ELSEVIER

Journal of Chromatography A, 926 (2001) 69–77

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Confirmatory analysis of residues of stanozolol and its major metabolite in bovine urine by liquid chromatography–tandem mass spectrometry

Rosa Draisci*, Luca Palleschi, Camilla Marchiafava, Emanuele Ferretti, Fernanda Delli Quadri

Veterinary Medicine Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

Abstract

A reliable method for the confirmation of the synthetic hormone stanozolol and its major metabolite, 16β-hydroxystanozolol, in bovine urine by liquid chromatography coupled with tandem mass spectrometry has been developed. [$^2H_3$]Stanozolol was used as internal standard. Sample preparation involved enzymatic hydrolysis, liquid–liquid extraction and purification on an amino solid-phase extraction column. The analytes were ionized using atmospheric pressure chemical ionization with a heated nebulizer interface operating in the positive ion mode, where only the protonated molecules, $[M+H]^+$, at m/z 329 and m/z 345, for stanozolol and 16β-hydroxystanozolol, respectively, were generated. These served as precursor ions for collision-induced dissociation and three diagnostic product ions for each analyte were identified for the unambiguous hormone confirmation by selected reaction monitoring liquid chromatography–tandem mass spectrometry. The accuracy ranged from 19.7 to 14.9% and from 18.9 to 13.2% for stanozolol and 16β-hydroxystanozolol, respectively. The precision ranged from 12.4 to 2.4% and from 13.1 to 1.8% for stanozolol and 16β-hydroxystanozolol, respectively. The limit of quantification of the method was 1 ng/ml in the bovine urine for both stanozolol and 16β-hydroxystanozolol. The developed method fulfils the European Union requirements for confirmatory methods.

Keywords: Hormones; Stanozolol

1. Introduction

Stanozolol (17-methyl-2′H-androst-2-eno[3,2-*c*]-pyrazol-17-ol; Stan) and its metabolites are synthetic anabolic steroids with a growth stimulating effect on humans and animals.

Although the use of anabolic steroids for cattle fattening is prohibited within the European Union (EU) [1] Stan was often found in injection sites taken at the slaughter house [2] and in cocktails for injection, whereas it has never been detected in tissues (kidney, fat, meat) or excreta (urine, feces) taken during regulatory inspections [3]. This could be due to the difference between the structure of Stan and the other anabolic steroids; a pyrazole ring fused to the androstane ring system, hampers the detection of Stan by the test methods used for multiresidues analyses of anabolic steroids [3]. Moreover, Stan is rapidly metabolised after administration, 16β-hydroxystanozolol (16β-OH-Stan) being the major metabolite in humans [4–7], horses [8,9] and cattle [3,10,11]. Particularly, 16β-OH-Stan was detected in

*Corresponding author. Tel.: +39-6-4990-2327; fax: +39-6-4990-2327 or +39-6-4938-7077.

E-mail address: draisci@iss.it (R. Draisci).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00837-8

veal calf urine until at least 14 days after injection of 200 mg Stan [3]. In addition to 16β-OH-Stan, the presence of other metabolites, such as 3-hydroxystanozolol [3,10,11], 4,16-dihydroxystanozolol [11] and 4β-hydroxystanozolol [3,12] has also been investigated in bovine urine.

The development of sensitive and specific methods able to detect its metabolites is therefore required for a successful control of the illegal use of Stan in meat production. An enzyme-linked immunosorbent assay (ELISA) test for the screening of Stan and 16β-OH-Stan in urine samples from horses pretreated with Stan has been developed by Douglas et al. [13]. High-performance thin-layer chromatography (HPTLC) has also been proposed, although it showed poor sensitivity and low specificity for Stan in comparison with other anabolic hormones [14]. For confirmatory purposes, the combination of gas chromatography with mass spectrometry (GC–MS) has been used, since the information on the molecular structure of the analyte, obtained by electron impact (EI) ionisation [11,15] or negative chemical ionisation (NCI) [3], results in higher specificity. On the other hand, GC–MS methods show several disadvantages for the detection of Stan and its metabolites at low concentration levels (ng/ml or ng/g), due to adsorption at the injector and bad derivatisation of the analytes. Liquid chromatography coupled to mass spectrometry (LC–MS) [12] or tandem mass spectrometry (LC–MS–MS) with an electrospray (ESI) [3,16] or atmospheric pressure chemical ionisation (APCI) [3,16] source could be a more reliable approach for the analysis at the lower concentration levels (ng/ml or ng/g), although a cumbersome sample preparation [3] and derivatisation steps [16] have been used for LC–MS–MS so far.

In this paper a new sensitive, specific and reliable LC–MS–MS method with an APCI source and a heated nebulizer (HN) interface for the unambiguous confirmation of Stan and 16β-OH-Stan in bovine urine, using a simplified sample preparation, using [$^2H_3$]stanozolol (Stan-d_3) as internal standard (I.S.), is reported. The method was successfully applied to determine Stan and 16β-OH-Stan in incurred bovine urine samples obtained from a proficiency study organised within the EU.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, butanol, ethanol, hexane, methanol, acetic acid, ammonium acetate, sodium acetate and sodium carbonate were LC or analytical grade and purchased from Carlo Erba (Milan, Italy). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

β-Glucuronidase/arylsulfatase (*Helix pomatia*) from Sigma (St. Louis, MO, USA) were used as supplied. A standard of Stan was provided by Sigma; Stan-d_3 and 16β-OH-Stan were obtained from Radian International (Austin, TX, USA). Individual hormone standard stock solutions of 0.1 mg/ml were prepared in ethanol. Individual and composite working standard solutions were prepared daily by appropriate dilution of the standard stock solutions with ethanol. All solutions were stored in the dark at −20°C and were stable for at least 1 month.

2.2. Samples

A series of lyophilised samples of blank (5 ml) and incurred bovine urine (5 ml) containing residues of Stan and 16β-OH-Stan were obtained during a proficiency study organised by the European Union Community Reference Laboratory RIVM/ARO within the European Union for the analysis of 16β-OH-Stan in bovine urine. These samples were assayed by selected reaction monitoring (SRM) LC–MS–MS and the absence of the analytes under investigation was verified. All samples were stored at −20°C until assayed. All lyophilised samples were reconstituted with 5 ml of distilled water and then stored at 4°C.

2.3. Sample preparation procedure

Calibration, validation and incurred samples were prepared in the same way.

An aliquot (2.5 ml) of urine was fortified with 25 ng of I.S., 1 ml of 2 *M* acetate buffer solution, pH 5.2, and with 200 μl of sulfatase enzyme solution (*Helix pomatia*) were added. The mixture was shaken on a vortex mixer and incubated for 12 h at 37°C. The pH was brought to 9 by adding 700 μl of 1 *M*

sodium carbonate solution. The solution was extracted with 10 ml of hexane–butanol (80:20, v/v) extraction solvent and centrifuged for 2 min at 3500 rpm. The organic phase was transferred to a 10-ml tube and the aqueous layer was re-extracted with fresh extraction solvent. The extracts were combined and the organic solvents were evaporated at 55°C under a soft nitrogen stream. The sample was reconstituted with 5 ml methanol–water (80:20, v/v).

The sample was purified by solid-phase extraction (SPE) using an NH_2 cartridge (Varian, NH_2, 500 mg, 3 ml cartridges) which had previously been conditioned with 5 ml of methanol–water (80:20, v/v). The methanolic extract was passed through the amino column whereby the eluate was collected in a 4-ml glass vial. The extract was evaporated to dryness under nitrogen, using an evaporation block at 55°C, and the sample was re-suspended in 250 μl methanol. A 5-μl aliquot of the solution was injected into the LC–MS–MS system.

2.4. LC–APCI-MS–MS equipment and conditions

Analyses were carried out with a Varian 9010 LC pump (Varian, Palo Alto, CA, USA) liquid chromatograph. A Rheodyne (USA) injection valve equipped with a 5-μl loop was used for injection by flow injection analysis (FIA)–MS, FIA–MS–MS and LC–MS–MS. Chromatographic separations were obtained under isocratic conditions using a reversed-phase Kingsorb C_{18} column (Phenomenex, Torrance, CA, USA) (250×2 mm I.D., 5 μm) at room temperature, with a mobile phase of acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate, and at a flow-rate of 100 μl/min.

Mass spectral analyses were performed on a PE-Sciex API III plus triple-quadrupole (PE-Sciex, Thornill, Canada) equipped with an APCI source and a HN operating in the positive ion mode. Ultra-high-purity nitrogen was used as curtain gas (0.6 l/min), while air was used as nebulizer gas (400 kPa) and auxiliary gas (1.5 l/min). The HN temperature was set at 350°C and the discharge current at 4 μA. The orifice potential voltage (OR) was set at 80 V for both Stan and 16β-OH-Stan and at 70 V for Stan-d_3. Full-scan mass spectra were acquired in the positive ion mode from m/z 200–400. In the MS–MS experiments, product ion mass spectra were acquired in positive ion mode by colliding quadrupole 1 (Q1) selected precursor-ion, with argon (gas thickness $300 \cdot 10^{13}$ molecules cm^{-2}) in quadrupole 2 (Q2) operated in radio frequency (RF)-only mode, and scanning the third quadrupole mass spectrometer, Q3, from m/z 50–400. The MS and MS–MS experiments were run with a resolution of 0.8 a.m.u. measured at half peak height for both the mass-resolving quadrupoles. A collision energy of 40 eV was chosen for the collision-induced dissociation (CID) experiments. The protonated molecule, $[M+H]^+$, at m/z 329 for Stan, at m/z 345 for 16β-OH-Stan and m/z 332 for Stan-d_3, was the precursor ion for CID and three product ions for both Stan and 16β-OH-Stan and two product ions for Stan-d_3 were identified to carry out SRM LC–MS–MS analyses. The precursor–product ion combinations of m/z 329/81, m/z 329/95 and m/z 329/107 were used for Stan, m/z 345/81, m/z 345/95 and m/z 345/107 for 16β-OH-Stan and m/z 332/81 and m/z 332/95 for Stan-d_3. The dwell time for each monitored transition was 150 ms. Peak-area ratios of the analyte to internal standard were computed using MacQuan version 1.3 software from PE-Sciex.

2.5. Calibration and quantification

In order to evaluate the extraction efficiency of the analytes, standard curves were prepared daily in the range 20–400 ng/ml by plotting peak area ratios of the analyte to I.S. (100 ng/ml) versus analyte concentrations using a least-squares linear regression model.

Calibration curves were prepared daily by spiking blank control samples with 25 ng of I.S. followed by mixtures of the hormones to obtain concentrations in the range 1–20 ng/ml. Calibration curves were constructed in the same way as standard curves and were used to interpolate concentrations of the analyte in the validation and incurred samples.

The validation samples, used to evaluate the precision and the accuracy of the method, were prepared independently in the same fashion as the sample for calibration. During the validation, three replicates of the validation samples were prepared

and analysed on each of 3 days for each concentration (i.e., 1.0, 5.0, 10.0 and 20.0 ng/ml).

3. Results and discussion

Positive-ion FIA–MS was initially performed by adopting a mixture of acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate at a flow-rate of 100 μl/min. Representative HN full-scan (mass range *m/z* 200–400) mass spectra as obtained by FIA in the MS positive ion mode for the analytes under investigation are shown in Fig. 1. The formation of the protonated molecule, $[M+H]^+$, of Stan (*m/z* 329), 16β-OH-Stan (*m/z* 345) and Stan-d_3 (*m/z* 332), was obtained under the above conditions.

Experiments were performed to obtain spectra with maximum intensities of the protonated molecular ion of each analyte by selected ion monitoring (SIM) FIA–MS analyses at *m/z* 329, 332, and 345. The effect of varying orifice potential voltage was investigated between the range 60–100 V. Finally, an OR of 70 V for Stan-d_3 and 80 V for Stan and 16β-OH-Stan was adopted as the best compromise in terms of the signal-to-noise ratio. Although the simplicity of the FIA–MS spectra are useful for the identification of the analytes based on their molecular ions, they do not provide further structural information. Tandem mass spectrometry was therefore used in order to obtain additional structural information by detecting diagnostic product ions obtained by CID of the precursor ion.

The protonated molecule, $[M+H]^+$, served as the precursor ion for CID in the MS–MS experiments, carried out by FIA–MS–MS on the individual hormone standard solutions. Fig. 2 shows the positive-product ion mass spectra (mass range *m/z* 50–400) of the protonated molecules, $[M+H]^+$, of Stan, 16β-OH-Stan and Stan-d_3. Comparison of the spectra reveals the production of the most abundant product ions at *m/z* 81, 95 and 107 for both Stan and 16β-OH-Stan, at *m/z* 81 and 95 for Stan-d_3. Transitions of the respective protonated molecules to these product ions were therefore selected according to the SRM technique.

In order to achieve targeted analyses and maximum sensitivity as well as for quantitative purposes SRM LC–MS–MS analyses were finally performed using a reversed-phase Kingsorb C_{18} column at room temperature, with a mobile phase of acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate, at a flow-rate of 100 μl/min.

Under the adopted conditions, Stan eluted at t_R = 17.8 min, Stan-d_3 at t_R = 17.7 min and 16β-OH-Stan at t_R = 10.4 min. Specificity of the SRM LC–MS–MS method was proved by processing and analysing blank control samples (Fig. 3A). No interference was noticed around the retention times of Stan and its metabolite extracted from urine samples.

Calibration graphs were constructed by plotting peak-area ratios versus hormone concentrations using a least-squares linear regression model; concentrations of the analyte in validation and incurred samples were subsequently interpolated from these curves. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) greater than 0.993 for all curves.

Validation samples at four different concentrations (i.e., 1.0, 5.0, 10.0 and 20.0 ng/ml) were prepared and analysed to determine the extraction efficiency of the analytes under investigation and to evaluate the accuracy and the repeatability of the analytical method in the bovine urine samples. Representative chromatograms of urine control samples fortified at 10.0 ng/ml of Stan, 16β-OH-Stan and Stan-d_3 (I.S.) are reported in Fig. 3B. The relative retention time, for each analyte, corresponded to that of the calibration standard within a tolerance of 0.5%.

The extraction efficiency of the analytes was determined by comparing the peak areas of the fortified samples with those of the standard and I.S., respectively. Three replicates at each of the four concentrations were analysed and the following mean percentages of extraction efficiency were estimated: 74.3±13.3% for Stan, 76.1±14.2% for 16β-OH-Stan and 74.7±13.1% for Stan-d_3. The data for accuracy and precision (repeatability) of the method were obtained by analysing three replicates of each fortification level (i.e., 1.0, 5.0, 10.0 and 20.0 ng/ml) on each of 3 days for bovine urine. The accuracy (relative error, RE, %) was determined by assessing the agreement between the measured and nominal concentrations of validation samples, while the precision was determined by calculating the relative standard deviation (RSD) for the repeated measure-

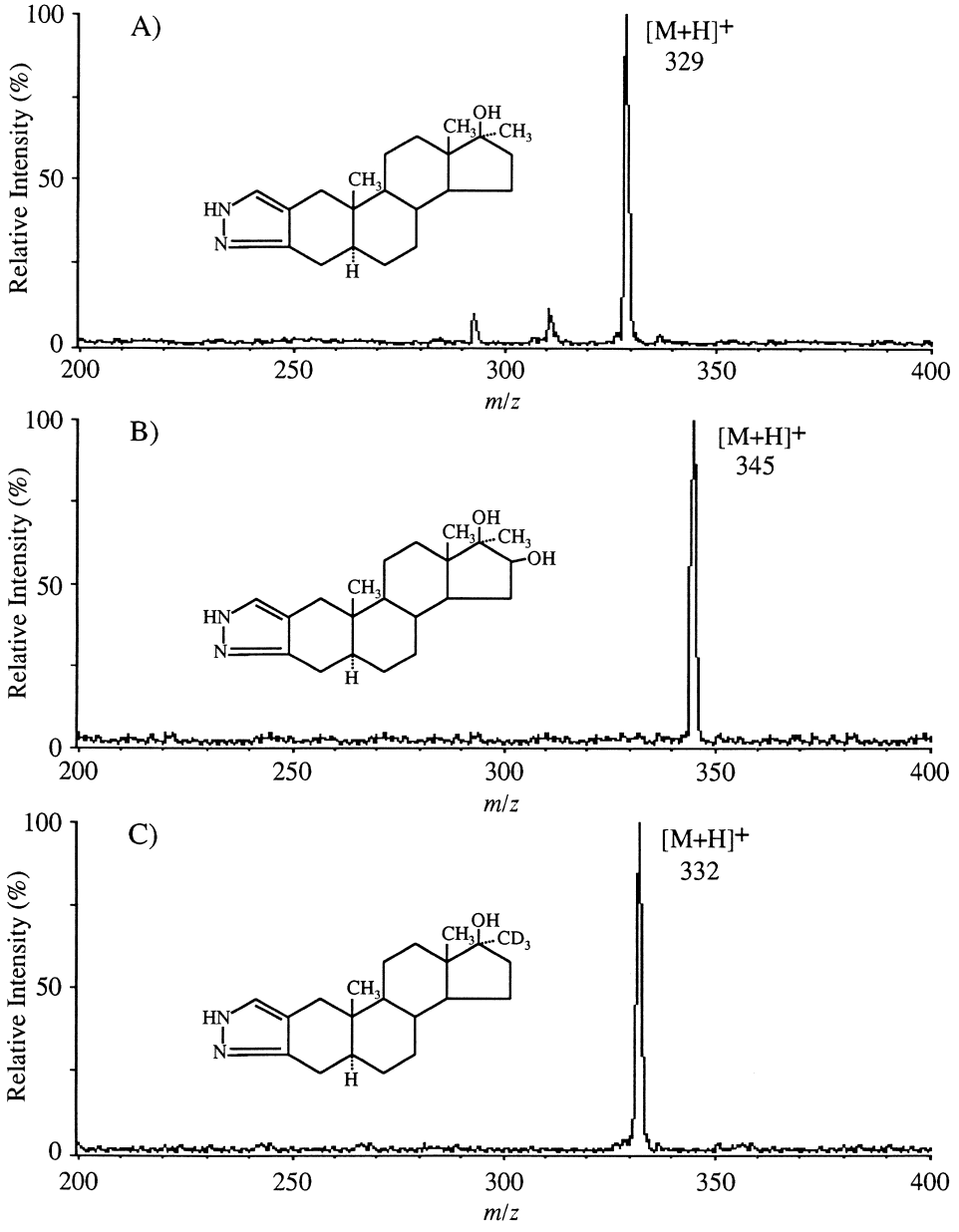

Fig. 1. Positive ion mass spectra of stanozolol (A), 16β-hydroxystanozolol (B) and stanozolol-d_3 (C). Conditions: FIA; mobile phase: acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate; flow-rate 100 μl/min; OR was set at 80 V for stanozolol and 16β-hydroxystanozolol and at 70 V for stanozolol-d_3.

ments (Table 1). The accuracy values ranged from 19.7 to 14.9% and from 18.9 to 13.2% for Stan and 16β-OH-Stan, respectively, while the precision ranged from 12.4 to 2.4% and from 13.1 to 1.8% for Stan and 16β-OH-Stan, respectively, over the four concentrations evaluated. These values were considered satisfactory, on account of the complexity of the biological matrices.

The limit of quantification (LOQ), defined as the lowest concentration of the identified analyte in the

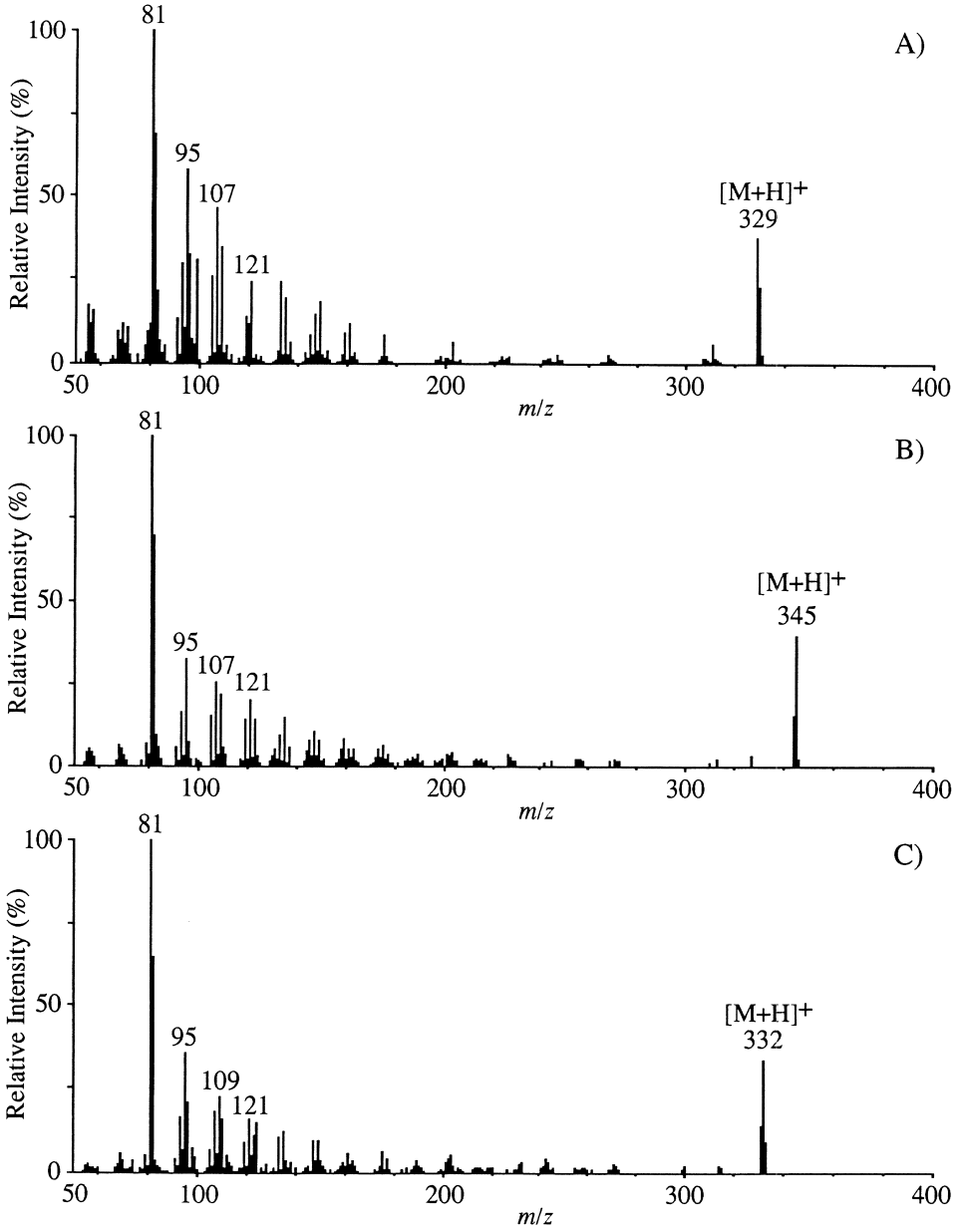

Fig. 2. Positive product ion spectra of stanozolol (A), 16β-hydroxystanozolol (B) and stanozolol-d_3 (C), with the $[M+H]^+$ ion as precursor, at m/z 329 for stanozolol, at m/z 345 for 16β-hydroxystanozolol and at m/z 332 for stanozolol-d_3. Conditions: FIA; mobile phase: acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate; flow-rate 100 μl/min; OR was set at 80 V for stanozolol and 16β-hydroxystanozolol and at 70 V for stanozolol-d_3. Argon was used as the collision gas. CID was carried out with a collision energy of 40 eV.

sample at which an acceptable accuracy and precision are obtained, was 1.0 ng/ml for both Stan and 16β-OH-Stan in bovine urine, which makes the procedure described suitable for control purposes.

The SRM LC–MS–MS method was then used to analyse incurred samples obtained as part of a proficiency study for 16β-OH-Stan in bovine urine. Representative SRM LC–MS–MS chromatographic

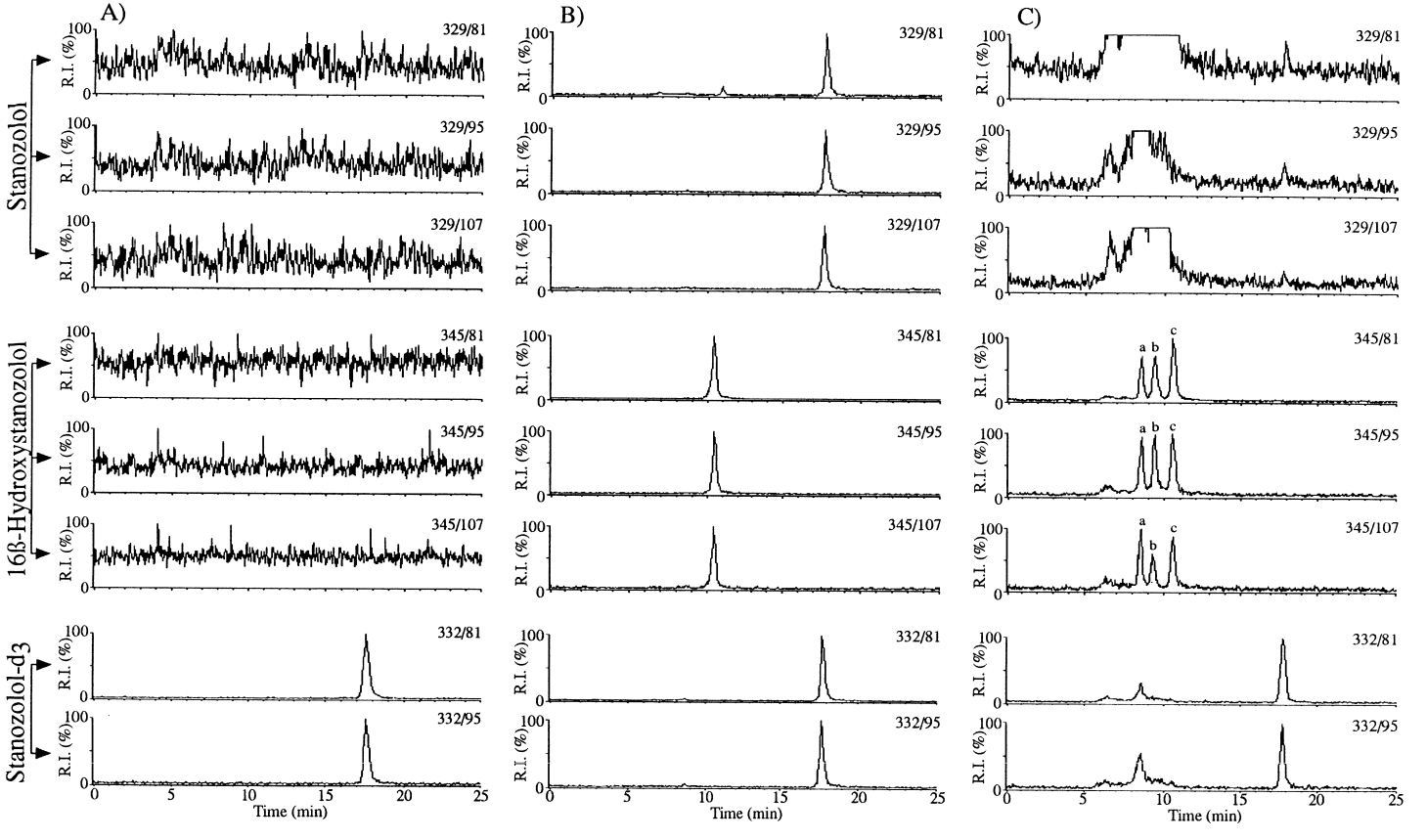

Fig. 3. SRM LC–MS–MS chromatograms of: (A) extract of blank control bovine urine spiked with 5 ng/ml of Stan-d_3 as I.S.; (B) extract of blank control bovine urine spiked with 10 ng/ml of Stan, 16β-OH-Stan and 5 ng/ml Stan-d_3; (C) extract of an incurred bovine urine sample, containing 16β-OH-Stan (8.2 ng/ml) and Stan (<LOQ). a, b: Metabolites of Stan; c: 16β-OH-Stan. R.I.: Relative intensity. Precursor–product ion combinations used in SRM detection are shown. Conditions: isocratic HPLC analysis; column reversed-phase Kingsorb C_{18} (250×2 mm, 5 μm); mobile phase: acetonitrile–water (60:40, v/v) containing 40 m*M* ammonium acetate; flow-rate 100 μl/min; OR was set at 80 V for Stan and 16β-OH-Stan and at 70 V for Stan-d_3. Argon was used as the collision gas. CID was carried out with a collision energy of 40 eV.

Table 1
Precision (RSD, %) and accuracy (RE, %) for Stan, 16β-OH-Stan and Stan-d_3 in bovine urine samples

Analyte	Spike level (ng/ml)	Measured concentration (mean±SD)* (ng/ml)	Accuracy (RE, %)*	Precision (RSD, %)*
Stanozolol	1.00	0.80±0.10	19.7	12.4
	5.00	4.11±0.43	17.8	10.5
	10.00	8.37±0.46	16.3	5.5
	20.00	17.02±0.41	14.9	2.4
16β-Hydroxystanozolol	1.00	0.81±0.11	18.9	13.1
	5.00	4.21±0.35	15.7	8.3
	10.00	8.46±0.40	15.4	4.7
	20.00	17.35±0.31	13.2	1.8
Stanozolol-d_3	1.00	0.81±0.10	19.1	13.0
	10.00	8.32±0.49	16.8	5.9

*Each analyte is the mean of nine samples (three/day for 3 days).

profiles of an incurred bovine urine sample are reported in Fig. 3C. The presence of Stan (<LOQ) and 16β-OH-Stan (8.2 ng/ml) was shown in this incurred sample through the detection of the peak at the same retention time (t_R=17.8 min and t_R=10.4 min, respectively) as for the fortified sample (Fig. 3B) in the SRM LC–MS–MS ion trace of the analyte. Interestingly, two chromatographic peaks (a, b) eluting at a retention time close to that of 16β-OH-Stan (c), having the same precursor–product ion pairs as 16β-OH-Stan, differing mainly in the ion relative intensities, were observed in the SRM LC–MS–MS ion traces of the incurred samples and were tentatively assigned to the metabolites of Stan, such as 4β-hydroxystanozolol and 3-hydroxystanozolol.

The suitability of SRM LC–MS–MS to unambiguously identify violative samples of Stan and 16β-OH-Stan in bovine urine was therefore shown.

4. Conclusion

The aim of this research was to develop a specific, sensitive and reliable LC–APCI-MS–MS method using a HN interface for the measurement of Stan and 16β-OH-Stan residues in bovine urine.

The unambiguous confirmation of the presence of the analytes in bovine urine in the proposed method results from the retention time information, the presence of the protonated molecule of the analyte, and the employment of a triple precursor–product ion reaction.

The developed method complies with the criteria proposed by the recent revision of the Commission Decision 93/256/EEC [17] for confirmatory methods of substances listed in Group A of Annex I of Council Directive 96/23/EC [18], such as anabolic steroids, requiring the identification of the analyte based on the presence of at least 1 precursor and two transition product ions for LC–MS–MS technique.

The ability to perform analyses involving simple extraction procedures and highly selective and sensitive determination by SRM LC–MS–MS, along with the wide-spread presence of benchtop LC–multiple MS apparatus in laboratories, makes this analytical method particularly valuable for routine control of the illegal use of Stan and its major metabolite in livestock production.

Research activities by our group are ongoing in order to obtain more detailed profiles of the Stan metabolites in the bovine urine, which would allow further development in the control strategies of illegal treatments.

References

[1] Commission of the European Communities, Council Directive 96/22/EC, Off. J. Eur. Communities: Legis. L125 (1996) 3.

[2] K. Van Oosthuyze, E. Daeseleire, A. Van Overbeke, C. Van Peteghem, A. Ermens, Analyst 119 (1994) 2655.
[3] H.F. De Brabander, K. De Wasch, L.A. van Ginkel, S.S. Sterk, M.H. Blokland, P. Delahaut, X. Taillieu, M. Dubois, C.J. 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.
[4] R. Massè, C. Ayotte, H.G. Bi, R. Dugal, J. Chromatogr. B 497 (1989) 17.
[5] W. Schanzer, G. Opfermann, M. Donike, J. Steroid Biochem. 36 (1990) 153.
[6] W. Schanzer, M. Donike, Anal. Chim. Acta 275 (1994) 23.
[7] W. Schanzer, P. Delahaut, H. Geyer, M. Machnik, S. Horning, J. Chromatogr. B 687 (1996) 93.
[8] W.M. Much, J.D. Henion, Biomed. Environ. Mass Spectrom. 19 (1990) 37.
[9] P. Teale, I. Grainger, E. Houghton, presented at the 12th International Mass Spectrometry Conference, Amsterdam, 26–30 August 1991, poster TuM-CO2.
[10] P. Delahaut, X. Taillieu, M. Dubois, K. De Wasch, H.F. De Brabander, P. Batjoens, D. Courtheyn, Arch. Lebensmittel. 49 (1998) 3.
[11] V. Ferchaud, B. Le Bizec, M.P. Montrade, D. Maume, F. Monteau, F. Andre, J. Chromatogr. B 695 (1997) 269.
[12] M.H. Blokland, S.S. Sterk, K.D. Hartog, L.A. van Ginkel, R.W. Stephany, in: L.A. van Ginkel, A. Ruiter (Eds.), Proceedings of the EuroResidue IV Conference, Veldhoven, RIVM, Bilthoven, 8–10 May 2000, p. 232.
[13] J.C. Douglas, A. McCormick, R.I. McConnell, J.V. Lamont, S.P. Fitzgerald, in: L.A. van Ginkel, A. Ruiter (Eds.), Proceedings of the EuroResidue IV Conference, Veldhoven, RIVM, Bilthoven, 8–10 May 2000, p. 371.
[14] F. Smets, H.F. De Brabander, P.J. Bloom, G. Pottie, J. Plan. Chromatogr. 4 (1991) 207.
[15] P. Batjones, H.F. De Brabander, F. Smets, G. Pottie, Analyst 119 (1994) 2607.
[16] M. Van de Wiele, K. De Wasch, J. Vercammen, D. Courtheyn, H.F. De Brabander, S. Impens, in: L.A. van Ginkel, A. Ruiter (Eds.), Proceedings of the EuroResidue IV Conference, Veldhoven, RIVM, Bilthoven, 8–10 May 2000, p. 371.
[17] Revision of Commission Decision 93/256/EEC, European Commission, Directorate General For Agriculture VI B II 2, in preparation.
[18] Commission of the European Communities, Council Directive 96/23/EC, Off. J. Eur. Communities: Legis. L125 (1996) 3.