Detection of Streptomycin Residues in Whole Milk Using an Optical Immunobiosensor

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The development of an assay for the detection of streptomycin residues in pasteurized whole milk using an optical biosensor (Biacore) is reported. Streptomycin−adipic hydrazide coupled to bovine thyroglobulin was used to produce a sheep polyclonal antibody. The antibody displayed excellent cross-reactivity with dihydrostreptomycin (106%). There was no significant cross-reaction with other aminoglycosides or common antibiotics. Streptomycin was also immobilized onto a CM5 sensor chip to provide a stable, reusable surface. The developed assay permitted the direct analysis of whole milk samples (∼3.5% fat) without prior centrifugation and defatting. Results were available in 5 min. The limit of detection of the assay was determined as 4.1 ng/mL, well below the European maximum residue limit (MRL) of 200 ng/mL. Repeatability (or coefficient of variation) between runs was determined as 3.5% (100 ng/mL; 0.5 × MRL), 5.7% (200 ng/mL; MRL), and 7.6% (400 ng/mL; 2 × MRL).

Keywords: *Biosensor; streptomycin; drug residues, milk*

INTRODUCTION

The antibiotic compound streptomycin and its close analogue dihydrostreptomycin (Figure 1) find wide application in modern agricultural practice. They are narrow-spectrum aminoglycosides mainly active against Gram-negative bacteria. They act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes. Susceptible strains include *Actinomyces bovis*, *Pasturella* spp., *Escherichia coli*, *Salmonella* spp., *Campylobacter fetus*, *Leptospira* spp., and *Brucella* spp. *Mycobacterium tuberculosis* is also sensitive (*1*).

Product licenses for all veterinary drugs state a withdrawal time that must be observed by producers before the treated animals can be sent for slaughter. Public health concerns exist based on the nonadherence to these withdrawal periods and on unlicensed use of the drugs. The presence of streptomycin residues in foodstuffs of animal origin can lead to allergic reaction following the ingestion of contaminated milk or meat (*2*). There is also concern about increasing bacterial resistance to antibiotics, which has been observed in livestock (*3*). The transmission of these resistance factors to man would be catastrophic, drastically reducing the effectiveness of antibiotics on human diseases. In the dairy industry, antibiotic-contaminated milk can result in the loss of cultured products (yogurt and cheese) due to the inhibition of starter cultures and inflict severe economic loss (*4*).

For consumer protection, regulatory authorities have established residue limits for streptomycin and dihydrostreptomycin in edible tissues and milk. In Europe (EC Regulation 2377/90), for bovine and ovine milk, the maximum residue limit (MRL) is 200 μg/kg for both streptomycin and dihydrostreptomycin. In the United States, the tolerance limit for dihydrostreptomycin in milk is set at 125 μg/kg. The Codex Committee has set a combined MRL of 200 μg/kg for streptomycin plus dihydrostreptomycin in milk.

A wide array of analytical methods exist for the detection and quantitative measurement of streptomycin. Microbial inhibition (agar diffusion) assays and immunoassays are commonly employed as screening tests (*5*−*7*). HPLC and GC-MS/LC-MS (*8*, *9*) are used for confirmatory analysis.

Biosensors are a recent addition to analytical instrumentation capable of performing residue testing and offer the potential for rapid, quantitative high-throughput analysis. The Biacore system (Biacore AB, Uppsala, Sweden) utilizes the optical phenomenon of surface plasmon resonance (SPR) to monitor biological interactions and is a versatile, robust instrument that has been shown to produce reliable residue screening test results from complex matrices with minimal sample preparation (*10*, *11*).

In the present study, a Biacore sensor was used to develop a procedure for rapid screening of whole milk (fat content = 3.5%) for streptomycin residues. The assay was constructed, with regard to sensitivity, to allow accurate determination of the streptomycin content below and at the regulatory residue limits.

MATERIALS AND METHODS

Equipment. An optical SPR Biosensor system (BIACORE 2000) was obtained from Biacore AB. BIACORE control software, version 3.1, was used for instrument operation and BIA evaluation software, version 3.0, for data handling.

Chemicals and Reagents. Streptomycin sulfate, adipic acid dihydrazide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimde (NHS), bovine thyroglobulin, Freund's complete adjuvant, and Freund's incomplete adjuvant were purchased from Sigma-Aldrich

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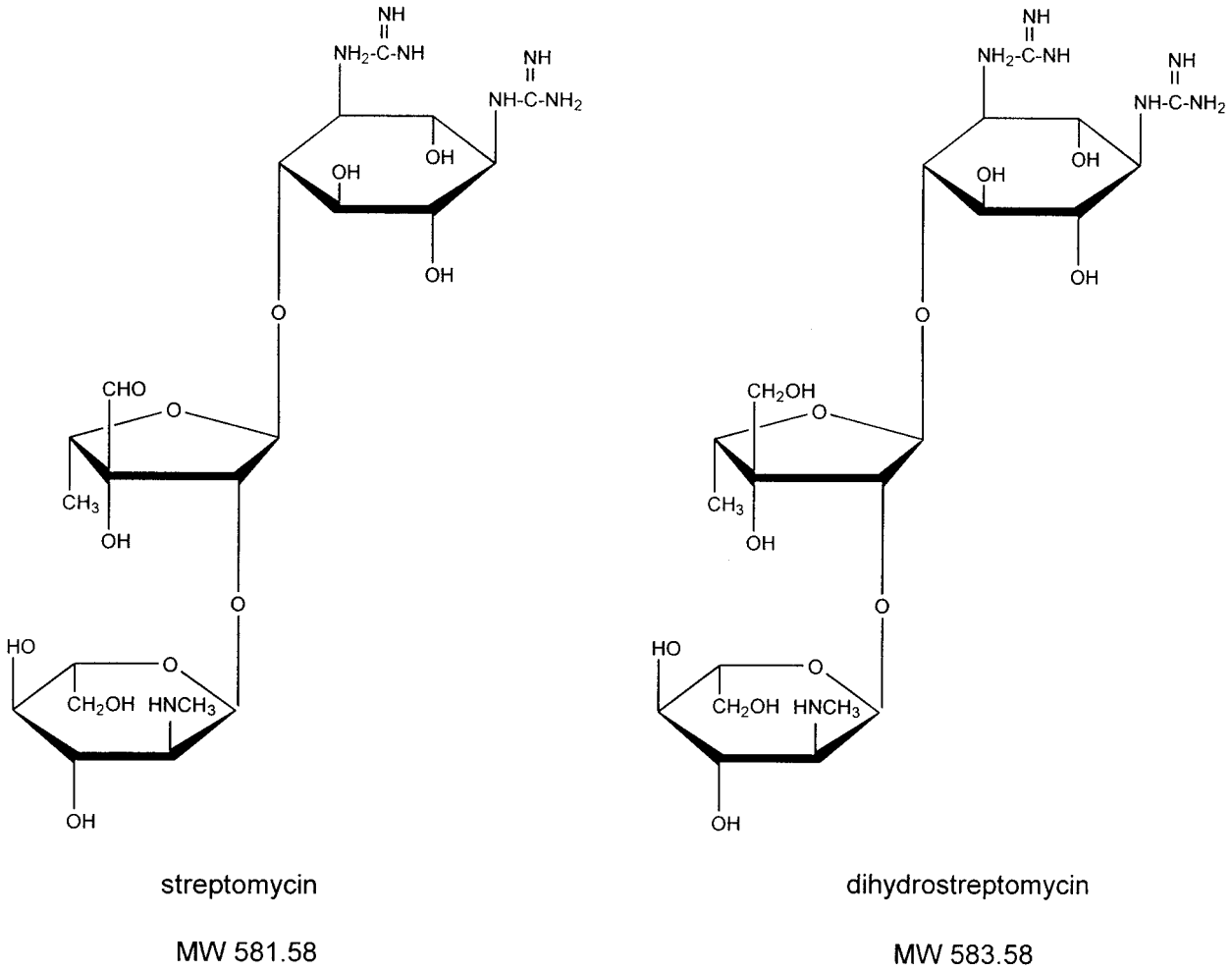

Figure 1. Structures of the aminoglycosides, streptomycin, and dihydrostreptomycin.

Co. Ltd. (Dorset, U.K.). CM5 sensor chips, HBS-EP buffer, and an amine coupling kit were obtained from Biacore AB. A streptomycin-amine derivative, suitable for immobilization to a CM5 sensor chip, was obtained from the Department of Veterinary Science, The Queen's University of Belfast (Northern Ireland). Whole milk samples, for use as control negatives, were obtained from local supermarkets and screened for the presence of antimicrobials by microbial inhibition (5) and by the DELVO test prior to use.

Preparation of Streptomycin—Adipic Hydrazide. A mixture of streptomycin (1 mM) and adipic acid dihydrazide (5 mM) was refluxed for 90 min in 70 mL of 40% v/v methanol/water. The reaction mixture was cooled to −20 °C and the product crystallized by the addition of cold methanol. The crystals were collected by vacuum filtration (Whatman No. 42), washed with a small amount of cold methanol, and then dried overnight in a desiccator.

Coupling of Streptomycin—Bovine Thyroglobulin Immunogen. EDC (10 mg) and NHS (2 mg) were added to a solution of bovine thyroglobulin, BTG (50 mg), dissolved in 2.5 mL of 1 mM sodium acetate and mixed for 5 min at room temperature. Streptomycin−adipic hydrazide (10 mg) dissolved in 400 μL of pyridine was added to the activated BTG solution. The reaction mixture was incubated overnight at room temperature before purification by extensive dialysis against saline (0.15 M sodium chloride). The purified immunogen was diluted with saline to a final concentration of 1 mg/mL protein and stored at −20 °C until used.

Antibody Production. A polyclonal antibody was raised in a sheep (reference S27) by intramuscular injection with 3 mg of the streptomycin−adipic hydrazide−BTG immunogen emulsified with Freund's complete adjuvant. Booster injections (3 mg of immunogen emulsified with Freund's incomplete adjuvant) were administered on a monthly basis. Test bleeds were collected 1 week after each booster injection and monitored for the presence of antibodies by immunoassay. After four booster injections, a large bleed was collected (200 mL) and used for this study. The γ-globulin fraction of this serum was prepared by precipitation using saturated ammonium sulfate and then desalted by extensive dialysis against saline. A cross-reactivity profile of antibody S27 in buffer and in milk was determined by the construction of calibration curves of dihydrostreptomycin and other structurally related compounds and common antibiotics (neomycin, virginiamycin, penicillin G, sulfamethazine, and chlortetracycline).

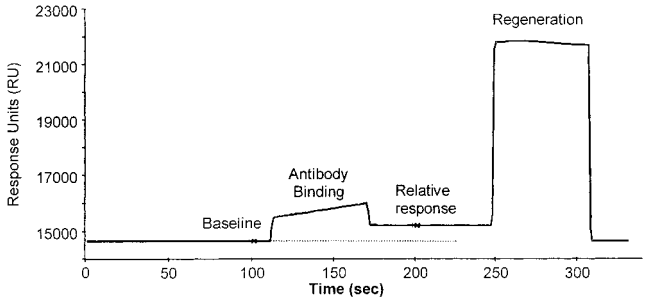

Figure 2. One complete analysis cycle showing the binding of antibody (sample response or relative response) and the regeneration of the sensor surface.

The concentration of streptomycin or other analyte at the midpoint of the relative response calibration curve (IC_{50}) was used to determine the cross-reactivity, which is expressed as a percent relative to streptomycin.

Immobilization of Streptomycin to the Surface of a CM5 Sensor Chip. Streptomycin was immobilized to the

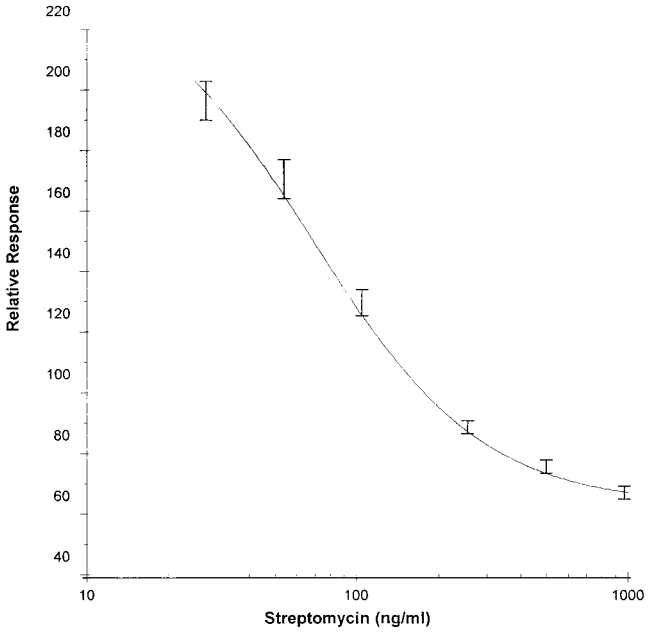

Figure 3. Calibration curve produced in whole milk. Error bars indicate the standard deviation over 3 days.

surface of a CM5 sensor chip (Biacore AB) externally from the instrument to avoid system contamination. The CM5 sensor chip consists of a glass slide coated with a thin layer of gold on one side. Carboxymethyldextran is covalently attached, through an inert linker layer, to the gold, providing a surface matrix to which a range of coupling chemistries can be employed to immobilize biomolecules (*12*, *13*).

Briefly, the chip surface was activated by contact with 40 μL of a 1:1 v/v mixture of 0.4 M EDC/0.2 M NHS for 20 min. The reactants were removed, and 40 μL of the streptomycin amine derivative (20 mg/mL in 10 mM sodium acetate, pH 4.5) added and allowed to remain in contact with the activated sensor chip surface for 1 h. The surface was then washed with deionized water. Unreacted sites were then "capped" by the addition of 40 μL of 1 M ethanolamine for 30 min. The sensor chip surface was then washed repeatedly with water and dried using a stream of nitrogen gas. The sensor chip was stored at 4 °C in a 50 mL centrifuge tube with desiccant when not in use.

Sample Preparation. Whole Milk (Pasteurized, Fat Content = 3.5%). Calibration standards were prepared from a stock standard (1 mg/mL streptomycin in water) by dilution, first with water to give an intermediate solution of 5 ug/mL and then by serial dilution with negative milk to give calibration points of 1000, 500, 250, 100, 50, and 25 ng/mL. Spiked samples were prepared in a similar manner at concentrations of 400, 200, and 100 ng/mL, representing 2 × MRL, MRL, and 0.5 × MRL.

Ten microliters of whole milk was diluted with 190 μL of anti-streptomycin antibody diluted 1/1000 in HBS−EP buffer. Twenty microliters of each sample was then injected over the sensor surface at a flow rate of 25 μL/min (48 s). Report points (in resonance units, see Figure 2) were recorded before and after each injection (i.e., baseline response as zero plus sample response). The surface was then regenerated with a 25 μL injection of 180 mM sodium hydroxide containing 20% acetonitrile (60 s). Including the wash programs, one full analysis cycle took ~5 min.

Assay Validation. The limit of detection (LOD) was calculated from the mean of the measured content of representative blank samples (n = 20) plus 3 times the standard deviation (s) of the mean (mean + 3s). Assay precision was defined by determining *intra-assay* (within run) and *interassay* (between runs) variation, expressed as coefficient of variation (CV). The interassay variation was evaluated over 3 days.

Table 1. Validation Data for the Milk Biacore Assay[a]

whole milk	100 ng/mL (0.5 × MRL)	200 ng/mL (MRL)	400 ng/mL (2 × MRL)
intra-assay			
mean value (n = 10)	99.4	211.8	384.7
s_r (n = 10), %	3.5	5.6	4.9
interassay			
mean value (n = 3)	99.9	212.7	402.0
s_r (n = 3), %	3.5	5.7	7.61

[a] Limit of detection (mean concentration n = 20 known negatives + 3s) = 4.1 ng/mL.

RESULTS

Cross-Reactivity Profile. The anti-streptomycin polyclonal antibody, S27, displayed high cross-reactivity with the close structural analogue dihydrostreptomycin in buffer (106%) and similarly in the chosen matrix, whole milk (106%). There was no significant cross-reaction (<0.1%) with the other aminoglycosides (neomycin and virginiamycin) or other common antibiotic compounds (penicillin G, chlortetracycline, and sulfamethazine) tested.

Assay Validation Results. Table 1 outlines a summary of the validation data calculated for the milk

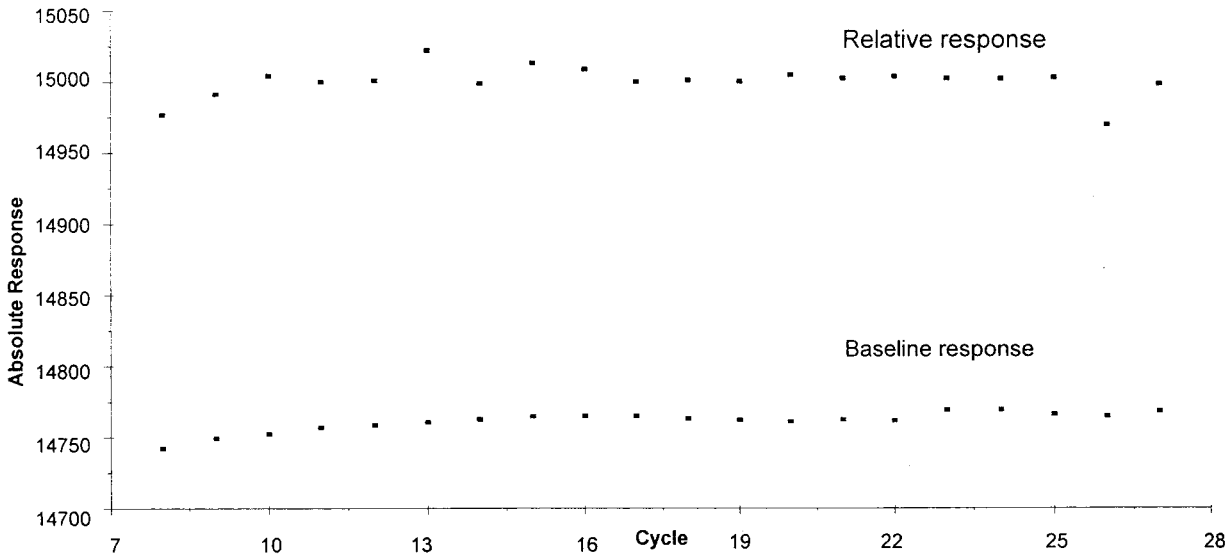

Figure 4. Baseline and relative response of 20 sequential analysis cycles of negative milk.

assay. The LOD of the direct assay was calculated as 4.1 ng/mL, well below the regulatory limits. A typical calibration curve is shown in Figure 3. The intra-assay CVs ranged from 3.5 to 5.6% and the interassay CVs from 3.5 to 7.6%.

The robustness of the SPR method, including the critical regeneration procedure, was checked by performing 20 sequential analyses of negative milk samples as shown in Figure 4. Minimal drift [<25 resonance units (RU)] was observed for the baseline response. The response values following the injection of each of the 20 samples varied over a range of 50 RU. More significantly, a single lane of the sensor chip was reused repeatedly, running in excess of 200 sample analysis cycles without any decrease in response.

DISCUSSION

Inside the BIACORE instrument, the sensor chip forms an interface between the optical unit and the sample medium. The light source is focused on the interface in a wedge-shaped beam giving a fixed range of incident angles. Under conditions of total internal reflection (TIR), an electromagnetic component of light (an evanescent wave) propagates away from the interface into the sample medium. SPR arises through interactions of the evanescent wave with delocalized surface electrons in the gold film. This interaction excites collective resonant oscillation of the electrons (or "plasmons") absorbing the light energy, causing a drop in the reflected light intensity and producing a sharp "shadow" in the reflected light wedge. The sensor monitors the angle of this shadow (the resonance angle), which is a direct measure of the refractive index of the medium into which the evanescent wave propagates. The resonance angle shifts when biomolecules bind to the chip surface and change the refractive index of the surface layer. The change in angle is directly proportional to the change of mass on the surface. Plotting the resonance angle against time displays the progress of the interaction at the sensor surface in the form of a "sensogram" (Figure 2), recording changes as they occur (in real time) and using resonance units to quantify the signal.

The biosensor method is an inhibition assay and detects the antibody when it binds to the streptomycin immobilized on the sensor chip surface. A fixed concentration of antibody is mixed with the sample prior to injection. Any streptomycin present in the sample will bind to the antibody and subsequently inhibit it from then binding to the surface of the sensor chip. The higher the concentration of streptomycin in the sample, the higher the level of inhibition and hence the lower the response of the biosensor. Each analysis cycle is recorded in the form of a sensogram with report points taken before and after each sample injection to quantify the surface binding. The first report point (baseline response) is then assumed as zero. The sample response value is then referred to as the relative response. The surface is then regenerated, ready for the next sample.

The biosensor method described is a direct assay; that is, there is no need for sample preparation prior to analysis. In the presented procedure, milk is simply mixed with a buffer solution containing the specific antibody. Published enzyme immunoassay procedures (*6*, *7*) have required some form of sample preparation (defatting following centrifugation), due to the interference caused by the lipid content of the sample. The detection limits claimed for these assays (6 and 5 ng/mL, respectively) were similar to that of the presented method (4 ng/mL). The time involved in sample preparation and the lengthy incubation periods (>2 h) required for the assays are a big disadvantage of these ELISA systems.

Using the biosensor technology, more rapid results can be obtained due to the lack of sample preparation and overall speed of analysis (~5 min per sample) without any loss in sensitivity. A further beneficial characteristic of this direct biosensor-based test is that it lends itself to being automated, offering the potential for high-throughput analysis.

The biosensor method described has been evaluated using pasteurized whole milk with a fat content of 3.5%. Skimmed and semiskimmed milk present no problems to the assay; however, further work is required on those samples that contain higher fat content values (i.e., raw milk samples as submitted to the dairy plant) to determine any interference.

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