JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of ELISA and Immunochromatographic Assay for the Detection of Gentamicin

YONG JIN, JIN-WOOK JANG, CHANG-HOON HAN,† AND MUN-HAN LEE*

Department of Biochemistry, College of Veterinary Medicine, Seoul National University, 151-742 Seoul, Republic of Korea

Competitive direct enzyme-linked immunosorbent assay (ELISA) and the immunochromatographic assay were developed using a monoclonal antibody to detect gentamicin in the animal plasma and milk. No cross-reactivity of the antibody was observed with other aminoglycosides based on competitive direct ELISA, indicating that the antibody is highly specific for gentamicin. On the basis of the standard curves, the detection limits were determined to be 0.9 ng/mL in phosphate-buffered saline (PBS), 1.0 ng/mL in plasma, and 0.5 ng/mL in milk, respectively. Recoveries of gentamicin from spiked plasma and milk at levels of 25−100 ng/mL ranged from 85 to 112%. The concentration of intramuscularly injected gentamicin was successfully monitored in the rabbit plasma through competitive direct ELISA. The detection limits were estimated to be about 6 ng/mL of gentamicin in PBS, plasma, and milk using the colloidal gold-based immunochromatographic assay, which is suitable for the simple screening of gentamicin residues in the veterinary field. Observed positives can be confirmed using a more sensitive laboratory method such as competitive direct ELISA. Therefore, the assays developed in this study could complement each other as well as veterinary field and laboratory findings.

KEYWORDS: Gentamicin; monoclonal antibody; competitive direct ELISA; immunochromatographic assay; plasma; milk

INTRODUCTION

Gentamicin, an aminoglycoside antibiotic produced by *Micromonospora purpurea*, is widely used in veterinary medicine to treat mastitis, bacillary diarrhea, and pneumonia (*1*). It is classified as a broad-spectrum antibiotic, which inhibits growth of *Pseudomonas aeruginosa* and *Serratia marcescens* (*2*). However, despite its impressive clinical successes, gentamicin is potentially ototoxic and nephrotoxic to human and animals (*3*, *4*); thus, monitoring of its residues in foods is essential for the maintenance of public health. For consumer protection, the European Agency for the Evaluation of Medical Products (EMEA) established maximum residue limits (MRL) for edible tissues and milk: 50 μg/kg for meat, 100 μg/kg for milk, and 50 μg/kg for porcine fat (*5*).

Therefore, reliable analytical methods are required to monitor gentamicin residue levels in the livestock products. Various techniques have been developed for the detection of gentamicin residues in milk, urine, blood, and tissue including microbioassay (*6*, *7*), gas chromatography (GC) (*8*), high-performance liquid chromatography (HPLC) (*9*−*11*), and immunoassay (*12*−*16*), among which immunoassay has become the most popular method for the detection of gentamicin in foods because of its high sensitivity and specificity.

In the veterinary fields, however, a more simple and rapid detection method is required. Recent studies have reported on the colloidal gold-based immunochromatographic assay for the detection of ricin (*17*) and for the detection of sennosides A and B (*18*). The method can be applied to field studies because of its rapid and simple screening procedure.

In this study, we developed a competitive direct enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody of gentamicin for the detection of gentamicin in the animal plasma and milk. In addition, an immunochromatographic assay, which can be used as a rapid and simple screening method for the detection of gentamicin in veterinary fields, was developed using colloidal gold-labeled antibody.

MATERIALS AND METHODS

Materials. Gentamicin sulfate, kanamycin sulfate, neomycin sulfate, streptomycin sulfate, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), horseradish peroxidase (HRP), goat anti-mouse IgG-horseradish peroxidase conjugate, *o*-phenylenediamine dihydrochloride (OPD), hydrogen peroxide, Freund's complete adjuvant, Freund's incomplete adjuvant, polyoxyethylene-sorbitan monolaurate (Tween 20), and colloidal gold particle were purchased from Sigma−Aldrich (St. Louis,

* To whom correspondence should be addressed. Telephone: +82-2-880-1268. Fax: +82-2-886-1268. E-mail: vetlee@snu.ac.kr.
† Present address: Brain Korea 21, School of Agricultural Biotechnology, Seoul National University, 151-742 Seoul, Republic of Korea.

10.1021/jf050484o CCC: $30.25
Published on Web 09/08/2005

MO). BALB/c mice and rabbits were purchased from Charles River Technology (Seoul, Korea). The high-flow nitrocellulose membrane was obtained from Millipore (Billerica, MA).

Preparation of KLH, BSA, and HRP Conjugates. Gentamicin was conjugated with KLH, BSA, or HRP according to the procedure described by Haasnoot et al. (*19*) using EDC.

Immunization of Mice. Immunization was performed as described previously (*20*). Briefly, four BALB/c female mice, 8−10 weeks of age, weighing approximately 18 g, were given three intraperitoneal injections every 2 weeks with gentamicin−KLH conjugate (100 μg/injection). A total of 10 days following the third injection, serum was collected from the retrobulbar plexus of each mouse and the highest sensitivity antiserum was determined by competitive indirect ELISA. The spleen from the mouse with serum showing optimum relative inhibition was used for the subsequent fusion. A total of 3 weeks after the third injection and 4 days prior to fusion, this mouse was given a fourth injection of conjugate in phosphate-buffered saline (PBS) (0.1 mL).

Monoclonal Antibody Production. Hybridoma cell lines were produced through the fusion of myeloma cells (Sp2/0) and spleen cells obtained from immunized mice using PEG 1500 as described previously (*21*). A total of 12 days after the fusion, competitive indirect ELISA was performed to screen for antibody-producing cells using the culture supernatant. A stable hybridoma-cell-producing antibody with the highest sensitivity to gentamicin was selected and cloned to 0.5 cells/well by limit dilution. The cultured hybridoma cells ($5-10^6$ cell) were injected intraperitoneally into the mice to produce monoclonal antibody in the ascites fluid. Immunoglobulin was prepared from the ascites fluid of each mouse as described previously (*21*).

Cross-Reactivity with Other Aminoglycosides. Cross-reactivity of the antibody with other aminoglycosides (kanamycin, neomycin, and streptomycin) was determined by competitive direct ELISA as described below. The B/B_0 value of 50% (CR_{50}) was calculated as described previously (*22*).

Competitive Direct ELISA. Competitive direct ELISA was developed using monoclonal antibody of gentamicin. Each well of the microtiter plates was coated with 100 μL aliquots of gentamicin antibody (diluted 1/2000 in PBS) and incubated for 3 h at 37 °C. Unbound antibody was removed from the plate with the washing solution (0.02% Tween 20 in PBS), and each well was blocked with 200 μL blocking solution (1% skim milk in PBS) at 37 °C for 1 h. Gentamicin standards (50 μL each; 1−1000 ng/mL) were added to each well and incubated with 50 μL diluted gentamicin−HRP conjugate (1/2000 in PBS) for 1 h at 37 °C. After the unbound gentamicin and gentamicin−HRP conjugate were removed with the washing solution, 100 μL of substrate solution was added to each well, which was then incubated for 20 min at 37 °C. Absorbance was measured at 490 nm using an ELISA reader (Emax, Molecular Devices, CA).

To prepare the calibration curves of gentamicin in the plasma and milk, gentamicin stock standard solutions (1000 μg/mL) were prepared by dissolving gentamicin in the rabbit plasma or bovine milk. The solutions were further diluted with the plasma or milk to 0, 10, 20, 50, 100, 250, 500, 1000, 2500, 5000, and 10 000 ng/mL, which were then diluted 10-fold in PBS. The standard curves of gentamicin in the plasma and milk were established by competitive direct ELISA. For recovery test, gentamicin-spiked solutions were prepared by dissolving gentamicin in the rabbit plasma or bovine milk to give final concentrations of 25, 50, and 100 ng/mL and then diluting 10-fold in PBS. The recoveries of gentamicin from the spiked rabbit plasma or bovine milk were calculated on the basis of the standard curves constructed by competitive direct ELISA. The detection limits were determined as the mean background levels plus 3 times standard deviation (SD).

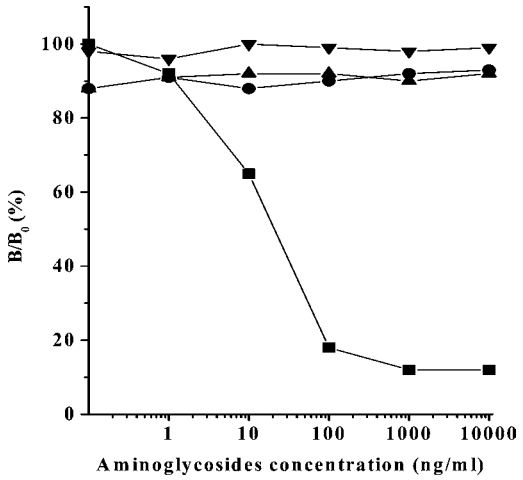

Figure 1. Cross-reactivity of aminoglycosides with the monoclonal antibody of gentamicin. Gentamicin (■), kanamycin (▲), neomycin (▼), and streptomycin (●), where B is the absorbance of the well containing aminoglycosides and B_0 is the absorbance of the well without aminoglycosides.

Monitoring of the Blood Gentamicin Concentration. Gentamicin was administered intramuscularly to rabbits at 8 mg kg^{-1} day^{-1} for 3 consecutive days. Blood samples were collected from the ear vein of each rabbit 2, 4, 6, 8, 10, and 12 h after the last injection of gentamicin and were centrifuged (2000*g*) for 10 min to obtain plasma. Plasma samples were diluted 10-fold in PBS and subjected to competitive direct ELISA to determine the gentamicin concentration in the blood.

Immunochromatographic Assay. Colloidal gold (40 nm in diameter) was conjugated with gentamicin monoclonal antibody as described previously (*17*). Briefly, 20 μg of purified monoclonal antibody was added to 1 mL of colloidal gold solution (pH 8.0). The mixture was allowed to stand for 10 min and was centrifuged at 15000*g* for 45 min. After centrifugation, the gold pellets were blocked with 1% BSA solution for 30 min. This gold-labeled antibody was supplied ready to use in 0.2 M Tris-HCl buffer (pH 8.7) containing 1% Triton X-100 at an optical density of 10 at 540 nm. Because it is quite concentrated, gold-labeled antibody was diluted 10-fold and was measured at 540 nm using spectrophotometer.

A total of 1 μL (3 μg of gentamicin−BSA) of gentamicin−BSA (3 mg/mL) conjugate was applied to one end of the nitrocellulose membrane strip (HF 135, 25 × 4.5 mm). After drying, the lower edge of the test strip was dipped into the well containing each concentration of gentamicin in 50 μL of buffer (0.2 M Tris-HCl at pH 8.7 and 1% Triton X-100) and 2 μL colloidal gold-labeled monoclonal antibody. After the mixture of the sample and the gold-labeled antibody rose up the membrane, the color intensities that decreased with an increasing concentration of gentamicin in the sample solutions were monitored.

RESULTS AND DISCUSSION

Cross-Reactivity. The purified gentamicin monoclonal antibody did not show any cross-reactivity with other aminoglycosides (kanamycin, neomycin, and streptomycin), indicating that the monoclonal antibody is highly specific for gentamicin (**Figure 1**). The specificity of the antibody can be explained by

Table 1. Background Levels of Gentamicin Determined in PBS, Plasma, and Milk ($n = 4$)

	mean background level[a] (ng/mL)	standard deviation (ng/mL)	limit of detection[b] (ng/mL)
PBS	0.48	0.14	0.90
plasma	0.41	0.2	1.01
milk	0.37	0.05	0.52

[a] Mean background levels were obtained from the standard curves of gentamicin in PBS, rabbit plasma, and bovine milk constructed by competitive direct ELISA. [b] Limit of detection = the mean background levels plus 3 times SD (Bo + 3 SD).

Table 2. Recoveries of Gentamicin from Spiked Rabbit Plasma and Bovine Milk ($n = 4$)

samples	level added[a] (ng/mL)	level found[b] ±SD (ng/mL)	recovery (%)
rabbit plasma	25	28.1 ± 1.5	112
	50	54.0 ± 0.6	108
	100	85.0 ± 2.2	85
bovine milk	25	21.8 ± 1.4	87
	50	51.9 ± 3.9	103
	100	109.2 ± 4.4	109

[a] Prepared by dissolving gentamicin in rabbit plasma or bovine milk to give final concentrations of 25, 50, and 100 ppb, which were then diluted 10-fold in PBS. [b] Calculated from the standard curves constructed by competitive direct ELISA.

the differences in the molecular structure of the aminoglycosides. All aminoglycosides consist of two or more amino sugars joined through a glycosidic linkage to a hexose nucleus, which is either streptose (found in streptomycin) or 2-deoxystreptamin (characteristic of all other aminoglycosides) (*2*); the aminoglycoside families are distinguished by the amino sugars attached to the nucleus. In gentamicin, two amino sugars are attached to its nucleus, whereas neomycin has three amino sugars attached. In addition, the molecular structure of amino sugars in gentamicin is different from those of streptomycin and kanamycin (*19*). These structural differences enable each antibody to recognize its own specific antigen.

Competitive Direct ELISA. To determine the detection limits of gentamicin in the plasma and milk, standard curves of gentamicin in PBS, rabbit plasma, and bovine milk were constructed by competitive direct ELISA (data not shown). Background levels of gentamicin determined in PBS, plasma, and milk were summarized in **Table 1**. The detection limits, which were defined as the mean background levels plus 3 times SD, were 0.9 ng/mL in PBS, 1.0 ng/mL in plasma, and 0.5 ng/mL in milk, respectively (**Table 1**). Recoveries of gentamicin from the plasma and milk spiked with different concentrations of gentamicin (25, 50, and 100 ng/mL), as determined by competitive direct ELISA based on the values obtained from the standard curves, were 112, 108, and 85% and 87, 103, and 109%, respectively (**Table 2**). The gentamicin concentration sharply increased to 1300 ng/mL after the intramuscular administration up to 2 h and then rapidly decreased to less than 300 ng/mL after 4 h of withdrawal (**Figure 2**). When aminoglycosides are administered into the body cavities, which contain serosal surfaces, extremely rapid and complete absorption takes place, whereas slow absorption can be observed when administered orally or rectally (*1*). In addition, Isoherranen and Soback (*8*) reported that aminoglycosides bind readily to tissue proteins and macromolecules via ionic bounds while less to the plasma proteins. They also showed that aminoglycoside accumulations in the renal proximal tubules were several fold higher than in the plasma or other tissues, and half-lives of

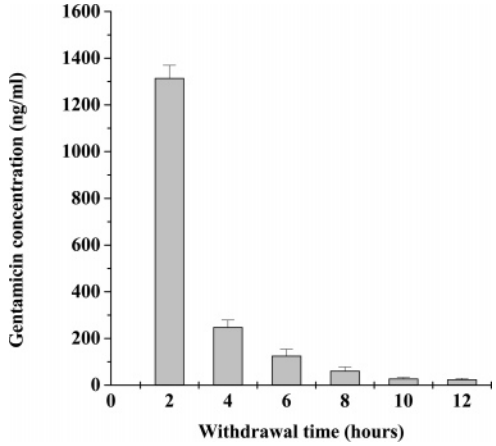

Figure 2. Plasma depletion profile of gentamicin after intramuscular administration of gentamicin. Gentamicin was administered intramuscularly to rabbits at 8 mg kg^{-1} day^{-1} for 3 consecutive days. Blood samples were collected from the ear vein of each rabbit at 2, 4, 6, 8, 10, and 12 h after the last injection of gentamicin. Plasma samples were diluted 10-fold in PBS and subjected to the competitive direct ELISA. Each value shows the mean (±SD) of the gentamicin concentration ($n = 4$).

aminoglycosides were 2−3 and 30−700 h in the plasma and tissues, respectively. Therefore, because of the longer and more variable half-lives of aminoglycosides in the tissues than in the plasma, our future study will focus on the estimation of the time-dependent concentration of gentamicin in tissues using monoclonal antibodies.

Immunochromatographic Assay. A schematic description of the colloidal gold-based immunochromatographic assay is illustrated in **Figure 3**; negative and positive results are judged by the appearance or disappearance of a red color band on the strip, respectively. The gold-labeled monoclonal antibody of gentamicin did not show any cross-reactivity with the other aminoglycosides tested as revealed through the immunochromatographic assay (**Figure 4**). PBS, rabbit plasma, and bovine milk spiked with gentamicin (0, 0.5, 1, 2, 4, 6, and 8 ng/mL) were tested by the immunochromatographic assay (**Figure 5**). The color intensity gradually decreased with an increasing concentration of gentamicin and disappeared completely at 6 ng/mL of gentamicin in the samples. Through the immunochromatographic assay, therefore, the detection limits were estimated to be about 6 ng/mL of gentamicin in PBS, plasma, and milk. Plasma samples collected from rabbits 2, 4, 6, 8, 10, and 12 h after intramuscular injection of gentamicin (8 mg kg^{-1} day^{-1} for 3 consecutive days) were subjected to the immunochromatographic assay (**Figure 6**). No color development was observed in all strips, which suggests that plasma samples contain higher than 6 ng/mL gentamicin. Subsequent ELISA gave the accurate numbers of the gentamicin concentration (**Figure 6**). Therefore, the immunochromatographic assay is suitable for the screening of gentamicin residues in the veterinary field. Observed positives can be confirmed using competitive direct ELISA. The application of the colloidal gold-based immunochromatographic assays has several advantages. First, the nanoparticles of colloidal gold show better mobility than other materials in the porous nitrocellulose membrane. The colloidal gold particles are also less susceptible to aggregation during the preparation of the test device. Finally, the gold-labeled antibody improves the assay sensitivity (*17*). In the present study, a compromise was made between the sensitivity and nonspecific binding of antigen−antibody reaction in the immunochromatographic assay. On the basis of the findings that

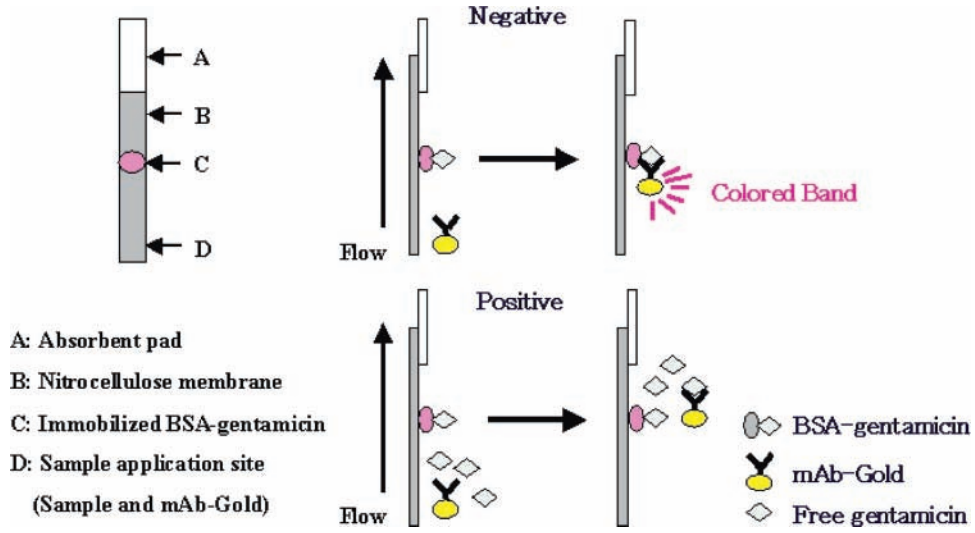

Figure 3. Schematic description of the colloidal gold-based immunochromatographic assay. In the absence of gentamicin in the sample, the gold-labeled monoclonal antibody can bind to the immobilized gentamicin–BSA conjugate on the membrane and develop a red color band. In the presence of gentamicin, however, free gentamicin competes with the immobilized gentamicin–BSA conjugate for the binding to the gold-labeled antibody.

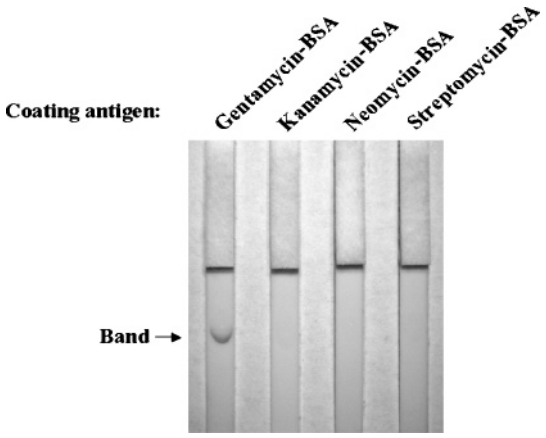

Figure 4. Cross-reactivity of aminoglycosides with the monoclonal antibody of gentamicin in immunochromatographic assay. A total of 3 μg of gentamicin–BSA, kanamycin–BSA, neomycin–BSA, or streptomycin–BSA conjugate was applied to each strip of nitrocellulose membrane. After the gold-labeled antibody rose up the membrane, the intensities of the red color band on each membrane strip were observed.

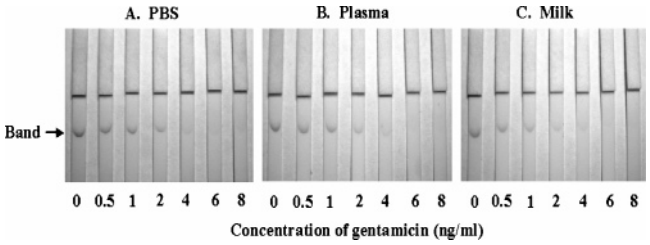

Figure 5. Immunochromatographic assay for the detection of gentamicin. A series of dilutions (0, 0.5, 1, 2, 4, 6, and 8 ng/mL) of gentamicin were prepared in PBS (A), rabbit plasma (B), and bovine milk (C).

the pH and the composition of the developing solution were important for better resolution, we optimized the assay conditions for better sensitivity without any cross-reactivity or nonspecific bindings.

In conclusion, the colloidal gold-based immunochromatographic assay could be applied to the detection of aminoglycosides in veterinary fields because of its rapid and simple procedure. For greater accuracy, however, the detection should

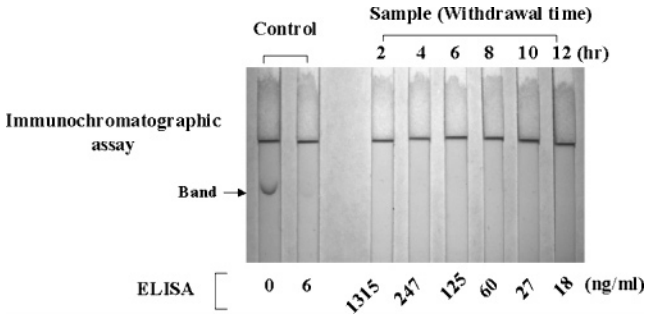

Figure 6. Comparison of competitive direct ELISA and immunochromatographic assay. Gentamicin was administered intramuscularly to rabbits at 8 mg kg^{-1} day^{-1} for 3 consecutive days as described in the Materials and Methods. Blood samples were collected from rabbits 2, 4, 6, 8, 10, and 12 h after the last injection of gentamicin. Plasma samples were subjected to immunochromatographic assay. Subsequent competitive direct ELISA determined the gentamicin concentration in the blood.

be supported by a more sensitive laboratory method such as the competitive direct ELISA method. The assays developed in this study could complement each other as well as the veterinary field and laboratory findings. Moreover, instead of slaughtering the animals to obtain test samples, the methods developed in the present study could be applied to determine the aminoglycoside concentration in the plasma of live animals.

ABBREVIATIONS USED

KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; MRL, maximum residue limits; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HRP, horseradish peroxidase; OPD, *o*-phenylenediamine dihydrochloride; Tween 20, polyoxyethylene-sorbitan monolaurate; CR_{50}, cross-reactivities at the B/B_0 value of 50%; PBS, phosphate-buffered saline.

LITERATURE CITED

(1) Riviere, J. E.; Spoo, J. W. Aminoglycoside antibiotics. In *Veterinary Pharmacology and Therapeutics*, 8th ed.; Adams, H. R., Ed.; Iowa State University Press: IA, 2001; 841−867.

(2) Reynolds, J. E. F. Antibacterial agents. In *Martindale: The Extra Pharmacopoeia*, 30th ed.; The Pharmaceutical Press: London, U.K., 1993; 109−113.
(3) Hewitt, W. L.. Gentamicin: Toxicity in perspective. *Postgrad. Med. J.* **1974**, *7*, 55−61.
(4) Ramsden, R. T.; Wilson, P.; Gibson, W. P. R. Immediate effects of intravenous tobramycin and gentamicin on human cochlear function. *J. Laryngcol. Otol.* **1980**, *94*, 521−531.
(5) The European Agency for the Evaluation of Medical Products (EMEA)/MRL/803/01-FINAL.
(6) Lantz, C. H.; Lawrie, D. J.; Witebsky, F. G.; Maclowry, J. D. Evaluation of plasma gentamicin assay procedure for a clinical microbiology laboratory. *J. Clin. Microbiol.* **1980**, *10*, 583−589.
(7) Rosner, A.; Aviv, H. Gentamicin bioautography assay vs. the microbiological disk test. *J. Antibiot.* **1980**, *6*, 600−603.
(8) Isoherranen, N.; Soback, S. Chromatographic methods of analysis of aminoglycoside antibiotics. *J. AOAC Int.* **1999**, *82*, 1017−1045.
(9) Stead, D. A.; Richards, R. M. E. Sensitive fluorimetric determination of gentamicin sulfate in biological matrices using solid-phase extraction, pre-column derivatization with 9-fluorenylmethyl chloroformate, and reversed-phase high-performance liquid chromatography. *J. Chromatogr., B: Biomed. Sci. Appl.* **1996**, *675*, 295−302.
(10) Stead, D. A.; Richards, R. M. E. Sensitive high-performance liquid chromatographic assay for aminoglycosides in biological matrices enables the direct estimation of bacterial drug uptake. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *693*, 415−421.
(11) Graham, A. E.; Speicher, E.; Williamson B. Analysis of gentamicin sulfate and a study of its degradation in dextrose solution. *J. Pharm. Biomed.* **1997**, *15*, 537−543.
(12) Tsay, Y. G.; Wilson, L.; Keefe, E. Quantitation of plasma gentamicin concentration by a solid-phase immunofluorescence method. *Clin. Chem.* **1980**, *26*, 1610−1612.
(13) Place, J. D.; Thompson, S. G.; Clements, H. M.; Ott, R. A.; Jensen, F. C. Gentamicin substrate-labeled fluorescent immunoassay containing monoclonal antibody. *Antimicrob. Aagents Chemother.* **1983**, *8*, 246−251.
(14) Berkowitz, D. B.; Webert, D. W. Enzyme immunoassay-based survey of prevalence of gentamicin in plasma of marketed swine. *J. AOAC Int.* **1986**, *69*, 437−441.
(15) Ploczekova, C.; Foldes, O. Immunochemical determination of gentamicin in serum III. The competitive ELISA. *Cesk. Epidemiol. Mikrobiol. Imunol.* **1992**, *41*, 346−354.
(16) Hanes, S. D.; Herring, V. L. Gentamicin enzyme-linked immunosorbent assay for microdialysis samples. *Ther. Drug. Monit.* **2001**, *23*, 689−693.
(17) Shyu, R. H.; Shyu, H. F.; Liu, H. W.; Tang, S. S. Colloidal gold-based immunochromatographic assay for detection of ricin. *Toxicon* **2002**, *40*, 255−258.
(18) Putalun, W.; Morinaga, O.; Tanaka, H.; Shoyama Y. Development of a one-step immunochromatographic strip test for the detection of sennosides A and B. *Phytochem. Anal.* **2004**, *15*, 112−116.
(19) Haasnoot, W.; Stouten, P.; Cazemier, G.; Lommen, A.; Nouws, J. F. M.; Keukens, H. J. Immunochemical detection of aminoglycosides in milk and kidney. *Aanalyst* **1999**, *124*, 301−305.
(20) Dixon, D. E.; Warner, R. L.; Ram, B. P.; Hart, L. P.; Pestka, J. J. Hybridoma cell line production of specific monoclonal antibody to the mycotoxins zearalenone and α-zearalenol. *J. Agric. Food Chem.* **1987**, 35, 122−126.
(21) Harlow, E. D.; Lane, D. Monoclonal antibodies. In *Antibodies*; Cold Spring Harbor Laboratory: New York, 1988; 196−214.
(22) Kitagawa, T.; Fujiwara, K.; Tomonoh, S.; Takahashi, K.; Koida, M. Enzyme immunoassays of kanamycin group antibiotics with high sensitivities using anti-kanamycin as a common anti-plasma: Reasoning and selection of a heterologous enzyme label. *J. Biochem.* **1983**, *94*, 1165−1172.

Received for review March 3, 2005. Revised manuscript received August 5, 2005. Accepted August 9, 2005. This study was supported by Brain Korea 21 project from the Ministry of Education and by Research Institute for Veterinary Science (RIVS) of the College of Veterinary Medicine in Seoul National University, Republic of Korea.

JF050484O