

# Gentamicin Sulfate Assay by HPLC with Charged Aerosol Detection

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## Key Words

Aminoglycoside Antibiotics, Sisomicin, Fermentation Products, Ion-Pairing Reagents, Ointments and Creams

## Introduction

Aminoglycoside antibiotics are proven medications for both human and veterinary use with broad-spectrum activity, particularly against gram negative bacteria.<sup>1</sup> Many of these antibiotics are manufactured by bacterial culture (fermentation) processes, and as such can be a mixture of active compounds and not a single chemical compound. One example of an antibiotic manufactured by fermentation is gentamicin, an aminoglycoside antibiotic that is produced by *Micromonospora echinospora* (*Micromonospora purpurea*). Gentamicin sulfate is a mixture of four major compounds: gentamicins C<sub>1</sub>, C<sub>1a</sub>, C<sub>2</sub>, and C<sub>2a</sub>. Additionally, gentamicin C<sub>2b</sub> is commonly present as a minor component. These gentamicin congeners are closely related structurally, as summarized in Figure 1.

In addition to the gentamicin complex, fermentation impurities and degradation products may be present.<sup>2-4</sup> Many of these compounds, such as sisomicin (Figure 2A), also have antibiotic activity. Other compounds, such as garamine, are common degradation products of numerous aminoglycoside antibiotics (Figure 2B).

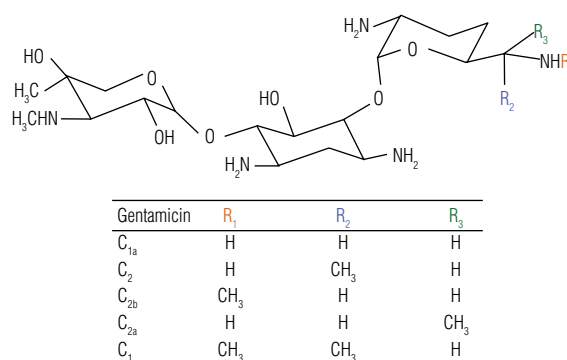


Figure 1. Gentamicin C congener structures

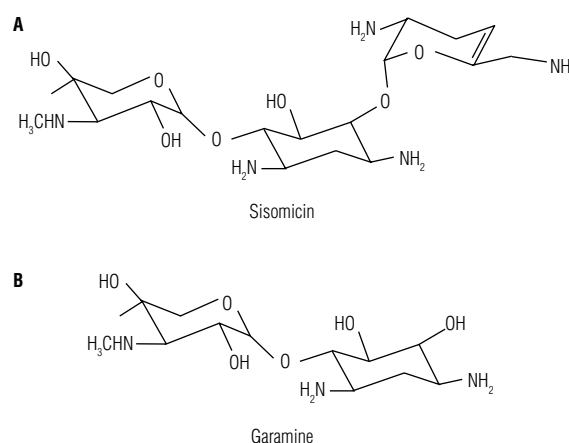


Figure 2. The structures of A) sisomicin and B) garamine: note the similarity of sisomicin to gentamicin C<sub>1a</sub>.

Aminoglycoside antibiotic assays, including those for gentamicin sulfate, are frequently microbial assays.<sup>5</sup> These assays measure activity; however, they cannot quantify impurities or determine content of specific compounds in a product. For this reason, chromatographic techniques are often favored for improved specificity and the ability to differentiate impurities that have the potential for both antibiotic activity and unintended side effects. Sisomicin, a common impurity in gentamicin sulfate, is also a prescribed antibiotic with similar activity but greater renal toxicity.<sup>1</sup>

The potential for such adverse events requires that impurities be determined. Aminoglycoside determination presents several challenges because the structural similarities in the compounds make separation of the individual components within gentamicin sulfate potentially difficult. The gentamicin C congeners differ by methylation at three potential sites, as shown in Figure 1. However, these otherwise hydrophilic compounds can be separated by reversed-phase high-performance liquid chromatography (HPLC) with strong ion-pairing reagents that assist in accentuating the small hydrophobicity differences.

In addition to separation challenges, aminoglycoside antibiotics do not contain chromophores, making UV detection insensitive. To compensate for this lack of a chromophore, derivatization techniques have been developed. The U.S. Pharmacopeia (USP) gentamicin sulfate monograph specifies content determination after gentamicin derivatization with *o*-phthalaldehyde.<sup>6</sup> After sample derivatization, the gentamicins are separated on a C18 reversed-phase column (USP L1) and quantified by UV detection. This method, although effective, is an indirect detection method, which requires additional preparation time and reagents for derivatization.

Other detection techniques are available for compounds that lack UV chromophores. This work investigates gentamicin sulfate sample analysis by reversed-phase HPLC with charged aerosol detection. This detection technique does not rely on the presence of a chromophore and does not require derivatization of samples for detection. Charged aerosol detection is based on nebulization of the column eluent and formation of analyte particles. These particles become charged by reacting with a stream of nitrogen gas that was previously charged after passing over a corona discharge needle. The charged particles then pass through an electrometer and the current is measured. This technique is ideal for nonvolatile analytes that do not have a chromophore and is compatible with many volatile ion-pairing reagents, which are needed to successfully separate the gentamicin congeners.

## Goal

Develop a rugged HPLC with charged aerosol detection method to separate and detect the gentamicin congeners in gentamicin sulfate drug substance and gentamicin sulfate-containing ointments, solutions, and creams

## Equipment

- Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system including:
  - SRD-3600 Integrated Solvent and Degasser Rack, 6 Channels (P/N 5035.9230)
  - HPG-3400RS Binary Rapid Separation Pump with Solvent Selector Valves (P/N 5040.0046)
  - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020)
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- Thermo Scientific Dionex Corona ultra RS Charged Aerosol Detector (P/N 70-9406)

Also used for identification of analytes:

- WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection (P/N 5825.0020)
- Thermo Scientific Q Exactive Benchtop LC-MS/MS mass spectrometer (P/N IQLAAEGAAPFALGMAZR)
- Vial Kit, 0.3 mL Polypropylene with Caps and Septa, 100 Each (P/N 055428)
- Filter Unit, 0.2  $\mu$ m nylon membrane, 1 L capacity (P/N 164-0020)
- Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software:
  - Version 7.1 was used for sample analysis data collection and processing
  - Version 6.8 was used for fraction collection
- Thermo Scientific Xcalibur 2.2 SP1 with Foundation 2.0 SP1 and Q Exactive™ Orbitrap MS instrument control software 2.0 SP1

## Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 M $\Omega$ -cm resistivity or better
- Acetonitrile (P/N A955-4)
- Heptafluorobutyric acid (HFBA) (Acros P/N 172800250)
- Trifluoroacetic acid (TFA), LC/MS grade (P/N A116-50)
- Gentamicin sulfate, USP grade (Sigma-Aldrich® P/N G1914)

## Sample

- Gentamicin sulfate samples were provided as a generous gift.

## Conditions

Column:	Thermo Scientific Acclaim RSLC PA2, 2.2 $\mu$ m Analytical (2.1 $\times$ 100 mm)
Mobile Phase A:	0.025:5:95 HFBA:acetonitrile: DI water
Mobile Phase B:	0.3:5:95 TFA:acetonitrile: DI water
Gradient:	From 0 to 3 min: 1–10% mobile phase B (99–90% mobile phase A)
	From 3 to 8 min: 10–100% mobile phase B (90–0% mobile phase A)
	From 8 to 11 min: 100 % mobile phase B
	4 min of equilibration at 99% mobile phase A before injection
Flow Rate:	0.45 mL/min
Inj. Volume:	1.0 $\mu$ L
Temperature:	15 $^{\circ}$ C
Detection:	Charged aerosol detector, low filter, 60 Hz data collection rate, nebulizer temperature, 15 $^{\circ}$ C
System Backpressure:	~450 bar
Noise:	~0.07 pA (charged aerosol detection)
Run Time:	11 min (4 min equilibration)

## Preparation of Solutions and Reagents

### Mobile Phase Preparation

**Mobile phase A:** Add 950 mL (950 g) of DI water to a 1 L glass bottle. Add 50 mL of acetonitrile to the DI water and mix the solution. Add 250  $\mu$ L of HFBA to the acetonitrile solution and mix well.

**Mobile phase B:** Add 950 mL (950 g) of DI water to a 1 L glass bottle. Add 50 mL of acetonitrile to the DI water and mix the solution. Add 3.0 mL of TFA to the acetonitrile solution and mix well.

**Alternate mobile phase preparation method:** If baseline distortion is observed, prepare the mobile phases as follows: Add 100 mL acetonitrile to 1900 mL DI water to prepare 2 L of 5:95 acetonitrile:water. Add 250  $\mu$ L HFBA to 1 L of this solution to prepare mobile phase A. Add 3.0 mL TFA to the remaining 1 L of this solution to prepare mobile phase B. Prepare the mobile phases in this way to minimize the differences in the amount of acetonitrile in each mobile phase, which leads to reduced changes in response due to organic mobile phase content at the charged aerosol detector. The preparation of the 5:95 acetonitrile:water solution may be scaled as needed.

### Standards and Sample Solutions

**Standards:** Weigh 16.73 mg of gentamicin sulfate into a preweighed glass vial. Dry the gentamicin sulfate in the glass vial for 1 h at 113  $^{\circ}$ C. Remove the vial and allow it to cool in a desiccator. Once the vial and gentamicin sulfate are cool, reweigh the vial to determine the mass of the dried gentamicin sulfate, which in this case is 14.68 mg. Add 2.94 mL of DI water to prepare a 5.0 mg/mL stock solution of gentamicin sulfate. Systematically elevated values for gentamicin content of samples will be observed if the standard is not dried before use. The amount of this error will depend on the amount of water the gentamicin sulfate standard has absorbed.

**Aqueous saline-based samples:** Dilute samples by a factor of 50 in DI water prior to analysis. Add 20.6 mg of sample to a 1.5 mL vial. Add 1.25 mL of DI water to the sample.

**Mineral oil-based samples (ointments and creams):** Add 102.67 mg of sample into a 1.5 mL microcentrifuge tube. To this sample add 100  $\mu$ L of ethyl acetate to disperse the oils. Vortex repeatedly until a suspension of sample is formed in the ethyl acetate. Add 500  $\mu$ L of DI water to the suspension and vortex again until the sample is well suspended in the water. Centrifuge at 16,000  $\times$  g rpm for 10 min to separate the DI water, ethyl acetate, and mineral oil from each other. Collect sample aliquots from the bottom aqueous layer in the microcentrifuge tube using a conical gel-loading pipette tip. Dilute the extracts from ointments 1:1 with DI water. Dilute ophthalmic ointment samples 1:3 with DI water. For cream samples, increase centrifugation time to 30 min. The phase separation will not be as stable as with ointment samples. After collection of the aliquots from the aqueous extract, dilute these samples 1:3 with DI water before injection.

### Data Processing: Group Integration

Integration of the gentamicin peaks is accomplished by group integration within the Chromeleon™ CDS software. To do this, create a group, such as gentamicin, in the processing method component table. Assign any peak that is part of the gentamicin complex to this group. In this work, the first major peak in the gentamicin complex, gentamicin C<sub>1a</sub>, is selected as the peak to contain the concentration values of all standards. The remaining individual components—C<sub>2</sub>, C<sub>2b</sub>, C<sub>2a</sub>, and C<sub>1</sub>—are given null concentration values. For calibration and sample analysis, the group amount is reported for any peak in the group. To add the group amount column to a table, right click on the table, chose *Insert Column*, and choose the *Group Amount* variable from the *Peak Results* categories. If desired, calculate amount values of individual components of the group based on the relative peak area of the individual component compared to the total peak area of the group. For this work, the amounts of the individual gentamicin congeners are not used for calculations.

### Precautions

After initial installation, allow the column to condition at 50% mobile phase A and 50% mobile phase B for a minimum of 4 h prior to injecting samples and connecting to the charged aerosol detector to achieve minimum background and stable retention times.

To minimize carryover at the autosampler, ensure that a DI water needle wash occurs both before and after an injection. Exercise care when collecting the samples to avoid introducing organic material into the aqueous samples. Due to the mineral oil matrix, a column wash of 80% acetonitrile may be necessary after several samples are injected if any organic phase from the extraction has been introduced into the injected sample.

Retention time reduction will be observed as the column ages and samples are injected. To extend column lifetime, a 20 min column wash of 80% acetonitrile in water is recommended at the end of each sequence to remove any residual sample components and TFA-based mobile phase from the column.

## Results and Discussion

### Separation

Multiple reversed-phase columns were investigated for the separation of gentamicin sulfate, including pentafluorophenyl and embedded polar phases. In each case, TFA was necessary for high-resolution separations. Substitution of all or part of the TFA with formic acid or trichloroacetic acid was not effective in resolving the individual compounds in the gentamicin complex.

Prior publications have shown separation of the gentamicin congeners by many methods, with run times ranging from 20 to 80 min.<sup>2-4,7</sup> A particularly effective method for retaining decomposition products was reported on pentafluorophenyl stationary phases using high concentrations of TFA (between 1 and 2%) for the separation.<sup>7</sup> Although the resolution of these separations is excellent, this concentration of TFA is damaging to the column, which can lead to detector contamination and is not recommended for long-term ruggedness. For this reason, separations that employ lower concentrations of TFA were investigated.

The lowest TFA concentration that resulted in consistent resolution of gentamicin components was 0.3% when using the Acclaim™ RSLC PolarAdvantage II (PA2) column. This column allowed good separation of the primary components of gentamicin as well as sisomicin, which elutes just before gentamicin C<sub>1a</sub>. As with previously published methods, a low concentration of HFBA was included in the gradient to improve retention of small degradation products of gentamicin, such as garamine.

Figure 3 shows the separation of USP-grade gentamicin sulfate on the Acclaim RSLC PA2 column with a HFBA/TFA gradient. The column temperature was maintained at 15 °C, which allowed greater separation between gentamicin C<sub>2b</sub> and gentamicin C<sub>2</sub>. Elution conditions at temperatures ranging from 15 to 35 °C were investigated while maintaining an analysis time of <10 min. The best resolution under these conditions was observed at 15 °C. Elution at higher temperatures led to loss of resolution between gentamicin C<sub>2</sub> and gentamicin C<sub>2b</sub>.

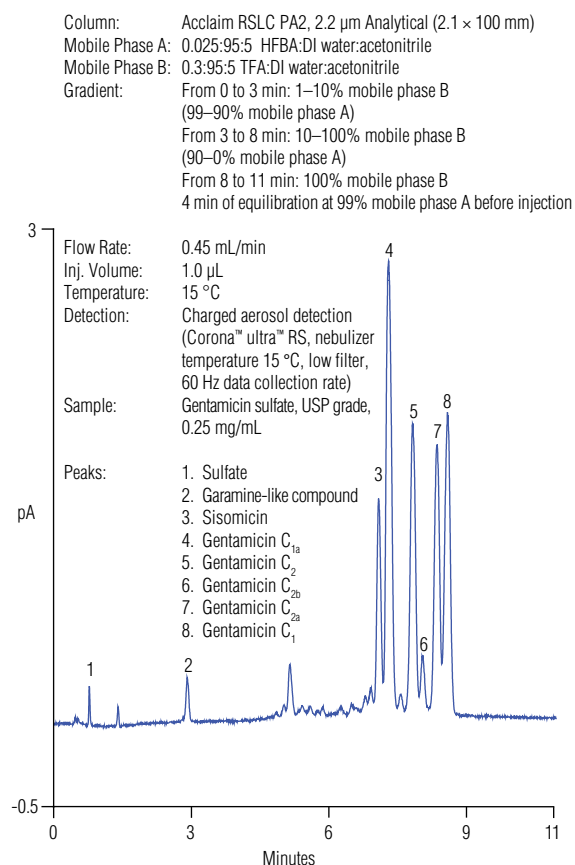


Figure 3. Separation of gentamicin sulfate on an Acclaim RSLC PA2 column

### Peak Assignments

Peak assignments were made by comparing the relative peak areas with the certificate of analysis provided with the product and analysis by mass spectrometry (MS). For MS identification, individual peaks were collected after separation on the Acclaim RSLC PA2 column using a WPS-3000 TBFC. These fractions were then lyophilized to remove the TFA and HFBA. Fractions were reconstituted in 25% acetonitrile with 1% formic acid in DI water (resulting sample concentrations ranged between 0.5 and 4.6 µg/mL) and infused using a syringe pump at a flow rate of 10 µL/min into a Q Exactive™ Benchtop MS/MS mass spectrometer with high-resolution accurate mass (HR/AM) detection.

The MS resolution was set at 70,000, which generates accurate measurement of protonated molecular and fragment ions with deviation typically <2 ppm. The Q Exactive was operated in full scan mode with data-dependent full scan of fragments and dynamic exclusion triggering. Identities of collected fractions were confirmed by matching measured accurate molecular mass and characteristic fragments.<sup>2-3</sup> Gentamicins C<sub>1a</sub>, C<sub>2</sub>, C<sub>2b</sub>, C<sub>2a</sub>, and C<sub>1</sub>, as well as sisomicin, were each confirmed by MS. Garamine was not present at high enough concentration in the suspected fraction to confirm by MS. This peak is assigned as a garamine-like compound, by comparison to literature, with retention similar to an ethylgaramine standard.

### Quantification Assay Linearity and Precision

Linearity of response for the gentamicin complex was investigated between 0.025 and 0.75 mg/mL of gentamicin sulfate (0.017–0.51 mg/mL as free gentamicin base). The quantification of samples was corrected by the certificate of analysis value of gentamicin base. Calibration data were fit with a quadratic model. The coefficient of determination (based on linear conversion of the data) was 0.997. Precision of the method was evaluated by measuring retention time and peak area RSDs for seven replicate injections of a 0.10 mg/mL gentamicin sulfate standard. Table 1 summarizes these precisions.

Retention time precision (as RSD) was excellent, ranging from 0.04 to 0.24. Peak area precision for individual gentamicin congeners was typically <3%, with exceptions for low-concentration components, such as gentamicin C<sub>2b</sub>, which will have inherently greater error. Using the peak height for gentamicin C<sub>1</sub>, the estimated limit of quantification (LOQ), based on a signal-to-noise ratio of 10, is 0.01 mg/mL (10 ng injected on column) of gentamicin sulfate. Using the peak height of the least prevalent congener, gentamicin C<sub>2b</sub>, the estimated LOQ is 0.075 mg/mL (75 ng injected on column) of gentamicin sulfate.

### Sample Analysis

Four drug product samples were evaluated for gentamicin base content. These samples comprised three different sample matrixes: saline solutions, topical creams, and topical ointments. The separations of four samples are shown in Figure 4. For each sample, the gentamicin complex is well resolved from other components in the sample. Matrix components from the samples may interfere with determination of weakly retained impurities; however, in this case, the peak that elutes similarly to ethylgaramine, potentially garamine, is well resolved from sample matrix components.

### Sample Analysis Precision and Accuracy

Table 2 lists the quantified amounts and recoveries for one day of triplicate sample analysis. As shown, the precision of analysis (as RSD) for individual replicates ranged between 0.77 and 4.86 for triplicate injections. The determined amounts in the samples were consistent with the label claim for gentamicin base present in the product. Recoveries for gentamicin sulfate (added to the samples after extraction) were good, ranging from 80 to 113%, indicating consistent response and absence of interfering compounds that elute with the gentamicin complex. Samples were analyzed for three days. Between-day analysis precisions are summarized in Table 3 for three representative sample matrixes: saline drops, ointments, and creams. Results for the assay are within 10% between analysis days, showing good precision. Additionally, results are within 90 to 135% of the label claim, which is the content range specified by the respective USP monographs,<sup>8-10</sup> further suggesting accuracy of the method.

Table 1. Precision for sequential injections (n = 7) of a 0.10 mg/mL gentamicin sulfate standard

Analyte	Retention Time (min)	Retention Time Precision (RSD)	Peak Area (pA*min)	Peak Area Precision (RSD)
Garamine-Like Compound	2.92	0.16	0.008	5.11
Sisomicin	7.12	0.06	0.600	1.53
Gentamicin C <sub>1a</sub>	7.33	0.05	0.169	0.64
Gentamicin C <sub>2</sub>	7.86	0.06	0.108	1.13
Gentamicin C <sub>2b</sub>	8.08	0.08	0.011	3.69
Gentamicin C <sub>2a</sub>	8.38	0.05	0.096	1.22
Gentamicin C <sub>1</sub>	8.62	0.40	0.114	0.14
Gentamicin Group	N/A	N/A	0.550	0.72

Column: Acclaim RSLC PA2, 2.2 µm Analytical (2.1 × 100 mm)  
 Mobile Phase A: 0.025:95:5 HFBA:DI water:acetonitrile  
 Mobile Phase B: 0.3:95:5 TFA:DI water:acetonitrile  
 Gradient: From 0 to 3 min: 1–10% mobile phase B (99–90% mobile phase A)  
 From 3 to 8 min: 10–100% mobile phase B (90–0% mobile phase A)  
 From 8 to 11 min: 100% mobile phase B  
 4 min of equilibration at 99% mobile phase A before injection  
 Flow Rate: 0.45 mL/min  
 Inj. Volume: 1.0 µL  
 Temperature: 15 °C  
 Detection: Charged aerosol detection (Corona ultra RS, nebulizer temperature 15 °C, low filter, 60 Hz data collection rate)  
 Samples:  
 A) Gentamicin sulfate cream  
 B) Gentamicin sulfate ophthalmic ointment  
 C) Gentamicin sulfate ointment  
 D) Gentamicin sulfate ophthalmic solution

Peaks:  
 1. Unretained ions from matrix  
 2. Garamine-like compound  
 3. Sisomicin  
 4. Gentamicin C<sub>1a</sub>  
 5. Gentamicin C<sub>2</sub>  
 6. Gentamicin C<sub>2b</sub>  
 7. Gentamicin C<sub>2a</sub>  
 8. Gentamicin C<sub>1</sub>

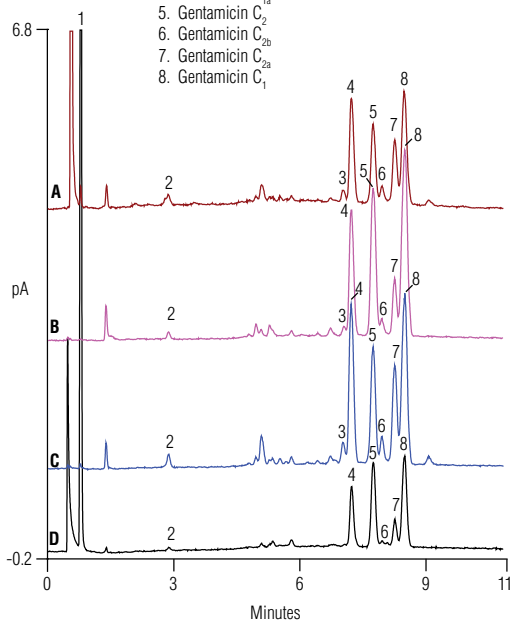


Figure 4. Separation of gentamicin in A) topical cream extract, B) ophthalmic ointment extract, C) topical ointment extract, and D) diluted ophthalmic solution (25% signal offset)

Table 2. Results for one day of replicate sample analysis

Sample	Measured Gentamicin Concentration (mg/mL)	Precision (RSD) n = 3	Label Claim (% Gentamicin Base)	Determined Sample Amount (% Gentamicin Base)	Amount Spiked (mg/mL)	Recovery (%)
Ointment (rep. 1)	0.12	0.81	0.1	0.1	0.080	103
Ointment (rep. 2)	0.14	4.86	0.1	0.1	0.080	111
Ointment (rep. 3)	0.13	2.63	0.1	0.1	0.080	113
Ophthalmic Ointment (rep. 1)	0.13	2.12	0.3	0.3	0.080	111
Ophthalmic Ointment (rep. 2)	0.13	0.77	0.3	0.3	0.080	92
Ophthalmic Ointment (rep. 3)	0.13	1.09	0.3	0.3	0.080	80
Cream (rep. 1)	0.087	1.87	0.1	0.1	0.010	101
Cream (rep. 2)	0.087	1.12	0.1	0.1	0.010	98
Cream (rep. 3)	0.073	1.79	0.1	0.1	0.010	98
Ophthalmic Solution (rep.1)	0.051	1.64	0.3	0.3	0.034	112
Ophthalmic Solution (rep.2)	0.052	3.02	0.3	0.3	0.034	91
Ophthalmic Solution (rep.3)	0.055	1.27	0.3	0.3	0.034	93

Table 3. Results for three days of triplicate analysis: analysis precision (as RSD) is provided for both the determined concentration of gentamicin base in the extracts and for the overall assay after calculating the amounts in the product, then correcting for the dilutions and the mass of product analyzed.

Sample (Day)	Determined Gentamicin Amount (mg/mL)	Intraday Precision (RSD)	Determined Gentamicin Base in Sample (mg/g)	Between-Day Precision (Determined Amount as RSD)	Between-Day Precision (Assay as RSD)
Ophthalmic Solution (Day 1)	0.069	1.9	3.4	11.8	6.7
	0.067		3.3		
	0.069		3.4		
Ophthalmic Solution (Day 2)	0.058	5.4	2.8		
	0.064		3.2		
	0.064		3.1		
Ophthalmic Solution (Day 3)	0.051	4.2	2.9		
	0.052		3.1		
	0.055		3.1		
Ophthalmic Ointment (Day 1)	0.14	12.3	3.1	10.9	6.5
	0.17		3.3		
	0.15		3.3		
Ophthalmic Ointment (Day 2)	0.16	0.4	3.0		
	0.16		3.2		
	0.16		3.2		
Ophthalmic Ointment (Day 3)	0.13	0.9	2.8		
	0.13		2.8		
	0.13		2.8		
Cream (Day 1)*	0.071	2.6	1.1	11.5	9.9
	0.073		1.1		
	0.075		1.2		
Cream (Day 2)	0.073	8.3	1.2		
	0.063		1.3		
	0.063		1.3		
Cream (Day 3)	0.087	9.8	1.0		
	0.087		1.0		
	0.073		1.1		

\*Determined gentamicin base concentrations for cream sample extracts have been corrected for dilution.



## Conclusion

In this work, a direct-detection reversed-phase HPLC chromatographic assay was developed for gentamicin sulfate. This method uses an Acclaim RSLC PA2 column, which allows the reduction of the concentration of TFA compared to published methods, making the method more suitable for long-term use for both the column and the detector. By using charged aerosol detection, derivatization is not necessary. Three sample matrixes containing gentamicin sulfate were analyzed to determine the content of gentamicin. The quantification of gentamicin base had good recoveries and was consistent with the label claim of these products, both indicators of an accurate assay. This method is faster than previously published methods, with excellent separation between the gentamicin congeners and resolution of many impurities that can be present in fermentation-manufactured products.

## Supplier

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 800-325-3010. [www.sigma-aldrich.com](http://www.sigma-aldrich.com)

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