

Development of Competitive Direct Enzyme-linked Immunosorbent Assay for the Detection of Gentamicin Residues in the Plasma of Live Animals*

Yong Jin, Jin-Wook Jang, Mun-Han Lee and Chang-Hoon Han**

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

ABSTRACT : Competitive direct ELISA was developed to detect gentamicin residues. Mice immunized with gentamicin-keyhole limpet hemocyanin (KLH) conjugate developed good antiserum titers, which gradually increased with booster injections, indicating immunization was successfully processed. Monoclonal antibody against gentamicin was prepared using hybridoma cells cloned by limit dilution of fused cells. IgG was purified from ascites fluid of hybridoma cell-injected mice through ammonium sulfate precipitation and Sephadex G-25 gel filtration. After the gel filtration, fractions of high antibody titer were further purified through affinity chromatography on protein A/G column. Monoclonal antibody against gentamicin was confirmed as IgG1, which has kappa light chain. Cross-reactivities (CR₅₀) of gentamicin monoclonal antibody to other aminoglycosides (kanamycin, neomycin, and streptomycin) were less than 0.005%, indicating the monoclonal antibody was highly specific for gentamicin. Standard curve constructed through competitive direct ELISA showed measurement range (from 80 to 20% of B/B₀ ratio) of gentamicin was between 1 and 40 ng/ml, and 50% of B/B₀ ratio was about 4 ng/ml. The gentamicin concentration rapidly increased to 1,300 ng/ml after the intramuscular administration up to 2 h, then sharply decreased to less than 300 ng/ml after 4 h of withdrawal, during which the elimination half-life (t_{1/2}) of gentamicin in the rabbit plasma was estimated to be 1.8 h. Competitive direct ELISA method developed in this study using the prepared monoclonal antibody is highly sensitive for gentamicin, and could be useful for detecting gentamicin residues in plasma of live animals. (*Asian-Aust. J. Anim. Sci.* 2005, Vol 18, No. 10 : 1498-1504)

Key Words : Monoclonal Antibody, Gentamicin, Aminoglycosides, Competitive Direct ELISA

INTRODUCTION

Gentamicin, an aminoglycoside antibiotic produced by *Micromonospora purpurea*, is widely used in veterinary medicine to treat mastitis, bacillary diarrhea, and pneumonia (Cleveland et al., 1990). The commercially available drug is a mixture of the sulfate salts of gentamicins C₁, C_{1a}, and C₂ (Figure 1), all of which appear to have similar antimicrobial activities (Abou-Zei and Shehata, 1977; Cleveland et al., 1990). Gentamicin is classified as a broad-spectrum antibiotic due to its growth inhibition of *Pseudomonas aeruginosa* and *Serratia marcescens* bacteria (Reynolds, 1993), and is known to perturb protein synthesis in Gram-negative bacteria by binding to the 30 S subunit of ribosomal RNA, which causes misreading of the genetic code, thereby inhibiting translation (Fourmy et al., 1998; Ren et al., 2002).

However, despite its impressive clinical successes, gentamicin is potentially ototoxic and nephrotoxic to human and animals (Hewitt, 1974; Ramsden et al., 1980); thus monitoring of the drug residues in foods, particularly meat, milk, and eggs, is essential for the maintenance of public health. To prevent harmful aminoglycoside residues from

entering the human food chain, international authorities recommend long withdrawal periods before slaughtering the animals for human consumption. Since 1996 the European Union (EU) has established provisional maximum residue limits (MRL) on edible tissues, fat, milk, and eggs: 0.1 ppm for milk, muscle, and fat, 0.2 ppm for liver, and 1 ppm for kidney (European Union regulation, 1996).

Simple and reliable analytical methods are thus required to monitor gentamicin residue levels in livestock products, and various techniques have been developed for the detection of gentamicin residues in milk, urine, blood, and tissue including microbioassay (Lantz et al., 1980; Rosner and Aviv, 1980), gas chromatography (GC; Isoherranen and Soback, 1999), and high-performance liquid chromatography (HPLC; Stead and Richards, 1996, 1997; Graham et al., 1997). Moreover, enzyme-linked immunosorbent assay (ELISA) using polyclonal (Tsay et al., 1980; Place et al., 1983) or monoclonal antibody (Berkowitz and Webert, 1986; Ploczekova and Foldes, 1992; Hanes and Herring, 2001) has been widely used for the detection of antibiotic residues due to its high sensitivity, simplicity, and ability to screen large number of small-volume samples. Even though monoclonal antibody against gentamicin was produced by other groups, it is not commercially available at this moment. In this study, we produced a monoclonal antibody against gentamicin, and developed a competitive direct ELISA for the detection of gentamicin residues in live animals.

* Brain Korea 21, School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea

** Corresponding Author: Chang-Hoon Han, Tel: +82-2-880-1240, Fax: +82-2-886-1268, E-mail: han57@snu.ac.kr

Received March 17, 2005; Accepted June 28, 2005

MATERIALS AND METHODS

Materials

Gentamicin 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/incomplete adjuvants, polyoxyethylene-sorbitan monolaurate (Tween 20), kanamycin, neomycin, and streptomycin sulfates were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic, polyethylene glycol 1,500 (PEG 1,500), hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidine (HT) media, microtiter plates, and microculture plates (96- and 24-well plates) were obtained from Gibco BRL (Rockville, MD, USA). BALB/c mice and rabbits were introduced from Charles River Technology (Seoul, Korea).

Preparation of KLH and HRP conjugates

Gentamicin was conjugated with KLH according to the procedure described by Haasnoot et al. (1999) using EDC. Coupling of gentamicin to KLH was accomplished through a drop-wise addition of EDC (300 mg in 1 ml PBS) into the KLH-gentamicin mixture (20 mg KLH and 90 mg gentamicin sulfate in 2 ml PBS) over a 10-min period with constant stirring. After a 2 h reaction at room temperature, the non-coupled gentamicin and EDC were removed by dialysis (over 3 days at 4°C) against 4 L PBS. The conjugated immunogen was further purified by gel filtration using Sephadex G-25 as described previously (Lewis et al., 1972). Coating antigen was prepared as mentioned above using BSA instead of KLH. The concentration of gentamicin-KLH or gentamicin-BSA conjugate was determined by Lowry method (1951). Aliquots of immunogen or coating antigen were stored at -70°C until use.

Gentamicin-HRP conjugate was prepared by the method of Haasnoot et al. (1999). Briefly, 45 mg gentamicin sulfate dissolved in 1.5 ml PBS (pH 7.4) was mixed with 10 mg HRP. Subsequently, 300 mg EDC dissolved in 1 ml water was added to the mixture drop-wise. The mixture was then incubated at room temperature for 1 h and stored at 4°C overnight. The solution was dialyzed against PBS over 4 days, and aliquots were stored at -70°C until used for competitive direct ELISA.

Immunization of mice

Immunization was performed as described previously (Dixon et al., 1987). Four BALB/c female mice, 8-10 weeks

of age, weighing approximately 18 g, were given four intraperitoneal injections with gentamicin-KLH conjugate (100 µg/injection). The first injection consisted of 0.2 ml of conjugate in a 1:1 ratio of saline and Freund's complete adjuvant. The second (day 14) and the third (day 28) injection consisted of 0.2 ml of conjugate in saline and Freund's incomplete adjuvant (1:1). Ten days following the third injection, serum was collected from the retrobulbar plexus of each mouse and titers were determined by indirect ELISA to determine the highest sensitivity antiserum. The spleen from the mouse with serum showing optimum relative inhibition was used for the subsequent fusion. Three weeks after the third injection and 4 days prior to fusion, this mouse was given a fourth injection of conjugate in PBS (0.1 ml).

Titration of antiserum

Ten days after each injection, blood was collected from the retrobulbar plexus of each mouse, and antibody titers were determined by indirect ELISA using serially diluted serum and peroxidase-conjugated goat anti-mouse IgG. Each well of the microtiter plates was coated with 100 µl gentamicin-BSA conjugate (5 µg/ml) in 50 mM sodium bicarbonate buffer (pH 9.6) and incubated for 3 h at 37°C. Unbound conjugate was removed from the plate using a washing solution (0.02% Tween 20 in PBS), and each well was blocked with 200 µl blocking solution (1% skim milk in 50 mM sodium bicarbonate buffer, pH 9.6) at 37°C for 1 h. Subsequently, 100 µl serially diluted serum obtained from each immunized mouse was individually added to each well and incubated for 1 h at 37°C. Unbound serum antibody was removed with the washing solution, and 100 µl goat anti-mouse IgG conjugated with peroxidase (diluted to 1/2,000 in PBS) was added to each well. The plates were then incubated for 1 h at 37°C and washed four times with the washing solution. To each well 100 µl enzyme substrate (0.04% OPD in citrate buffer, pH 5.0, containing 0.01% H₂O₂) was added, and the plates were incubated for 20 min at 37°C. The color developed was measured at 490 nm using an ELISA reader (Emax, Molecular Devices, CA, USA).

Monoclonal antibody production

Hybridoma cell lines were produced through the fusion of myeloma cells (Sp2/0) and spleen cells obtained from the immunized mice using PEG 1,500 as described previously (Harlow and Lane, 1988). Four days after the final injection, the mouse with the highest antibody titer was sacrificed by cervical dislocation. The spleen was removed aseptically, teased apart with 1-ml syringe needles in plastic petri dishes containing 10 ml DMEM (pH 7.2, containing 1% antibiotic-antimycotic), producing a single-cell suspension, which was then combined with 10⁷ myeloma cells (Sp2/0). The

combined cells were centrifuged, and the cell pellet was resuspended in 100 μ l DMEM medium, and fused using 1.5 ml PEG 1,500. PEG 1,500 was added slowly over 1 min to the cell pellet while resuspending the cells by stirring with the end of the pipet. Stirring was continued for an additional minute. One milliliter of medium without serum was added to the cell suspension over the next minute. Then additional 9 ml of medium was added to cell suspension slowly over the next 2 min with stirring. The fused cells were harvested by centrifugation at 400 \times g for 5 min.

The fused cells were suspended in 40 ml HAT medium containing thymocytes (5×10^6 cells/ml) as the feeder cells, and 400 μ l cell suspension (1×10^5 cells) was pipetted into the 96-well cell culture plates, which were then incubated at 37°C under an atmosphere of 8% CO₂.

Twenty-four hours after the fusion, half of the HAT medium (0.2 ml) was removed from each well through aspiration and thereafter replaced with a fresh medium every 3 days for 12 days. The cultures were then fed HT medium for 7 days, and were cultured in 20% FBS-DMEM thereafter. Twelve days after the fusion, competitive indirect ELISA was performed to screen for antibody-producing cells using the culture supernatant. One stable hybridoma cell producing an antibody that had the highest binding capacity and sensitivity to gentamicin was selected, and cloned to 0.5 cells/well by limit dilution. Cloning was performed twice to obtain stable cell lines. The selected hybridoma (1×10^7 cell) was stored in 1 ml FBS-DMSO (9:1) and frozen in liquid nitrogen. To produce monoclonal antibody, the cultured hybridoma cells (5×10^6 cell) were injected intraperitoneally into the mice and the ascites fluid was harvested.

Immunoglobulin was prepared from the ascites fluid of each mouse by ammonium sulfate precipitation (Harlow and Lane, 1988). The precipitate was redissolved in 1 ml PBS (pH 7.4) and applied to PD-10 column (Amersham Biosciences, Uppsala, Sweden). Subsequently, the fractions of high antibody titer were confirmed by indirect ELISA, and the pooled fraction was applied to Protein A/G column (Pierce, IL, USA). After washing, the bound antibody was eluted from the column with 10 ml elution buffer (100 mM glycine-HCl, pH 2.5). The fractions of high antibody titer fractions were then confirmed by indirect ELISA, and the pooled fraction was stored in a freezer for subsequent tests.

Isotype determination

The isotype class and subclass of the secreted antibody were determined using a mouse monoclonal antibody isotyping kit (Pierce). Each well of the microtiter plates was coated with 100 μ l gentamicin-BSA conjugate (5 μ g/ml) in 50 mM sodium-bicarbonate buffer (pH 9.6) and incubated for 3 h at 37°C. Unbound conjugate was removed from the plate with the washing solution (0.02% Tween 20 in PBS),

and each well was blocked with 200 μ l blocking solution (1% skim milk in 50 mM sodium bicarbonate buffer, pH 9.6) at 37°C for 1 h. After incubation with 50 μ l cell culture supernatant, 50 μ l each of anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, kappa light chain, or lambda light chain produced in rabbit was individually added to each well and incubated for 1 h at 37°C. After washing, all wells were then further incubated with 50 μ l goat anti-rabbit IgG conjugated with peroxidase for 1 h. After washing, 100 μ l ABTS (2,2-azino-di [3-ethyl benzthiazoline sulfonic acid]) substrate solution was added to each well. Subsequently, the plates were incubated for 30 min at room temperature, and the color developed was measured at 405 nm using an ELISA reader.

Cross-reactivity with other aminoglycosides

To determine the specificity of gentamicin antibody, cross-reactivity of the antibody with other aminoglycosides (kanamycin, neomycin, and streptomycin) was determined by competitive indirect ELISA. Using this method, the cross-reactivity at B/B₀ value of 50% (CR₅₀) was calculated as described previously (Kitagawa et al., 1983). Where B is the OD value obtained in the presence of aminoglycosides, and B₀ is the OD value obtained in the absence of aminoglycosides.

Competitive direct ELISA

Competitive direct ELISA was developed using monoclonal antibody and gentamicin-HRP conjugate. Each well of the microtiter plates was coated with 100 μ l aliquots of gentamicin antibody (diluted 1/3,000 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 μ l blocking solution (1% skim milk in PBS) at 37°C for 1 h. Gentamicin standards (50 μ l each) ranging from 1 to 1,000 ng/ml were individually added to each wells, and incubated with 50 μ l of gentamicin-HRP conjugate (diluted 1/2,000 in PBS) for 1 h at 37°C. After removing the unbound gentamicin and gentamicin-HRP conjugate with the washing solution, 100 μ l substrate solution (0.04% OPD in citrate buffer, pH 5.0, containing 0.01% H₂O₂) was added to each well and incubated for 20 min at 37°C. Absorbance was measured at 490 nm using an ELISA reader.

Monitoring of gentamicin concentration in blood

Gentamicin was administered intramuscularly to rabbits at 8 mg/kg/day 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, and were centrifuged (2,000 \times g) for 10 min to obtain plasma. Plasma samples were diluted 10-fold in PBS and subjected to the

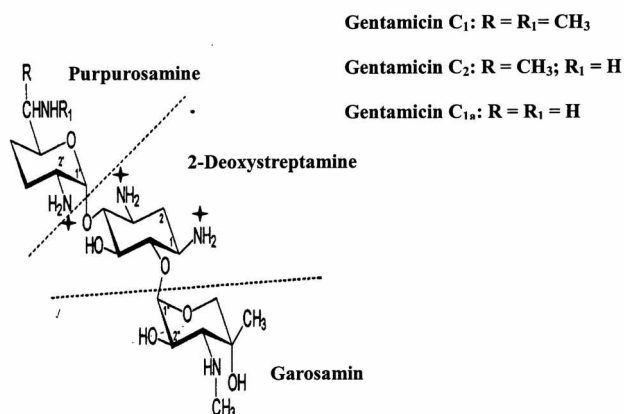


Figure 1. Structures of the gentamicin complex components; primary amines of gentamicin, presumed coupling sites of EDC, are shown (asterisks).

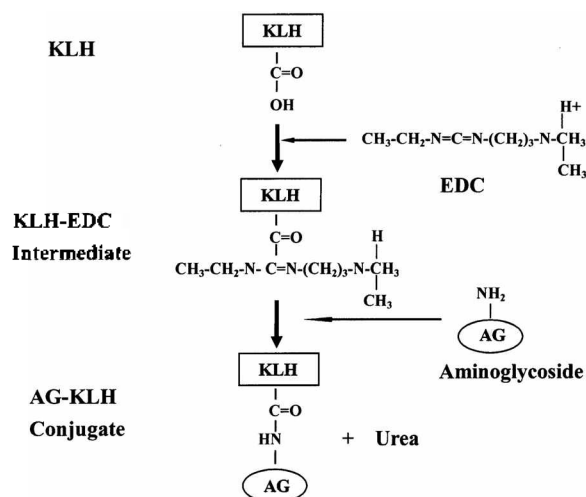


Figure 2. Procedure of aminoglycoside-KLH conjugation. Primary amines of gentamicin were conjugated with the carboxylic groups of KLH using EDC as a coupling reagent.

competitive direct ELISA to determine the gentamicin concentration in the blood. The slope of the plasma concentration-time curve was estimated based on the linear-regression of the depletion curve, under the assumption that tissue gentamicin is eliminated by the first-order kinetics. The elimination half-life ($t_{1/2}$), defined as the time required for the body to eliminate 50% of the remaining drug, was calculated using the following equation: $t_{1/2} = 0.693/k$, where k , the overall elimination constant, is the slope of the plasma concentration-time curve.

RESULTS AND DISCUSSION

Preparation of gentamicin-KLH conjugate

To generate the gentamicin-specific antibody, gentamicin-KLH conjugate was synthesized and used as an

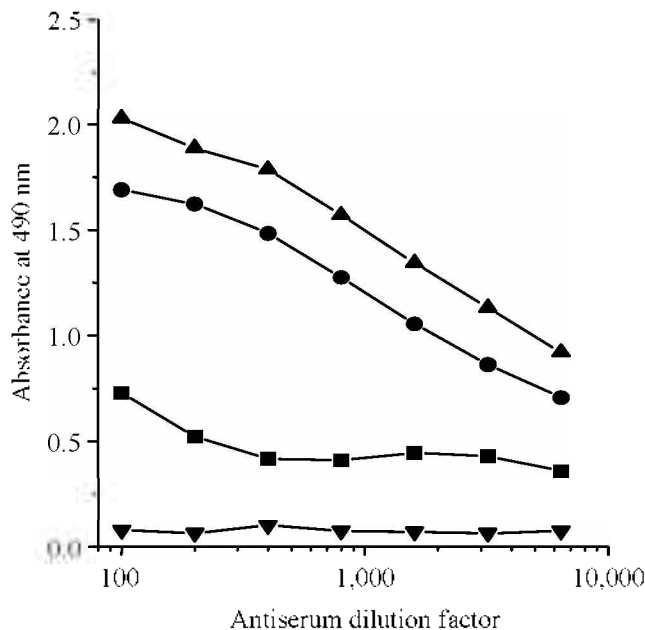


Figure 3. Titration of antisera against gentamicin using indirect ELISA after immunization of mouse with gentamicin-KLH conjugate. One hundred micrograms of immunogen was injected intraperitoneally three times at 14-day interval. Sera were collected 10 days after primary immunization (■), primary boosting (●), and secondary boosting (▲), and the antibody titers were compared with those of the pre-immune plasma (▼). End point titers for all sera were 1:6,400.

immunogen. Primary amines of gentamicin were conjugated with the carboxylic groups of KLH using EDC as a coupling reagent (Figure 2). Gentamicin, like most other antibiotics, is a low-molecular weight organic compound and thus is devoid of any antigenicity. Nevertheless, through the conjugation of gentamicin with a protein or polypeptide carrier, an antibody against the haptenic group can be obtained. Due to its high molecular weight (400-3,000 kDa) and a large number of functional groups, KLH is a useful carrier protein for the production of immunogen conjugate (Harlow and Lane, 1988). EDC reacts with the carboxylic groups of KLH to form highly reactive and short-lived *O*-acylisourea derivatives. Subsequently, the activated KLH reacts with the primary amines of gentamicin to form amide linkages (Bauminger and Wilchek, 1980).

Titration of antiserum

To determine whether the immunization protocol adequately stimulated plasma cells to secrete gentamicin-specific antibody, mouse serum was collected 10 days after each immunization, and titers were determined by indirect ELISA using gentamicin-BSA as the coating antigen. The mice immunized with gentamicin-KLH developed good antiserum titers, which were gradually increased by repeating the booster injections, an indication that the

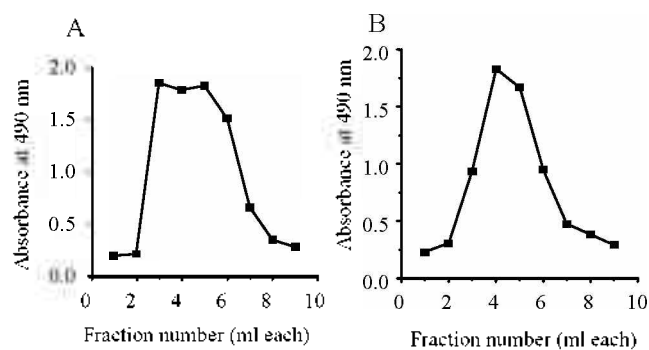


Figure 4. Purification of the monoclonal antibody of gentamicin through Sephadex G-25 molecular exclusion chromatography (A) and Protein A/G affinity chromatography (B). For each fraction, antibody titration was performed through indirect ELISA, and absorbance was measured at 490 nm using an ELISA reader.

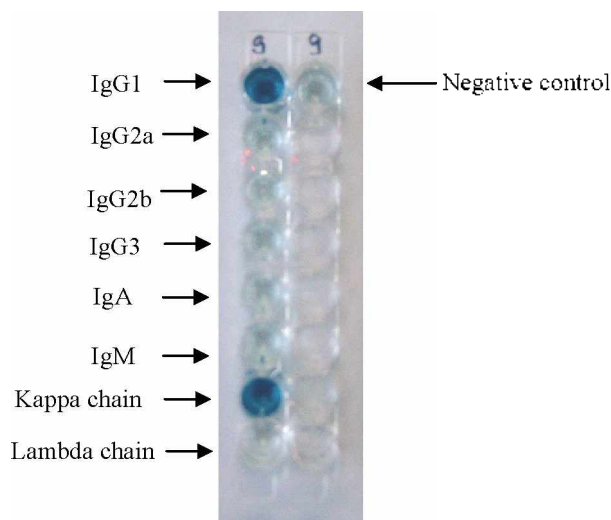


Figure 5. Determination of monoclonal antibody isotype. Rabbit antisera specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, kappa light chain, and lambda light chain were added to each well and detected with goat anti-rabbit IgG conjugated with peroxidase. Negative control includes only pre-immune serum.

immunization was successfully processed (Figure 3).

Monoclonal antibody production

Ammonium sulfate precipitation and Sephadex G-25 gel filtration were performed to purify IgG from the ascites fluid of hybridoma cell-injected mice. After Sephadex G-25 gel filtration, the fractions of high antibody titer (fractions 3-6) were collected and pooled (Figure 4A). Subsequently, the pooled fraction was subjected to affinity chromatography on a protein A/G column, and the fractions of high antibody titer (fractions 3-6; Figure 4B) were collected and stored for subsequent use. Based on an isotyping kit assay, the monoclonal antibody of gentamicin was confirmed to be a IgG1 which has kappa light chain (Figure 5).

Table 1. Cross-reactivity (CR_{50}) of the gentamicin monoclonal antibody to other aminoglycosides

Aminoglycosides	CR_{50} (%)
Gentamicin	100
Kanamycin	<0.005
Neomycin	<0.005
Streptomycin	<0.005

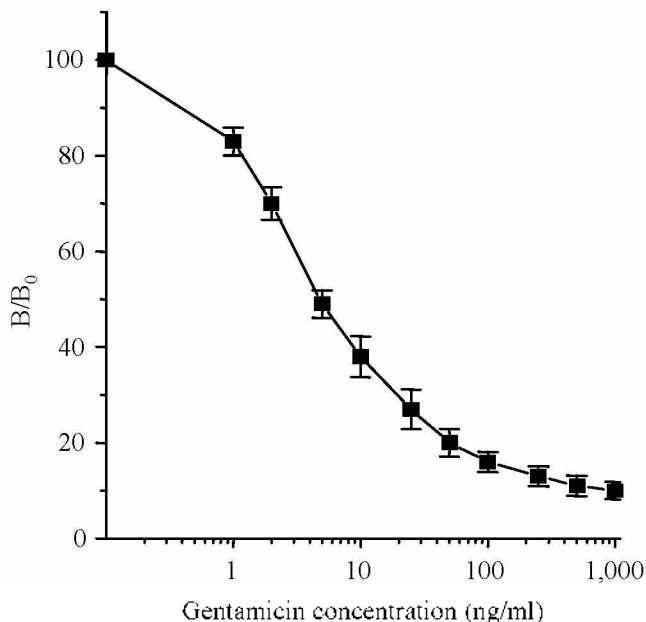


Figure 6. Standard curve of gentamicin constructed through competitive direct ELISA. B is the OD value obtained in the presence of free gentamicin, and B₀ is the OD value obtained in the absence of free gentamicin. Error bars show the mean of B/B₀ ± SD. (n=4).

Cross-reactivity with other aminoglycosides

The purified gentamicin monoclonal antibody, upon cross-reactivity testing with other aminoglycosides (kanamycin, neomycin, and streptomycin), showed cross-reactivities (CR_{50}) of less than 0.005%, indicating that the monoclonal antibody is highly specific for gentamicin (Table 1). The specificity of the antibody can be explained by the differences in the molecular structure of the aminoglycosides; they all consist of two or more amino sugars joined with a glycosidic linkage to a hexose nucleus, either streptose (found in streptomycin) or 2-deoxystreptamin (characteristic of all other aminoglycosides) (Reynolds, 1993). The aminoglycoside families are distinguished by the amino sugars attached to this nucleus. There are two amino sugars attached to the nucleus of gentamicin, whereas neomycin has three amino sugars attached. In addition, the molecular structures of amino sugars in kanamycin are quite different from those in neomycin, gentamicin, and streptomycin (Haasnoot et al., 1999). These structural differences enable each antibody to recognize its specific antigen.

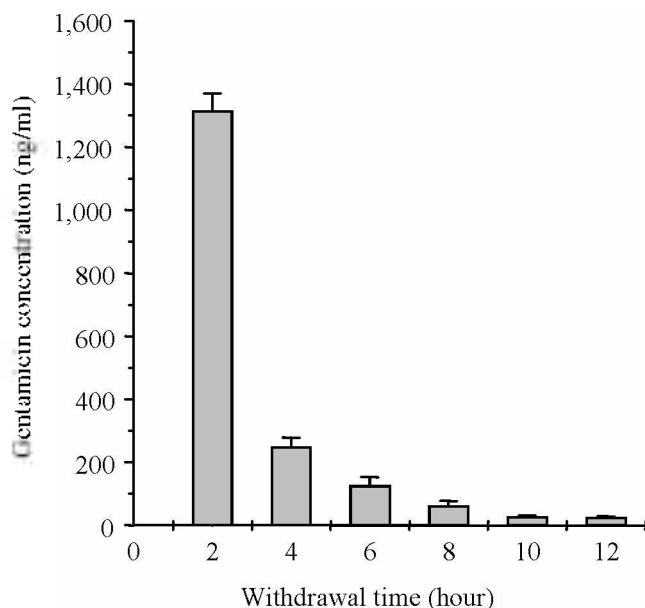


Figure 7. Depletion profile of gentamicin in rabbit serum after intramuscular administration of gentamicin. Gentamicin was administered intramuscularly to rabbits at 8 mg/kg/day for 3 consecutive days. Blood samples were collected from the ear vein of each rabbit 2, 4, 6, 8, 10, and 12 h after the last injection of gentamicin. Plasma samples were diluted 10-fold in PBS and subjected to the competitive direct ELISA to determine gentamicin concentration. Error bars show the means of gentamicin concentration \pm SD. (n=4).

Competitive direct ELISA

To determine the detection limits of gentamicin, standard curves of gentamicin were constructed by competitive direct ELISA (Figure 6): measurement range (from 80 to 20% of B/B_0 ratio) of gentamicin was between 1 and 40 ng/ml, and 50% B/B_0 ratio was about 4 ng/ml. In the present study, we produced a monoclonal antibody against gentamicin and developed a competitive direct ELISA, which is a useful method for the detection of gentamicin in a small-volume sample. Through this simple procedure, detection of gentamicin as low as 1 ng/ml was possible using 50 μ l sample. Gentamicin is commonly found in the form of a nonvolatile sulfate salt, thus, analysis through gas chromatography is difficult. Moreover, it cannot be readily detected spectrophotometrically without prior derivatization, because it does not possess a chromophore. HPLC method was, therefore, developed to detect gentamicin in the animal plasma and tissue through the fluorescence derivatization of gentamicin (Stead and Richards, 1996; Posyniak et al., 2001). However, this method required extensive sample purification prior to derivatization.

Recently, ELISA methods using polyclonal or monoclonal antibody have been widely used for the detection of antibiotics in plasma and milk, including the quantitative analysis method for gentamicin in

microdialysis samples as reported by Hanes and Herring (2001). Loomans et al. (2003) prepared a neamin antibody, and developed a generic ELISA for the detection of gentamicin, kanamycin, and neomycin in milk. However, their method overestimated the concentration of neomycin when lower than 100 ng/ml in milk. In addition to being a more rapid and simple procedure than the competitive indirect ELISA method, which is used for the screening of antibiotics, competitive direct ELISA method developed in the present study has high sensitivity as well as no cross-reactivity with other aminoglycosides.

Monitoring of gentamicin concentration in blood

The gentamicin concentration rapidly increased to 1,300 ng/ml after the intramuscular administration up to 2 h, then sharply decreased to less than 300 ng/ml after 4 h of withdrawal (Figure 7), during which the elimination half-life ($t_{1/2}$) of gentamicin in the rabbit plasma was estimated to be 1.8 h. 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 (Riviere and Spoo, 2001). In addition, Isoherranen and Soback (1999) reported that aminoglycosides bind readily to tissue proteins and macromolecules via ionic bonds, while less to the plasma proteins. They also showed that aminoglycoside accumulation in the renal proximal tubules were several-fold higher than in the plasma or other tissues, and half-lives of aminoglycosides were 2-3 and 30-700 h in the plasma and tissues, respectively. Therefore, due to 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.

In conclusion, the purified monoclonal antibody of gentamicin was IgG1, which has kappa light chains. The antibody showed no cross-reactivity with other aminoglycosides, indicating that the antibody is highly specific for gentamicin. Moreover, competitive direct ELISA method developed in this study using the monoclonal antibody is highly sensitive for the detection of gentamicin. Concentration of gentamicin was successfully monitored in the rabbit plasma through competitive direct ELISA after intramuscular injection of gentamicin. Therefore, instead of slaughtering the animals to obtain test samples, this method could be a useful tool for the detection of gentamicin residues in the plasma of live animals.

ACKNOWLEDGEMENT

This study was supported by the Brain Korea 21 project of the Ministry of Education, and Research Institute for

Veterinary Science (RIVS) of College of Veterinary Medicine, Seoul National University, Korea.

REFERENCES

- Abou-Zeid, A. A. and Y. M. Shehata. 1977. Gentamicins. *Zbl. Bakt. Parasit. Infekt. Hyg.* 132:97-108.
- Bauminger, S. and M. Wilchek. 1980. The use of carbodiimides in the preparation of immunizing conjugates. *Method Enzymol.* 70:151-159.
- Berkowitz, D. B. and D. W. Webert. 1986. Enzyme immunoassay-based survey of prevalence of gentamicin in plasma of marketed swine. *J. AOAC Int.* 69:437-441.
- Cleveland, C. B., D. E. Francke, W. M. Heller, J. A. Kepler, G. P. Provost and M. J. Reilly. 1990. Anti-Infective Agents. In *AHFS Drug Information 90*. American society of hospital pharmacists press, Bethesda, MD, pp. 51-67.
- Dixon, D. E., R. L. Warner, B. P. Ram, L. P. Hart and J. J. Pestka. 1987. Hybridoma cell line production of specific monoclonal antibody to the mycotoxins zearalenone and α -zearalenol. *J. Agric. Food Chem.* 35:122-126.
- European Union regulation no. 1140/96, L151 (26-6-1996).
- Fourmy, D., S. Yoshizawa and J. D. Puglisi. 1998. Paromomycin binding induces a local conformational change in the A-site of 16S rRNA. *J. Mol. Biol.* 277:333-345.
- Graham, A. E., E. Speicher and B. Williamson. 1997. Analysis of gentamicin sulfate and a study of its degradation in dextrose solution. *J. Pharmaceut. Biomed.* 15:537-543.
- Haasnoot, W., P. Stouten, G. Cazemier, A. Lommen, J. F. M. Nouws and H. J. Keukens. 1999. Immunochemical detection of aminoglycosides in milk and kidney. *Analyst* 124:301-305.
- Hanes, S. D. and V. L. Herring. 2001. Gentamicin enzyme-linked immunosorbent assay for microdialysis samples. *Ther. Drug Monit.* 23(6):689-93.
- Harlow, E. D. and D. Lane. 1988. Monoclonal antibodies. In *Antibodies: A Laboratory Manual*. Cold spring harbor laboratory, New York, NY, pp. 196-214.
- Hewitt, W. L. 1974. Gentamicin: toxicity in perspective. *Postgrad. Med. J.* 7:55-61.
- Isoherranen, N. and S. Soback. 1999. Chromatographic methods of analysis of aminoglycoside antibiotics. *J. AOAC Int.* 82:1017-1045.
- Kitagawa, T., K. Fujiwara, S. Tomonoh, K. Takahashi and M. Koida. 1983. Enzyme immunoassays of kanamycin group antibiotics with high sensitivities using anti-kanamycin as a common antiplasma: reasoning and selection of a heterologous enzyme label. *J. Biochem.* 94:1165-1172.
- Lantz, C. H., D. J. Lawrie, F. G. Witebsky and J. D. MacLowry. 1980. Evaluation of plasma gentamicin assay procedure for a clinical microbiology laboratory. *J. Clin. Microbiol.* 10:583-589.
- Lewis, J. E., J. C. Nelson and H. A. Elder. 1972. Radioimmunoassay of an antibiotic: gentamicin. *Nature New Biology* 239:214-216.
- Loomans, E. E. M. G., J. V. Wiltenburg, M. Koets and A. V. Amerongen. 2003. Neamin as an immunogen for the development of a generic ELISA detecting gentamicin, kanamycin, and neomycin in milk. *J. Agric. Food Chem.* 51:587-593.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Place, J. D., S. G. Thompson, H. M. Clements, R. A. Ott and F. C. Jensen. 1983. Gentamicin substrate-labeled fluorescent immunoassay containing monoclonal antibody. *Antimicrob. Agents Chemother.* 8:246-251.
- Ploczekove, C. and O. Foldes. 1992. Immunochemical determination of gentamicin in serum. III. The competitive ELISA. *Cesk Epidemiol Mikrobiol Imunol.* 41(6):346-354.
- Posyniak, A., J. Zmudzki and J. Niedzielska. 2001. Sample preparation for residue determination of gentamicin and neomycin by liquid chromatography. *J. Chromatogr. A.* 914:59-66.
- Ramsden, R. T., P. Wilson and W. P. R. Gibson. 1980. Immediate effects of intravenous tobramycin and gentamicin on human cochlear function. *J. Laryngol. Otol.* 94:521-531.
- Ren, Y. G., J. Martinez, L. A. Kirsebom and A. Virtanen. 2002. Inhibition of klenow DNA polymerase and poly(A)-specific ribonuclease by aminoglycosides. *RNA* 8:1393-1400.
- Reynolds, J. E. F. 1993. *Martindale: The extra pharmacopoeia 30th edition*. The pharmaceutical press, London, UK, pp. 109-113.
- Riviere, J. E. and J. W. Spoo. 2001. Aminoglycoside antibiotics. In *Veterinary Pharmacology and Therapeutics, 8th edition*; (Ed. H. R. Adams); Iowa State University Press, Iowa, pp. 841-867.
- Rosner, A. and H. Aviv. 1980. Gentamicin bioautography assay vs. the microbiological disk test. *J. Antibiot.* 6:600-603.
- Stead, D. A. and R. M. E. Richards. 1996. 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* 675:295-302.
- Stead, D. A. and R. M. E. Richards. 1997. Sensitive high-performance liquid chromatographic assay for aminoglycosides in biological matrices enables the direct estimation of bacterial drug uptake. *J. Chromatogr. B* 693:415-421.
- Tsay, Y. G., L. Wilson and E. Keefe. 1980. Quantitation of plasma gentamicin concentration by a solid-phase immunofluorescence method. *Clin. Chem.* 26:1610-1612.