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Determination of gentamicin in bovine milk using liquid chromatography with post-column derivatization and fluorescence detection

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Abstract

A method capable of separating and quantifying the three major and one minor components of gentamicin in milk has been developed. The method is capable of detecting 15 ng/ml gentamicin, based on a total of the four components. Milk samples are centrifuged at 4°C, the fat layer removed, and the samples deproteinated with 30% trichloracetic acid. After a second centrifugation, the supernatant is passed through a C_{18} solid-phase extraction column. The column is washed with water, water–methanol (50:50) and methanol. Ammonium hydroxide (16%) in methanol is used to elute the gentamicin. The eluent is evaporated to near dryness and taken up with water. An aliquot of the sample is then mixed with an ion-pairing reagent for chromatography. Separation is achieved using pentanesulfonic acid in a water–methanol mobile phase on a C_{18} reversed-phase column. The *o*-phthalaldehyde fluorescence derivatives of gentamicin are formed post-column and are detected with excitation at 340 nm and emission at 430 nm. The percent recovery of gentamicin averaged 72, 78 and 88% from milk samples fortified at 15, 30 and 60 ng/ml, respectively.

Keywords: Gentamicin

1. Introduction

Gentamicin is an aminoglycoside currently approved for use in the USA in veterinary medicine [1]. However, gentamicin is not approved for use in lactating dairy cows, and a tolerance has not been established for gentamicin residues in bovine milk [2]. Due to concerns about potential misuse, the U.S. Food and Drug Administration (FDA) needs a method capable of detecting and quantifying gentamicin in bovine milk. To ensure the safety of the milk supply, the FDA has determined that the method must be capable of detecting and quantifying residues at a concentration of 30 ng/ml.

Gentamicin consists of three major components, gentamicin C_{1a} (C_{1a}), gentamicin C_1 (C_1), and gentamicin C_2 (C_2). One minor component gentamicin C_{2a} (C_{2a}), is known to be a significant fraction of total gentamicin in some formulations [3,4]. The four components are shown in Fig. 1. Methods developed for the determination of gentamicin in biological matrices were recently reviewed [5]. The official method for the determination of gentamicin in tissue is a microbiological assay [2]. Agarwal [6] reported a method for the determination of gentamicin in milk; however, it could not detect

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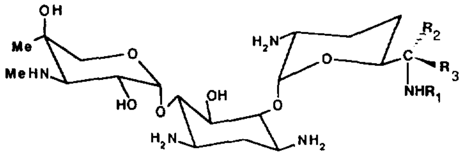

Fig. 1. Structure of gentamicin. For C_{1a}, $R_1=R_2=R_3=H$; for C_{2a}, $R_1=R_3=H$, $R_2=Me$; for C_2, $R_1=R_2=H$, $R_3=Me$; for C_1, $R_1=R_3=Me$, $R_2=H$.

gentamicin at a concentration of 30 ng/ml. A gas chromatography-based method has also been reported for the determination of gentamicin in milk [7].

Recently Shaikh and Jackson [8] developed a method for the determination of Neomycin B in bovine kidney, based on ion-pairing chromatography with post-column derivatization and fluorescence detection [8]. The chromatography used in this published method has been adapted for the determination of gentamicin in milk.

2. Experimental

2.1. Materials

Gentamicin Sulfate Standard, with a potency of 682 μg/mg and a content consisting of 19% C_{1a}, 46% C_2, and 35% C_1 as assayed by Schering (QC lot # 112285, Schering Corporation, Union, NJ, USA), was provided. Sodium sulfate (Fisher Pesticide Analysis grade), 1-pentanesulfonic acid sodium salt mono hydrate (Fisher, HPLC grade), and trichloroacetic acid (reagent grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol (HPLC grade) was obtained from Burdick and Jackson, Distilled in Glass (Muskegan, MI, USA). Reagent-grade ammonium hydroxide and glacial acetic acid were obtained from J.T. Baker (Phillipsburg, NJ, USA). Reagent-grade water, distilled, deionized to >17 mΩ cm resistivity, and treated to remove traces of organic compounds, was prepared in-house (Barnstead Nanopure II system with OrganicPure, Sybron/Barnstead, Boston, MA, USA). All references to water refer to reagent-grade water. The *o*-phthalaldehyde (OPA) post-column derivatization reagent solution was obtained from Pierce Chemical (Rockford, IL, USA). The 6 ml solid-phase extraction (SPE) columns containing 0.5 g of C_{18} were purchased from Supelco (Bellefonte, PA, USA). Polypropylene autosampler vials (1 ml) with open-top caps and Teflon disks were purchased from National Scientific Company (Lawrenceville, GA, USA).

2.2. Equipment

The liquid chromatograph consisted of a quaternary pump with autosampler (Series 410 and ISS 200, Perkin-Elmer, Norwalk, CT, USA), a post-column reaction system with a 1 ml post-column reaction coil constructed of tefzel with a 0.01 in. I.D. maintained at a temperature of 33°C (PCR 520 and Spectroflow 400 pump, Applied Biosystems, Foster City, CA, USA), and a fluorescence detector (LS-4, Perkin-Elmer). The post-column reaction system had an internal pulse dampener prior to the mixing tee. Both the analytical and guard columns were in a column heater (CH-30, Eppendorf, Milwaukee, WI, USA) set at 32.5°C. All external tubing between the pump, post-column reaction system, and detector was insulated to protect from fluctuations in room temperature. A refrigerated centrifuge (Sorvall RC5C with a HS-4 rotor, #38002 buckets, DuPont, Medical Products, Wilmington, DE, USA), set at 4°C, was used for sample preparation.

2.3. Ion-pair concentrate

A 0.11 *M* pentanesulfonic acid, sodium salt, in 1% acetic acid solution was added to samples and standard solutions at a ratio of 9:1 sample/standard:ion pair concentrate.

2.4. Chromatographic conditions

Separation was achieved using a PhaseSep Spherisorb ODS2, 5 μm particle size, 15 cm×4.6 mm (Bodman, Aston, PA, USA, or equivalent) with a guard column (2 cm×4.6 mm) containing the same packing (Alltech Associates, Wakegeen, IL, USA). The mobile phase consisted of 0.011 *M* pentanesulfonic acid, sodium salt, 0.0056 *M* sodium sulfate and 0.1% acetic acid in water–methanol (82:18). It was filtered through a 0.45 μm Nylon 66 filter before use. The flow-rate was 1.5 ml/min with the OPA added post-column at a flow-rate of 0.9 ml/min. Both the mobile phase and the OPA were allowed to come to room temperature before use, and the system was typically run for 2 h prior to analysing the first standard, to ensure system stability. The excitation and emission wavelengths were 340 and 430 nm, respectively, with a slit width of 10 nm.

2.5. Standard preparation

The gentamicin standard was dried for 3 h under vacuum (0.67 kPa) at 60°C. A 100 μg/ml stock standard solution was prepared by dissolving 0.01466 g of gentamicin sulfate standard in 100 ml of water; the weight used was adjusted based on the potency of the standard. A 1.0 μg/ml standard solution was prepared from the stock. The 1.0 μg/ml solution was used to prepare fortified control milk samples and to prepare 240, 120, 60 and 30 ng/ml solutions for instrument calibration. As the samples are concentrated by a factor of 4, these solutions are equivalent to 60, 30, 15 and 7.5 ng gentamicin/ml of milk. All the standard solutions were prepared with water in polypropylene volumetric labware. Prior to analysis, ion-pair concentrate was added to each standard solution used for calibration.

2.6. Milk samples

Incurred milk, i.e., milk from a cow dosed with gentamicin, was generated at this facility from a Holstein cow treated with 5 mg/kg body weight of gentamicin. The complete experimental details are being published elsewhere [9]. Control milk, i.e., milk containing no gentamicin, was obtained from a Holstein dairy cow that did not receive any antibiotic treatment.

2.7. Sample preparation

Polypropylene labware was used in sample preparation as gentamicin adsorbs to glass. Whole milk (10.0 ml) was centrifuged at 4°C and 3100 *g* (4000 rpm using the equipment listed above) for 30 min. The solidified fat on the top of the milk sample was removed with a spatula. Trichloracetic acid (1.0 ml of 30%) was added to the defatted milk sample, and the samples are mixed briefly and centrifuged for 30 min at 4°C and 3100 *g* to deproteinate [10].

C_{18} SPE columns are prepared by washing with 5 ml of methanol followed by 5 ml of water. All of the deproteinated supernatant from each sample is passed through the SPE column. The SPE columns are washed with 10 ml each of distilled water, methanol–distilled water (1:1), and methanol (in order) at a moderately fast flow-rate, 2–3 drops/s. The columns should not be allowed to go dry. The gentamicin is eluted at a moderately fast flow-rate from the SPE columns with 7 ml of 16% ammonium hydroxide in methanol and collected in a 15 ml graduated polypropylene centrifuge [11]. The samples are evaporated to <0.3 ml with a gentle stream of nitrogen on the N-evaporator. The samples are not allowed to go dry. Water is added to the sample (final volume of 2.5 ml). Samples may be stored refrigerated at this point for up to two months.

2.8. Sample analysis

On the day of analysis, each sample (540 μl) is mixed with 60 μl of ion-pair concentrate, and 500 μl of each sample is injected onto the LC for analysis. Prior to the start of analysis, duplicate 240 ng/ml standards were injected to determine system reproducibility. A control milk sample and a control milk sample fortified at 30 ng gentamicin/ml are analyzed with each set of samples to verify system performance. The amount of gentamicin in each sample was calculated by using an external calibration curve generated with standards to calculate the amount of each component present. The results were calculated using both peak height and area. The results were comparable; however, for the data presented in this paper, peak height was used. The total gentamicin present was calculated by summing the amount of each component present, then dividing by four, the concentration factor.

2.9. Gentamicin component identification and percent in standards

The identity of each component is tentative, as individual reference standards of each component were not available. The official assay of the standard lists only three components; however, four components were present in the standard. The presence of four gentamicin components in the standard has been confirmed by mass spectrometry [12]. The elution order of the gentamicin was assigned by the following procedure: The relative abundance of each component signal was compared with the percent of the component given in the assay of the standard. The relative response factors of the OPA derivatives of gentamicin C_1, C_{1a} and C_2 have been shown to be within 5% of each other [13]. The listed percentage for C_2 in the standard was taken as representing the amount of C_2 and C_{2a} present; this approach is consistent with mass spectrometric analysis of the standard. The proposed elution order was compared with published separations of gentamicin using similar conditions [14]. Additionally, the proposed elution order was evaluated to determine if it was consistent with the structural variations of the gentamicin components (see Fig. 1). However, it was not possible to unambiguously identify the peak associated with C_2 from the peak associated with C_{2a}. The larger of the two peaks was assigned to C_2.

3. Results and discussion

A series of analyses was completed to validate the performance of the method in the range of 15–60 ng gentamicin/ml milk over an 8 day period. Seven control milk samples, seven fortified milk samples at each of three concentrations, 15, 30 and 60 ng/ml, and five incurred milk samples from each of two milkings were analyzed. Data from the analysis of one incurred milk sample was shown statistically to be an outlier [15] and is not reported. Fig. 2 shows the chromatograms from the analysis of a control milk sample and an incurred milk sample containing 37 ng/ml of gentamicin. No interferences were seen in the control milk except for a small peak that had a retention time slightly longer than the C_{2a} peak in 2 samples. However, this interference was not greater than allowable under FDA Center for Veterinary Medicine guidelines. Table 1 lists a summary of the results of the analyses of fortified milk samples. The average recoveries for samples fortified at 15, 30 and 60 ng/ml gentamicin was 72%, 78% and 88%, respectively. Table 2 lists the results of the analyses of incurred milk samples; milk from two milkings was analyzed. Milk from one milking (second milking post dose) contained an average of 5.9, 4.3, 10.5 and 10.0 ng/ml of C_{1a}, C_{2a}, C_2 and C_1, respectively, for a total of 31 ng/ml gentamicin. The coefficient of variation was 14.1% for C_{1a}, 13.3% for C_{2a}, 11.7% for C_2 and 12.4% for C_1. Milk from another milking (third milking post dose) contained an average of 2.4, 1.9, 4.3 and 3.9 ng/ml of C_{1a}, C_{2a}, C_2 and C_1, respectively, for a total of 12 ng/ml gentamicin. The coefficient of variation was 8.9% for C_{1a}, 12.9% for C_{2a}, 9.3% for C_2 and 10.0% for C_1.

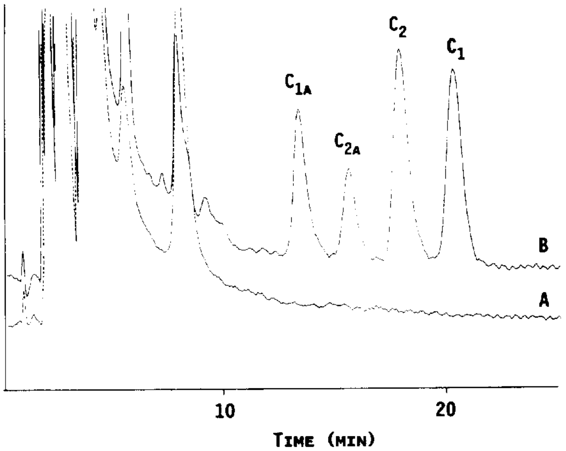

Fig. 2. Chromatogram of: (A) a control milk sample, and (B) a milk sample from a cow treated with gentamicin. The proposed elution order for the four components of gentamicin is given. The amount of each component present in (B) is 5.5 ng/ml for C_{1a}, 3.5 ng/ml for C_{2a}, 9.5 ng/ml for C_2 and 9.3 ng/ml for C_1.

The external calibration curves generated for gentamicin were linear over the range of the method. Typically, r^2 for the linear regression of the calibration curves was greater than 0.995. No systematic bias was evident in the residuals, the difference between an amount of standard injected and the calculated amount based on the resulting standard curve. This is an additional indication that the external calibration curves were linear. The absolute values of all calculated intercepts were less than 10% of the signal of the lowest standard analyzed; most were less than 5%. In reviewing the calculated intercepts of all of the calibration curves generated during method validation, no trends, positive or negative, were observed in the data.

Table 3 lists for each component the limit of detection as calculated based on the definition of

Table 1
Summary of the recovery of gentamicin from fortified milk samples[a]

	Gentamicin C_{1a} (C_{1a})		Gentamicin C_{2a} (C_{2a})		Gentamicin C_2 (C_2)		Gentamicin C_1 (C_1)		Total Gentamicin ($C_{1a}+C_{2a}+C_2+C_1$)	
	ng/ml	% Rec[b]	ng/ml	% Rec	ng/ml	% Rec	ng/ml	% Rec	ng/ml	% Rec
15.2 (ng/ml)[c]										
AVG (n=7)	2.2	71	1.8	79	3.7	71	3.3	72	11.0	72
S.D.	0.37	12.2	0.36	16.0	0.52	9.8	0.36	7.9	1.47	9.7
C.V.		17.3		20.3		13.9		10.9		13.4
30.3 (ng/ml)[c]										
AVG (n=7)	4.6	76	3.9	87	8.0	76	7.2	78	23.8	78
S.D.	0.59	9.7	0.83	18.4	0.92	8.7	0.80	8.7	2.9	9.7
C.V.		12.8		21.1		11.4		11.1		12.4
60.6 (ng/ml)[c]										
AVG (n=7)	10.4	85	8.2	91	18	88	16	90	54	88
S.D.	1.6	14	1.3	15	3	14	3	14	8	14
C.V.		16		16		16		16		16

[a] The averages are a combination of intra- and inter-day results. The same control milk sample was used for all fortified samples; however, each individual sample was fortified separately.

[b] Recovery.

[c] Concentration of gentamicin in fortified milk sample.

Table 2
Results of the analysis of milk from a cow treated with gentamicin[a]

	Gentamicin C_{1a} (C_{1a}) (ng/ml)	Gentamicin C_{2a} (C_{2a}) (ng/ml)	Gentamicin C_2 (C_2) (ng/ml)	Gentamicin C_1 (C_1) (ng/ml)	Total Gentamicin ($C_{1a}+C_{2a}+C_2+C_1$) (ng/ml)
Milk A[b]	4.7	4.8	8.9	8.3	26.7
Milk A	6.3	3.9	11.1	10.7	32.0
Milk A	5.5	3.5	9.5	9.3	27.8
Milk A	6.8	4.8	11.9	11.5	35.0
Milk A	6.4	4.3	10.9	10.3	31.9
Milk B	2.4	2.2	4.8	4.3	13.7
Milk B	2.1	1.9	3.9	3.5	11.4
Milk B	2.6	1.9	4.1	3.6	12.2
Milk B	2.3	1.6	4.5	4.1	12.5

Milk from two milkings was analyzed.

[a] The data are a combination of intra- and inter-day results from replicate analysis of the same milk sample.

[b] The incurred milk was generated from a single treatment of one cow. Milk from the second and third milkings post-dose was used for method validation. Complete details are given in [9].

Table 3
Determination of the limit of detection (LOD)

Peak	LOD[a] (ng/ml)
C_{1a}	0.3
C_{2a}	0.4
C_2	0.4
C_1	0.4

[a] Estimates based on the average response of control sample plus three times the standard deviation of the average response.

Keith et al. [16]. The signal-to-noise ratio for the C_{2a} peak (smallest peak, 14% of total) was about 30:1 for a 120 ng gentamicin/ml standard. The performance of the method is considered acceptable according to FDA guidelines.

During the development of the method, several areas were identified that are critical to the performance of the method. If the temperature of the post-column reaction coil is not constant, the variability of the signal is unacceptably large. The column temperature must be kept constant or the baseline and retention time will drift. Gentamicin in solution will adsorb to glassware. To prevent such losses, all labware that comes in contact with samples or gentamicin solutions should be made of plastic; polypropylene is recommended. To maintain acceptable chromatography, it is essential that standard recommended practices be followed such as a periodic flushing of the analytical column [8].

During the development of the method, a series of common veterinary drugs, including other aminoglycosides, penicillins, tetracyclines, macrolides, sulfa drugs, chloramphenicol and novobiocin were tested for interference. Neomycin, another aminoglycoside antibiotic, was the only compound tested that will be detected if present in the milk. The primary component of neomycin is only partially resolved from the gentamicin C_{2a} peak. However, neomycin can be distinguished from gentamicin by its pattern of peaks. Neomycin will only have one primary peak (neomycin B) in the chromatogram with a small peak, of less than 10% (neomycin C), eluting before the primary peak [8]. As seen in Fig. 2, a chromatogram of a milk containing gentamicin has 4 peaks (3 if the minor component C_{2a} is not present), with peak heights typically within a factor of 3.

The retention time of four components of gentamicin is dependent on the percent methanol in the sample and can vary from column to column, even when columns from the same manufacturer are used. Eighteen percent methanol is suggested as a starting point as it gives adequate separation on most columns tested. Small (1–2%) changes in the methanol component of the mobile phase will cause large (5–10) min shifts in the retention time of gentamicin. The retention time of the C_{1a} peak, the first peak to elute, initially should be at least 15 min. The retention time will decrease as the column ages. This decrease is not significant on a daily basis. However, the user will need to periodically decrease the amount of methanol in the mobile phase to maintain adequate separation, and prevent the first peak from eluting on the shoulder of the sample matrix.

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