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JMS Letters

Dear Sir,

Ion Trap High-performance Liquid Chromatography/Multiple Mass Spectrometry in the Determination of β-Agonists in Bovine Urines

β-Agonists are well known compounds, widely used in the veterinary treatment of pulmonary diseases because of their bronchodilator activity, due to the interactions with specific adrenergic receptors.[1,2] On the other hand, when these drugs are administered at concentrations higher than therapeutic doses, they are able to mime anabolic activity, improving the protein-to-fat ratio and allowing leaner carcasses to be produced.[2,3]

In view of their ascertained toxic activity towards humans, the European Economic Community (EEC) at first allowed the use of clenbuterol only as a bronchodilator, but as a consequence of its increasing fraudulent use throughout Europe, the EEC has more recently completely banned the use of all β-agonists even as therapeutic agents (Directive No. 96/22).[4] Consequently, analytical methods able to detect their presence, even at residue levels, in the target organs or biological fluids of some animal species,[5] and also in foodstuffs, are of great interest.

Several analytical procedures have been proposed,[6] and are currently employed, for confirmatory analyses; they are based on gas chromatographic/mass spectrometric (GC/MS) and high-performance liquid chromatographic (HPLC)/MS procedures, the chromatographic methods with usual detectors not exhibiting sufficiently high specificity. In the past, GC/MS methods were preferred, owing to the advanced technological development reached by this technique. With such an approach, valid results can be achieved once a preliminary derivatization step has been performed in order to increase the volatility of these compounds (mostly by trimethylsilylation or perfluorination).

More recently, the development of effective HPLC/MS interfaces, particularly atmospheric pressure ionization systems such as electrospray ionization (ESI)[7] and atmospheric pressure chemical ionization (APCI),[8] allowed β-agonists to be determined without any derivatization and with good sensitivity, by single ion monitoring of the protonated molecules (MH^+). However, when isobaric ions are present, interferences can occur. For this reason, systems able to produce, from ESI-generated MH^+ ions, fragments diagnostic from the structural point of view, are of interest, allowing the development of more specific analytical methods. For this purpose, collisionally induced dissociation (CID) is particularly effective. This approach can be efficiently achieved using multi-sector or triple-quadrupole mass spectrometers, but these are very expensive and unavailable in most common analytical laboratories. An interesting alternative method is that based on the variation of the voltage between the sample cone and the skimmer, even if, in presence of co-eluting species, it can lead to misleading results.

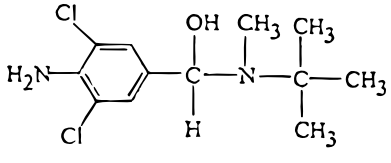
The ion trap, developed by Paul and co-workers,[9] appeared in the 1980s, showing particularly interesting sensitivity and effectiveness in collisional experiments. Even if the energy deposited in the selected precursor ion is different from those observed in high-energy collision experiments or in those obtained with triple quadrupoles,[10] the high yield of product ions (of the order of 80–90%), and the capabilities for sequential MS/MS experiments,[11] make this instrument unique.

In this letter, we report the data achieved with a commercially available ion trap-based HPLC/MS system (Finnigan LCQ) for the determination of β-agonists in bovine urine samples. Three characteristic β-agonists were chosen as being the commercially most relevant and representative of their internal classes: clenbuterol (CBT) as an arylamino derivative and salbutamol (SBT) and terbutalin (TBT) as phenolic derivatives.

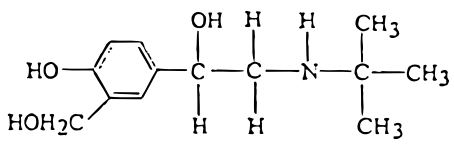
Samples were purchased from Sigma (St Louis, MO, USA) and stock standard solutions (1 mg ml^{-1}) and dilute working standard solutions (0.05–1.0 μg ml^{-1}) were prepared by dissolving each compound in methanol or preparing their mixtures in the HPLC mobile phase.

The ESI mass spectrum obtained by direct infusion of a mixture of the three β-agonists under investigation shows the production of MH^+ ions, at *m/z* 277 for CBT containing two ^{35}Cl, *m/z* 240 for SBT and *m/z* 226 for TBT, together with minor peaks at *m/z* 259 and 222, due to a primary water loss from the protonated molecules of CBT and SBT, respectively. Spectra were obtained on the Finnigan LCQ instrument; the spray was generated by use of a sheath gas (nitrogen) at a flow rate of 60 (arbitrary units, a.u.) and of an auxiliary gas (nitrogen) at a flow rate of 5 a.u. Ionization was performed applying the following parameters: spray voltage, 4.5 kV; capillary temperature, 255 °C; and capillary voltage, 27 V. Ions were collimated applying a tube lens offset of 15 V, an octapole 1 offset of −3.75 V, a lens voltage of −15.80 V, an octapole 2 offset of −7.50 V and an octapole amplitude of 400 V (peak to peak). Spectra were collected in the mass range from *m/z* 200 to 300.

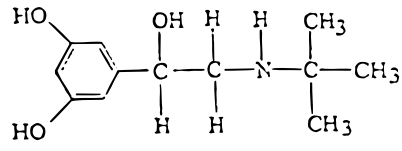
Some of the collisional spectra of the MH^+ species described above are reported in Fig. 1. They were obtained by the use of a supplementary r.f. field applied to the end-caps of the trap, with a frequency corresponding to the period motion of the selected species. In the case of CBT, the MH^+ decomposition is already observed at 15% of the maximum supplementary r.f. voltage (15 V peak-to-peak); under these conditions the most intense peak is detected at *m/z* 259, corresponding to $[MH - H_2O]^+$ species, while MH^+ has virtually disappeared. This gives an idea of the fairly high energy deposition in CID experiments performed with an ion trap. In fact, as reported[12] in collisional experiments performed by increasing the cone voltage up to 20 V in an APCI single quadrupole instrument, the MH^+ ion still represents the most abundant species. On increasing the supplementary r.f. voltage up to 40% of the maximum value, a further peak at *m/z* 203 is detected, due to the primary loss of C_4H_8 [see Fig. 1(a)]. Both of these decomposition pathways have been already described as originating by different ionization methods[12] and consequently they are structurally highly diagnostic for this class of compounds.



CLENBUTEROL (CBT)



SALBUTAMOL (SBT)



TERBUTALIN (TBT)

CCC 1076–5174/97/070781–04 $17.50

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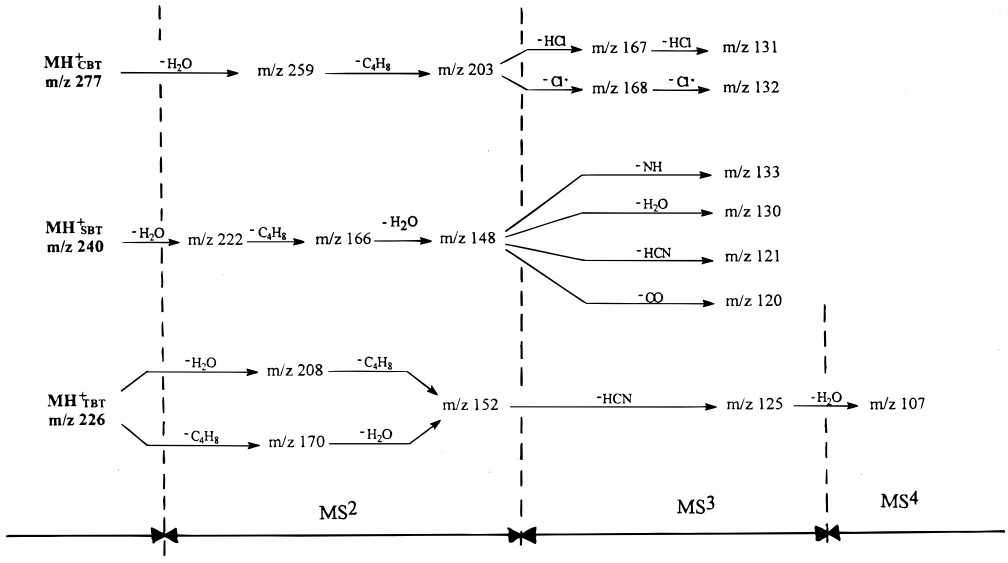

Scheme 1

In fact, the MH^+ species of SBT shows analogue fragmentation channels [Fig. 1(b)], with the production of $[MH - H_2O]^+$ ions at *m/z* 222, $[MH - H_2O - C_4H_8]^+$ ions at *m/z* 166 and $[MH - 2H_2O - C_4H_8]^+$ ions at *m/z* 166 and $[MH - 2H_2O - C_4H_8]^+$ ions at *m/z* 148. Analogously, MH^+ of TBT [Fig. 1(c)] decomposes through losses of H_2O, C_4H_8 and (C_4H_8, H_2O), leading to ions at *m/z* 208, 170, and 152, respectively. These results are summarized on the left-hand side of Scheme 1.

The observed dependence of ion abundances on collision energy indicates that a compromise between specificity and sensitivity requirements has to be found: in fact, an abundant fragmentation allows the characterization of each compound to be improved but, at the same time, part of the total ion current is lost, with a consequent decrease in sensitivity. According to our experience, the percentages of the maximum supplementary r.f. voltage that can be chosen are 15% for TBT, 30% for SBT and 25% for CBT.

Passing to MS^3 experiments, diagnostically relevant ionic species can be further detected. Thus, by colliding fragment ions at *m/z* 203 for CBT, ionic species at *m/z* 167, 168, 132 and 131 are produced, due to sequential losses of HCl and Cl· (see the right-hand side of Scheme 1). Analogously (see Scheme 1), the ion at *m/z* 148 for SBT generates further fragments at *m/z* 133, 130, 121 and 120, while the ion at *m/z* 152 for TBT shows the formation of species at *m/z* 125. MS^4 experiments on these last ionic species activate a further H_2O loss, leading to ions at *m/z* 107.

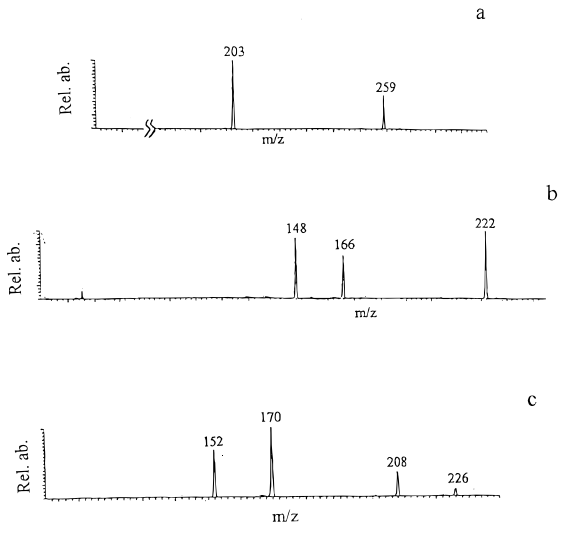

Figure 1. CID mass spectra of (a) MH^+ of clenbuterol (*m/z* 277), (b) MH^+ of salbutamol (*m/z* 240) and (c) MH^+ of terbutalin (*m/z* 226).

The results described above are, in our opinion, a good example of the effectiveness of the ESI/ion trap method in MS^n experiments. The reproducibility of these data is particularly good: a day-to-day variation of the relative abundance of product ions of $<3\%$ has usually been observed.

After this preliminary investigation on the qualitative behaviour of the analytes, we performed quantitative measurements on them. Just to check the linearity of the instrumental response, without any consideration of recovery problems, urine samples were purified and subsequently spiked with the compounds of interest in the range 0.5–50.0 ng mL^{-1}; chromatograms were acquired in the single ion monitoring (SIM) mode on the MH^+ monoisotopic species.

Urine samples were treated according to a previously standardized procedure.[10] Each sample (7 mL) was buffered at pH 5 and deconjugated using β-glucuronidase (100 µl per 100 ml) (Sigma) at 37 °C for 3 h. The urine samples were adjusted to pH 7–8 with a few drops of NaOH 1N and loaded on Sep-Pak Vac 6 cc, 500 mg silica cartridges (Waters, Milford, MA, USA) preactivated with 5 mL of water. The resin was washed with water (4 mL) and acetonitrile (4 mL) and finally elution was performed with methanol–1% triethylamine (5 mL). Solvents were removed on a 40-place Turbovap LP evaporator (Zymark, Hopkinton, MA, USA). The residue was dissolved in the HPLC mobile phase (70 µL).

The separation was carried out on a Nova-Pak C_{18} cartridge column (3.9 × 150 mm, 4 µm, 6.0 nm) (Waters) using a mobile phase consisting of acetonitrile–water (9 : 1) containing ammonium acetate (7 mM, pH 3.9). The injection volume was 20 µL. The flowrate of 0.5 mL min^{-1} was controlled by using a P4000 pump (Thermoseparation Products, San José, CA, USA). UV detection was carried out at 254 nm with a Shimadzu (Kyoto, Japan) detector. As an example, the SIM chromatograms of a urine sample spiked at 1 ng mL^{-1} are presented in Fig. 2. The equations for the calibration graphs were $y = 6626.3x - 3999.6$ ($r^2 = 0.9987$) for CBT, $y = 4876.1x + 1809$ ($r^2 = 0.9982$) for TBT and $y = 3377.3x + 3313.5$ ($r^2 = 0.9989$) for SBT (y = absorbance; x = concentration in mg ml^{-1}). The high correlation coefficients show the high linearity of response achieved by the method.

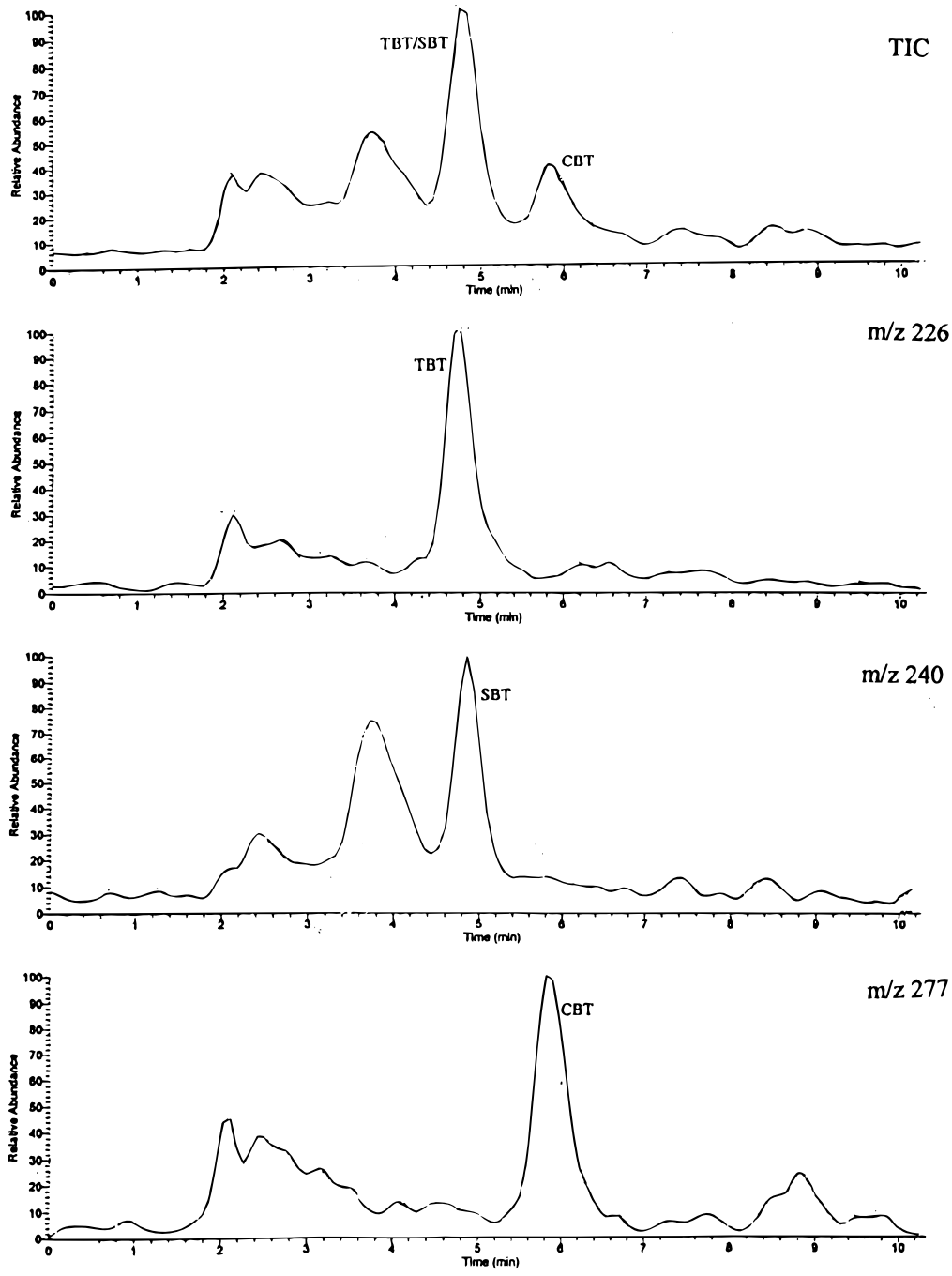

Figure 2. SIM chromatogram of a urine sample spiked with 1 ng ml^{-1} of the compounds of interest. The ionic species monitored were those at *m/z* 277 for CBT, *m/z* 240 for SBT and *m/z* 226 for TBT.

In order to evaluate the possible increase in the selectivity of the method, samples spiked in the concentration range tested for linearity were also analysed in the selected reaction monitoring (SRM) mode, by monitoring the fragment ions typical for each analyte. The variation of the relative abundance of the chosen fragment ions was calculated in the case of samples spiked at 0.5 and 50.0 ng ml^{-1}; results are presented in Table 1.

Considering these results, some urine samples spiked at 1 ng mL^{-1} were purified and analysed in both the SIM and SRM modes. Owing to the complexity of the matrix and to non-quantitative recoveries during the purification step, a limit of quantitation of not better than 1.0 ng mL^{-1} was found in both modes.

Finally, some real samples of urine collected in cowshed from animals treated with clenbuterol and confirmed to be positive by previous enzyme-linked immunosorbent assay (ELISA)[13] and GC/MS tests (which showed an analyte level of the order of 2–20 ng mL^{-1}) were analysed according to the suggested procedure. A comparison of the chromatograms obtained by HPLC/MS in the SIM and SRM modes with that for the same sample obtained by GC/MS showed that high signal-to-noise ratios are obtained in both cases, but the best results are achieved in the SRM mode.

In conclusion, the results obtained show that the determination of β-agonists in urines is not an easy task, because of the complexity of the matrices and because of the low concentration at which these compounds are present in real samples.

Table 1. Relative abundances and related variance [RSD = relative standard deviation (%)] of the product ions significant for TBT, SBT and CBT[a]

Compound and concentration	Relative abundance (%) *m/z* 170	*m/z* 152	RSD	*m/z* 208	RSD
TBT standard, 100 ng ml^{-1}	100	63.1	0.3	40.0	0.5
TBT spiked at 0.5 ng ml^{-1}	100	59.3	7.9	33.0	10.1
TBT spiked at 50.0 ng ml^{-1}	100	64.3	0.9	36.7	4.2
	m/z 222	*m/z* 166	RSD	*m/z* 148	RSD
SBT standard, 100 ng ml^{-1}	100	19.5	0.7	12.1	1.0
SBT spiked at 0.5 ng ml^{-1}	100	22.1	12.7	9.9	13.5
SBT spiked at 50.0 ng ml^{-1}	100	20.7	2.8	11.3	5.1
	m/z 259	*m/z* 203	RSD		
CBT standard, 100 ng ml^{-1}	100	12.0	1.1		
CBT spiked at 0.5 ng ml^{-1}	100	8.9	10.6		
CBT spiked at 50.0 ng ml^{-1}	100	11.3	5.1		

[a] For each compound the estimated standard deviations of the slope and intercept are as follows: for TBT, $s_a = 167.0$ and $s_b = 4196.4$; for SBT, $s_a = 144.7$ and $s_b = 3637.7$; for CBT, $s_a = 80.6$ and $s_b = 2024.7$.

European legislation has already defined that the GC/MS-based approach represents the best analytical system for the unequivocal identification of β-agonists or other drugs commonly used in the veterinary field. However, the possibility of overcoming the difficulties in the derivation step of the waste of time and non-quantitative derivatization reactions before GC/MS analysis, can be provided by the development of effective HPLC/MS-based procedures. In this paper, the applicability of an HPLC/MS system with an ion trap analyser was investigated in the detection of β-agonists in urine samples and the results obtained seem to encourage the application of this technique. A low detection limit (0.5 ng mL^{-1}) and good linearity in the range 0.5–50.0 ng mL^{-1} were found. Furthermore, the possibility of performing experiments with detection of either the protonated molecules MH^+ or the characteristic collisionally generated fragment ions increases the selectivity of the method and improves the confidence of results in confirmation analyses.

Also, important structural information can be achieved by performing MS^n experiments and should be particularly useful in preliminary studies of the detection of new molecules. In particular, both the ease of performing such experiments and the reproducibility of the product ion spectra make this technique extremely effective in the recognition of unknown compounds, allowing different relative abundances of the product ions to be obtained by varying the supplementary r.f. voltage amplitude.

The main limitation at present seems to be the limit of quantitation: 1 ng mL^{-1} is good but it is desirable to reach lower limits. This could be achieved either by improving the yields or the performance of the purification step, or by enhancing the selectivity of the final detection, for example by performing MS^3 analyses.

Yours,

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