ).

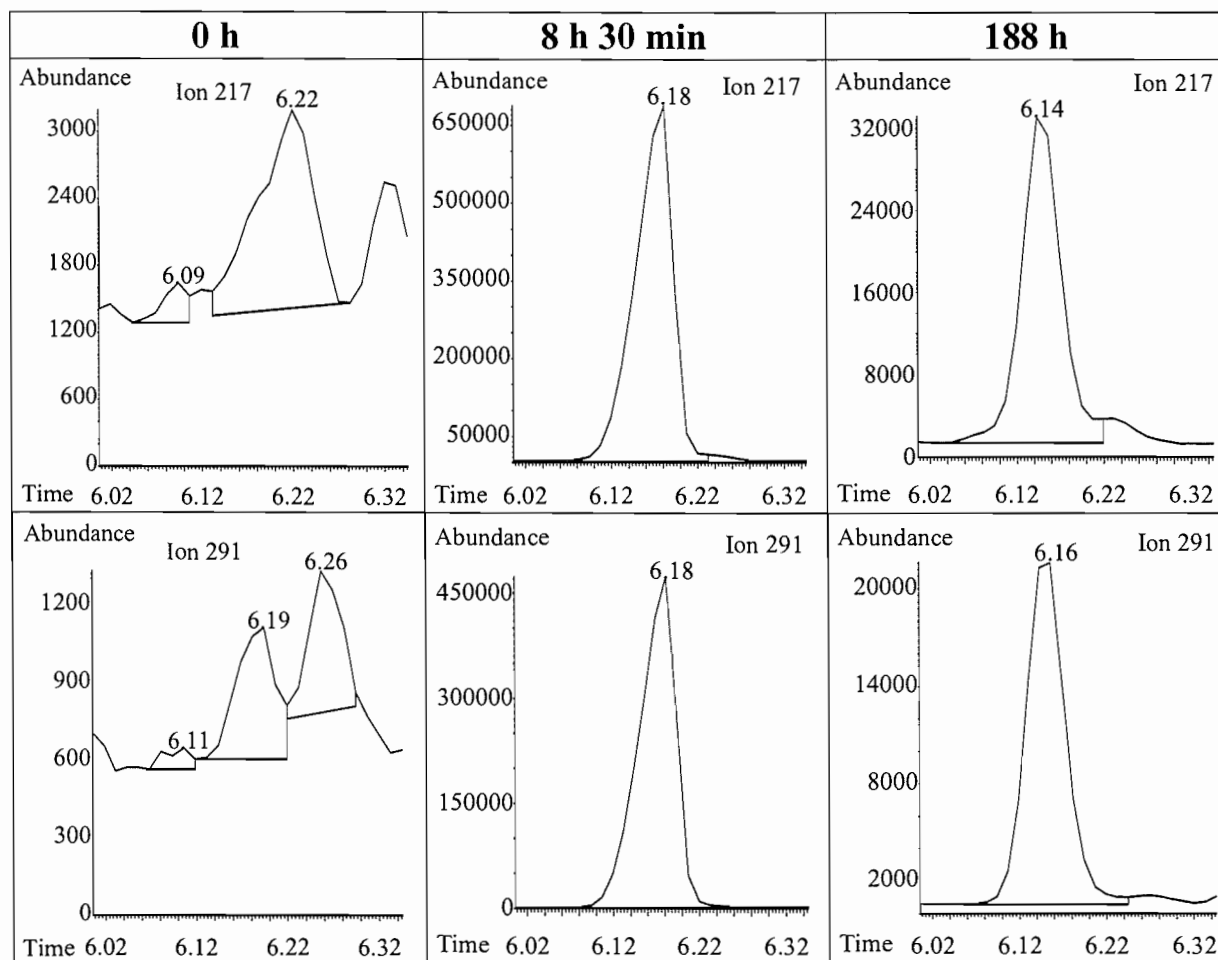


Fig 5: Blank urine and two samples, positive for letrozole, taken at different times after administration (screening for anabolic steroids, SIM mode)

#### Summary/Conclusion

- For the detection of letrozole misuse screening of the letrozole metabolite bis-4-cyanophenyl-methanol is an excellent tool.
- For screening and confirmation purposes the fragmentation pattern of the TMS derivative shows suitable ions with high intensity [ $m/z$  217 (base peak),  $m/z$  291 ( $M^+ - 15$ ) and  $m/z$  306 ( $M^+$ )].

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