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Multiresidue confirmation of β-agonists in bovine retina and liver using LC-ES/MS/MS

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Abstract

Misuse of numerous β-agonist drugs for their growth promoting effects in livestock production requires significant regulatory enforcement activities worldwide. The proof of illegal drug use needed for regulatory action usually requires the high degree of specificity derived from mass spectrometric analysis of suspect tissues and body fluids. In this paper, we describe a multiresidue screening method for confirmation of nine β-agonist compounds in bovine liver and retina. A wide range of analyte structures was selected in order to demonstrate applicability to other chemically related β-agonists for which standards are not currently available. The class-specific method, which is based on mixed mode cation exchange/reverse phase solid phase extraction, reverse phase gradient LC separation using a cyanopropyl-silica phase, and tandem mass spectrometry (MS/MS) in the multiple reaction monitoring (MRM) mode, yields high analyte recoveries at the target level of 1 ppb (ng/g). In addition, acquisition of multiple MRM transitions for each analyte permits simultaneous confirmation of β-agonists at the level of 1 ppb in liver and retina by using intensity ratios between fragment ions and protonated molecules. Estimated values for the limit of quantification (LOQ) for individual β-agonists were 0.08–0.3 ppb in liver and 0.02–0.5 in retina; the estimated limits of confirmation, using accepted criteria from international regulatory agencies, were 0.25–0.8 ppb in liver and 0.1–1 ppb in retina. This method should be useful in supporting regulatory enforcement programs that monitor β-agonist misuse.

Keywords: β-Agonists; Mass spectrometry; Retina; Clenbuterol; Ractopamine

1. Introduction

The use of β-adrenergic receptor agonists as growth stimulants provides a significant competitive advantage to livestock producers due to repartitioning of carcass composition to decreased fat deposition and increased muscle mass [1]. For this reason, several β-agonist drugs have received regulatory approval for use in livestock (e.g., ractopamine in swine and zilpaterol in cattle and swine). However, the use of potent illegal β-agonists continues to demand regulatory scrutiny because of documented adverse health effects (e.g., cardiovascular and CNS) in consumers of contaminated meats [2]. The high potency of growth promoting effects and rapid metabolism of β-agonists require highly sensitive analytical methods for quantification and confirmation of residues in livestock tissues for regulatory enforcement programs. While screening of livestock tissue residues can be done inexpensively using rapid immunochemical tests, accurate quantification and confirmation require the sensitivity and specificity of mass spectrometry coupled with a chromatographic procedure. Pharmacokinetic differences among tissue types have been used to maximize screening efficiency because typical β-agonists are eliminated in the following order: plasma > urine > liver ≫ retina and hair [3–5]. The accumulation of β-agonists in retinal tissue has been particularly useful in identifying residues of β-agonists at long withdrawal times (weeks to months) after administration of the drug [4–6].

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We have previously demonstrated the usefulness of LC with API/MS detection for the quantitative and confirmatory analysis of individual or a few structurally related β-agonists in bovine tissues at trace levels [6]. In the present study, we use LC with electrospray tandem mass spectrometry (LC-ES/MS/MS) in the multiple reaction monitoring mode (MRM) to simultaneously confirm the presence of a diverse set of nine representative β-agonists (Fig. 1) in bovine liver and retina at the 1 ppb level, which is appropriate for monitoring legal and illegal use of selected target compounds in livestock production.

2. Experimental

2.1. Reagents

Salbutamol, terbutaline, fenoterol, clenbuterol and sulfatase from *H. pomatia* (type H-5) were purchased from Sigma Chemical Co. (St Louis, MO). Cimaterol and mabuterol were obtained from Boehringer-Ingelheim (St. Joseph, MO), zilpaterol from Hoechst Roussel Vet (Clinton, NJ), ractopamine from Eli Lilly (Indianapolis, IN). Brombuterol and ring-labeled $^{13}C_6$-clenbuterol were synthesized in-house and characterized spectroscopically (UV, NMR) and chromatographically (LC-ES/MS). Control bovine liver and retina samples were obtained at a commercial slaughterhouse and were generously provided by Dr. Chander Dev (FSIS-USDA, Omaha, NE). All solvents were HPLC grade and Milli-Q water was used throughout.

2.2. Tissue treatment

A 200 mg portion of liver was homogenized on ice in 2 ml of 25 mM sodium citrate buffer (pH 5) and 2 ng ^{13}C-labeled clenbuterol was added as an internal standard (10 ppb). Enzymatic hydrolysis of glucuronide/sulfate conjugates was achieved by the addition of 20 μg of sulfatase/glucuronidase and incubation at 37 °C for 1 h. Alternatively, a 200 mg portion of retina was homogenized on ice in 3 ml 0.01 M HCl without enzyme treatment. After centrifugation of the tissue samples the supernatant was carefully decanted and purified using the following SPE method.

2.3. Solid phase extraction

SPE was carried out using mixed mode HCX 96-well array cartridges (100 mg, 1 cc, Argonaut Technologies, Foster City, CA) under reduced pressure. The cartridges were activated with 2 × 1 ml of elution solvent (5% NH_4OH in MeOH) followed by 2 × 1 ml washes of MeOH. The cartridge was equilibrated with 2 × 1 ml washes of 10 mM NH_4OAc buffer (pH 5). Aliquots corresponding to 200 mg equivalents of liver or retina samples were then loaded onto the cartridges. The cartridge was then sequentially washed with 400 μl of the 10 mM NH_4OAc buffer (pH 5), 400 μl of 1 M formic acid

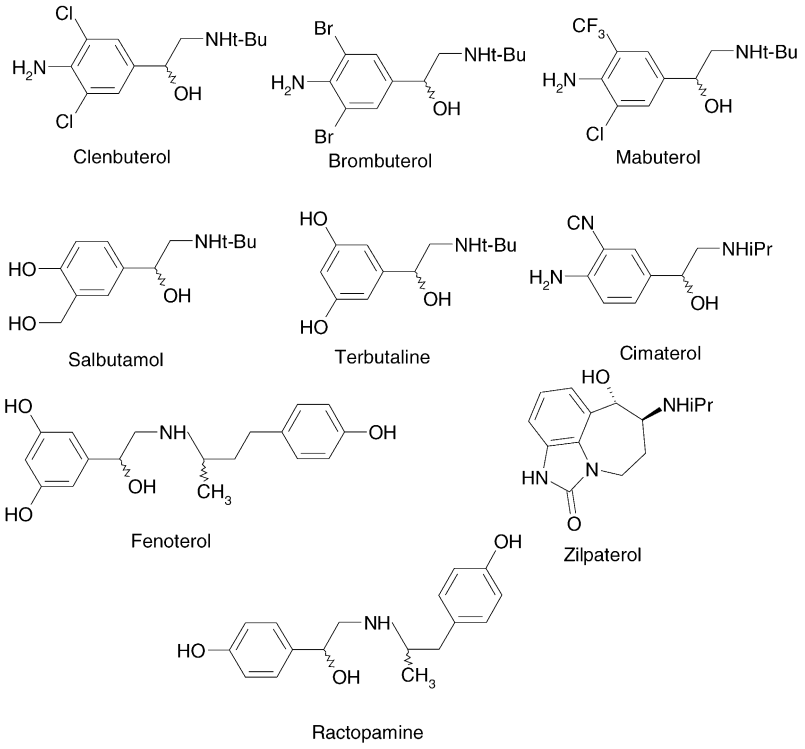

Fig. 1. Structures of selected β-agonists. t-Bu: *tert*-butyl; iPr: isopropyl.

(aq), dried for 30 s, and finally washed with 400 μl of MeOH. Analytes were eluted with 3 × 400 μl washes of 5% NH_4OH in MeOH. The eluate was reduced to dryness using a centrifugal vacuum concentrator and reconstituted into 200 μl of 5% MeOH in 0.1% formic acid (aq).

2.4. Liquid chromatography

LC was performed using a Waters 2795 liquid handling system (Waters Assoc., Milford, MA). Chromatographic separation was achieved on a Betamax Base analytical column (2 mm × 100 mm, 5 μm particles, Thermo Hypersil-Keystone, Bellefonte, PA) equipped with a C_{18} Security Guard cartridge (2 mm × 4 mm, 2 μm particles, Phenomenex, Torrance, CA) at a flow rate of 0.3 ml/min. The mobile phase consisted of 0.1% formic acid (aq) and acetonitrile. Initial gradient conditions were set to 5% ACN and held for 2.5 min before incorporating a linear gradient increasing to 65% ACN at 8.5 min. At 8.6 min the gradient was programmed to initial conditions to reequilibrate the column for 3.4 min (total run time 12 min). Injection volumes were 50 μl and all separations were performed at ambient temperature. Analysis of sample sets included interspersed blank tissue samples and standards to ensure that no carryover of analyte or changes in instrument response, respectively, occurred. No evidence for carryover was observed.

2.5. Mass spectrometry

The entire column effluent was directed into either a Quattro Premier or a Quattro Ultima triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface. Positive ions were acquired in the multiple reaction monitoring (MRM) mode using a desolvation temperature of 400 °C and a source temperature of 120 °C. The chromatographic run was split into two scan events, the first monitored three MRM transitions for each of the first four compounds eluting from 0 to 4.5 min while the second monitored three MRM transitions for each of the last five compounds eluting from 4.6 to 11 min plus the major transition for ^{13}C-labeled clenbuterol internal standard. The base peak in each compound spectrum was the protonated molecule $[M + H]^+$ and was subsequently used as the precursor ion for the resulting MRM transitions. Table 1 shows the Quattro Premier operating parameters while Table 2 shows the corresponding details for the Quattro Ultima; the strongest transi-

Table 1
Quattro Premier mass spectrometer parameters

Compound	MRM transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Salbutamol	240.2 → 222.2	0.1	19	11
	240.2 → 166.1			14
	240.2 → 148.1			19
Zilpaterol	262.2 → 244.2	0.1	20	13
	262.2 → 202.1			18
	262.2 → 185.1			25
Terbutaline	**226.2 → 152.0**	0.1	23	16
	226.2 → 125.0			24
	226.2 → 107.0			31
Cimaterol	220.2 → 202.2	0.1	16	10
	220.2 → 160.0			16
	220.2 → 143.0			24
Fenoterol	304.2 → 286.2	0.1	25	14
	304.2 → 135.0			17
	304.2 → 107.0			32
^{13}C- clenbuterol	283.2 → 209.1	0.1	19	17
Clenbuterol	**277.2 → 203.1**	0.1	19	17
	277.2 → 168.1			28
	277.2 → 132.1			27
Ractopamine	302.2 → 284.2	0.1	20	12
	302.2 → 164.1			16
	302.2 → 107.0			30
Brombuterol	367.1 → 349.1	0.1	20	12
	367.1 → 293.0			19
	367.1 → 212.0			31
Mabuterol	311.2 → 293.2	0.1	22	12
	311.2 → 237.1			18
	311.2 → 217.1			25

The major MRM transition is indicated in bold font. A collision gas cell pressure of 4.0×10^{-3} mbar Ar was used.

Table 2
Quattro Ultima mass spectrometer parameters

Compound	MRM transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Salbutamol	239.9 → 221.9	0.1	40	10
	239.9 → 165.9			13
	239.9 → 147.9			19
Zilpaterol	261.9 → 244.0	0.1	40	13
	261.9 → 202.0			19
	261.9 → 185.0			24
Terbutaline	**225.9 → 151.9**	0.1	50	19
	225.9 → 124.9			26
	225.9 → 107.0			31
Cimaterol	219.9 → 202.0	0.1	35	10
	219.9 → 160.0			15
	219.9 → 143.0			22
Fenoterol	304.0 → 285.9	0.08	40	14
	304.0 → 134.9			19
	304.0 → 106.9			29
^{13}C- clenbuterol	**283.0 → 208.9**	0.08	45	17
Clenbuterol	**277.0 → 202.9**	0.08	45	17
	277.0 → 168.0			28
	277.0 → 131.9			26
Ractopamine	302.0 → 284.0	0.08	50	13
	302.0 → 164.0			17
	302.0 → 107.0			30
Brombuterol	367.0 → 348.9	0.08	30	12
	367.0 → 292.9			19
	367.0 → 211.9			31
Mabuterol	311.0 → 292.9	0.08	35	11
	311.0 → 236.9			18
	311.0 → 216.9			25

The major MRM transition is indicated in bold font. A collision gas cell pressure of 2.2×10^{-3} mbar Ar was used.

tion, which is shown in bold font in Tables 1 and 2, was the one used for quantification. Resolution was set to give peak widths at half-height of 0.9 Th for product and precursor ions.

The Premier has a much faster scanning capability (i.e., required shorter interscan delay times) than the Ultima so it was possible to use dwell times of 0.1 s throughout the method compared to the 0.08 s dwell times for the second scan function on the Ultima. Although the faster scanning capabilities of the Premier would have accommodated all MRM transitions in a single time window, two time functions were used to maintain consistency with the Ultima data. Optimal collision energies were similar for the two instruments, but due to a different cone design the optimal cone voltages were different. All liver analyses were conducted using the Pre-

Table 3
Inter-day variability of β-agonist recoveries and suppression in bovine liver

Compound	Transition	Day 1 suppression (% of standard)	Day 1 recovery (%)	Day 2 suppression (% of standard)	Day 2 recovery (%)
Salbutamol	240 → 148	17	65	35	63
Zilpaterol	262 → 185	18	88	58	92
Terbutaline	226 → 152	19	54	41	56
Cimaterol	220 → 160	37	97	68	89
Fenoterol	304 → 135	42	91	64	81
Clenbuterol	277 → 203	43	102	59	96
C_{13}-clenbuterol	283 → 208	55	95	72	95
Ractopamine	302 → 164	21	96	45	91
Brombuterol	367 → 212	30	98	49	95
Mabuterol	311 → 217	42	106	61	93

The recovery of each β-agonist was determined by comparing the signals for liver spiked at 1 ppb before SPE cleanup with those from a blank liver extract fortified with β-agonists at 1 ppb immediately before analysis. Suppression was determined by comparing signals from the fortified sample with those from neat standards.

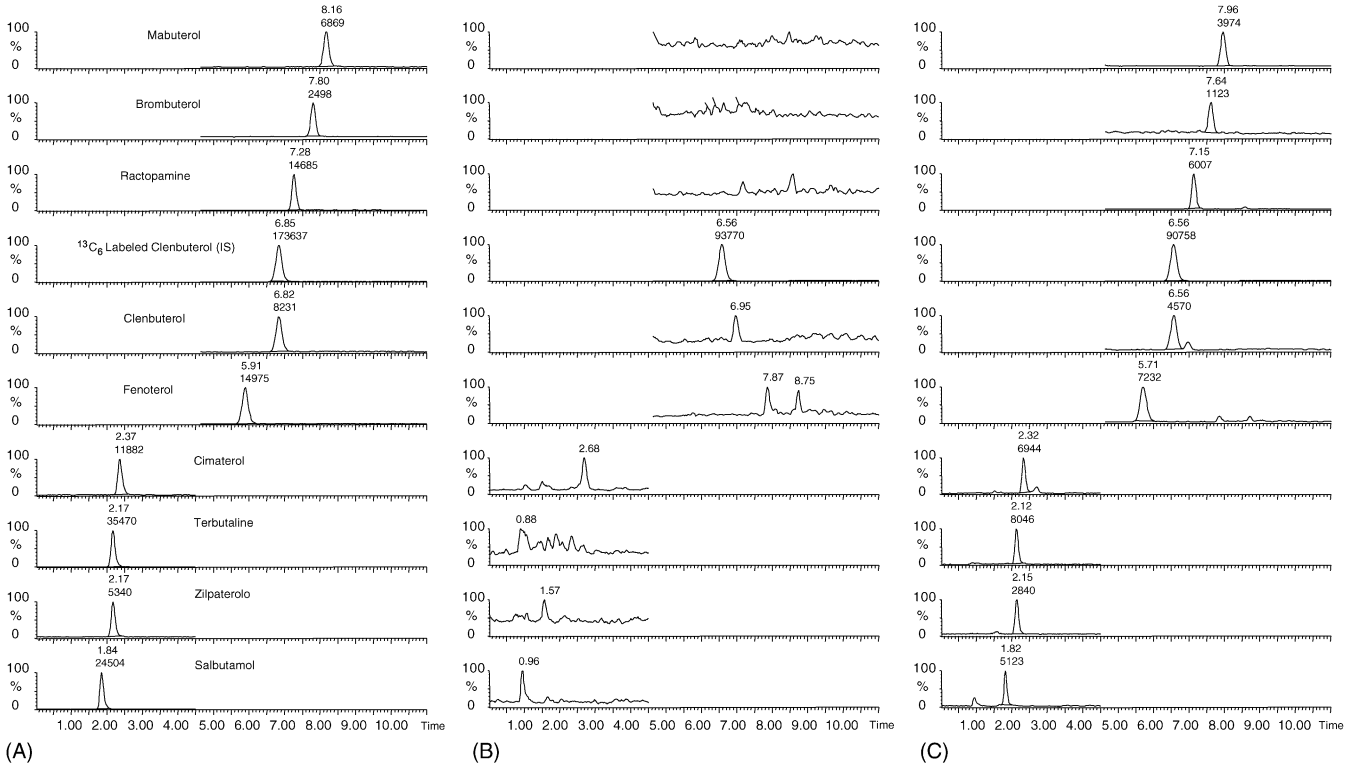

Fig. 2. LC-ES/MS/MS multi-component analysis of various β-agonists. (A) Standards (50 pg) with $^{13}C_6$ labeled clenbutero internal standard (IS, 500 pg). (B) Untreated bovine liver (50 mg equivalents) spiked with 10 ppb IS. (C) Bovine liver (50 mg equivalents) spiked with nine β-agonists at 1 and 10 ppb IS. The MRM transitions from top to bottom correspond to mabuterol (311.0 → 216.9), brombuterol (367.0 → 211.9), ractopamine (302.0 → 164.0), clenbuterol IS (283.0 → 208.9), clenbuterol (277.0 → 202.9), fenoterol (304.0 → 134.9), cimaterol (219.9 → 160.0), terbutaline (225.9 → 151.9), zilpaterol (261.9 → 185.0) and salbutamol (239.9 → 147.9).

mier while retina samples were analyzed using the Ultima. Detailed comparisons of β-agonists on the two instruments showed essentially identical responses.

3. Results

3.1. Method performance

A gradient LC-ES/MS/MS method was developed to separate, quantify using a single internal standard, and confirm the presence of β-agonists in liver and retina tissue using a simple and rugged mixed mode SPE procedure to eliminate interferences. Three MRM transitions were monitored for each compound. Reconstructed chromatograms from the highest intensity transitions, based on signal-to-noise ratio, were used initially to detect the compounds of interest (Figs. 2 and 3) and the other two MRM transitions were used to provide confirmation of their presence when compared to an authentic standard (Fig. 4). Fig. 2 shows chromatograms for nine β-agonists in a standard, blank liver and spiked liver from the strongest transitions. Fig. 3 shows the analogous responses from retina spiked at 1 ppb. Zilpaterol and terbutaline were not resolved chromatographically, but neither was affected because of the specificity of the MRM transitions. A small interference peak was observed in the blank retina close to the retention time for ractopamine but the response was so low as to be negligible.

Tables 3 and 4 show the inter-day recoveries and suppressions observed for liver and retina, respectively. Significant ion suppression was observed for all compounds in fortified liver or retina samples (i.e., blank tissue processed through the SPE procedure to which an authentic standard was added immediately prior to analysis) when compared with an equivalent amount of standards dissolved in the reconstitution solvent. Recoveries were determined by comparing the peak areas of the spike samples to those of the fortified samples. Analyte recoveries were greater than 80% for seven out of the nine compounds analyzed, with salbutamol and terbutaline, the two most polar compounds, being the only exceptions. The recoveries of these compounds were in the range of 50–60% for both liver and retina.

3.2. Method validation

The method was validated for nine β-agonists by replicate analysis ($n = 3$–4) through the SPE and LC-ES/MS/MS procedures on different days using 200 mg of untreated bovine liver or retina spiked at 1 ppb. Injection volumes were kept to 50 μl resulting in 50 mg tissue equivalents being injected

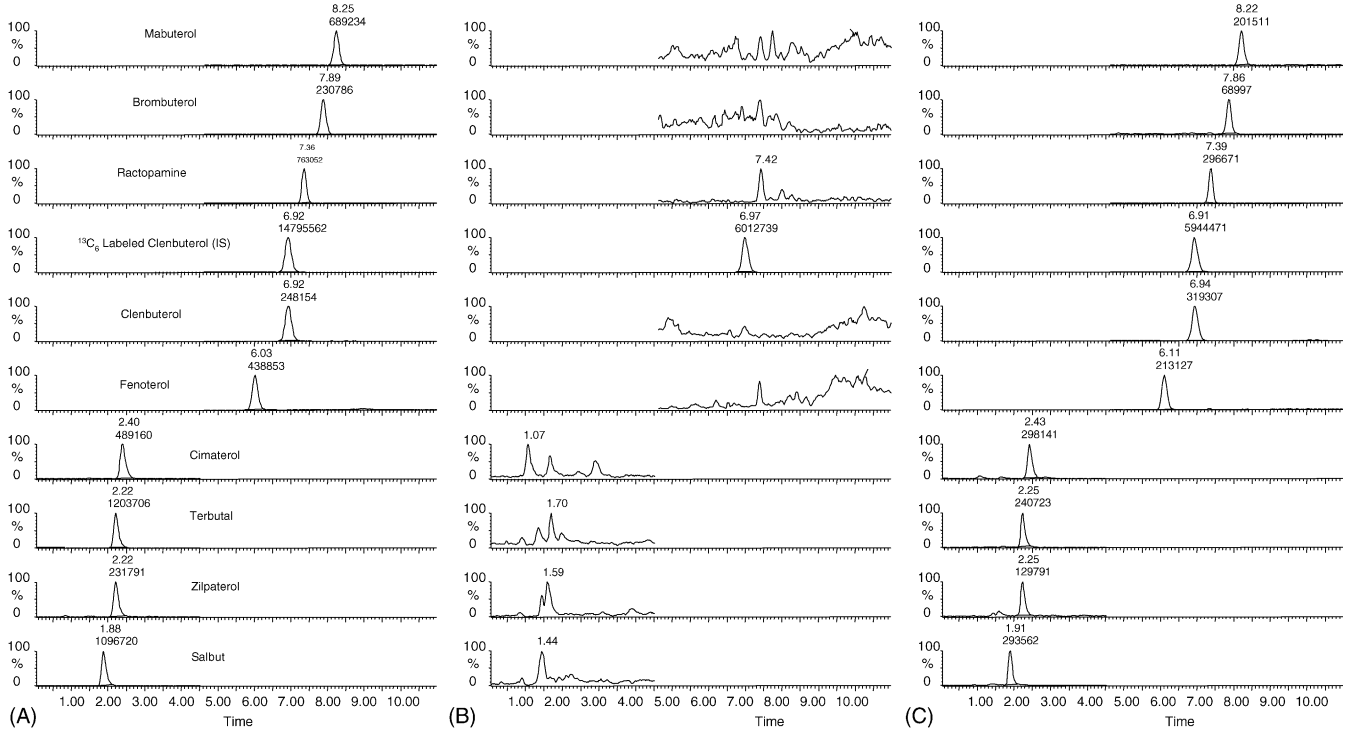

Fig. 3. LC-ES/MS/MS multi-component analysis of various β-agonists. (A) Standards (50 pg) with $^{13}C_6$ labeled clenbuterol internal standard (IS, 500 pg). (B) Untreated bovine retina (50 mg equivalents) spiked with 10 ppb IS. (C) Bovine retina (50 mg equivalents) spiked with nine β-agonists at 1 and 10 ppb IS. The MRM transitions from top to bottom correspond to mabuterol (311.0 → 216.9), brombuterol (367.0 → 211.9), ractopamine (302.0 → 164.0), clenbuterol IS (283.0 → 208.9), clenbuterol (277.0 → 202.9), fenoterol (304.0 → 134.9), cimaterol (219.9 → 160.0), terbutaline (225.9 → 151.9), zilpaterol (261.9 → 185.0) and salbutamol (239.9 → 147.9).

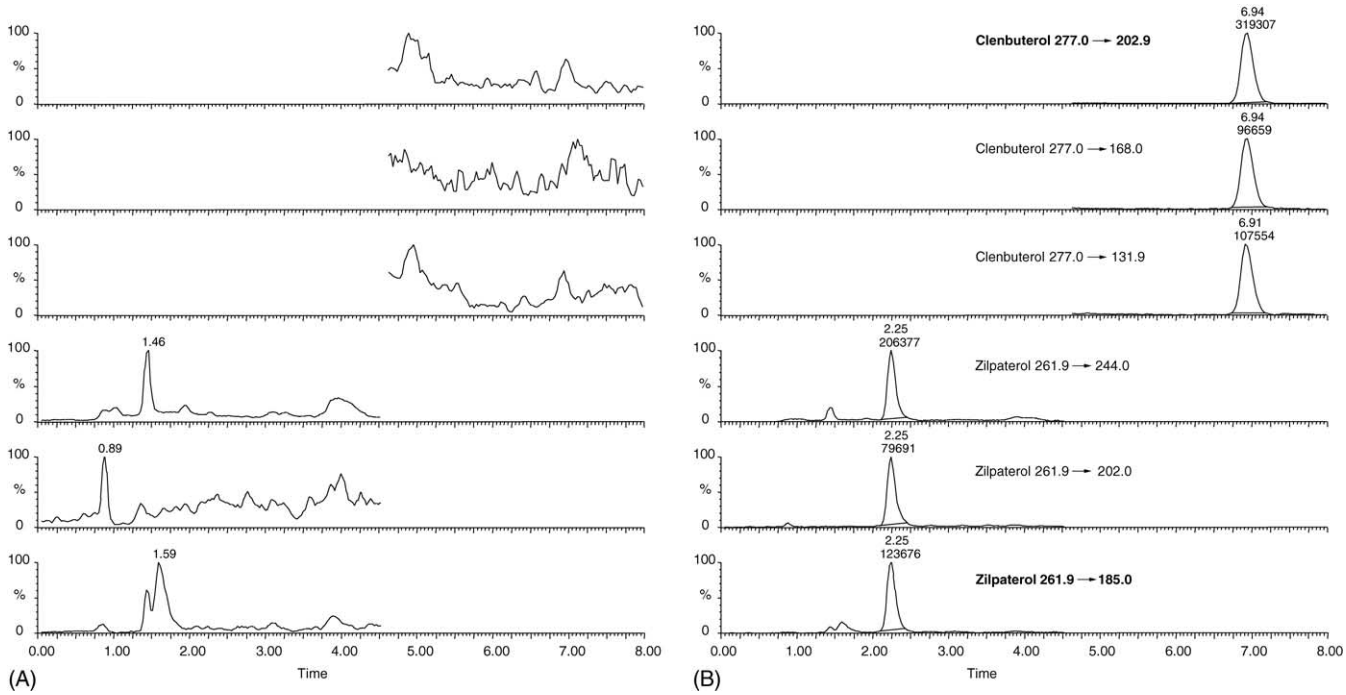

Fig. 4. Confirmatory analysis of zilpaterol and clenbuterol in bovine retina. (A) Three MRM transitions for zilpaterol and clenbuterol in control retina. (B) Three MRM transitions for zilpaterol and clenbuterol in retina spiked at 1 ppb.

Table 4
Inter-day variability of β-agonist recoveries and suppression in bovine retina

Compound	Transition	Day 1 suppression (% response)	Day 1 recovery (%)	Day 2 suppression (% response)	Day 2 recovery (%)
Salbutamol	240 → 148	55	65	44	65
Zilpaterol	262 → 185	80	92	79	92
Terbutaline	226 → 152	51	58	50	57
Cimaterol	220 → 160	78	91	84	96
Fenoterol	304 → 135	79	95	76	88
Clenbuterol	277 → 203	51	96	48	104
^{13}C-clenbuterol	283 → 208	69	94	59	103
Ractopamine	302 → 164	47	98	42	83
Brombuterol	367 → 212	33	99	18	95
Mabuterol	311 → 217	29	100	18	98

The recovery of each β-agonist was determined by comparing the signals for retina spiked at 1 ppb before SPE cleanup with those from a blank retina extract fortified with β-agonists at 1 ppb immediately before analysis. Suppression was determined by comparing signals from the fortified sample with those from neat standards.

onto the LC column. Simultaneous quantification and confirmation were based on the acquisition of multiple product ions. All compounds were quantified using the response ratio of their strongest transition compared to the internal standard, $^{13}C_6$-clenbuterol. As shown in Tables 5 and 6, acceptable inter- and intra-day precision and accuracy were obtained for all compounds spiked into liver and retina at 1 ppb (i.e., R.S.D. 1.2–9.6%, accuracy 58–108%). The accuracy for quantitation of salbutamol and terbutaline deviated significantly from the nominal because the recoveries were lower than the essentially quantitative recovery for the internal standard. For all other analytes, acceptable accuracy was observed.

The basis for MS confirmation involved the acquisition of three MRM transitions for each compound. The product ion spectra for β-agonists are dominated by fragment ions resulting from sequential losses of H_2O, the *N*-alkyl group (e.g., *tert*-butyl or isopropyl), and further fragmentation of the remaining groups as previously reported [6,7]. The strongest transition for each compound was used for computing response ratios of integrated peak areas in relation to the other two transitions. These response ratios were then compared to those produced by an authentic standard for confirmation. Fig. 4 shows representative MRM chromatograms for zilpaterol and clenbuterol in control retina and retina spiked at 1 ppb with each compound. Results for all nine compounds in liver are shown in Table 7 while the corresponding results for retina are shown in Table 8. The transitions shown in bold correspond to the signal to which the others were compared. Table 7 shows one transition from two compounds that were not usable. Firstly the terbutaline transition from 226 → 125 showed a large interference peak from the liver that was present on both days. Also the weaker transition for fenoterol from 304 → 286 was not detectable on day 1, however, was measurable on day 2 even though the approximate signal-to-noise was about 3:1. The confirmatory accuracy for MRM ratios was observed for all other compounds, with both transitions agreeing with standard ratios to within 20% of the absolute value. Intra-day precision of individual ratios was highly reproducible (i.e., relative standard deviations of <12% for liver).

Table 8 shows that in spiked retina samples the ratios for all transitions accurately agreed with the ratios obtained from an authentic standard on both days. The accuracies between sample- and standard-derived ratios were in the range of 1–5% absolute, while the intra-day precision showed relative standard deviations in the range of 1–6% in retina.

Table 5
Liver method validation summary

Compound	Day 1 ppb (R.S.D.)	Day 2 ppb (R.S.D.)
Salbutamol	0.68 ± 0.053 (7.7%)	0.69 ± 0.028 (4%)
Zilpaterol	0.93 ± 0.050 (5.4%)	1.00 ± 0.032 (3.2%)
Terbutaline	0.56 ± 0.031 (5.5%)	0.61 ± 0.032 (5.3%)
Cimaterol	1.01 ± 0.035 (3.5%)	0.97 ± 0.022 (2.3%)
Fenoterol	0.95 ± 0.081 (8.5%)	0.89 ± 0.022 (2.4%)
Clenbuterol	1.09 ± 0.047 (4.4%)	1.05 ± 0.045 (4.3%)
Ractopamine	1.02 ± 0.065 (6.4%)	1.00 ± 0.023 (2.3%)
Brombuterol	1.06 ± 0.055 (5.2%)	1.04 ± 0.063 (6%)
Mabuterol	1.14 ± 0.065 (5.7%)	1.02 ± 0.035 (3.5%)

Untreated bovine liver was spiked at 1 ppb with nine β-agonists on two separate days (results show day 1, $n = 3$: day 2, $n = 4$). The values in ppb were determined based on response ratios with the internal standard and represent means ± S.D. with the relative standard deviation (R.S.D.).

Table 6
Retina method validation summary

Compound	Day 1 ppb (R.S.D.)	Day 2 ppb (R.S.D.)
Salbutamol	0.70 ± 0.016 (2.3%)	0.65 ± 0.047 (7.1%)
Zilpaterol	0.99 ± 0.050 (5.1%)	0.93 ± 0.033 (3.6%)
Terbutaline	0.63 ± 0.013 (2%)	0.57 ± 0.019 (3.4%)
Cimaterol	0.98 ± 0.027 (2.7%)	0.96 ± 0.041 (4.3%)
Fenoterol	0.94 ± 0.080 (8.5%)	0.77 ± 0.033 (4.3%)
Clenbuterol	1.03 ± 0.016 (1.5%)	1.05 ± 0.028 (2.7%)
Ractopamine	1.06 ± 0.022 (2.1%)	0.84 ± 0.080 (9.6%)
Brombuterol	1.06 ± 0.013 (1.2%)	0.95 ± 0.12 (12%)
Mabuterol	1.07 ± 0.030 (2.8%)	1.01 ± 0.051 (5%)

Untreated bovine retina was spiked with 1 ppb concentrations of the various β-agonists on two separate days ($n = 4$). The values in ppb were determined based on response ratios with the internal standard and represent means ± S.D. with the relative standard deviation (R.S.D.).

Table 7
Confirmation of β-agonist residues in bovine liver samples

Compound	Transition	Standard	Liver day 1	Liver day 2
Salbutamol	**240 → 148**	1	1	1
	240 → 222	0.37 ± 0.01 (3%)	0.39 ± 0.03 (6%)	0.33 ± 0.03 (7%)
	240 → 166	0.27 ± 0.01 (2%)	0.23 ± 0.05 (20%)	0.22 ± 0.01 (5%)
Zilpaterol	**262 → 185**	1	1	1
	262 → 244	1.53 ± 0.01 (1%)	1.51 ± 0.06 (4%)	1.71 ± 0.05 (3%)
	262 → 202	0.61 ± 0.02 (4%)	0.78 ± 0.09 (12%)	0.66 ± 0.01 (1%)
Terbutaline	**226 → 152**	1	1	1
	226 → 125	0.27 ± 0.01 (2%)	n/a	n/a
	226 → 107	0.27 ± 0.01 (2%)	0.27 ± 0.01 (2%)	0.27 ± 0.01 (2%)
Cimaterol	**220 → 160**	1	1	1
	220 → 202	1.02 ± 0.02 (2%)	1.08 ± 0.01 (1%)	1.03 ± 0.05 (5%)
	220 → 143	0.94 ± 0.01 (1%)	0.94 ± 0.02 (2%)	0.96 ± 0.03 (3%)
Fenoterol	**304 → 135**	1	1	1
	304 → 286	0.13 ± 0.03 (2%)	n/a	0.14 ± 0.01 (5%)
	304 → 107	1.02 ± 0.02 (2%)	1.03 ± 0.04 (4%)	1.04 ± 0.02 (2%)
Clenbuterol	**277 → 203**	1	1	1
	277 → 168	0.39 ± 0.01 (3%)	0.42 ± 0.02 (5%)	0.40 ± 0.02 (5%)
	277 → 132	0.52 ± 0.02 (4%)	0.49 ± 0.03 (7%)	0.52 ± 0.03 (5%)
Ractopamine	**302 → 164**	1	1	1
	302 → 284	0.51 ± 0.01 (1%)	0.53 ± 0.03 (6%)	0.49 ± 0.03 (5%)
	302 → 107	0.74 ± 0.01 (1%)	0.77 ± 0.04 (5%)	0.74 ± 0.04 (6%)
Brombuterol	**367 → 212**	1	1	1
	367 → 349	1.31 ± 0.04 (3%)	1.32 ± 0.07 (5%)	1.21 ± 0.05 (4%)
	367 → 293	2.12 ± 0.13 (6%)	2.41 ± 0.03 (1%)	2.21 ± 0.08 (3%)
Mabuterol	**311 → 217**	1	1	1
	311 → 293	0.86 ± 0.03 (4%)	0.85 ± 0.04 (5%)	0.77 ± 0.03 (4%)
	311 → 237	2.42 ± 0.06 (2%)	2.38 ± 0.10 (4%)	2.37 ± 0.06 (3%)

The ratios of peak areas for the respective β-agonists for the MRM transitions, relative to that for the major transition (bold text), are shown for liver samples spiked at 1 ppb and an authentic standard (200 pg). The values shown are means ± S.D. (day 1, $n = 3$; day 2, $n = 4$) with relative standard deviations (R.S.D.).

Table 9 shows the estimated limits of quantitation (LOQ) and the limits of confirmation (LOC) for both the liver and retina analysis. Both the LOQ and LOC results were obtained by extrapolating to a signal-to-noise ratio of 10:1, the only difference being that the LOQ uses the strongest transition whereas the LOC uses the weakest of the transitions, both based on the signal-to-noise ratios. The values shown in Table 9 represent the three-transition procedure for all compounds in retina and all but terbutaline, zilpaterol and fenoterol in liver where only two transitions were available. This results in the LOC for these three compounds being closer to the LOQ. In all cases the LOQ was $\leq$0.5 ppb and the LOC was $\leq$1 ppb.

4. Discussion

The β-agonists chosen for this study were primarily those available from commercial sources, including some known to be used legally in specific livestock species in some countries (ractopamine and zilpaterol), and some known to be used illegally based on previous regulatory investigations (e.g., clenbuterol, brombuterol). It was also deemed important to include compounds with a wide range of chemical structures (e.g., halogenated aromatics like clenbuterol and phenolics like ractopamine) with a wide range of polarities over which to validate a generally applicable method for cleanup and detection of β-agonists in tissues. Liver was selected as a tissue for analysis because previous studies that showed that β-agonists could be detected shortly after withdrawal from dosing [3,4,7]. Liver homogenates were treated with β-glucuronidase/sulfatase enzymes to hydrolyze conjugated metabolites of the hydroxylated β-agonists in order to maximize signals due to total β-agonist residues as previously described for ractopamine [7,8]. No evidence was previously observed for ractopamine conjugate formation in retina so enzymatic treatment was not used in this study [7]. Retina was selected because it is a well-established long-lived reservoir for β-agonist residues that is useful for screening weeks and months after drug withdrawal [4,7]. In order to facilitate regulatory enforcement efforts, 1 ppb was chosen as a target tissue level for this method because this level is relevant to β-agonist residues previously found in tissues at extended withdrawal times [7].

When designing a rugged and reliable multi-component screening method for analysis of a wide variety of β-agonists,

Table 8
Confirmation of β-agonist residues in bovine retina samples

Compound	Transition	Standard	Retina day 1	Retina day 2
Salbutamol	**240 → 148**	1	1	1
	240 → 222	0.54 ± 0.01 (1%)	0.53 ± 0.02 (3%)	0.53 ± 0.02 (3%)
	240 → 166	0.33 ± 0.001 (0.4%)	0.33 ± 0.01 (3%)	0.33 ± 0.01 (2%)
Zilpaterol	**262 → 185**	1	1	1
	262 → 244	1.63 ± 0.05 (3%)	1.64 ± 0.03 (2%)	1.59 ± 0.05 (3%)
	262 → 202	0.62 ± 0.01 (1%)	0.63 ± 0.01 (2%)	0.63 ± 0.02 (3%)
Terbutaline	**226 → 152**	1	1	1
	226 → 125	0.35 ± 0.01 (3%)	0.35 ± 0.003 (1%)	0.36 ± 0.02 (5%)
	226 → 107	0.37 ± 0.01 (4%)	0.36 ± 0.01 (2%)	0.37 ± 0.01 (2%)
Cimaterol	**220 → 160**	1	1	1
	220 → 202	1.46 ± 0.03 (2%)	1.43 ± 0.02 (1%)	1.39 ± 0.05 (4%)
	220 → 143	0.81 ± 0.01 (1%)	0.81 ± 0.02 (2%)	0.80 ± 0.02 (3%)
Fenoterol	**304 → 135**	1	1	1
	304 → 286	0.29 ± 0.01 (2%)	0.29 ± 0.01 (5%)	0.29 ± 0.01 (2%)
	304 → 107	1.05 ± 0.03 (2%)	1.04 ± 0.03 (3%)	1.05 ± 0.03 (3%)
Clenbuterol	**277 → 203**	1	1	1
	277 → 168	0.30 ± 0.01 (2%)	0.31 ± 0.01 (3%)	0.32 ± 0.01 (3%)
	277 → 132	0.33 ± 0.004 (1%)	0.34 ± 0.01 (2%)	0.34 ± 0.02 (5%)
Ractopamine	**302 → 164**	1	1	1
	302 → 284	0.87 ± 0.01 (1%)	0.87 ± 0.01 (2%)	0.85 ± 0.04 (4%)
	302 → 107	0.68 ± 0.01 (2%)	0.64 ± 0.03 (4%)	0.63 ± 0.03 (5%)
Brombuterol	**367 → 212**	1	1	1
	367 → 349	1.75 ± 0.04 (2%)	1.69 ± 0.04 (2%)	1.79 ± 0.1 (6%)
	367 → 293	2.95 ± 0.06 (2%)	2.94 ± 0.04 (1%)	2.98 ± 0.09 (3%)
Mabuterol	**311 → 217**	1	1	1
	311 → 293	0.94 ± 0.02 (2%)	1.03 ± 0.01 (1%)	0.99 ± 0.05 (5%)
	311 → 237	2.22 ± 0.05 (2%)	2.31 ± 0.05 (2%)	2.30 ± 0.07 (3%)

The ratios of peak areas for the respective β-agonists for the MRM transitions, relative to that for the major transition (bold text), are shown for retina samples spiked at 1 ppb and an authentic standard (200 pg). The values shown are means ± S.D. ($n = 4$) with relative standard deviations (R.S.D.).

the large range of polarities selected was a significant challenge to both SPE cleanup and LC separation. Initial attempts to use a reversed phase SPE method suggested that pH control is of paramount importance because of the differing pK_a values. It was observed that a slight change in pH results in increased recoveries for some of these compounds but a dramatic reduction for others. Attempts to use a cation exchange method showed that not enough sample cleanup resulted. In our method we used a mixed mode SPE cartridge (HCX) that combines strong cation exchange and C_8 reversed phase interactions and allows for both high recoveries and efficient removal of tissue interferences. The use of a HCX-3 cartridge which encompasses a C_{18} rather than a C_8 reversed phase was tested but the suppression and interference, presumably from non-polar components, was even greater than that observed with the HCX cartridge. On the other hand, the C_4 HCX-5 cartridge was also evaluated but the recoveries for the early eluting compounds were

Table 9
Estimated limits of quantitation (LOQ) and confirmation (LOC) for β-agonists in liver and retina (50 mg tissue equivalents injected)

Compound	Liver LOC (ppb)	Liver LOQ (ppb)	Retina LOC (ppb)	Retina LOQ (ppb)
Salbutamol	0.6	0.3	0.4	0.06
Zilpaterol	0.5	0.2	0.4	0.1
Terbutaline	0.5[a]	0.1	0.4	0.05
Cimaterol	0.25	0.08	0.1	0.1
Fenoterol	0.25[a]	0.2	0.5	0.02
Clenbuterol	0.4	0.25	0.2	0.04
Ractopamine	0.8	0.1	0.15	0.02
Brombuterol	0.6	0.5	1	0.5
Mabuterol	0.25	0.15	0.3	0.15

[a] Matrix interferences from liver obscured the third transition for terbutaline and fenoterol so confirmation was based on the two strongest transitions (three ions). All other confirmations were based on three transitions (four ions).

unacceptably low. Although high recoveries were obtained from liver and retina tissues, significant signal attenuation resulting from ion suppression was observed when compared with neat standards. Despite the suppression effects, excellent sensitivities were observed for all compounds in tissue extracts, which reflects the very high responses observed with ES/MS/MS for these basic drugs. The analytical sensitivity achieved using this method exceeds that previously reported for β-agonists using either immunoassays [1,3,4], GC/MS [5], LC with fluorescence detection [8], or LC/MS [6].

The Betamax base analytical column utilizes a cyanopropyl-bonded stationary phase to provide alternative selectivity to traditional alkyl-bonded columns by increasing the polar interactions with basic analytes. This column enabled the use of 0.1% formic acid (aq) to enhance the sensitivity of positive ion electrospray ionization mass spectrometry and also provided adequate retention and resolution of the β-agonists. This column also provided narrower peak widths compared to the reversed phase columns evaluated and thus improved the overall method sensitivity.

Analysis of β-agonists in both liver and retina gave good quantitation results in terms of precision and accuracy; however, retina samples contained fewer interfering peaks and less suppression was observed when compared to the corresponding liver samples (Tables 3 and 4). These factors made the analysis of β-agonists in retina somewhat more sensitive than that for liver, as shown by the respective inter- and intra-day precision and accuracy data for quantitative determinations. Estimated LOQ and LOC data were similarly affected and overall shows slightly better results for retina than for liver (Table 9).

The U.S. FDA and the EU describe proposed regulations for confirmatory LC/MS methods that must be attained. These criteria state that when using MS/MS in the MRM mode the accuracy determination for a real sample using one ratio (i.e., two transitions involving three ions) must be within 10% of the absolute value produced by an authentic standard to be sufficient for regulatory confirmation; however, if two ratios are used (i.e., three transitions involving four ions) accuracy can be within 20%. The retina samples for all compounds using three transitions met these criteria on both days. For the liver samples, analysis on day 1 showed that the third transitions for zilpaterol, terbutaline and fenoterol fell outside of this 20% limit. The second transitions for all three of these compounds did, however, meet the 10% criteria. The day 2 results were better in that only terbutaline failed in the third transition agreement criterion but its second transition again met the within 10% criterion.

These procedures have been used in conjunction with the USDA-FSIS Western Regional Laboratory to identify, quantify, and confirm β-agonist residues in ovine, bovine, and porcine retina samples that had previously been analyzed and found positive using an ELISA screening procedure (generic bronchodialators, Neogen Co., Lexington, KY). Ractopamine was frequently observed in porcine retina (58–195 ppb), consistent with its approved use in the United States for finishing hogs; however, other unapproved β-agonists were also confirmed (e.g., clenbuterol: 0.2–1.7 ppb; cimaterol: 1.4 ppb; salbutamol: 0.06 ppb; data not shown).

5. Conclusions

This paper describes a rugged and reliable LC-ES/MS/MS method for the simultaneous quantification and confirmation of nine diverse target β-agonists in livestock tissue. This list of β-agonists includes some that are approved for use in food-producing animals by regulatory agencies in some countries, but whose residues are illegal in other countries, as well as others whose use is not approved anywhere. Approximate limits of quantification (S/N ratio 10:1) for all compounds analyzed were at or below 0.5 ppb. The sensitivity obtained for the retina analysis was much better in all aspects compared to those for the liver, probably due to the presence of fewer interferences. Using the FDA and EU regulatory guidelines for confirmatory MS methods based on MRM transition ratios, the limits of confirmation observed were at or below 1 ppb for nine representative β-agonists in both liver and retina. This ability to confirm multiple target β-agonist residues in tissues at this level of sensitivity should be useful in regulatory screening programs to monitor anabolic drug misuse in animals.

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