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Journal of Chromatography B, 691 (1997) 357–370

JOURNAL OF CHROMATOGRAPHY B

Quantitative multi-residue determination of β-agonists in bovine urine using on-line immunoaffinity extraction–coupled column packed capillary liquid chromatography–tandem mass spectrometry

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Received 29 May 1996; revised 9 September 1996; accepted 10 September 1996

Abstract

This report demonstrates the potential of on-line immunoaffinity extraction and coupled column packed capillary liquid chromatography–ion spray tandem mass spectrometry for multi-residue determination of five β-agonists, clenbuterol, mabuterol, mapenterol, methylclenbuterol, and tolubuterol, in bovine urine using an automated column switching system. Trace enrichment and preliminary sample cleanup was performed on-line using bovine urine diluted with phosphate-buffered saline. The column switching process involves trapping the target analytes onto a mini-bore immunoaffinity column, whereupon the target analytes are released from the immunoaffinity column onto a trapping column and subsequently eluted onto a packed capillary analytical column. The latter packed capillary column was used to provide the optimum sensitivity for ion spray LC–MS–MS analyses. The three-column system consists of a 2.0 mm I.D. immunoaffinity column, a 1 mm I.D. reversed-phase trapping column and a 320 μm I.D. packed capillary analytical column. Both qualitative and quantitative results are presented for the multi-residue determination of the target β-agonists from the complex urinary matrix. Using tolubuterol as an internal standard, the quantitative data showed good linear response within the concentration ranges studied. Lower levels of quantitation were 50 part per trillion (ppt) for clenbuterol and methylclenbuterol, 20 ppt for mabuterol and 10 ppt for mapenterol. The bovine renal elimination is described using the technique for one of the β-agonists, clenbuterol. The concentration of clenbuterol was detectable 15 days after the cessation of oral administration.

Keywords: β-Agonists; Clenbuterol; Mabuterol; Mapenterol; Methylclenbuterol; Tolubuterol

1. Introduction

β-Agonists have been used as bronchodilators in human and veterinary medicine as therapeutic drugs for the treatment of pulmonary diseases [1,2]. They are the agents of first choice in the treatment of asthma. When the dosage is 5 to 10 times higher than those used for therapeutic treatments of bronchial diseases, clenbuterol acts like an anabolic steroid which helps developing muscle tissue at the expense of fat [3–6]. Extended withdrawal periods result in a compensatory growth and a reversal of the repartitioning effect in the animal [5–7]. The β-agonist, clenbuterol, is approved for therapeutic use in animals in some countries. It is not licensed for any use in the United States. Recently, there has been evidence that clenbuterol is being illegally used on calves as a growth-promoting agent in Europe and

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PII S0378-4347(96)00433-1

the United States [8,9]. Several other analogs have emerged over the past few years, including mabuterol, mapenterol and salbutamol. Interest in these compounds stems from their high anabolic potency [10,11].

When growth-promoting doses of the drug are administered without a withdrawal period, high concentrations of β-agonists accumulate in the various tissues of treated animals [12,13]. The retina has been found to contain the highest concentration of the drug [14–17]. Among all the edible tissues, liver appears to contain the highest concentration throughout the withdrawal period and, therefore, has been considered the most suitable edible tissue for β-agonist residue detection [13,18–20]. Clenbuterol was detectable (0.35 ng/g) in liver up to the 56th day of withdrawal [14,19]. Muscle samples of the same animals revealed much lower residue concentrations. β-Agonists seem to accumulate preferably in pigmented tissues [21]. High levels of β-agonists present in edible tissues could exert pharmacological effects on consumers. The concentration of β-agonists in tissues are not reduced by various cooking processes except for deep frying using extreme temperature conditions [22].

β-Agonists are excreted in urine as the parent drugs following administration [23]. Among the samples that can be taken from live animals, it is reasonable to anticipate that urine should contain the detectable levels of β-agonists post-withdrawal and may therefore be the sample of choice. High concentrations of clenbuterol in urine were found prior to the removal of the drug from the feed. The urinary levels are reduced to below the level of detection of enzyme immunoassay (EIA) 5 days after the withdrawal of growth-promoting dose [12,24]. Recently, clenbuterol has been found to accumulate in thr hair of treated animals [21,25], particularly black hair.

The dangers of abuse of β-agonists are underscored by several human poisoning incidences where consumption of animal food products containing clenbuterol residues were implicated [26–28]. The clinical profile of clenbuterol poisoning in humans is characterized by tachycardia, tremors, headache, dizziness, nausea, fever and chills, and in acute cases, breathing interruptions can occur. Two major outbreaks occurred in 1990 and 1992 in Spain where 135 and 140 victims, respectively, became ill after consuming bovine liver or meat containing clenbuterol residue [26,27]. A similar incident was reported in France which involved 22 illnesses [28]. The illegal use of β-agonists is a major threat to the reputation of the beef industry and a potential risk to consumers. It is important to develop sensitive analytical methods for monitoring such abusive use.

Various analytical methods have been reported for the determination of β-agonist residues in animal tissue and body fluids. These include enzyme immunoassay (EIA) [12,14,18–20,24,29–33], enzyme-linked immunosorbent assay (ELISA) [34], radioimmunoassay (RIA) [35,36], gas chromatography coupled with mass spectrometry (GC–MS) [13,37–43] or tandem mass spectrometry (GC–MS–MS) [44], HPLC methods with UV [45] or mass spectrometric detection (LC–MS) [46–48], capillary electrophoresis–mass spectrometry (CE–MS) [49] and planar chromatography [50].

Although immunoassay techniques are very sensitive, the potential lack of specificity is a great drawback. GC–MS and GC–MS–MS methods can provide definitive qualitative and quantitative results but require time-consuming derivatization steps for polar analytes like β-agonists. LC–MS and LC–MS–MS techniques allow direct determination of polar drugs compounds without derivatization. The goal of this study was to develop a method that is sensitive and selective enough to confirm the presence of β-agonists long after the growth promotion effect of the drug has disappeared. The success of such analytical methods could discourage the abusive use of these compounds and protect the consumers of meat products.

Immunoaffinity extraction using antibody–antigen interactions can provide high specificity for targeted analytes [41,43,51–53]. Although the possibility of obtaining false-positive results has limited its use to screening, immunocross-reactivity can be advantageous when used as a technique for sample preparation in multi-residue analysis [43,54]. Immunoaffinity extraction combined on-line with coupled column HPLC in conjunction with mass spectrometry [52,55,56] and tandem mass spectrometry [57] have been demonstrated. This report describes the quantitative multi-residue determination of β-agonists in

bovine urine by on-line immunoaffinity extraction and coupled column liquid chromatography, combined with ion spray tandem mass spectrometry.

2. Experimental

2.1. Chemicals and solvents

Clenbuterol hydrochloride was purchased from Sigma (St. Louis, MO, USA). Tolubuterol hydrochloride and mapenterol were kindly provided by Dr. L. Leyssens (Dr. L. Willems-Instituut, Belgium) and Dr. J. van der Greef (Center for Bio-Pharmaceutical Sciences, The Netherlands), respectively. Mabuterol and methylclenbuterol were generously supplied by Dr. M.-P. Montrade (Ecole Nationale Veterinaire, France). The antibody against clenbuterol was supplied by Central Veterinary Laboratory (New Haw, Addlestone, UK). Deionized water was prepared in a Barnstead NANOpure analytical type D4700 deionization system (Dubuque, IA, USA). All solvents and chemicals used for the HPLC mobile phases were from Fisher Scientific (Rochester, NY, USA).

The adsorption and washing buffers were phosphate-buffered saline (PBS) containing 0.01 *M* sodium phosphate and 0.15 *M* sodium chloride (pH 7.4). The elution solvent for the desorption of IgG from the protein G column was 2% (v/v) acetic acid in deionized water. The stripping solution for removal of the residual IgG from the protein G between analyses was 20% (v/v) acetic acid in deionized water.

HPLC solvents were mixtures of acetonitrile–methanol–water (A=2.5:2.5:95, B=47.5:47.5:5, v/v/v). Both solvents contained 0.1% acetic acid and 5 m*M* ammonium acetate. Isocratic elution was performed for quantitative analysis using 28% A and 72% B. For qualitative analysis, a linear gradient elution was used from 90% A/10% B to 100% B in 4 min.

2.2. Animal treatment and urine specimens

A 12 week old bull calf (93 kg) was treated over a period of three days with an oral dose of clenbuterol corresponding to 20 μg/kg of body weight per day. The drug was added in the morning ration of milk replacer. The animal was housed in the Equine Isolation Building at Cornell University, College of Veterinary Medicine during the study. Urine samples were collected at regular intervals during the period of experiment via preputial massage. The animal was euthanized at the end of the study.

Ten random calf urine samples were also obtained from a slaughterhouse (Penn Quality Meats, Bloomville, NY, USA). The urine specimens were collected from the bladders of slaughtered calves using 50-ml syringes equipped with 18-gauge needles. The calves had been formula-fed and were approximately 16 weeks old at the time of slaughter. A fresh syringe and needle were used for the collection of urine from each animal.

Control blank bovine urine known to be free of β-agonists was obtained from the Cornell University Large Animal Teaching Lab. Stock solutions of the β-agonists were prepared as 1 μg/μl solutions of the free base in methanol. Working standards were prepared by diluting the stock solution with PBS and adding to control bovine urine to provide the appropriate concentrations. All urine samples were kept frozen at −20°C until used and were diluted with PBS (1:4, v/v) before being loaded onto the immunoaffinity column.

Studies have shown that there is no effect from enzymatic hydrolysis on the quantitative level of clenbuterol [43]. In addition, no data are available which suggest the conjugation of clenbuterol in animal species. Therefore, no urine sample in this study was subjected to enzymatic digestion prior to analysis. In addition, no direct determination of drug conjugates were undertaken in this work.

2.3. Coupled column HPLC

Automated on-line sample clean-up and enrichment was performed using an Integral™ micro analytical workstation (PerSeptive Biosystems, Framingham, MA, USA). The automated column switching system is equipped with three ten-port switching valves, an autosampler, two isocratic pumps for binary gradient and a reagent pump. An external pump (ABI 140A solvent delivery system, Applied Biosystems, Foster City, CA, USA) was

incorporated into the system to deliver the HPLC mobile phases for gradient elution. The Integral™ was configured as shown in Fig. 1A–C to perform the complete process of on-line sample enrichment for LC–MS–MS analysis.

Three different columns were used in the column switching system. The immunoaffinity extraction (IAE) column was a 10×2.0 mm I.D. guard column cartridge (Upchurch Scientific, Oak Harbor, WA, USA) packed with protein G packing (PerSeptive Biosystems). Antibodies against clenbuterol were noncovalently bound to the protein G column. The trapping column was a self-packed 20×1.0 mm I.D. guard column packed with 30–40 μm Pellicular C_8 (Upchurch Scientific, Oak Harbor, WA). The analytical column was either a 320 μm×150 mm I.D. packed capillary column packed with 3-μm Spherisorb C_{18} (LC Packings, Amsterdam, The Netherlands) with the flow-rate maintained at 4 μl/min, or a 100×1.0 mm I.D. microbore column packed with 5-μm Spherisorb C_{18} (Keystone Scientific, Bellefonte, PA, USA) maintained at 60 μl/min flow-rate. When the packed capillary analytical column was used, a flow-split was achieved through a 1/16-inch polyetheretherketone (PEEK) tee (Upchurch Scientific) and a microbore HPLC column (Spherisorb C_8, 3 μm, 150×1 mm I.D., ISCO, Lincoln, NE, USA).

The sample analysis process can be divided into three steps. Fig. 1A shows the valve positions for the first step, in which the protein G column was preconditioned by pumping 20 column volumes of PBS through it. Fifty microliters of PBS-diluted antibodies (antiserum–PBS, 1:9, v/v) were injected via the autosampler. Non-IgG components were removed with another 20 column volumes of PBS. Fifty milliliters of PBS-diluted bovine urine were loaded onto the IAE column at a 4 ml/min flow followed by the pumping of 20 column volumes of PBS through to remove any remaining non-binding constituents in the bovine urine. Concomitantly, both the trapping and analytical columns were equilibrated with HPLC mobile phase.

During the second step, the valve position was changed (Fig. 1B) and the flow from the IAE was directed to the trapping column. The trapping column was conditioned with 100 column volumes of phosphate buffer (10 m*M* sodium phosphate, pH 7.4). The analytes preconcentrated in the IAE column during the first step were then eluted with 20 column volumes of 2% acetic acid and trapped at the beginning of the trapping column. The flow-rate during the second step was 200 μl/min.

During the third step, the valves were switched once again to allow back-flushing of trapped analytes from the beginning of the trapping column into the analytical column (Fig. 1C). During this step the LC–MS–MS determination took place. In the mean time, the IAE column was regenerated by flushing 20 column volumes of 20% acetic acid to strip residual antibodies and analytes off the IAE column and re-equilibrated with 50 column volumes of PBS for the next sample.

2.4. Mass spectrometry

The mass spectrometer used in this study was a Perkin-Elmer Sciex API-300 triple quadrupole mass spectrometer equipped with an ion spray interface (Thornhill, Ontario, Canada). The ion spray interface was maintained at 5.0 kV. The declustering energy (the potential difference between the orifice and the AC rods) for both MS and MS–MS experiments was maintained at 36 V. Nitrogen was used as collision gas in the second quadrupole. Each analyte was monitored by its specific reaction pathway during one injection.

2.5. Quantitative analysis

Calibration curves were constructed for clenbuterol, mabuterol, methylclenbuterol and mapenterol, using tolubuterol (5 ppb) as the internal standard. The calibration curves were generated by analyzing control bovine urine spiked with targeted β-agonists and the internal standard using the packed capillary analytical column. The mass spectrometer was operated in the selected reaction monitoring mode (SRM) and peak area ratios (analyte/internal standard) were calculated using Sciex MacQuan software. The concentration of the drug in the urine specimens was determined from the calibration curve. The accuracy of the coupled column chromatographic system was determined prior to, during and at the end of the analysis by analyzing quality control urine (QC) samples containing known con-

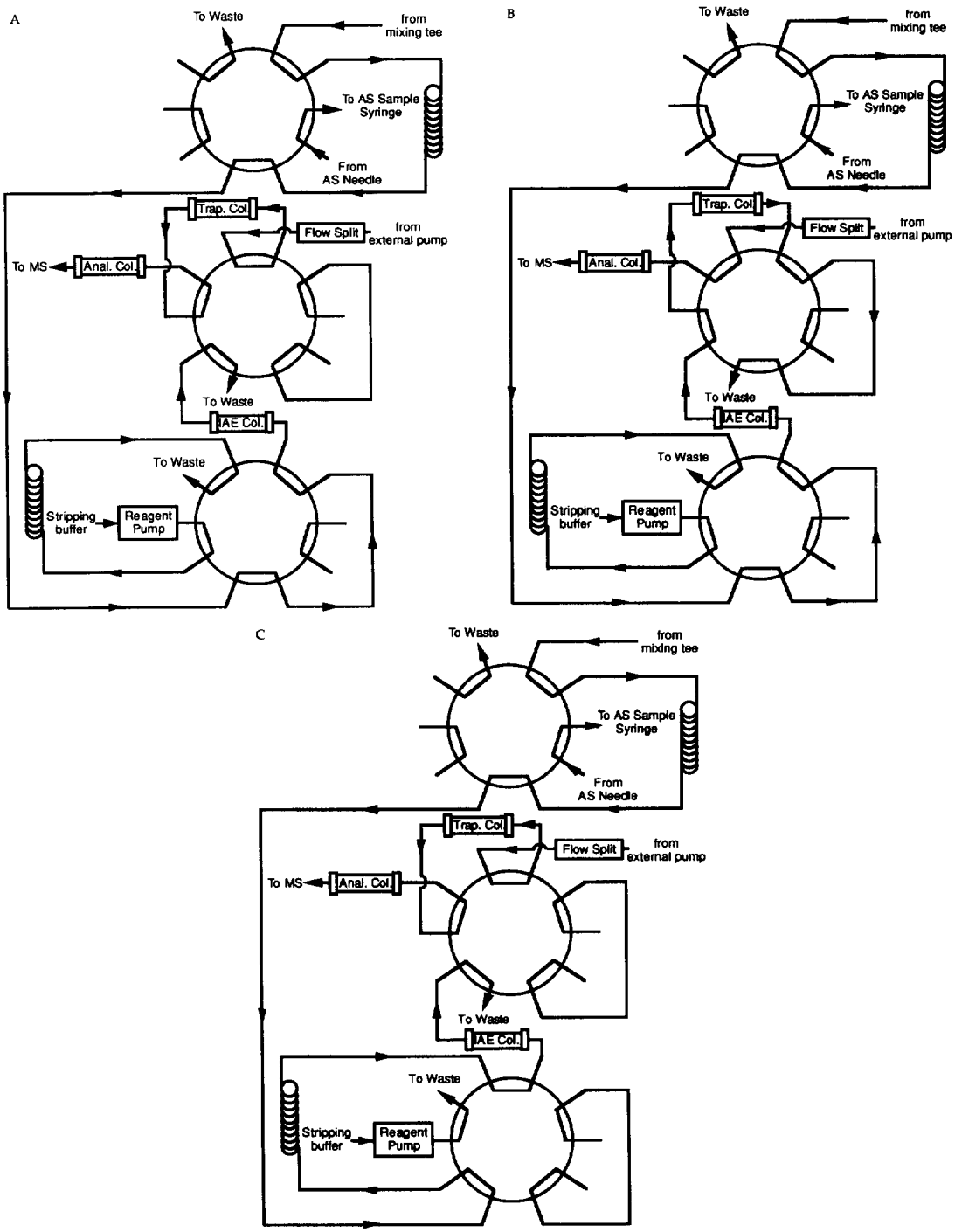


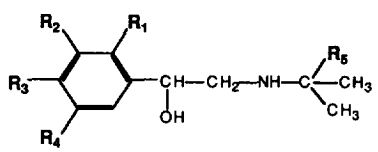
Fig. 1. Schematic of plumbing system for Integral™ Microanalytical Workstation. (A) Valve positions for loading urine onto the immunoaffinity column. (B) Valve positions for desorption of analytes and antibodies from the immunoaffinity column while loading onto the trapping column. (C) Valve positions for regenerating the immunoaffinity column while eluting the analytes from the trapping column onto the analytical column for LC–MS–MS analysis.

centrations of the drug. Typically, the percentage deviation was less than 15% as determined from the measurement of these QC samples which were prepared at low, medium and high concentrations within the range of the calibration curve.

3. Results and discussion

3.1. Mass spectrometric characteristics

The structure and molecular masses of the β-agonists selected in this study are shown in Fig. 2. The fragmentation characteristics of these β-agonists were studied in order to determine the proper mass spectrometric parameters for the IAE–LC–LC–MS–MS analysis. Collision-induced dissociation product ion mass spectra have been obtained for these compounds. Fig. 3 shows the full-scan product ion mass spectra of the five β-agonists investigated in this work. The scan range was from m/z 50 to 340. Typical fragmentation pathways for the general structure of β-agonists consist of inductive cleavage to give a loss of water molecule from the benzylic hydroxyl group, and the rearrangement of a methyl group hydrogen atom to the protonated secondary amine function followed by the loss of the N-substituted alkene moiety. The major common fragmentation pathways of the β-agonists included in this study are summarized in Table 1 and Fig. 4. These results are consistent with those reported by others [47,48,58]. The expected fragment ions at m/z 57 (for clenbuterol and mabuterol) or 71 (for methylclenbuterol and mapenterol) were not observed under the experimental conditions. The reaction pathway (a)→(d) shown in Fig. 4 was found to be the most prominent transition occurring in the collision cell for the β-agonists selected in this study. Therefore, this transition was monitored in all the



	MW	R_1	R_2	R_3	R_4	R_5
Tulobuterol	227	Cl	H	H	H	CH_3
Clenbuterol	276	H	Cl	NH_2	Cl	CH_3
Mabuterol	310	H	CF_3	NH_2	Cl	CH_3
Methyl-Clenbuterol	290	H	Cl	NH_2	Cl	C_2H_5
Mapenterol	324	H	CF_3	NH_2	Cl	C_2H_5

Fig. 2. Structures and molecular masses of β-agonists.

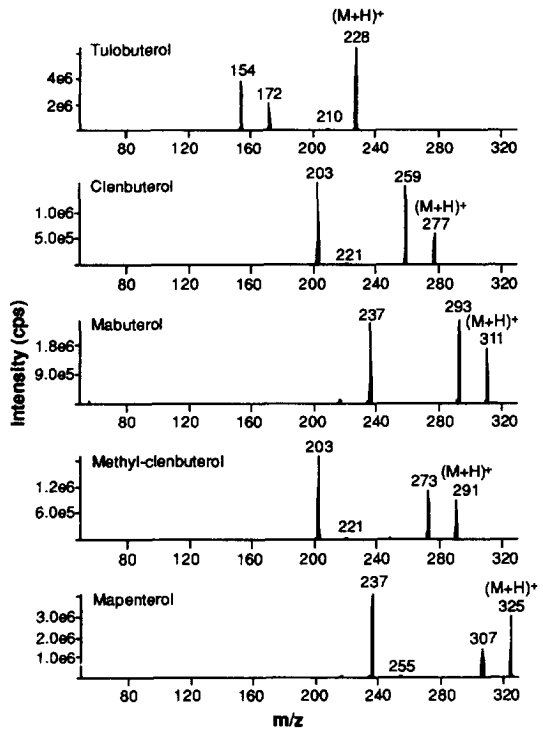


Fig. 3. Product ion mass spectra of β-agonists obtained by infusion of a methanolic solution of the β-agonists (1 ng/μl) at 4 μl/min.

Table 1
Major common product ions of β-agonists.

Compound	Product ions			
	(a)	(b)	(c)	(d)
Tolubuterol	228	210	172	154
Clenbuterol	277	259	221	203
Mabuterol	311	293	255	237
Methylclenbuterol	291	273	221	203
Mapenterol	325	307	255	237

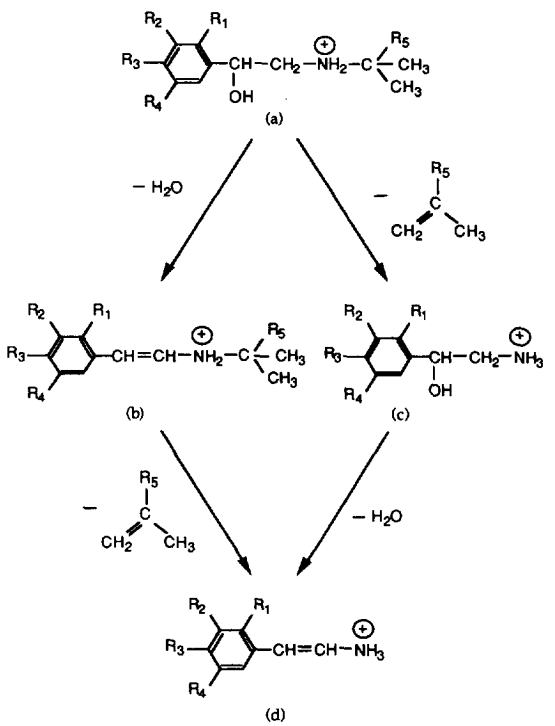


Fig. 4. Proposed fragmentation pathways of β-agonists.

SRM–LC–LC–MS analyses for maximum sensitivity.

3.2. Analysis of spiked bovine urine

Bovine urine spiked with the five β-agonists were analyzed using the on-line IAE–LC–LC–MS–MS approach described herein. Analytical columns with two different dimensions were evaluated. Fig. 5A–C shows the chromatograms of a blank bovine urine (Fig. 5A), as well as a bovine urine spiked with the five β-agonists at 100 ppt (Fig. 5B) and 500 ppt levels (Fig. 5C) using SRM–LC–LC–MS and a 1 mm I.D. microbore analytical column. Fig. 6A–C shows the corresponding chromatograms obtained with a packed capillary analytical column. Each β-agonist was differentiated by both the retention time and the reaction pathway specific for the compound. Some variation in the peak intensities correponding to the individual β-agonist is observed. In each case, direct comparisons of response should be avoided since several experimental factors may affect the ion current response observed. This may be due to a combination of differing ion current response, or a difference in trapping efficiency for each compound under these experimental conditions. In the chromatograms obtained with a control blank urine, the arrow-pointed regions are the expected retention times of the spiked β-agonists. No interferences from endogenous urine components were observed in these regions.

The above results demonstrate the feasibility for the simultaneous detection of each β-agonist in fortified bovine urine using the described system. The advantages of using a packed capillary analytical column are also shown. Upon inspection of the chromatograms, the gain in sensitivity with the packed capillary over the 1 mm I.D. microbore column was about 2- to 4-fold. This is less than the theoretical value of approximately 10-fold [59]. Extra-column volumes and the relatively large sample sizes (~15 μl) injected onto the analytical column during the last switching event may be the main contributors to this reduced gain in sensitivity.

3.2.1. Calibration graphs

Calibration curves for clenbuterol were constructed in the two concentration ranges: 0–10 ppb and 10–250 ppb using tolubuterol as internal standard (Fig. 7). The inset in Fig. 7 shows the calibration curve in the concentration range between 0.05 and 10 ppb levels for clenbuterol. Simultaneous quantitation of several other β-agonists was carried out using tolubuterol as an internal standard. Fig. 8 shows the calibration curves for the three β-agonists: mabuterol, methylclenbuterol and mapenterol. Good linearity is observed for all the calibration curves using linear least squares regression. Blank bovine urine showed no evidence for the target drugs.

3.2.2. Statistical analysis

Precision and accuracy of the analytical method were determined by analyzing two sets of quality control urine samples containing known concentrations (2.5 and 35 ppb) of clenbuterol. Six replicates were obtained for each concentration. The statistical data for the quantitation of clenbuterol are presented in Table 2. The expected concentrations in Table 2 are the levels at which the quality control samples were spiked. The observed concentrations were

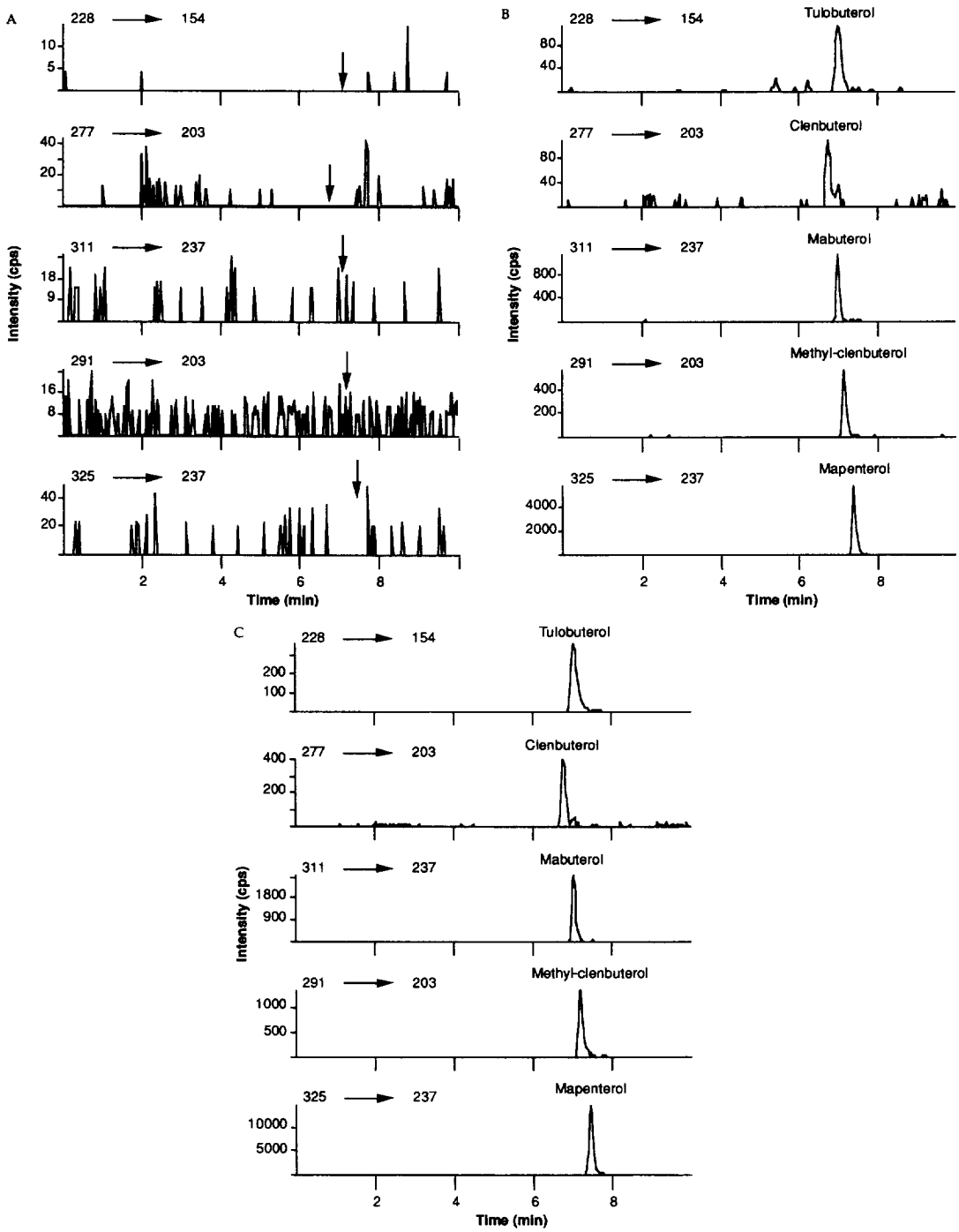


Fig. 5. SRM/IAE–LC–LC–MS of bovine urine using a 1 mm I.D. microbore analytical column. (A) Blank urine. (B) Urine spiked with five β-agonists at 100 ppt levels. (C) Urine spiked with five β-agonists at 500 ppt levels.

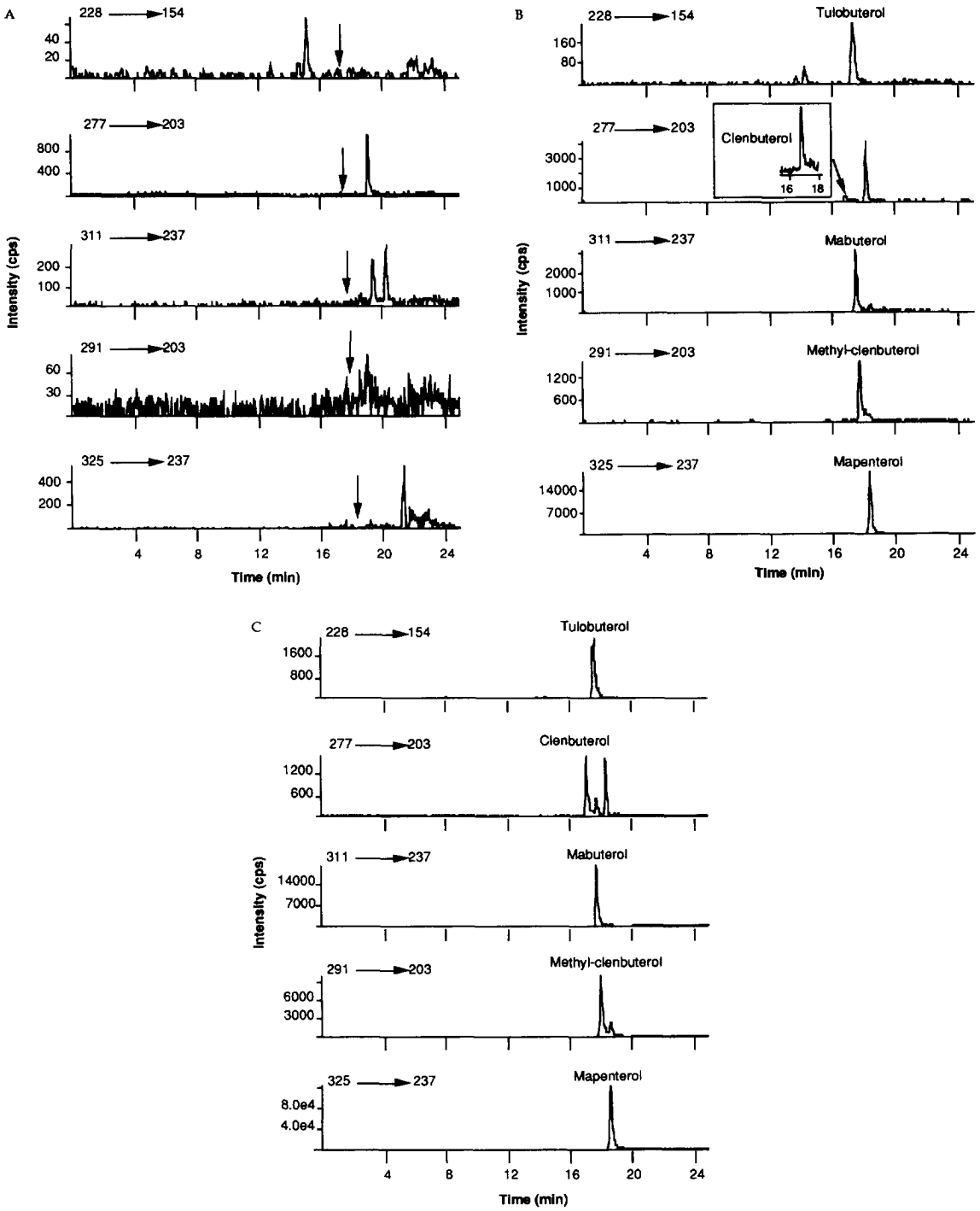


Fig. 6. SRM/IAE–LC–LC–MS of bovine urine using a packed capillary analytical column. (A) Blank urine. (B) Urine spiked with five β-agonists at 100 ppt levels. (C) Urine spiked with five β-agonists at 500 ppt levels.

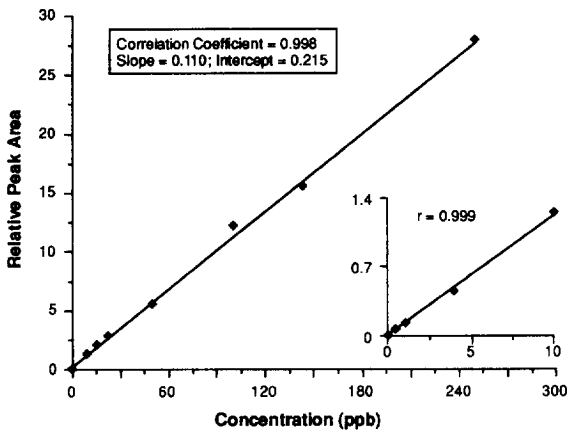


Fig. 7. Calibration curves for clenbuterol in bovine urine by SRM/IAE–LC–LC–MS. The inset shows the calibration curve for clenbuterol at concentrations below 10 ppb level.

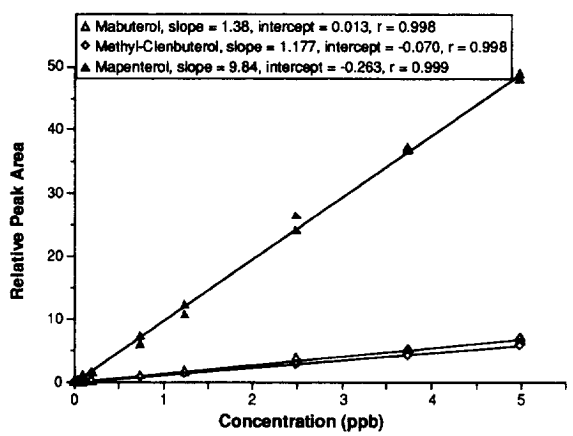


Fig. 8. Calibration curves for mabuterol, methylclenbuterol, and mapenterol in bovine urine by SRM/IAE–LC–LC–MS.

Table 2
Precision and accuracy for the determination of clenbuterol in bovine urine by SRM/IAE–LC–LC–MS

Replicates	Expected (ppb)	Observed (ppb)	Error (%)	C.V. (%)
6	35	37.80	8.0	5.67
6	2.5	2.34	−6.0	17.10

computed from the standard curve. The percentage error is the percentage difference between the observed and the expected concentrations relative to the expected concentration.

3.2.3. Lower level of quantitation

A signal-to-noise ratio ≥6 was used as the criterion for a significant response in quantitative analyses. Fig. 9 shows the chromatograms obtained at the lower level of quantitation (LLQ) for the four β-agonists: clenbuterol (50 ppt), mabuterol (20 ppt), methylclenbuterol (50 ppt) and mapenterol (10 ppt) in bovine urine.

3.3. Urinary elimination of clenbuterol in a treated animal

A 12-week-old bull calf was treated orally with clenbuterol for 3 days at 20 μg/kg daily. Table 3 and Fig. 10 show the concentrations of clenbuterol in the urine collected from the calf at various times during and after the administration. QC samples at

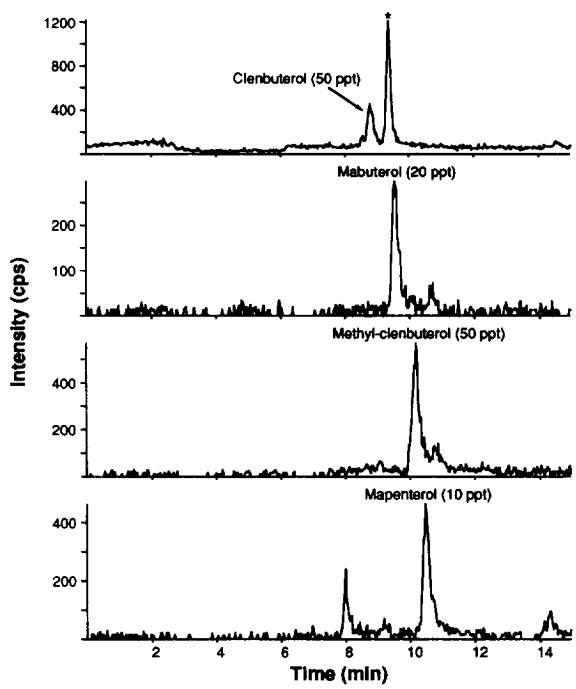


Fig. 9. SRM/IAE–LC–LC–MS chromatograms of β-agonists in spiked bovine urine at LLQ levels. The peak marked as * is from an endogenous component in bovine urine.

Table 3
Urinary concentration of clenbuterol of a calf treated orally with clenbuterol for three days

Day	Time (h)	Concentration (ppb)	Note
1	0.000	0.0000	Collected prior to the treatment
	QC sample	2.301 (2.000)[a]	% error=15.0
	1.000	30.963	First clenbuterol treatment
	2.000	94.981	
	4.000	96.561	
	6.000	92.933	
	8.500	138.79	
	13.75	183.71	
	QC sample	173.37 (200.00)[a]	% error=−13.3
2	24.00	82.262	Second clenbuterol treatment
	25.00	39.302	
	26.00	47.229	
	30.00	90.982	
	QC sample	114.13 (100.00)[a]	% error=14.1
	32.30	50.382	
	37.80	26.756	
3	48.00	58.668	Third clenbuterol treatment
	50.00	47.704	
	QC sample	14.289 (15.000)[a]	% error=−4.7
	54.00	50.355	
	56.30	55.400	
	62.00	45.336	
4	72.00	57.615	
	80.30	57.666	
5	96.00	40.198	
	QC sample	28.881 (30.000)[a]	% error=−3.7
6	144.0	20.069	
7	168.0	7.445	
8	192.0	4.583	
9	216.0	4.225	
10	240.0	3.226	
14	336.0	0.906	
16	384.0	0.328	
18	432.0	0.081	
20	480.0	0.000	End of the experiment

[a] Actual concentrations of the QC samples are in parentheses.

five different concentrations were analyzed along with the unknown samples to confirm the reliability of the instrumental setup. The percentage error for each QC sample is included in Table 3. The density of the urine specimens collected during the study was constant, as measured by the specific gravity (1.043±0.007). A significant increase in the urinary concentration of clenbuterol was observed following the oral administration, arrows indicate the time of the administration. The samples collected during the administration contained high levels of clenbuterol (inset of Fig. 10). As expected, the concentration of clenbuterol declined towards low ppt levels during the withdrawal period. By day 18, 15 days after the cessation of the administration, the urinary concentration of the drug was measured to be 81 ppt, which was close to the lower level of quantitation of the method. The urinary half-life for clenbuterol was

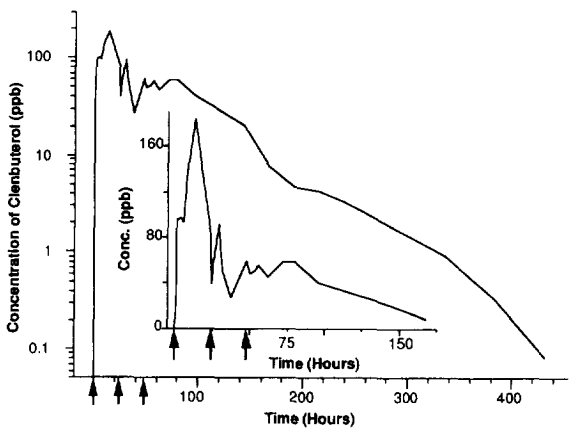


Fig. 10. Semi-log plot for the renal elimination of clenbuterol in a calf treated orally with clenbuterol. The inset shows the linear plot for the urinary concentrations of clenbuterol during the first 168 h of the study. The calf was treated with clenbuterol at 20 μg/kg daily for three days. Arrows denote the time when the consecutive oral administrations took place.

determined to be 42.2 h from the slope of the regression line fitted to the log of urinary concentration vs. time, using the linear least-square method as shown in Fig. 11 [60]. This is consistent with the reported value of 1–3 days [23]. The maximum urinary concentration was 183.71 ppb, which was reached 13.75 h after the first treatment.

3.4. Analysis of random samples from a slaughterhouse

Ten random urine samples obtained from a slaughterhouse were analyzed for the presence of the β-agonists. Only the five β-agonists known to be recognized by the antibodies used in this method were monitored. Control experiments were conducted between the analyses of unknowns to confirm reliable performance of the instrumental system. In these controlled experiments, either blank bovine urine (Fig. 12A) or blank urine spiked with 50 ppt levels of the five β-agonist (Fig. 12B) were analyzed. As shown in Fig. 12B, good sensitivity was achieved with the described system. Each of the slaughterhouse urine specimen tested negative for the five targeted β-agonists. Fig. 13 shows typical chromatograms obtained from a representative slaughterhouse urine sample. Comparing Fig. 13 with the chromatograms obtained with a control blank urine as shown in Fig. 12A, it is clear from these data that this limited sampling from a slaughterhouse showed no abusive use of these targeted β-agonists.

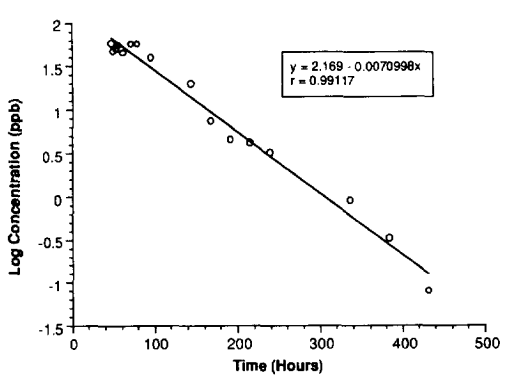


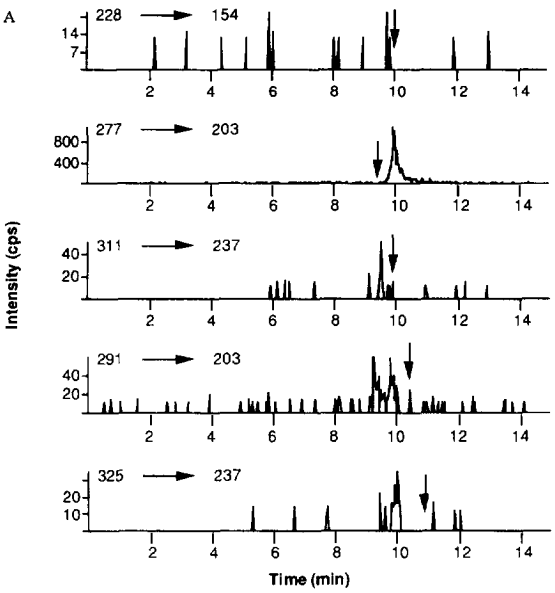
Fig. 11. Linear regression plot fitted to the log of concentration of clenbuterol vs. time for the determination of the urinary half-life for clenbuterol in a calf treated orally with clenbuterol.

4. Conclusions

Simultaneous determination of five β-agonists in spiked bovine urine is demonstrated using an automated column switching system for on-line immunoaffinity extraction and coupled column HPLC/tandem mass spectrometry. Quantitative results are presented for this multi-residue analysis from the complex urinary matrix. The quantitative data showed good linear response within the concentration ranges studied. The bovine renal elimination is described for one of the β-agonists, clenbuterol. The concentration of clenbuterol was detectable 15 days after the cessation of the administration with a lower level of quantitation of 50 ppt.

Acknowledgments

Several organizations and individuals have provided financial and material support for this work. Financial support from Advanced Bioanalytical Services is gratefully acknowledged. We thank PE Sciex for the loan of the API-300 triple quadrupole mass spectrometer and PerSeptive Biosystems for the loan of the Integral micro analytical workstation. We acknowledge LC Packings for the donation of sever-



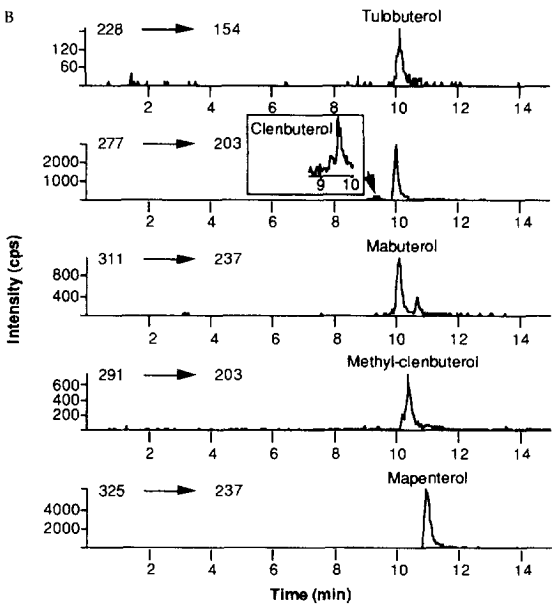


Fig. 12. SRM/IAE–LC–LC–MS chromatograms of representative blank and fortified bovine urine samples obtained with a packed capillary analytical column. (A) Blank bovine urine. Arrow-pointed regions denote the expected retention times of the five β-agonists. (B) Blank bovine urine spike with the five β-agonists at 50 ppt levels.

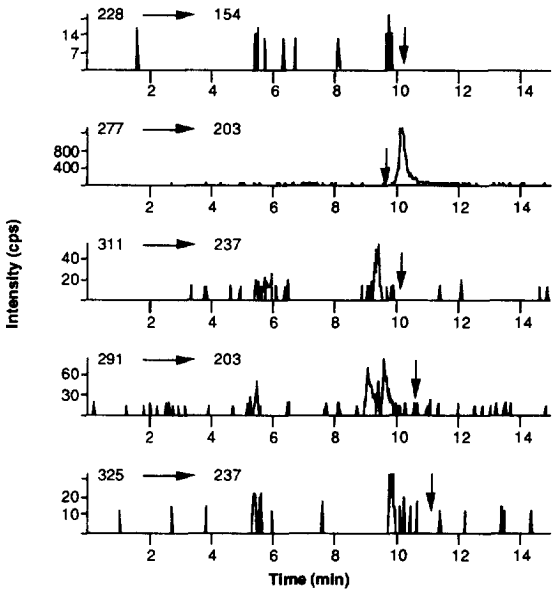


Fig. 13. SRM/IAE–LC–LC–MS chromatograms of a randomly selected slaughterhouse urine sample obtained using a packed capillary analytical column. Arrow-pointed regions denote the expected retention times of the five β-agonists.

al packed capillary HPLC columns used in this study. We are also grateful to R. van Soest of LC Packings for helpful suggestions, Dr. J. Hurley and A. Vonderchek of the College of Veterinary Medicine at Cornell for the assistance with the clenbuterol administration and renal elimination study. We would like to thank Dr. M.-P. Montrade of Ministere de l'Agriculture, France, Dr. J. van der Greef of the Center for Bio-Pharmaceutical Sciences, The Netherlands and Dr. L. Leyssens of the Dr. L. Willems-Instituut, Belgium for the donation of several β-agonist standards, Dr. M.J. Sauer of Central Veterinary Laboratory, UK for the clenbuterol antibodies and Dr. A. Chaudhry at the USDA for the slaughterhouse random calf urine samples. We also thank Dr. Tim Wachs for his invaluable technical support.

References

[1] I. Ziment, Chest, 107 (1995) 198S–205S.
[2] D. Spina, in C.P. Page and W.J. Metzger (Editors), Advances in Clinical Pharmacology: Drugs and the Lung, Raven Press, New York, NY, 1994, pp. 1–45.
[3] M.F. Miller, D.K. Garcia, M.E. Coleman, P.A. Ekeren, D.K. Lunt, K.A. Wagner, M. Procknor, T.H. Welsh Jr. and S.B. Smith, J. Anim. Sci., 66 (1988) 12–20.
[4] C.A. Ricks, R.H. Dalrymple, P.K. Baker and D.L. Ingle, J. Anim. Sci., 59 (1984) 1247–1255.

[5] J.P. Hanrahan, J.M. Fitzsimmons, J.M. McEwan, P. Allan and J.F. Quirke, in J.P. Hanrahan (editor), Beta-agonists and Their Effects on Animal Growth and Carcass Quality, Elsevier Applied Science, London, 1987, pp. 106–108.
[6] T.J. Prince, R.H. Dalrymple and D.N. Marple, J. Anim. Sci., 61 (1985) 301–309.
[7] R.W. Jones, R.A. Easter, F.K. McKeith, R.H. Dalrymple, H.M. Maddock and D. Becktel, J. Anim. Sci., 61 (1985) 905–913.
[8] D.P. Puzo, in The Los Angeles Times, Los Angeles, CA, 1994, p. 1.
[9] D. Hajewski, in Milwaukee J. Sentinel, Milwaukee, WI, 1995, p. 1.
[10] L.A. van Ginkel, H.J. van Rossum and R.W. Stephany, in Proceedings of the EC Workshop, RIVM, Bilthoven, April 22–26, 1991.
[11] G.J. Garssen, G.H. Geesink, A.H. Hoving-Bolink and J.C. Verplanke, Meat Sci., 40 (1995) 337–350.
[12] C.T. Elliott, W.J. McCaughey and D.H. Shortt, Food Addit. Contam., 10 (1993) 231–244.
[13] M.P. Montrade, B. Le Bizec, F. Monteau and F. Andre, Food Addit. Contam., 12 (1995) 625–636.
[14] C.T. Elliott, J.D.G. McEvoy, W.J. McCaughey, D.H. Shortt and S.R.H. Crooks, Analyst, 118 (1993) 447–448.
[15] C.T. Elliott, Vet. Res. Commun., 17 (1993) 459–468.
[16] C.T. Elliott, W.J. McCaughey, S.R.H. Crooks, J.D.G. McEvoy and D.G. Kennedy, Vet. Q., 17 (1995) 100–102.
[17] C.T. Elliott, J.D. McEvoy, W.J. McCaughey, S.R.H. Crooks and S.A. Hewitt, Vet. Rec., 132 (1993) 301–302.
[18] M.J. Sauer, R.J.H. Pickett and A.L. MacKenzie, Anal. Chim. Acta, 275 (1993) 195–203.
[19] M.J. Sauer, R.J.H. Pickett, S. Limer and S.N. Dixon, J. Vet. Pharmacol. Ther., 18 (1995) 81–86.
[20] H.H.D. Meyer, L. Rinke and I. Dursch, J. Chromatogr., 564 (1991) 551–556.
[21] I. Duersch, H.H.D. Meyer and H. Karg, J. Anim. Sci., 73 (1995) 2050–2053.
[22] M.D. Rose, G. Shearer and W.H.H. Farrington, Food Addit. Contam., 12 (1995) 67–76.
[23] P. Delahaut, M. Dubois, O. Pri-Bar, G. Buchanan, G. Degands and F. Ectors, Food Addit. Contam., 8 (1991) 43–53.
[24] G. Degand, A. Bernes-Dyckaerts and G. Maghuin-Rogister, J. Agric. Food Chem., 40 (1992) 70–75.
[25] A. Polettini, J. Segura, G. Gonzalez, R. de la Torre and M. Montagna, Clin. Chem., 41 (1995) 945–946.
[26] J.F. Martinez-Navarro, Lancet, 336 (1990) 1311.
[27] T.A. Galmes, S.A.C. Mariano, P.J. Oliver, P.F. Gonzalez and C. De La Calleja, Alimentaria, 33 (1995) 23–25.
[28] C. Pulce, D. Lamaison, G. Keck, C. Bostvironnois, J. Nicolas and J. Descotes, Vet. Human Toxicol., 33 (1991) 480–481.
[29] D. Courtheyn, V. Bakeroot, F. de Volder and J. Vercammen, Food Agric. Immun., 6 (1994) 131–139.
[30] G. Degand, A. Bernes-Dyckaerts, P. Delahaut and G. Maghuin-Rogister, Anal. Chim. Acta, 275 (1993) 241–247.
[31] I. Dursch, H.H.D. Meyer and S. Jager, Anal. Chim. Acta, 275 (1993) 189–193.
[32] L. Howells, M. Sauer and R. Sauer, Anal. Chim. Acta, 275 (1993) 275–278.
[33] R.J.H. Pickett and M.J. Sauer, Anal. Chim. Acta, 275 (1993) 269–273.
[34] R. Angeletti, M.P. Oriundi, R. Piro and R. Bagnati, Anal. Chim. Acta, 275 (1993) 215–219.
[35] S. Collins, M. O'Keeffe and M.R. Smyth, Analyst, 119 (1994) 2671–2674.
[36] D. Boyd, M. O'Keeffe and M.R. Smyth, Analyst, 119 (1994) 1467–1470.
[37] R.T. Wilson, J.M. Groneck, K.P. Holland and A.C. Henry, J. Assoc. Off. Anal. Chem. Int., 77 (1994) 917–924.
[38] J.A. Garcia-Regueiro, B. Perez and G. Casademont, J. Chromatogr. A, 655 (1993) 73–76.
[39] F. Andre, B. Le Bizec, M.-P. Montrade, D. Maume, F. Monteau and P. Marchand, Analyst, 119 (1994) 2529–2535.
[40] M.C. Dumasia and E. Houghton, J. Chromatogr., 564 (1991) 503–513.
[41] H. Hooijerink, R. Schilt, E.O. van Bennekom and F.A. Huf, J. Chromatogr. B, 660 (1994) 303–313.
[42] M.-P. Montrade, B. Le Bizec, F. Monteau, B. Siliart and F. Andre, Anal. Chim. Acta, 275 (1993) 253–268.
[43] L.A. van Ginkel, R.W. Stephany and H.J. van Rossum, J. Assoc. Off. Anal. Chem. Int., 75 (1992) 554–560.
[44] L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, J. Chromatogr., 564 (1991) 515–527.
[45] C. Eddins, J. Hamann and K. Johnson, J. Chromatogr. Sci., 23 (1985) 308–312.
[46] W.J. Blanchflower and D.G. Kennedy, Biomed. Environ. Mass Spectrom., 18 (1989) 935–938.
[47] D.R. Doerge, S. Bajic and S. Lowes, Rapid Commun. Mass Spectrom., 7 (1993) 462–464.
[48] D.R. Doerge, S. Bajic, L.R. Blankenship, S.W. Preece and M.I. Churchwell, J. Mass Spectrom., 30 (1995) 911–916.
[49] M.H. Lamoree, N.J. Reinhoud, U.R. Tjaden, W.M.A. Niessen and J. van der Greef, Biol. Mass Spectrom., 23 (1994) 339–345.
[50] A. Posyniak, J. Niedzielska, S. Semeniuk and J. Zmudzki, J. Planar Chromatogr., 8 (1995) 157–159.
[51] S.E. Katz and M. Siewierski, J. Chromatogr., 624 (1992) 403–409.
[52] E. Davoli, R. Fanelli and R. Bagnati, Anal. Chem., 65 (1993) 2679–2685.
[53] S.J. Shahtaheri, M.F. Katmeh, P. Kwasowski and D. Stevenson, J. Chromatogr., 697 (1995) 131–136.
[54] L.A. van Ginkel, J. Chromatogr., 564 (1991) 363–384.
[55] G.S. Rule and J.D. Henion, J. Chromatogr., 582 (1992) 103–112.
[56] G.S. Rule, A.V. Mordehai and J. Henion, Anal. Chem., 66 (1994) 230–235.
[57] J. Cai and J. Henion, Anal. Chem., 68 (1996) 72–78.
[58] L. Debrauwer and G. Bories, Anal. Chim. Acta, 275 (1993) 231–239.
[59] G.J. de Jong, N. Lammers, F.J. Spruit, C. Dewaele and M. Verzele, Anal. Chem., 59 (1987) 1458–1461.
[60] M.O. Amdur, J. Doull and C.D. Klaassen, Casarett and Doull's Toxicology: the Basic Science of Poisons, 4th ed., Pergamon Press, New York, NY, 1991.