

LC-MS/MS measurement of ampicillin residue in swine tissues at 5 days after in-feed administration

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ABSTRACT. We assessed ampicillin (ABPC) concentrations of kidney, muscle and intestine after a 5-day withdrawal period in 2 male and a female young Large White pigs fed the diet containing ABPC (ABPC medicated feed, 24 mg/kg/day) for a week. The ABPC residues were measured with liquid chromatography–tandem mass spectrometry, and the mean recoveries and quantitation limits ranged from 91.8 to 97.2% and from 0.1 to 0.12 ng/g, respectively. The residual ABPC concentrations were ≤ 1.18 ng/g for the muscle, ≤ 0.53 ng/g for the kidney and ≤ 1.93 ng/g for the intestine, suggesting below the Japanese provisional maximum residue limits. These results reveal that the analytical method is developed for residual ABPC and that the withdrawal period is appropriate.

KEY WORDS: ampicillin, LC-MS/MS, medicated feed, residue level, swine tissue

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Ampicillin (ABPC, CAS: 69-53-4) is widely used as a β -lactam antibiotic for veterinary practice in Japan. ABPC is usually administered to swine at 3–12 mg/kg/day for 1–7 days; in addition, ABPC is able to be administered double the daily dose in the case of a serious illness. The Japanese provisional maximum residue limits (MRLs) for ABPC in swine were established as 0.06, 0.009 and 0.01 μ g/g (ppm) in the muscle, kidney and intestine, respectively [10].

High-performance liquid chromatography after pre-column derivatization [13], liquid chromatography–mass spectrometry (LC-MS) [14] and liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods [6] have been evaluated for ABPC residue quantification in tissues of domestic animals. However, a sensitive analytical method for determination of ABPC in swine tissues has not been reported.

According to the application for approval of the original ABPC product, it has been shown that residual levels of muscle, kidney and intestine are higher than those of other swine tissues in the residue study following forced oral administration of ABPC. In this study, we aimed to develop a simple and sensitive determination method for ABPC in swine muscle, kidney and intestine and to assess a 5-day withdrawal period of ABPC in swine after oral administration through medicated feed at the maximum dose.

Three young Large White pigs (approximately 3 months

old, weighing 33.3 and 42.8 kg for 2 males and 33.5 kg for a female) were used for the ABPC residue study after medicated feeding of 24 mg (potency)/kg/day ABPC for a week. An ABPC product sold as the “ampicillin powder KS” (Kyoritu Seiyaku, Co., Ltd., Tokyo, Japan) was used for dispensing the medicated feed. Since subjects of Japan MRL regulation include all edible tissues from both male and female domestic animals, we conducted the study using both sexes of swine. The pigs were fed SD feed for swine (drug-free feed, Nippon Formula Feed Manufacturing Co., Ltd., Yokohama, Japan) twice a day (at 9 am and at 2 pm) during the 22-day preliminary breeding period and after the administration period. The pigs drank water *ad libitum* in this study. The medicated feed prepared by mixing response to 24 mg/kg ABPC with 200 g of SD feed just before administration was administered to the pig completely, and then, an animal was fed about 600 g of non-ABPC-containing SD feed at 9 am for a week. Pigs were sacrificed at 5-day withdrawal period, and their tissues (longissimus muscle, renal parenchyma of both kidneys and intestine (jejunum and ileum)) were divided into lumps of 5 g as soon as possible. The divided tissues were immediately stored at -80°C until ABPC determination. All animal experimental procedures were performed in accordance with the Guidelines for Regulation of Animal Experimentation issued by the National Veterinary Assay Laboratory (2005).

The ABPC standard for official assay methods of veterinary medical ABPC product was obtained from the National Veterinary Assay Laboratory (Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan). Isotopically labeled ABPC (ABPC-d5), used as the internal standard (IS), was purchased from Hayashi Pure Chemical Industries, Co., Ltd. (Osaka, Japan). LC-MS-grade formic acid, methanol and ultrapure water (water) were supplied by Wako Pure Chemical Industries (Osaka, Japan).

The LC-MS/MS system consisted of a Nanospace SI-2 series (Shiseido Co., Ltd., Tokyo, Japan) and TSQ Quan-

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tum Discovery Max apparatus (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). The LC-MS/MS conditions were as follows: analytical column Hypurity C18 (particle size: 3 μm , 2.1 mm \times 50 mm, Thermo Fisher Scientific) connected to a Sumipax filter PG-ODS precolumn (SCAS, Osaka, Japan) was maintained at 40°C; mobile phases 0.05% formic acid in water (solvent A, v/v) and 0.05% formic acid in methanol (solvent B, v/v) flowed gradient (0–100% solvent B; 0.5–7 min); ESI (negative ion mode); spray voltage 3 kV; sheath gas 50; auxiliary gas pressures 20; fragment ions were m/z 348.1/207.0 for ABPC and 353.1/212.0 for IS (16eV).

Two working standard solutions containing 1 and 100 ng/ml ABPC were produced by diluting the ABPC stock solution (10.0 mg/100 ml) with water and were stored at -20°C . An IS working solution (25 $\mu\text{g}/\text{ml}$) was prepared by diluting the IS stock solution (0.1 mg/ml) with water and was stored at -20°C .

A matrix spike sample in each tissue, in which the concentration of ABPC was 7 ng/g, was prepared to spike ABPC in the blank tissue for determining the recovery of ABPC. Provisional MRLs of ABPC in swine have been established in Japan in 2006; therefore, the provisional MRL will be revised in the future. In this study, the concentration of matrix spiked sample (7 ng/g) was selected as a medium level between the lowest ABPC MRL in Europe (0.004 ppm) [3] and the ABPC tolerance in U.S.A. (0.01 ppm) [4]. Muscle, kidney and intestine samples (5 g) were added to 0.1 ml of the IS working solution and 5 ml of water and homogenized for 1 min with a homogenizer (Physcotron, Microtec Co., Ltd., Chiba, Japan). After centrifugation of the homogenized samples at $2,073 \times g$ (3,000 rpm) for 15 min at 5°C , the supernatants of the muscle and intestine samples were re-centrifuged at $14,475 \times g$ (14,000 rpm) for 20 min at 5°C . For the kidney samples, the supernatant fluid was re-centrifuged at $14,475 \times g$ for 40 min at 5°C . Approximately 400 μl of the second supernatant fluids were transferred to the filter units (Ultracel YM-10, Nihon Millipore Co., Ltd., Tokyo, Japan), which had been prewashed by adding 200 μl each of 1.0% Tween 20 and water, and the filter units were centrifuged at $14,475 \times g$ (14,000 rpm) for 30 min at 5°C . Each filtrated fluid was filtered through a 0.45- μm membrane filter (Eki-crodisc 13, Nippon Genetics Co., Ltd., Tokyo, Japan). The sample solution (50 μl) was injected into the LC-MS/MS system.

Typical chromatograms of ABPC and IS in blank swine intestine spiked with ABPC (7 ng/g) and blank swine intestine are shown in Fig. 1. No interaction peak was observed in the blank tissues under the present assay conditions. Linearity of the standard calibration curves (r) was >0.999 for ABPC levels of 0.1–50 ng/ml.

In our LC-MS/MS method for determining ABPC, mean extraction recoveries at 7 ng/g in the three tissues ranged from 91.8 (kidney) to 97.2% (intestine). Intraday and interday precisions in recovery were no more than 8.2% as coefficients of variation, and additionally, the intraday (4.9%) and interday precisions in kidney (8.2%) are larger than those in muscle (1.7 and 2.8%, respectively). The differences in

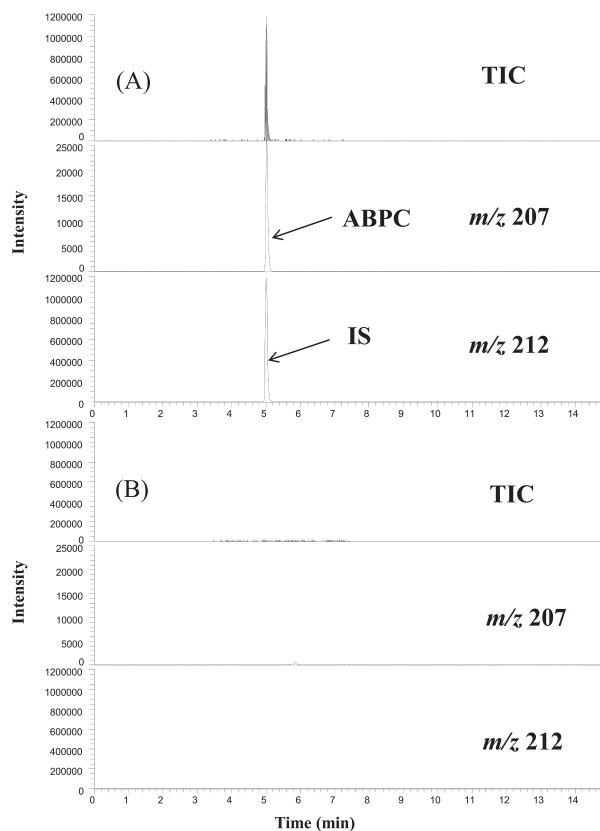


Fig. 1. Typical chromatograms of ampicillin (ABPC) and IS (ABPC-d5) in swine intestines, (A) blank intestine spiked with ABPC (7 ng/g) and IS, (B) blank intestine.

the mean recovery between kidney and intestine and in the precisions between kidney and muscle could be attributed to difference in biological matrixes contained in each tissue. These data were fulfilled Guideline 49 of the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH GL49) [8]. Limits of detection (LODs) (0.01–0.04 ng/g) and limits of quantitation (LOQs) (0.1–0.12 ng/g) of ABPC in our method were better than those in other methods for animal tissues, e.g., the porcine muscle tissue (LOD=3–5 ng/g and LOQ=25 ng/g) [13], porcine kidney (LOQ=50 ng/g) [11] and porcine tissues (LOD=9.8–11.1 ng/g) [12]. The LOD and LOQ mean the lowest analyte concentration likely to be reliably distinguished from noises in blank chromatogram and the lowest concentration of a measurand that can be reliably measured by the analytical method, respectively.

Good stabilities of ABPC in a miscellaneous tray at -70°C and in the muscle at -75°C have been reported for 52 weeks [7] and over 8 months [14], respectively. The samples in this study were stored at an appropriate temperature (-80°C) according to its storage term.

ABPC levels after the withdrawal period following the last administration were determined to be 0.09–1.18 ng/g in 2 muscles, 0.53 ng/g in 1 kidney and ≤ 1.93 ng/g in 3 intes-

Table 1. Residual ampicillin (ABPC) concentrations in the muscles, kidneys and intestines after a 5-day withdrawal period in two male and a female young Large White pigs fed the diet containing ABPC (ABPC medicated feed, 24 mg/kg/day) for a week

Animal No. (sex, body weight)	Residual ABPC concentration		
	Muscle (ng/g)	Kidney (ng/g)	Intestine (ng/g)
1 (female, 33.5 kg)	[0.09] ^{a)}	0.53	0.53
2 (male, 42.8 kg)	ND ^{b)}	ND ^{b)}	0.21
3 (male, 33.3 kg)	1.18	ND ^{b)}	1.93
Provisional maximum residue limit ^{c)}	60	9	10

a) ABPC was detectable, and the residual concentration was below the limit of quantitation; b) Not detected (<the limit of detection); c) See the Ministry of Health, Labour and welfare (MHLW) Ministerial Notification No.499 (November 29, 2005) 2005. <http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228/>

times; however, concentrations in other analyzed tissues were below the LOD (Table 1). All these quantitative values were lesser than the Japanese provisional MRL for each tissue. The differences in residual ABPC concentrations among 3 pigs can be attributed to individual differences; however, we cannot discuss any factor for the individual differences, because of a limited number of experimental animals.

Oral bioavailability of amoxicillin in swine has been reported 31% [2] or 47% [9]; however, that of ABPC has not been shown in swine. Since it has been reported that oral bioavailability of ABPC is approximately a half of amoxicillin bioavailability in many species of animals [15], oral bioavailability of ABPC in swine is expected 15–24%. Serum protein binding rate of ABPC has been reported 17% in both bovine and human [1].

In this study, the residue concentrations of ABPC in swine intestines were determined higher than those in other tissues. It suggests that high ABPC concentration in swine intestine results from the release of bile including ABPC into the small intestine, because Galtier *et al.* have reported that biliary ABPC excretion rate is $3.55 \pm 0.66 \mu\text{g/kg/min}$ after intravenous administration of 20 mg/kg to swine [5].

In conclusion, the analytical method for residual ABPC in swine tissues is developed in this study. Our results determined by the analytical method prove that the 5-day withdrawal period in swine administered in medicated feed at the maximum dose and administration period of ABPC (24 mg/kg/day within 1 week) is appropriate to ensure that ABPC concentrations are below the MRLs. It is revealed that the withdrawal period of 5 days in swine established for forced oral administration of the ABPC product is also assigned to that for the medicated feed administration.

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