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Application of ion-exchange cartridge clean-up in food analysis VI. Determination of six penicillins in bovine tissues by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A multiresidue analytical method was developed for the quantification of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC) in bovine tissues using liquid chromatography– electrospray ionization tandem mass spectrometry (LC–ESI MS/MS) with a multiple reaction monitoring technique. Using the deuterated PCG and NFPC as internal standard was effective for improvement of repeatability and accuracy. We chose $[M - H\text{-}141]^-$ as a monitor ion of MRM analysis and $[M - H]^-$ as a precursor ion for each penicillin. Combination of an ion-exchange cartridge clean-up and ion-pair LC enable us to determine the residual penicillins using the standard curves made from standard solutions without the influence of sample matrix on the MS. The average recoveries of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC from bovine liver, kidney and muscle at the same concentrations as the tolerance levels of PCG (50 μg/kg) ranged from 77 to 101% with the coefficients of variation ranging from 0.7 to 4.2% ($n = 5$). The limits of quantification for the six penicillins were 2–10 μg/kg in bovine muscle, liver and kidney (S/N ratio >10).

Keywords: Food analysis; Antibiotics; Penicillins

1. Introduction

Penicillin antibiotics have been widely used for livestock as veterinary drugs to prevent and treat infectious diseases. Such use is regulated because of the concerns about their possible effects on human health. In Japan, the same maximum residue limit (MRL) of 50 μg/kg has been established as WHO, FDA and EU for benzylpenicillin (PCG) in edible animal tissues to protect consumers [1]. In order to evaluate possible improper and illegal drug uses, a determination method for the monitoring of these residues in livestock products has been required for food sanitation and safe manufacturing practice.

A number of authors have reported multiresidue methods for the determination of penicillins in food by liquid chromatography–mass spectrometry (LC MS) or LC MS/MS techniques [2–9]. However, only a few of these methods is applicable to the determination of penicillins in edible animal tissues at levels equal to or below MRLs [2,7]. Moreover, most of the methods do not report the use of appropriate internal standards (I.S.), which are important for reliable quantitation and method validation. The use of phenethicillin, nafcillin (NFPC) and phenoxymethylpenicillin (PCV) as I.S. has been reported, respectively [2,4,8], however, these compounds are also one of the beta-lactam family and may also be applied in veterinary medicine, which questions the suitability of these compounds as valid I.S. Using a stable isotopically labeled I.S. is reasonable and suitable for mass spectrometric analysis, because there is no difference in the physicochemical behavior from a corresponding compound except for molecular weight. Riediker and Stadler [5] developed a simultaneous determination method of five penicillins in milk using a deuterated PCG as an I.S., however, there was some doubt whether this method is applicable to the determination of residual penicillins in animal tissues, which include larger amounts

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of matrix than the case of milk. Moreover, they used only one I.S., it is not sufficient to determine the five penicillins having different chemical behaviors individually.

In our previous studies [10–13], we have reported the applicability of sample clean-up by an ion-exchange cartridge in combination with ion-pair HPLC for the analysis of ionable compounds in foods. Using this technique, we have already developed a confirmation method for six cationic penicillins, PCG, PCV, oxacillin (MPIPC), cloxacillin (MCIPC), NFPC and dicloxacillin (MDIPC), in bovine tissues by electrospray ionization liquid chromatography–tandem mass spectrometry (LC–ESI MS/MS) [10]. Because of their effective clean-up and satisfactory separation, it can remove the influence of sample matrix on the MS response. So we can decide this confirmation method is applicable for quantification using simple and appropriate calibration curve.

As described above, use of the stable isotopically I.S. is essential to develop the precise quantitative method of residual penicillins in bovine tissues by LC–ESI MS/MS. We can find two deuterated compounds on the market in Japan; deuterated PCG (PCG-d5) and NFPC (NFPC-d6). These are very suitable for our purpose, because the polarities of these six penicillins are higher in the following order; MDIPC, NFPC, MCIPC, PCV and PCG, and we can use PCG-d5 for porlar penicillins and NFPC-d6 for the less porlar penicillins.

In this paper, we report in detail the simultaneous analysis of residual six penicillins in bovine tissues using multiple reaction monitoring (MRM) with two kinds of stable isotopically I.S. under LC–ESI MS/MS conditions.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile and distilled water (H_2O) were HPLC grade reagents, and other chemicals were analytical grade reagents. Di-*n*-butylammonium acetate (DBAA) was the ion-pairing reagent purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Bond Elut C_{18} had a 6 ml capacity packed with a 500 mg solid phase and was purchased from Varian (Harbor City, CA, USA). Sep-Pak Accell Plus QMA had a 3 ml capacity packed with a 500 mg solid phase and was obtained from Waters (Milford, MA, USA).

PCG potassium salt, PCV potassium salt, MPIPC sodium salt, MCIPC sodium salt, NFPC sodium salt and MDIPC sodium salt were purchased from Sigma (St. Louis, MO, USA). PCG-d5 potassium salt (chemical purity >99.0%) and NFPC-d6 (chemical purity >93.0%) were obtained from Hayashi Pure Chemical Industries (Osaka, Japan). These chemicals labeled with stable isotope were used as I.S. Separate stock solutions of each penicillin and I.S. were prepared by dissolving 5 mg of each compound in 5 ml of water. Subsequent dilutions of six penicillins were made using the mixture of acetonitrile–water (30:70 (v/v)) containing 50 m*M* DBAA, and those of two I.S. were made with water. All of the working standards were prepared fresh daily.

2.2. Sample preparations

The sample solution was prepared according to our previous method [10], except for using a mixed I.S. aqueous solution (0.25 μg/ml each of PCG-d5 and NFPC-d6). A brief summary of the extraction procedure was as follows. A 5 g of sliced and homogenized muscle sample was added 0.5 ml of a mixed I.S. aqueous solution and 60 ml of 2% NaCl aqueous solution. For liver and kidney samples, 5% sodium tungstate aqueous solution and 0.17 *M* sulfuric acid aqueous solution were added to the extraction solution, 2% NaCl, as deproteinization reagents. The extraction was repeated three times.

2.3. LC–ESI MS/MS conditions

The LC–ESI MS/MS system consisted of an Quattro II triple quadrupole tandem mass spectrometer (Micromass UK Ltd., Altrincham, UK) equipped with a Z-spray API source and an HP1100 series LC (Hewlett Packard, Palo Alto, CA, USA).

Chromatographic conditions used in the present study were described in detail in our previous paper [10], therefore, a brief statement is given here. Separation was performed on a TSKgel ODS-80Ts column (5 μm, 150 × 4.6 mm,

Table 1
Compound-specific ESI MS/MS parameters for the six penicillins and the two internal standards

Penicillins	Precursor ion (*m*/*z*) $[M - H]^-$	Monitor ion[a] (*m*/*z*) $[M - H\text{-}141]^-$	Cone voltage (V)	Collision energy (eV)	Retention time window (min)
Benzylpenicillin	333	192	23	13	2.50–3.50
Benzylpenicillin-d5	338	197	23	16	2.50–3.50
Phenoxymethylpenicillin	349	208	23	10	3.51–4.20
Oxacillin	400	259	20	13	4.00–5.00
Cloxacillin	434	293	20	13	5.01–5.80
Nafcillin	413	272	23	13	5.60–6.50
Nafcillin-d6	419	278	20	13	5.60–6.50
Dicloxacillin	468	327	23	13	7.00–7.80

[a] Product ion.

i.d.; TOSOH, Tokyo, Japan) at 30 °C. LC solvent A was a mixture of acetonitrile–water (30:70, v/v) whereas solvent B was a mixture of acetonitrile–water (50:50, v/v), both mobile phase containing 2 m*M* DBAA. The flow rate was 1.0 ml/min (split ratio = 1:4). The gradient conditions were as follows, based on times (*t*) set at the pump: from $t = 0$–3 min, hold B% = 0; $t = 3.1$–8 min, ramp linearly to B% = 100.

The compound-specific mass spectrometric parameters for MRM analysis are summarized in Table 1. MRM data of PCG, NFPC, PCG-d5 and NFPC-d6 were collected at 0.5 s of dwell time, and the other MRM data were collected at 1.0 s of dwell time. The other conditions were described in detail in our previous paper [10].

3. Results and discussion

3.1. Selection of I.S.

As described above, we could find two deuterated compound on the market, namely, PCG-d5 and NFPC-d6. So, we measured the full-scan ESI tandem mass spectra of these compounds to confirm the applicability for I.S. Each compound gave three kinds of product ions ($[M - H]^-$, $[M - H–CO_2]^-$ and $[M - H\text{-}141]^-$), which were the same as six penicillins. Since the all differences of mass numbers of each three product ion between PCG and PCG-d5 are five, we can surely confirm that the substituted atoms of PCG are five of all hydrogen atoms on the side chain, not beta-lactam ring [8]. Similarly, we can also confirm the labeled part of NFPC-d6. All of the precursor ions (molecular ion species) and their isotopic ions of PCG, NFPC and their deuterides were compared with each other, because the overlapping of the ions could lead to serious errors in MRM determination at the same retention time windows. Both the precursor ions of PCG and PCG-d5 and those of NFPC and NFPC-d6 did not overlap each other including their isotopic ions. Consequently, we were able to decide to use the commercially available PCG-d5 and NFPC-d6 as I.S. in this study. ESI MS/MS conditions (precursor ion, monitor ion, cone voltage and collision energy; listed in Table 1) of PCG-d5 and NFPC-d6 were optimized in the same manner as described in detail in our previous paper [10].

3.2. Sample preparation

In the case of using stable isotopically labeled I.S., we can usually make correction for the sample loss in the sample preparation, when the I.S. are immediately added a weighed sample. In order to use the I.S. for this purpose, it is necessary that the behavior of each PCG-d5 and NFPC-d6 in the sample preparation is similar to that of corresponding penicillins. So, we investigated the recoveries and the coefficient of variation (C.V.) of PCG-d5 and NFPC-d6. Bovine muscle samples were fortified with PCG-d5 and NFPC-d6 (50 μg/kg, each) and were analyzed according to our previous determination method using UV-HPLC ($n = 5$) [12]. The average recoveries for PCG-d5 and NFPC-d6 were 79 and 85% with the C.V. 6.4 and 4.4%, respectively. They are very similar to the recoveries and the C.V. of the six penicillins using same method (77–90%, 3.9–6.4%). Based on the results of these preliminary experiments, we judged that the sample loss was able to be corrected using PCG-d5 and NFPC-d6 as I.S. In order to make sure of the repeatability and the accuracy of determination of the residual penicillins around or less than tolerance level, we selected to add 0.5 ml of the mixture of the I.S. (250 ng/ml each of PCG-d5 and NFPC-d6) to a 5 g sample.

Many published determination methods using LC MS required the matrix matched calibration curves owing to matrix interference. However, our selected combination of sample clean-up method and LC separation was successful in reducing the sample matrix to a minimum [10]. Accordingly, we can decide to use the standard solution prepared by the 30% acetonitrile aqueous solution containing 50 m*M* DBAA same as the final sample solution of our clean-up method for the purpose of keeping the accuracy of LC separation. Calibration curves were constructed by peak-area ratios of penicillins to I.S. (PCG-d5 for PCG, PCV and MPIPC, and NFPC-d6 for MCIPC, NFPC and MDIPC), and were linear over the range of 2.5–500 ng/ml ($r^2 > 0.999$). These results suggest that the proposed conditions are applicable for a determination method of the penicillins.

Table 2
Recoveries of penicillins from bovine muscle

Penicillins	Without I.S.			With I.S.					
	Fortified (μg/kg)	Recovery[a] (%)	C.V. (%)	Fortified (μg/kg)	Recovery[a] (%)	C.V. (%)	Fortified (μg/kg)	Recovery[a] (%)	C.V. (%)
Benzylpenicillin	50	95	11	50	102	1.5	10	95	2.2
Phenoxymethylpenicillin	50	88	8.7	50	95	4.7	10	95	1.4
Oxacillin	50	81	9.6	50	87	5.3	10	110	3.9
Cloxacillin	50	76	7.5	50	84	6.0	10	115	12
Nafcillin	50	69	13	50	100	5.5	10	99	1.9
Dicloxacillin	50	66	10	50	77	5.4	10	81	11

[a] $n = 5$.

Table 3
Recoveries of penicillins from bovine liver and kidney

Penicillins	Liver			Kidney		
	Fortified (μg/kg)	Recovery[a] (%)	C.V. (%)	Fortified (μg/kg)	Recovery[a] (%)	C.V. (%)
Benzylpenicillin	50	93	3.4	50	101	1.5
	10	105	3.1	10	100	3.7
Phenoxymethylpenicillin	50	100	5.5	50	114	4.4
	10	107	10	10	107	9.4
Oxacillin	50	86	8.8	50	105	7.9
	10	88	9.6	10	114	11
Cloxacillin	50	96	5.7	50	107	5.0
	10	119	10	10	123	11
Nafcillin	50	96	1.5	50	98	6.8
	10	85	2.1	10	87	2.9
Dicloxacillin	50	91	8.9	50	103	6.0
	10	99	7.6	10	103	15

[a] $n = 5$.

3.3. Recoveries

For the purpose of certification of the applicability of this method, the recoveries of six penicillins from bovine muscle, liver and kidney were examined. Samples, which were previously found to be negative for penicillins using bioassay screening, were fortified with the six penicillins (50 or 10 μg/kg each), and analyzed according to the procedure described in Experimental Section in this paper. The recoveries and corresponding C.V. are listed in Tables 2 and 3. The average recoveries for the penicillins from muscle ranged from 77 to 115% with the C.V. ranging from 1.4 to 12%.

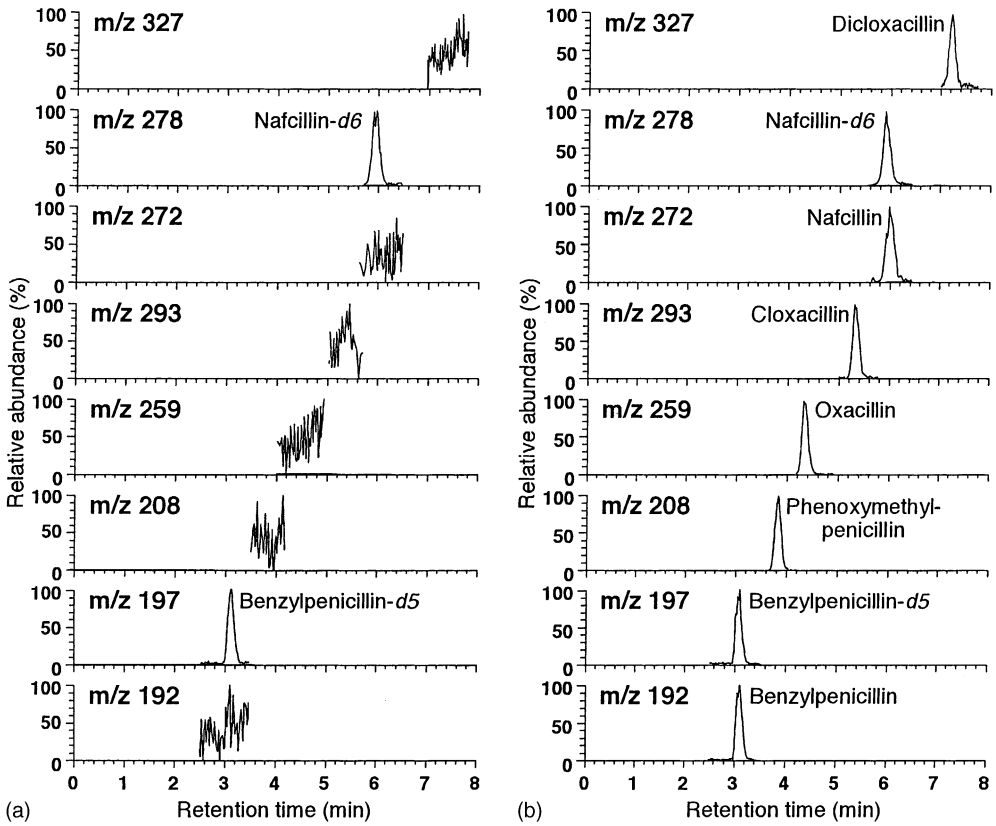

Fig. 1. Typical MRM chromatograms of bovine liver samples under LC–ESI MS/MS conditions: (a) bovine liver (control); (b) fortified with the six penicillins at the concentration of 10 μg/kg. Each sample was fortified with benzylpenicillin-d5 and nafcillin-d6 as internal standard at the concentration of 25 μg/kg. For conditions see Section 2 and Table 1.

For the liver, the average recoveries ranged from 85 to 119% with the C.V. ranging from 2.1 to 10%. And for the kidney, the average recoveries ranged from 87 to 123% with the C.V. ranging from 1.5 to 15%. In the cases of the C.V. at lower concentration, especially for liver and kidney, there are clear differences in these six penicillins between having their own I.S. or not. These phenomenon are able to be explained that the differences of the behavior in sample preparation between I.S. and penicillins except for PCG and NFPC and the influence of slight sample matrix are observed at low concentration. We estimate that our presented method will be improved the accuracy and the sensitivity of these four penicillins using their corresponding stable isotopically compounds as their I.S. In order to confirm the effect of I.S., we calculated the average recoveries and the C.V. from the same data obtained by recovery tests of muscle samples fortified at 50 μg/kg using same standards data without I.S. (shown in Table 2). Comparing these results, both recoveries and C.V. calculated with I.S. are better than those calculated without I.S. So we can recognize the importance of using I.S. to the improvement of repeatability and accuracy of the quantification. Fig. 1 shows typical MRM chromatograms of the fortified liver (b) and the corresponding blank control (a). On the bases of the results of above recovery tests and the MRM chromatograms of the fortified samples at 10 μg/kg, the limits of quantification have been estimated at 2 μg/kg for PCV, 5 μg/kg for PCG, MPIPC, MCIPC and NFPC, and 10 μg/kg for MDIPC in bovine muscle and at 3 μg/kg for PCV, 5 μg/kg for MPIPC and MCIPC, and 10 μg/kg for PCG, NFPC and MDIPC in bovine liver and kidney (S/N ratio >10), respectively. To investigate the availability of this method, we analyzed twenty samples (10 muscles and 5 each of liver and kidney) which were previously found to be negative for penicillins using bioassay screening. The obtained MRM chromatograms were almost the same that each corresponding blank sample. These results clearly indicated that the method proposed here has satisfactory repeatability, recovery and accuracy for the simultaneous determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine muscle, liver and kidney.

4. Conclusions

A LC–ESI MS/MS method for the determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine tissues was developed with the following characteristics: (1) use of deuterated PCG and NFPC as I.S. greatly contributes to the improvement of repeatability and accuracy of the quantification. (2) Combination of an ion-exchange cartridge clean-up and ion-pair LC is effective in removing the influence of sample matrix on the MS response. (3) The quantification limits of penicillins in bovine tissues satisfy the MRLs established by WHO, FDA, EU and Japan.

Because of these characteristics, we strongly recommend the analytical method presented in this paper for the precise determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine tissues.

References

[1] Food Sanitation Law, Article No. 7, Law No. 233, 24 December 1947, Standards or Requirements of Foods or Additives, Ministry of Health and Welfare Notification, 28th December 1959, revised on 26th November 1999.
[2] K. Hatano, J. Food Hyg. Soc. Jpn. 44 (2003) 1.
[3] D.M. Holstege, B. Puschner, G. Whitehead, F.D. Galey, J. Agric. Food Chem. 50 (2002) 406.
[4] F. Bruno, R. Curini, A.D. Corcia, M. Nazzari, R. Samperi, J. Agric. Food Chem. 49 (2001) 3463.
[5] S. Riediker, R.H. Stadler, Anal. Chem. 73 (2001) 1614.
[6] E. Daeseleire, H.D. Ruyck, R.V. Renterghem, Rapid Commun. Mass Spectrum. 14 (2000) 1404.
[7] V. Hormazabal, M. Yndestad, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 3099.
[8] W.J. Blanchflower, S.A. Hewitt, D.G. Kennedy, Analyst 119 (1994) 2595.
[9] R. Straub, M. Linder, R.D. Voyksner, Anal. Chem. 66 (1994) 3651.
[10] Y. Ito, Y. Ikai, H. Oka, H. Matsumoto, Y. Miyazaki, K. Takeba, H. Nagase, J. Chromatogr. A 911 (2001) 217.
[11] Y. Ito, Y. Ikai, H. Oka, H. Matsumoto, T. Kagami, K. Takeba, J. Chromatogr. A 880 (2000) 85.
[12] Y. Ito, Y. Ikai, H. Oka, T. Kagami, K. Takeba, J. Chromatogr. A 855 (1999) 247.
[13] Y. Ito, Y. Ikai, H. Oka, J. Hayakawa, T. Kagami, J. Chromatogr. A 810 (1998) 81.