**NOTE**

Pharmacology

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**LC-MS/MS measurement of ampicillin residue in chicken tissues at 2 days after in-feed administration**

Kouko HAMAMOTO1) and Yasuharu MIZUNO1)

1)National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1, Tokura, Kokubunji, Tokyo 185-8511, Japan

**ABSTRACT.** We assessed ampicillin (ABPC) concentrations of liver, kidney, and skin at a 2-day withdrawal period in ten male and ten female White Leghorn chickens fed the diet containing ABPC (ABPC medicated feed 40 mg/kg body weight/day) for a week. The ABPC residues were measured with liquid chromatography–tandem mass spectrometry and the mean recoveries and quantitation limits ranged from 93.0% to 102.7% and from 0.1 to 1.4 ng/g, respectively. The residual ABPC concentrations were ≤7.82 ng/g for the skin and ≤0.64 ng/g for the kidney, suggesting below the Japanese provisional maximum residue limits. These results reveal that the analytical method is developed for residue ABPC and that the withdrawal period is appropriate.

**Key words:** ampicillin, chicken tissue, LC-MS/MS, medicated feed, residue level

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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 orally administered to chicken at 5–20 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 chicken were established as 0.02, 0.02, 0.03, 0.02 and 0.02 mg/kg (ppm) in the muscle, fat, liver, kidney and other edible tissues including skin, respectively [9].

Various analytical methods have been evaluated for the quantification of ABPC residue in organs, tissues, milk and eggs of domestic animals. However, a sensitive analytical method for determination of ABPC in chicken tissues has not been reported. According to the application for approval of the original ABPC product, it has been shown that liver and kidney are higher levels than other chicken tissues in the residue study following forced oral administration of ABPC. At the time of this application, it was shown that residual concentrations of liver and kidney on the withdrawal period (2 days) following forced oral administration of ABPC were <limit of detection (LOD: 0.03 ppm) and <LOD (0.02 ppm), respectively. However, residue chicken skins have not been confirmed sufficiently, and the effect of food intake on the oral absorption of ABPC in chicken has not been investigated in the oral ABPC product. It is known that food intake affects the bioavailability of ABPC in human [13]; therefore, it was needed to clarify the effect of in-feed administration to chicken on the withdrawal period of the oral ABPC product for food safety by measuring ABPC residue concentrations of only livers and kidneys as long residual tissues and skins at 2 days after in-feed administration. In this study, we aimed to assess a 2-day withdrawal period of ABPC in chicken after oral administration through medicated feed at the maximum dose.

Ten male and ten female clinically healthy White Leghorn chickens (mean ± standard deviation body weight during medication; 2.0 ± 0.3 kg) were used for the ABPC residue study after medicated feeding about a 40 mg (potency)/kg/day dose of ABPC product, which is sold as a “ampicilline powder KS” (Kyoritu Seiyaku, Co., Ltd., Tokyo, Japan). The product was uniformly mixed at 1.2 mg ABPC/g with drug-free feed (SD feed for chicken, Nippon Formula Feed Manufacturing Co., Ltd., Yokohama, Japan). 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 chicken. Animals were fed medicated feed ad libitum for a week, and feed intake was measured once a day. The average feeding intake of the chickens during medication was 76.5 g/day per a chicken, and the mean ABPC concentration in medicated feed was 1.206 mg/g measured by the modified LC-MS/MS method; therefore, chickens were administered at 45.7 mg/kg body weight per day for a week. Chickens were sacrificed at 2-day withdrawal period, and their livers, both kidneys and skins were divided into parts of 5 g as soon as possible. The divided tissues were immediately stored at –80°C until ABPC determination. Three samples of liver and kidney or six samples of skin for residual ABPC analysis were randomly selected from samples of 20 chickens used for this examination. All animal experimental procedures were performed in accordance...
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with the Guidelines for Regulation of Animal Experimentation issued by the National veterinary Assay Laboratory (2005). Chemicals, reagents, instruments and chromatographic conditions in ABPC residue analysis are used according to previous LC-MS/MS method of ABPC in swine tissues [5].

Kidney and skin removed fat as hard as possible (5 g) were added to 0.1 ml of the working IS (25 µg/ml of the deuterium-labeled ABPC (ABPC-d5, molecular weight: 354.4) in water) solution and 5 ml of water and homogenized for 1 min with a homogenizer (Phylocitrone, Microtec Co., Ltd., Chiba, Japan). In the case of a skin sample, the sample solution was kept cool in ice-water bath during sample homogenization to prevent temperature increase. After centrifugation of the homogenized samples at 2,073 xg (3,000 rpm) for 15 min at 5°C, the supernatant of skin sample was re-centrifuged at 14,475 xg (14,000 rpm) for 20 min at 5°C. For the kidney samples, the supernatant fluid was re-centrifuged at 14,475 xg for 40 min at 5°C. Approximately 400 µl of the supernatant fluid of each sample was transferred to the filter units (Ultraceyl 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 centrifuged again at 14,475 xg (14,000 rpm) for 30 min at 5°C. Each filtrated fluid was filtrated by a 0.45 µm membrane filter (Eckocidisc 13, Nippon Genetics Co., Ltd., Tokyo, Japan).

Liver (5 g) was added to the IS working solution and Millivaine buffer (pH 7.0, 5 ml). The mixture was homogenized for 1 min using the homogenizer and centrifuged at 2,073 xg (3,000 rpm) for 15 min at 5°C. The supernatant (ca. 3–4 ml) was separated and centrifuged again at 14,000 rpm for 20 min at 5°C and filtrated by 0.45 µm filter (p25 mm, Millex-HPF HV, Millipore, Temecula, MA, U.S.A.). The filtrate (2 ml) was loaded onto a solid-phase extraction (SPE) cartridge (Bond elute C18, 500 mg/6 ml, Agilent Technologies Inc., Santa Clara, CA, U.S.A.), which was prewashed with acetonitrile (6 ml) and water (10 ml). After sample loading, the SPE cartridge was washed with water (3 ml), and the analytes were eluted with an 85% acetonitrile solution in water (6 ml). The first 13 drops of the eluant from the cartridge were discarded in order to concentrate the sample solution sufficiently in next evaporation step, following which the eluat was collected. The cartridge eluant was evaporated at 30°C until its volume reached 0.7 ml, diluted with water (1 ml) and filtered using the 0.45 µm membrane filter. All samples (50 µl) were injected onto an LC–MS/MS system.

The LC-MS/MS system consisted of a HPLC Nanospace series (Shiseido Co., Ltd., Tokyo, Japan) and a TSQ Quantum Discovery Max apparatus (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Samples were maintained at 4°C in the autosampler and injected onto a Hypersil C18 column (particle size: 3 µm, 2.1 mm ×50 mm, Thermo Fisher Scientific) with aSumipax filter PG-ODS pre-column (SCAS, Osaka, Japan), which was maintained at 40°C. LC-MS/MS mobile phases consisted of 0.05% formic acid in water (solvent A, v/v) and 0.05% formic acid in methanol (solvent B, v/v) for gradient elution and flowed 0.2 ml/min. The gradient program was set as: time (min)% mobile phase A: 0.5/100, 6.5/0, 2/100 and 6/100. The MS/MS condition was set as: ESI (negative mode), splay voltage 3 kV, capillary temperature 300°C, sheath gas 50 a.u., auxiliary gas 20 a.u., precursor >product ion m/z 348.1>207.0 (ABPC) and m/z 353.1>212.0 (IS), collision energy (CE) 16 eV and collision gas (argon) 1.2 mTorr.

Typical chromatograms of ABPC and IS in blank chicken skin spiked with ABPC (7 ng/g) and blank chicken skin are shown in Fig. 1. Provisional MRLs of ABPC in chicken 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) [2] and the ABPC tolerance in U.S.A. (0.01 ppm) [3]. No interaction peak was observed in the blank tissues under the present assay conditions. The ABPC standard (858 µg (potency)/mg) for the Japanese official assay methods applied to veterinary medicine ABPC products was obtained from the National Veterinary Assay Laboratory (Tokyo, Japan). Linearity of the standard calibration curves (r) for kidney and skin was >0.999 for ABPC levels of 0.1 (excluding the calibration curve for kidney), 0.5, 2.5, 5, 10, 25 and 50 ng/ml in water. It suggests a matrix effect in liver samples that peak areas of ABPC and IS in the liver samples were less than half of them (33–38%) in the calibration standards; therefore, matrix-matched calibration standards were used for the determination of ABPC in chicken liver. Linearity of the standard curves (r) for liver was >0.999 for ABPC levels of 2.5, 5, 10, 25 and 50 ng/ml with chicken liver matrixes. It is a first report of highly sensitive analytical methods for ABPC in chicken skin, kidney and liver using LC-MS/MS. Especially, chicken liver contains a lot of fat; therefore, residual concentration in chicken liver was measured simple and high sensitive by extracting with the Millivaine buffer instead of water.

In our LC—MS/MS method for determining ABPC, extraction recoveries, LODs (signal to noise ratio (S/N)=3), limits of quantitation (LOQs, S/N=10), intraday and interday precisions at 7 ng/g in the three tissues are shown in Table 1. LODs (0.01–0.06 ng/g) and LOQs (0.1–0.2 ng/g) of ABPC in kidney and skin in our method were better than other method for chicken muscle tissue (LOD=0.6 ng/g and LOQ=1.5 ng/g) [8]. These recoveries and precisions fulfilled Guideline 49 of the International Cooperation on Harmonization of Technical Requirements for Evaluation of Veterinary Products (VICH GL49) [6].

ABPC levels after the withdrawal period following the last administration were determined to be 0.64 ng/g in one kidney and 6.30 ± 1.5 ng/g in six skins; however, ABPC residues were not detected in two kidneys and in 3 livers (Table 2). ABPC was detected in one kidney sample, whereas it was not detected in other kidney samples. We cannot discuss any factor for the individual differences of ABPC residue concentration within kidneys, because of a limited number of samples (n=3).

In this study, skin samples were determined highest residue concentration among three kinds of chicken tissue samples. In rat, it has been reported that ABPC concentrations in skin samples (0.39 ± 0.19 µg/g) are about 10 times higher than those in plasma samples (0.04 ± 0.02 µg/g) at 8 hr following intravenous administration of an ABPC injection [10]. The beta half-life of ABPC following intravenous administration to chicken has been reported 1.96 hr [7]. It indicates that the elimination of ABPC from a central compartment in chicken is fast. As for differences among tissue distributions, it is known that biological factors

doi: 10.1292/jvms.15-0350
affecting drug distribution are blood flow to tissue and capillary porosity [4, 11–12, 14]. Drugs enter rapidly high perfused tissues with discontinuous capillaries (e.g. liver), while drug distribution into the tissues with low perfusion rates and with continuous capillaries (e.g. skin) is slow. Higher ABPC concentrations in skin samples than those in liver and kidney samples at the withdrawal period in the present study seem to be caused by slow drug transfer between skin and plasma depending on the low blood perfusion in skin.

Fig. 1. Typical chromatograms of ampicillin (ABPC) and IS (ABPC-d5) in chicken skins, (A) blank skin spiked with ABPC (7 ng/g), (B) blank skin.
Alhendi et al. have reported that the mean of the ABPC residue concentrations in the kidney of broiler chicken after 2 days following oral administration of two dose levels of 1 and 2 mg/kg body weight/day for 40 days was 250 ± 30 and 310 ± 40 ng/g, respectively [1]. These higher residue concentrations in their study than our results are presumed to result from their long administration period (about 6 times longer than our study) and the difference of analytical method (Alhendi et al.’s analytical method: bioassay, LOD=50 ng/g).

In conclusion, the analytical method for residual ABPC in chicken liver, kidney and skin is developed in this study. Our results determined by the analytical method prove that the 2-day withdrawal period in chicken administered in medicated feed at the maximum dose and administration period of ABPC (40 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 2 days in chicken established for forced oral administration of the ABPC product is also assigned to that for the medicated feed administration.

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### Table 1. Recovery, precision and accuracy of the method for the determination of ABPC in chicken liver, kidney and skin by LC-MS/MS (n=3)

| Tissue   | Concentration in control tissues (ng/g) | Mean recovery (%) | Intraday coefficient of variation | Interday coefficient of variation | Limit of detection (ng/g) | Limit of quantitation (ng/g) |
|----------|----------------------------------------|-------------------|-----------------------------------|-----------------------------------|--------------------------|----------------------------|
| Liver    | 7                                      | 93.8              | 2.0                               | 5.7                               | 0.41                     | 1.35                      |
| Kidney   | 7                                      | 102.7             | 2.5                               | 8.7                               | 0.06                     | 0.20                      |
| Skin     | 7                                      | 93.0              | 2.5                               | 2.7                               | 0.01                     | 0.10                      |

### Table 2. Residual ampicillin (ABPC) concentration in the liver, kidney and skin after 2-day withdrawal period fed the diet containing ABPC (ABPC medicated feed, 40 mg/kg/day) for a week (Liver and kidney: n=3, skin: n=6)

| Animal No. | Liver (ng/g) | Kidney (ng/g) | Skin (ng/g) |
|------------|--------------|---------------|-------------|
| 1 (male)   | ND a)        | ND a)         | 7.64        |
| 2 (male)   | ND a)        | ND a)         | 6.91        |
| 3 (female)| ND a)        | 0.64          | 6.43        |
| 4 (male)   | ND b)        | ND b)         | 7.82        |
| 5 (female)| ND b)        | ND b)         | 5.43        |
| 6 (female)| ND b)        | ND b)         | 3.56        |

Mean ± SD 6.30 ± 1.5

Provisional maximum residue limit<sup>c)</sup> 30 20 20

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<sup>a)</sup> ND: Not detected (<the limit of detection); <sup>b)</sup>: No data; <sup>c)</sup> 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/.
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