Gentamicin in Tissue and Whole Milk: An Improved Method for Extraction and Cleanup of Samples for Quantitation on HPLC

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A new, inexpensive, and rapid procedure for the extraction of gentamicin from animal tissue and whole milk samples is described. Tissue samples from gentamicin-treated calves were homogenized in a pH 8.8 extraction buffer, mixed, filtered, and centrifuged. The resulting supernatant fractions were extracted using bifunctional hydrophobic/ion exchange solid phase cartridges. The isolated gentamicin was derivatized with a chromophore and analyzed using high-performance liquid chromatography. Whole milk samples were similarly extracted and analyzed. The resulting chromatograms showed exceptionally clean samples with no interfering peaks in the area of gentamicin elution. This allowed good separation and detection of all four isomers of gentamicin. The detection limit for gentamicin was 0.6 μg/mL in whole milk and 1 μg/g in tissue samples. The coefficients of variation were 2.5% for whole milk and 8.1% for tissue samples. The efficiency of recovery was greater than 90%.

Keywords: *Gentamicin; HPLC/UV; solid phase extraction; tissue; milk*

INTRODUCTION

Gentamicin is a broad spectrum antibiotic widely used in treating bacterial infections in dairy cattle and calves. Numerous studies (Brown et al., 1988; Haddad et al., 1987) have shown that the drug may have an extended retention time in many tissues. Although gentamicin has not been approved by the Food and Drug Administration (FDA) for use in cattle, its use is permissible when prescribed by a licensed veterinarian in accordance with the FDA extra-label policy. When animals destined for use as food products are treated with gentamicin under the extra-label policy, it is important to establish withdrawal times to ensure that the meat or dairy products obtained are gentamicin-free when they reach the consumer. Even though tolerance levels for gentamicin in beef have not currently been established, the tolerance levels for pork are 0.1–0.4 ppm, depending on the tissue, and the tolerance levels for uncooked turkey meat are 0–0.1 ppm. The "safe levels" for gentamicin in milk are 0–30 ppb (*Code of Federal Regulations*, 1994). The present method was developed to facilitate the detection of gentamicin in calf tissue and whole milk using HPLC. Although a considerable volume of work has been published on the detection of gentamicin using HPLC (Peng et al., 1977; Rumble et al., 1987; Semchuk et al., 1993; Shaikh and Allen, 1985), most of it deals with quantitation of the drug in matrices such as serum and urine. Adaptation of these methods to whole milk and tissue extracts was unsatisfactory because of the complexity of the matrices and the high fat and protein contents of the extracts. Previously published methods of tissue sample preparation for the analysis of gentamicin content on HPLC have included lengthy and cumbersome extraction procedures. These methods also yielded samples that produced interfering coeluting peaks when they were analyzed on HPLC (Agarwal, 1989). In addition, tissue or whole milk sample preparation methods had low analyte recovery as a result of the need to deproteinize and defat these samples (Agarwal, 1989; Scheurer and Moore, 1992). Many of these difficulties can be circumvented if the analysis is performed on a laser-based polarization detection system (Bobbit and Ng, 1992; Brown and Baird, 1988; Brown et al., 1990; Ng et al., 1991) or if a radioammunoassay (RIA) technique is used (Haddad et al., 1986a,b, 1987) since these techniques do not require extensive sample cleanup. HPLC, however, is the conventional method for the quantitation of drugs in biological fluids, necessitating the development of an improved method of sample cleanup for use with this system.

The purpose of this study was to develop a simple and rapid method for the extraction of gentamicin from tissue samples and to develop an inexpensive cleanup procedure for tissue extracts and whole milk samples, which would yield a very high rate of analyte recovery and cleaner samples for analysis on HPLC.

MATERIALS AND METHODS

Material. All solvents were of analytical or HPLC grade. Gentamicin sulfate and netilmicin were purchased from Schering Corp. (Kenilworth, NJ). The hydrophobic ion exchange solid phase cartridges (C_{18}COOH copolymer packing, 300 mg) were donated by Chemical Separations Corp. (King of Prussia, PA), and the 1-heptanesulfonic acid (sodium salt), *o*-phthalaldehyde, and mercaptoacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

Instrumentation. Analyses of the samples were performed on a Hewlett-Packard HPLC system (Model 1090A) equipped with a three-channel solvent delivery system, autoinjector, autosampler, and diode array detector interfaced with a Chem Station Analyzer. The column used in the analysis

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0021-8561/95/1443-1849$09.00/0

was a Zorbax SB-C18, 2.1 mm × 150 mm (Mac Mod Analytical Inc., Chadds Ford, PA).

Reagents. *Extraction buffer* components were 0.1 M potassium phosphate/0.1 M sodium sulfate. The pH was adjusted to 8.8 with the use of 50% potassium hydroxide.

The elution buffer (100 mL) consisted of 500 mg of 1-heptanesulfonic acid (sodium salt), dissolved in 20 mL of 0.4 M potassium borate (pH 10.4) before the addition of 80 mL of methanol (MeOH).

The mobile phase was 1-heptanesulfonic acid (HSA; sodium salt, 5 g) dissolved in acetic acid–water–MeOH (50:250:700). The mobile phase was degassed with helium prior to use.

The derivatizing reagent was 100 mg of *o*-pthalaldehyde (OPA) dissolved in 0.5 mL of HPLC grade MeOH. A 9.5 mL volume of 0.4 M potassium borate (pH 10.4) and a 0.2 μL volume of mercaptoacetic acid (MAA) were then added. The pH was adjusted to 10.4 with 50% potassium hydroxide solution. The reagent was filtered using a 0.45 μm disposable filter (Sigma) and stored at <0 °C in a UV-shielded container for up to 1 week.

Methods. *Treatment and Sample Collection.* Six lactating cows were administered 1 mg/kg of gentamicin as an intramammary infusion (10 mg/mL) in the right front quarter. Milk samples were collected from treated and untreated quarters at designated times beginning at 10 min post-treatment and continuing to 120 h post-treatment. Samples were stored frozen at −20 °C until analyzed. One-week-old calves were administered 4.4 mg/kg of gentamicin in multiple intravenous injections at 0, 8, 24, 36, and 48 h. The calves were then euthanized on designated days following the last drug administration, and tissue samples were collected and frozen until analyzed. HPLC analysis was performed on samples of heart, liver, spleen, muscle, and kidney obtained from gentamicin-treated calves and on samples of milk obtained from the treated and untreated quarters of lactating cows.

Tissue Extraction. A 400 μg/mL solution of netilmicin in HPLC grade water (J. T. Baker Inc., Phillipsburg, NJ) was prepared for use as an internal standard (IS). Fifty microliters (20 μg) of this IS was added by repeat pipetter to each gram of thawed tissue sample. The sample was homogenized (Polytron PT 3000, Kinematica AG) in a 25 mL volume of the extraction buffer for 30 s at medium speed. The resulting homogenate was mixed for 1 h using a rotator at room temperature and then centrifuged at 17 000 rpm (4 °C) for 30 min. The supernatant was filtered through a Kim wipe to remove the visible fat globules and then decanted for solid phase cleanup procedures. The supernatant was not deproteinized, thus eliminating the problem of significant loss of the target analyte.

Solid Phase Cleanup of Whole Milk and Tissue. The IS, netilmicin (20 μg), was added as above to 1 mL aliquots of each whole milk sample or standard, and the tubes were placed in a water bath (35 °C). The samples were then extracted on solid phase cartridges using a Vac-Elut SPS-24 vacuum manifold (Analytichem International, Harbor City, CA). The solid phase cartridge was first preconditioned with 2 mL of MeOH and 2 mL of deionized (DI) water, and then the 1 mL sample of prewarmed milk was added and slowly eluted to waste. The cartridge was then washed with 10 mL of deionized water (35 °C) and 10 mL of MeOH and dried under vacuum for 2 min. The analyte (gentamicin) was eluted with 2 mL of the elution buffer. The eluate was then evaporated to dryness in a water bath (65 °C) under air pressure or using a SpeedVac A-290 concentrator (Savant Instruments Inc., Farmingdale, NY). The dried sample eluate was reconstituted with DI water (150 μL) and used for analysis on the HPLC. Gentamicin was similarly recovered from tissue sample extracts, with the entire supernatant (25 mL) directly applied onto the solid phase cartridge for extraction.

Preparation of Standards. An aqueous stock solution of gentamicin was prepared (1.0 mg/mL), and this stock solution was used in preparing milk and tissue standards. Gentamicin-free whole milk was spiked to yield standard concentrations of gentamicin of 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 μg/mL. After the addition of the IS, the spiked gentamicin standards were extracted on solid phase cartridges as described above and analyzed on HPLC, and a standard curve was constructed from the results obtained. Gentamicin-free tissue samples were similarly fortified with gentamicin and IS, allowed to incubate overnight at 4 °C, and then extracted. The extracts were subjected to cleanup procedures using SPE and HPLC analysis as described above, and a standard curve was generated from the results.

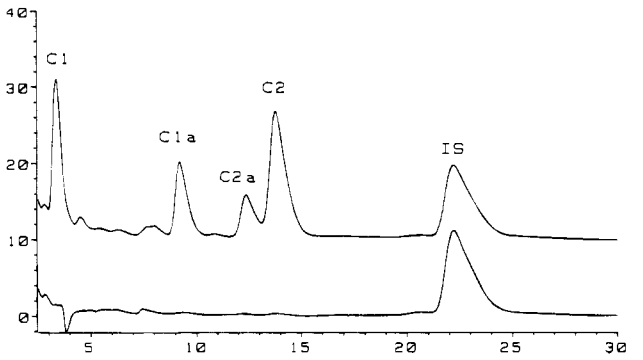


Figure 1. Overlay of two HPLC chromatograms plotting response (*Y* axis) vs time (*X* axis). The bottom tracing is a chromatogram of a solid phase extracted (SPE) sample of gentamicin-free whole milk (control). The top tracing is a chromatogram of a 40 μg/mL gentamicin-spiked whole milk standard. The IS peak represents the internal standard, netilmicin.

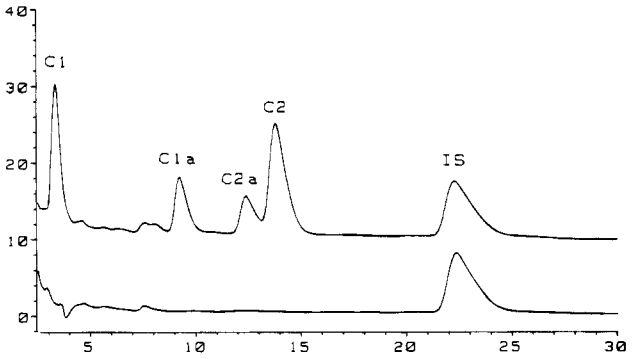


Figure 2. Overlay of two HPLC chromatograms plotting response (*Y* axis) vs time (*X* axis). The bottom tracing is a chromatogram of a solid phase extracted (SPE) sample of gentamicin-free beef muscle (control). The top tracing is a chromatogram of a 100 μg/g gentamicin-spiked beef muscle standard.

Analysis of the Samples on HPLC. All samples were derivatized with OPA in the presence of MAA and analyzed on HPLC according to the method of Albracht and DeWit (1987). Since the column employed was a low volume 2.1 mm i.d. column, the following modifications were necessary: the flow rate and the injection volume were 0.5 mL/min and 10 μL, respectively, for this study rather than 1.0 mL/min and 20 μL. The elution was isocratic.

RESULTS

HPLC analysis of control whole milk and tissue extracts, subjected to the SPE cleanup procedures described in this study, produced chromatograms free from interfering peaks in the area of interest (Figures 1–3). The derivatization of gentamicin with OPA in the presence of MAA, as detailed by Albracht and DeWit (1987), allowed HPLC analysis with UV detection at 330 nm. This procedure yielded a gentamicin derivative that permitted separation of all four isomers of gentamicin, provided better quantitation results, and was more stable than OPA–mercaptoethanol-derivatized gentamicin employed in fluorescence detection (Agarwal, 1989). Since UV detection is very reliable and

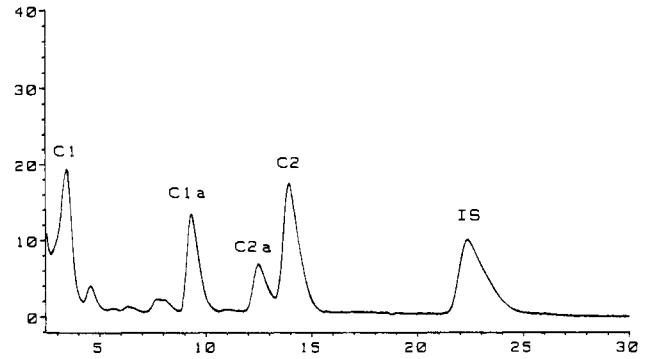


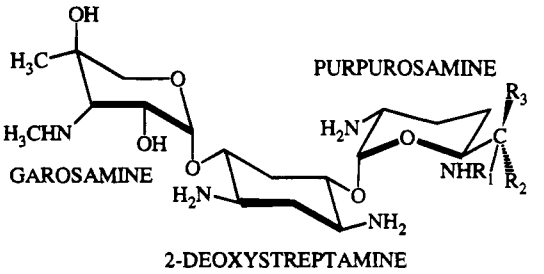
Figure 3. Chromatogram of a 40 μg/mL aqueous gentamicin standard.

readily available with most HPLC instruments, UV detection was chosen over fluorescence detection for this study. Analysis of gentamicin standard, gentamicin-spiked milk and tissue standards, and experimental samples yielded four distinct peaks (Figures 1–3).The relative concentrations, as indicated by peak area (peak 1 > peak 4 > peak 2 > peak 3), corresponded to those reported by Albracht and DeWit (1987), using UV detection and similar HPLC conditions. Thus, it would appear that the order of elution was also the same: C_1, C_{1a}, C_{2a}, and C_2 and peaks were tentatively identified as such. Since it was not relevant to this study to establish unequivocal identification of these isomers, no further analysis of individual peaks was performed. Standard curves were constructed for eight extracted gentamicin-spiked milk or tissue samples (0.625–80 μg/mL for milk and 1–80 μg/g for tissue) by plotting the area of the eluted peak versus the sample concentration. Standards were extracted and a standard curve constructed once a week when a new batch of derivatizing reagent was used in the assay. The coefficients of determination (r^2) were greater than 0.99 as calculated by the linear least-squares method. The detection limits for gentamicin samples were 0.6 μg/mL in whole milk and 1 μg/g in tissue samples. The detection limit for the LC was about 5 ng of gentamicin. Tissue samples fortified with 100 μg/g and milk samples fortified with 45 μg/mL of gentamicin were extracted and analyzed in replicate ($n = 6$). The coefficients of variation were 2.5% for milk and 8.1% for tissue. The recovery rate for gentamicin from spiked tissue and whole milk samples, when quantitated using the above standard curves, was in excess of 90%. The sensitivity of this assay was more than 500 times greater than that reported by Albracht and DeWit (1987) for samples analyzed using UV detection and similar HPLC procedures. Concentrations of gentamicin obtained from representative samples of whole milk and tissue are shown in Tables 1 and 2, respectively. The results of the study involving the pharmacokinetic model for predicting gentamicin withdrawal in cows and calves will be published elsewhere.

DISCUSSION

Gentamicin is an aminoglycoside antibiotic complex, consisting of four major components designated C_1, C_{1a}, C_2, and C_{2a}. These components differ only minimally by substitutions at the R_1, R_2, or R_3 position (Figure 4). The chemistry of gentamicin allowed the present method to employ a bifunctional separation phase consisting of weak cation exchange and ion pair elution.

The reason for the multiplicity of the approaches used in the present study was the difficulty in achieving



	R_1	R_2	R_3
C_1	$-CH_3$	H	$-CH_3$
C_{1a}	H	H	H
C_2	H	H	$-CH_3$
C_{2a}	H	$-CH_3$	H

Figure 4. Structure of gentamicin and its analogs, C_1, C_{1a}, C_2, C_{2a}.

Table 1. Concentrations of Gentamicin Recovered from Whole Milk Samples (Treated Quarter)

time	gentamicin (μg/mL)	time	gentamicin (μg/mL)
10 min	1200	5 h	313
15 min	1095	6 h	182
30 min	830	8 h	106
45 min	750	12 h	45
1 h	770	24 h	6.1
2 h	525	48 h	(0.2)[a]
3 h	399	96 h	ND[b]
4 h	339	120 h	ND

[a] The detection method is not reliable for values below 0.6 μg/mL in whole milk. [b] ND, none detected.

Table 2. Concentrations of Gentamicin Recovered from Calf Tissue Samples (7 Days Postadministration)

tissue sample	gentamicin (μg/g)	tissue sample	gentamicin (μg/g)
spleen	2.2	kidney	69.6
liver	4.5	kidney cortex	103.0
lung	1.9	kidney medulla	25.0
muscle	1.3		

sufficient separation of gentamicin from the matrix components. This difficulty may be due, in large measure, to the subtle differences in chemical potential that these matrix components and gentamicin exhibit relative to one another. Thus, while most of the existing methodologies take advantage of a single predominant retention property of these molecules (i.e. charge effects of amine function in ion exchange separations, hydrophobicity, and/or ion pair in reversed phase separations) to effect the required separation, the resolving power of these phases between some matrix components and gentamicin was not sufficient to accomplish the task. The end result was "dirty" extracts, which made further analysis difficult or impossible. The existence of these interfering matrix components with chromatographic properties similar to the desired analyte and with small differences in chemical potential requires a stationary phase that simultaneously discriminates a solute with regard to charge neutralization, parity of total energy, and steric effects. Thus, the choice of a hydrophobic/ion exchanger for the SPE procedure used in this study was highly justified.

This study presents a rapid method for the extraction of gentamicin from tissue samples with an excellent recovery rate. The solid phase extraction procedure employed in the cleanup of the samples was inexpensive and very successful. The procedure yielded very clean

samples for quantitation of gentamicin on HPLC, which enabled all four isomers of gentamicin to be detected in tissue extracts. No published reports that claimed similar results for HPLC analysis of gentamicin in tissue have been found. The results obtained were more sensitive than previous studies using UV detection and similar HPLC procedures.

ACKNOWLEDGMENT

We thank Chemical Separations Corp. (King of Prussia, PA) for donating the solid phase cartridges used in this study for the method development. This study was supported by a research grant from the Pennsylvania Department of Agriculture.

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Received for review August 19, 1994. Accepted April 27, 1995.[⊗]

JF940482W

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.