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High-throughput analysis of tetracycline and penicillin antibiotics in animal tissues using electrospray tandem mass spectrometry with selected reaction monitoring transition

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Abstract

A simple, rapid, and simultaneous analysis method for oxytertracycline, tetracycline, chlortetracycline, penicillin G, ampicillin, and nafcillin in meat has been developed by using electrospray ionization tandem mass spectrometry. The sample preparation was performed by homogenizing with water followed by a centrifugal ultrafiltration, after addition of internal standards (demeclocycline, penicillin G-d5, ampicillin-d5 and nafcillin-d6). The MS/MS analysis involves the combined use of sample enrichment on the short column and a multiple reaction monitoring technique. The overall recoveries from animal (bovine and swine) muscle, kidney, and liver fortified at the levels of 0.05 and 0.1 ppm ranged from 70 to 115% with the coefficients of variation ranging from 0.7 to 14.8% ($n = 5$). Analysis time, including sample preparation and determination, is only 3 h per eight sample and detection limits for all antibiotics are 0.002 ppm. The method is considered to be satisfactory for the rapid screening of the tetracycline and penicillin antibiotic residues in meat.

Keywords: Tetracycline; Penicillins; Animal tissues; ESI LC–MS/MS

1. Introduction

Tetracycline (TC) and penicillin (PC) antibiotics (Fig. 1) are commonly used all over the world as veterinary medicines and feed additives because of their economical advantages [1,2]. In Japan, oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), ampicillin (ABPC), penicillin G (PCG), and nafcillin (NFPC) are approved to use for domestic animals, and they are often used for treatment of mastitis, pneumonia, bacterial diarrhea, and bacterial arthritis in food-producing animals. Such wide utilization may lead to residue problems in livestock production, therefore, maximum residue limits (MRLs) have been established for OTC, TC, and CTC of 0.2–1.2 μg/g in edible animal tissues being sum of them and for PCG of 0.05 μg/g. For ABPC and NFPC, the MRLs of 0.005–0.3 μg/g are going to be established in edible animal tissues to protect the consumers in Japan. One of the major role as public health agencies is to provide safe products for consumers through quantification of these residues in livestock products.

Microbiological assays have been most commonly used to analyse such residues, but they are complicated, time consuming, and non-specific. In contrast, high-performance liquid chromatography (HPLC) is a fast and reliable technique with high sensitivity, so a number of HPLC methods for TC and PC antibiotics in animal tissues have been reported up to date [3–8], yet the simultaneous multi-residue analysis has not been reported, for some properties of these compounds make the multiresidue analysis difficult, as will be described later. Therefore, we wished to establish a simple, rapid, and simultaneous analysis method of tetracycline and penicillin antibiotics in animal tissues.

TC antibiotics have unfavorable properties for developing the method, such as formation of chelate complex with metal ions and binding silanol groups in the stationary phase [1]. However, as we have previously reported in the analytical method of TC antibiotics in animal tissues, we could solve these problems by adding oxalic acid into the mobile phase of HPLC system and adding ethylenediaminetetraacetic acid sodium salt into the extraction solvent adjusted to pH 4.0 [1,9,10]. On the other hand,

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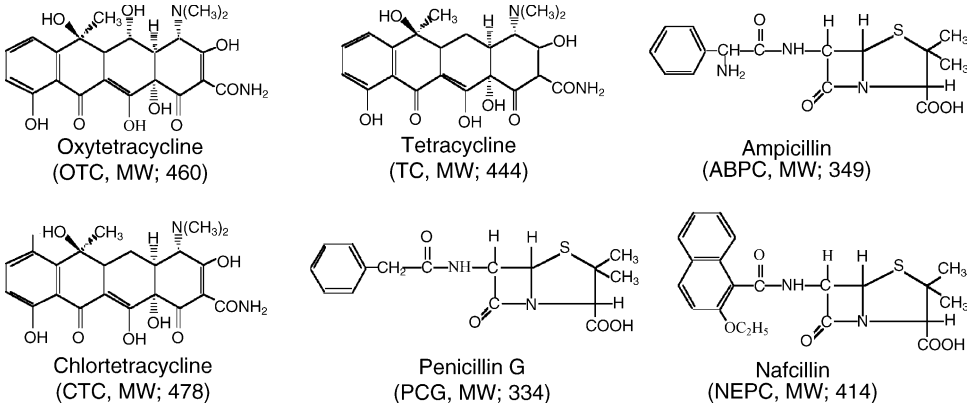

Fig. 1. Tetracycline and penicillin antibiotics.

PC antibiotics decomposes under acidic or basic condition, so we used the neutral mobile phase in LC and purified under the neutral conditions to analyse PC antibiotics [2,11–14]. This makes the simultaneous multiresidue analysis for both antibiotics difficult. Nonetheless, it is possible to establish the multiresidue method if we could obtain the optimal condition by giving a careful thoughts to the chemical properties of the TC and PC antibiotics.

In this paper, we describe in detail the high-throughput simultaneous analysis of TC and PC antibiotics in animal tissues using electrospray tandem mass spectrometry (ESI-MS/MS).

2. Experimental

2.1. Chemicals and reagents

Methanol, distilled water, and formic acid were of HPLC analysis grade.

Potassium salts of PCG, ABPC, and NFPC were obtained from Sigma (St. Louis, MO, USA) and potassium salts of PCG-d5, ABPC-d5, NFPC-d6 from Hayashi (Osaka, Japan). OTC, TC, CTC, and demeclocycline (DMCTC) were supplied by Pfizer (Tokyo, Japan). Each stock solution of the antibiotics and their internal standards were prepared by dissolving 50 mg in 50 mL of distilled water. They were stored in 10 mL light-resistant vials at 5 °C and were stable for up to 1 week.

Amicon Ultrafree-MC and Microcon YM were purchased from Millipore (Bedford, MA, USA).

2.2. Apparatus

The HPLC system consisted of an HP1100 series binary pump, a column compartment and an auto sampler (Hewlett-Packard, Palo Alto, CA, USA). The MS/MS system consisted of a Quattro II triple quadrupole tandem mass spectrometer (Micromass UK, Altrincham, UK) equipped with a Z-spray API source.

2.3. Sample enrichment

Sample enrichment was performed on a TSK-Guardgel ODS-80 Ts column (5 μm, 15 mm × 3.2 mm I.D.; Tosoh, Tokyo, Japan) at 30 °C. The mobile phase consisted of a stepwise gradient. Mobile phase A was distilled water containing 0.05% formic acid. Mobile phase B was methanol containing 0.05% formic acid. The flow rate was 0.2 mL/min. The gradient conditions were as follows, base on time (t) set at the pump: t = 0.00–0.50 min, hold %B = 0; t = 0.51–6.00, hold %B = 100.

2.4. ESI-MS/MS conditions

The desolvation gas (nitrogen) temperature and flow-rate were set at 200 °C and 370 L/h, respectively. The ion source temperature was set at 100 °C. The instrument was operated in the positive and negative ion modes for the tetracyclines and penicillins, respectively, because more abundant ions were observed for tetracyclines in positive mode and for penicillins in negative mode. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 1.9×10^{-3} mbar in the collision cell. The other mass spectrometric parameters are summarized in Table 1.

2.5. Sample preparation

A 5 g aliquot of a representative sample was weighed into a 50 mL centrifuge tube and was added 0.5 mL of a mixed internal standard solution (0.5 μg/mL of PCG-d5, ABPC-d5, NFPC-d6, and DMCTC aqueous solution). The mixture was blended with 5 mL of ultra pure water for 2 min using a high speed blender. After centrifugation (13,000 rpm, 5 °C, 20 min), a 350 μL aliquot of the supernatant was put into the ultrafilter unit (Ultrafree-MC/PB, NMWL = 10,000) that was prewashed by adding 400 μL each of 1.0% Tween 20 and ultra pure water, and centrifuged (13,000 rpm, 20 °C, 30 min). An 50 μL of the filtrate was injected into the LC–ESI-MS/MS system.

2.6. Quantitation

Calibration curves were constructed by peak–area ratios of the antibiotics to internal standards (PCG-d5, ABPC-d5, and NFPC-d6 were used for PCG, ABPC, and NFPC, respectively, and DMCTC was used for OTC, TC, and CTC). Recoveries were

Table 1
ESI MS/MS conditions of the tetracycline and penicilin antibiotics

Antibiotics	Precursor ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Monitor ion (*m*/*z*)	Retention time window (min)
Oxytetracycline	461 $[M+H]^+$	25	20	426 $[M+H–NH_3–H_2O]^+$	3.30–4.10
Tetracycline	445 $[M+H]^+$	25	22	410 $[M+H–NH_3–H_2O]^+$	3.30–4.10
Chlortetracycline	479 $[M+H]^+$	30	20	462 $[M+H–NH_3]^+$	3.30–4.10
Demeclocycline	465 $[M+H]^+$	30	20	448 $[M+H–NH_3]^+$	3.30–4.10
Penicilin G	333 $[M-H]^-$	20	12	192 $[M-H-141]^-$	4.20–5.10
Penicilin G-d5	338 $[M-H]^-$	20	12	197 $[M-H-141]^-$	4.20–5.10
Ampicillin	348 $[M-H]^-$	20	13	207 $[M-H-141]^-$	3.30–4.10
Ampicillin-d5	353 $[M-H]^-$	20	13	212 $[M-H-141]^-$	3.30–4.10
Nafcilin	413 $[M-H]^-$	20	10	272 $[M-H-141]^-$	4.20–5.10
Nafcilin-d6	419 $[M-H]^-$	20	10	278 $[M-H-141]^-$	4.20–5.10

calculated as the ratio of the peak–area ratio of the analyte to the internal standard from the fortified samples to the corresponding peak–area ratio of standard solutions.

3. Results and discussion

In order to develop the simultaneous analysis of TC and PC antibiotics, we ploted our strategy as follows: after the internal standards are spiked into the sample, the antibiotics are extracted from tissue with appropriate solvent. This solvent should not conflict with the unfavorable properties described above. And then, the antibiotics are purified by ultrafiltration. Usually, chromatographic techniques are applied during the sample purification step, however, it is not suited for simultaneous multiresidue analysis due to the conflicting issues of unfavorable properties mentioned earlier. Therefore, the ultrafiltration technique was applied for purification in the present study. For the detection of TC and PC antibiotics, we use electrospray tandem mass spectrometry with sample enrichment using short column.

3.1. Internal standards

In order to improve reproducibility and accuracy, it is essential to use an internal standard. Especially, a stable isotopically labeled internal standard can correct various influences including suppressing or promoting ionization of analyte by sample matrix and extraction efficiency of analytes from sample [15–21]. In this study, there is the possibility of a decrease of the relative contribution of the analyte to the total ion current, because we choose a simple clean up without chromatographic techniques for the purpose of development of a rapid multiresidue analysis, and also extraction efficiency may be decreased, due to extraction of analytes having conflicted chemical properties each other. So we decided to use the commercially available antibiotics labeled with stable isotopes. However, only PCG-d5, ABPC-d5, and NFPC-d6 are commercially available. For tetracycline analysis, we used DMCTC as an internal standard, because we have already reported its effect in our previous paper [22]. All of the precursor ions (molecular ion species) and isotopic ions of the antibiotics and their internal standards were compared with each other, because the overlapping of the ions could lead to serious errors in selected reaction monitoring (SRM) determination using ESI-MS/MS. Both the precursor ions of the antibiotics and those of the corresponding internal standards did not overlap each other, including their isotopic ions. Therefore, we decided that DMCTC was chosen as internal standard for TC antibiotics, and deuterium labeled PC antibiotics (penicillin G-d5, ampicillin-d5, and nafcillin-d6) for PC analysis.

3.2. Sample preparation

Distilled water was used for extraction solvent after careful consideration of properties of the both antibiotics. It is well known that PC antibiotics are easily extracted into water from animal tissues as described in our previous reports [2,11–14]. On the other hand, TC antibiotics are more soluble in acidic condition than in the neutral condition [1], so their extraction efficiencies with water are less than 50% in our preliminary experiments. However, using DMCTC as internal standard will compensate the less effective extraction of TC antibiotics in distilled water used for an extraction solvent.

Next, we compared several types of membranes used in the ultrafiltration and showed the difference in recoveries of the antibiotics, because some membranes sometimes cause irreversible adsorption of the antibiotics. As shown in Table 2, Ultrafree MC/PL membranes gave satisfactory recoveries, however, PB and YM membranes showed poor recoveries on TC, DMCTC, and NFPC. The rate of filtration using MC/PL with nominal molecular weight limit (NMWL) of 10,000 was the fastest. Therefore, we selected MC/PL membrane with NMWL of 10,000 for the ultrafiltration.

3.3. ESI-MS/MS with sample enrichment using short column

For the determination of the antibiotics, we chose ESI-MS/MS that is well suited for ionization of polar compounds with excellent reproducibility. First attempt was made to detect both antibiotics by flow-ESI-MS/MS. However, the sensitivity was not so great in this technique, so we decided to couple short column directly with mass spectrometer and to enrich sample concentration using short column before introduction of the sample into mass spectrometer. As mentioned above, it is

Table 2
Comparison of the membranes on the recoveries of the antibiotics

Membrane	NMWL[a]	Recovery (%)[b]						
		OTC	TC	CTC	DMCTC	PCG	ABPC	NFPC
Ultrafree-MC/PB	5000	84	50	89	69	100	100	61
Ultrafree-MC/PB	10000	98	59	102	58	98	99	70
Ultrafree-MC/PL	5000	100	89	102	95	99	99	89
Ultrafree-MC/PL	10000	100	92	99	98	98	100	92
Microcon YM-3	3000	90	58	82	63	90	97	75
Microcon YM-10	10000	107	68	101	67	85	98	71

[a] NMWL, nominal molecular weight limit.
[b] $n = 3$, A 0.5 mL of a standard solution containing 0.5 μg each of anatibiotics was put into the ultrafilter units pretreated by adding 400 μL of 1% Tween 20 and centrifuged at 13,000 rpm for 5 min.

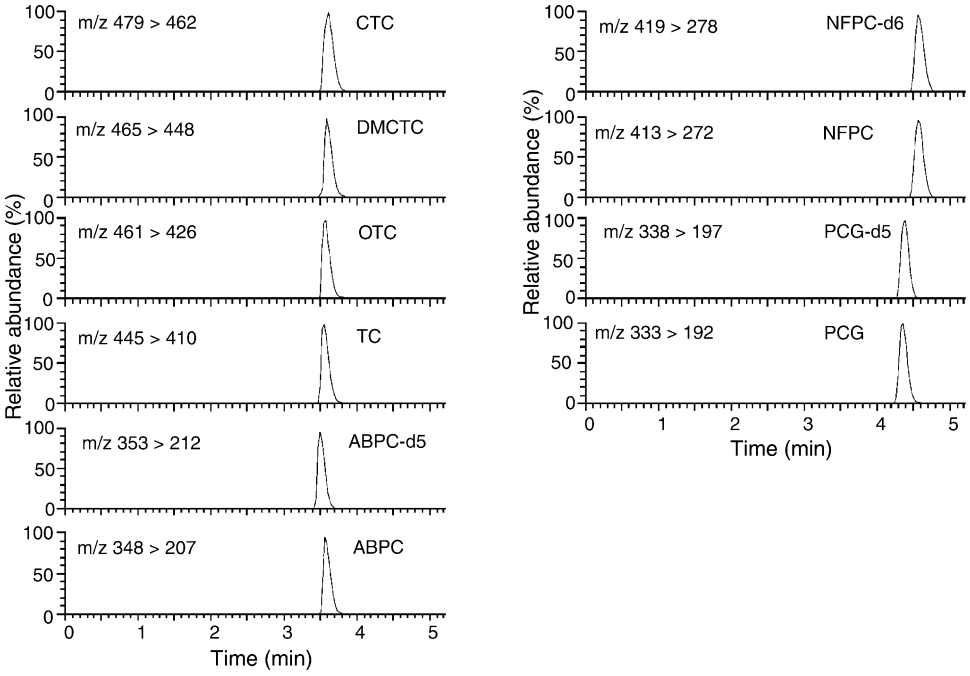

Fig. 2. SRM chromatograms of standards of tetracycline and penicillin antibiotics (each of 0.1 ppm) under ESI LC–MS/MS.

Table 3
Recoveries of tetracycline and penicillin antibiotics from bovine tissues

Antibiotics	Fortified (ppm)	Muscle		Kidney		Liver	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
OTC	0.05	102.9	2.3	70.4	2.0	78.0	2.6
	0.1	99.6	2.3	79.8	3.0	85.3	2.2
TC	0.05	102.1	2.3	67.9	1.6	80.5	2.8
	0.1	97.0	2.4	82.9	4.3	83.7	4.0
CTC	0.05	88.2	2.7	88.4	2.9	105.6	3.8
	0.1	88.3	2.9	97.3	2.8	101.5	4.3
ABPC	0.05	103.1	5.2	96.1	2.4	102.3	9.8
	0.1	104.4	3.8	103.8	4.5	106.6	5.7
NFPC	0.05	96.2	3.4	96.1	3.0	100.1	2.9
	0.1	99.4	1.5	98.7	2.2	98.2	2.2
PCG	0.05	95.9	5.1	97.2	1.9	96.4	2.9
	0.1	99.6	1.7	98.7	2.6	97.9	4.5

$n = 5$.

Table 4
Recoveries of tetracycline and penicillin antibiotics from swine tissues

Antibiotics	Fortified (ppm)	Muscle		Kidney		Liver	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
OTC	0.05	73.6	2.8	72.3	2.3	75.8	2.2
	0.1	79.2	1.9	83.6	2.5	79.0	4.1
TC	0.05	67.1	3.6	69.3	1.3	72.7	2.7
	0.1	73.3	2.4	85.4	4.0	72.6	3.2
CTC	0.05	99.6	1.5	91.6	3.7	115.9	14.8
	0.1	101.7	0.7	85.1	3.9	110.7	0.9
ABPC	0.05	99.2	2.1	100.4	5.3	96.5	7.9
	0.1	99.2	3.3	103.6	5.9	100.9	3.2
NFPC	0.05	97.5	1.6	98.8	2.2	102.6	4.2
	0.1	97.4	0.8	99.7	2.8	101.2	1.7
PCG	0.05	100.4	2.2	94.4	0.3	97.5	4.6
	0.1	97.7	1.4	97.2	0.9	98.6	1.2

$n = 5$.

hard to analyse TC and PC antibiotics simultaneously by chromatographic techniques, because their chemical properties are not compatible. This is why the simultaneous analytical method has not been reported so far. We again investigated relationship between their separation behaviors and the chemical properties. As the results, it was clear that TC antibiotics gave symmetrical peaks below pH 3.0 [1,23] and PC antibiotics were stable above pH 3.0 for 5 min [2]. Therefore, minimum requirements for the simultaneous analysis are to elute the antibiotics in 5 min from column using a mobile phase with pH 3.0. Since we use MS/MS in the present study, it is not necessary to obtain complete separation of all antibiotics in liquid chromatography, it requires only the enrichment of sample concentration. So, we focused on how to enrich sample concentration in this experiment. After various experiments, we found the optimum conditions where all antibiotics elute without peak tailing or decomposition of PC

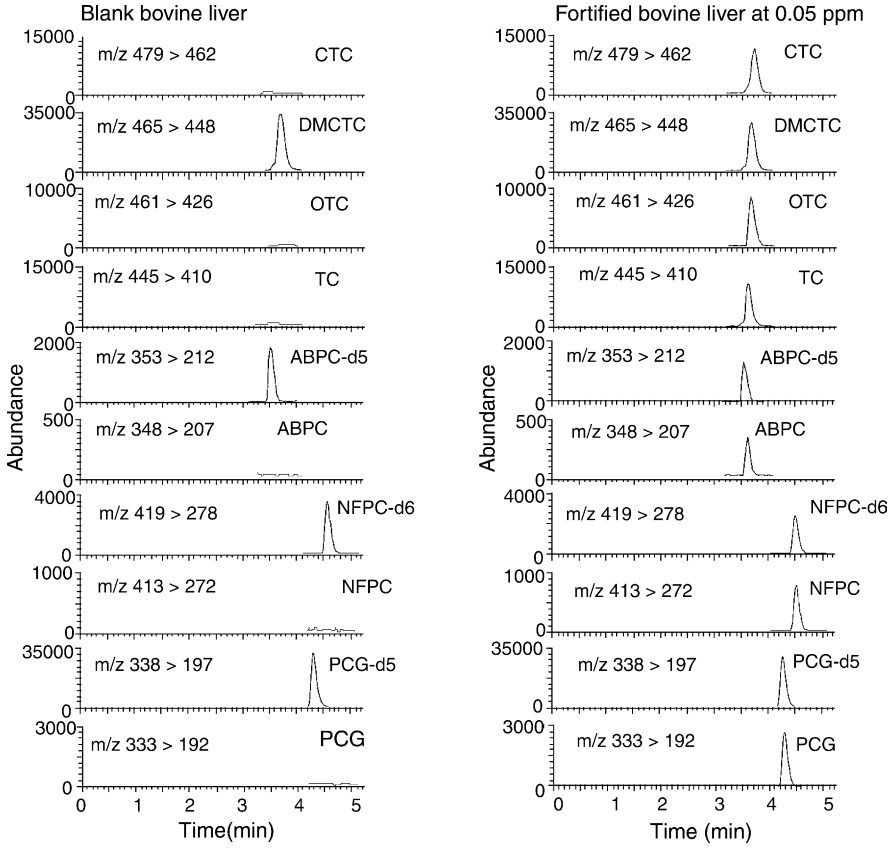

Fig. 3. Typical SRM chromatograms of the fortified and blank bovine livers.

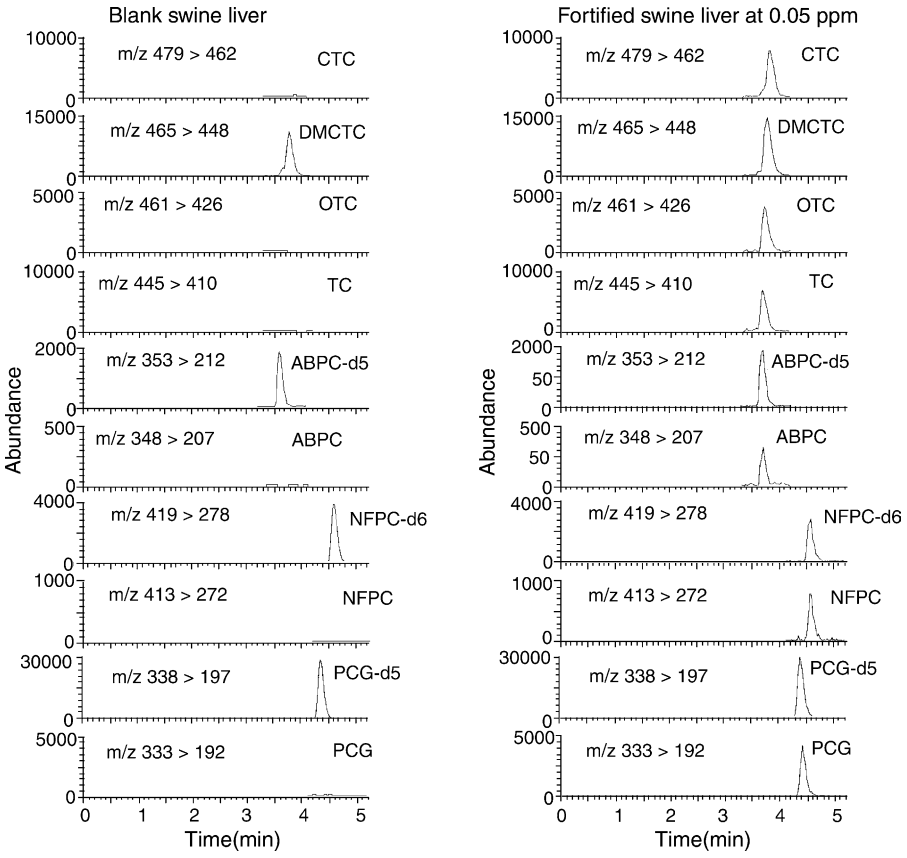

Fig. 4. Typical SRM chromatograms of the fortified and blank swine livers.

antibiotics into mass spectrometer within 5 min. The conditions are: after injection of 50 μL of sample solution into this short column, the column was washed with 0.05% formic acid for 30 s and then all antibiotics were eluted into the mass spectrometer from the column using 0.05% formic acid in methanol.

Fig. 2 shows SRM chromatograms of standard TC and PC antibiotics. All of the antibiotic peaks were clearly observed at retention time of 3.5 min for all TC antibiotics and ampicillin, and at 4.5 min for penicillin G, nafcillin and their deuterated compounds. Calibration curves were constructed using peak–area ratio of the antibiotics to their internal standards, and good linearity was observed between 0.001 and 0.2 ppm for all of the antibiotics with correlation coefficients over 0.999.

3.4. Recovery

Based on the above experimental results, we established the multiresidue analytical method for TC and PC antibiotics. After weighing 5 g of the sample, internal standards are spiked into the sample, then the antibiotics are extracted from the tissue in 5 mL of distilled water. After centrifuged at 13,000 rpm for 20 min, the supernatant was filtered through ultrafiltration membrane. The filtrate was introduced into ESI-MS/MS from sample enrichment short column.

Following this procedure, bovine muscle, liver, and kidney and swine muscle, liver, and kidney fortified with TC and PC antibiotics at concentration of 0.05 and 0.1 ppm were analysed. Tables 3 and 4 show the recovery corrected by internal standards and RSD. The overall recoveries ranged from 70 to 115% and RSD ranged from 0.7 to 14.8% and the detection limits were 0.002 ppm for all antibiotics calculated by S/N ratio of 3. These values indicate that the method presented here provides sufficient recoveries with good repeatability for determination of TC and PC antibiotics in animal tissues.

Figs. 3 and 4 show typical SRM chromatograms of the fortified bovine and swine liver samples and its corresponding blank liver samples. For blank liver, there were no interfering peaks of endogenous compounds at the retention time of the antibiotics, it can be said that the presented method is highly specific due to the SRM.

4. Conclusions

We have established a simple, rapid, and simultaneous analytical method for tetracycline and penicillin antibiotics in animal tissues using ESI-MS/MS. The method involves the following steps: addition of internal standards, extraction with distilled water, purification with ultrafiltration, and mass spectrometric determination with sample enrichment on the short column. Recoveries, RSD, detection limits, and analysis time are to be 70–115%, 0.7–14.8%, 0.002 ppm, and 3 h per eight samples, respectively.

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