Application of ELISA for the Detection of Penicillin Antibiotic Residues in Live Animal

H. J. Lee, M. H. Lee* and In K. Han[1]

College of Veterinary Medicine, Seoul National University, Suwon 441-744, Korea

ABSTRACT : Penicillin antibiotics such as penicillin G, ampicillin and amoxicillin have been widely used in the pig industry to control salmonellosis, bacterial pneumonia, and urinary tract infections. Extensive use of antibiotics in veterinary clinics has resulted in tissue residues and bacterial resistance. To prevent unwanted drug residues entering the human food chain, extensive control measures have been established by both government authorities and industries. The demands for reliable, simple, sensitive, rapid and low-cost methods for residue analysis of foods are increasing. In this study, we established a rapid prediction test for the detection of pigs with unacceptable tissue residues of penicillins. The recommended therapeutic doses of three penicillins, penillin G (withdrawal time, 7 days), ampicillin (withdrawal time, 7 days) and amoxicillin (withdrawal time, 14 days), were administered to three groups of 20 pigs each. Blood was sampled before drug administration and during the withdrawal period. The concentration of penicillins in plasma, determined by a semi-quantitative ELISA, were compared to that of internal standard, 4 ppb, which corresponded to the Maximum Residue Limit in milk. The absorbance ratio of internal standard to sample (B/Bs) was employed as an index to determine whether drug residues in pig tissues were negative or positive. That is, a B/Bs ratio less than 1 was considered residue positive, and larger than 1 negative. All 60 plasma samples from pigs were negative to three penicillins at pretreatment. Penicillin G could be detected in the plasma of the treated pigs until day 4 post-treatment and ampicillin until day 2, whereas amoxicillin could be detected until day 10 of its withdrawal period. The present study showed that the semi-quantitative ELISA could be easily adapted to detect residues of penicillin antibiotics (penicillin G, ampicillin and amoxicillin) in live pigs. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 11 : 1604-1608*)

Key Words : Live Animal Screening Test, Penicillins, ELISA, Pig, Plasma

INTRODUCTION

With the ever-growing world population, animal production practices have become more intensive and efficient, accompanied by increasing demands for drug treatments. Currently, approximately 80% of all food animals receive medication for part or most of their lives (Sternesjö et al., 1998). In the near future, nearly all animals produced in the world for food will have received a chemotherapeutic and prophylactic agent of some type (Booth, 1988). A survey in 1993 of all carcasses violating statutory limits for contaminants in the United States revealed that the drugs most frequently causing residues were penicillin (20%), streptomycin (10%), oxytetracycline (10%), and sulfamethazine (9%) (Paige, 1994). According to the Canadian federal meat inspection testing programs, penicillins were the most frequently detected residues in tissues from pigs (Korsrud et al., 1998). In Korea, Department of Veterinary Service, Ministry of Agriculture & Forestry has conducted National Residue Program (NRP) to investigate drug residues of livestock products from slaughtering establishments, and from import shipments at the port of entry in

1986. In 1997, a total of 45,000 samples comprising 10,000 beef, 23,000 pork, and 11,000 poultry meats were analyzed for five antibiotics (penicillins and tetracyclines) and six sulfonamides residues and the results showed violative residues of tetracyclines, sulfonamides, and aminoglycosides in beef and pork meat.

Penicillin G, ampicillin and amoxicillin are the major penicillin antibiotics used in veterinary medicine (Nielsen et al., 1994). A few cases of minor allergic reactions (e.g., skin rashes) in previously sensitized individuals due to penicillin G residues from milk and meat have been documented. Also, widespread agricultural use of antibiotics might be linked to an increase of antibiotic resistance to the animal and human pathogens (Dewdney et al., 1984; Franco et al., 1990; Huber, 1971; Kindred et al., 1993; Mitchell et al., 1995; Ormerod et al., 1987).

Therefore, it is a very important problem for the public health to prevent antibiotic residues in foods. Reliable, simple, sensitive, rapid and low-cost methods for detecting residues in foods are needed (Mitchell et al., 1998). Various of ELISA technologies have been developed and adopted for detecting the generic groups of chemical residues in milk, urine, blood, and meat samples (Gardner et al., 1996; Szekacs, 1994). Enzyme-linked immunosorbent assay (ELISA) has become the most popular method for chemical residue detection in food due to its extreme sensitivity,

---

* Address reprint request to M. H. Lee. Tel: +82-331-290-2741, Fax: +82-331-293-0084, E-mail: yetlee@snu.ac.kr.
[1] College of Agriculture & Life Sciences, Seoul National University, Suwon 441-744, Korea.
Received June 27, 2000; Accepted August 18, 2000

simplicity, and ability to screen a large number of samples (Clifford, 1985; Gardner et al., 1996; Szekacs, 1994).

We have established an ELISA method to predict penicillin G, ampicillin, and amoxicillin residues in swine tissues by examining the drug depletion profile from blood plasma during the withdrawal period. The method established can be applied to live animals at farms, or at slaughterhouses before slaughtering.

MATERIAL AND METHODS

Materials

Animals used in this study were sixty healthy pigs weighing an average of 65 kg with no previous history of antibiotic treatment. Penicillin Injection (300,000 IU/ml procaine penicillin G) was purchased from Green Cross Animal Pharmaceutical Company (Seoul, Korea). Binotal Injection (100 mg/ml ampicillin natrium) was obtained from Bayer Korea Ltd. (Seoul, Korea). Clamoxyl L.A. Injection (150 mg/ml amoxicillin trihydrate) was obtained from Pfizer Korea Ltd. (Seoul, Korea). ELISA kits for β-lactams, manufactured by Idetek, were purchased from Korea Media Ltd.

Drug administration and samples

Penicillin G was administered intramuscularly to 20 pigs at a rate of 4000 IU per kg body weight per day for four consecutive days, ampicillin intramuscularly to 20 at a rate of 10 mg per kg body weight per day for four consecutive days, and amoxicillin intramuscularly to 20 at a rate of 15 mg per kg body weight per day for four consecutive days. Blood samples were collected from all pigs before administration of the drugs and on day 1, 2, 4, 6, 8, and 10 after the last administration. From the pigs treated with amoxicillin blood samples were also collected on day 14 after withdrawal. Ten ml of blood from each pig was collected in heparinized tubes and centrifuged at $4500 \times g$ for 10 minutes to collect the plasma.

Preparation of standard curves

Stock standard solutions of 100 μg/ml of each of penicillin G, ampicillin, and amoxicillin were prepared using USP standards in saline. These stock solutions were further diluted with saline to prepare 1, 2, 5, and 10 μg/ml working standard solutions. Standard curves of each antibiotics were constructed using the standard solutions fortified into plasma to estimate the detection limit for the ELISA kit.

Analysis of penicillins in plasma

ELISA test methods for ß-lactams were applied to each plasma sample in duplicate using a modified methodology described by Boison et al. (1995), in which the manufacturer's protocol for milk screening was adapted for plasma screening. Briefly, 250 μl of the internal standard solution (equivalent to 4 ppb penicillin G) was pippetted into a test tube containing immobilized antibodies against β-lactams. The plasma (250 μl, diluted 1:10 w/PBS) was pippetted into individually labeled tubes. An equal volume of tracer solution (enzyme conjugate, lyophilized horseradish peroxidase-labelled β-lactams conjugate with preservative) was added, and the test tubes were incubated at room temperature for 3 minutes with continuous shaking. The excess sample and conjugate reactants were then washed out with saline. A color developer (0.5 ml, enzyme substrate) made up of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide in citrate buffer was added to the test tubes; the mixture was incubated at room temperature for 3 minutes with continuous shaking. Diluted sodium dodecyl sulfate solution (0.5 ml) was added to each test tube to stop the reaction. The absorbance was read at the wavelength of 405 nm with a photometric detector (Idetek Reader, Awareness Technology, Inc., USA, operated in the 0.9 ratio mode) and compared with that of the internal standard (4 ppb). Samples with absorbance greater than that of the internal standard were considered to be negative (ß-lactams drug free), and those with absorbance less than that of the internal standard were considered as positive. In this analysis, no more than 5 samples were processed simultaneously, and the assay was completed within 10 minutes (Boison et al., 1995; Cullor et al., 1994).

RESULTS

Standard curves and detection limits

The standard curves of penicillin G, ampicillin and amoxicillin were constructed to determine the detection limits of each drug. The detection limits of the drugs were less than 1 ppb based on the B/Bo ratio of 0.8 in the ELISA system (figure 1).

Live animal test for penicillins

Penicillin G. Results of plasma analysis are shown in table 1. As the absorbance ratios of the 20 pigs before injection were greater than 1.0, the concentrations of penicillin in the diluted plasma ($\times 10$) of this group were less than 4 ppb and showed a negative result. On day 1 of withdrawal, 7 of the 20 samples were found positive. The number of positive samples on day 2 was 4, and was 2 samples on day 4 of withdrawal. All samples showed negative reaction after day 5 of withdrawal (B/Bs ratio ≥ 1.0).

Ampicillin. Results of plasma analysis are shown in table 2. Absorbance ratios before injection were all greater than 1.0, that is the concentrations of
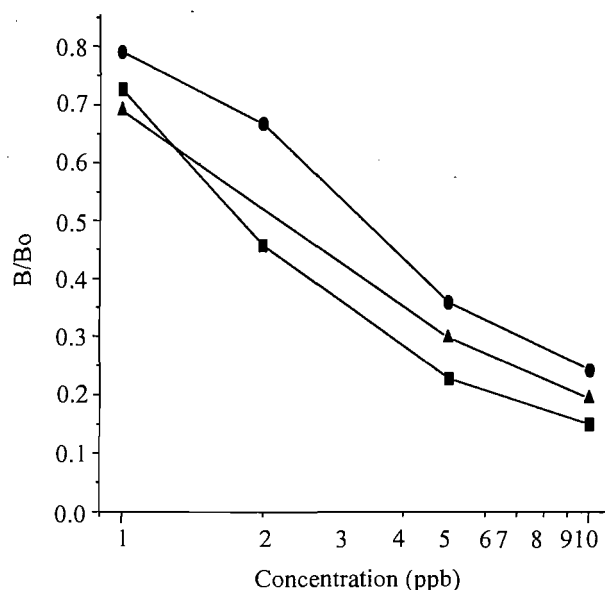
Detection limit of penicillins was calculated as less than 1 ppb in saline. The detection limit of ELISA kit was decided as the point of B/Bo ratio of 0.8. B/Bo: Absorbance ratio of standard (Bo) and saline or control serum (B).

▲: Penicillin G, ■: Ampicillin, ●: Amoxicillin.

Figure 1. Standard curves of penicillins in saline

ampicillin in the diluted plasma (×10) of this group were less than 4 ppb, showing negative. On day 1 of withdrawal, 8 of the 20 samples were found positive. The number of positive samples on day 2 was 4. All samples showed a negative reaction after day 4 of withdrawal (B/Bs ratio ≥ 1.0).

Amoxicillin. Results of plasma analysis are shown in table 3. Absorbance ratios before injection were all

Table 2. Depletion profile of ampicillin in plasma during the withdrawal period

Withdrawal (days)	No. positive	No. negative	B/Bs ratio (Mean ± SE)
Control*	0	20	1.361 ± 0.134
1	8	12	1.013 ± 0.191
2	4	16	1.262 ± 0.318
4	0	20	1.303 ± 0.240
6	0	20	1.343 ± 0.095
8	0	20	1.362 ± 0.086
10	0	20	1.411 ± 0.124

* Blood was collected before administration of ampicillin. The drug was administered intramuscularly with 10 mg/kg body weight once daily for four consecutive days, and blood samples were collected from pigs during the withdrawal period. Concentration of ampicillin in plasma was analyzed using LacTek ELISA kit. B is absorbance of sample and Bs is absorbance of internal standard (4 ppb).

Table 1. Depletion profile of penicillin G in plasma during the withdrawal period

Withdrawal (days)	No. positive	No. negative	B/Bs ratio (Mean ± SE)
Control*	0	20	1.472 ± 0.085
1	7	13	1.214 ± 0.341
2	4	16	1.362 ± 0.425
4	2	18	1.389 ± 0.191
6	0	20	1.413 ± 0.095
8	0	20	1.449 ± 0.327
10	0	20	1.501 ± 0.204

* Blood was collected before administration of penicillin G. The drug was administered intramuscularly with 4000 IU/kg body weight once daily for four consecutive days, and blood samples were collected from pigs during the withdrawal period. Concentration of penicillin G in plasma was analyzed using LacTek ELISA kit. B is absorbance of sample and Bs is absorbance of internal standard (4 ppb).

greater than 1.0, so the concentrations of amoxicillin in the diluted plasma (×10) of this group were less than 4 ppb (negative). On day 1, 2, 4, and 6 of withdrawal, all samples showed positive. After day 8 of withdrawal, 11 of the 20 samples were found positive. All samples showed a negative reaction after day 14 of withdrawal (B/Bs ratio ≥ 1.0).

DISCUSSION

To prevent unwanted drug residues from entering the human food chain, both the government authorities and the industries have established extensive control measures (Sternesjö et al., 1998). A variety of rapid

Table 3. Depletion profile of amoxicillin in plasma during the withdrawal period

Withdrawal (days)	No. positive	No. negative	B/Bs ratio (Mean ± SE)
Control*	0	20	1.746 ± 0.202
1	20	0	0.165 ± 0.059
2	20	0	0.211 ± 0.068
4	20	0	0.377 ± 0.165
6	20	0	0.563 ± 0.216
8	11	9	0.985 ± 0.269
10	1	19	1.482 ± 0.373
14	0	20	1.652 ± 0.342

* Blood was collected before administration of amoxicillin. The drug was administered intramuscularly with 15 mg/kg body weight once daily for four consecutive days, and blood samples were collected from pigs during the withdrawal period. Concentration of amoxicillin in plasma was analyzed using LacTek ELISA kit. B is absorbance of sample and Bs is absorbance of internal standard (4 ppb).

screening tests have been developed and applied for determining drug contamination of animal products on farms and slaughterhouses.

The Swab Test On Premises (STOP), a nonspecific microbial inhibition test, has been used in abattoirs in the United States and Canada for over 10 years to screen for antibiotic residues in tissues from slaughtered animals (Korsrud et al., 1998). The test requires overnight incubation, and results are not ready until the following day. In the United States, however, the Live Animal Swab Test (LAST), which tests urine of live animals, is used to screen for antibiotic residues. This test, like STOP, requires overnight incubation, and results are not ready until the following day. Sweeney et al. (1993) developed a model to predict the number of days for sulfamethazine concentration to fall below 0.1 ng/g of tissue residues in various organs from analysis of the urine of pigs. This prediction model provided the practical basis for the current SOS test in which swine urine is used for screening sulfonamide residues in animal tissues in federally inspected abattoirs of the United States, Canada, and Korea. With the correlation between residue levels in tissue and urine established, the urine residue is used as an indicator of sulfamethazine in animal tissue (Boison et al., 1995). Though, unlike STOP and LAST, the SOS test provides same-day results, it is able to detect only sulfonamides. Boison et al. (1995) conducted experiments to determine whether penicillin residues in the plasma of live animals can be used as an indicator of penicillin residues in tissues of food-producing animals at slaughter. According to the results, penicillin G in the plasma did not correlate with that in tissues. To estimate withdrawal time for penicillin G, Korsurd et al. (1998) analyzed tissue, plasma, and muscle from healthy pigs injected intramuscularly with procaine penicillin G and slaughtered after withdrawal from the drug for various periods of time. Penicillin G was not detected in liver after 1 day of withdrawal, in muscle and fat after 2 days of withdrawal, in plasma after 4 days of withdrawal. And the correlation between residue levels in tissues and plasma was not shown. With the administered dosage taken into consideration, plasma concentrations profiles of penicillin antibiotics in our study were similar to the above studies. As the withdrawal time of a drug is established based on the tolerance level in tissue and elimination rate of the drug, and blood is a central pool of drug distribution to body compartments and elimination from tissues through biological fluids (Booth, 1988), it may be able to predict the residue of drugs in tissue by examining the blood drug depletion profile during withdrawal period (Korsrud et al., 1995; Boison et al., 1995).

According to our results, the developed methods can be adapted easily to detect residues of penicillin antibiotics in live pigs using diluted (×10) blood plasma with the modified ELISA test kits. To increase the sensitivity of the ELISA test kit the concentration of its internal standard solution was modified to 2 ppb and the plasma samples were retested. Because 10 of the 60 samples showed a positive reaction it was not possible to lower the concentration of the internal standard (data not shown).

It is conceivable that the veterinary inspector in the abattoir may be able to use this method to screen for penicillin antibiotics in plasma from live pigs in holding pens prior to slaughter, or on farm, and obtain same-day results. Pigs that show positive can then be held in the pens until retest results come up negative before they are slaughtered.

REFERENCES

Boison, J. O., G. O. Korsrud, M. G. Papich and J. D. MacNeil. 1995. Comparison of four commercially available rapid test kits with liquid chromatography for detecting penicillin G residues in bovine plasma. J. Assoc. Off. Anal. Chem. Int. 78:1144-1152.

Booth, N. H. 1988. Toxicology of drug and chemical residues. In: Veterinary Pharmacology and Therapeutics. (Ed. N. H. Booth and L. E. McDonald). Iowa State University Press, Ames, Iowa. pp. 1149-1205.

Clifford, M. N. 1985. The history of immunoassays in food analysis. In: Immunoassays in Food Analysis. Elsevier Applied Science Publishers, New York. pp. 3-10

Cullor, J. S., A. Van Eenennaam, I. Gardner, L. Perani, J. Dellinger, W. L. Smit, T. Thompson, M. A. Payne, L. Jensen and W. M. Guterbock. 1994. Performance of various tests used to screen antibiotic residues in milk samples from individual animals. J. Assoc. Off. Anal. Chem. Int. 77(4):862-870.

Dewdney, J. M. and R. G. Edwards. 1984. Penicillin hypersensitivity is milk a significant hazard?: a review. J. Rech. Soc. Med. 77:866-877.

Franco, D. A., J. Webb and C. E. Taylor. 1990. Antibiotic and sulfonamide residues in meat: implications for human health. J. Food Prot. 53:178-185.

Gardner, I. A., J. S. Cullor, F. D. Galey, W. Sischo, M. Salman, B. Slenning, H. N. Erb and J. W. Tyler. 1996. Alternatives for the validation of diagnostic assays used to detect antibiotic residues in milk. J. Am. Vet. Med. Assoc. 209:46-52.

Huber, W. G. 1971. The impact of antibiotic drugs and their residues. Adv. Vet. Sci. Comp. Med. 15:101-132.

Kindred, T. P. and W. T. Hubbert. 1993. Residue prevention strategies in the United States. J. Am. Vet. Med. Assoc. 202:46-49.

Korsrud, G. O., C. D. C. Salisbury, A. C. E. Fesser and J.

D. MacNeil. 1995. Laboratory evaluation of the Charm Farm test for antimicrobial residues in meat. J. Food Prot. 58:1129-1132.

Korsrud, G. O., C. D. C. Salisbury, C. S. Rhodes, M. G. Papich, W. D. G. Yates, W. S. Bulmer, J. D. MacNeil, D. A. Landry, G. Lambert, M. S. Yong and L. Ritters. 1998. Depletion of penicillin G residues in tissues, plasma and injection sites of market pigs injected intramuscularl with procaine penicillin G. Food Add. Contam. 15(4):421-426.

Mitchell, J. M. and A. J. Yee. 1995. Antibiotic use and transfer of drug resistance: does it mean we should stop treating animals with these drugs? Dairy Food Environ. Sanit. 15:484-487.

Mitchell, J. M., M. W. Griffiths, S. A. McEwen, W. B. McNab and A. J. Yee. 1998. Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. J. Food Prot. 61(6):742-56.

Nielsen, P. and N. J. Gyrd-Hansen. 1994. Bioavailability of penicillin V after oral administration to fed and fasted pigs. J. Vet. Pharmacol. Ther. 17(2):160-162.

Ormerod, A. D., T. M. S. Reid and R. A. Main. 1987. Penicillin in milk-its importance in urticaria. Clin. Allergy. 17:229-234.

Paige, J. C. 1994. Analysis of tissue residues. FDA Vet. 9(6):4-6.

Sternesjö, Å. and G. Johnsson. 1998. A novel rapid enzyme immunoassay (fluorophos betaScreen) for detection of β-lactam residues in ex-farm raw milk. J. Food Prot. 61(7):808-811.

Sweeney, R. W., P. C. Bardalaye, C. M. Smith, L. R. Soma and C. E. Uboh. 1993. Pharmacokinetic model for predicting sulfamethazine disposition in pigs. Am. J. Vet. Res. 54(5):750-754.

Szekacs, A. 1994. Development of enzyme-linked immunosorbent assay (ELISA) systems for environmental monitoring. Acta Biol. Hung. 45:77-80.