

Application of ELISA for the Detection of Oxytetracycline Residue in Live Animals

H. J. Lee, M. H. Lee* and In K. Han¹

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

ABSTRACT : Oxytetracycline has been widely used in the cattle industry to control pneumonia, shipping fever, foot rot, bacterial enteritis, and uterine infections. Extensive use of antibiotics in veterinary clinics has resulted in residues in tissue and bacterial resistance to antibiotics. To prevent unwanted drug residues from 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 test for tissue residues of oxytetracycline in cattle. The recommended therapeutic dose of oxytetracycline (withdrawal time, 14 days) was administered to 10 cattle. Blood samples were collected from each cow before drug administration and during the withdrawal period. The concentration of oxytetracycline in plasma, determined by a semi-quantitative ELISA, was compared to that of the internal standard, 10 ppb. The absorbance ratio of internal standard to sample (B/Bs) was employed as an index to determine whether the residues in cattle tissues were negative or positive. That is, a B/Bs ratio less than 1 was considered as residue positive and that greater than 1 as negative. Based on this criterion, all plasma samples from cattle were negative to oxytetracycline at pre-treatment. Oxytetracycline could be detected in the plasma treated cattle until day 14 post-treatment. The present study showed that the semi-quantitative ELISA could be easily adapted in predicting tissue residues for oxytetracycline in live cattle. (*Asian-Aus. J. Anim. Sci.* 2000. Vol. 13, No. 12 : 1775-1778)

Key Words : Live Animal Screening Test, Oxytetracycline, 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. Antimicrobial agents are widely used in animal husbandry for prophylactic and therapeutic purposes. 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 chemotherapeutic and prophylactic agents of some type (Booth, 1988). A survey in 1993 of all violative carcasses in the United States revealed that the drugs most frequently causing residues were penicillin (20%), streptomycin (10%), oxytetracycline (10%), sulfamethazine (9%), and tetracycline (4%) (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, the Department of Veterinary Service, Ministry of Agriculture & Forestry has conducted a 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.

Since the tetracyclines are extensively used and have been reported to prevail in coliform multiresistant organisms, veterinarians should consider dosage regimens very carefully and anticipate the likelihood of dealing with these organisms (Booth, 1988). Also, it is a very important problem for 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). There is a need to develop rapid tests providing same-day results that will also enable detection of antimicrobials in biological fluids, obtained preferably from live animals, which then can be used by primary producers in the field or in abattoirs. Various of enzyme immunoassay technologies have been developed and adopted for detecting the generic groups of chemical residues in milk, urine, blood, and meat samples (Szekacs, 1994; Gardner et al., 1996). ELISA has become the most popular method for chemical residue detection in food due to its extreme sensitivity, simplicity and ability to screen large numbers of samples (Clifford, 1985; Szekacs, 1994; Gardner et al., 1996). Most residue assays commercially available for field applications are qualitative or semi-quantitative screening assays, which provide a reliable and accurate indication that the analyte of interest is not present in the sample at unsafe or violative levels. This requires that screening

* Address reprint request to M. H. Lee. Tel: +82-331-290-2741, Fax: +82-331-293-0084, E-mail: vetlee@snu.ac.kr.

¹ College of Agriculture & Life Sciences, Seoul National University, Suwon 441-744, Korea.

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assays be developed with a detection level optimized below the unsafe or violative levels (maximum residue limits) so that a violative sample will have a high probability of causing a positive test result (Gardner et al., 1996).

We have established an ELISA method to predict oxytetracycline residue in cattle tissue by examining the drug depletion profile from blood plasma during the withdrawal period. The established method here can be applied to live animals at farms or at abattoirs before slaughtering.

MATERIAL AND METHODS

Materials

Ten female cattle, 7 to 8 months old (mean weight 200 kg), of Holstein breed were used. Terramycin Injectable Solution (50 mg/ml oxytetracycline hydrochloride) was purchased from Pfizer Korea Ltd. (Seoul, Korea). ELISA kits for tetracyclines, manufactured by Idetek, were purchased from Korea Media Ltd.

Drug administration and blood samples

Oxytetracycline was administered intramuscularly to all the cattle at the rate of 20 mg per kg body weight per day for four consecutive days. Blood samples were collected from all of cattle before administration of the drugs and on day 1, 3, 5, 7, 10, and 14 after the last administration. Ten ml of blood from each cattle was collected in heparinized tubes and centrifuged at 4500 \times g for 10 minutes to collect the plasma.

Preparation of standard curves

Stock standard solution of 1000 μ g/ml of oxytetracycline was prepared using USP standards in phosphate buffer solution and control serum. This stock solution was further diluted with phosphate buffer solution and serum to prepare 1, 2, 5, 10, 20, 50, 100, 500, and 1000 μ g/ml working standard solutions. A standard curve was constructed using the standard solutions mixed into serum to estimate the detection limit for the ELISA kit.

Analysis of oxytetracycline in plasma

ELISA test methods for oxytetracycline were applied to each plasma sample in duplicate using the methodology described by Boison et al. (1995), which modified the manufacturers protocol for milk screening and adapted it for plasma screening. Briefly, 250 μ l of the internal standard solution (equivalent to 3 ppb oxytetracycline) was pipetted into a test tube containing immobilized tetracyclines antibodies. The plasma (250 μ l, diluted 1:10 w/PBS) was pipetted into individually labelled tubes. An equal volume of tracer solution (enzyme conjugate, lyophilized

horseradish peroxidase-labelled tetracyclines conjugate with preservative) was added, and the test tubes were incubated at room temperature for 3 minutes with continuous shaking, and then the excess sample and conjugate reactants were washed out with saline. After 0.5 ml colour developer (enzyme substrate), made up of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide in citrate buffer was added, the mixture was incubated at room temperature for 3 minutes with continuous shaking. Finally, 0.5 ml of the dilute sodium dodecyl sulfate solution 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 (3 ppb). Samples with absorbance greater than the absorbance of the internal standard were considered to be negative (tetracyclines drug free), whereas samples with absorbance less than that of the internal standard were considered positive. In this analysis, no more than 5 samples were processed simultaneously, and the assay was completed within 10 minutes (Cullor et al., 1994; Boison et al., 1995).

RESULTS

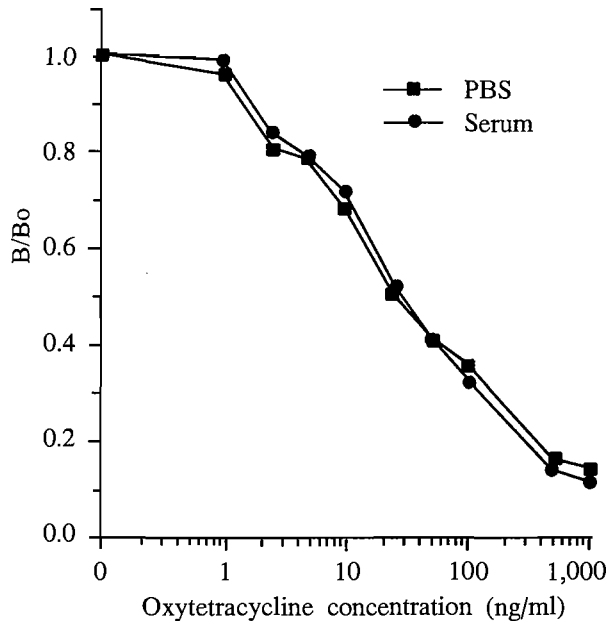
Standard curves and detection limits

The standard curve of oxytetracycline was constructed to determine the detection limit of the drug, which was found to be less than 3 ppb based on the B/Bo ratio of 0.8 in the ELISA system (figure 1).

Live animal test for oxytetracycline in plasma

Results of analysis for oxytetracycline in plasma are shown in table 1. As the absorbance ratios of the control group were greater than 1.0, the concentrations of oxytetracycline in the diluted plasma ($\times 10$) of this group were greater than 3 ppb, thus the group tested negative. All samples tested positive on day 1 of withdrawal. On days 3, 5, and 7, one sample tested negative. The number of positive samples was 6 on day 10 of withdrawal, and on day 14, the last day of withdrawal, three showed positive reaction (B/Bs ratio ≤ 1.0).

To prevent false-positive reaction by ELISA kit for oxytetracycline, we modified the concentrations of ELISA internal standards to 10 and 20 ppb, and compared the B/Bs ratio of oxytetracycline-medicated plasma on day 14 of withdrawal against 20, 10, and 3 ppb internal standards. Three of the 10 cattle tested positive at the 3 ppb internal standard and one positive at the 10 ppb internal standard, but none of the samples tested positive at the 20 ppb internal standard (table 2). Therefore, we adjusted the concentration of the internal standard to 10 ppb. If the



Detection limit of oxytetracycline was calculated as less than 3 ppb. The detection limit of ELISA kit was decided with the point of B/Bs ratio 0.8 B/Bo. The absorbance ratio of standard (Bo) and saline or control serum (B).

Figure 1. Standard curve of oxytetracycline in PBS and serum.

animal tests positive, its slaughter should be postponed.

DISCUSSION

To prevent unwanted drug residues from entering the human food chain, both government authorities and industries have established extensive control measures (Sternesjö et al., 1998). A variety of rapid 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 non-specific microbial inhibition test, has been used in abattoirs in the United States and Canada for more than 10 years to screen for antibiotic residues in tissues of slaughtered animals (Korsrud et al., 1998). The test requires overnight incubation, thus results are not available until the following day. In the United States, a variant of STOP, the Live Animal Swab Test (LAST), which tests urine of live animals, is used to screen for antibiotic residues. This test, like STOP, also requires overnight incubation. Sweeney et al. (1993) developed a model to predict from urine the number of days for sulfamethazine concentration to fall below 0.1 ng/g of tissue in various organs of in pigs. This predictor model provided a practical basis for current SOS test, in which swine urine is used for screening sulfonamide residues in animal tissue in federally inspected abattoirs of the United States, Canada, and Korea. As the correlation between the residue levels in tissue and urine has been established, the urine residue can be used as an indicator of sulfamethazine in animal tissue (Boison et al., 1995). Landoni et al. (1992) studied the tissue distribution of a long-acting oxytetracycline formulation in muscle, kidney, fat, urine, bile, saliva, and serum after intramuscular administration to calves at a dose rate of 20 mg/kg. Observed concentrations were higher than the minimal inhibitory concentration for the majority of pathogens in all of the analyzed tissues for at least 72 hours post-injection. MacNeil et al. (1989) studied the ability of the in-plant screening test (STOP) and laboratory assays (TLCB, HPLC) to detect oxytetracycline residues in calves. Oxytetracycline was detected in the serum from all treated calves 2 days after the

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

Withdrawal (days)	No. of positive	No. of negative	B/Bs ratio (Mean \pm SE)
Control*	0	10	1.215 \pm 0.054
1	10	0	0.232 \pm 0.087
3	9	1	0.524 \pm 0.189
5	9	1	0.754 \pm 0.273
7	9	1	0.926 \pm 0.361
10	6	4	1.042 \pm 0.392
14	3	7	1.135 \pm 0.414

* Blood was collected before administration of oxytetracycline.

Concentration of oxytetracycline in plasma was analyzed using LacTek ELISA kit. B is absorbance of sample and Bs is absorbance of internal standard (3 ppb).

Table 2. Comparison of B/Bs ratios for oxytetracycline in plasma on day 14 of withdrawal between 20, 10 and 3 ppb reference standards

No. of Animals	Concentration of internal standard		
	3 ppb	10 ppb	20 ppb
1	1.012	1.244	1.556
2	0.847	1.010	1.263
3	0.897	1.140	1.425
4	1.028	1.155	1.444
5	2.223	1.329	1.662
6	1.182	1.100	1.326
7	1.085	1.179	1.422
8	0.716	0.916	1.104
9	1.116	1.244	1.499
10	1.243	1.317	1.589
No. of negative	7	9	10
No. of positive	3	1	0

last injection and also detected in the serum after 19 days of withdrawal. The LAST was compared with the quantitative oxytetracycline assay of urine samples and tissue specimens to determine the accuracy of the LAST in detecting oxytetracycline in bovine urine and predicting violative residues in tissues (TerHune et al., 1989). The LAST was inconsistent in detecting urine oxytetracycline in low concentrations and therefore failed to accurately predict oxytetracycline residues in tissues. Taking into consideration the administered dosage, plasma concentration profiles of oxytetracycline 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 (Booth, 1988), it may be possible to predict the residues of drugs in tissue by examining the blood drug depletion profile during the withdrawal period (Boison et al., 1995; Korsrud et al., 1995).

According to our results, the developed methods can be adapted easily for use in prediction of tissue residues of oxytetracycline in live cattle by screening blood plasma with the modified ELISA test kits. The veterinary inspector in the abattoir may be able to use this method to screen for oxytetracycline in plasma of live cattle in holding pens prior to slaughter and obtain same-day results. Thus, cattle testing positive can be held in the pens and retested before they are slaughtered.

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