Simultaneous Determination of 14 Veterinary Drug Residues in Pork by Liquid Chromatography-Time-of-Flight Mass Spectrometry (LC-TOF/MS)

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Abstract [**Objectives**] This study was conducted to establish an analytical method for the determination of 14 veterinary drug residues in pork using liquid chromatography-time-of-flight mass spectrometry (LC-TOF/MS). [**Methods**] The separation was performed on a Phenomenex Kinetex® column. Qualitative analysis was conducted in multiple reaction monitoring (MRM) mode, and quantification was carried out using the matrix-matched external standard method. [**Results**] Under optimized conditions, the retention times of the 14 prohibited veterinary drugs ranged from 1.2 to 9.0 min. Within the mass concentration range of 5－200 μg/ml, all analytes exhibited good linearity, with correlation coefficients (r) ≥ 0.999 1. The limits of detection (LOD, $S/N=3$) ranged from 0.07 to 0.78 μg/kg, and the limits of quantification (LOQ) ranged from 0.25 to 1.98 μg/kg. The average recoveries at three spiking concentration levels ranged from 82.4% to 104.8%, with relative standard deviations ($n=6$) between 2.8% and 6.9%. [**Conclusions**] This method is simple and accurate, and enables rapid qualitative and quantitative analysis of 14 prohibited veterinary drug residues in pork.

Key words Pork; Liquid chromatography-time-of-flight mass spectrometry (LC-TOF/MS); Prohibited veterinary drugs

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Veterinary drugs refer to substances used for the prevention, treatment, and diagnosis of animal diseases, or for the intentional regulation of animal physiological functions. Prohibited veterinary drugs are substances that are harmful to human health and also cause harm to the environment. There are numerous types of veterinary drugs that may remain as residues in beef, exhibiting diverse physicochemical properties and varying pharmacological effects[1－2]. Therefore, establishing a screening method for prohibited veterinary drug residues in beef can better regulate food safety. On January 6, 2020, the Ministry of Agriculture and Rural Affairs of the People's Republic of China issued Announcement No. 250, which redefined the list of prohibited drugs and chemicals in animal-derived foods, aligning with the standards of the European Union and other countries. The detection methods for veterinary drug residues in animal-derived foods need to make breakthroughs in several aspects. First, in view of the limited variety and number of drugs, traditional detection methods targeting single classes or individual drugs can no longer meet the needs of monitoring. The focus in residue analysis has gradually shifted toward the development of multi-residue detection methods. Second, considering the speed and efficiency of sample pretreatment technologies, high throughput, high efficiency, and simplicity are the current directions for the development of pretreatment techniques[3－5].

In China, fundamental research on detection technologies and relevant limit standards for prohibited veterinary drug residues is still in its initial stages, and a national standard for simultaneous multi-residue detection has yet to be established. Currently, the primary detection methods for prohibited veterinary drug residues include colloidal gold immunochromatography (GICT), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), chromatography-mass spectrometry, and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)[6]. The GICT method has low sensitivity, and a relatively high detection limit, and its results are susceptible to human and environmental factors, resulting in poor reproducibility. Th ELISA method exhibits low specificity, cannot analyze multiple components simultaneously, and is prone to false positives. Liquid chromatography (LC) involves cumbersome procedures, and is time-consuming. It has high costs, and demands high levels of expertise from both the instrumentation and the testing personnel. LC-TOF/MS offers higher sensitivity and improved detection limits, enabling simultaneous analysis of multiple residual compounds while meeting the requirements for trace residue analysis in complex matrices. It is therefore a preferred method for the analysis of prohibited veterinary drug residues[7－10]. In this study, LC-TOF/MS was applied to determine the residues of 14 prohibited veterinary drugs in pork, aiming to provide a reference for standard revision and effective monitoring of such residues in pork.

Materials and Methods

Materials and reagents

Pork (commercially available).

Standard substances of 14 prohibited veterinary drugs: clen-

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buterol, ractopamine, salbutamol, terbutaline, ronidazole, tinidazole, methaqualone, α-zearalanol, β-zearalanol, dienestrol, diethylstilbestrol, hexestrol, sodium pentachlorophenoxide, chloramphenicol (purity ≥ 99.0%, Analysis Capability Assessment System of CAIQ); formic acid, acetonitrile, NH_4AC (chromatographic grade, Sigma-Aldrich, USA); BLC-1 organic filter membrane (0.22 μm, Beijing Guohuan Gaoke Automation Technology Research Institute).

Instruments and equipment

TSQ Altis LC-TOF/MS (Thermo Fisher Scientific); VM-0005M multi-function vortex mixer (LabYeah Biotechnology); Avanti JXN-30 high-speed refrigerated centrifuge (Beckman Coulter); AN-12 automatic nitrogen blowing concentrator (Guangzhou Gedanna Instrument Co., Ltd.).

Experimental methods

Chromatographic conditions The LC separation was performed using a Phenomenex Kinetex® F5 column with an injection volume of 5 μl and a column temperature of 40 ℃. The mobile phase A consisted of an aqueous solution with 0.1% formic acid, and B consisted of acetonitrile with 0.1% formic acid. Gradient elution conditions were as follows: 0.0 – 0.5 min, 5% B; 0.5 – 1.0 min, 5% B; 1.0 – 7.0 min, 30% B; 7.0 – 10.0 min, 95% B; 10.0 – 13.0 min, 5% B. The flow rate was 0.30 ml/min.

Mass spectrometry conditions An electrospray ionization source (ESI^+) was used as in positive mode. The capillary voltage was set at 2.50 kV. The ion source temperature was set at 200 ℃, and the desolvation temperature was set at 520 ℃. Gas flow rates were as follows: desolvation gas at 1 000 L/h, nebulizing gas at 3.00 L/min, and drying gas at 15.00 L/min. The scan time was 0.1 s. Data acquisition was performed in multiple reaction monitoring (MRM) mode.

Preparation of standard solutions Aliquots of 100 μl were accurately pipetted from individual standard solutions of each banned veterinary drug at a concentration of 1 000 μg/ml. They were diluted to a final volume of 10 ml with formic acid to obtain a mixed standard stock solution at a concentration of 10 μg/ml, which was stored in a refrigerator at 4 ℃. Matrix-matched mixed standard working solutions at concentrations of 5, 10, 50, 100, and 200 μg/ml were prepared by diluting the stock solution with a blank sample solution and were used immediately after preparation.

Sample pretreatment Edible portions of pork samples were collected, cut into pieces, and uniformly homogenized using a food processor. The homogenized samples were then added into containers and stored at – 20 ℃ for further analysis.

A 10.0 g pork sample was weighed into a 50 ml centrifuge tube. A mixed standard solution was added (external standard method), followed by 15 ml of 5% formic acid-acetonitrile (1 : 99, V/V) and 0.06 ml of β-glucuronidase. The mixture was vortexed for 10 min and ultrasonicated for 5.0 min, and then subjected to enzymatic hydrolysis in a water bath at 37 ℃ in the dark for 10 h. The inner tube (containing 5 zirconia beads of 4 mm, 200 mg of PSA, 500 mg of anhydrous magnesium sulfate, and 100 mg of C_{18}) was tightly sealed. The sample was then centrifuged at 10 000 r/min for 5 min. The residue was re-extracted with 20 ml of 5% formic acid-acetonitrile by repeating the above operation once. The extracts were combined and evaporated to near dryness using a rotary evaporator in a 50 ℃ water bath, followed by complete drying under a nitrogen stream. The obtained residue was accurately dissolved with 2 ml of 2% formic acid, and the resulting solution was vortexed for 1 min, and filtered through a 0.22 μm microporous membrane prior to analysis.

Results and Analysis

Optimization of chromatographic conditions

The use of an efficient chromatographic column is a prerequisite for detecting residues of prohibited veterinary drugs. In this study, the separation effects of two columns, Phenomenex Kinetex® (100 mm × 2.1 mm, 2.6 μm) and T3 (100 mm × 2.1 mm, 1.8 μm), on the target analytes were investigated. The results showed that the Phenomenex Kinetex® column exhibited strong retention for all 14 prohibited veterinary drugs, with good peak shapes and complete separation of the components, as shown in Fig. 1. Therefore, the Phenomenex Kinetex® column was selected for the analysis of the target analytes in this study.

The separation performance of 0.1% formic acid in acetonitrile and acetonitrile-water as mobile phases was investigated, so as to evaluate their elution effects on the 14 prohibited veterinary drugs. The results showed that when acetonitrile was used as the organic phase, the peak times of the compounds were delayed and the response values decreased. In contrast, 0.1% formic acid in acetonitrile exhibited lower viscosity and stronger elution capability than acetonitrile-water, and resulted in sharper peak shapes. Therefore, acetonitrile was selected. Mobile phases of (1) acetonitrile, (2) 0.1% formic acid in acetonitrile, (3) water, and (4) 0.1% formic acid in water were prepared separately. The experimental results showed that when 0.1% formic acid in acetonitrile was used as the mobile phase, both the response and peak shape of each target analyte were optimal. Under the optimal elution conditions, the retention times of the 14 β-agonists ranged from 1.2 to 9.0 min.

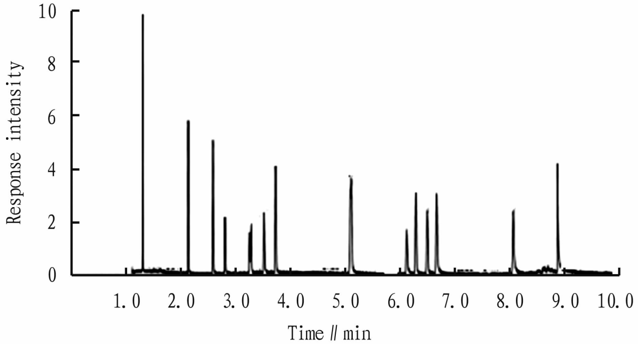

Fig. 1 LC-TOF/MS total ion chromatogram of the 14-standard mixture at a mass concentration of 0.2 μg/ml

Optimization of MS conditions

Standard solutions of the 14 prohibited veterinary drugs at a concentration of 200 μg/L were individually infused into the MS system via a syringe pump for parameter optimization. Full scanning was performed in positive ion mode over a mass range of m/z 200 – 500, and MS spectra of the 14 prohibited veterinary drugs were obtained. The ion with the highest response was selected as the precursor ion, and the declustering potential (DP) was optimized to achieve the strongest signal for the precursor ion. Two ions with relatively high responses were chosen as the quantitative and qualitative ions. In multiple reaction monitoring (MRM) mode, the collision energy (CE) was optimized. The mass spectrometry parameters are shown in Table 1.

Table 1 MS parameters of the 14 prohibited veterinary drugs

Compound	m/z		Declustering potential//V	Collision energy//eV	Retention time//min
	Precursor ion	Product ion			
Clenbuterol	277.2	202.1 *, 135.7	45	50, 20	1.22
Ractopamine	301.4	152.0 *, 124.8	24	36, 24	2.27
Salbutamol	239.3	149.1 *, 221.6	46	15, 25	2.60
Terbutaline	255.3	105.2 *, 139.4	62	29, 51	2.93
Ronidazole	200.2	178.9 *, 106.4	58	22, 47	3.35
Tinidazole	247.3	159.2 *, 200.3	53	19, 30	3.69
Methaqualone	250.3	205.4 *, 262.8	65	16, 25	3.98
α-Zearalanol	322.4	149.8 *, 189.6	60	15, 22	5.36
β-Zearalanol	322.4	246.7 *, 209.3	70	33, 23	6.21
Dienestrol	266.3	180.3 *, 205.1	25	15, 21	6.43
Diethylstilbestrol	268.4	168.4 *, 225.8	15	14, 28	6.67
Hexestrol	270.4	289.6 *, 294.5	20	22, 17	6.99
Sodium pentachlorophenoxide	288.3	246.1 *, 201.5	20	12, 19	8.35
Chloramphenicol	323.1	126.9 *, 90.9	17	10, 25	8.92

* indicates the quantitative ion.

Method validation

Linear range, limit of detection, and limit of quantification

A series of standard solutions at concentrations of 5, 10, 50, 100, and 200 μg/ml were prepared. Standard curves were plotted with mass concentration as the abscissa and peak area of each analyte as the ordinate. The linear regression equations and correlation coefficients are presented in Table 2. The results showed that the 14 prohibited veterinary drugs exhibited good linear relationships within the concentration range of 5 – 200 μg/ml, with correlation coefficients (r) all ≥ 0.999 1. The limit of detection (LOD) was calculated based on a signal-to-noise ratio of 3 ($S/N = 3$), and the limit of quantification (LOQ) was calculated based on a signal-to-noise ratio of 10 ($S/N = 10$). The results showed that the LOD values for the 14 prohibited veterinary drugs ranged from 0.07 to 0.78 μg/kg, and the LOQ values ranged from 0.25 to 1.98 μg/kg (Table 2).

Table 2 Linear relationships, correlation coefficients, limits of detection, and limits of quantification for the 14 prohibited veterinary drugs

Compound	Linear regression equation	Correlation coefficient (r)	LOD//μg/kg	LOQ//μg/kg
Clenbuterol	$y = 5.984x - 1.254$	0.999 4	0.20	0.83
Ractopamine	$y = 6.325x + 1.985$	0.999 2	0.18	0.70
Salbutamol	$y = 5.021x + 0.189$	0.999 3	0.21	1.64
Terbutaline	$y = 8.965x - 1.458$	0.999 2	0.47	1.34
Ronidazole	$y = 9.365x + 1.269$	0.999 5	0.07	0.25
Tinidazole	$y = 6.354x - 1.851$	0.999 6	0.11	1.35
Methaqualone	$y = 8.365x + 1.327$	0.999 2	0.18	0.88
α-Zearalanol	$y = 5.214x + 0.684$	0.999 7	0.78	1.98
β-Zearalanol	$y = 8.369x - 1.985$	0.999 3	0.73	1.61
Dienestrol	$y = 5.214x + 1.351$	0.999 8	0.19	0.93
Diethylstilbestrol	$y = 7.365x + 1.148$	0.999 9	0.08	0.76
Hexestrol	$y = 6.325x - 1.864$	0.999 4	0.54	1.01
Sodium pentachlorophenoxide	$y = 8.365x - 1.395$	0.999 3	0.51	1.28
Chloramphenicol	$y = 7.637x + 1.189$	0.999 1	0.33	1.56

Recovery and precision of the method The 14 prohibited veterinary drug standards were spiked into blank pork sample matrices at three concentration levels: 5.0, 50.0, and 100.0 μg/kg, with three replicates prepared for each level. Each concentration level was measured six times, and the relative standard deviation (*RSD*) of the measured values was calculated. As shown in Table 3, the average recoveries at each spiked concentration level ranged from 82.4% to 104.8%, with RSDs between 2.8% and 6.9%. The results indicate that this method essentially meets the requirements for the rapid determination of prohibited veterinary drug residues in routine pork samples.

Table 3 Recoveries and precision (*RSD*) of the 14 prohibited veterinary drugs ($n=6$)

Compound	Spiking level//5.0 μg/kg		Spiking level//10.0 μg/kg		Spiking level//100.0 μg/kg	
	Recovery//%	*RSD*//%	Recovery//%	*RSD*//%	Recovery//%	*RSD*//%
Clenbuterol	84.6	5.4	89.2	5.1	94.4	4.4
Ractopamine	83.8	6.2	88.3	5.7	95.7	5.2
Salbutamol	84.5	4.8	91.7	4.1	104.8	3.1
Terbutaline	86.6	5.8	92.1	5.3	97.1	4.7
Ronidazole	82.4	6.3	89.6	6.0	96.2	4.2
Tinidazole	84.2	4.9	93.0	4.1	102.7	3.8
Methaqualone	85.2	6.5	89.9	5.8	94.8	5.0
α-Zearalanol	84.8	5.1	98.7	4.4	104.5	2.8
β-Zearalanol	84.3	4.8	97.4	4.6	102.4	3.7
Dienestrol	87.2	6.0	96.1	5.8	101.9	4.7
Diethylstilbestrol	85.3	5.2	97.7	4.7	103.3	3.4
Hexestrol	84.8	6.8	88.3	6.1	98.8	4.1
Sodium pentachlorophenoxide	85.9	6.9	94.1	5.2	97.8	4.8
Chloramphenicol	84.9	5.9	99.5	4.7	101.2	3.8

Conclusions and Discussion

In this study, 0.1% formic acid in acetonitrile was used as the extraction solvent, achieving the optimal extraction efficiency for prohibited veterinary drug residues in pork. The Phenomenex Kinetex® chromatographic column enabled complete separation of the 14 prohibited veterinary drugs, showing good peak shapes and minimal interference from impurities.

Through the optimization of chromatographic and MS conditions, an LC-TOF/MS method for the determination of 14 prohibited veterinary drugs in pork was established. Within the mass concentration range of 5 – 200 μg/ml, the prohibited veterinary drugs exhibited good linear relationships, with correlation coefficients (r) all $\geqslant 0.9991$. The *LOD* values ranged from 0.07 to 0.78 μg/kg, and the *LOQ* values ranged from 0.25 to 1.98 μg/kg. The average recoveries were between 82.4% and 104.8%, with RSDs ($n=6$) ranging from 2.8% to 6.9%. This method is characterized by simple operation, high sensitivity, and good reproducibility. Its precision and accuracy meet the required methodological criteria, making it suitable for the simultaneous determination of residues of the 14 prohibited veterinary drugs in pork.

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