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Screening and confirmatory GC-MS methods for the detection of trenbolone in bovine urine

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Abstract

Sensitive and selective methods for the screening (GC-MS) and confirmatory analysis (GC-MS/MS) of 17α- and 17β-trenbolone in bovine urine were developed. In the first stage of the analysis, the enzymatic hydrolysis of trenbolone metabolites with glucuronidase AS-HP in acetate buffer (pH 5.2) solution was carried out. Free compounds were extracted from urine with diethyl ether. For the purification of the extract solid phase, extraction with C18 and NH2 columns was applied. The evaporated extract was subjected to two derivatisation steps; the first with MSTFA/I2 solution and second with MSTFA. The separation of the analytes on HP-5 ms capillary column was conducted. The methods were validated according to the Commission Decision 2002/657/EC. For GC-MS method, CCα and CCβ were 0.21–0.36 μg L^{-1} for 17α- trenbolone and 0.20–0.34 μg L^{-1} for 17β-trenbolone, while for GC-MS/MS method the values were lower and amounted to 0.15–0.25 μg L^{-1} for 17α- trenbolone and 0.20–0.34 μg L^{-1} for 17β-trenbolone. Method recoveries in spiked samples ranged from 86%–111% with standard deviation lower than 25% for both detection techniques.

Key words: bovine urine, trenbolone, validation, gas chromatography, mass spectrometry.

Introduction

Trenbolone acetate (TBA) is a synthetic steroid with strong anabolic properties. In animals, TBA alone or in combination with 17ß-oestradiol, is used to improve weight gain and feed efficiency. TBA is administered by subcutaneous implantation in the ear. The dosage of TBA varies with manufacturer of the implant, ranging between 40 (Revalor G) and 300 mg (Finaplix) per animal. After implantation, TBA is metabolised to 17β-trenbolone (TBOH), the most biologically potent compound. In the bovine species, the 17β-epimer is the major metabolite occurring in muscle while the 17α-epimer is the major metabolite occurring in the excreta, bile, and liver. Elimination in the urine occurs following conjugation, predominately to glucuronic acid (13, 19). TBA is one of the six hormones permitted in the U.S. in fattening animals. Administration of natural and synthetic steroids as growth promoters in food-producing animals has been banned within the European Union (4). For effective protection of consumers against unwanted residues of anabolic hormones, efficient surveillance and monitoring of the ban are necessary, which require the availability of validated analytic methods for screening and confirmation of hormone residues in urine and other sample matrices. Therefore, there is a need to develop a highly sensitive and specific analytical method for the determination of hormones in biological samples of animal origin. Literature data indicates that currently liquid chromatography is the preferred method for trenbolone analysis, because LC-MS techniques are simpler without a time-consuming derivation procedure (2, 3, 9). However, this method also has drawbacks; matrix effects in liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have quickly become a major concern in biological samples analysis (16, 20). Gas chromatography mass spectrometry (GC-MS) is an alternative analytical technique available for the last 40 years for hormone steroids analysis (8, 12, 18). Despite the need for hormones derivatisation, GC-MS has the advantage

over LC-MS/MS due to better chromatographic resolution, particularly epimers separation and high sensitivity. For many years determination of trenbolone by GC-MS was difficult. Due to its structure (Fig. 1), TBOH causes problems using the common derivatising agents. The effective derivatisation procedure for trenbolone based upon N-methyl-N-(trimethylsilyl) trifluoro-acetamide-iodine (MSTFA-I_2) derivatisation was first presented by Maume *et al.* and Marchand *et al.* (15, 17). This derivative gives specific mass spectra for trenbolone, and sensitive signals on high mass ions and reproducible gas chromatograms. In the present study GC-MS screening and GC-MS/MS confirmatory methods have been developed for determination of 17α- and 17β-trenbolone in bovine urine. The two acquisition modes of the GC-MS system - selective ion monitoring (SIM) and tandem mass spectrometry (MS/MS) were compared.

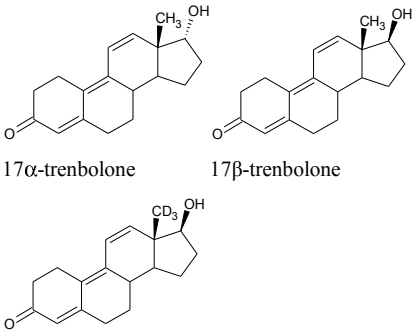


Fig. 1. Structural formulas of investigated compounds

Material and Methods

Reagents and chemicals. Standards of 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and internal 17β-trenbolone-d3 (17β-TBOH-d3) were purchased from the Netherlands National Institute for Public Health and Environment-RIVM (Bilthoven, the Netherlands). Primary standard stock solutions were prepared in methanol at a concentration of 10 μg mL^{-1}. Working solutions were obtained by ten-fold dilution of primary standard solutions to the concentration of 1 μg mL^{-1} in methanol. The standards were stored in a freezer (<-18°C) according to the recommendations of the certificate.

Solvents: methanol (HPLC, resi grade), ethanol, ethyl acetate (HPLC grade), and acetone (resi grade) were purchased from J.T. Baker (the Netherlands); isooctane (GC grade) was obtained from Merck (Germany); diethyl ether, n-hexane, and methanol (analytical grade) were obtained from POCh (Poland). Other chemicals: concentrated acetic acid (99.5% purity), hydrochloric acid (0.1 M), anhydrous sodium sulphate (99.5% purity), sodium hydrogen carbonate (99.5% purity), and sodium acetate anhydrous (99.0% purity) were of analytical grade and obtained from POCH (Poland). Sodium carbonate (99.8% purity) was provided by Sigma-Aldrich (Germany). Purified water was achieved with a Milli-Q apparatus (Millipore, USA). The β-glucuronidase (23 U mL^{-1})/aryl sulfatase (68 U mL^{-1}) Helix Pomatia and Tris (hydroxymethyl)-aminomethane buffer substance (analytical grade) were purchased from Merck (Germany). SPE columns (Bakerbond® C_{18} 100 mg/mL and NH_2 500 mg/3 mL) were supplied by Mall Baker (Deventer). Acetate buffer (0.05 M), pH 4.8, was prepared by mixing 40 mL of 0.05 M acetic acid solution (1.42 mL in 500 mL of water) with 60 mL of 0.05 M sodium acetate solution (4.1 g in 1000 mL of water) and adjusting the pH to 4.8. Acetate buffer (0.04 M), pH 5.2, was prepared by mixing 25 mL of 0.04 M acetic acid solution (1.2 mL in 500 mL of water) with 75 mL of 0.04 M sodium acetate solution (3.28 g in 1000 mL of water) and adjusting the pH to 5.2. Tris buffer (20 mM), pH 8.5, was prepared by dissolving Tris solid substance (4.8 g) in water (500 mL), then by mixing 50 mL of prepared solution with 9 mL of hydrochloric acid (0.1 M), diluting with water to 200 mL, and adjusting the pH to 8.5. Ten percent sodium hydrogen carbonate and 10% sodium carbonate in water were prepared by dissolving solid substances (100 g) in water (900 mL). A carbonate buffer was prepared by mixing 10% sodium hydrogen carbonate solution (100 mL) with 10% sodium carbonate solution (500 mL) and adjusting the pH to 10.25. Derivatisation reagents: N-methyl-N- (trimethylsilyl) trifluoro-acetamide (MS TFA) (GC grade) was purchased from Sigma Aldrich (Germany) and iodine - I_2 (analytical grade) was obtained from Merck (Germany). Derivatisation mixture - MSTFA: I2, (1000:10, V/m) was prepared by dissolving of 10 mg I_2 in 1000 μL of MSTFA, and was stored below -18°C; storage periods did not exceed one month.

GC-MS analysis. An HP 6890 gas chromatograph fitted with a HP 7683 auto sampler and connected to a HP 5973 single quadrupole mass-selective detector (all from Agilent Technologies) was used for GC-MS analysis. GC separation was achieved on an Agilent HP 5 ms capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The programme temperature was: 120°C initial temperature and held for 2 min, ramped at 18°C/min to 280°C and held for 4 min, ramped at 30°C/min to 310°C, and then held for 3 min. Samples (2 μL) were injected in the pulsed splitless mode. The injector temperature was set at 250°C. The helium carrier gas was maintained at 0.9 mL/min constant flow. The quadrupole and ion source temperatures were set at 150°C and 230°C, respectively. Acquisitions were performed in selected ion monitoring (SIM) mode. The mass spectrometer was operated in the EI mode at 70 eV, using the Agilent MSD ChemStation D.01.00 software.

GC-MS/MS analysis. An Agilent 7890N GC with Agilent 7000 triple quadrupole mass detector and

MassHunter software were used for the analysis. Chromatographic separation of trenbolone was achieved on the same non-polar capillary column using 1 mL min^{-1} flow of helium. The oven temperature was kept constant at 120ºC for 2 min and was then increased by 20ºC per min to 200ºC, a further increase in temperature was 4ºC per min to 280ºC and then held for 3 min. The samples (2 µL) were injected in pulsed splitless mode in 250ºC. The MS source and Quadrupole temperatures were set at 250 and 150ºC, respectively. The GC-MS apparatus was operated in positive electron impact (EI) ionisation mode, the data was acquired in multiple reactions monitoring (MRM) mode. The ions monitored and transitions used for detection of 17α- and 17β-trenbolone are presented in Table 1.

Sample preparation. Ten millilitres of urine, centrifuged and filtered through membrane filters (ø25 mm, 0.45 µm) for clarification of aqueous solutions (Millex®-HA, Millipore, Ireland) were adjusted to pH 5.2 by adding 5 mL of acetate buffer and a few droplets of glacial acetic acid, or carbonate buffer, if needed. Afterwards, an overnight (16–20 h) enzymatic hydrolysis at 37°C (±2°C) with glucuronidase AS – HP (160 µL) was performed. After cooling the sample to the room temperature, 10 µL of internal standard – TBOH-d3 at a concentration of 1 µg mL^{-1} was added to obtain a final concentration of 1 µg L^{-1}. The isolation of free hormones from urine by double liquid-liquid extraction (30 and 20 mL) with diethyl ether was performed. The combined ether phase was washed successively with 20 mL of carbonate buffer and 20 mL of distilled water, then passed through the layer of anhydrous sodium sulphate and evaporated to dryness under of stream of nitrogen at 60°C. The residue was dissolved in 3 mL of acetate buffer (0.05 M, pH 4.8) and was passed through the C_{18} SPE column previously conditioned with 2 mL of methanol and 2 mL of TRIS buffer/methanol mixture (80:20, v/v). The column was washed with 2 mL of TRIS buffer/methanol mixture (80:20, v/v) and with 2 mL of methanol/water mixture (40:60, v/v), then was dried under vacuum for 2 min. Elution of analytes was carried out with 3 mL of acetone. The eluate was passed through the NH_2 SPE column previously conditioned with 5 mL of methanol/acetone (40:60, v/v), and evaporated to dryness under nitrogen. The residues were dissolved in ethanol, transferred to derivatisation vial and evaporated to dryness again. Then, derivatisation was applied, first with 30 µL MSTFA/I_2 (1000 µL: 10 mg) solution for 3 min at room temperature, and after evaporation of the mixture to dryness with 30 µL of MSTFA (60°C ± 2°C, 40 min). After addition of 50 µL of isooctane, the derivative was injected into GC-MS system.

Validation. The method developed was submitted to a validation process in accordance with Commission Decision 2002/657/EC requirements (6). The experiments were conducted by the software "ResVal" (v 2.0) (1), which was intended for the validation of analytical methods. Validation studies included five experiments. For both GC-MS and GC-MS/MS methods the validation process globally included 174 assays performed on urine samples. The specificity, precision (repeatability and within-laboratory reproducibility), recovery, decision limit (CCα), detection capability (CCβ), and uncertainty of the method were evaluated. The validation process included the step of analysis of 10 blank urine samples for checking signal specificity, simultaneously with 10 samples of urine fortified with 17α- and 17β-trenbolone at a concentration equal to 1.0 µg L^{-1}, and with higher dose (5 µg L^{-1}) of testosterone an 19-nortestosterone, which are good representatives for hormones, that may be present endogenously in the urine of animals. Within three days, three sets of analyses were performed. Each series consisted of a blank urine sample and six blank urine samples fortified with trenbolone at each concentration level 0.5, 1.0, and 1.5 µg L^{-1}. In addition, spiking levels 2.0 and 5.0 µg L^{-1} were performed. The linearity was evaluated on the basis of four standard calibration curves consisting at least of five points, and on three matrixes matched calibration curves covering the range of concentrations from CCα to 5.0 µg L^{-1}. Additionally, eight samples in robustness studies were analysed.

Results

The summary of the validation results of 17α- and 17β-trenbolone in urine samples is presented in the Tables 2, 3.

Table 1. GC-MS monitored ions (SIM) and GC-MS/MS ion acquisition parameters (MRM) mode used for identification of trenbolone

Compound	GC-MS mass monitored (m/z)	GC-MS/MS MRM transition (m/z)	Collision energy (eV)
17α-trenbolone	323 380* 442 449 539	442.0 > 352.0 380.2 > 322.9*	27 26
17β-trenbolone	323 380 442 524 539	442.0 > 352.0 380.2 > 322.9	30 18
17β-trenbolone-d3	326 383 445 527 542	445.9 > 355.0 383.2 > 325.9	26 20

*ions and transitions in bold were used for quantification

Table 2. Calibration parameters

Compound	Linear range (µg L^{-1})	Slope ± s_b	y-Intercept ± s_a	Correlation coefficient	Standard error
GC-MS					
Standard calibration curve					
17α-trenbolone	0.20 - 5	0.2932 ± 0.0731	-0.0012 ± 0.0177	0.9906	0.0534
17β-trenbolone	0.20 - 5	0.4436 ± 0.0057	0.0187 ± 0.0156	0.9984	0.0380
Matrix matched calibration curve					
17α-trenbolone	0.21 - 5	0.2546 ± 0.0773	0.0486 ± 0.0174	0.9985	0.2089
17β-trenbolone	0.20 - 5	0.4545 ± 0.0070	0.0492 ± 0.0228	0.9990	0.0591
GC-MS/MS					
Standard calibration curve					
17α-trenbolone	0.10 - 5	1.2132 ± 0.1329	-0.1864 ± 0.1041	0.9964	0.2296
17β-trenbolone	0.10 - 5	1.1404 ± 0.1391	-0.1287 ± 0.0813	0.9940	0.2809
Matrix matched calibration curve					
17α-trenbolone	0.15 - 5	1.0416 ± 0.0610	-0.1838 ± 0.0570	0.9952	0.2089
17β-trenbolone	0.10 - 5	0.8718 ± 0.2248	0.0317 ± 0.0538	0.9994	0.0591

Table 3. Performance characteristics of the presented methods

Compound	Spiking level (µg L^{-1})	Recovery (%) (n=18)	Repeatability (R.S.D., %) (n=18)	Within-lab reproducibility (R.S.D., %) (n=18)	Measurement uncertainty at 1µg L^{-1} (U, µg L^{-1})	Decision limit (CCα, µg L^{-1})	Detection capability (CCβ, µg L^{-1})
GC-MS							
17α-TBOH	0.5	111.0	16.9	16.9	0.36	0.21	0.36
	1	86.3	15.7	20.8			
	1.5	99.7	13.3	20.2			
17β-TBOH	0.5	95.4	22.6	22.6	0.33	0.20	0.34
	1	94.0	12.3	17.0			
	1.5	101.3	6.2	12.1			
GC-MS/MS							
17α-TBOH	0.5	92.5	6.8	32.3	0.39	0.15	0.25
	1	88.7	7.9	21.6			
	1.5	86.2	15.4	22.7			
17β-TBOH	0.5	92.5	5.3	23.6	0.32	0.10	0.17
	1	87.7	7.3	17.8			
	1.5	87.6	14.6	23.2			

The linear regression parameters of standard and matrix matched calibration curves were correct for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) exceeding 0.99 value. The calculated concentrations of CCα and CCβ of both compounds were much lower than the proposed recommended concentration limit set at 2 µg L^{-1} (5). The method is characterised by a good recovery, above 85% and correct precision. The coefficients of within day and day to day variations (CV) were less than 20% and 25%, respectively. The specificity studies revealed no interferences in the range of the retention times of the analytes as shown in Figs 2, 3. It was also demonstrated that there was no interference of either testosterone or 19-nortesto-sterone in the responses obtained with the trenbolone.

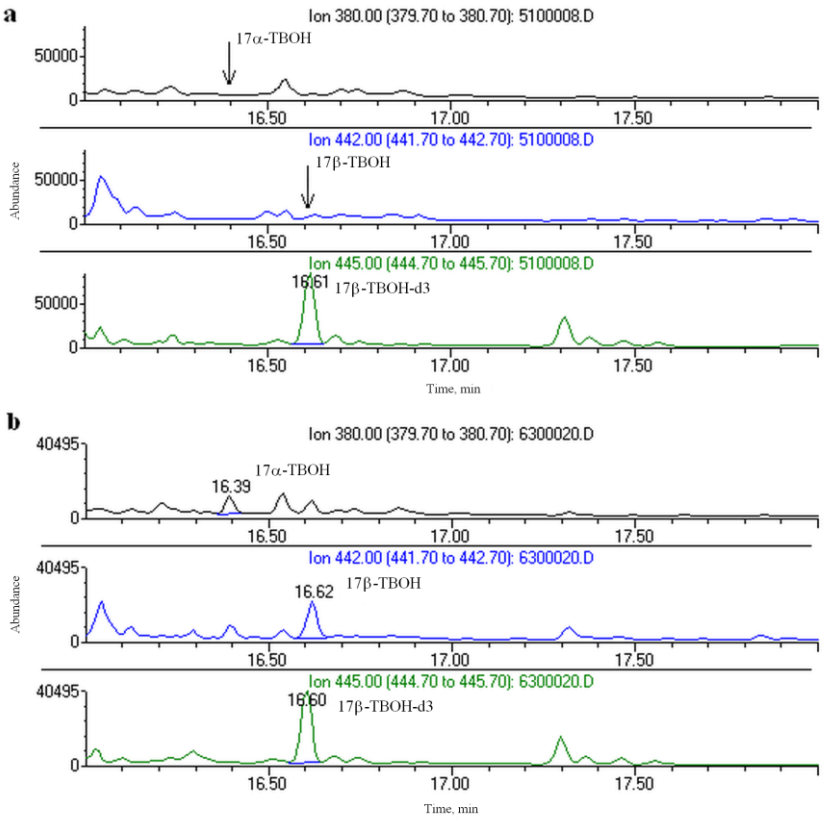


Fig. 2. GC-MS (EI) chromatogram of: a) blank urine sample, b) spiked bovine urine sample with 17α- and 17β- trenbolone at 1 µg L^{-1}

Seven different variables (minor changes) including pH of extraction, temperature, and time of extract evaporation, composition of washing solutions for C_{18} SPE column, serial numbers of C_{18} cartridges, and temperature of derivatisation reaction chosen from the sample preparation step were taken into account in the evaluation of method ruggedness. It was demonstrated that all selected factors did not significantly affect the analytical performance.

Discussion

For the detection of steroid hormones in biological material gas chromatography coupled to single or multiple mass spectrometries are commonly used techniques (10, 14, 22). In the study both methods: GC-MS for screening and GC-MS/MS for confirmatory purposes were applied. Optimisation of the mass spectrometric conditions was performed following a two-step process. In the first step, a full-scan spectrum was obtained for each of the standard derivative. Then, candidates for diagnostic ions in GC-MS SIM mode, precursor and product ions in the multiple reactions monitoring (MRM) method were chosen. Table 1 summarises the results on the optimal conditions found for each compound. Performance of GC-MS methods depends on the correct choice of the derivative. Trenbolone compound is difficult to analyse by gas chromatography. The difficulty in obtaining derivative stems comes from the structure of a molecule containing three double bonds, and the thermal instability in the gas chromatograph (11, 21). Four most commonly used derivatisation reagents and approaches were tested for the evaluation of the best derivatisation method of trenbolone before GC-MS analysis. For each approach, three assays of 30 ng hormone standards were performed using GC-MS full scan mode. Out of examined methods, one with heptafluorobutyric anhydride, and three approaches with different silylation reagents, such BSTFA and MSTFA, and a catalyst such as ammonium iodide (NH_4I) or iodine (I_2) were tested. Only in the case of silylation mixtures consisting of MSTFA and iodine, a high signal of trenbolone was observed, a small signal was detected for BSTFA. For the rest of derivatisation agents no signal was found. However, in the case of trenbolone, the most advantageous was two-step derivatisation performed successively with a mixture of MSFTA/I2 at room temperature, then with the MSTFA at 60°C. The obtained derivatives of α-and β-trenbolone are suitable for GC-MS analysis due to high ions intensity and the required fragmentation, different for the two epimeric forms of this compound. As a result of such derivatisation a molecule is formed by TMS-enolisation of the ketone group at the third carbon, silylation of the hydroxyl group at C17 and the connection N(CH3)COCF3 to C4 atom (Fig. 4).

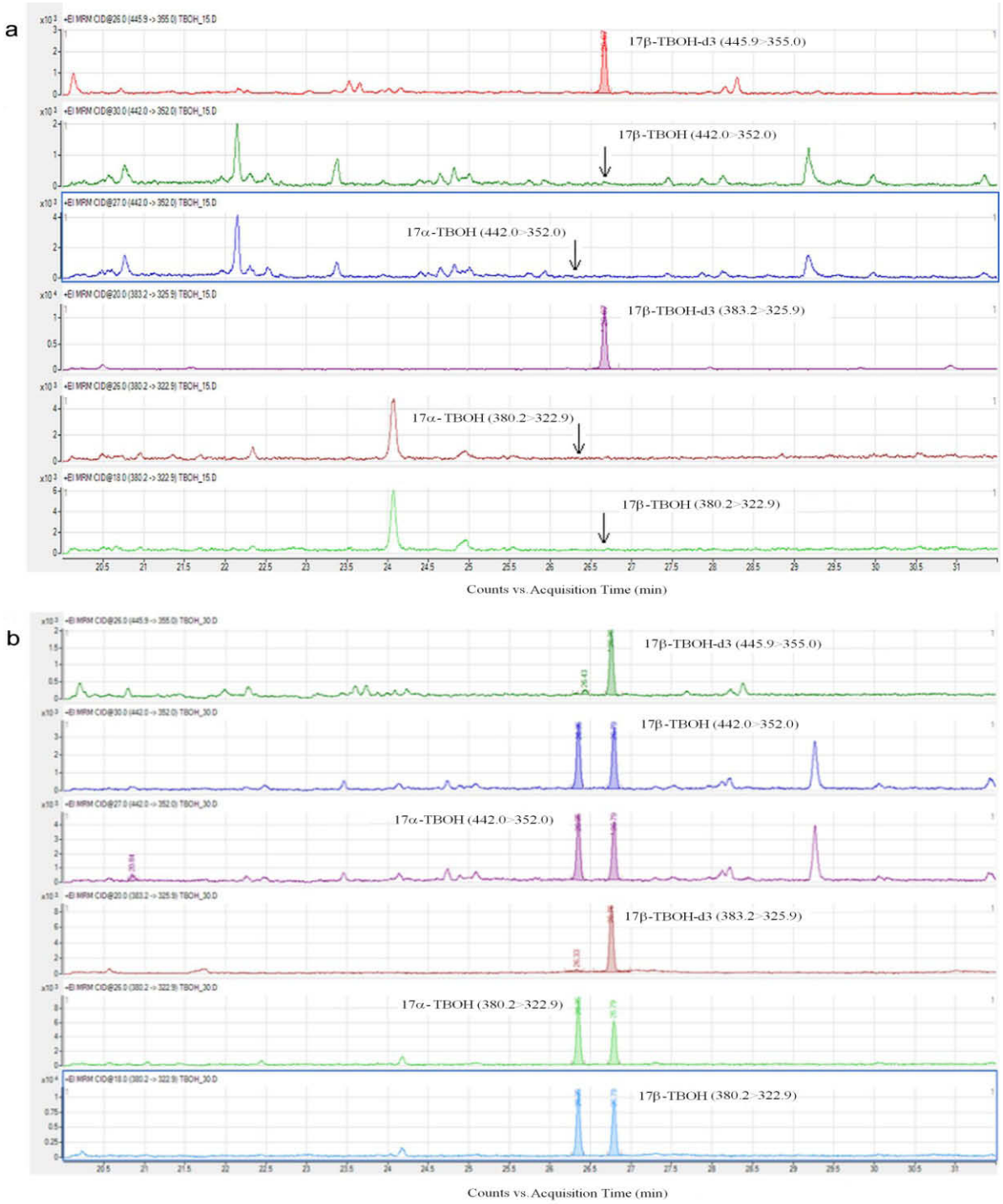


Fig.3. GC-MS/MS, MRM acquisition mode. Ion chromatograms of: a) blank urine sample, b) spiked bovine urine sample with 17α- and 17β-trenbolone at 1 µg L^{-1}

This highly efficient way of derivatisation allows detecting trenbolone at low levels, lower than 1 µg L^{-1}. The stability of trenbolone derivatives was performed for standard solutions containing 10 ng of analyte, and for urine samples fortified at 1 µg L^{-1}. The study showed that MSTFA derivatives of trenbolone are not stable, since after one week the compounds lost their properties both: in standard solution and in urine samples, and after two weeks a two-fold decrease in the signal intensity was observed. A good separation of trenbolone on non-polar HP -5 ms was achieved in one of the most frequently used capillary column containing a 5% phenyl liquid phase. The simplest way to separate steroid hormones from the matrix components is a liquid-liquid extraction (LLE) with organic solvents. To verify the efficiency of extraction of analytes from enriched to a concentration of 20 µg L^{-1} urine samples, solvents such as diethyl ether, t-butyl methyl ether, and chloroform were tested. The best results were obtained for diethyl ether (97% recovery), which was applied in the method.

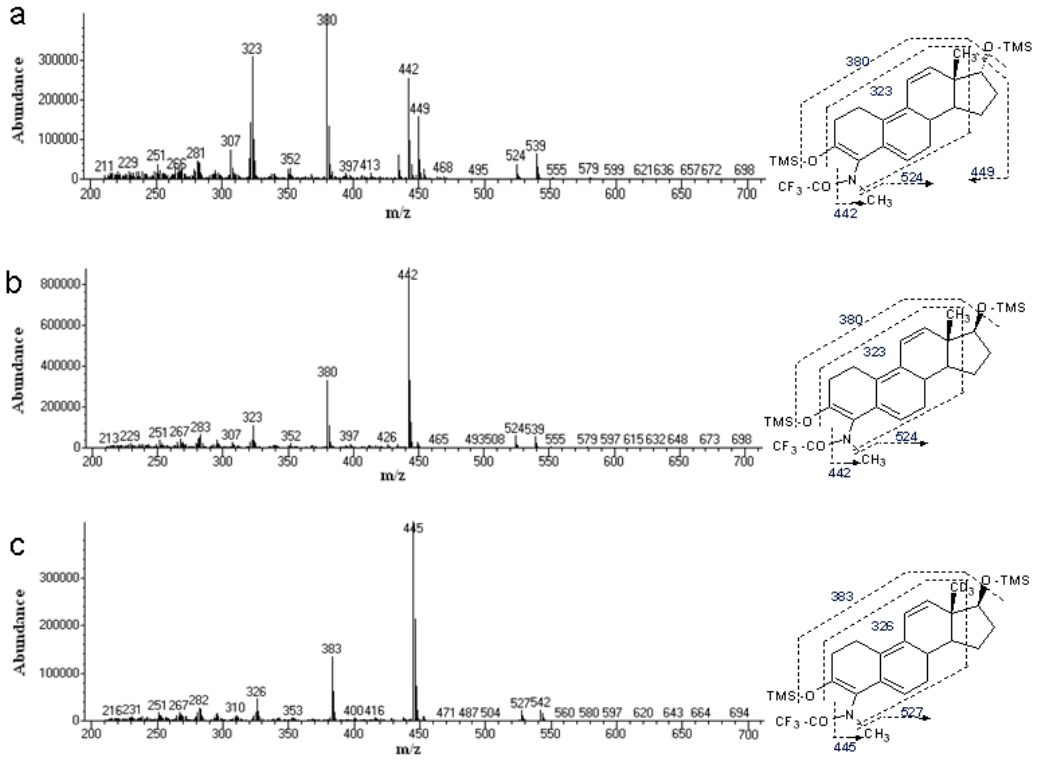


Fig. 4. GC-MS full mass spectra and the proposed schemes of (-TMS) derivatives fragmentation of: a) 17α-TBOH, b) 17β-TBOH, c) 17β-TBOH-d3

For the other solvents, recovery was lower and amounted to 81% for t-butyl methyl ether and 62% for chloroform, respectively. For further purification of the extract, a solid phase extraction was selected and its advantages are as follows: significant reduction of organic solvent, concentration of the extract, reproducibility improvement, reduction in time analysis, simplicity, and safety. The usefulness of six types of SPE columns such as: SiOH, C_8, NH_2, C_6H_5, SBD_1, and C_{18} for analyte separation and purification of biological extracts was evaluated. The effectiveness of hormones adsorption on SPE column, with standard solutions diluted in appropriate phase was performed. For each type of SPE sorbent the standard treatment approach was applied. The experiment was repeated three times for each column. The recoveries of above 85% were obtained for C_{18}, NH_2, and C_6H_5, in the case of SiOH and C_8 they amounted to about 50%, and for SDB_1 25% only. Further optimisation of experiment involving the impact of the matrix on the purification of extracts was performed on columns, on which the highest recovery was obtained. In the studies, extracts obtained from 20 mL of urine were used. The extracts were initially purified using liquid-liquid extraction with diethyl ether. The organic phase was evaporated under a stream of nitrogen, and the residue was dissolved in a suitable phase and fortified to a concentration of hormones of 20 μg L^{-1}. The presence of matrix components had adversely affected the recovery of trenbolone from the phenyl column, which decreased almost twice, so this column was excluded from further study. The application of two columns, C_{18} and NH_2 for the extract purification allowed obtaining pure extracts. The absence of any interfering peaks around the analytes retention times demonstrated that the clean-up procedure was suitable for the purpose.

The methods were validated according to the criteria specified in Commission Decision 2002/657/EC. The performance of the method was investigated with respect to various parameters, such as specificity, recovery, precision, decision limit, detection capability, and ruggedness.

The specificity of the methods was demonstrated by the analysis of 10 different blank bovine urine samples and samples fortified with analytes at 1 μg L^{-1} in the presence of potentially interfering compounds. No interfering peaks from endogenous compounds were found in the retention time of the target analytes. For each of the spiked samples chromatograms showed a significant increase in peak area corresponding to each analyte; therefore, the methods were found to be specific for compound investigated (Figs 2, 3).

Calibration curves were constructed by plotting peak-area ratios *versus* hormone concentrations using a least-square linear regression model. The linearity was good for both analytes in the whole range of tested concentrations, as proven by the correlation coefficients (r^2) exceeding 0.99 value for all curves. Recovery for both GC-MS and GC-MS/MS were very similar, and exceeded the values of 86%.

Application of stable isotope-labelled analogue of the analyte as an internal standard compensates for the loss of the analyte during sample preparation, and can achieve satisfactory recovery. The repeatability of GC-MS/MS method was slightly better than the GC-MS, but reproducibility was comparable, and for the level of 1 μg L^{-1} did not exceed 22%.

The values of CCα and CCβ were significantly lower than actually recommended concentration for trenbolone established at 2 μg L^{-1}. The uncertainty for 17α- and 17β-trenbolone for validation level of 1 μg L^{-1} was calculated as the sum of variances of reproducibility at levels 1–1.5 μg L^{-1} and variance of the matrix effects, by using ResVal software. The combined uncertainty was multiplied by a coverage factor of 2 to estimate expanded uncertainty, which ranges from 33% to 36% for GC-MS and from 32% to 39% for GC-MS/MS method.

According to the guidelines of Commission Decision 2002/657/EC for the confirmation of banned substances listed in Group A of Annex I of Directive 96/23/EC (7), a minimum of four identification points (IP) shall be required, which means that for GC-MS technique, four diagnostic ions for tested analyte have to be identified, and their relative intensities should fall within certain ranges of tolerance. For GC-MS/MS one precursor and two daughter ions (4IP) or two precursor ions, each with one daughter (5IP) must be earned.

Over 50% of samples analysed by GC-MS during validation did not meet the criteria for confirmatory method; therefore this method is used for screening purposes.

In the case of GC-MS/MS method used for detection of both forms of trenbolone, two precursor ions, each with one daughter ion, were detected, so five identification points were obtained. Additionally, the criteria concerning ion ratio were fulfilled in concentration range CCα -5 μg L^{-1} over 95%. Therefore, this method is suitable for the confirmatory purposes.

The method performance has been successfully evaluated by the participation in Proficiency Testing organised by Progetto Trieste 2011, 1st round - Veterinary Drug Residues, showing our data with Z-scores of -0.42 and -1.09 for 17α-trenbolone and 17β-trenbolone. These results prove good accuracy and reproducibility of the developed methods.

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