Simultaneous Determination of Salbutamol, Ractopamine, and Clenbuterol in Animal Feeds by SPE and LC–MS

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method for the simultaneous determination of salbutamol, ractopamine, and clenbuterol in commercial feeds was developed. Samples were extracted with phosphoric acid–methanol solution, and further clean-up was achieved with a C18 cation exchange mixed mode cartridge. Separation of analytes was developed on a C18 column with 0.01M aqueous ammonium formate solution (pH 3.8)–acetonitrile by gradient program, and characterized by LC–MS on a quadrupole detector, in electrospray positive ion mode. This method provides average recoveries for salbutamol, ractopamine, and clenbuterol of 83–110% and coefficients of variation of 1.5–11% in feeds spiked in the range of 0.5–500 mg/kg. The limits of detection and quantification in feeds were 0.01 mg/kg and 0.05 mg/kg, respectively. Such limits are well below the dose of 2–25 mg/kg feed proposed as effective.

Introduction

Phenethanolamine (β-adrenergic agonists) was developed for the therapeutic treatment of asthma and preterm labor in humans and has been used in domestic livestock for the same purposes (1). The prior studies have shown that inclusion of some β-adrenergic agonists, such as clenbuterol and ractopamine, in the diets of meat production animals improved growth performance, carcass characteristics, and efficiency of feed utilization; the beneficial effects on carcass composition were the marked expansion of the muscular mass, together with a decrease in fat accumulation (2,3). As the demand from consumers for production of lean meat increases because of economic advantages, the use of β-adrenergic agonists at doses above therapeutic levels as feed additives for farm animals has increased (4,5). The concentration of β-adrenergic agonists in medicated feeds varies by feed types, which are complete feed, concentrated feed, and premixed in the Chinese market. The complete feed could be fed to the animal directly. For β-adrenergic agonist drugs, the approved doses as additives in complete feed ranged from 2 to 25 mg/kg. Concentrated feed is used to produce complete feed by only adding corn and/or other grains, and the premix is mainly composed of vitamins, trace minerals, and other feed additives. The percentages of concentrated feed and premixed included in complete feed are usually 25–40% and 1–10%, respectively. So the effective inclusion levels of these drugs in concentrated feed and premix should be 2.5–4.0 and 10–100 times higher than that in complete feeds, respectively. The illicit use of these drugs as growth promoters in animals can pose a risk for food safety and consumer protection (6,7). For instance, clenbuterol had led to several cases of intoxication outbreaks in humans in the European Union and China. On the basis of such epidemiological evidence, the use of β-adrenergic agonists is either prohibited in the European Union countries or strongly regulated as in the United States, Canada, and Australia (8,9). The entire class of β-adrenergic agonists is also strictly prohibited for use in feeds and drinking water in China (10). Therefore, it is very imperative to develop a simple and accurate method for the detection of these drugs in animal feed, so producer compliance with the regulations could be effectively monitored.

In the scientific literature, few papers deal with the determination of phenethanolamines in feeds. A method about extraction, clean-up, and gas chromatographic–mass spectrometry (GC–MS) characterization of zilpaterol as a feed additive in fattening cattle was reported by Bocca et al. (2003) (11). One immunoaffinity chromatography (IAC)–GC–MS method for the detection and identification of β-agonists (clenbuterol, ractopamine, and cimaterol) in biological samples and animal feed was reported by Ginkel et al. (12). The other methods for the determination of salbutamol, ractopamine, and clenbuterol in feeds published have mostly been with single drug by high-performance liquid chromatography or GC–MS (13,14,15). Regarding the determination of salbutamol, ractopamine, and clenbuterol, most previously published methods are related to their residues in tissues (16,17,18). However, there have been no reports, so far, on the simultaneous determination of salbutamol, ractopamine, and clenbuterol in feeds by liquid chromatography (LC)–MS. Actually, the matrix of feeds is more variable, especially for concentrated feed and premix, which have high concentrations of vitamins, minerals, and other additives. The previous research results have shown that the extraction and clean-up procedures are the key steps of drug analysis in feeds (19).

The aim of this work was to develop and validate a precise, reliable, and sensitive method that could simultaneously detect and confirm salbutamol, ractopamine, and clenbuterol in feeds, and special attention was paid to sample preparation. This method is used to detect salbutamol, ractopamine, and clenbuterol in complete feed, concentrated feed, and premix for growing-finishing pigs.

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Experimental

Materials and reagents

Sample of drug free feeds for swine were used in the spiked experiments, and all the feeds were made at the pilot workshop of the Ministry of Agriculture Feed Industry Center in Beijing, China.

All chemicals and solvents were analytical or LC grade. They are phosphoric acid, metaphosphoric acid, ammonia solution, formic acid, sodium sulfur, acetic acid, hydrochloric acid, methanol, and acetonitrile. Analytical standards of salbutamol and clenbuterol were bought from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ractopamine analytical standard was a gift from the Ministry of Agriculture Test Centre of Veterinary Drug Residues (Beijing, China).

The stock standard solutions of salbutamol, ractopamine, and clenbuterol were prepared by dissolving in methanol. All the stock standard solutions were comprised of 1 mg/mL each pure standard product. All solutions were left in an ultrasonic bath for 5 min. Spiking standard solutions were obtained by diluting stock solutions 1:10 with 2% acetic acid solution to get 100 µg/mL of solutions. Working standard solutions comprised of 10 µg/mL of each drug were prepared by diluting spiking standard solutions 1:10 with 2% acetic acid solution. The stock standard solutions were stored at + 4°C in the absence of light, and spiking standard solutions and working standard solutions were prepared fresh daily.

Extracted solution was prepared by diluting 3.92 g of concentrated phosphoric acid with 200 mL of water, then diluting with methanol to 1,000 mL. For this solution, the ratio of 0.2 mol/L phosphoric acid solution to methanol was 1:4.

Solid-phase extraction (SPE) columns, Oasis MCX 1 cc (30 mg) purchased from Waters (China), were used for clean-up.

Chromatographic and MS conditions

LC–MS system used for method development consisted of a Waters Alliance 2690 Separations Module, Micromass ZQ, and MassLynx 4.0 software (Waters Company). The LC–MS column 2.1 mm × 150 mm was used in the method development packed with 3.5 µm particles of Symmetry MS C18 from Waters. The mobile phase for LC–MS was 0.01M aqueous ammonium formate solution (pH 3.8, adjusted with formic acid)–acetonitrile. Separation was optimized by changing the composition of the mobile phase in gradient (Table I). The flow rate was at 0.2 mL/min, injection volume was 20 µL. The MS was set to collect pseudo-molecular ion data in positive ion mode (ESI^+) at *m/z* 240 for salbutamol, *m/z* 302 for ractopamine, *m/z* 277 for clenbuterol, respectively, and quantification by external standard. Nitrogen was used as desolvation gas at flow-rate of 300 L/h. Source and desolvation temperatures were 120°C and 300°C, respectively. Potentials applied on the capillary and on the cone were 3 kV and 25 V, respectively.

Table I. Mobile Phase Linear Gradient Program

Time (min)	Mobile phase A*	Mobile phase B†	Gradient curve shape
0	98	2	
5	70	30	linear
15	50	50	linear
16	98	2	linear
25	98	2	

* Mobile phase A: 0.01M aqueous ammonium formate solution (pH 3.8, adjusted with formatic acid).
† Mobile phase B: acetonitrile (LC grade).

Sample preparation and extraction

Typical drug-free commercial feeds for growing-finishing pigs were used in this study, including complete feed, concentrated feed, and premix. Each sample (1,000 g) was processed through the sample splitter until a portion weighing ca 200 g was obtained; then it was ground until all passed a 0.25-mm mesh screen. Unused sample portions may be refrigerated for up to two months.

The described feed sample (complete feed, 5 g; concentrated feed, 3 g; premix, 1 g) was weighed into a 50-mL polypropylene centrifuge tube. Certain amounts of salbutamol, ractopamine, and clenbuterol were spiked into the tube with mixed spiking standard solution, and left to stand at room temperature for 1 h to allow drug-matrix interaction. Next, a volume of 40 mL of 0.2 mol/L phosphoric acid–methanol (1:4) extraction solution was added. The tube was shaken with a vortex mixer until it was homogenized, then centrifuged at 3,500 rpm for 10 min after being shaken on a "wrist action" shaker for 20 min. The supernatant was filtered through a quantitative filter paper into 100-mL volumetric flasks, and then the sediment was re-extracted with two additional 30 mL amounts of 0.2 mol/L phosphoric acid–methanol (1:4) extraction solution. All of the supernatant was combined and diluted to volume with 0.2 mol/L phosphate acid–methanol (1:4) extraction solution. For concentrated feed, the described solution should be further diluted 1:2 with 0.2 mol/L phosphate acid–methanol (1:4) extraction solution, respectively. An aliquot of 4 mL filtrate from complete feed or concentrated feed was decanted into a 10-mL tube and evaporated into dryness at 55°C under a gentle nitrogen stream. Two milliliters of 2% aqueous acetic acid solution was added to the tube, and the tube was shaken with a vortex mixer for 30 s. The solution was further used for clean-up with an SPE column. For premix, 0.1 mL of 1 g/L sodium sulfur ($Na_2S{\cdot}9H_2O$) solution was added into 0.2 mL extract and mixed with a vortex. Next, 4 mL of 2% aqueous acetic acid solution was added and mixed again with a vortex, then centrifuged at 3,500 rpm for 10 min. The supernatant was further used for clean-up with an SPE column.

SPE column clean-up

The clean-up system consisted of a vacuum manifold processing station of Agilent Technologies and a vacuum pump (Agilent Technologies, Palo Alto, CA). The centrifuge used was a Biofuge 22R from Heraeus (Sepatech, Osterode, Germany). The mostatic bath was purchased from Shanghai Jinghong Laboratory Equipment Ltd. Corp. (Shanghai, China).

The column was conditioned with 1 mL of methanol, followed by 2 × 1 mL of water before the extract. One milliliter of sample

solution was prepared as described in the "Sample preparation and extraction" section, then washed and eluted with the corresponding solutions. The flow rate through the SPE column was controlled at *ca.* 2 drops/s by applying positive pressure at the inlet. When the eluting solvent passed through, the column was suctioned to dryness. The eluate was captured in a 10-mL tube and evaporated to near dryness at 55°C in water bath under a gentle stream of nitrogen gas, then 1 mL of 2% aqueous acetic acid solution was added to the tube, and the suitable solution was used for analysis by LC–MS.

Determination of salbutamol, ractopamine, and clenbuterol

The linearities of salbutamol, ractopamine, and clenbuterol were evaluated by spiked feed samples with a mixture of standard solutions to yield 0.5~30 mg/kg. The standard calibration curves were generated by running working standard solution, then plotting recorded peak area versus the corresponding mass of analyte in injected volumes. The precision was expressed with relative standard deviation (RSD) by means of injection of working standard solutions as well as spiked feed samples, and the determination was repeated seven times. The recoveries were evaluated using four levels of concentration lying within the linearity range. These assays were also repeated seven times. The quantification and identification of salbutamol, ractopamine, and clenbuterol were analyzed by injecting 2 µL of the calibration standard solutions and sample extracts for LC–MS.

All samples exceeding the linear calibration range were diluted appropriately and re-injected on the same day of analysis.

Results and Discussion

LC–MS

The chromatogram of the three kinds of β-adrenergic agonists at select ion recording mode is shown in Figure 1. Under moderate ionization condition, abundant pseudo-molecular ions and fragments of analyte were acquired, which is expected for identification purpose in full scan mode as well as quantification purpose in selected ion mode. Figure 2 shows the mass spectra (full scan mode) of salbutamol, ractopamine, and clenbuterol. They were identified according to the retention time of the chromatogram and the mass characterization on ions *m/z* 240, 222, 166 for salbutamol; *m/z* 302, 284, 164 for ractopamine, and *m/z* 277, 259, 203 for clenbuterol.

The molecules of β-adrenergic agonists were singly protonated to form pseudo-molecular ions $(M+H)^+$. Furthermore, $(M+H-H_2O)^+$ fragment ion was obtained with neutral loss of H_2O. The subsequent fragmentation pathways were: clenbuterol, loss of isobutene; ractopamine, loss of 1-hydroxy-3-vinyl; salbutamol, loss of isobutene and H_2O, successively (Table II). Otherwise, *m/z* 279, 261, and 205 for clenbuterol are the isotope peaks of *m/z* 277, 259, and 203, respectively.

The pseudo-molecular ions, 240 for salbutamol, 302 for ractopamine, and 277 for clenbuterol, were chosen in SIR mode for quantification purpose.

Choice of extract solvent and clean-up

Salbutamol, ractopamin, and clenbuterol all belong to the group of adrenergic agonist drugs and have a common phenethanolamine on the molecules, but there is a big difference among their polarities. According to the *pK* of the functional groups present in the molecules studied, salbutamol, ractopamine, and clenbuterol are all easy to resolve in an acidic solution. Methanol has a good solubility to most substances whether polar or non polar. Therefore, the typical inorganic acids and methanol were chosen as main solvents. The efficiencies of the extract solutions were compared among the four solutions, which were 5% hydrochloric acid methanol solution, 0.5% metaphosphoric acid–methanol (1:4, v/v), 0.2 mol/L phosphoric acid–methanol (1:1, v/v), and 0.2 mol/L phosphoric acid–methanol (1:4, v/v). All methods were evaluated using solutions of all drugs at concentration 10 µg/mL. The results indicated that 0.2 mol/L phosphoric acid solution–methanol (1:4, v/v) was the best extraction solvent, the average recoveries for salbutamol, ractopamine, and clenbuterol were 83~110%, which

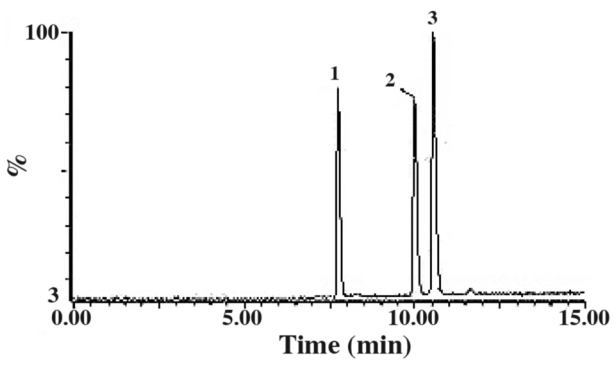

Figure 1. Chromatogram of β-adrenergic agonists at select ion recording mode, simultaneous detection of *m/z* 240, 302, 277 (peak numbers: 1, salbutamol; 2, ractopamine; 3, clenbuterol). Chromatographic and MS conditions: column was 2.1 mm × 150 mm, packed with 3.5 µm particles of Symmetry MS C18; mobile phase was 0.01M aqueous ammonium formate solution (pH 3.8)–acetonitrile; the flow rate was 0.2 mL/min; source and desolvation temperature were 120°C and 300°C, respectively; capillary and cone voltage were 3.0 kV and 25 V, respectively.

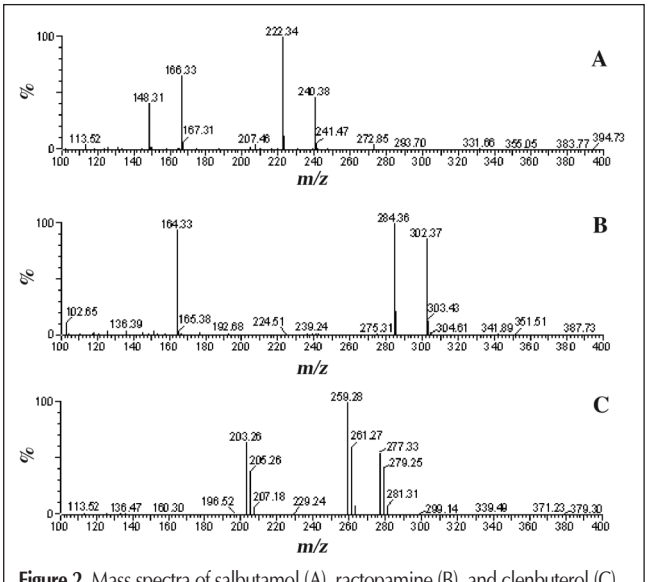

Figure 2. Mass spectra of salbutamol (A), ractopamine (B), and clenbuterol (C).

were much higher than those of the other three kinds of extract solutions.

Feed samples contain significant amounts of salt and proteins that can interfere with detection, especially for premix and concentrated feeds, which are high in vitamins, minerals, and other additives. A significant amount of metal ion, heavy metal ion, and protein present in feedstuffs are precipitated in the presence of phosphoric acid and organic solvent. The other fraction of interferences could be removed in subsequent clean-up procedures by SPE. In this case, the mixed-mode cation exchange column (Oasis MCX) has been chosen for clean-up because the amine of phenethanolamine exits with positive charge under acidic condition. The recoveries of salbutamol, ractopamine, and clenbuterol all exceeded 95% with MCX column for the standard solutions. For feed samples, the results showed that a high percentage of methanol in the extraction filtrate make it difficult to get excellent recoveries of the drugs. Thus, 4 mL of the extraction filtrate from complete feed or concentrated feed was decanted into a 10-mL tube and evaporated into dryness at 55°C under a gentle nitrogen stream, then 2 mL of 2% acetic acid solution was added into the tube, and the tube was shaken with a vortex mixer for 30 s. This solution was further used for clean-up with the SPE column. For extraction filtrate from premix, it contains not only high levels of analytes, but also great amounts of metal ions with two positive charges. To make sure of the excellent clean-up efficiency, the volume of extraction filtrate loaded on column was reduced compared with complete feed or concentrated feed. In order to delete the effect of metal ions with two positive charges on the efficiency with column, disodium ethylenediamine tetraacetate (Na_2EDTA) and sodium sulfur were chosen for this purpose. Each volume (0.05, 0.1, 0.15, and 0.2 mL) of 1 g/L sodium sulfur solution and disodium ethylenediamine tetraacetate solution was added to 0.2 mL of premix extraction filtrate and mixed with a vortex; next, 4 mL of 2% aqueous acetic acid was added and mixed again with a vortex, then centrifuged at 3,000 rpm for 10 min. The supernatant was used for clean-up with an SPE column. The results showed that clean-up efficiency was improved mostly by adding 0.1 mL of 1 g/L sodium sulfur solution to 0.2 mL of premix extraction filtrate. The steps of clean-up with a column are shown in Table III. There are no interferences present on the retention times of salbutamol, ractopamine, and clenbuterol after purified by SPE. The clean-up proves to be effective in removing possible interferences from different types of matrices.

Table II. The *m/z* and Corresponding Fragment Ions of Salbutamol, Ractopamine, and Clenbuterol*

	Characteristic ions (*m/z*)		
Clenbuterol	$(M+H)^+$ 277	$(M+H-H_2O)^+$ 259	$(M+H-H_2O-C_4H_8)^+$ 203
Ractopamine	$(M+H)^+$ 302	$(M+H-H_2O)^+$ 284	$(M+H-H_2O-C_8H_8O)^+$ 164
Salbutamol	$(M+H)^+$ 240	$(M+H-H_2O)^+$ 222	$(M+H-H_2O-C_4H_8)^+$ 166
	$(M+H-H_2O-C_4H_8-H_2O)^+$ 148		

* Electric spray in positive ion mode (ESI^+); capillary voltage, 3.0 kV; source temperature, 120°C; cone voltage, 25 V; desolvation temperature, 300°C; desolvation gas flow, 300 L/h; cone gas flow, 50 L/h.

Table III. Steps and Purposes of SPE in Feed Clean-up

Steps	Operating	Purposes
Equilibration	1 mL methanol/1 mL water	Prepares sorbent for use
Load	Dryness extraction and spiked 1 mL 2% acetic acid solution	Basic analytes are retained under acidic condition by ion exchange mechanism
Wash 1	1 mL of 0.1 M hydrochloric acid solution	Removes protein
Wash 2	1 mL of methanol	Removes non-polar organic interference
Elute	1 mL of 10% ammonia methanol solution	Elutes analytes of salbutamol, ractopamine, and clenbuterol

Table IV. Recoveries of β-Adrenergic Agonists from Spiked Feeds for Growing-Finishing Pig

Feeds	Drugs*	Spiked levels (mg/kg)	Recoveries (*n* =7) Mean (%)	RSD (%)
Complete feed	Sal.	0.5	95.8	3.9
	Rap.	0.5	85.2	4.6
	Clen.	0.5	84.4	5.3
Complete feed	Sal.	2	109.6	4.2
	Rap.	2	86.3	4.7
	Clen.	2	83.3	5.1
Complete feed	Sal.	10	116.0	3.2
	Rap.	10	101.2	3.5
	Clen.	10	103.2	3.9
Concentrated feed	Sal.	50	96.8	10.3
	Rap.	50	107.8	5.3
	Clen.	50	103.6	3.3
Premix	Sal.	500	100.0	10.3
	Rap.	500	94.4	3.6
	Clen.	500	91.9	10.2

* Sal. = Salbutamol; Rap. = Ractopamine; Clen. = Clenbuterol.

Linearity and accuracy

Regression analysis of the data obtained by running 0.05~10 µg/mL working solutions and injecting seven replicates showed the detector response to be linear ($R^2 > 0.998$). The samples were spiked with different levels of β-adrenergic agonists to yield 0.5, 1, 5, 10, 20, 30 mg/kg. The linearities were determined by comparing the analyses of spiked complete feed with those of standard solutions of salbutamol, ractopamine, and clenbuterol. The regression coefficients of salbutamol, ractopamine, and clenbuterol were 0.9950, 0.9973, and 0.9980 in the spiked range

0.5~30 mg/kg, respectively. The results suggested that the linearity of the method analysis could be quantitative by extra-standard method.

The accuracy of the method was evaluated by analyzing three sets of seven replicated 1 to 5 g samples from each feed. Each set was previously spiked with three drugs at different levels based on using dose in animal production (Table IV). Recoveries of these three drugs in feeds were satisfactory. The mean recoveries are all over 90% when the spiked level of the drugs was over 10 mg/kg. The recoveries of the drugs ractopamine and clenbuterol were ~ 85% and 83% at 2 mg/kg and 0.5 mg/kg spiked levels, respectively; but for salbutamol, the recoveries at these levels were relatively higher.

Limits of determination

The limits of detection and quantitation were 0.01 mg/kg and 0.05 mg/kg for salbutamol, ractopamine, and clenbuterol in the feeds, respectively. Such limits are well below the β-adrenergic agonist doses from 2 to 25 mg/kg used as effective in complete feed.

For this proposed method, the limit of detection is the same or lower than that of the methods for the determination of clenbuterol and salbuterol in feeds by GC–MS, which are 0.01 and 0.02 mg/kg, respectively (13,14). The limit of quantitation is lower than that of the method for determination of ractopamine in feeds by LC, which is 0.5 mg/kg (15).

Application

In order to ensure that the proposed method could be used for the determination of ractopamine, salbuterol, and clenbuterol in real feed samples, 15 commercial feed samples for growing-finishing pigs were collected in the Chinese market and detected. The results showed that all samples were negative, which are the same as those detected at the same time with other published methods for single drugs in feeds (13,14,15).

Conclusion

In this study, the extraction process developed was suitable for salbutamol, ractopamine, and clenbuterol in feeds, and it resulted in good acceptable yield and a low amount of co-extracts. Furthermore, no matrix mismatches were observed between different commercial products. Data with good precision and accuracy were obtained by external standard. The method proposed for the determination of salbutamol, ractopamine, and clenbuterol in growing-finishing pig feeds was demonstrated to be highly specific and sensitive, taking into account the doses proposed for feed addition.

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