1. INTRODUCTION

This method describes the analysis of stanozolol, 4ß-hydroxy-stanozolol and 16ß-hydroxy-stanozolol in urine for a screening and a confirmation method. The screening method describes a clean-up step and concentration step done by using Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE), amino (NH_2). The samples are analysed with High Performance Liquid Chromatography Mass-Spectrometric (HPLC-MS). For purpose of quantification, deuterated internal standards are used. The limit of detection is 0,5 ng/ml for stanozolol and 1 ng/ml for 4ß-hydroxy-stanozolol and 16ß-hydroxy-stanozolol. The confirmation method is only suitable for 16ß-OH-stanozolol and contains the same clean up and concentration steps. The samples are analysed with selected reaction monitoring without using internal standards.

2. MATERIALS

Reference to a company and/or product is for purpose of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and The Environment (RIVM) to the exclusion of others, which might also be suitable.

All chemicals are pro analyse quality or better, unless stated otherwise.

The water used was Milli-Q water.

Prepared solutions are stored at room temperature and expire after 1 year.

2.1. Stanozolol and metabolites

Stanozolol and its metabolites are synthetic anabolic steroids, which have a growth stimulating effect on man and animal. These anabolic steroids are used illegally for growth promotion purposes in slaughter animals and can be found in injection sites.
K. van Oosthuyze (8.1) concluded in 1993 that 63% of the injection sites contained stanozolol. The structure of stanozolol differs from other anabolic steroids because a pyrazole moiety is attached to the A-ring (figure 1).

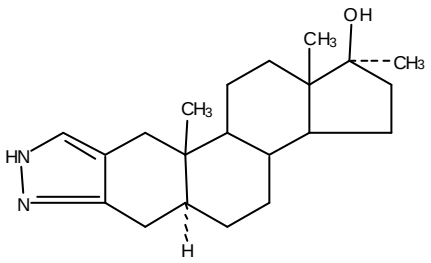


Figure 1: Structure stanozolol

W.Schanzer et al. (8.2) studied extensively the excretion of stanozolol in human urine. They noticed that 97% of stanozolol and the mono-hydroxy metabolites were excreted as conjugates. Only 3 % were detected unconjugated in urine. The major metabolites found in human urine are 4ß-hydroxy-stanozolol and 16ß-hydroxy-stanozolol (figure 2).

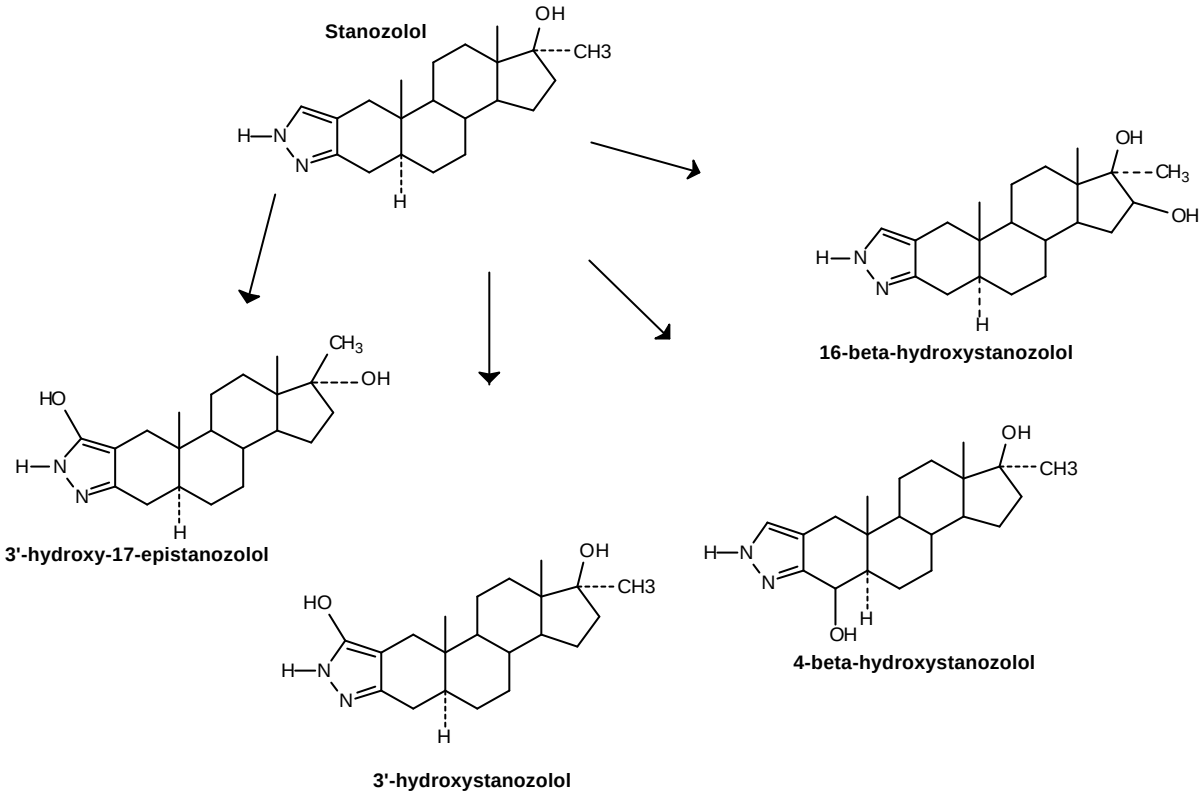


Figure 2: Metabolites of stanozolol.

2.2. Standards

2.2.1.	Stanozolol	($C_{21}H_{32}N_2O$),	Abbreviation: St,	MW:328.50
2.2.2.	Stanozolol-d3	($C_{21}H_{29}D_3N_2O$),	Abbreviation: St-d3,	MW: 331.47
2.2.3.	4ß-Hydroxy-stanozolol	($C_{21}H_{32}N_2O_2$),	Abbreviation: 4ß-OH-St,	MW: 344.50
2.2.4.	16ß-Hydroxy-stanozolol	($C_{21}H_{32}N_2O_2$),	Abbreviation: 16ß-OH-St,	MW: 344.50

Stanozolol, Stanozolol-d3 and 16ß-hydroxy-stanozolol were obtained from Radian, 4ß-hydroxy-stanozolol was a kind gift of W.Schanzer. From these standards stock solutions are prepared containing 1 mg/ml or 100 ng/ml in ethanol. These solutions are registered and stored in the dark at approximately -20°C (not higher then -10°C) for a maximum period of 5 years. Working solutions are prepared by 10 fold dilutions of the stock solutions. These solutions are stored in the dark at approximately 4°C (range 1-10°C) for a maximum period of 6 months. Quality control of the solid standards includes the registration of a mass spectrum (identity) and a HPLC Diode array chromatogram/UV-spectrum.

2.3. Samples

Samples are stored in the dark at approximately -20°C, but not higher than -10°C, until analysis. If analysis will take place within 2 days, the samples can be stored at approximately 4°C (range 1-10°C).

2.4. Chemicals

2.4.1. Standard mix: contains 0,1 ng/µl of the compounds stanozolol (2.2.1), 16ß-hydroxy-stanozolol (2.2.4) and 4ß-hydroxy-stanozolol (2.2.3).

2.4.2. Internal Standard: contains 0,1 ng/µl of stanozolol-d3 (2.2.2)

2.4.3. Acetic acid (Baker, 6152)

2.4.4. Sodium acetate (Merck, 6268)
2.4.5. Acetate buffer, 2 mol/l, pH 5.2. Dissolve 25.2g acetic acid (2.4.3) and 129.5 g sodium acetate (2.4.4) in 800 ml water. Adjust the pH, with a pH meter (2.5.2), at 5.2 ± 0.1 and add water to a final volume of 1000 ml.
2.4.6. Beta-glucuronidase/sulfatase containing 100.000 units ß-glucuronidase and 100.000 units sulfatase per ml (suc d'Helix Pomatia). (Industrie Biologique, France, IBR 213583))
2.4.7. Sodium carbonate anhydrous (Merck-Vel, 1720)
2.4.8. Carbonate solution 1 mol/l. Dissolve 10.6g sodium carbonate (2.4.7) in 100 ml water.
2.4.9. Hexane (Baker, 8668)
2.4.10. 1-Butanol (Merck, 101990)
2.4.11. Methanol (Baker, 8045)
2.4.12. Ethanol (Baker, 8006)
2.4.13. Ammonium acetate (Baker, 0390)
2.4.14. Eluens A. Dissolve 0,35 g ammonium acetate (2.4.13) in 900 ml water and 100 ml methanol (2.4.11).

2.5. Equipment

Standard laboratory glassware and equipment is used, with the addition of:

2.5.1. Plastic tubes 50 ml (Omnilabo, 800516).
2.5.2. pH-meter CG 837 (Schott).
2.5.3. Centrifuge Varifuge 3.0R with rotor 5315 (Heraeus).
2.5.4. Glass tubes 15 ml (Rennes, custom made).
2.5.5. Evaporation block (TurboVap® LV, Zymark).
2.5.6. SPE Extract-Clean NH_2 (Alltech, 211153).
2.5.7. Minishaker ms1 (IKA).
2.5.8. Glass tubes 10 ml (Rennes, custom made).
2.5.9. Glass derivatisation vials (HP, 5182-0553).
2.5.10. Inserts 50 µl (Alltech, 98024).
2.5.11. HPLC-column Alltima C18, 15 cm x 2.1 mm 5 µm, (Alltech, 88370).
2.5.12. LC-MS equipment. The LC-MS used for this method is a HP-1100 system with a LC-MSD detector and HP LC-MSD chemstation software, which is used for control and data processing.

3. ANALYTICAL PROCEDURE

3.1. Sample preparation

A set samples consists of 2 blank control samples and the other samples.

3.1.1. Pipette accurately ± 5 ml of the urine sample in a 50 ml tube.
3.1.2. Spike the blank control sample and the other samples according to table 1.

Table 1*:* Preparation of a sample sequence.

Identification (ID)	µl Standard Mix (2.4.1) (0,1 ng/µl)	µl Internal Standard (2.4.2) (0,1 ng/µl)
Blank urine	0	250
Blank urine + spike 2 ng/g	100	250
Sample(s)	0	250

3.2. Hydrolyses

3.2.1. Add 2 ml 2M acetate buffer (2.4.5) to a 5 ml urine sample (pH=5.2). Check the pH with a pH-meter (2.5.2) and if necessary adjust with concentrated acetic acid (2.4.3) or 1 M carbonate solution (2.4.8).

3.2.2. Add 50 µl of Helix-Pomatia (2.4.6).

3.2.3. Shake the mixture and hydrolyse overnight at 37°C.

3.3. LLE extraction

3.3.1 Bring the pH of the hydrolysed urine samples to ± 9 by adding as much as necessary of a 1M carbonate solution (2.4.8). Check the pH with a pH-meter (2.5.2).

3.3.2 Extract the solution with 10 ml hexane/butanol (80:20 v/v-%).

3.3.3 Centrifuge for 3 minutes at 3600 rpm.

3.3.4 Transfer the organic phase to a clean 10 ml tube.

3.3.5 Evaporate a part of the organic solvent at 55°C under a soft nitrogen stream.

3.3.6 Repeat step 3.3.2 to 3.3.4 and combine both hexane/butanol extracts.

3.3.7 Evaporate the organic solvent at 55°C under a soft nitrogen stream.

3.3.8 Reconstitute the sample with 5 ml methanol/water (80/20 v/v-%)

3.4. Solid Phase Extraction (SPE)

3.4.1. Condition the amino SPE column with 5 ml of 80/20 v/v-% methanol/water. During clean-up the SPE columns should not run dry!

3.4.2. Pass the methanol/water extract through the amino column whereby the eluant is collected in a 10 ml tube.

3.4.3. Evaporate the extract under a soft nitrogen stream to dryness using an evaporation block at 55°C.

3.4.4. Resuspend the dried extract in 500 µl ethanol and mix by use of a Vortex mixer for 30 seconds.

3.4.5. Transfer the ethanol mixture to a HPLC injection vial.

3.4.6. Evaporate the extract under a soft nitrogen stream to dryness using an evaporation block at 55°C.

3.4.7. Resuspend the sample in 25 µl methanol/water (40/60 v/v-%) and mix with a Vortex mixer for 30 seconds.

3.4.8. Transfer the methanol/water mixture in to a 50 µl insert and place this insert in the HPLC injection vial.

3.5. LC-MS detection

Calibration curve

3.5.1. Prepare a calibration curve according to table 2 by pipetting the indicated amounts into a HPLC injection vial.

Table 2: Preparation calibration curve

Amount (absolute)	µl Standard mix (2.4.1) (0.1 ng/µl)	µl Internal standard-d3 (2.4.2) (0.1 ng/µl)
0.0 ng	0	250
1.0 ng	10	250
2.5 ng	25	250
5 ng	50	250
10 ng	100	250
25 ng	250	250

3.5.2. Evaporate to dryness under a soft nitrogen stream on an evaporation block at 55°C.

3.5.3. Resuspend the dried extract in 25 µl methanol/water (40/60 v/v-%) and mix with a Vortex mixer for 30 seconds.

3.5.4. Transfer the water/methanol mixture into a 50 µl insert.

Gradient and HPLC system

3.5.5. The described gradient in table 3 is used. Eluens A is 5 mmol/l ammonium acetate in a solution of methanol/water (10/90 v/v-%) (2.4.14). Eluens B is a solution of methanol/water (90/10 v/v-%).

Table 3: HPLC gradient

Time (min)	Flow (ml/min)	Percentage eluens A	Percentage eluens B
0	0.3	40	60
20	0.3	20	80
21	0.3	10	90
23	0.3	10	90
23.1	0.3	40	60

3.5.6. Detection is performed in SIM mode whereby the following ions are monitored.

Table 4**:** Ions monitored

ID.	M/Z
Stanozolol	329
Stanozolol-d3	332
4ß-Hydroxy-stanozolol	327
16ß-Hydroxy-stanozolol	345

The instrument is operated in APCI in positive mode and is optimised for optimum performance for the analysed compounds.

The following conditions are used as a starting point for the analyses:

Gas Temp : 350°C
Vaporiser temp : 325°C
Drying Gas : 10.0 l/min
Neb Pres : 30 psig
Vcap : 2000 V
Corona : 5 µA
Gain : 25
Fragmentor : 120 V

3.5.7. To check the HPLC system a standard of 10 ng should be injected. The signal to noise ratio for stanozolol must be greater then 6.

Sequence

3.5.8. When the system check is performed and the criteria are fulfilled, analysis can be carried out. A sequence should include the following injections:

- Standards 0 - 25 ng absolute
- Standard 0 ng
- Control samples (Blank and spike 2 ng/ml)
- Standard 0 ng
- Samples
- Standard 0 - 25 ng absolute

4. INTERPRETATION AND CALCULATION

4.1. Calculation of quantitative results

Quantitative results are obtained by constructing a calibration curve of the Response Factor (RF) versus the concentration (absolute). For calculation the linear regression program Calwer© is used.

The RF is calculated for each compound:

- RF 4ß-OH-St = peak area 4ß-OH-St / peak area St-d3
- RF 16ß-OH-St = peak area 16ß-OH-St / peak area St-d3
- RF St = peak area St / peak area St-d3

The concentration of compounds in the sample is calculated through correction of the calculated amount by the volume of sample taken for the analysis.

Quantification is only valid if:
- The signal originating from the analyte has a S/N ratio > 6.
- The coefficient of correlation of the constructed calibration curve is > 0.99.

5. VALIDATION OF THE PROCEDURE

The described method is validated according to SOP ARO/425 (3) and according to the definitions and criteria of the European Commission decision 96/23/EC (4). The compounds can only be identified by their mass and retention time. Therefore this method is a screenings method. This is a method used to establish if a sample is negative for the analyte at a predetermined level or might contain the analyte at or above this level. For identification another technique is used: selected reaction monitoring (see chapter 7: confirmation).

5.1. Decision Limit (CCα)/Detection Capability (CCß)

The amount of analyte necessary for analysis is determined by fortifying blank urine around the MPL (maximum permitted level) in equidistant steps. This was performed with concentrations from 0 till 3 ng/ml in steps of 0.5 ng/ml.

Table 5: Decision Limit (CCα)/Detection Capability (CCß)

Analyte	CCa Decision limit		CCß Detection Cap.	
	With outlier	Without outlier	with outlier	without outlier
Stanozolol	0.36	0.11	0.60	0.18
16ß-OH-St	0.33	0.20	0.55	0.32
4ß-OH-St	0.41	0.18	0.68	0.30

Discussion: The outlier was a sample that was cloudy during the sample preparation. Because of this, the following was decided: when used as a screening method, no action will be taken when a sample is cloudy. However used as a quantification method the cloudy sample must be reanalysed.

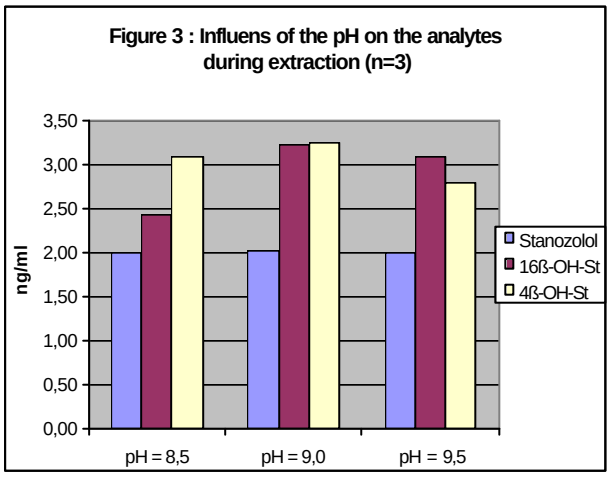
Conclusion: Based on these results the detection capability for respectively stanozolol, 16ß-hydroxy-stanozolol and 4ß-hydroxy-stanozolol will be 0.5 ng/ml, 1 ng/ml and 1 ng/ml.

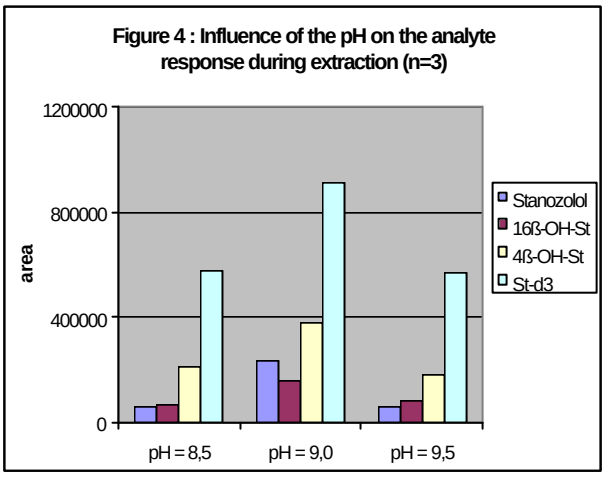
5.2. Selectivity/specificy

The metabolites of stanozolol are already established in earlier studies. They don't interfere with each other. Other veterinary drugs or contaminants likely to be used in combination with stanozolol weren't discovered. Therefore only interference of the matrix was investigated by analysing 20 representative blank urine samples. These samples were checked for any interference in the region of interest where stanozolol and its metabolites were expected to elute. Results show there is no interference at or nearby the expected retention times of the compounds to be analysed.

5.3. Ruggedness

For this method, the pH and the LLE extraction were identified as the critical factors that could influence the results.
Blank urine samples were spiked with a mix containing 0.5 ng/ml stanozolol, 1 ng/ml 16ß-hydroxy-stanozolol and 2 ng/ml 4ß-hydroxy-stanozolol.
The pH influence was determined by extracting at 3 pH's, namely 8.5, 9.0 and 9.5.
For the LLE extraction the percentage organic modifier was varied, namely
70/30 v/v-% hexane/butanol, 80/20 v/v-% hexane/butanol and 90/10 v/v-% hexane/butanol.





5.3.1. pH influence (n=3).

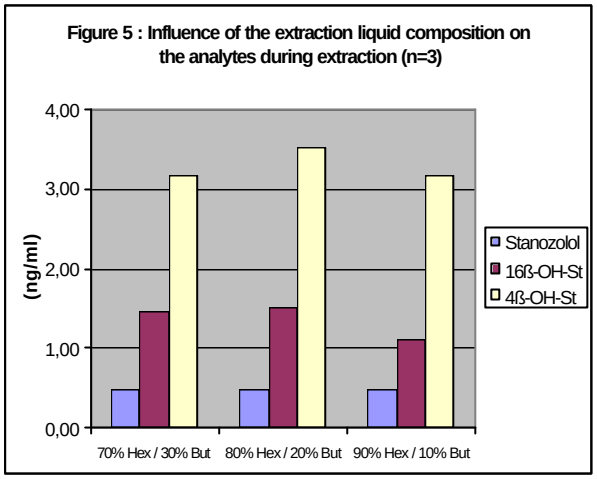
Conclusion: As shown in figure 3, the pH has no great impact on the results in terms of ng/ml. In terms of peak area (figure 4) however, it is shown that there is a optimum at pH=9.0. This was to be expected because in a earlier study this parameter was determined to be pH=9.0.

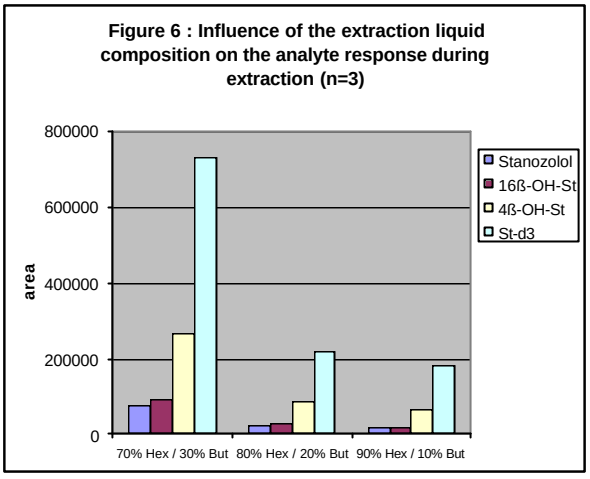
5.3.2. Influence of the extraction liquid composition (n=3).

Conclusion: As shown in figure 5, the composition of the extraction liquid has no great impact on the results in terms of ng/ml. There seems to be an optimum at 80% hexane / 20% butanol.

In terms of peak area (figure 6) however, it is shown that there is a great impact at 70% hexane / 30 % butanol. Because in this study the postulate limits were achieved further study wasn't performed.

Overall conclusion: This method is considered to be stable for it's purpose.





5.4. Precision

5.4.1. Repeatability

The repeatability was determined by spiking 10 times a blank urine sample at the detection capability level. This was done 3 times on 3 different days.

Accuracy (%) (n=10)	06.06.1999		
	Stanozolol	**16ß-OH-St**	**4ß-OH-St**
Average	111.4	162.0	174.8
CV (%)	20.8	17.1	22.1

Accuracy (%) (n=10)	07.06.1999		
	Stanozolol	**16ß-OH-St**	**4ß-OH-St**
Average	101.9	152.3	156.5
CV (%)	7.2	15.1	20.6

Accuracy (%) (n=10)	08.06.1999		
	Stanozolol	**16ß-OH-St**	**4ß-OH-St**
Average	99.6	140.2	200.1
CV (%)	4.0	7.4	13.3

Accuracy (%) (n=10)	total survey		
	Stanozolol	**16ß-OH-St**	**4ß-OH-St**
Average	104.3	151.5	177.2
CV (%)	14.1	15.0	20.6

Conclusion: The overall co-variation is below 20% for stanozolol and 16ß-OH-St, for 4ß-OH-St the co-variation is slightly higher. However the accuracy for 16ß-OH-St and 4ß-OH-St is higher then 120%. This is due to lack of a suitable deuterated internal standard for these compounds. Therefore the use of a deuterated internal standard for 16ß-OH-St and 4ß-OH-St is highly recommended.

5.4.2. Reproducibility

This parameter will be established during the investigating through close co-operation with other laboratories.

5.4.3. Within-laboratory reproducibility

This parameter will be established during following projects. Hereby the results are gained by monitoring control samples, using Shewart maps.

6. Chromatogram urine sample

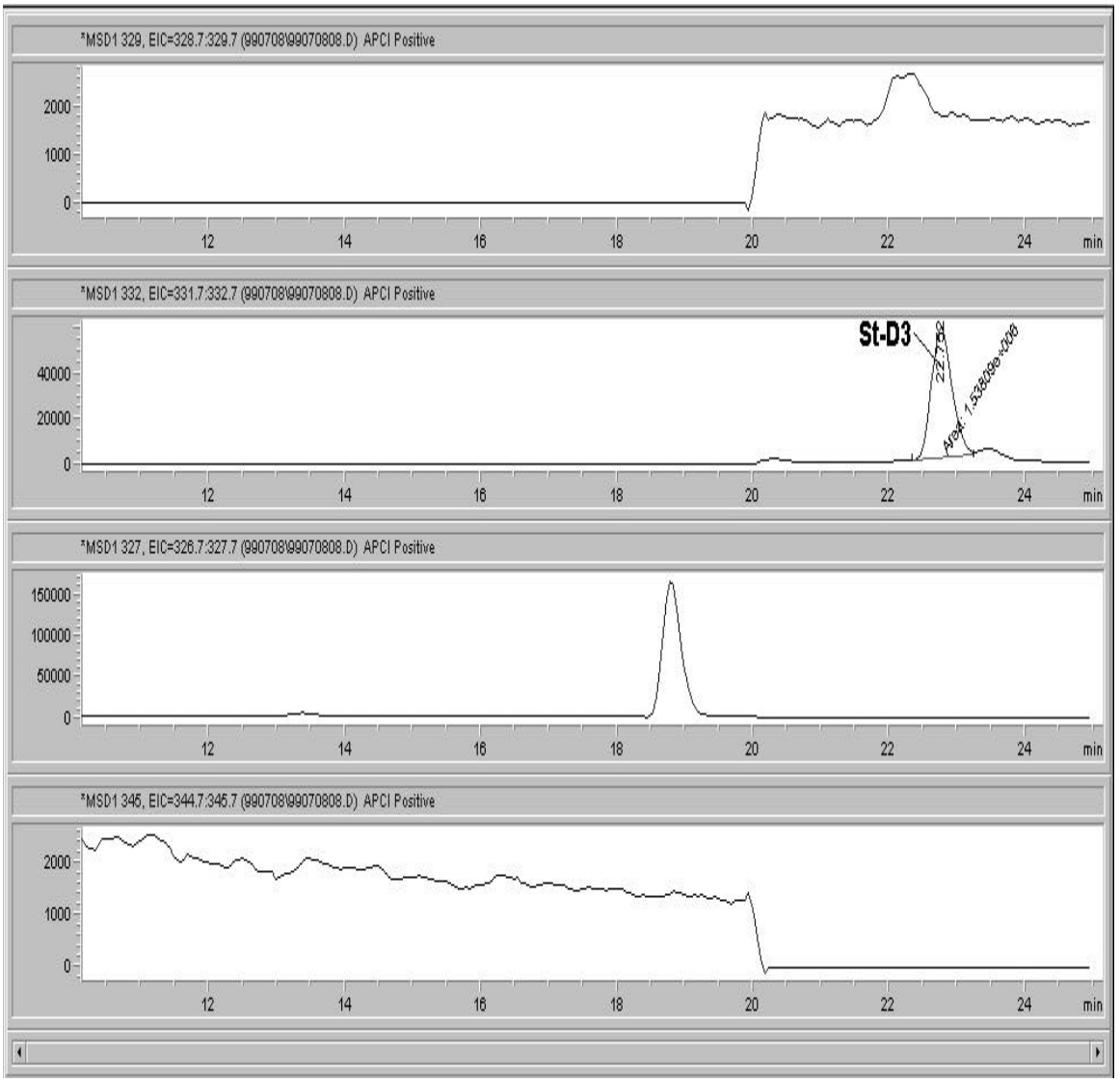


Figure 7: Blank urine sample with internal standard St-d3.

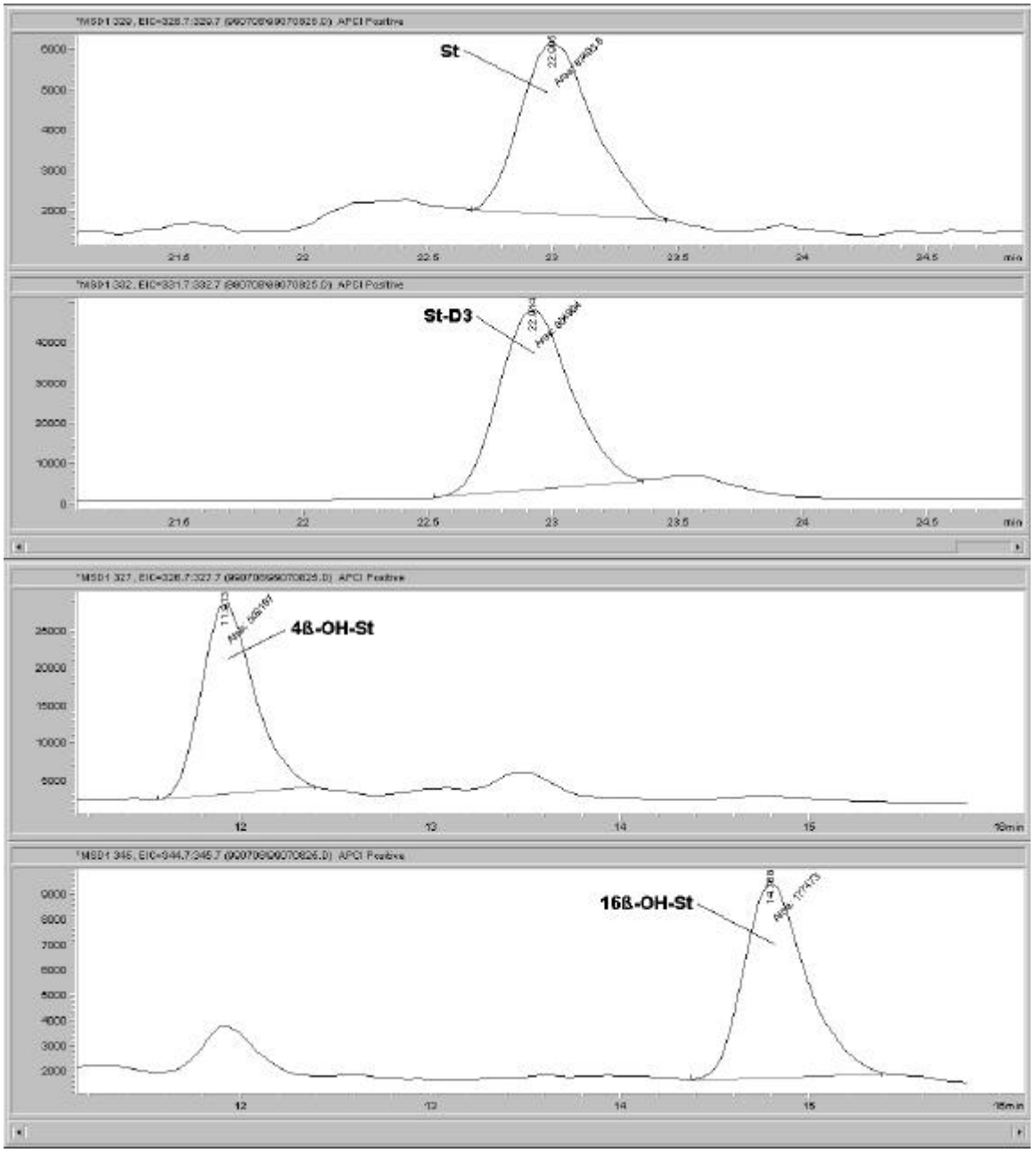


Figure 8: Blank urine sample spiked with a mixture of stanozolol, 16ß-OH-St and 4ß-OH-St. The concentration levels are 0.5 ng/ml for stanozolol, 1 ng/ml for 16ß-OH-St and 2 ng/ml for 4ß-OH-St.

7. CONFIRMATORY ANALYSIS

7.1. Method of analysis

7.1.1. Hydrolysis, extraction and Clean-up as described for LC-MSD (see section 4) ***without*** adding internal standard.

7.1.2. Evaporate the final extraction solvent under a stream of nitrogen at 50°C.

7.1.3. Redissolve the residue in 50 µl methanol and vortex for 30 seconds.

7.1.4. Add 200 µl milli-Q water and vortex for 30 seconds.

7.1.5. Inject 4/5 of the final sample extract and the standard solutions into the LC-MS^n (Finnigan LCQ) system.

7.2. Confirmation with LC-MS^n

Calibration curve

7.2.1. Prepare a calibration curve according to table 5 by pipetting the indicated amounts into a HPLC injection vial or spiking the indicated amounts to blank urine control samples before hydrolyses.

Table 5*:* Preparation calibration curve

Amount (absolute)	µl Standard mix (0.1 ng/µl)
0.0 ng	0
2.5 ng	25
10 ng	100
25 ng	250

7.2.2. Evaporate the final extraction solvent under a stream of nitrogen at 50°C.
7.2.3. Redissolve the residue in 50 µl methanol and vortex for 30 seconds.
7.2.4. Add 200 µl milli-Q water and vortex for 30 seconds.
7.2.5. Inject 4/5 of the final sample extract and the standard solutions into the LC-MS^n (Finnigan LCQ) system.

Gradient and HPLC system

7.2.6. HPLC-column: Super ODS 2 micron, L= 5.0 cm, id = 4.6 mm.
7.2.7. The described gradient in table 6 is used.
Eluens A is solution of Acetonitrile/ methanol/water (20/20/60 v/v/v-%).
Eluens B is a solution of acetonitrile/ methanol/water (50/20/30 v/v/v-%).
Eluens C is 100% methanol.

Table 6*:* HPLC gradient

Time (min)	Flow (ml/min)	Percentage eluens A	Percentage eluens B	Percentage eluens C
0	1.0	100	0	0
4	1.0	0	100	0
4.01	1.0	0	0	100
5	1.0	0	0	100
5.01	1.0	100	0	0

7.2.8. Detection is performed by ion trap MS^2 in APCI(+) mode whereby for 16ß-hydroxy-stanozolol, the transition ions in table 7 are monitored.

Table 7: Ions monitored

ID.	Transitions ions monitored for LC-MS^n			
16ß-OH-St	159	227	255	329

Table 8: Acquisition parameters

Vaporiser	450°C
Capillary	150°C
Nitrogen (high purity)	70 psi

Ions are detected in the APCI (+) MS^2 mode.

For 16ß-hydroxy-stanozolol measurements by LC-MS^n, at least two transition ions have to be monitored and the ratio between the two recorded fragment ions has to be calculated and compared with the ratio obtained for either standards or fortified control samples. The calculation and comparing is done automatically by using a program called confirmation.

8. RELATED DOCUMENTS

8.1 K. van Oosthuyze, E. Daeseleire, A. van Overbeke, C. van Peteghem: Survey of the hormones used in cattle fattening based on the analyses of Belgian injection sites. *Analyst,* **119** (1994) 2655-2657.

8.2 W. Schanzer, G. Opfermann, M. Donike: Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J. Steroid Biochem,* **36** (1990) 153-174.

8.3 A.A.M. Stolker, Procedure for the validation of analytical methods, SOP ARO/425, revision 1, 28 april 1997, RIVM.

8.4 Report: European Commission Decision laying down requirements for analytical methods to be used for detecting certain substances and residue thereof in live animals and animal products according to Council Directive 96/23/EC (Revision of Commission Decision 93/256/EC).

9. CONCLUSIONS

The described method is a rather simple clean-up combined with a LC-MS (APCI, positive mode) analyses. The clean-up consist of a LLE extraction and a SPE amino extraction. After clean-up are the samples injected on a C18 HPLC column. From the gained results it is obvious that a (semi) quantitative analyses can be performed for stanozolol, 4ß-hydroxy-stanozolol and 16ß-hydroxy-stanozolol. Proven is that positive samples on 16ß-OH-Stanozolol can be confirmed by use of selected reaction monitoring.

10. ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionisation
ARO	Laboratory for Residue Analysis
EU	European Union
HPLC	High Performance Liquid Chromatography
LLE	Liquid-Liquid Extraction
MS	Mass-Spectrometer
MW	Molecular Weight
RF	Response Factor
SOP	Standard operating procedure
SPE	Solid Phase Extraction