Journal of Chromatography B, 877 (2009) 1915–1922

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous detection of residues of β-adrenergic receptor blockers and sedatives in animal tissues by high-performance liquid chromatography/tandem mass spectrometry

Jing Zhang[a], Bing Shao[a,b,*], Jie Yin[c], Yongning Wu[a], Hejun Duan[a]

[a] Central Laboratory, Beijing Center for Disease Control & Prevention, Beijing 100013, China
[b] School of Public Health and Family Medicine, Capital Medical University, Beijing 100089, China
[c] Department of Nutrition and Food Hygiene, School of Public Health, Peking University, Beijing 100083, China

ARTICLE INFO

Article history:
Received 26 February 2009
Accepted 16 May 2009
Available online 21 May 2009

Keywords:
β-blocker
Sedative
LC–MS/MS
Animal tissues

ABSTRACT

A method using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) was developed to screen and confirm residues of multi-class veterinary drugs in animal tissues (porcine kidney, liver, muscle; bovine muscle). Thirty target drugs (19 β-blockers, 11 sedatives) were determined simultaneously in a single run. Homogenized tissue samples were extracted with acetonitrile and purified using a NH_2 solid-phase extraction cartridge. An Acquity UPLC™ BEH C18 column was used to separate the analytes, followed by tandem mass spectrometry using an electrospray ionization source in positive mode. Recovery studies were done at three fortification levels. Overall average recoveries in pig muscle, kidney, and liver fortified at three levels from 76.4% to 118.6% based on matrix-fortified calibration with coefficients of variation from 2.2% to 19.9% ($n = 6$). The limit of quantification of these compounds in different matrices was 0.5–2.0 μg/kg. This method was successfully applied in screening and confirming target drugs in >200 samples.

1. Introduction

Drugs are being used on a large scale for different reasons in modern farming. β-adrenergic receptor blockers (e.g., carazolol, propranolol, metoprolol) and sedatives are often used to reduce the stress of animals (particularly pigs) during transport to the slaughterhouse. Such stress usually results in a loss of meat quality and even in premature death [1–3]. Sedatives are also illicitly used in animal husbandry to enhance the feed conversion ratio by reducing animal activity. The most frequently used sedatives in animal husbandry are benzodiazepines (e.g., diazepam, nitrazepam), phenothiazines (e.g., chlorpromazine, acepromazine) and butyrophenones (e.g., azaperone) [4,5].

Illicit administration of drugs before slaughter gives rise to drug residues in edible animal tissues because these sedatives and β-blockers are frequently injected just a few hours before slaughter. The health hazard presented by these veterinary drugs in food-producing animals is even more critical than that of other drugs [5]. Previous studies have suggested that sedatives such as chlorpromazine have possible genotoxic activity [6]. Some countries have set maximum residue limits (MRLs) for β-blockers, and prohibited sedatives in foods of animal origin to protect consumer health. The European Commission issued a MRL of 50 μg/kg for azaperone and azaperol (biotransformation product of azaperone) in animal muscles, and a MRL of 5 μg/kg for carazolol in porcine/bovine muscle; it recommends that chlorpromazine should be banned [7].

Detection of the residues of sedatives and β-blockers in biological samples has been reported. Early techniques included radioimmunoassay for screening of carazolol in samples of urine and blood [8], and thin-layer chromatography used in detection of these agents in kidney tissue [9,10]. High-performance liquid chromatography–ultra violet (HPLC–UV) with detection wavelengths in the range 220–254 nm and fluorescence detection (usually for azaperol and carazolol) of tranquillizers in muscle, kidney and liver have been reported [11–13]. Mass spectrometry is becoming the most effective technique because the ionization produces fragments that enable structures to be characterized. Gas chromatography coupled with mass spectrometry (GC–MS) with or without derivatization before injection has also been used to detect tranquillizers (carazolol, azaperone, azaperol, haloperidol, xylazine, phenothiazines) in urine and meat [14,15]. Assays based on liquid chromatography–mass spectrometry (LC–MS), or tandem mass spectrometry (MS/MS), have been frequently used to detect these drugs in animal-based foods in recent years [15–19]. One or two drugs (e.g., azaperone, carazolol) are usually involved in

* Corresponding author at: Beijing Center for Disease Control and Prevention, Beijing 100013, China. Tel.: +86 10 64407191; fax: +86 10 64407178.
E-mail address: shaobingch@sina.com.cn (B. Shao).

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doi:10.1016/j.jchromb.2009.05.025

these methods [16,17]. Multi-residue methods for the detection of carazolol and several sedatives (including azaperone, haloperidol, chlorpromazine, propionylpromazine and xylazine) in animal production by LC–MS/MS were established by Delahaut et al. [18,19]. Lu et al. presented a rapid method for detection of β-blockers in urine samples by capillary electrochromatography–electrospray ionization–mass spectrometry (CEC–ESI–MS) [20]. Based on literature review, a method to simultaneously detect the residues of more than 10 sedative drugs in animal tissues using LC–MS is lacking. Multi-class β-blockers and sedatives may be being used illicitly in veterinary breeding, so developing a detection method is necessary for surveillance purposes.

The aim of this study was to develop a comprehensive method for simultaneous determination of 11 sedatives and 19 β-blockers in porcine muscles, bovine muscles, the porcine kidney and the porcine liver.

Table 2
Target compound concentration by LC–MS/MS in incurred samples.

Test pig	Compound	Residues in tissues (μg/kg)		
		Muscle	Liver	Kidney
Pig 1	Timolol	105.5	35.6	252.4
	Propranolol	65.1	17.9	109.6
	Penbutolol	94.6	11.2	32.9
Pig 2	Oxprenolol	24.2	7.3	50.9
	Metoprolol	90.8	19.8	262.9
Pig 3	Xylazine	42.3	28.8	88.1
	Nitrazepam	72.3	N.D.	1.2
Pig 4	Haloperidol	158.4	313.9	249.8
	Diazepam	23.3	4.9	1.9

N.D.: not detectable.

2. Materials and methods

2.1. Reagents and material

HPLC-grade methanol, acetonitrile, and acetone were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (99%) was from Acros Organics (Morris Plains, NJ, USA). Ultra-pure water was obtained using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). Anhydrous sodium sulfate of analytical purity was obtained from Beijing Chemical Company (Beijing, China).

Solid-phase extraction (SPE) cartridges such as Oasis HLB (150 mg, 6 mL), C18, NH_2 and silica cartridges (500 mg, 6 mL) were from Waters Corporation (Milford, MA, USA).

2.2. Standards

The standards of 19 β-blockers (sotalol, atenolol, pindolol, nadolol, timolol, acebutolol, metoprolol, bunolol, carazolol, celiprolol, oxprenolol, labtalol, bisoprolol, propranolol, alprenolol, betaxolol, carvedilol, nebivolol, penbutolol) were purchased from Dr. Ehrenstorfer Gmbh (Augsburg, Germany). Azaperone, xylazine, chlorpromazine, and acepromazine were from Sigma–Aldrich (St. Louis, MO, USA). Droperidol and haloperidol were obtained from Waco Corporation (Tokyo, Japan). The other drugs (nitrazepam, estazolam, fluphenazine, oxazepam, diazepam) were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All these chemicals were analytical grade >96% purity.

Individual stock standard solution (1000 mg/L) was prepared in methanol and stored in the dark at −18 °C. Serial working solutions were obtained by combining aliquots of stock solutions followed by subsequent dilution with methanol.

2.3. Sample preparation

Aliquots (about 5 g) of homogenized sample (porcine and bovine muscles; porcine kidney and liver) were weighed and transferred into a 50 mL polypropylene centrifuge tube. About 8–12 g of anhydrous sodium sulfate was added until free-flowing powder could be obtained after vortex-mixing. Acetonitrile (10 mL) was added to the mixture, which was vortex-mixed for 2 min. It was then sonicated for 20 min at 40 °C. After cooling to room temperature, the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was decanted into a conical flask. Residues were extracted with another 10 mL of acetonitrile. Supernatants were combined and concentrated to dryness by a rotary evaporator at 40 °C. Residues were re-dissolved for further purification.

Two types of conventional SPE cartridges (reversed-phase (Oasis HLB and C18) and normal-phase (silica and NH_2 cartridge), were used in screening for purification purposes. The operation conditions are summarized in Table 1. The eluate was evaporated to dryness under a gentle stream of nitrogen, and reconstituted with 1 mL of methanol for LC–MS–MS analysis.

2.4. LC–MS/MS analysis

Chromatographic separation was carried out on a Waters Acquity UPLC™ system (Waters Corporation) using an Acquity UPLC™ BEH C_{18} column (50 mm × 2.1 mm; particle size, 1.7 μm). The column oven was 40 °C, the flow rate was 0.45 mL/min, and the injection volume was 3 μL. The mobile phase consisted of water containing 0.1% formic acid (A) and methanol (B). The initial composition was 95% A and 5% B. A gradient elution was carried out whereby phase B was increased linearly to 70% in the first 8 min, increased to 100% in another 1 min, held for 2 min, and returned to the initial composition and equilibrated for 3 min before the next injection.

Mass spectrometry was carried out on a Waters Quattro Ultima Pt mass spectrometer (Waters Corporation, Manchester, UK) using the multiple reaction monitoring (MRM) mode and positive ESI mode. Capillary voltage, extractor voltage, RF lens voltage, and multiplier voltage were set at 3.0 kV, 3.0 V, 0 V and 650 V, respectively. The source temperature and desolvation temperature were held at 100 °C and 350 °C, respectively. The desolvation gas and cone gas were set at a flow of 550 L/h and 50 L/h, respectively. The collision gas by ultra-high-purity argon was held at 0.06 mL/min to maintain the pressure of the collision chamber at 3.4×10^{-3} mbar. MS–MS parameters were optimized in direct flow-injection mode.

Table 1
SPE conditions for purification after acetonitrile extraction upon different cartridges.

Cartridge	HLB	C18	NH_2	Silica
Re-dissolved solution	50 mL 5% Methanol–water solution		0.5 mL Methanol	
Condition	6 mL Methanol and 6 mL water		6 mL Methanol	
Flow rate of sample loading	2–3 mL/min		1 mL/min	
Rinsing solution	6 mL Water		No rinsing	
Eluting solution	6 mL Methanol		5 mL Methanol + acetone (1 + 1) and 5 mL acetone	

Table 3
Mass acquisition parameters for the 30 target compounds.

Compound	MRM transition[a]	Cone voltage (V)	Collision energy (eV)
Sotalol	273.0>255.0	55	9
	273.0>212.8		16
Atenolol	267.1>189.7	50	16
	267.1>115.8		16
Pindolol	249.1>115.8	50	14
	249.1>171.8		14
Nadolol	310.0>254.0	35	15
	310.0>235.9		17
Timolol	317.0>261.1	50	13
	317.0>73.5		19
Acebutolol	337.2>115.8	40	18
	337.2>319.1		14
Metoprolol	268.1>115.8	45	14
	268.1>190.7		14
Bunolol	292.1>235.9	45	19
	292.1>200.9		19
Carazolol	299.2>115.8	50	16
	299.2>221.9		17
Celiprolol	380.2>251	45	20
	380.2>307.2		17
Oxprenolol	266.3>71.5	40	15
	266.3>115.8		15
Labtalol	329.2>311.1	45	10
	329.2>294.2		16
Bisoprolol	326.0>115.7	60	15
	326.0>221.9		11
Propranolol	260.2>115.8	50	16
	260.2>182.7		15
Alprenolol	250.1>115.8	45	15
	250.1>71.8		17
Betaxolol	308.1>115.8	50	17
	308.1>97.7		19
Carvedilol	407.0>223.7	50	19
	407.0>99.8		26
Nebivolol	406.0>150.8	55	27
	406.0>122.8		36
Penbutolol	292.2>236.0	35	13
	292.2>73.7		18
Azaperone	328.1>164.8	40	18
	328.1>120.8		17
Xylazine	221.1>89.7	40	19
	221.1>163.7		19
Droperidol	380.1>193.7	50	13
	380.1>164.8		24
Haloperidol	376.0>164.8	45	21
	376.0>122.7		31
Nitrazepam	282.1>235.9	50	20
	282.1>207.3		28
Acepromazine	327.1>85.8	50	17
	327.1>253.8		21
Estazolam	294.9>266.9	45	22
	294.9>191.6		19
Fluphenazine	438.0>170.9	65	21
	438.0142.9		25
Oxazepam	286.9>269	50	13
	286.9>240.9		18
Chlorpromazine	319.0>85.8	45	15
	319.0>245.9		19
Diazepam	284.9>153.6	40	22
	284.9>192.7		25

[a] The quantitation ion transitions are underlined.

2.5. Method validation

During LC–MS–MS analysis, the electrospray response of the analyte in the biological matrix could be affected by non-drug-related co-eluting components in the original biological sample, even though these components themselves do not possess an electrospray response. This effect usually results in signal suppression or, less frequently, in enhancement of the analyte response [21–23].

Three types of standard calibration curves were prepared to determine signal suppression and absolute recoveries: (1) neat standards curve (plotted by using methanol-dissolved standard solutions from 0.5 μg/L to 100 μg/L); (2) matrix-matched standard curves (plotted by using standards spiked in extracts of blank samples spiked before LC–MS/MS analysis); and (3) matrix-fortified standard curves (plotted by using extracts of blank samples spiked before pretreatment). Signal suppression was carried out following the strategy applied by Matuszewski et al. [24]: the ratio between the slope of matrix-matched standard curves and the

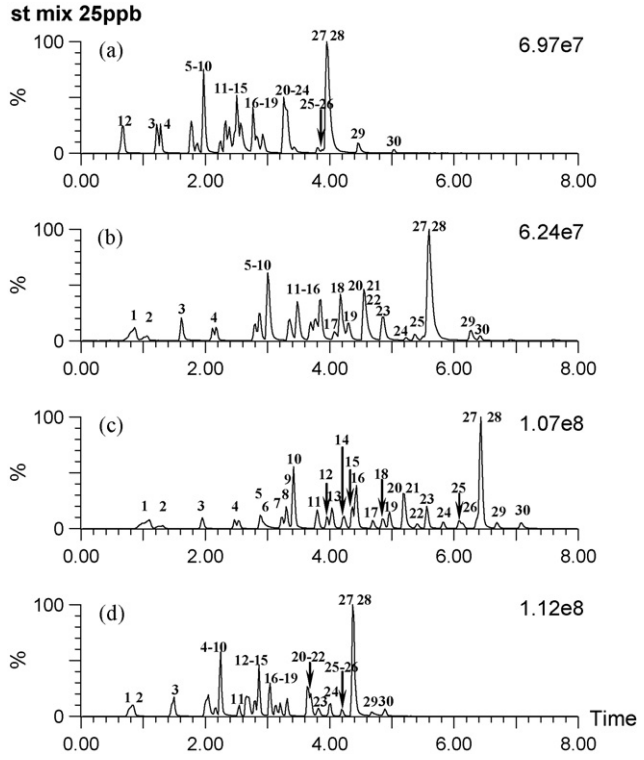

Fig. 1. Total-ion chromatograms of 30 target compounds using different mobile phases: (a) water-acetonitrile, (b) water-methanol, (c) water containing 0.1% formic acid-methanol, and (d) water containing 0.1% formic acid-acetonitrile. (1. sotalol, 2. atenolol, 3. pindolol, 4. nadolol, 5. azaperone, 6. xylazine 7. timolol, 8. metoprolol, 9. acebutolol, 10. bunolol 11. carazolol, 12. celiprolol, 13. oxprenolol, 14. labtalol 15. droperidol 16. bisoprolol, 17. propranolol, 18. alprenolol, 19. betaxolol, 20. nitrazepam, 21. carvedilol, 22. haloperidol, 23. acepromazine, 24. estazolam, 25. oxazepam, 26. nebivolol, 27. chlorpromazine, 28. penbutolol, 29. diazepam, 30. fluphenazine)

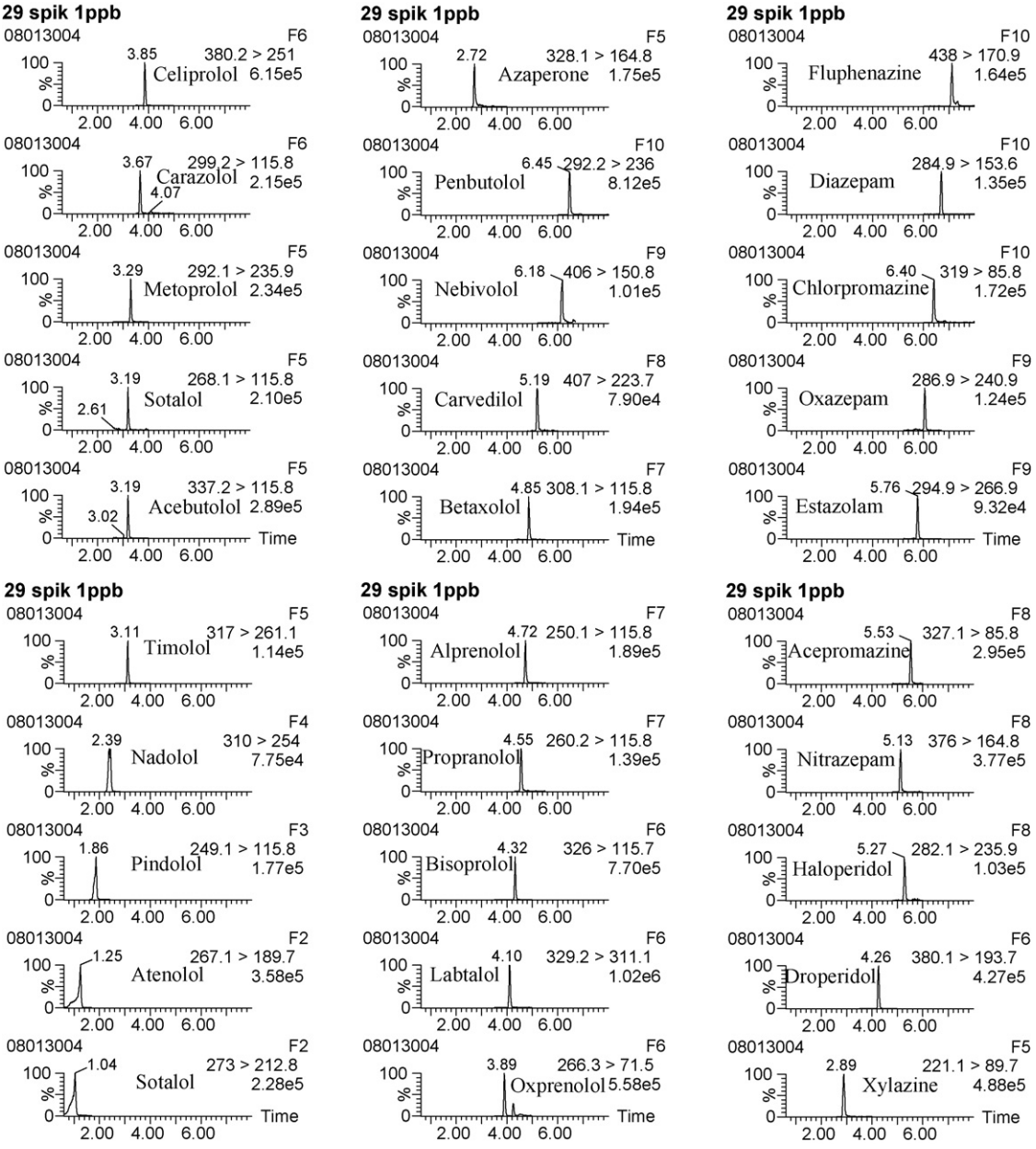

Fig. 2. LC–MS/MS chromatograms of 30 target compounds in a spiked sample of porcine liver.

slope of standard solution curves were subtracted and multiplied by 100 to obtain a percentage. Absolute recoveries were used to evaluate the efficiency of sample preparation, calculated as the slope of matrix-fortified standard curves, divided by the slopes of matrix-matched standard curves, and multiplied by 100 to obtain a percentage.

2.6. Incurred samples and control samples

Control samples and incurred samples were obtained from the hoggery at China Agricultural University (Beijing, China). For incurred samples, four pigs were injected (i.m.) with two or three drugs (0.5 mg/kg body weight (bw); Table 2) and slaughtered 2.5 h later. The care and use of the animals in this study were approved by the Animal Experiment Committee at China Agriculture University.

3. Results and discussion

3.1. Optimization of LC–MS/MS

Mass spectrometric parameters were initially optimized by full scan and daughter scan under positive and negative mode for each compound using direct flow-injection. The $[M+H]^+$ ion was chosen as the precursor ion for all analytes. Table 3 lists the characteristic ions and collision energy for each compound during MRM acquisition.

Mobile phase compositions (i.e., water–methanol and water–acetonitrile) and the concentration of formic acid, usually employed in reversed-phase chromatography and positive ESI mode, were optimized to achieve maximal sensitivity. Results indicated that higher sensitivity and good chromatographic behavior (less tailing) can be achieved if 0.1% formic aid was used (Fig. 1). This

may be because formic acid in water improves the protonation of target compounds during LC–ESI–MS/MS analysis. Comparing the system of 0.1%-formic acid–methanol (Fig. 1c) with that of 0.1%-formic acid–acetonitrile (Fig. 1d), the former exhibited a higher chromatographic separation efficiency than the latter. This was due to the stronger elution capability of acetonitrile onto the C18 column. With respect to sensitivity and resolution, water containing 0.1% formic acid–methanol was more desirable than the others and it was therefore selected as the mobile phase.

Following the conditions described above, LC–MS/MS chromatograms of 30 target compounds in MRM mode are shown Fig. 2 (a spiked sample of porcine liver).

3.2. Sample preparation

3.2.1. Extraction

Octanol–water partition coefficients ($\log K_{ow}$; calculated by Kowwin in EPI suite software, Version 3.11) for the 30 target compounds were from 0.12 (atenolol) to 5.20 (chlorpromazine), demonstrating a wide range of polarity. Acetonitrile, a popular polar organic solvent, was therefore used as the extractant to ensure good extraction efficiencies for all analytes. Anhydrous sodium sulfate was used to dry the sample and improve the extraction efficiency of hydrophobic drugs (e.g., chlorpromazine, diazepam). It was also helpful for the next evaporation procedure.

We tried to directly use concentrated acetonitrile extracts for the analysis to save time and expense. Acceptable results were obtained when samples of porcine muscle and bovine muscle were used. The situation with samples of liver and kidney was quite different: several compounds, including sotalol, atenolol, and azaperone could not exhibit detectable peaks even matched at 20 μg/L, which

Table 4
Matrix effect of target compounds in porcine liver sample extracts using different treating procedure.

Compound	Acetonitrile extracted	NH_2 cartridge purified	Silica cartridge purified
Sotalol	77.9	35.6	45.9
Atenolol	88.6	33.3	30.6
Pindolol	48.7	12.2	21.6
Nadolol	60.9	16.2	20.2
Timolol	58.3	22.8	31.7
Acebutolol	44.8	24.3	30.3
Metoprolol	27.2	12.7	10.1
Bunolol	47.4	33.6	30.0
Carazolol	41.5	27.3	31.7
Celiprolol	52.4	11.8	22.5
Oxprenolol	50.7	16.7	27.6
Labtalol	61.0	13.3	15.9
Bisoprolol	47.9	21.2	24.0
Propranolol	34.5	15.7	24.7
Alprenolol	41.0	0	0
Betaxolol	57.6	15.6	19.6
Carvedilol	47.9	22.9	36.2
Nebivolol	44.1	19.4	24.6
Penbutolol	50.7	0	0
Azaperone	75.5	33.3	36.2
Xylazine	77.9	27.5	28.8
Droperidol	48.3	31.4	39.7
Haloperidol	51.7	24.9	30.0
Nitrazepam	37.9	15.5	36.4
Acepromazine	51.0	31.6	36.9
Estazolam	41.9	21.6	24.7
Fluphenazine	39.9	26	29.7
Oxazepam	41.0	16.4	12.8
Chlorpromazine	34.1	0	19.9
Diazepam	26.4	0	0

Table 5
Recoveries and RSDs of 30 target compounds in different animal tissues ($n = 6$).

Compound	Spiked level (μg/kg)	Porcine muscle		Porcine liver		Porcine kidney		Bovine muscle	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Sotalol	2.0	87.6	13.5	105.5	19.8	98.1	18.2	93.7	8.9
	4.0	90.0	7.9	107.5	19.2	98.4	6.8	100.5	8.6
	8.0	101.6	10.3	101.1	7.9	103.4	13.9	110.5	11.0
Atenolol	2.0	94.4	3.5	98.9	19.2	95.8	10.2	103.6	4.5
	4.0	103.6	11.7	89.7	16.4	100.1	17.4	93.7	10.2
	8.0	94.4	6.6	85.8	7.6	94.2	14.4	100.7	11.1
Pindolol	0.5	98.4	4.6	87.4	12.9	89.9	12.2	94.6	14.6
	1.0	102.8	7.9	84.1	5.5	98.9	12.7	89.6	16.9
	2.0	90.0	6.5	95.8	11.2	103.4	16.1	95.0	14.2
Nadolol	0.5	97.2	7.8	89.8	18.7	100.0	19.3	100.4	9.1
	1.0	94.4	7.6	92.5	8.6	85.7	17.7	97.4	7.9
	2.0	97.6	12.1	90.0	13.3	92.1	11.8	87.7	7.1
Timolol	0.5	96.8	5.8	90.8	9.9	87.4	17.1	84.2	13.0
	1.0	99.6	6.3	99.0	11.8	91.7	11.7	94.0	8.8
	2.0	97.6	9.1	96.0	5.9	99.2	15.5	96.3	4.5
Acebutolol	0.5	109.6	4.4	93.7	18.9	98.4	7.6	98.9	13.2
	1.0	96.8	13.5	100.5	8.6	103.6	14.2	98.6	8.3
	2.0	98.4	7.9	110.5	11.0	104.8	14.9	100.1	11.0
Metoprolol	0.5	99.6	6.7	103.6	19.0	105.6	11.1	102.3	9.6
	1.0	96.4	14.2	93.7	10.2	97.9	16.5	103.3	5.5
	2.0	96.0	5.7	100.7	19.1	100.5	12.1	110.5	8.3
Bunolol	0.5	96.0	9.5	94.6	14.6	101.6	13.9	113.3	6.0
	1.0	98.4	3.9	89.6	16.9	95.8	11.4	102.0	8.0
	2.0	92.0	12.0	95.0	14.2	102.1	11.9	90.7	13.9
Carazolol	0.5	94.8	6.3	87.2	16.4	87.2	13.8	96.2	15.1
	1.0	99.2	7.4	85.3	4.5	91.3	12.1	93.8	4.5
	2.0	101.6	8.8	90.1	7.8	102.1	9.2	97.1	4.2

Table 5 (*Continued*)

Compound	Spiked level (μg/kg)	Porcine muscle		Porcine liver		Porcine kidney		Bovine muscle	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Celiprolol	0.5	96.4	19.3	83.1	15.2	92.5	8.3	87.1	7.9
	1.0	90.4	8.2	89.5	9.2	91.8	6.9	90.6	8.1
	2.0	106.4	10.5	93.5	10.2	95.6	7.8	91.2	6.0
Oxprenolol	0.5	93.5	17.7	103.2	10.5	89.1	18.1	98.2	15.7
	1.0	93.9	12.7	95.2	8.4	88.5	17.4	94.8	14.8
	2.0	91.7	12.6	94.7	6.9	105.6	18.2	101.7	6.2
Labtalol	0.5	91.8	10.1	94.5	16.7	94.2	11.3	105.2	17.6
	1.0	91.0	13.3	97.8	8.1	84.0	19.9	101.3	8.3
	2.0	93.1	9.4	93.2	11.5	86.1	13.2	102.7	5.7
Bisoprolol	0.5	94.3	8.9	87.9	8.6	95.4	12.3	94.1	3.5
	1.0	93.5	9.6	92.8	11.4	76.4	18.7	92.3	7.8
	2.0	87.2	9.8	97.4	9.8	97.3	6.5	97.7	13.4
Propranolol	0.5	93.7	9.3	79.4	13.6	96.0	10.6	86.4	10.3
	1.0	97.1	8.6	85.9	19.7	105.9	10.5	91.3	12.9
	2.0	89.6	10.8	97.2	19.2	95.0	12.5	90.7	11.8
Alprenolol	0.5	91.3	9.2	110.2	8.2	92.3	8.8	106.9	8.9
	1.0	91.4	10.3	103.7	12.7	94.8	10.7	103.5	7.5
	2.0	93.7	9.0	104.2	9.4	89.6	10.6	97.5	6.7
Betaxolol	0.5	91.0	9.4	91.7	10.8	80.3	16.0	98.2	17.2
	1.0	94.5	10.8	86.2	9.2	102.1	14.3	93.7	8.1
	2.0	93.7	9.4	89.9	7.6	93.5	6.7	96.5	7.5
Carvedilol	0.5	94.6	10.1	96.2	8.7	105.1	7.0	86.9	11.5
	1.0	90.3	10.2	89.1	8.0	103.7	11.4	94.5	15.6
	2.0	93.0	8.8	103.2	8.9	103.6	11.3	101.3	12.0
Nebivolol	0.5	93.8	8.4	101.7	12.2	92.6	13.5	95.6	19.1
	1.0	94.9	11.8	90.7	10.6	99.7	9.5	89.8	14.6
	2.0	90.3	13.3	93.0	14.3	102.4	5.6	94.9	10.6
Penbutolol	0.5	89.0	12.1	103.0	13.3	76.8	11.4	77.5	11.5
	1.0	93.2	9.1	82.5	18.2	86.7	8.3	97.4	16.4
	2.0	94.0	10.6	97.4	16.9	106.2	7.0	95.7	12.6
Azaperone	0.5	84.6	13.0	91.0	7.9	98.0	2.2	101.2	15.0
	1.0	90.5	17.0	109.8	9.1	117.2	6.5	111.4	5.4
	2.0	96.0	6.9	76.6	10.3	115.2	10.2	99.4	4.8
Xylazine	0.5	107.8	7.6	105.1	13.9	112.4	6.2	109.5	17.6
	1.0	96.7	9.3	96.8	9.1	98.0	10.6	101.3	15.1
	2.0	113.1	10.3	100.7	8.5	115.6	8.9	95.1	14.3
Droperidol	0.5	104.1	12.7	99.9	14.5	105.4	9.5	113.3	16.4
	1.0	89.7	12.7	105.6	14.8	94.3	6.6	92.7	7.1
	2.0	112.7	13.2	99.5	11.1	92.9	12.4	100.8	6.9
Haloperidol	0.5	99.1	17.2	79.2	9.8	101.3	10.5	102.5	13.6
	1.0	78.4	11.2	94.4	12.1	85.0	7.7	94.9	16.6
	2.0	113.3	4.9	89.9	10.3	103.2	17.0	99.8	13.1
Nitrazepam	0.5	96.3	11.0	84.8	13.4	81.1	15.2	110.8	14.1
	1.0	93.9	15.3	99.5	9.2	79.2	9.2	113.9	9.1
	2.0	106.0	10.5	102.2	15.3	91.2	6.8	94.9	17.6
Acepromazine	0.5	108.6	12.3	86.1	12.7	96.6	14.3	95.5	9.3
	1.0	89.4	10.2	98.6	10.8	108.6	10.6	96.7	8.2
	2.0	114.7	14.7	95.9	14.2	95.1	16.9	96.3	4.3
Estazolam	0.5	99.5	6.9	97.3	19.9	100.0	14.4	95.9	11.0
	1.0	84.3	19.0	84.8	14.1	93.5	10.5	90.8	8.4
	2.0	82.3	10.3	96.0	9.0	98.1	10.9	97.3	8.2
Fluphenazine	0.5	106.8	11.3	96.2	13.3	101.0	14.4	92.0	7.7
	1.0	97.2	8.7	89.8	16.8	92.4	11.3	98.8	14.6
	2.0	95.8	9.8	87.6	16.7	109.0	15.9	116.4	11.3
Oxazepam	0.5	92.5	12.5	100.1	14.1	117.2	11.8	98.9	11.1
	1.0	87.2	18.5	96.0	8.7	96.6	10.0	100.5	7.7
	2.0	113.0	9.4	96.2	13.7	110.0	12.9	93.1	10.8
Chlorpromazine	0.5	98.5	6.1	85.1	9.2	98.5	12.1	97.9	7.2
	1.0	79.2	13.7	79.5	14.8	105.0	14.5	108.7	8.2
	2.0	113.3	8.8	92.1	8.3	85.1	8.0	118.6	3.1
Diazepam	0.5	88.1	12.7	95.2	17.7	96.5	16.1	93.1	11.8
	1.0	93.7	10.2	85.3	12.5	92.0	19.3	93.8	6.7
	2.0	108.1	13.0	93.9	14.1	95.5	14.7	99.3	9.3

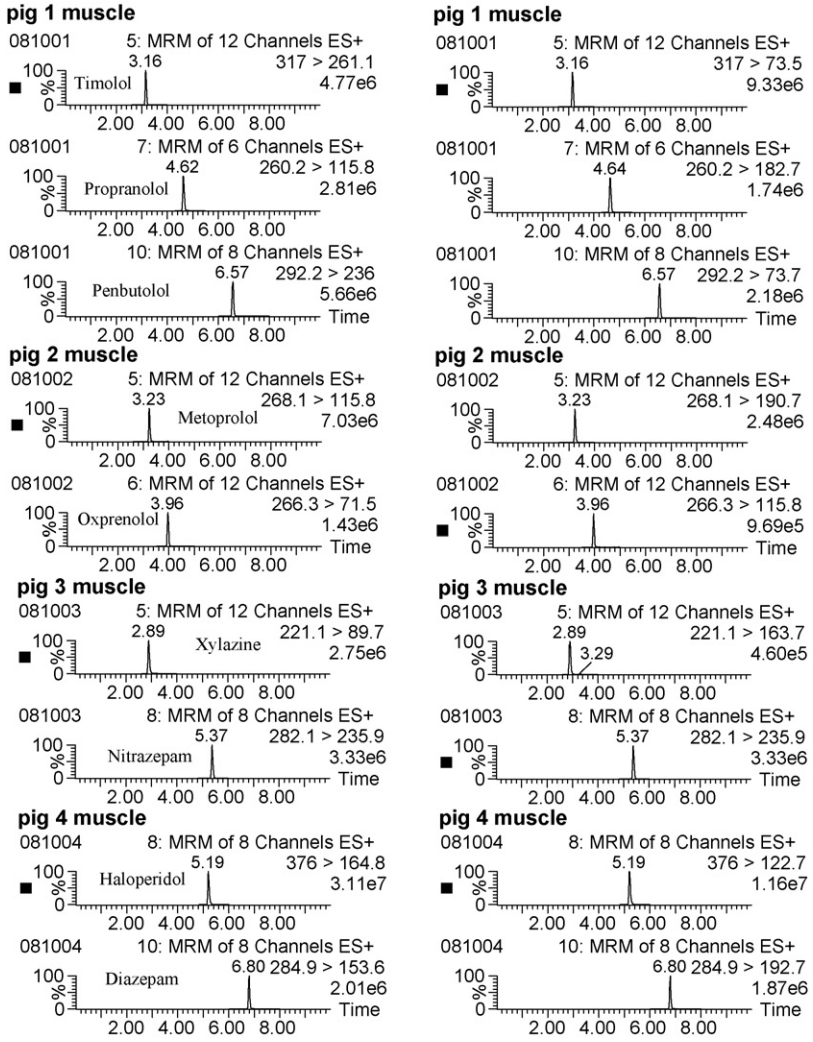

Fig. 3. LC–MS/MS chromatograms of nine analytes in incurred porcine muscle samples.

is 10–50 times higher than the detection limit of the instrument. This can be explained by the high matrix effect of liver and kidney extracts. More than 40% of ion suppression was observed for most of analytes in a porcine liver matrix (Table 4). An additional cleanup procedure was therefore used.

3.2.2. Purification

Several conventional SPE cartridges mentioned in Sections 2.1 and 2.3 were screened for purification. At first, 50 mL 5% methanol–water-dissolved mixture standard (0.5 μg/L for C18 and HLB cartridge) and 0.5 mL methanol-dissolved mixture standard (50 μg/L for NH_2 and silica cartridge) were prepared for the recovery test. Satisfactory recoveries (78–104%) of 30 compounds were observed for all four cartridges.

These cartridges were used in the analysis of spiked liver samples. For the reverse-phase cartridges (C18 and HLB), poor recoveries (<10%) were obtained for chlorpromazine, fluphenazine, diazepam, and penbutolol. This was not attributable to their bonding with the absorbents because no analytes were found in an additional elution using 6 mL of methanol. We found a high fraction of these compounds in the residues of re-dissolved acetonitrile extracts (loading solution). This resulted from drug adsorption (chlorpromazine, fluphenazine, diazepam, penbutolol) onto the matrix residues due to their high hydrophobicity. Increasing the proportion of methanol in the loading buffer may help improve the recoveries of the four analytes, but it will reduce the retention of hydrophilic compounds (e.g., sotalol, atenolol). C18 and HLB were therefore unsuitable for the purification.

Acceptable results for each analyte were achieved using the normal-phase cartridges (NH_2 and silica). In terms of the matrix effect listed in Table 4, the NH_2 cartridge is preferable.

3.3. Method validation

As mentioned in Section 2.5, the effect of co-eluting residual matrix components may result in the suppression or, less frequently, in enhancement of the analyte response. Suppression of the signal was <40% in spiked samples after SPE purification for all compounds; matrix-fortified standard curves for quantitative analysis were therefore prepared from the spiked control sample. Linearity was tested in the range 0.5–50 μg/kg except for sotalol and atenolol (2–100 μg/kg). All showed correlation coefficient (r) values of >0.986 (data not shown), indicating a good correlation for each target compound.

The limit of detection (LOD; defined as the concentration that yields a signal-to-noise (S/N) ratio of 3) was 0.2 μg/kg except for sotalol and atenolol (0.6 μg/kg). The limit of quantification (LOQ; defined as the concentration that yields an S/N ratio of 10 for the chromatographic response) was 0.5 μg/kg except for atenolol and sotalol (2.0 μg/kg).

The procedure of sample preparation was evaluated using a standards spiking test at three levels in four matrices (porcine muscle and bovine muscle; porcine liver and porcine kidney. Recoveries of analytes from the spiked sample ranged from 76.4% to 118.6%. The reproducibility of this method was represented by the relative standard deviation (RSD) percentage at each fortification level for each compound (Table 5). The precision of the method was within 20%.

3.4. Method application

We collected 174 samples of porcine muscle, 16 porcine livers, 7 porcine kidneys, and 29 beef samples from three local markets in Beijing municipality and five local markets in Hebei Province. These samples were analyzed using our developed method. Chlorpromazine and diazepam were found simultaneously in five samples of porcine muscle, with a concentration range of 14.3–93.6 μg/kg and 6.1–41.3 μg/kg, respectively. These drugs have been prohibited for animal feeding in China since 2002 [25]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Union also recommended that chlorpromazine should not be used in food-producing animals [7]. Our results demonstrated that chlorpromazine and diazepam are being used in animal-producing food in China. The highly potent neuroleptic drug haloperidol was detected in two samples of porcine muscle at 8.5 μg/kg and 0.5 μg/kg. Detection of β-blockers was relatively rare: only metoprolol was found in one pork sample at 3.5 μg/kg.

Incurred samples were obtained by treating four pigs by intramuscular injection of each drug (0.5 mg/kg bw) to validate the method (Table 2). Two-and-a-half hours after administration, injected drugs were detected in muscle samples at 23.3–158.4 μg/kg. Concentration levels in incurred liver samples were much lower than that in corresponding kidney samples except for haloperidol and diazepam. Nitrazepam was undetectable in the liver matrix, whereas its residues in muscle and kidney were 72.3 μg/kg and 1.2 μg/kg, respectively. MRM chromatograms of target compounds in incurred muscle samples are shown in Fig. 3.

4. Conclusion

A rapid LC–ESI–MS/MS method was developed for simultaneous analyses of 30 compounds (19 β-blockers and 11 sedatives) in animal tissues. The preparation procedure comprised a simple acetonitrile extraction step, followed by a SPE cleanup using NH_2 cartridges. The method has been fully validated, and is now routinely used in our laboratory for the determination of multi-residues of sedatives and β-blockers in food of animal origin.

Acknowledgments

The authors gratefully acknowledge financial support from the National Key Technology R&D Program (grant number 2006BAK02A27) and the Key Projects of the Scientific Olympics in the Beijing Science & Technology Special Program (grant number Z07001000560708).

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