LC-MS-MS to detect and identify four beta-agonists and quantify clenbuterol in liver†

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Received 30th June 1998, Accepted 23rd October 1998

European legislation forbids the use of beta-agonists as growth-promoting substances in cattle raised for human consumption. However, the use of beta-agonists is allowed as a therapeutic treatment of tocolysis for female cattle during calving and of respiratory diseases and tocolysis for horses not raised for human consumption. A maximum residue limit (MRL) of 0.5 μg kg^{-1} for clenbuterol in the liver of cattle and horses is proposed by law. Residues of beta-agonists in liver are identified with LC-MS-MS. Using ion trap technology, it was possible to identify each analyte without the need to resolve completely the chromatographic peaks. For each analyte, specific fragment ion spectra were obtained. The coeluting or incompletely resolved peaks were separated mass spectrometrically. For tulobuterol, bromobuterol and mabuterol, qualitative information was obtained. All beta-agonists could be detected up to a concentration of 0.1 μg kg^{-1}. For clenbuterol, a limited quantitative validation was performed. A working range was defined for which the method was applicable. Quantification was based on the integration of the response of the analytes in spiked blank liver samples. The mean recovery was 15%. The relative standard deviation (RSD) values at different concentrations were below the maximum allowed RSD. The limit of detection of clenbuterol was 0.11 μg kg^{-1}. The limit of quantification was 0.21 μg kg^{-1}. It was possible to quantify clenbuterol below one-half of the MRL. The advantage of this method is the ease of use of the mass spectrometric separation to qualify and quantify the presence of four beta-agonists in liver.

1. Introduction

The growth-promoting effects of the use of beta-agonists in slaughter animals (live weight-gain, improvement of feed conversion and increase of the ratio of muscle to fat tissue) are of economical importance. These (side) effects are obtained when the applied doses are 5–10 times higher than necessary for therapeutic treatment.[1,2]

The dangers of residues from the abuse of beta-agonists have been underscored by several human poisoning incidences where the consumption of animal food products containing clenbuterol residues has been implicated.[3,4]

Literature data indicate that the liver is, among all the edible tissues, the most suitable target organ for monitoring the use of beta-agonists, because it contains the highest concentration throughout the withdrawal period.[5–8] A maximum residue limit (MRL) of 0.5 μg kg^{-1} for clenbuterol in the liver of cattle and horses is proposed by law.

Various analytical methods for the determination of residues of beta-agonists in animal tissue and body fluids have been described.[9–15] For the identification of these components, HPLC methods with UV detection can be employed; also, the use of GC-MS is very common.[9–12] In GC-MS, the derivatisation is a critical step to improve the poor gas chromatographic performance of beta-agonists. The combination of LC and MS simplifies the development of analytical methods for polar molecules since derivatisation is not required.[13,14] With electrospray (ES) and atmospheric pressure chemical ionisation interfaces (APCI), mostly only the $(M + H)^+$ ion for beta-agonists is formed. For an unequivocal identification of the analyte, more analytical information from fragmentation is necessary. Increased fragmentation and more diagnostic ions may be obtained by collision induced dissociation using single quad LC-MS. In this case, there is no isolation of a parent ion; all incoming ions, including background ions, are fragmented by the applied energy and disturb the diagnostic spectrum.

For the determination of beta-agonists in liver, LC-MS_2 or LC-MS-MS offers a number of advantages over single quad LC-MS. LC-MS_n provides discrimination between analytes on the basis of chromatographic properties (retention time), parent ion (MS_1) and daughter ion(s) (MS_2) detection. Structurally specific information may be obtained from the MS-MS spectrum. Low cost benchtop LC-MS_n apparatus (in comparison with the classical MS-MS machines) is constructed using ion trap technology. Moreover, methods may be simplifed by the separation of coeluting compounds mass spectrometrically. The aim of this study was to develop a simple method to identify the residues of four beta-agonists and to quantify clenbuterol in liver, using a combination of off-line solid phase extraction clean-up and a primary HPLC separation with electrospray ionisation (ESI) in positive ion mode and MS-MS full scan detection.

2. Experimental

2.1. Chemicals and purification column

All solvents used were HPLC grade and other chemicals were analytical grade from Merck (Darmstadt, Germany). Clenbuterol and tulobuterol were obtained from Sigma (St. Louis, MO, USA). Mabuterol, bromobuterol and deuterated [$^2H_6$]clenbu-

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

terol (internal standard) were a gift from the State Institute for Quality Control of Agricultural Products (RIKILT, Wageningen, Netherlands). Standard stock solutions (200 ng μl^{-1}) were prepared in 0.1 M HCl. Spiking standards were prepared in ethanol (100 pg μl^{-1}) and working standards in mobile phase (100 pg μl^{-1}). The standard solutions were stored at −24 °C. For the purification step, Chem Elut 1020 CE (50 ml) disposable columns were used from Varian (California, USA).

2.2. Instrumentation

The elution of clenbuterol, tulobuterol, bromobuterol, mabuterol and the internal standard, clenbuterol-d_6, was performed on a Symmetry C18 column (5 μm, 150 × 2.1 mm, Waters, Milford, USA) using a model P4000 pump and a model AS3000 autosampler (TSP, San Jose, USA). An isocratic system, 22 : 78 methanol–0.032% trifluoro acetic acid, was used as the mobile phase at a flow rate of 0.3 ml min^{-1}. The injection volume of the sample was 100 μl. The total run time was 20 min.

The mass spectrometer and the data system used included an LCQ Ion Trap Mass Analyzer (Finnigan MAT, San Jose, USA), with an electrospray interface and a Navigator 1.0.1, respectively.

The instrument was operated in MS-MS-full scan positive ion mode. The maximum injection time was set at 400 ms with a total of two microscans per scan. An electrospray voltage of 4.5 kV and a capillary temperature of 220 °C were used. The isolation width of the parent ion was 2 mass to charge units.

2.3. Extraction and clean-up

The extraction and clean-up procedures have been described previously by Courtheyn *et al.*[15] To 11 g of liver, 12 ml of 0.5 M HCl (saturated with ethyl acetate) was added. The sample was enriched with a mixture of clenbuterol, tulobuterol, bromobuterol, mabuterol (at different concentrations according to the calibration line) and the internal standard (0.5 μg kg^{-1}) and homogenised by means of a blender (Ultra Turrax); 10 ml of 0.5 M HCl (saturated with ethyl acetate) was added and the mixture was centrifuged at 10 000 rpm for 20 min. The supernatant was adjusted to pH 12 using NaOH 33%. It was centrifuged again at 10 000 rpm for 5 min. The supernatant from the second centrifugation was transferred onto ChemElut columns. They were left to react for 45 min. The columns were eluted with 3 × 20 ml of toluene; 150 μl of 0.1 M HCl was brought into the toluene eluate. The mixture was centrifuged for 20 min at 1500 rpm. The analytes were concentrated into a 0.1 M HCl drop of 150 μl; 100 μl of the final extract was injected into the LC-MS system.

2.4. Optimisation of the mass spectrometer

The different instrument parameters (electrospray voltage, tube lens offset, capillary voltage, auxiliary and sheet gas flow, first and second octapole offset, inter-octapole lens) were optimised for clenbuterol (parent ion 277) using a built-in syringe pump. A concentration of 10 ng μl^{-1} of the mixture was continuously infused in the eluent flow (0.3 ml min^{-1}) at a flow rate of 5 μl min^{-1}. The tune file was used in the experimental method for further MS analysis.

During infusion, the collision energy to induce fragmentation of the parent ion (MH^+) in the ion trap was also optimised for the different analytes, in order to obtain the highest abundance of fragment ions. This collision energy was then specified in the different segments of the experimental method.

3. Results and discussion

3.1. ESI LC-MS-MS full scan

Ten microlitres of a 100 pg μl^{-1} standard mixture (clenbuterol, tulobuterol, bromobuterol, mabuterol and clenbuterol-d_6) was analysed by LC-MS to obtain retention time data. Relative retention time data (ratio of the retention time of the analyte to the retention time of the internal standard) are given in Table 1. The average and standard deviation were calculated for about 200 QC samples.

The chromatographic peaks of tulobuterol, bromobuterol and mabuterol were not completely resolved. The bad chromatographic separation was backed up by a mass spectrometric separation in MS-MS full scan. For this purpose, the chromatogram was divided into two segments. In the first segment, clenbuterol and clenbuterol-d_6 were detected. The first segment was subdivided into two scan events. In each scan event, the parameters (parent mass, relative collision energy, scan range) for clenbuterol and the internal standard were specified. In the second segment, tulobuterol, bromobuterol and mabuterol eluted. The MS-MS parameters for these three beta-agonists were given in the three scan events of the second segment. The experimental method is summarised in Table 2 and illustrated in Fig. 1. During and after acquisition, the different mass traces per segment can be filtered out using Navigator software. The mass spectra can be used to generate a reconstructed ion chromatogram (RIC) which will enable the quantitation of two or more coeluting analogues to be carried out.

The fragment ions are summarised in Table 3. The areas of these daughter ions are also used for quantification purposes.

The structures of the analytes are summarised in Fig. 2.

Based on the fragment ions, a fragmentation pathway is proposed in Fig. 3.[16,17] The most intense daughter ion is formed

Table 1 Relative retention time data of the four beta-agonists. (Clen, clenbuterol; Tulo, tulobuterol; Bbu, Bromobuterol; Mabu, mabuterol)

	Relative retention time			
	Clen	Tulo	Bbu	Mabu
Average	1.02	1.60	1.78	1.98
Standard deviation	0.01	0.03	0.04	0.04

Table 2 Summary of the experimental method

Segment	Scan event	Component	Parent ion (*m/z*)	Scan range daughter ions (*m/z*)	Relative collision energy (%)
Segment 1	Scan event 1	Clenbuterol	277.1	200–285	20
	Scan event 2	Clenbuterol-d_6	283.1	200–290	20
Segment 2	Scan event 1	Tulobuterol	228	100–230	20
	Scan event 2	Bromobuterol	367	250–360	20
	Scan event 3	Mabuterol	311	200–320	20

by the loss of water for the above-mentioned beta-agonists. The intensity of the ion formed by the loss of $C(CH_3)_3$ is much lower, except for tulobuterol where the loss of $C(CH_3)_3$ forms the base fragment ion.

3.2. Validation of ESI LC-MS-MS full scan of clenbuterol in bovine liver

The parameters described below were only tested within the laboratory. No interlaboratory evaluation has been performed. The calculation of the different validation parameters was based on Verwaal *et al.*[18]

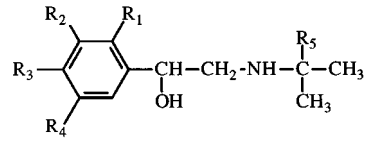
Firstly a range of application had to be established for which the analysis method was suitable. This range of application is related to the MRL and the concentration level at which the analyte is present. In this case, analyte concentrations are lower than 10^{-6} μg kg^{-1} and the MRL is 0.5 μg kg^{-1}. The range of application (described by Verwaal *et al.*)[18] is: 0.5 μg kg^{-1} ± 0.5 μg kg^{-1} (2 max RSD/100). The maximum RSD_r was calculated as follows: max RSD = $(2 \times 2^{(1-0.5 \log C)})/(1.6 \times 1.6)$. With a maximum RSD of 39%, the range of application for which a calibration line will be drawn is 0.11 to 0.89 μg kg^{-1}.

Calibration curves were generated using spiked liver samples and clenbuterol-d_6 as an internal standard. In each experiment, the internal standard was added at a concentration of 0.5 μg kg^{-1}. The calibration line was based on five concentrations of fortified liver samples: 0.1, 0.2, 0.5, 0.7 and 0.9 μg kg^{-1}. A single injection was made at the levels of 0.2 and 0.7 μg kg^{-1}. The lowest and highest concentrations were injected in triplicate and the concentration at the MRL was injected twice. The calibration curve obtained for clenbuterol was linear and gave a coefficient of correlation R^2 = 0.9910 (y = 1.06702x + 0.00326079). (Fig. 4).

Since it is possible to perform the quantitation using different ions or combinations of ions, the validation and/or quantitation parameters were based on the ratio of the sum of the areas of the diagnostic fragment ions of clenbuterol to those of clenbuterol-d_6.

The limit of detection (LOD) was calculated as the ratio of three times the relative standard deviation to the slope of the calibration line. For clenbuterol, this value is 0.11 μg kg^{-1}. Clear spectra of tulobuterol, bromobuterol and mabuterol could also be obtained at a concentration of 0.1 μg kg^{-1} (Fig. 5).

The limit of quantification (LOQ) was calculated as the ratio of six times the relative standard deviation to the slope of the calibration line. The LOQ for clenbuterol was 0.21 μg kg^{-1}, which is less than one-half of the MRL.



	R_1	R_2	R_3	R_4	R_5
clenbuterol	H	Cl	NH_2	Cl	CH_3
tulobuterol	Cl	H	NH_2	Cl	CH_3
bromobuterol	H	Br	NH_2	Br	CH_3
mabuterol	H	CF_3	NH_2	Cl	CH_3

Fig. 2 Structures of the different analytes.

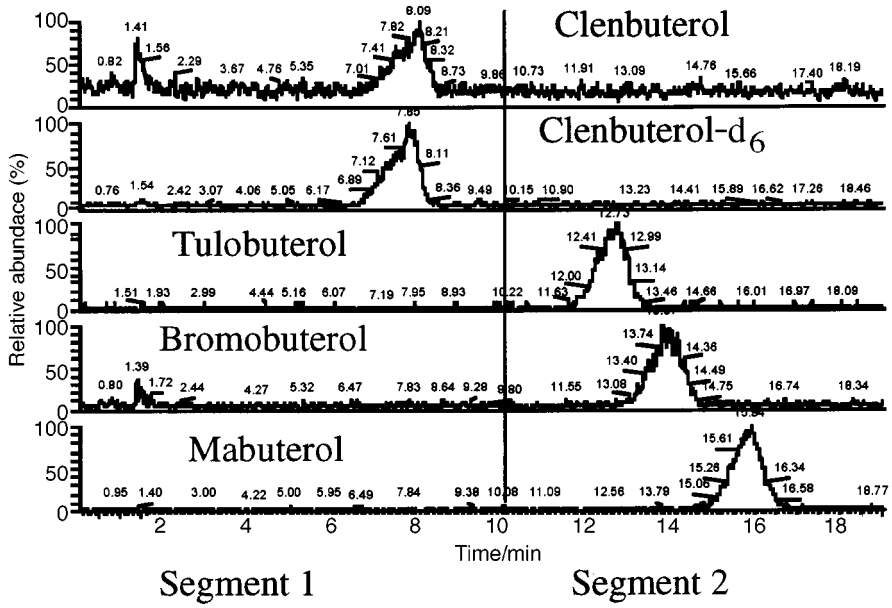


Fig . 1 LC-MS run of 1 ng clenbuterol, clenbuterol-d_6, tulobuterol, bromobuterol and mabuterol on column.

Table 2 Summary of the experimental method

	Analyte										
	Clenbuterol		Clenbuterol-d_6		Tulobuterol			Bromobuterol		Mabuterol	
Daughter ions (*m/z*)	203	259	204	265	154	172	210	237	349	237	293
Relative intensity (%)	10	100	5	100	75	100	10	10	100	20	100

The recovery was calculated at the MRL . A series (n = 6) of blank liver samples was enriched with 0.5 μg kg^{-1} of clenbuterol at the beginning of the extraction procedure. A second series of blank liver samples was enriched with 0.5 μg kg^{-1} just before LC-MS_2 analysis. In both series, the internal standard was added after the extraction procedure. The recovery was calculated by comparing the average area of the analyte internal standard peak area ratio of the extracted samples with those of the unextracted standard mixtures representing 100% recovery. The mean recovery of clenbuterol was 15% + 1%.

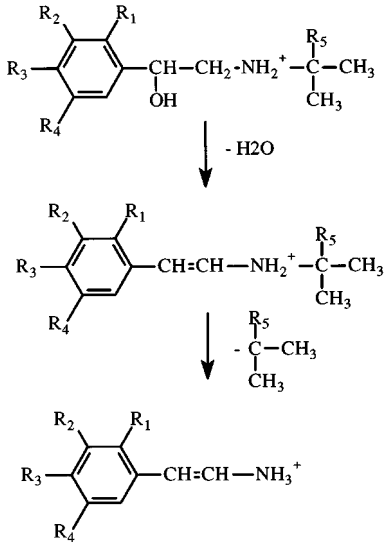


Fig. 3 Proposed fragmentation pathway of beta-agonists.

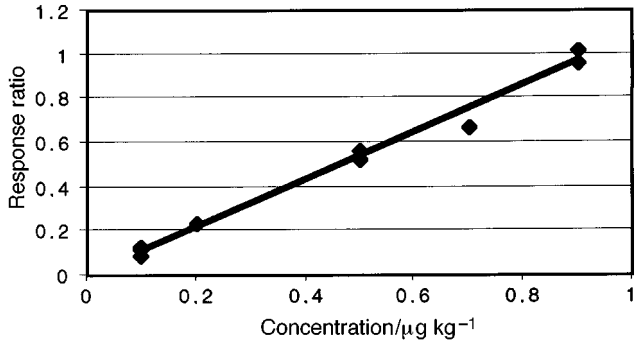


Fig. 4 Calibration curve of clenbuterol.

The within-assay variation was determined at 0.1, 0.5 and 0.89 μg kg^{-1} by extraction and analysis, on the same day, of ten aliquots of spiked liver samples for each concentration.

The within-assay variation RSD levels are reported in Table 4. The results fulfil the requirement of the RSD being less than 20%, and are smaller than the maximum RSD of the application range calculated earlier (39%).

An *F*-test was performed to determine whether the variances of the data sets of the lowest and highest values of the range of application were significantly different. The test proved that both sets of data belonged to the same population, so that they could be joined. The accuracy of the range of application can be calculated as the average of the ratio of the difference between the observed concentration and the expected concentration to the expected concentration. The accuracy for the range of application is 20%.

4 Summary and conclusion

The ESI LC-MS-MS method described in this paper provides a sensitive and reliable procedure for the qualitative analysis of tulobuterol, bromobuterol and mabuterol and for the quantitative analysis of clenbuterol in liver. The poor chromatographic separation was partly replaced by a mass spectrometric separation. The low limit of detection and quantification, despite the low recovery of the extraction and clean-up procedures, and the high selectivity make the method suitable as a confirmation technique in residue analysis. This method is used on a routine basis for the verification of positive results obtained by screening methods.

Acknowledgement

The authors are indebted to W. Derycke for the skilful operation of the LCQ Ion Trap Mass Analyzer.

Table 4 Within-assay variation of clenbuterol determinations in liver.

Added concentration /μg kg^{-1}	Mean concentration found (n = 10) /μg kg^{-1}	RSD (%)
0.1	0.12	13.69
0.5	0.55	11.69
0.89	1.03	11.89

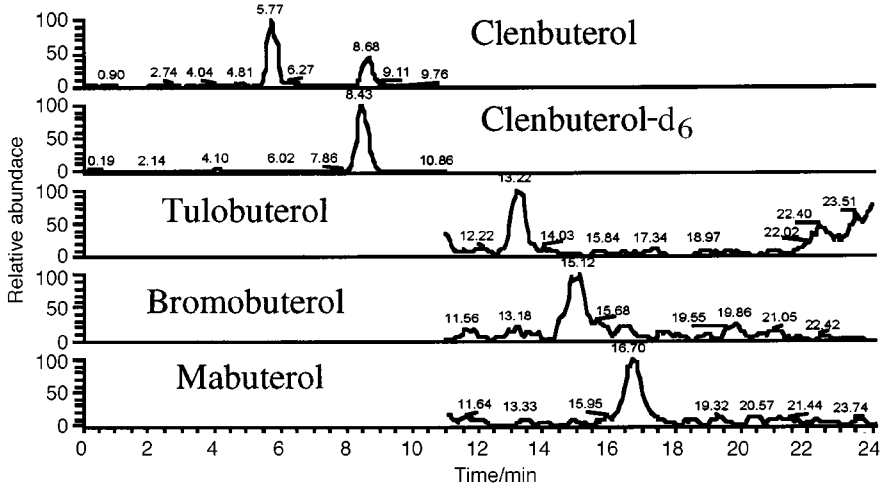


Fig. 5 LC-MS-MS chromatogram of 0.1 μg kg^{-1} beta-agonists in liver.

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Paper 8/05039B