ELSEVIER

Journal of Chromatography A, 904 (2000) 203–209

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of 16β-hydroxystanozolol in urine and faeces by liquid chromatography–multiple mass spectrometry ☆

M. Van de Wiele[a,*], K. De Wasch[b], J. Vercammen[a], D. Courtheyn[a], H. De Brabander[b], S. Impens[b]

[a]*State Laboratory, Braemkasteelstraat 59, B-9050 Gentbrugge, Belgium*

[b]*Laboratory of Chemical Analysis, Department of Veterinary Food Inspection, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*

Received 9 February 2000; received in revised form 27 August 2000; accepted 8 September 2000

Abstract

This paper describes the optimisation of the detection of stanozolol and its major metabolite 16β-hydroxystanozolol in faeces and urine from cattle. Faeces are extracted directly with diisopropyl ether. Urine is first submitted to an enzymatic hydrolysis and then extracted over a modified diatomaceous earth column (Chem-Elut) with a mixture of diisopropyl ether–isooctane. In a final step an acidic back extraction is performed. For the LC–MS–MS detection two approaches are discussed. In a first approach the final extract is detected without derivatization, while the second approach makes use of a derivatization step for 16β-hydroxystanozolol. While the MS–MS spectrum without derivatization exhibits extensive fragmentation, the spectrum of the derivative shows two abundant diagnostic ions with much more reproducible ion ratios. The derivatization method and the method without derivatization enable the detection of 16β-hydroxystanozolol up to 0.03 μg l^{-1} in urine and 0.07 μg kg^{-1} in faeces. Until now there is no literature available for the detection of 16β-hydroxystanozolol in faeces and urine at the ppt level.

Keywords: Hydroxystanozolol; Stanozolol; Steroids

1. Introduction

Stanozolol was first synthesised by Clinton and co-workers [1,2]. Several authors describe the anabolic effects of stanozolol. Clinically it is used in cases of osteoporosis and deficiency in protein synthesis [3]. In spite of its prohibiton by the International Olympic Committee (IOC) since 1974, this androgenic anabolic was often abused in sport by athletes. Also in horse-races, stanozolol was used to enhance performances. This anabolic steroid is illicitly used for growth promoting purposes in cattle. The discovery of high concentrations of stanozolol in injection sites taken at the slaughterhouse revealed the illegal use as growth promoter in cattle despite the ban in the European Union (EU) since 1988. Until 1999 it was never officially detected in tissues or excreta from animals. Several methods for stanozolol based on gas chromatography–mass spec-

☆Presented at the 6th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Brugge, 9–11 February 2000.

*Corresponding author.

E-mail address: mieke.vandewiele@rug.ac.be (M. Van de Wiele).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00945-6

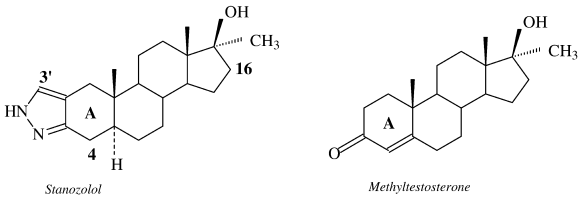

Fig. 1. Structure of stanozolol and methyltestosterone.

trometry (GC–MS) are capable of detection and identification [4–7]. Stanozolol has bad gas chromatographical properties because the analyte derivatises with difficulty and adsorbs at parts of the injector.

There is an important structural difference with other anabolic steroids. Stanozolol resembles methyltestosterone best. Instead of the 3-keto group, there is a pyrazole ring condensed to the androstane ring system (Fig. 1). Furthermore, stanozolol is almost completely metabolised. As is described by De Brabander et al., laboratories in Belgium and the Netherlands joined their efforts in a multi-laboratory study to develop an analytical strategy to detect the major metabolite 16β-hydroxystanozolol (16-OH-Stan). In earlier experiments LC–MS^n proved to be the method of choice for the detection of stanozolol and its metabolites at the lower ppb level [8,9].

2. Experimental

2.1. Reagents and chemicals

All chemicals used were of analytical grade from Merck. Helix pomatia juice with enzymatic activity of β-glucuronidase and sulfatase was purchased from Merck. A phenylboronic acid (Janssen Chimica, 98%) solution in methanol (1 mg ml^{-1}) was used for derivatization. Stanozolol (Stan) and its deuterated ($[^2H_3]$) internal standard (stanozolol-d_3) were obtained from Sigma (St. Louis, MO, USA). 16β-hydroxystanozolol (16-OHstan) was obtained from Radian International (Austin, TX, USA). Stock solutions (1 mg 100 ml^{-1}) and working standard solutions (1 ng μl^{-1}) were prepared in ethanol and stored refrigerated. Chem-Elut 1010 columns were supplied by Analytichem International (Harbor City, CA, USA); Clean Screen extraction columns (6 ml-500 mg) by BAS Technicol (Cheshire, UK) and Bakerbond spe octadecyl (C_{18}) extraction columns 7020 (6 ml-500 mg) by J.T. Baker (Philipsburg, PA, USA).

2.2. Samples

The samples were frozen on arrival and were kept at −20°C until analysis.

2.3. Extraction and clean-up

2.3.1. Urine

Urine (5 ml) was hydrolysed overnight at 52°C with 50 μl *Helix pomatia* juice after correcting the pH of the sample to 5.2 (±0.2) with acetic acid 2 *M*. Following the pH of the sample is adjusted to alkaline pH 11 (±0.2) by addition of 120 μl sodium hydroxide solution (5 *M*) and fine tuning with sodium carbonate solution (10%, w/v). The basic solution obtained was transferred onto a Chem-Elut 1010 column and allowed to stand for 10 min. The column was eluted with 50 ml diisopropyl ether–isooctane (50:50, v/v). A volume of 250 μl hydrochloric acid (0.5 *M*) was added to the tube and was then shaken vigorously by hand for 1 min. The sample was placed in an ultrasonic bath for 10 min so that the HCl drop was emulsified in the solution prior to centrifugation. After centrifugation at 3400 rpm for 10 min a clear drop of HCl containing the analyte was formed. Two hundred μl of the drop was taken up with a syringe and depending on the approach directly injected into the LC–MS system or derivatized.

2.3.2. Faeces

To faeces (2 g), 20 μl sodium hydroxide solution (32%) were added. The analyte was extracted with 15 ml diisopropyl ether. Non-activated sodium sulphate (5 g) was added to remove water. The sample was shaken for 10 min. After centrifugation at 3400 rpm for 10 min, 250 μl HCl (0.5 *M*) were added to the supernatant. Again the sample was shaken, sonicated for 10 min and centrifuged at 3400 rpm for 10 min.

2.4. Detection with LC–MS–MS

2.4.1. Approach without derivatization

Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150×2.1 mm, Waters, Milford, MA, USA). The mobile phase consisted of a mixture of methanol–0.077% formic acid (55:45, v/v) (A) pumped at a rate of 0.3 ml/min. A linear gradient was run as follows: 45% A (maintained for 10.8 min) to 40% A in 2.2 min (maintained for 7 min). In between samples there was an equilibration time of 10 min at initial conditions.

In this experiment a 1100 series quaternary pump and an autosampler from Hewlett-Packard (Palo Alto, CA, USA) were used. The MS detector was a Finnigan LCQ deca ion trap MS of ThermoQuest (San José, CA, USA) equipped with an electrospray interface in positive ion mode MS–MS full scan.

2.4.1.1. Experimental method

In the first time segment 16-OHStan was isolated and fragmented. In the second time segment stanozolol and deuterated stanozolol coeluted. In this segment two scan events allowed a mass spectrometrical separation. The experiment method and fragment ions are summarised in Table 1.

2.4.2. Approach with derivatization

After taking up the HCl drop with the concentrated analyte, the extract was evaporated to dryness with a vacuum Speedvac system (Life Sciences International, Cheshire, UK) (60°C). One hundred μl of ethanol was added and evaporated. The residue was redissolved with a phenylboronic acid solution in methanol (1 mg ml^{-1}) and this was injected into the LC–MS system. A model P4000 pump (TSP, San Jose, CA, USA) coupled to an LCQ Ion Trap Mass Analyser (Finnigan-MAT, San Jose, CA, USA) with an atmospheric pressure chemical ionisation (APCI) interface was used. The instrument was operated in the positive ion mode. Chromatographic separation was achieved using an Alltima C_{18} 100A column (5 μm, 250×3.2 mm, Waters) coupled with a pre-column Alltima C_{18} (5 μm, 7.5×3.2 mm, Waters). The mobile phase consisting of methanol was maintained at a flow-rate of 0.75 ml min^{-1} for 3.8 min.

3. Results and discussion

3.1. Clean-up

For the solid-phase extraction (SPE) clean-up of urine, different columns were tested: a C_{18} column, a Clean Screen Dau column, a combination of both and a modified diatomaceous earth column (Chem-Elut). Loading hydrolysed urine onto a Chem-Elut column and an extraction with an organic solvent followed by an extraction in acid conditions gives the best results. Purification on a C_{18} column by loading in alkaline conditions (pH 9), and elution in acidic conditions, rendered very dirty extracts. Purification on Clean Screen Dau columns, by application in acidic conditions (pH 6) and elution in alkaline conditions also resulted in clear extracts, comparable with these obtained on Chem-Elut. As the signal-to-noise ratio for Clean Screen Dau was lower than that with the Chem-Elut columns, the last was preferred. For faeces, the approach was very similar: Extraction with an organic solvent followed by an acidic extraction.

3.2. Detection without derivatization

First of all the detection method was optimised

Table 1
Experiment method for MS–MS detection

Analyte	Retention time (min)	Parent ion	Fragment ions	Collision energy (%)[a]
16β-Hydroxystanozolol	10.6±0.7	345	327, 309, range[b]	40
Stanozolol	19±0.6	329	311, range[b]	40
Stanozolol-d_3	19±0.6	332	314, range[b]	40

[a] The collision energy is presented by the software in a relative way (percentage).
[b] The range of specific ions is illustrated in Fig. 3.

using standard working solutions. Electrospray ionisation and APCI were compared. Since electrospray gave a more intense total ion current in MS full scan, this ionisation technique was used for further development. Using flow injections three different electrolytes added to the mobile phase were tested. For these flow injections working solutions of standards were prepared at a concentration of 1 ng μl^{-1}. Ten microliters were injected into a 20 μl loop. A 1% acetic acid and 0.02 *M* formic acid at pH 2.7 were compared with a neutral pH (pH 6.8) obtained with 0.5% ammonium acetate. The response using ammonium acetate was negligible to the response when acetic acid or formic acid was used. As illustrated in Fig. 2 the flow injections using formic acid were twice as good as when acetic acid was used. Also the best response was obtained for working solutions in mobile phase.

For the extraction procedure we started initially with 10 ml of urine but since the detection method could detect low enough concentrations, the procedure was downscaled to 5 ml. The extract after elution with organic solvent was evaporated to dryness. The residue was reconstituted in 66 μl of methanol and 54 μl of 0.02 *M* formic acid and 60 μl was injected on column. Although the extract was very dirty, we could still detect 16-OHStan up to 1 $\mu g\ l^{-1}$ in urine.

The only problem was that this way of working could not be used as a routine application. The dirty matrix was responsible for early deterioration of the chromatographic column and did block the heated capillary (entrance to the intermediate vacuum region of the mass spectrometer) after 10 or 15 injections. The extra step, the acidic back extraction, produced a very clear extract. The matrix interferences were largely eliminated, allowing a much lower limit of detection. Of the droplet, 60 μl was injected directly on column. Using the principle of on-column concentration we were able to inject a large volume. If the extraction procedure was used without the final acidic step diethyl ether could be used instead of toluene–dichloromethane. With the extra step the analytes did prefer to stay in the diethyl ether phase.

Working in MS–MS without derivatization a large number of diagnostic ions was obtained. The three

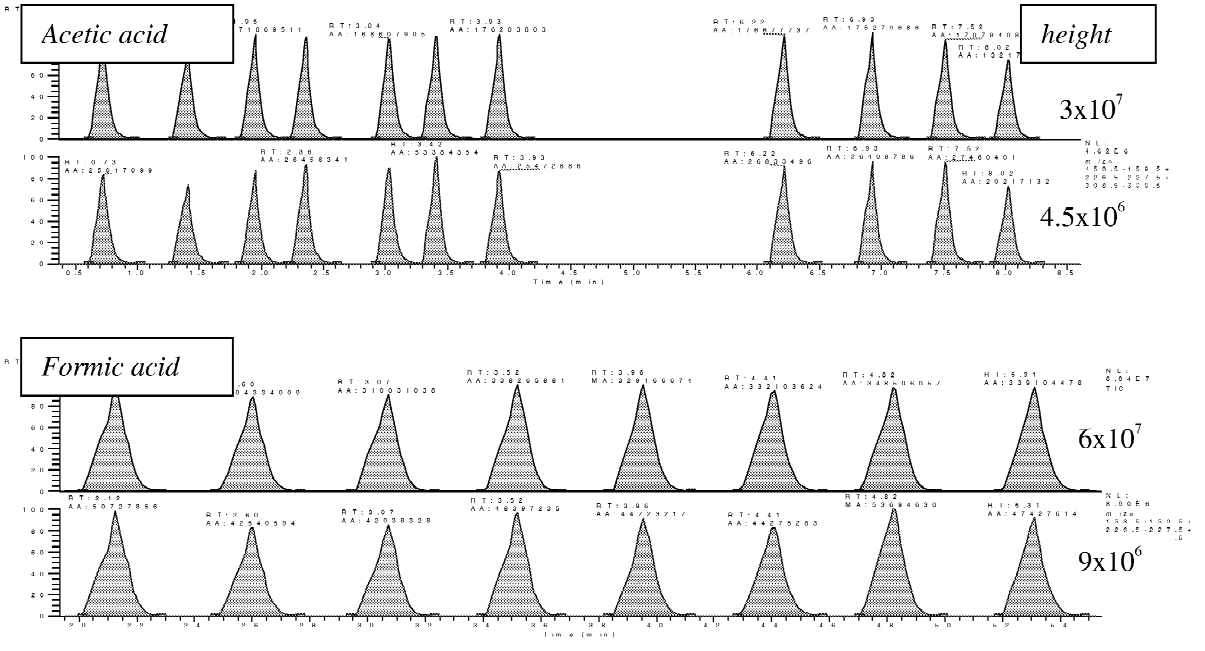

Fig. 2. Comparison of the height of the flow injections for formic acid and acetic acid (the first mass trace is the total ion current, the second mass trace is the sum of the diagnostic ions).

analytes showed a similar fragmentation pattern. For standard injections the diagnostic ions 327 and 309 of 16-OHStan, and 311 for Stan were most abundant but when working with matrices the signal-to-noise ratio of the ions was lower than 3 at concentrations below 1 μg kg^{-1} due to matrix interferences. Therefore the range of clustered ions was used for the integration and for qualitative interpretation. An LC–MS chromatogram and spectrum is presented in Fig. 3.

3.3. Detection with derivatization

For the clean-up of 16-OHStan a phenylboronic acid column, specific for binding diol containing compounds, was tested. The use of immobilized

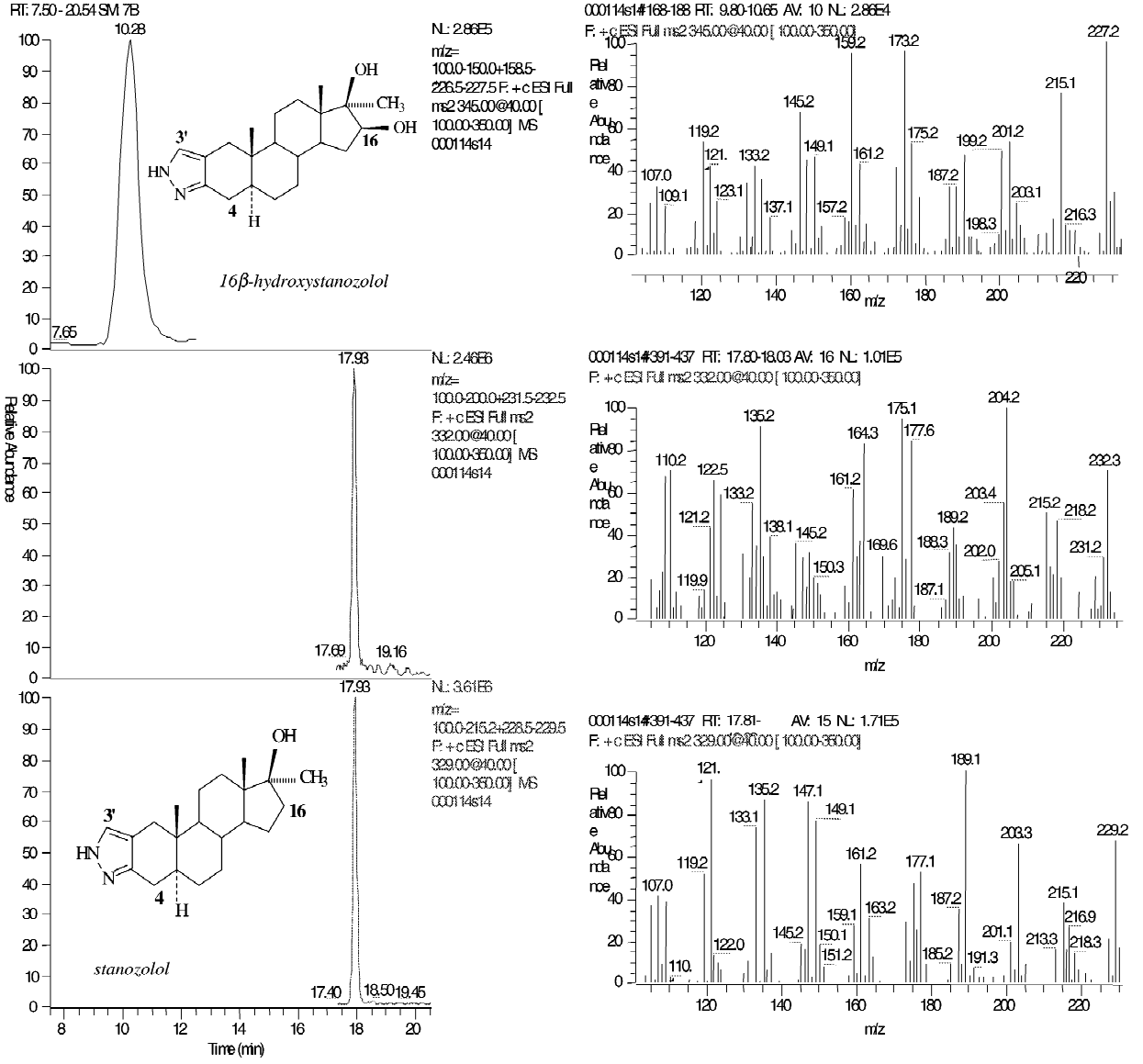

Fig. 3. LC–MS chromatogram and spectrum of 16-OHStan, Stanozolol-d_3, Stan.

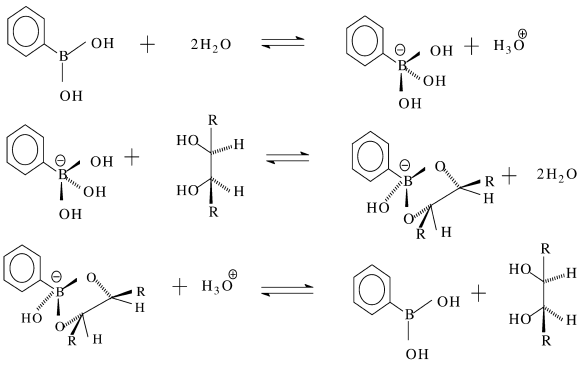

Fig. 4. The three step process of boronate binding including equilibration, retention and elution (Stolowitz).

phenylboronic acid has attracted yet considerable interest [10–14]. The mechanism of boronate binding is illustrated in Fig. 4. The immobilised phenylboronic acid is first equilibrated with an alkaline solution to obtain the reactive boronate form $RB(OH)_3^-$. The diol containing compound is next applied and is covalently bound with the concomitant release of water. Once the compound is retained, contaminants can be washed of from the bonded phase. Finally the compound of interest is eluted by acidification of the boronate complex which releases the diol containing compound and renders the immobilised phenylboronic acid neutral $[RB(OH)_2]$ [15]. The interaction between the metabolite and phenylboronic acid however was so strong that no solvent could elute the compound. All elution mixtures, as described in [13,14], and trials to elute the compound with EtOH–0.5 *M* HCl (90:10 v/v); 0.3% trifluoroacetic acid solution solved in ethanol; all failed. A very stable binding must have been formed. Clean-up by this way seemed impossible. We tested the reaction in solution in order to gain more specificity for the target metabolite and to evaluate the fragmentation of the reaction product. The approach was successful. Redissolving the residue with a solution of phenylboronic acid in MeOH resulted in a compound, which produced in APCI mode one very intense ion 431 (the molecular ion). A MS–MS scan of 16-OHStan shows 6 diagnostic ions: 327 and 309, the most abundant and 271, 255, 227, 201 less abundant (Fig. 5). Derivatization of 16-OHStan is favourable because the number of diagnostic ions is limited and the quality criteria according to EC 93/256 can be fulfilled much more easily as ion ratios are more stable.

The very specific extraction conditions combined with the specific reaction between 16-OHStan and

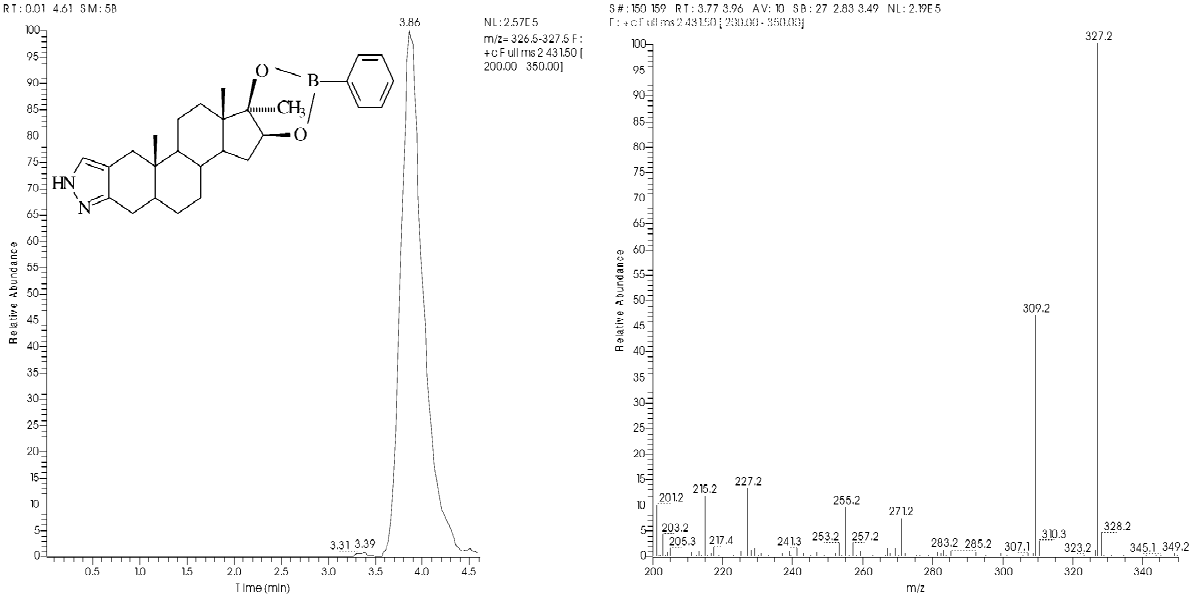

Fig. 5. MS–MS scan of the phenylboronic acid derivative of 16β-hydroystanozolol — parent ion 431.

phenylboronic acid, renders chromatograms with no interference of the matrix. The limit of detection, calculated with a S/N ratio = 3, is a 0.03 μg l^{-1} in urine and 0.07 μg kg^{-1} in faeces.

4. Conclusion

The two approaches discussed allow the detection of 16-OHStan at the ppt level. The advantage of the method without derivatisation is that no extra step is needed and that stanozolol can also be determined at a level of 0.3 μg l^{-1} in urine and 0.1 μg kg^{-1} in faeces. The advantage of the derivatisation reaction is that it produces a spectrum with more stable ion ratios. A combination of both approaches will give an unequivocal result of a suspect sample.

Acknowledgements

This work was financially supported by a grant from the Belgian Federal Ministery of Agriculture and the Institute of Veterinary Inspection (S5860-section 2).

References

[1] R.O. Clinton, A.J. Manson, F.W. Stanner, A.L. Beyler, G.O. Potts, A. Arnold, J. Am. Chem. Soc. 81 (1959) 1513.
[2] R.O. Clinton, A.J. Manson, F.W. Stanner, H.C. Newmann, R.G. Christansen, R.L. Clarke, J.H. Akermann, D.F. Page, J.W. Dean, W.B. Dickinson, C. Carabateas, J. Am. Chem. Soc. 83 (1961) 1478.
[3] C.H. Chesnut, J.L. Ivery, H.E. Guber, M. Mattews, W.B. Nelp, Metabolism 32 (1983) 571.
[4] V. Ferchaud, B. Le Bizec, M.-P. Montrade, D. Maume, F. Monteau, F. André, J. Chromatogr. B 695 (1997) 269.
[5] R. Massé, C. Ayotte, H. Bi, R. Dugal, J. Chromatogr. 497 (1989) 17.
[6] W. Schänzer, P. Delahaut, H. Geyer, M. Machnik, S. Horning, J. Chromatogr. B 687 (1996) 93.
[7] H.-Y.P. Choo, O.-S. Kwon, J. Park, J. Anal. Toxicol. 14 (1990) 109.
[8] H.F. De Brabander, K. De Wasch, L.A. Van Ginkel, S.S. Sterk, M.H. Blokland, Ph. Delahaut, X. Tailleu, M. Dubois, C.J.M. Arts, M.J. van Baak, L.G. Gramberg, R. Schilt, E.O. van Bennekom, D. Courtheyn, J. Vercammen, R.F. Witkamp, Analyst 123 (1998) 2599.
[9] Ph. Delahaut, X. Tailleu, M. Dubois, K. De Wasch, H.F. De Brabander, D. Courtheyn, Arch. Lebensmittelhyg. 49 (1998) 1.
[10] Application Note M2339, Determination of Catecholamines from Urine, Varian Inc., 1–6.
[11] C.R. Benedict, J. Chromatogr. 385 (1987) 369.
[12] T. Huang, J. Wall, P. Kabra, J. Chromatogr. 452 (1988) 409.
[13] M.S. Stoll, E.F. Hounsell, Biomed. Chromatogr. 2 (1988) 249.
[14] H. Echizen, R. Itoh, T. Ishizaki, Clin. Chem. 35 (1989) 64.
[15] M.L. Stolowitz, Application Note M1843, Varian Inc., 41–43.