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Screening for Anastrozole in Doping Analysis

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Abstract

Anastrozole (2,2´[5-(1H-1,2,4-triazol-1-ylmethyl)-1.3-phenylene]bis(2-methylpropionitrile)) is therapeutically used as non-steroidal aromatase inhibitor [Arimidex®] 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. An excretion study was carried out by oral application of Arimidex® (3 mg). Urine samples from women suffering from metastatic breast cancer and treated with anastrozole were also collected. The analysis was performed by liquid chromatography-mass spectrometry (LC-MS/MS). Anastrozole was identified by comparison of its mass spectrum and retention time with that of a reference substance. Anastrozole parent compound is detectable in the screening procedure of selected anabolic steroids for more than 24 days. The product ion scan, diagnostic ions and a validation of the method for the analysis of anastrozole are presented.

Introduction

Aromatase (estrogen synthetase) is an enzyme that catalyses the last step of the estrogen biosynthesis in the conversion of androgens to estrogens. Anastrozole is a potent and selective non-steroidal aromatase inhibitor. It 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. Anastrozole binds competitively the iron atom present in the heme group of the cytochrome P450 subunit of the enzyme. This process was shown to be controlled by FSH and to take place in female and male organisms. The drug significantly lowers serum estradiol concentration in post-menopausal women and has no detectable effect on formation of adrenal corticosteroids or aldosterone. Anastrozole is known to have no androgenic, estrogenic nor progestrogenic effect. In estrogen-dependent tumours estrogen deprivation causes growth arrest and possibly tumour cell death. It is proofed to be a successful drug in the treatment of metastatic breast cancer in post menopausal women. The recommended therapeutical dose for anastrozole is 1 mg per day [1]. The orally administered drug is well absorbed into the systemic circulation. Anastrozole has a mean terminal half-life of approximately 30 to 60 hours [2,3]. 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 [2,4]. The drug is eliminated via hepatic metabolism and renal excretion. Anastrozole is metabolized by N-dealkylation, hydroxylation and glucuronidation. Three metabolites of anastrozole have been identified in human plasma and urine [4]. The known metabolites are triazole, a glucuronide conjugate of hydroxy-anastrozole and a glucuronide of anastrozole itself. The pharmacological activity is primarily due to the parent drug [4]. It was shown that Arimidex® boosts testosterone excretion [5]. 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 [6]. At present a clinical study is performed in senior males to determine if the aromatase inhibitor anastrozole is as effective as testosterone replacement in improving muscle mass, muscle strength, sexual function, memory and bone health. Preliminary results of the research have shown that inhibition of estrogen production in males results in an increase in testosterone levels. The project started in August 2004 and the completion is expected for February 2007 [7]. In the underground literature anastrozole is advertised 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 gynaecomastia while using moderate amounts of such steroids”[8]. 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 female athletes the use of aromatase inhibitors is forbidden since January 2005. Previous investigations have shown that the detection of anastrozole monitoring the ions at *m/z* 209, 224 and 293 in the GC-MS screening procedure of anabolic steroids was possible [9]. Further characterisation of this screening method has shown a detection limit of 80 ng/ml for anastrozole. Resulting from its low dosage (1 mg per day) a GC-MS detection of anastrozole in clinical urine samples was not possible. Regarding this insufficient detection of anastrozol itself, a more appropriate screening analysis was developed.

Experimental

Chemicals and reagents: All solvents and reagents were of analytical grade purity (Baker, Deventer, The Netherlands; Fluka, Buchs, Switzerland; Riedel de Haen, Seelze, Germany; Merck, Darmstadt, Germany).Tert-butyl methyl ether (TBME) was purchased from KMF Laborchemie (St. Augustin, Germany) and distilled before use. ß-Glucuronidase from Escherichia coli was supplied by Roche Diagnostics GmbH (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoracetamide (MSTFA) was obtained from Macherey & Nagel (Düren, Germany). The synthetic reference materials were anastrozole (Thinker Chemical, Hangzhou, China) and Arimidex® (Astra Zeneca UK Ltd., Macclesfield, United Kingdom).

As internal standard 17α-methyltestosterone (Serva, Heidelberg, Germany) was used. All solutions and buffers were prepared using deionised water (Water Lab System, Millipore, Eschborn, Germany).

Sample preparation [10,11,12]: Conjugated and unconjugated anabolic steroids were extracted from 3 ml of urine at pH 9.6 with 6 ml TBME following enzymatic hydrolysis at pH 7 with ß-glucuronidase from E.coli. Two ml of the organic layer (corresponding to 1 ml of urine) was separated and evaporated to dryness. The dry residue was dissolved in 60 µl of methanol (MeOH)/ammonium acetate buffer (1:1) and transferred into HPLC vials.

Ten µl were injected into the LC-MS/MS system.

Liquid chromatography-mass spectrometry: Analyses were performed on an Agilent 1100 liquid chromatograph (Waldbronn, Germany) interfaced to an Applied Biosystems API 2000 triple quadrupole mass spectrometer (Darmstadt, Germany). LC was done on a Merck Puropher®Star RP-18 endcapped column (i.D. = 4 mm, length = 55 mm; particle size 3 µm). The mobile phase was A: 5 mM ammonium acetate buffer containing 1% of glacial acetic acid (pH = 3.5) and B: acetonitrile. The flow rate was 0.8 ml/min with a post-column split ratio of 10 to1. The gradient was 10% B to 90% B in 20 min with a re-equilibration time of 4 min at 10% B. The injected volume was 10 µl. Positive ionisation was accomplished by atmospheric pressure chemical ionisation (APCI) at an interface temperature of 475°C. Declustering potential was set to 31 V, and collision energies were optimised individually for each ion transition. Nitrogen (obtained from a K75-72-727 Whatman nitrogen generator) was used as collision gas at a collision cell pressure of 2.2e-5 torr.

Excretion urine samples: An excretion study was performed in a healthy male volunteer who has given his written consent. The excretion study was approved by the local ethical committee. One mg anastrozole was orally administered per day over a time period of 3 days. Each urine sample was collected for 7 days and thereafter morning urine samples over a time period of 17 days. In total urine samples over 24 days were collected. Additionally untimed urine samples from women suffering from metastatic breast cancer, treated with anastrozole, were made available from Dr. Wolfgang Kauffels (Gynaecology and Obstetrics, Hannover Medical School).

Validation: The validation was performed according to the existing in house standard operating procedure which was developed from:

- DIN 38402, part 51 (German industry norm, chapter 51)
- DIN 32645, edition May 1994
- International Standard for Laboratories, WADA (ISL: 5.4.4.2 Validation of Methods)
- Guidance for industry for bioanalytical method validation [13]

The evaluation of the data was processed with an EXCEL program which has been developed according to DIN 38402, part 1, edition May 1986.

Calibration curve: A calibration curve was generated with the concentrations 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 ng of anastrozole per ml of urine. As internal standard 17α-methyltestosterone was used (500 ng/ml). Each calibration point was prepared and analyzed once. For the evaluation the areas of the extracted ion transitions m/z 294/225 (analyte) and m/z 303/109 (internal standard) were used.
Lower limit of detection and quantification: The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were calculated from the signal to noise ratio of 10 blank urine samples and 10 specimens spiked with 0,1 ng anastrozole per ml.
Accuracy: Ten negative control urine samples spiked with 50 ng anastrozole per ml were used. Also a calibration curve was prepared. The concentrations of anastrozole in the spiked urine samples were determined and the deviations of the respective mean values from the true values calculated.
Precision: Ten negative control urine samples spiked with 5, 50 and 90 ng anastrozole per ml each were used. For the calculation of the intra-day precision the coefficient of variation of each concentration level was calculated. The corresponding inter-day precision was calculated from samples prepared and analysed at 3 different days.
Recovery: For the determination of the recovery 12 urine samples were prepared. Each urine sample was spiked with anastrozole (50 ng/ml). Six urine samples were spiked with anastrozole in the beginning of the sample preparation. The internal standard (500 ng/ml) was added to the TBME layer after separation of the conjugated fraction. In the other 6 urine samples anastrozole and the internal standard were added the TBME-layer after separation of the conjugated fraction.
Specificity / Robustness: For the determination of the specificity / robustness 10 urine samples were selected by random. Each was spiked with anastrozole (10 ng/ml) and prepared according to the sample preparation of anabolic steroids. The presence of anastrozole was confirmed according to IOC/WADA regulations [14] by comparison of the intensities of at least 3 ions from spiked urine samples with those of a pure standard.
Hydrolysis experiments:

I. The amount of freely excreted anastrozole was determined by simple extraction of different clinical urine samples with TBME.
II. The sum of conjugated and unconjugated anastrozole was determined by analysis of different clinical urine sample after enzymatic hydrolysis.
III. The kinetic of the hydrolysis of the conjugated anastrozole was investigated. Aliquots of 3 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 peroids (0, 5, 15, 30, 60, 120 and 240 minutes).

The sample preparation in II. and III. was performed according to the standard operating procedure (SOP) for the screening of anabolic steroids [12].

Extraction at different pH-values: Aliquots of 3 ml blank urine spiked with 50 ng/ml anastrozole were prepared according to the SOP for the screening procedure of selected anabolic androgenic steroids [12]. After hydrolysis the urine samples were adjusted to different pH-values: 7, 9.6 and 14 and analysed as described above [12].

Purification with n-pentane: Extraction with n-pentane instead of TBME is often used for confirmation methods of anabolic steroids (e.g. T/epiT, 19-norandrosterone) to exclude polar coeluting substances [12,15]. Purification tests with blank urine samples spiked with 50 ng/ml anastrozole were performed.

Results and Discussion

Fragmentation pattern of anastrozole

A product ion scan of anastrozole with a collision energy of 50 eV and the precursor ion *m/z* 294 was generated (Fig.1). The product ion scan shows the significant fragment ions at *m/z* 225, 210, 195, 142 and 130. The ion at *m/z* 225 is generated by a neutral loss of triazole. The loss of a methyl radical leads to *m/z* 210.

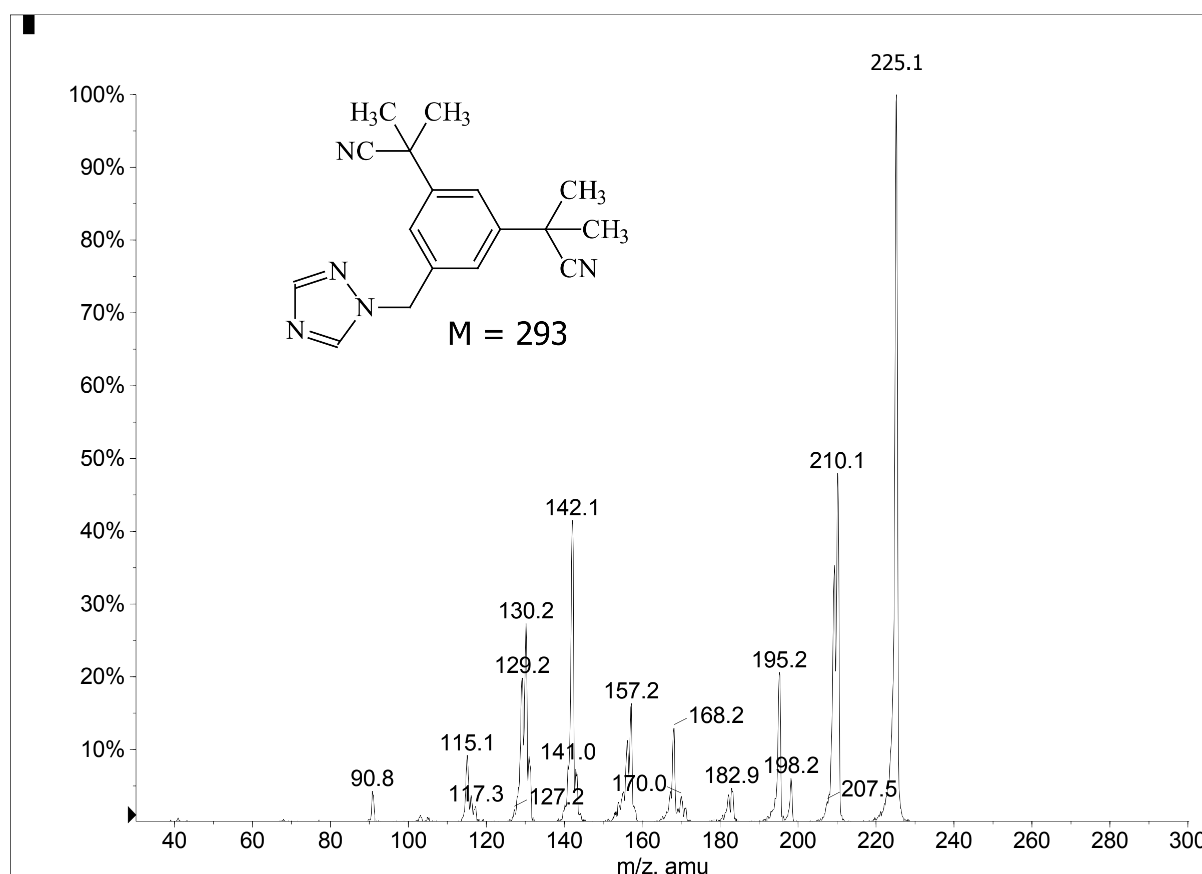

Fig 1: Chemical structure and product ion scan of anastrozole.

Calibration curve anastrozole (5 – 90 ng/ml)

Ten urine samples were spiked with anastrozole from 5 ng/ml up to 90 ng/ml, respectively. The integration of peak areas obtained by extraction of the ion-transitions *m/z* 294/225 for the analyte and *m/z* 303/109 for the internal standard and subsequent calculation of their ratio (response) yielded a linear and homoskedastic calibration curve for anastrozole (y = 1.0604 x + 0.1476) according to analysis of variance (ANOVA) over the range of interest.

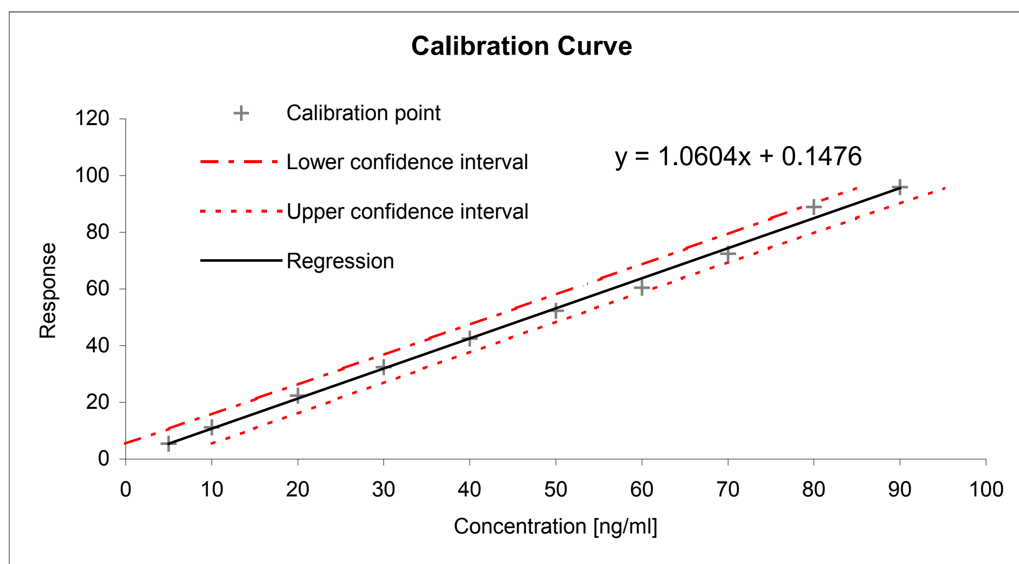

Fig 2: Calibration curve for anastrozole (5 – 90 ng/ml)

Lower limit of detection and quantification

For the determination of the lower limit of detection (LLOD) a signal to noise ratio of 3:1 is recommended. The lower limit of quantification (LLOQ) is characterized by a signal to noise ratio of 10:1. The LLOD was determined to 0.02 ng/ml and the LLOQ to 0.06 ng/ml.

Accuracy

The accuracy was determined using 10 measurements at a concentration level of 50 ng/ml. The deviation of the mean value from the true value was 0.4 %.

Precision

Intra-batch precision was determined using 10 analyses at 3 concentration levels within the working range. The corresponding inter-day precision was calculated from samples prepared and analyzed at 3 different days. Concerning intra-batch precision, the coefficient of variation (CV) was 2.8 %, 1.9 % and 8.1 % for the concentration levels at 5 ng/ml, 50 ng/ml and 90 ng/ml anastrozole, respectively. Inter-day precision yielded coefficients of variation for the low level at 11.1 %, for the medium level at 4.9 % and for the higher level at 6.1 %.

Recovery

The recovery was determined to be 97 %.

Specificity / Robustness

Anastrozole was detected according to the IOC/WADA regulations in each urine sample. The most appropriate ion transitions are *m/z* 294/225, 294/210, 294/142 and 294/130. The method is robust.

Hydrolysis experiments

Approximately 50 % of anastrozole was excreted non conjugated. The hydrolysis was completed within 5 minutes.

Extraction at different pH-values

Similar extraction yields for anastrozole in urine samples extracted at pH 7, 9.6 and 14 were found.
Since extraction at pH 7 results in interfering background an extraction at pH 9.6 is preferred.

Purification with n-pentane

Yield for n-pentane extraction of anastrozole was 2.6 %.
For confirmatory analysis of anastrozole the extraction with n-pentane may be used as an additional clean up.

Screening for anastrozole

For the detection of anastrozole misuse its inclusion into the screening for selected anabolic androgenic steroids via LC-MS/MS is recommended [12]. Suitable ion transitions are *m/z* 294/225, 294/210, 294/142 and 294/130.
After application of 3 mg Arimidex® anastrozole was detected over a time period of 24 days. Fig. 3 shows this excellent detection for a clinical spot urine sample containing approximately 5 ng/ml anastrozole.

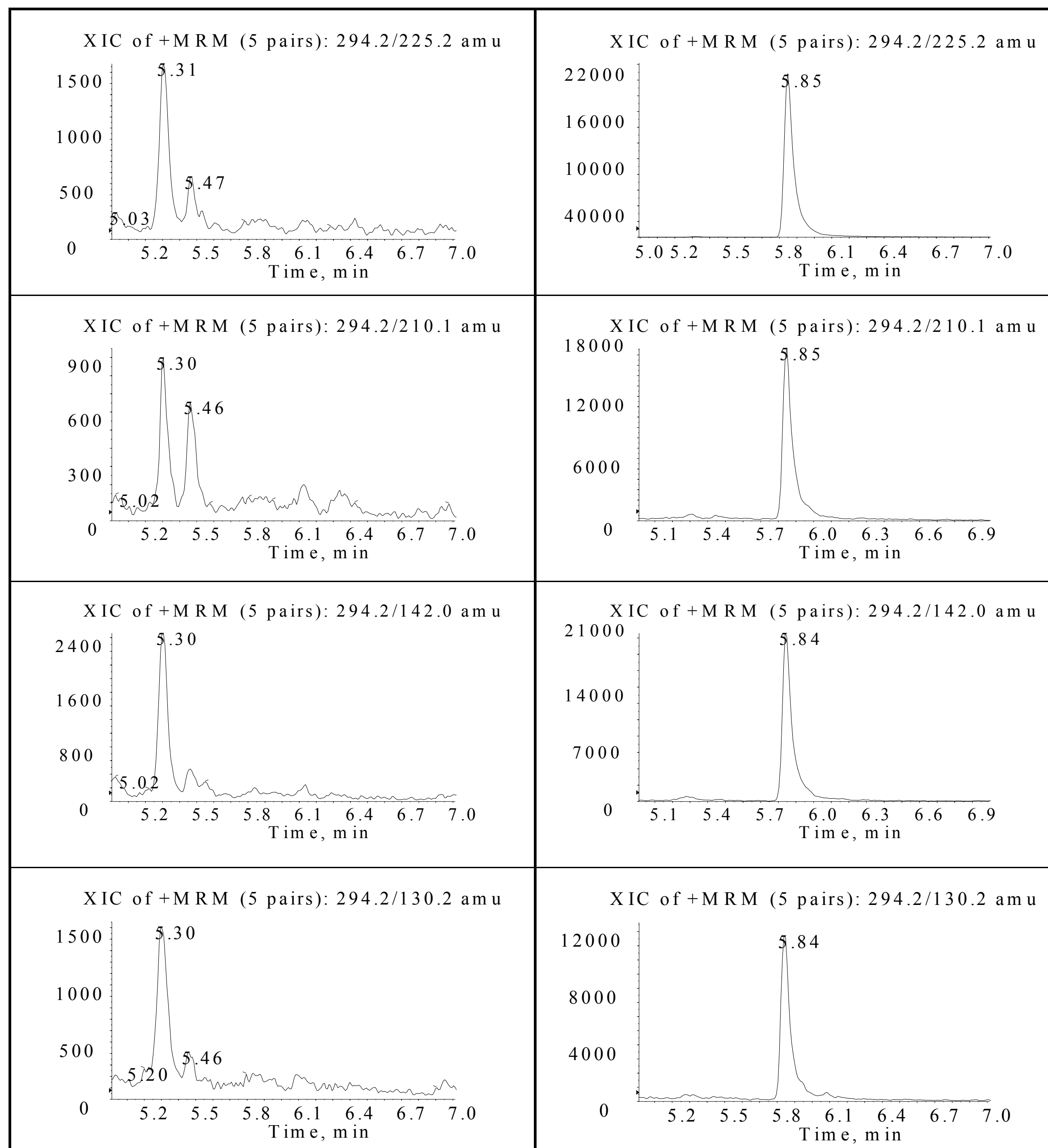

Fig 3: Screening for selected anabolic androgenic steroids via LC-MS/MS
Left: blank urine
Right: clinical untimed urine containing ~ 5 ng /ml anastrozole

Summary/Conclusion

- For the detection of anastrozole misuse screening for the parent compound via LC-MS/MS is a useful tool.
- The fragmentation pattern shows suitable ion transitions at *m/z* 294/225, 294/210, 294/142 and 294/130.
- Inclusion of anastrozole into the screening procedure 9 (selected anabolic androgenic steroids [11]) is recommended.
- The validation of the method shows a linear and homoskedastic calibration curve with a detection limit of 0.02 ng anastrozole per milliliter as well as high accuracy and precision.
- The standard operating procedure for selected anabolic androgenic steroids via LC-MS/MS is a robust screening method with a recovery of 97 % for anastrozole.

Acknowledgements

The authors wish to thank Dr. Wolfgang Kauffels from Gynaecology and Obstetrics, Hannover Medical School, for supplying with urine samples from patients. The authors are grateful to the World Anti-Doping Agency (WADA) and the Manfred Donike Society e.V. for their financial support.

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