Original Article

Rapid screening of toxic salbutamol, ractopamine, and clenbuterol in pork sample by high-performance liquid chromatography–UV method

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ABSTRACT

A rapid and simple high-performance liquid chromatography–UV method was developed for the separation and quantification of salbutamol, ractopamine, and clenbuterol in pork. A mixture of acetonitrile–formic acid–ammonium acetate was used as the mobile phase to separate three β-agonists on a C18 column with gradient. The effects of the addition of formic acid and ammonium acetate to mobile phases on the separation of β-agonists were investigated. These additives can greatly improve the resolution and sensitivity. Under the optimized chromatographic condition, this separation does not need extra sample preparation. Complete baseline separation of three β-agonists was achieved in < 20 minutes; the linear range is 0.2–50 μg/L with a correlation coefficient R^2 value of > 0.99. Excellent method reproducibility was found by intra- and interday precisions with a relative standard deviation of < 3%. The detection limit (S/N = 3) was found to be <0.05 μg/L; this method can be used for routine screening of the β-agonist residues in foods of animal origin before being identified by confirmatory methods.

1. Introduction

Βντρ-agonists are phenyl ethanolamines with different substituents on the aromatic ring and on the terminal amino group. These molecules have a powerful pharmacological activity [1]. The main applications in human and veterinary medicine are as tocolytic and bronchodilator agents [2]. However, these compounds are also used as growth promoters in livestock to increase the lean meat-to-fat ratio and improve feed conversion efficiency [3]. Numerous literatures

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have reported various poisoning effects and potential hazards of β-agonists, including cardiac palpitation, tachycardia, nervousness, muscle tremors, and confusion [4]. Therefore, no β-agonists have been permitted in most countries for growth-promoting purposes in farm animals. Therefore, inspection of β-agonists is very important in the food industry.

A survey found that the levels of residues of β-agonists have decreased over the years due to optimization of administration schemes and application of comedication to avoid detection. Many laboratories now use a combination of screening and confirmatory methods to cope with a relatively large number of samples and to increase the reliability of the final result [5]. Confirmatory methods include liquid chromatography/tandem mass spectrometry [6–8] and gas chromatography–mass spectrometry [9,10]. These confirmatory methods based on chromatographic approaches can provide both qualitative and quantitative detection results with satisfactory sensitivity and reproducibility. However, they require expensive instrumentation, and thus are not suitable for routine inspection.

As alternatives of confirmatory methods, various routine screening methods for the determination of β-agonist residues in animal tissue and body fluids have been developed. These methods use immunoassay [11–14], capillary electrophoresis [15], electrochemical sensors [11,16,17], surface plasmon resonance [18], supercritical fluid chromatography [19], chemiluminescence [20], molecularly imprinted polymers [21–23], and high-performance liquid chromatography (HPLC)–UV methods [24]. Among them, HPLC–UV is one of the most widely used methods in all the inspection centers and laboratories, but the current established methods need sample clean-up steps or are able to determine only one β-agonist in one chromatographic run [24–27].

This work was to develop a simple HPLC–UV analytical method for simultaneous determination of several β-agonists without extra sample clean-up steps for routine analysis, which can be used as a screening method for the detection of β-agonists before being identified by confirmatory methods. The linearity of calibration curve, recovery, precision, and lower limit of detection were studied to evaluate the developed method. The method was successfully used to determine the content of β-agonist residues in pork.

2. Methods

2.1. Chemicals, standard solutions, and materials

Three β-agonist standards, including salbutamol (SAL), ractopamine (RAC), and clenbuterol (CLB), were purchased from the laboratories of Dr. Ehrenstorfer (Augsburg, Germany). Methanol and acetonitrile (HPLC grade) were purchased from the Thermo Fisher Scientific Inc (Fairlawn, NJ, USA); β-glucuronidase/aryl sulfatase was purchased from Roche Diagnostica GmbH (Mannhein, Germany). Formic acid (HPLC grade) was obtained from Tedia Company Inc. (Fairfield, OH, USA). Hydrochloric acid and sodium hydroxide were obtained from Sinopharm Chemical Reagents Co., Ltd (Shanghai, China). Ammonium acetate and sodium acetate were obtained from Tianjin Chemical Reagents Company (Tianjin,

China). Deionized water was prepared using a Milli-Q water filtration system (EMD Millipore Corp., Billerica, MA, USA). All solvents for HPLC were filtered through 0.45 mm filters (Millipore Corp.) and degassed in an ultrasonic bath.

Stock solutions of the three β-agonists were prepared by dissolving 5 mg of each compound in 50 mL methanol. The standard solutions were prepared by diluting the stock solutions to 1 μg/mL. As sample matrixes, pork were obtained from supermarkets, free markets, and street vendors, and preserved at −20°C until use.

2.2. Sample preparation from tissue

In pork, β-agonists were extracted using a previously reported method with minor modification [10]. Briefly, aliquots of 2 g finely homogenized pork were transferred into 50 mL conical glass flasks. Eight milliliters of 0.2 mol/L sodium acetate and 50 μL of β-glucuronidase/aryl sulfatase were added and mixed. It was incubated at 37°C for 12 hours. The homogenate was then centrifuged at 132 000 g for 10 minutes, and the supernatant was recovered and transferred to a 50 mL flask. The tissue residue was washed with 5 mL sodium acetate, repeated twice. Then 5 mL 0.1 mol/L perchloric acid was added into 4 mL supernatant, and the pH was adjusted to 1.0 with 1.0 mol/L perchloric acid. The mixture was centrifuged at 132 000 g for 10 minutes again. The supernatant was transferred into another 50 mL centrifuge tube, and the pH was adjusted to 11 with 10 mol/L sodium hydroxide. The mixture was completely extracted with a mixed solution of 10 mL saturated sodium chloride solution and isopropanol–ethyl acetate (6:4, v/v). Then the supernatant was centrifuged at 132 000 g for 10 minutes. The organic phase was transferred into another centrifuge tube, dried under a gentle nitrogen stream, and reconstituted to 1.0 mL with methanol/water containing 0.1% formic acid (1/9, v/v) followed by filtering through 0.22 μm organic filters for HPLC analysis.

2.3. Analysis of β-agonists by HPLC

The analyses were carried using an Agilent Technologies HP1200 series HPLC system equipped with a quaternary gradient pump, a diode array detector, and a Chemstation data-analysis system (Agilent, Palo Alto, CA, USA). Chromatographic separation was performed at 25°C on Agilent Eclipse C18 (250 mm × 4.6 mm i.d., 5 μm particle) analytical columns (Agilent) at a flow rate of 0.8 mL/min via a ternary gradient. Eluent A consisted of 0.1% formic acid, eluent B is 0.1% formic acid in acetonitrile solution, and eluent C is 50 mmol/L ammonium acetate aqueous solution. Separation was performed with a gradient, as shown in Table 1. The injection volume was 20 μL, and the detection wavelength of the detector was set at 225 nm [28].

Table 1 − Gradients used for liquid chromatography.

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0.01	71	4	25
6	55	20	25
19	52	23	25

2.4. Linear range and limit of detection

The linear correlation and dynamic range of β-agonist detection were determined from calibration curves generated by serial analysis of β-agonist standards. A calibration curve for each compound was calculated based on peak areas obtained by serially injecting 20 μL of methanol-diluted solutions of β-agonist standards. Stock solutions of individual β-agonists were serially diluted and spiked into matrix solution extracted from the pork. The lowest detection limits were determined as concentrations corresponding to three times of noise ($S/N = 3$).

2.5. Precision

The precision of the method was evaluated using β-agonist-spiked samples. Five extractions of the same β-agonist-spiked tissue sample were tested consecutively to determine intraday relative standard deviations (RSDs). For interday precision, five extractions of the same β-agonist-spiked tissue sample were tested in three sequences over 5 days.

### 2.6. Recovery

The recovery of the assay was determined by spiking known amounts of standards into test samples. Nine β-agonist-spiked samples and nine control samples were compared. For pork samples, 2 μg, 0.8 μg, or 0.16 μg of the three β-agonists were directly spiked into 2.0 g tissues. The extraction of β-agonists was performed as described in the "Sample preparation from tissue" section. The control samples were subjected to the same procedure as test samples.

3. Results

3.1. Method specificity

Complete baseline separation of three β-agonists was achieved in < 20 minutes. The chromatogram of standards is shown in Figure 1A. The retention times for SAL, RAC, and CLB were 7.92 minutes, 13.09 minutes, and 16.88 minutes, respectively. The optimized gradient is shown in Table 1.
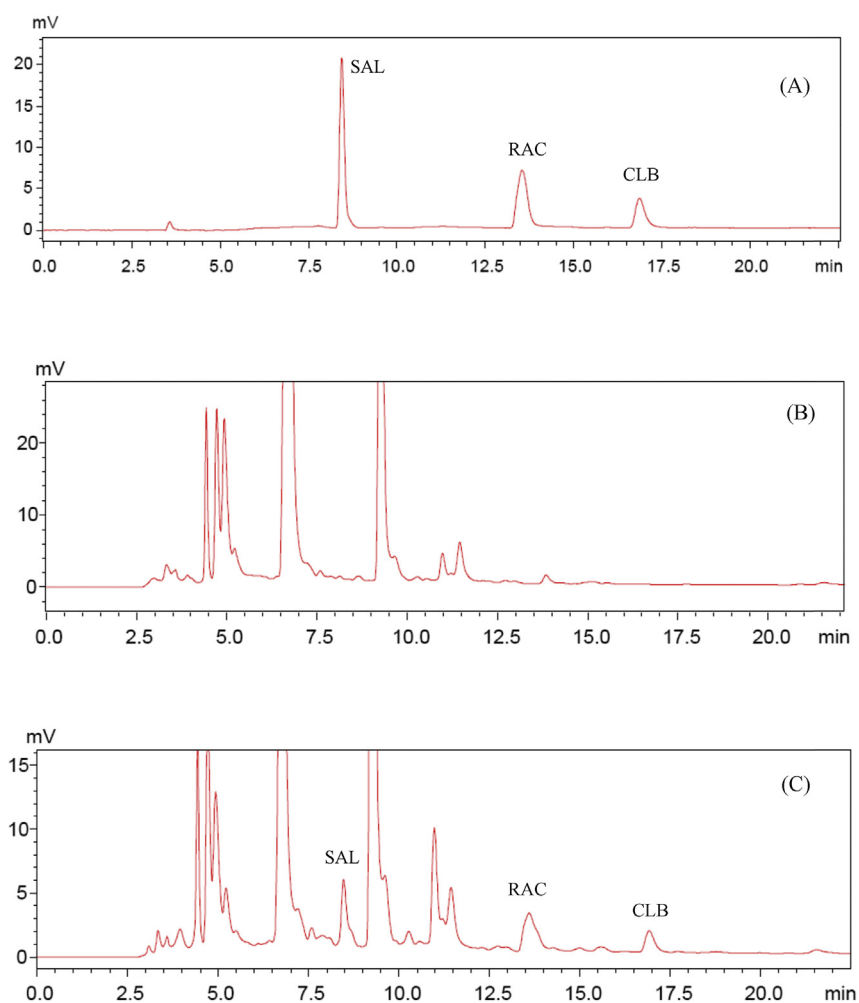
Figure 1 – Representative chromatograms for (A) standards, (B) blank samples, and (C) spiked sample. Mobile phase: eluent A, 0.5% formic acid; eluent B, 0.5% formic acid in acetonitrile; and eluent C, 50 mmol/L ammonium acetate. The gradients are shown in Table 1. CLB = clenbuterol; RAC = ractopamine; SAL = salbutamol.

Table 2 – Linear correlation, ranges, and lower limit of detection of β-agonists selected.

Standard	Regression equation	Coefficient (R^2)	Linear range (µg/L)	Limit[a] (µg/L)
SAL	$y = 739.36x + 9907.5$	0.9970	0.2−25	0.02
RAC	$y = 632.53x - 10048$	0.9992	0.4−50	0.05
CLB	$y = 713.86x - 12933$	0.9901	0.4−50	0.05

CLB = clenbuterol; RAC = ractopamine; SAL = salbutamol.
[a] S/N = 3.

Typical chromatograms of the blank and spiked samples are shown in Figures 1B and 1C. The β-agonists were not detected in blank samples, and no interference peaks were observed at the retention time of β-agonist standards. All the spiked β-agonist standards were well separated and detected with low-abundance peaks from matrix partially overlapped with the β-agonists. The quantitative results were still satisfactory because interference from the overlapped peaks was minimal.

3.2. Linear range and limit of detection

Under optimized conditions, the linear ranges were 0.2−25 µg/L for SAL and 0.4−50 µg/L for RAC and CLB. The correlation coefficients of calibration curves were > 0.99. The lower limits of detection were calculated based on a signal-to-noise ratio of 3, and the values varied from 0.02 µg/L to 0.05 µg/L (Table 2). The method provides sensitive detection with a wide linear range.

3.3. Precision

Five samples extracted from the same β-agonist-spiked tissue (0.008 µg/g) were analyzed consecutively and also in different days ($d = 5$, $n = 5$). The precision was high for both intraday (RSD values 1.61−1.95%) and interday (RSD values 2.74−2.98%) assays. The results are summarized in Table 3. Both inter- and intraday RSDs are < 3%, indicating a high degree of assay precision and reproducibility.

3.4. Recovery

Different samples were selected for maximum variability with respect to provenance. Spiked samples at three levels (1.0 µg/g, 0.4 µg/g, or 0.008 µg/g of tissue) were analyzed to evaluate the recovery (Table 4). Excellent recoveries of 84.9−91.9% for spiking 1.0 µg/g samples and less satisfactory recoveries of 90.1−82.5% and 79.2−88.4% for spiking, respectively, 0.4 µg/g and 0.008 µg/g samples were obtained.

3.5. Application to samples

The method was used to analyze 142 pork samples from the local Food and Drug Administration. The samples were randomly obtained from supermarkets, free markets, and street vendors of the entire Shaanxi Province in China, according to the local government monitoring plan. Five samples collected from free markets were detected to contain β-agonist residues. The SAL contents of two positive samples were 0.14 µg/kg and 1.13 µg/kg. The RAC contents of two positive samples were 0.27 µg/kg and 0.87 µg/kg. The CLB content of one positive sample was 0.65 µg/kg. The results were consistent with that of the local Food and Drug Administration and showed that this method was able to screen samples for β-agonists routinely before performing confirmatory tests.

4. Discussion

SAL and CLB have a similar molecular structure except for different substituent groups on the aromatic ring, but have a significant difference with RAC. These lead to their various hydrophobicity scales, making them difficult to be separated in a short high-performance liquid chromatography program. In this study, mixtures of acetonitrile−formic acid−ammonium acetate were evaluated for their potential to separate β-agonists in a complex matrix. When taking water and acetonitrile as the mobile phase, all the peaks of β-agonist were severely trailing and split into two or more peaks (Figure 2A). The possible reason for multiple peaks, besides a hydrophobic interaction, is that three agonists in this mobile phase can be charged, leading to an ion exchange interaction. Two different interactions lead to the split peaks. No trailing of CLB peak was observed and the peak became

**Table 3 – Analysis precisions[a] for each β-agonist compound.**

Standard	Intraday RSD (%, $n = 5$)	Interday RSD (%, $n = 5$)
SAL	1.61	2.74
RAC	1.83	2.85
CLB	1.95	2.98

CLB = clenbuterol; RAC = ractopamine; RSD = relative standard deviation; SAL = salbutamol.
[a] Spiking 0.008 µg/g in the tissue sample.

Table 4 – Recovery of β-agonists from pork.

Standard	% Recovery		
	Spiking 1.0 µg/g	Spiking 0.4 µg/g	Spiking 0.08 µg/g
SAL	91.9 ± 7.8	90.1 ± 7.1	88.4 ± 6.5
RAC	89.3 ± 5.4	88.3 ± 5.2	85.6 ± 4.8
CLB	84.9 ± 4.7	82.5 ± 5.5	79.2 ± 5.9

CLB = clenbuterol; RAC = ractopamine; SAL = salbutamol.
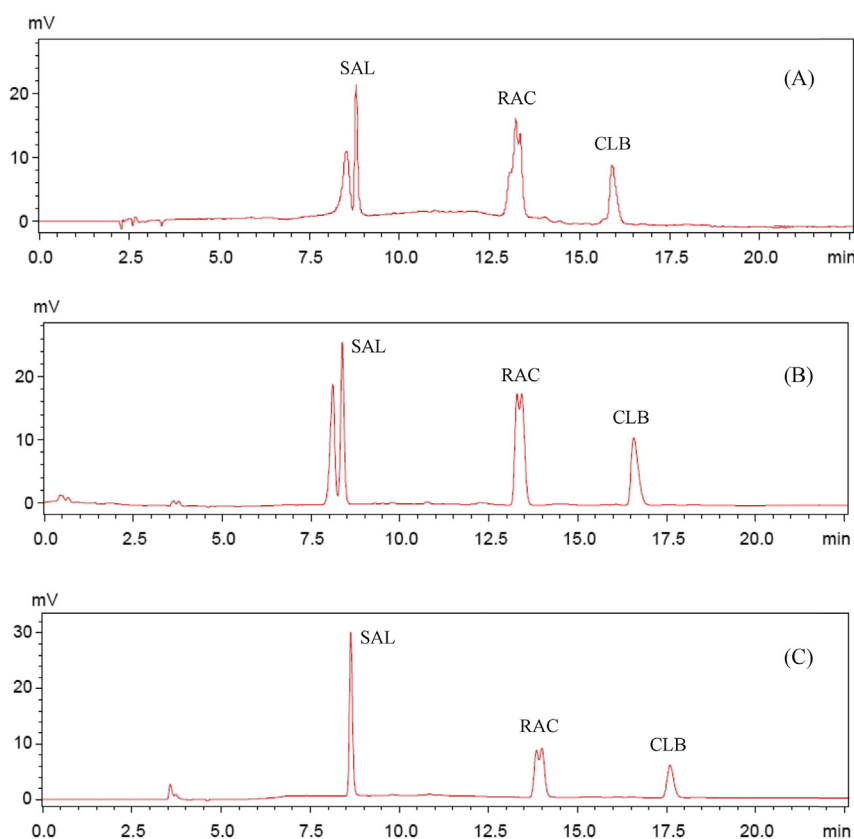
Figure 2 — Chromatograms of β-agonist standards by different mobile phases. Mobile phase: (A) eluent A, water; eluent B, acetonitrile; and eluent C, water; (B) eluent A, 0.05% formic acid; eluent B, 0.05% formic acid in acetonitrile; and eluent C, 0.05% formic acid; (C) eluent A, 0.5% formic acid; eluent B, 0.5% formic acid in acetonitrile; and eluent C, 0.5% formic acid. The gradients are shown in Table 1. CLB = clenbuterol; RAC = ractopamine; SAL = salbutamol.

sharp after addition of 0.05% formic acid (final concentration), although the peaks corresponding to SAL and RAC were still split into two peaks (Figure 2B). With the increasing the formic acid concentration to 0.5%, the ion exchange interaction also became weak, and the peaks of SAL and CLB both became sharper (Figure 2C). We continued to increase formic acid concentration to improve the RAC peak shape, but as evident from the results, higher concentrations of formic acid had no further effect on it. Therefore, we added ammonium acetate into the mobile phase in order to improve the RAC peak shape. Ammonium acetate solution was used as the third eluent. When the concentration of ammonium acetate was 50 mmol/L, RAC also showed one peak (Figure 1A). It is worth noting that ammonium acetate caused broadening of the peaks of SAL and CLB, and decreased their sensitivity.

Most of the reported HPLC methods for β-agonist separation used inorganic acids or salts as an additive to improve the peaks' resolution or shape. In this method, we used formic acid and ammonium acetate as the mobile phase additive, which are easily soluble in organic solvents. When the proportion of water in the mobile phase is low, inorganic acids or salts easily precipitate from the mobile phase; these very tiny

precipitate particles can damage the pump head of HPLC instrument [29]. Therefore, it is very important to HPLC instrument to use organic additives that can be soluble in solvents. In addition, organic additives are compatible with mass spectrum and can be applied in the liquid chromatography—mass spectrometry or gas chromatography—mass spectrometry. Compared with this method, most of the published HPLC—UV and other methods need extra sample clean-up steps, such as solid-phase extraction [24] and solid-phase microextraction [28]; these complex methods need more sample preparation time and may reduce the recovery. After the optimization of the method, sensitivity and quantification limits are slightly higher than the other HPLC—UV methods [26—28].

The extraction procedure for β-agonists did not need extra sample clean-up step, which reduced the sample preparation time. Moreover, as an additive in the mobile phase, formic acid and ammonium acetate can improve the resolution and sensitivity of three β-agonists. Quantitative data showed good precision, linear range, and limit of detection. The method can also be successfully applied to the 142 samples. Results suggested that this method can be used as a screening method to detect β-agonists.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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