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Application of ion-exchange cartridge clean-up in food analysis IV. Confirmatory assay of benzylpenicillin, phenoxymethylpenicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in bovine tissues by liquid chromatography–electrospray ionization tandem mass spectrometry

Yuko Ito[a,*], Yoshitomo Ikai[a], Hisao Oka[a], Hiroshi Matsumoto[a], Yutaka Miyazaki[a], Kazue Takeba[b], Hisamitsu Nagase[c]

[a]*Aichi Prefectural Institute of Public Health, 7-6 Nagare, Tsuji-machi, Kita-ku, Nagoya 462-8576, Japan*
[b]*The Tokyo Metropolitan Research Laboratory of Public Health, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan*
[c]*Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-8585, Japan*

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Abstract

A multiresidue analytical method was developed for the confirmation of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC) in bovine tissues using electrospray ionization liquid chromatography–tandem mass spectrometry (LC–ESI-MS–MS) with a product ion scan mode. All penicillins gave $[M-H]^-$, $[M-H-CO_2]^-$ and $[M-H-141]^-$ as the product ion, when $[M-H]^-$ was selected as the precursor ion. Combination of an ion-exchange cartridge clean-up and the LC–ESI-MS–MS method can reliably identify all of these penicillins fortified at a concentration of 0.05 mg/kg in bovine tissues, including liver, kidney and muscle. The limits of confirmation satisfy the maximum residue limits for each of the penicillins established by the World Health Organization, US Food and Drug Administration, European Union and Japan.

Keywords: Food analysis; Benzylpenicillin; Phenoxymethylpenicillin; Oxacillin; Cloxacillin; Nafcillin; Dicloxacillin

1. Introduction

Penicillin antibiotics have been widely used for livestock as veterinary drugs to prevent and treat infectious diseases. Such use may lead to problems with residues in the livestock products. In Japan, the same maximum residue limit (MRL) of 0.05 mg/kg has been established as the World Health Organization (WHO), US Food and Drug Administration (FDA), European Union (EU) for benzylpenicillin (PCG) in edible animal tissues to protect consumers [1]. One of the major roles as public health agencies

*Corresponding author. Tel.: +81-52-911-3111; fax: +81-52-913-3641.
E-mail address: yuuko_1_itou@mail.pref.aichi.jp (Y. Ito).

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is to provide safe products for consumers through the monitoring of these residues in livestock products.

Development of reliable confirmation methods for antibiotic residues has been required for food sanitation and safe manufacturing practice. There has not been reported, however, a reliable method for confirmation of the residual penicillins. Because mass spectrometric techniques can confirm penicillins with high sensitivity and selectivity, high-performance liquid chromatography–mass spectrometry (HPLC–MS) appears to be best suited for this purpose. Although some LC–MS methods have been published for the analysis of residual penicillins in milk [2–8], only a few methods have been reported for the simultaneous analysis of penicillins in animal tissues [9–12]. Furthermore, the reported electrospray ionization (ESI) LC–MS methods for PCG, phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC) and dicloxacillin (MDIPC) in muscle and kidney were less sensitive than UV–HPLC methods. Recently, tandem mass spectrometry (MS–MS) with a product ion scan mode in combination with LC–ESI-MS has been recognized as a rapid, sensitive and selective analytical method for the confirmation of various compounds. We, therefore, decided to employ LC–ESI-MS–MS to develop a highly sensitive and selective confirmation method for residual penicillins in bovine tissues.

In our previous studies [13–16], 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 quantitative determination method for PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine tissues. This clean-up method and HPLC conditions [14] are not directly applicable to LC–ESI-MS–MS, because the sample solution and the mobile phase contained nonvolatile compounds. These problems, however, can be overcome by careful consideration of the followings: a volatile ion-pair reagent in the mobile phase for the separation of penicillins, an elution solvent for the cartridges used for clean-up, and detailed conditions of LC–MS–MS. By investigating how to solve the above mentioned problems, we have developed a highly sensitive confirmation method for the residual penicillins in bovine tissues.

2. Experimental

2.1. Chemicals and reagents

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

Bond Elut C18 (Lot No. 071302 and 073061) 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 (Lot No. W8117J1) 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). Separate stock solutions of each penicillin were prepared by dissolving 50 mg of each compound in 50 ml of water. Subsequent dilutions were made using the mixture of acetonitrile–water (30:70, v/v) containing 50 m*M* DBAA. All of the working standards were prepared fresh daily.

2.2. Sample preparations

The sample solution was prepared according to our previous method [14,15], except for using 2 ml of acetonitrile–water (30:70, v/v) containing 50 m*M* DBAA as elution solvent from the QMA cartridge.

2.3. Apparatus

The HPLC system consisted of an HP1100 series binary pump, a degasser, a column compartment and an auto sampler (Hewlett Packard, Palo Alto, CA, USA). The LC–MS–MS system consisted of an Quattro II triple quadrupole tandem mass spectrometer (Micromass UK, Altrincham, UK) equipped with a Z-spray atmospheric pressure ionization (API) source. For optimization of the Z-spray probe position and the MS parameters, a Model 100 syringe pump (KD Scientific, Boston, MA, USA) was connected to the LC–MS–MS system.

2.4. Chromatographic conditions

Separation was performed on a TSKgel ODS-80Ts column (5 μm, 150×4.6 mm I.D.; TOSOH, Tokyo, Japan) at 30°C. The mobile phase consisted of a binary gradient. Mobile phase A was a mixture of acetonitrile–water (30:70, v/v) containing 2 m*M* DBAA. Mobile phase B was a mixture of acetonitrile–water (50:50, v/v) with the same concentration of DBAA. The flow-rate was 1.0 ml/min (split ratio=1:4). A smaller portion of the eluent was introduced into the ESI interface. 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; *t*=8.1–9 min, ramp back to %B=0; *t*=9–14 min, hold %B=0 to reequilibrate the system.

2.5. Mass spectrometric conditions

The desolvation gas (nitrogen) temperature and flow-rate were set at 400°C and 370 l/h, respectively. The ion source temperature was set at 100°C. The instrument was operated in the negative ion mode. Full scan data of product ions were collected from 150 to 500 *m/z* at a rate of 0.17 scans/s. The other different mass spectrometric parameters are summarized in Table 1. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 1.9×10^{-3} mbar in the collision cell. Position of the Z-spray probe was optimized using a 10 μg/ml solution of PCG in mobile phase A at a flow-rate of 200 μl/min by a syringe pump.

3. Results and discussion

3.1. Optimization of HPLC conditions

As described above, the HPLC conditions previously used in our laboratory could not be directly applied to LC–ESI-MS analysis, because the mobile phase contained the nonvolatile ion-pair reagent and buffer solution. As volatile ion-pair reagents for cation substances, triethylamine and DBAA were examined. Addition of triethylamine to the mobile phase without adjusting the pH was previously reported for the confirmation of penicillins by LC–ESI-MS [11]. In our experiments, however, we found that it imposed the heavy burden not only for the life of the LC column but also for the HPLC system and that the addition caused significant losses in the sensitivity by increasing the pH of the mobile phase to 13. On the other hand, the pH of the mobile phase containing 5 m*M* of DBAA was maintained around 7.7 and the addition of DBAA did not cause damages to the LC column and losses in the sensitivity like that of triethylamine. DBAA was, therefore, chosen as the ion-pair reagent.

Next, selected concentrations of this ion-pair reagent (DBAA) ranging from 1 to 5 m*M* were examined to obtain the optimal separation of the penicillins and the highest sensitivity for the penicillin analysis in LC–ESI-MS. The higher the DBAA concentration became, the longer the retention of each penicillin became, losing the sensitivity of detection. Although the DBAA concentration of 1 m*M* was found to yield a good separation of the six penicillins and the highest sensitivity in the LC–MS

Table 1
Diagnostic ions and ESI-MS–MS conditions for penicillins analysis

Penicillin	Product ions (*m/z*)			Precursor ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time window (min)
	$[M-H]^-$	$[M-H-CO_2]^-$	$[M-H-141]^-$	$[M-H]^-$			
Benzylpenicillin	333	289	192	333	23	8	2.50–3.40
Phenoxymethylpenicillin	349	305	208	349	23	8	3.41–4.00
Oxacillin	400	356	259	400	20	10	4.01–5.00
Cloxacillin	434	390	293	434	20	9	5.20–6.25
Nafcillin	413	369	272	413	23	9	6.26–7.00
Dicloxacillin	468	424	327	468	23	9	7.01–8.00

system, there was a considerable shift in the retention times between samples and standards when spiked samples were injected. With the concentrations over 2 m*M*, this phenomenon was not observed, hence, the DBAA concentration of 2 m*M* was used for the further analysis in this study.

Using isocratic HPLC, the peaks of NFPC and MDIPC became broad because the hydrophobicity of the penicillins increases in the following order: PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC. In order to carry out the highly sensitive LC–MS analysis for all of the six penicillins, we tried to apply gradient HPLC to sharpen the peaks of NFPC and MDIPC. After a series of preliminary experiments, we decided to use a binary gradient mobile phase; mobile phase A was a mixture of acetonitrile–water (30:70, v/v) containing 2 m*M* DBAA and mobile phase B was a mixture of acetonitrile–water (50:50, v/v) with the same concentration of DBAA. Using the gradient conditions described in the Section 2.4, the HPLC system gave the most favorable peak shapes and sensitivity, for instance, the sensitivity of MDIPC was over five-times higher than that under isocratic elution.

3.2. Optimization of ESI-MS–MS conditions

Electrospray ionization mass spectra recorded for the six penicillins gave $[M-H]^-$, $[M-H-CO_2]^-$ and $[M-H-141]^-$ (Table 1). This means that $[M-H-CO_2]^-$ and $[M-H-141]^-$ were generated as product ions and they are very useful for the confirmation of penicillins, when $[M-H]^-$ serves as a precursor ion for MS–MS.

In order to examine the effects of selected ion source temperature (80 to 140°C) and desolvation temperature (200 to 400°C), the intensity of $[M-H]^-$ was monitored at m/z 333 using PCG solution made by the mobile phase A. Increase in the ion source temperature was found to increase the ion intensity, however, the increase over 100°C decreased the intensity. On the other hand, only the increase of the ion intensity was observed in association with the increase in desolvation temperature. The optimal temperatures were 100°C for ion source temperature and 400°C for desolvation temperature.

Next, cone voltage was adjusted for each penicillin by the same procedure described above and the voltages which gave the highest intensities of $[M-H]^-$ for these penicillins are shown in Table 1.

In order to obtain the optimal MS–MS conditions for the analysis of penicillins, we measured the full-scan ESI tandem mass spectra of the six penicillins under the selected collision energies (8 to 12 eV). All of the three product ions for each penicillin were observed, especially, $[M-H-141]^-$ was observed as the most abundant ion. Furthermore, the intensity of $[M-H-CO_2]^-$ for PCG and PCV was inferior to that of $[M-H]^-$; whereas, the intensity of $[M-H-CO_2]^-$ for the other four penicillins was superior to that of $[M-H]^-$. Judging from these results, we chose a collision energy for each penicillin which produces all of the product ions with over 10% of relative abundance. The collision energies for respective penicillins are shown in Table 1.

3.3. Sample extraction

Suppression of ionization efficiency is a well known phenomenon of LC–ESI-MS when interfering substances are co-eluted with the target compound from LC column. Because this phenomenon decreases the sensitivity of LC–MS analysis, it is obvious that using Bond Elut C18 cartridge alone is not a sufficient clean-up method for highly sensitive LC–MS analysis. In our previous study, Sep-Pak Accell Plus QMA cartridge clean-up following Bond Elut C18 cartridge preclean-up was shown to be an effective clean-up method for the determination of these six penicillins. It is desirable to use an HPLC mobile phase as an elution solvent for the clean-up cartridge (Sep-Pak Accell Plus QMA) for good reproducibility of HPLC determination. We investigated whether or not the mobile phase described above can be used as the elution solvent. Bovine muscle extract spiked with all of the penicillins (0.5 mg/kg, each) was applied to a combination of Bond Elut C18 and Sep-Pak Accell Plus QMA clean-up. When 2 ml of the mobile phase were used, recoveries of the penicillins detected at 220 nm by using UV–HPLC were less than 2%. Accordingly, we tried to elute penicillins using an elution solvent having the different concentrations of DBAA from the mobile phase. Effects of the selected concentrations (10 to 70 m*M*) of DBAA on the recoveries were examined by UV–HPLC (Fig. 1). A DBAA

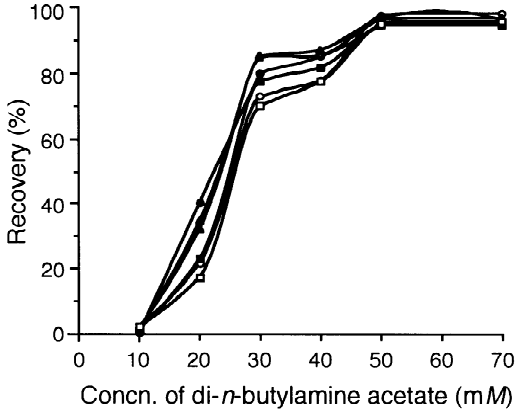

Fig. 1. Effect of the concentration of di-*n*-butylamine acetate in elution solvent on the recoveries of penicillins from the QMA cartridge. Operating conditions, column: TSKgel ODS-80Ts (150×4.6 mm I.D.); mobile phase: acetonitrile–water (3:7) containing 2 m*M* di-*n*-butylamine acetate; flow-rate: 1.0 ml/min; detection: UV at 220 nm; column temperature: 30°C. Results of three replicates. (●) PCG, (○) PCV, (▲) MPIPC, (△) MCIPC, (■) NFPC, (□) MDIPC.

concentration greater than 50 m*M* gave recoveries of all of the penicillins over 95%, however, the higher the concentrations of DBAA became, the more interfering substances were eluted. Based on the results of these preliminary experiments, we decided to use the 30% acetonitrile aqueous solution containing 50 m*M* DBAA as the elution solvent for the extracted samples.

3.4. LC–ESI-MS–MS of penicillins in bovine tissues

Bovine liver, kidney and muscle samples were fortified with the six penicillins at the concentration of 0.05 mg/kg (same as the MRL of PCG), and were analyzed according to the procedure described in Section 2.2. Fig. 2 shows typical mass chromatograms of the fortified liver sample (a) and the corresponding blank control (b) monitored at $[M-H-141]^-$ under LC–ESI-MS–MS conditions, respectively. Tandem mass spectra of the penicillins recorded at the tops of each peak on the mass chromatograms of fortified liver sample were shown in Fig. 3. All of these mass chromatograms and tandem mass spectra of fortified samples were almost the same as the respective standards. Good reproducibility of the present method was confirmed by the repeated analyses of the fortified samples at 0.05 mg/kg (each of five different samples of liver, kidney and muscle, and different days). The relative standard deviations (RSDs) of the retention times and the ion intensities of the penicillins monitored at $[M-H-141]^-$ were less than 1.3% and 9.5% (five different samples), and less than 2% and 14% (different 5 days), respectively. In order to examine the lower limits of confirmation of the present method, we analyzed bovine tissues fortified with the penicillins at a concentration of 0.02 mg/kg (same as the detection limit of UV–HPLC method). $[M-H-141]^-$ of all of the penicillins in bovine liver, kidney and muscle under the LC–MS–MS conditions was clearly detected (*S*/*N* ratio; 4.5–over 10). In the analysis of the muscle extract, the other two product ions ($[M-H]^-$ and $[M-H-CO_2]^-$) were also observed clearly (*S*/*N* ratio; 3.1–7.1), however, in the analysis of the kidney and liver extract, *S*/*N* ratios of the product ions were sometimes less than 3.0. On the bases of these findings, the lower limit of confirmation of the present method for muscle sample was estimated to be 0.02 mg/kg for all six penicillins, and those for liver and kidney were between 0.02 and 0.03 mg/kg.

4. Conclusions

An LC–ESI-MS–MS method for the confirmation of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in bovine tissues was developed with the following characteristics: (1) combination of Sep-Pak Accell Plus QMA cartridge clean-up and Bond Elut C18 cartridge preclean-up is effective in increasing the sensitivity and reproductivity of LC–ESI-MS–MS. (2) A mobile phase of HPLC containing the volatile ion-pair reagent can be used without causing clogging problems and the suppression of ionization efficiency. (3) The confirmation limits of penicillins in bovine tissues satisfy the MRLs established by the WHO, FDA, EU and Japan.

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

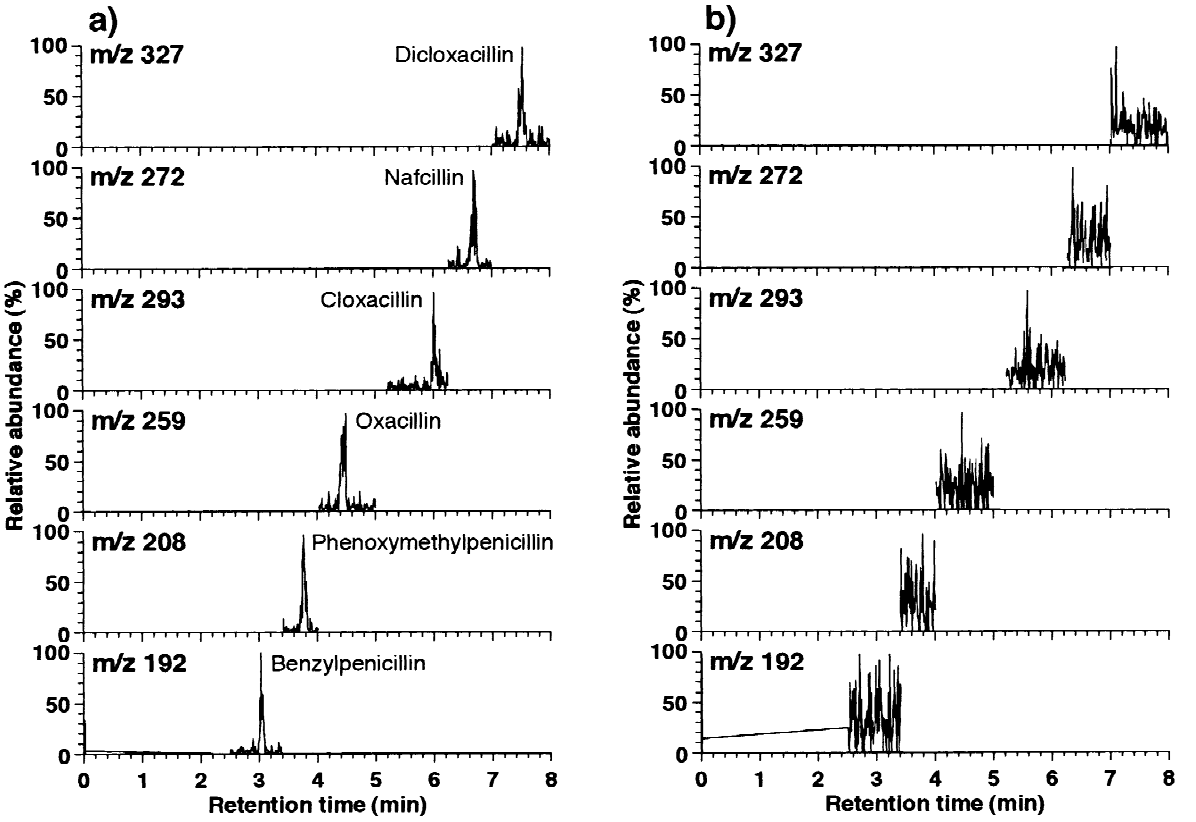

Fig. 2. Typical mass chromatograms of the fortified liver sample monitored at $[M-H-141]^-$ under LC–ESI-MS–MS conditions. (a) Fortified with the six penicillins at the concentration of 0.05 mg/kg; (b), bovine liver (control).

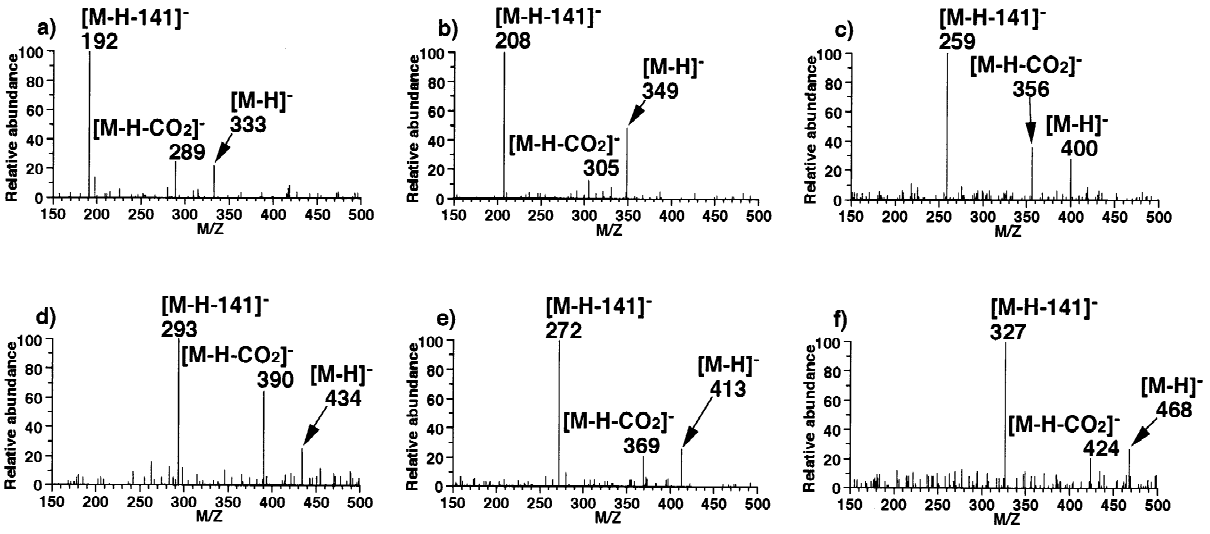

Fig. 3. ESI tandem mass spectra of penicillins fortified at a concentration of 0.05 mg/kg in bovine liver. (a) Benzylpenicillin; (b) phenoxymethylpenicillin; (c) oxacillin; (d) cloxacillin; (e) nafcillin; (f) dicloxacillin.

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