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Determination of trenbolone and its metabolite in bovine fluids by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method has been developed for the determination of trenbolone in bovine urine and serum. The aim was a control of the misuse of trenbolone in food-producing animals. The procedure involved, in both cases, a preliminary solid-phase clean-up followed by a liquid–liquid extraction for urine samples after a preliminary enzymatic hydrolysis. The extracts have been directly analysed by reversed-phase LC–MS–MS in selected reaction monitoring (SRM), acquiring two diagnostic product ions from the chosen precursor $[M+H]^+$. The procedures were validated across the concentration range of 1–1500 ng/ml. The linearity, the inter- and intra-day accuracy and precision have been determined. The procedure was specific and the accuracy values were better than 20% at the limit of quantitation of spiked samples. The limit of quantification (LOQ) and the limit of detection (LOD) were, respectively, 1 ng/ml and 350 pg/ml for urine and serum. According to the draft, SANCO/1805/2000, we determined the decision limit CCα and the detection capability CCβ. The recovery values for urine ranged from 87 to 128%, and for plasma the recovery was 70±4%. The procedure proved to be simple and suitable for routine and confirmatory purposes such as those developed for residue studies.

Keywords: Trenbolone; Steroids

1. Introduction

Synthetic anabolic steroids, such as trenbolone, speed muscle development in animals, but are known to be hazardous to man [1–3]. Administration of anabolic steroids for fattening livestock has been prohibited in the European Union since 1988 as they were identified as being possibly carcinogenic [4,5]. Reproducible, selective and sensitive analytical procedures are therefore required on account of the illegal use of these compounds. The identification of anabolic hormones is complicated by their low concentration levels. Illicit administration of trenbolone would probably be in doses that result in ppb levels or lower concentrations in animal tissues and fluids [6,7].

Such concentrations are difficult to detect, identify and quantify with adequate accuracy against the

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usual high background of co-extractive interferences. Analysys of steroids is frequently carried out in urine where hormone concentration is usually higher than in serum and remains high for several weeks if the sample is properly stored [8,9]. The anabolic androgenic steroids in urine are metabolised mostly as glucuronides and sulfates. Trenbolone is usually administered to cows as acetate, alone or in combination with other estrogenic compounds [10], and it is excreted mainly as 17α- and 17β-trenbolone in urine. The biotrasformations of TBA in cow and humans have been elucidated [11,12].

Several techniques have been developed, such as radioimmunoassay (RIA), enzyme immunoassay (EIA), high-performance liquid chromatography (HPLC), gas chromatography (GC), and GC–mass spectrometry (MS) to detect trenbolone in biological fluids. RIA and EIA are quite sensitive and useful for screening purposes; however, they can give false-positive results [8,13]. HPLC with different detectors has been used to detect trenbolone in tissues [8,14]. Trenbolone in spiked urine was analysed by normal-phase HPLC with on line UV detection at 350 nm [15], and by HPLC-fluorescence detection in bovine muscle and liver [16].

GC–MS is a sensitive and suitable technique for assay of hormones, but it is time-consuming because it requires derivatization due to their polarity and thermal instability. Due to its structure, trenbolone gives difficulties with most common derivatizing agents [17]. The instability of trenbolone in the derivatizing mixture causes the formation of other species over time [18,19]. The best alternative to GC–MS is HPLC–MS, and HPLC–MS–MS in SRM mode is the best choice to reach acceptable detection limits and to increase specificity. Liquid chromatography–thermospray mass spectrometry [20] has been used for detection of trenbolone in bovine bile and faeces up to a limit of detection of 0.5 ng/g.

Recently several steroids, found in the kidney, have been analysed by liquid chromatography–tandem mass spectrometry using atmospheric pressure chemical ionisation (APcI) in SRM mode [21]. For trenbolone a limit of detection of 1 ppb has been reached. In another work, trenbolone has been detected in bovine muscle and liver by LC-electrospray with a single quadrupole [22]. In this paper we report a study to confirm and quantify the presence of trenbolone in calf urine and serum by LC–MS–MS with atmospheric pressure ionisation (API) in positive ionisation in SRM mode.

The sample preparation for urine consisted of enzymatic hydrolysis of steroid glucuronides and sulfates followed by solid-phase clean-up and liquid–liquid extraction. For serum samples the procedure is simpler, the matrix being cleaner than urine.

The results obtained suggest that it is possible to detect illegal trenbolone treatment according to the limit decision of 2 ng/ml fixed by EEC [23].

2. Experimental

2.1. Chemicals and reagents

17β-Trenbolone and methyltestosterone (ISTD) standards were purchased from Sigma (Milan, Italy). 17α-Trenbolone was kindly supplied by FMSI Anti-doping Laboratory of Rome (Rome, Italy) in solution (concentration 10 ng/μl).

Methanol (HPLC grade), acetic acid and ammonium formate were obtained from Carlo Erba (Milan, Italy), and *tert*.-butylmethylether (TBME, HPLC grade) and formic acid were from Fluka (Sigma–Aldrich, Milan, Italy). All the other reagents were of analytical grade.

Ammonium formate 2.88 m*M* (pH 7.0) was prepared by dissolving 181.4 mg of ammonium formate in 1000 ml of water. Ultrapure water was produced with a Pure Lab system (USF Elga, Ransbach-Baumbach, Germany).

Acetate buffer (pH 5.5) was prepared by mixing 11 ml of 0.2 *M* acetic acid, and 89 ml of 0.2 *M* sodium acetate.

The enzyme β-glucuronidase aryl sulfatase (type H-2) was purchased from Boehringer (Mannheim, Germany).

Sep-Pack C_{18} (360 mg) and Oasis HLB (3 cc) cartridges were obtained from Waters (Milan, Italy). Stock standard solutions of 17β-trenbolone and methyltestosterone (1 mg/ml) in methanol were prepared monthly and stored at −20 °C in the dark.

Working solutions were daily prepared in mobile phase by appropriate dilution.

2.2. Instrumentation

HPLC analysis was carried out by a Shimadzu two-pump system LC-10 AD (Shimadzu, Kyoto, Japan) at a flow-rate of 150 μl/min under isocratic elution: mobile phase, methanol–ammonium formate (2.88 m*M*) 65:35. HPLC column (equipped with a guard column, Phenomenex C_{18} L=4 mm, 2 mm I.D.) 250×2.1 mm Hypersil C_{18}, 5 μm (Shandon, UK) was slurry packed in our lab. The injector was a Rheodyne 8125 with a sample loop of 5 μl.

MS and MS–MS analyses were performed on a PE-Sciex API 365 (Perkin-Elmer Sciex Instruments, Foster City, CA, USA) equipped with a turbo ion spray interface in positive mode.

The API source voltage was set at +4 kV. The orifice potential (OR) was set at 30 V and the ring potential (RNG) to 250 V. Nitrogen was used as nebulizing gas, as curtain gas and as collisional gas. The settings for the nebulizer, curtain and collision gas were 8, 8 and 3 (arbitrary units). The collisional energy was adjusted by the variation of the voltage difference between the high-pressure entrance quadrupole (Q0) and the collisional cell quadrupole RO_2 (−52 V) and it was found to give the highest sensitivity for the analyte at −42 eV. The vaporizer was set at 450 °C.

Acquisition parameters were optimised in ion spray mode by direct continuous pump infusion of standard working solution of the analyte (10 ng/μl in MeOH–ammonium formate 2.88 m*M*, 65:35) at a flow-rate of 10 μl/min in the mass spectrometer.

Data acquisition were performed preliminarily on the standard compounds of 17α- and 17β-trenbolone in full scan, in positive mode (mass range, 50–350 Da) using the first quadrupole to choose an abundant precursor (m/z 271 $[M+H]^+$). MS–MS product ion scans were then recorded from m/z 50–300 Da. Finally all the analyses, both on standard and on samples, were carried out by LC–MS–MS in SRM mode monitoring the product ions m/z 199.2, 227.2 and 253.3 from the precursor m/z 271.2 to obtain a high specificity and sensitivity. For the I.S. methyltestosterone the precursor ion m/z $[M+H]^+$ 303.1 and product ions m/z 109.1 and 97.0 were chosen for the SRM experiments.

2.3. Sample preparation

Five ml of urine (blank, fortified, incurred urine) fortified with 100 ng of ISTD were adjusted to pH 5.5 by adding acetate buffer and a few droplets of glacial acetic acid, and then hydrolysed with 50 μl of β-glucuronidase-aryl sulfatase from *Helix pomatia* (overnight, t=37 °C). The digested sample was cooled at room temperature and loaded on a Sep-Pack C_{18} SPE column (previously conditioned with 5 ml of methanol and washed with 10 ml of water). After a washing step with 5 ml of water, the sample was eluted with 3 ml of MeOH. The extract was then evaporated at 45 °C with a nitrogen stream to dryness. The residue, dissolved with 1.5 ml of acetate buffer at pH 5.5, was extracted with 3 ml of TBME twice. The organic layers were collected and evaporated to dryness under a nitrogen stream at 45 °C. The residue was dissolved in 100 μl of a MeOH–water (1:1) mixture, and aliquots of the solution (5 μl) analysed using the LC–API-MS–MS system.

Spiked serum samples (1 ml each) fortified with 100 ng of ISTD were loaded on a cartridge (Oasis HLB; previously conditioned with 3 ml of methanol and washed with 3 ml of water). After a washing step with 3 ml of water, the analyte was eluted with 2 ml of MeOH. The extract was then evaporated at 45 °C under a nitrogen stream to dryness. The residue was dissolved in 100 μl of a MeOH–water (1:1) mixture and analysed directly LC–MS–MS (5 μl).

All samples (urine and serum) were prepared in double aliquots.

3. Validation of the method

3.1. Recovery

The recovery of these methods was tested on a pool of calf blank urine and serum spiked with the 17β-trenbolone standard compound using LC–MS–MS in SRM positive mode. Urine samples were

hydrolysed as described above. For the determination of the recovery, the samples were compared with calibration standard solutions (analyte+ISTD dissolved directly in MeOH–H_2O, 50:50) which were not treated by SPE and TBME extraction. Two aliquots of both spiked samples and standard solution (with the same final concentration in mobile phase) were run in triplicate (six injection for each sample). Standards graphs were prepared daily by plotting peak area ratios of 17β-trenbolone (m/z 271→199) to I.S. (m/z 303→109) versus analyte concentration using a weighted ($1/x$) regression model. The curves were employed to evaluate the extraction efficiency of the analyte. Spiked urine were prepared at concentrations from 1 to 10 ng/ml of 17β-trenbolone, corresponding to final concentrations in MeOH, respectively, of 50 and 500 pg/μl. The ISTD concentration, added prior of LC–MS analysis was 1 ng/μl. For serum the recovery was tested using LC–MS–MS on a calf blank serum spiked with 5 ng/ml, corresponding to a final concentration of 50 pg/μl.

3.2. Calibration and quantitation procedure

Calibration curves were obtained by LC–SRM-MS–MS analyses of extracts of serum and urine blank control samples spiked with a mixture of 17β-trenbolone and I.S. prepared as above (see Section 2.3). The samples were prepared in duplicate and injected in triplicate into the LC–MS–MS system. The amount of 17β-trenbolone in the calibration samples was chosen according to the estimated concentration of the 17α- and 17β-trenbolone in real samples. Graphs were calculated by least-squares linear fitting of the peak area ratio of the analyte to I.S. (using the most abundant ions) versus 17β-trenbolone concentration and were used to interpolate the concentration of both the analytes in the fortified, incurred and validation samples. Estimates of the amounts of 17α-trenbolone in samples were interpolated from the calibration curve of 17β-trenbolone considering that the two analytes have the same behaviour at the mass spectrometer, and the amount of available 17α-trenbolone standard was not enough for the quantitative calibration curve.

3.3. Linearity, accuracy, precision and specificity

The validation of the analytical procedure was performed over 3 days. The linearity of the analytical procedure was evaluated by plotting the detector response (peak area ratio analyte/ISTD) versus the nominal concentration of 17β-trenbolone present in the fortified samples.

To ensure that the method produced satisfactory results in terms of precision and accuracy, urine extracts obtained from fortified samples at different known concentrations were prepared (validation samples).

Reproducibility of the injections of samples was evaluated by several injections. The precision was determined by calculating the relative standard deviation (RSD) for repeated measurements by determining the intra- and inter-day RSD values. The intra-day precision is referred as the repeatability of the assay and determined by calculating the RSD for the repeated measurements, while the inter-day precision is referred as the intermediate precision of assay. The accuracy of the method was determined by assessing the agreement between the measured and nominal concentrations of validation samples. The accuracy is expressed as the relative error of measurements (RE, %). The intra- and inter-day accuracies were evaluated. Specificity of the procedure was evaluated by analysing daily blank samples coming from different calves.

3.4. Decision limit, detection capability, limit of detection and quantification

According to the draft, SANCO/1805/2000 [24], we determined the decision limit CCα and the detection capability CCβ. CCα is the limit from which point on it can be decided that a sample is truly violative with an error probability of α. CCβ is the smallest content of the analyte that can be detected, identified and/or quantified in a sample with an error probability of β. The error should be less or equal to 5%. Decision limit CCα was calculated using 20 blank urines to calculate the signal-to-noise ratio at the retention time of the analyte. Three times the signal-to-noise ratio was used as decision limit. CCβ was determined fortify-

ing 20 blank urines at the decision limit. In this case, the concentration level where only <5% false-negative results remain is the detection capability of the method. Only one sample is allowed to give a false-negative result.

The limit of detection (LOD) was calculated with spiked urine and serum on the basis of a signal-to-noise ratio (S/N) 3:1.

The limit of quantification (LOQ) was estimated as the sample concentration of 17β-trenbolone resulting in a signal to noise of 10.

4. Results and discussion

4.1. Chromatographic conditions

Preliminary experiments were performed to select the column most suitable for our purpose: the separation of the epimers α- and β-trenbolone. Four C_{18} columns were tried in the following order: Nucleosil, Spherisorb, Kromasil and Hypersil ODS. The different columns showed different selectivity due to the different degree of silanization and different carbon percentage.

Nucleosil and Spherisorb, did not give a good separation, even when changing the composition of the mobile phase. Kromasil and Hypersil ODS columns gave satisfactory results in terms of separation, but the final choice was for Hypersil ODS columns because, under the same experimental conditions (isocratic 35% MeoH–65% H_2O), the retention times were shorter and the peaks were sharper than those obtained on the Kromasil column.

Under these conditions 17α- and 17β-trenbolone are eluted in less than 15 min with acceptable separation (R=1.5), and ISTD within 25 min.

4.2. LC–MS and LC–MS–MS analysis

First acquisitions with mass spectrometry were made on quadrupole 1 (Q1) in full scan mode on 17α- and 17β-trenbolone standard in continuous infusion. The development of the SRM-LC–MS–MS method in general firstly requires experiments carried out by infusion-MS on standard solution to determine suitable API parameters for absolute sensitivity and S/N ratio, as well as to determine the molecular related ions. The API mass spectrum of 17β-trenbolone is shown in Fig. 1A. The spectrum is dominated by protonated molecular ions, which easily provide the molecular mass. No significant fragments ions were observed in these mass spectra due to the extremely mild API conditions.

Acquisitions with a heated nebulizer (HN)-APCI source in positive mode were also tried, but there was no advantage. Fig. 1B shows the MS–MS collision-induced decomposition (CID) full-scan spectrum of the m/z 271 fragment ions of 17β-trenbolone standards under the same experimental conditions. Experimental parameters were chosen in order to reduce the intensity of the precursor ion to about 20%.

The quasi-molecular ion m/z 271 $[M+H]^+$ was chosen as a precursor for this experiment. Two product ions, m/z 199 $[M+H-C_4H_8O]^+$ and m/z 227 $[M+H-C_2H_4O]+$, were monitored in the SRM successive analysis. MS and MS–MS spectra of 17α-trenbolone are reported in Fig. 1C,D. A comparison between them shows that the two analytes 17α and 17β give the same fragments with the same relative intensities (with a tolerance of 10–20%).

Fig. 2A,B shows, respectively, the MS spectrum and the MS–MS spectrum of the I.S., methyltestosterone, in continuous infusion. The ion m/z 303 $[M+H]^+$ was chosen as a precursor and the ions m/z 109 $[M+H-C_{13}H_{22}O]^+$ and m/z 97 were monitored in the SRM analysis. The ions used for quantitation were, respectively, m/z 199 for trenbolone and m/z 109 for I.S.

For qualitative purposes EU criteria [25] were used (retention times, number of diagnostic ions and ion ratios). Our analyses showed, after the application of the criteria for confirmation, that the samples fulfilled these requirements within a day. Figs. 3 and 4A show, respectively, the product ion chromatogram of a urine fortified by 17α and 17β (respectively, 750 and 25 ng/ml) and of an incurred sample of bovine urine (found positive by a previous RIA analysis), monitoring the two product ions m/z 199 and m/z 227 of the precursor ion m/z 271 in SRM analysis. The amount of the metabolite 17α-trenbolone is much higher than 17β-trenbolone in urine according to the literature data [3]. Fig. 4B presents

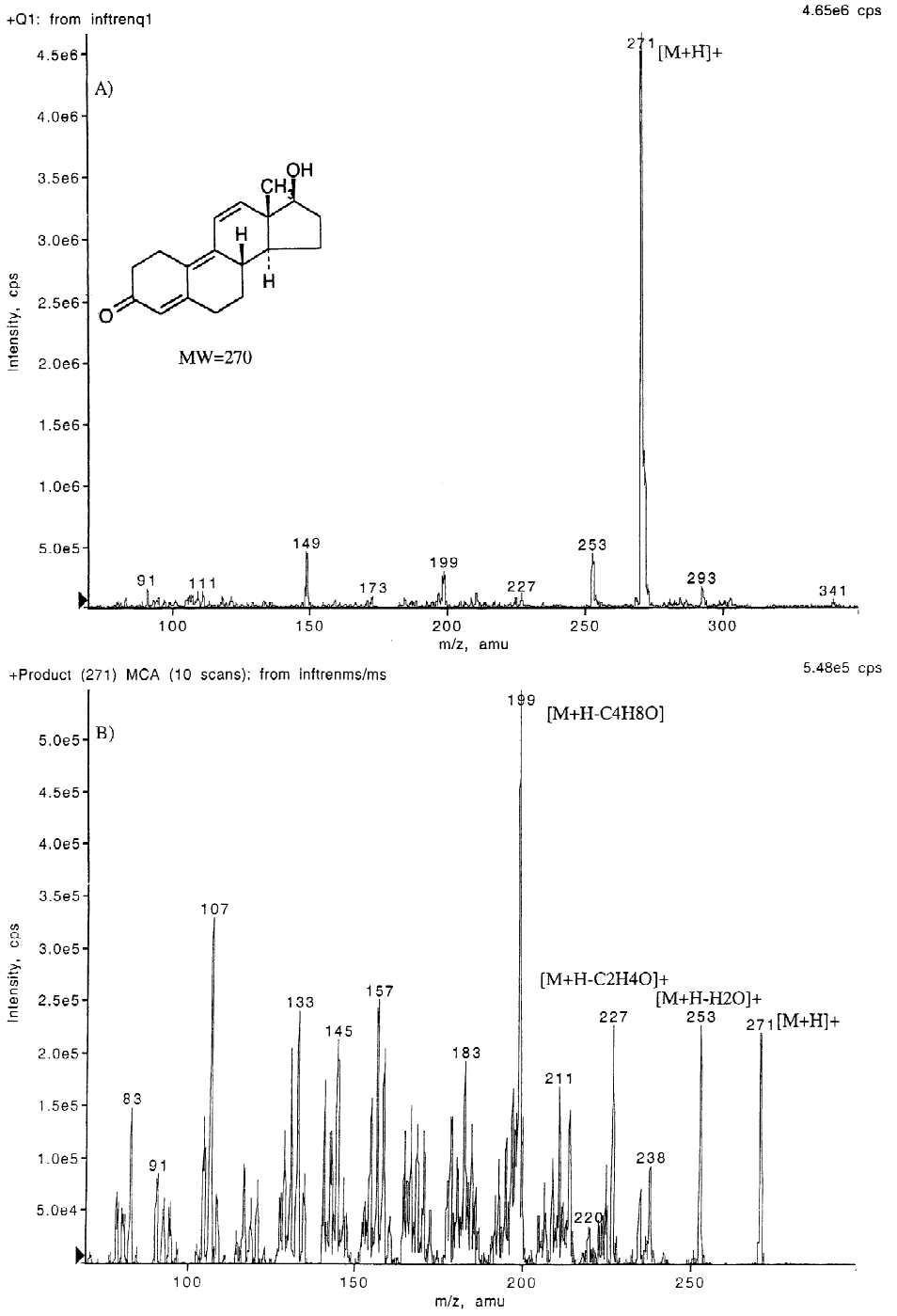

Fig. 1. (A) Full-scan API-MS spectrum, positive ions, of 17β-trenbolone standard. MS conditions: I.S.=4000 V, OR=30 V, RNG=250 V. (B) Full-scan MS–MS spectra of 17β-trenbolone standard. Precursor ion *m/z* 271. RO2=−52 V, IQ3=−67 V, RO3=−57 V. The spectra were obtained by infusion (10 μl/min) of the standard 20 ng/μl in MeOH–ammonium formate 2.88 m*M*. (C) Full-scan API-MS spectrum, positive ions, of 17α-trenbolone standard in the same MS conditions of β-trenbolone (see A). (D) Full-scan MS–MS spectra of 17α-trenbolone standard under the same conditions as 17β-trenbolone (see B).

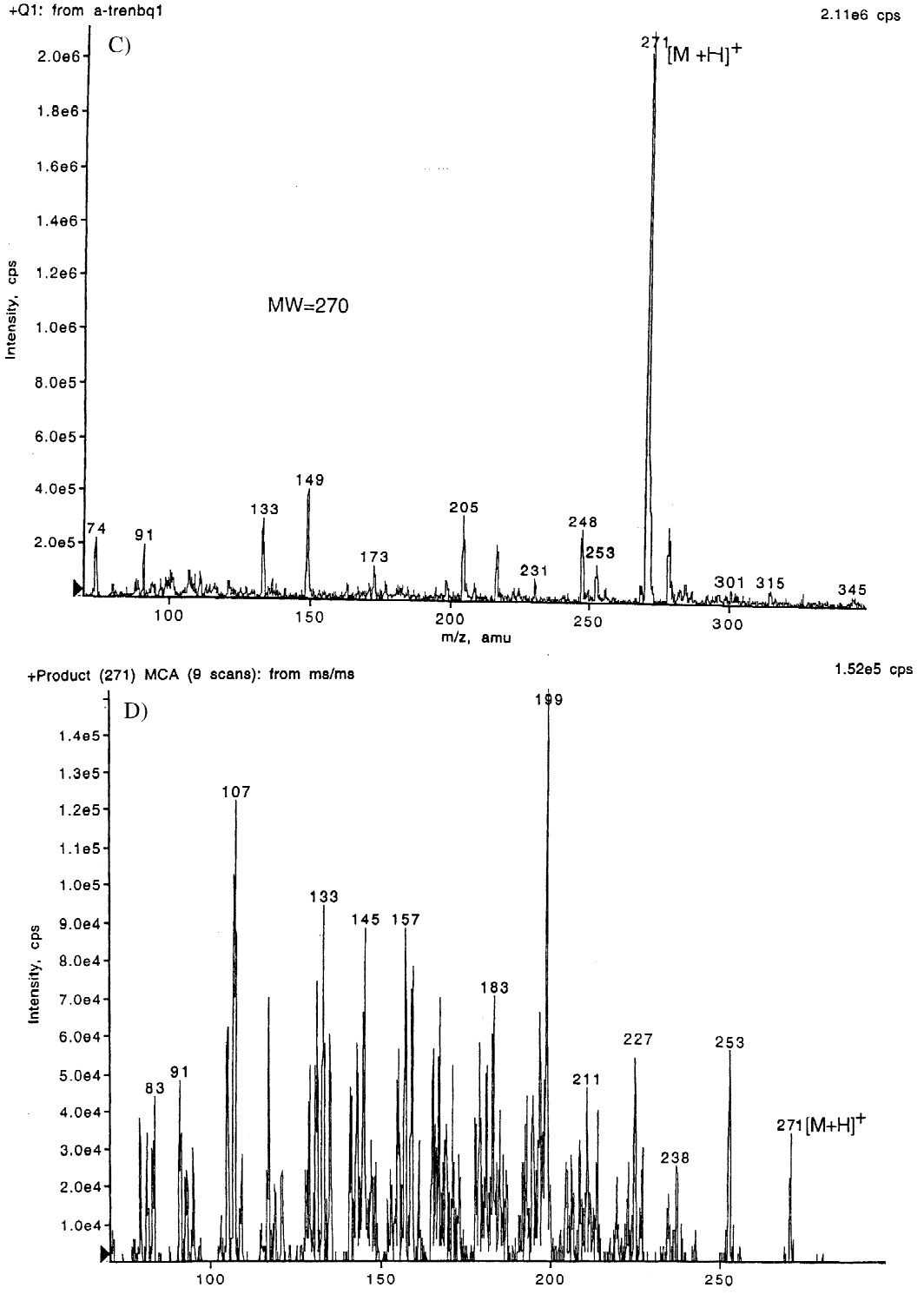

Fig. 1. (*continued*)

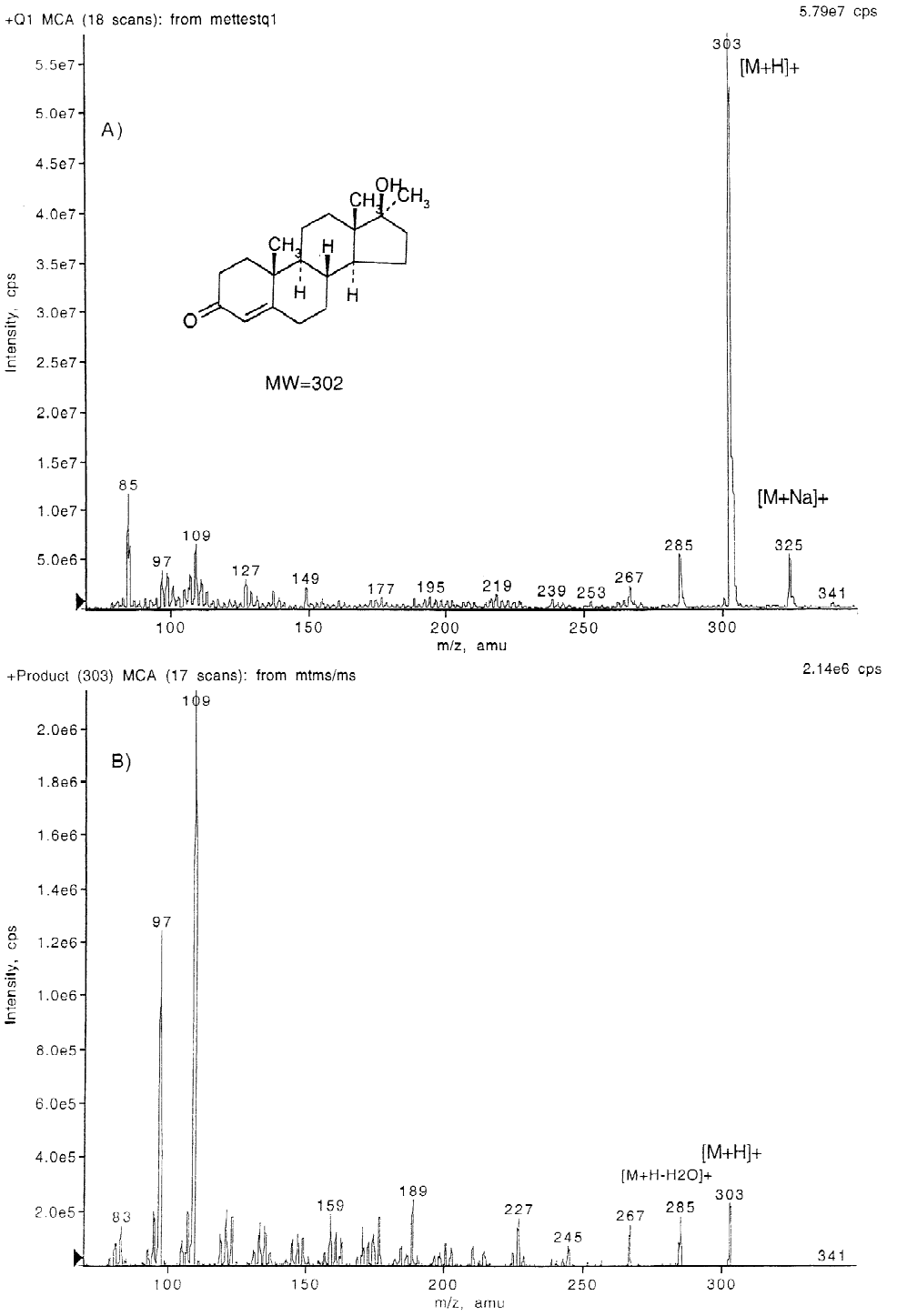

Fig. 2. (A) Full-scan API-MS spectrum, positive ions, of the ISTD methyltestosterone standard. MS conditions as in Fig. 1A. (B) Full-scan MS–MS spectrum of methyltestosterone standard. Precursor ion m/z 303. MS–MS condition as in Fig. 1B. Standard was infused as in Fig. 1.

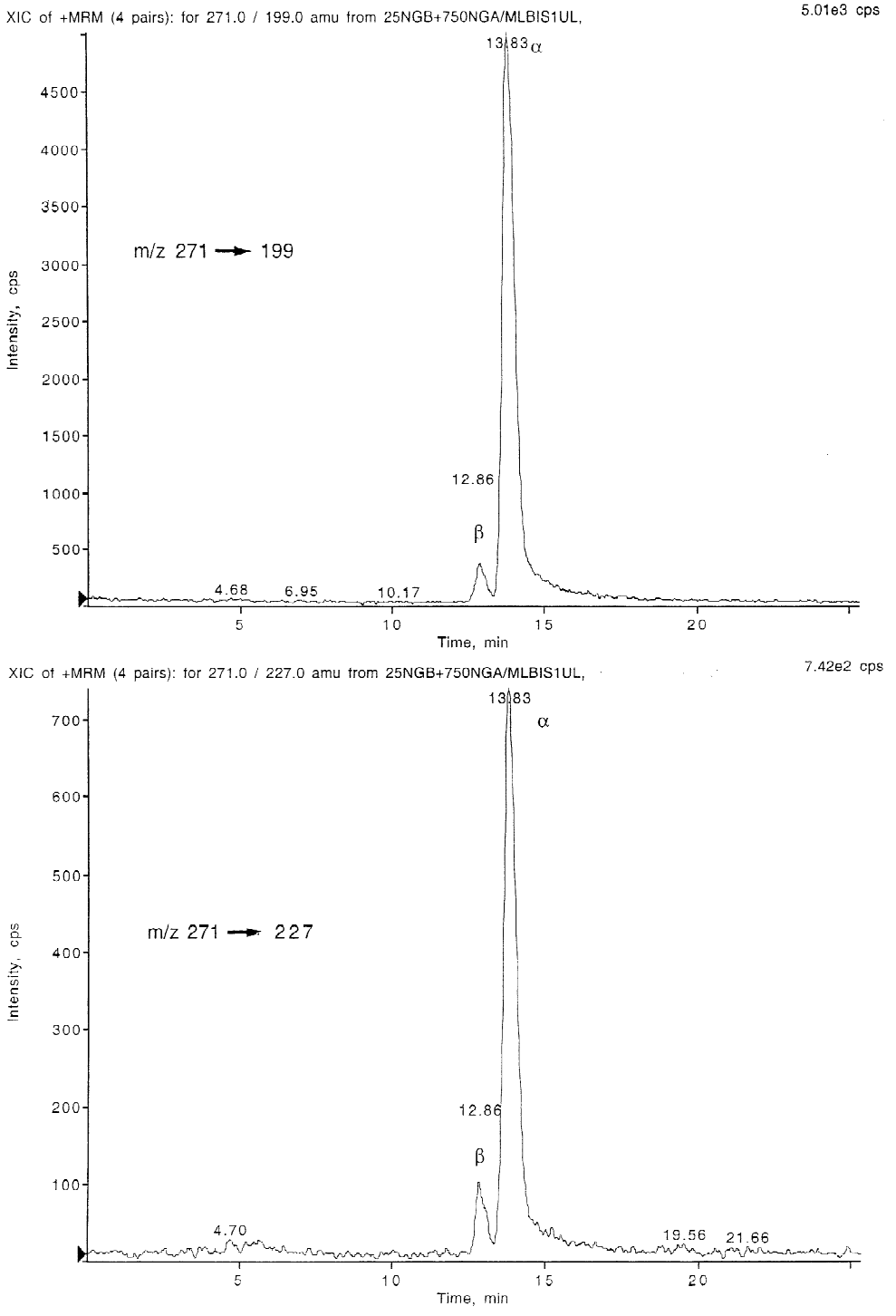

Fig. 3. SRM chromatogram in positive ionization of a spiked urine (17β-trenbolone 25 ng/ml, 17α-trenbolone, 750 ng/ml). HPLC conditions: column, C_{18} Hypersil 250×2.1 mm I.D; mobile phase, MeOH–ammonium formate, 2.88 m*M* (65:35). Flow=150 μl/min. MS–MS conditions as in Fig. 1B.

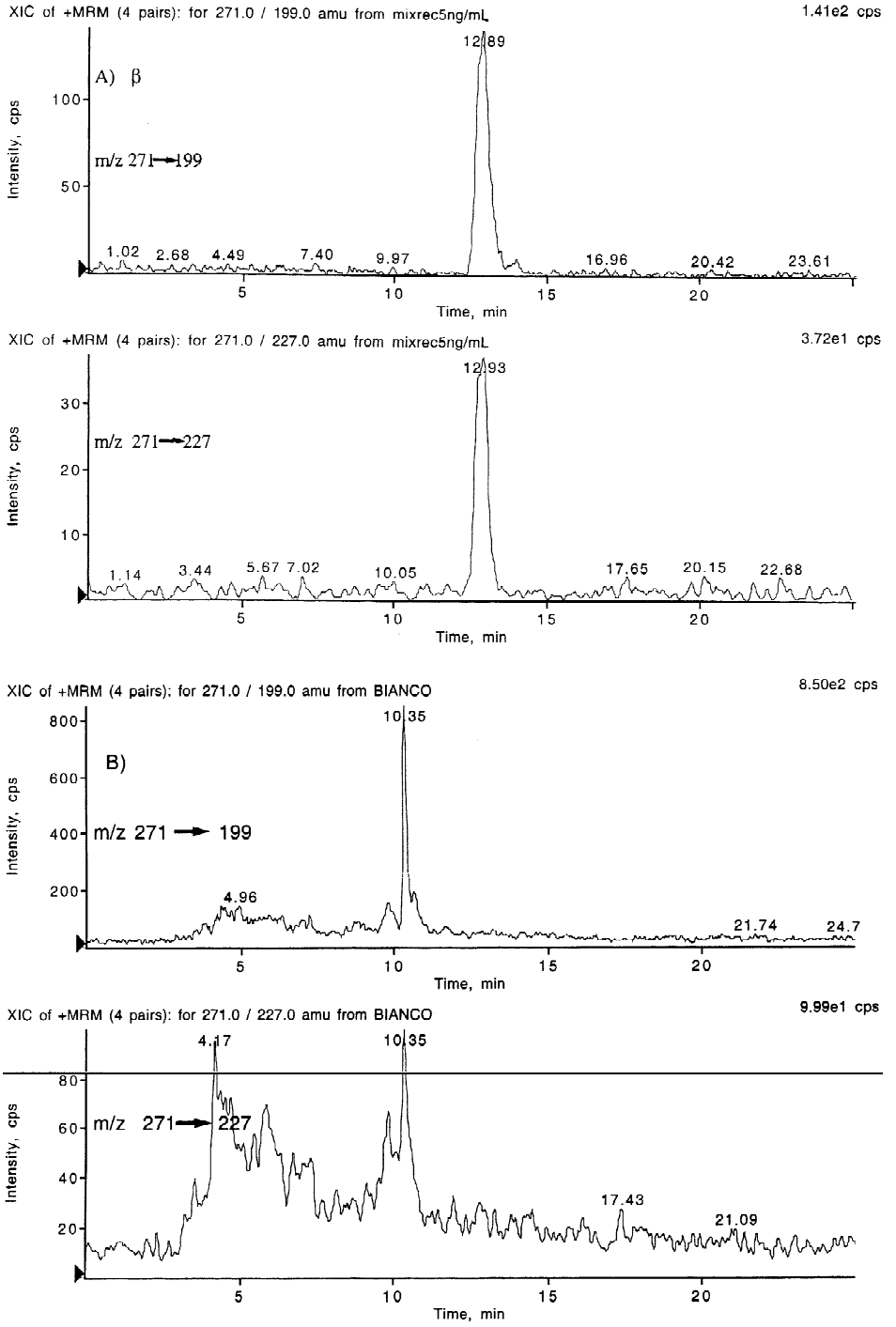

Fig. 4. (A) SRM chromatogram in positive ionization of an incurred urine sample of a calf. (B) SRM chromatogram in positive ionization of urine blank of a calf. Experimental conditions as in Fig. 3.

the results obtained, under the same experimental conditions, from the blank hydrolyzed bovine urine, showing no interfering peaks present at the trenbolone retention times. As these figures show, working in positive SRM, we obtained a very high selectivity and sensitivity and the analyses were fast.

Selectivity is due to reduced chemical noise obtained by selecting the protonated precursor ion and appropriate fragment ions for the analyte and internal standard. In this way only these ions are mass analysed while all other ions are not observed.

Also, selected ion monitoring (SIM) of the m/z 271 ion on the Q1 resulted in a good sensitivity for the detection of trenbolone. However, an endogenous compound in trenbolone-free urine interfered with its detection in the biological sample, as shown in Fig. 5.

For serum the extraction was performed on an Oasis HLB cartridge instead of C_{18} as we had better recovery, because Oasis has a higher retention power than C_{18}. Fig . 6A shows a SRM profile under the same experimental condition of spiked serum sample (5 ng/ml). Also, in this case blank bovine serum (not reported) did not give any interfering peaks at the retention time of trenbolone.

Fig. 6B shows the product ion chromatogram of the internal standard methyltestosterone (1 ng/μl) monitoring the two product ions m/z 109 and m/z 97 of the precursor ion m/z 303 in SRM analysis.

Table 1 shows the recovery results in urine by HPLC–SRM-MS–MS in positive mode ($n=6$). The average recovery calculated in urine samples was 100±3%; as shown, any significant differences in the extraction efficiency were observed at the lowest concentration. In serum, average recovery by HPLC–MS–MS was 70±4%.

Linearity was good for the analyte in the whole range of tested concentrations (see Section 3.3). Precision data were evaluated from these calibration curves, injecting the extracts 20 times each for three consecutive days. Fresh extracts were processed daily from a urine sample containing 17α- and 17β-trenbolone. The intra-day precision, ranged from 1 to 4% using HPLC–SRM-MS–MS. Table 2 summarizes the intra- and inter-day accuracy and precision of the analytical procedure at three different validation sample levels. As seen for the inter- and intra-day precision, the relative standard deviation was less than 10%. The procedure was accurate for all the concentrations tested, including the LOQ, and ranged from 5 to 12%. CCα was 0.5 ng/ml and CCβ, calculated by fortifying 20 blank bovine urines at 0.5 ng/ml, gave four false-negative results. Therefore CCβ was tested at a concentration of 1 ng/ml where no false-negative results were achieved. So CCβ is ≤1 ng/ml. LOD by LC–MS–MS in SRM was 350 pg/ml and LOQ was 1 ng/ml both in serum and urine samples. The minimum detectable amount (LOD) by LC–UV, also tested, was 30 ng/ml ($S/N=3$) and the limit of quantification (LOQ) was 100 ng/ml. These results show that HPLC–SRM-MS–MS has a limit a detection about 100 times lower than HPLC–UV. The LOD/LOQ values by LC–MS–MS characterising the described procedure is sufficient for food screening and confirmation and for residue depletion studies involving trenbolone.

4.3. Quantitation results

Nine calibration levels, in double aliquots, were used injecting, three times, 5 μl of extracts of urine blank control samples spiked with mixture of 17β-trenbolone and I.S. (1 ng/μl) prepared as above at the following concentrations 1, 8, 15, 25, 35, 50, 210, 750, 1500 ng/ml. The wide range was divided into two parts: low concentrations (1–50 ng/ml) for quantitate 17β-trenbolone and high concentrations (50–1500 ng/ml) to quantify 17α-trenbolone. The relative calibration graphs are given, respectively, by the equations: $y=(-0.0013\pm0.008)+(0.0052\pm0.0001)x$ with $r^2=0.998$ and $y=(0.228\pm0.1798)+(0.0048\pm 0.0001)x$ with $r^2=0.997$.

Two aliquots of the incurred urine sample of a bovine treated orally by trenbolone acetate were prepared and injected three times each. For 17α-trenbolone, the average of six injections ($n=6$) gave 843±4.3 ng/ml and for 17β-trenbolone gave 30±0.9 ng/ml.

Both calibration curves were used to evaluate precision and accuracy.

5. Conclusions

The aim of this work was to develop a specific sensitive and reliable LC–API-MS–MS method for the measurement of anabolic hormone residue 17β-

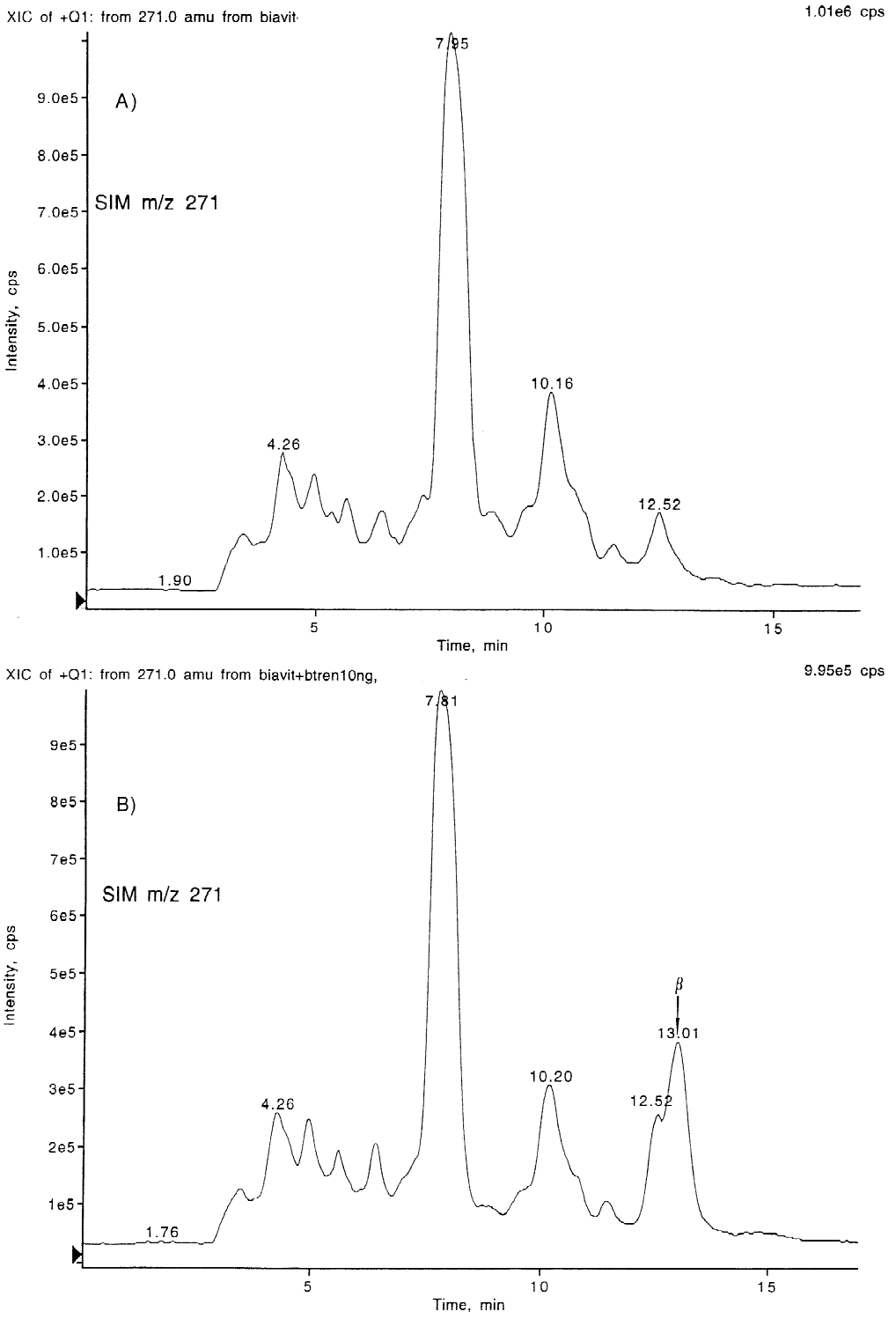

Fig. 5. HPLC–MS-SIM in positive ionization of (A) bovine urine blank (B) bovine 17β-trenbolone-spiked urine.

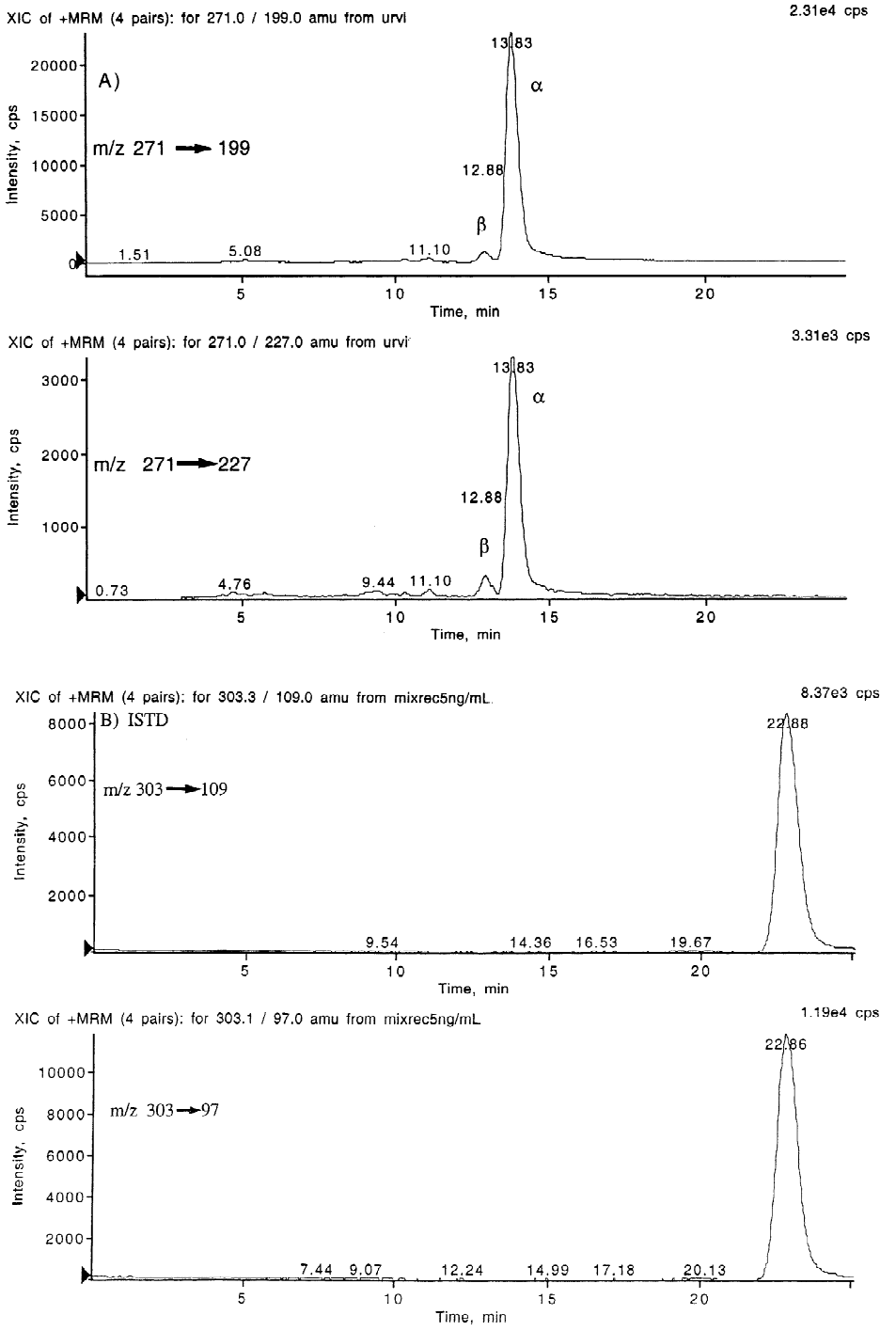

Fig. 6. (A) SRM chromatogram in positive ionization of a spiked serum sample of calf (5 ng/ml). (B) SRM chromatogram in positive ionization of methyltestosterone (ISTD) (1 ng/μl). Experimental conditions as in Fig. 3.

Table 1
Recovery values for 17β-trenbolone spiked urine by HPLC–SRM-MS–MS

Urine conc. (ng/ml)	Found[a] (ng/ml)	Recovery[b] (mean%± RSD)
1.0	1.28±0.06	128±5
1.5	1.44±0.07	96±5
2. 0	1.74±0.07	87±4
4.0	3.80±0.14	95±4
10.0	10.0±0.12	100±1

[a] Mean±SD.
[b] The mean recovery for each level was calculated from three 17β-trenbolone spiked replicates. RSD, relative standard deviation.

trenbolone and its metabolite 17α-trenbolone in bovine serum and urine. The method described is easy, highly specific and sensitive (LOD is 100 times lower than in HPLC–UV). Data obtained by this method showed a good precision and accuracy. On the other hand, with LC–MS–MS there are no problems, as for GC, due to the derivatization, and possible interferences typical of immunoassay are avoided.

The results obtained suggest that it is possible to detect illegal trenbolone treatment according to the limit decision fixed by the EEC [23], and the developed method complies with the EU criteria [25], requiring the identification of the analyte based on retention time information and on the presence of at least one precursor and two transition product ions to have the strong evidence of the drug treatment. This method is therefore suitable for laboratories involved in official controls.

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Table 2
Evaluation of intra- and inter-day accuracy and precision of HPLC–SRM-MS–MS

Parameters	Validation sample level HPLC–MRM-MS–MS					
	1 ng/ml (LOQ)		15 ng/ml		210 ng/ml	
	α-Trenb.	β-Trenb.	α-Trenb.	β-Trenb.	α-Trenb.	β-Trenb.
Intra-day precision RSD (%)	3.8	1.6	4.8	1.2	0.8	4.0
Intra-day accuracy RE (%)	−11	−12	−10	−3.3	−0.4	−1
Inter-day precision RSD (%)	9	2	3.2	2.5	4.5	9.3
Inter-day accuracy RE (%)	−11	−5	−7	−5	−6	−2

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[23] Tab 1 Piano Nazionale 2001 (PNR) per la ricerca dei residui negli animali ed in alcuni prodotti di origine animale in applicazione delle direttive 96/23/CE del Consiglio Europeo del 23/04/96, p. 14.
[24] Draft SANCO/1805/2000 Rev.3 Comission Decision. Laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC.
[25] Draft Revision of Commision decision 93/256/EC (1999).