A Gentamicin Sulfate Assay Using HPLC-Charged Aerosol Detection with an Ion-Pairing Reagent Gradient

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Abstract

Purpose: Gentamicin sulfate is a mixture of four major compounds: gentamicins C_1, C_{1a}, C_2, and C_{2a}. In addition, gentamicin C_{2b} is commonly present as a minor component. These gentamicins, as well as fermentation impurities and degradation products, are structurally similar and do not possess chromophores, making chromatographic separation and subsequent detection challenging. Alternate detection techniques are available for compounds that are not chromophoric. In this work, pharmaceutical products containing gentamicin sulfate are analyzed by reversed-phase HPLC with charged aerosol detection. This detection technique does not rely on the presence of a chromophore, does not require sample derivatization for detection, is compatible with volatile ion-pairing reagents, and readily detects nonvolatile analytes such as gentamicin.

Method: This method uses an aqueous extraction followed by sample separation on a polar-embedded stationary phase for determination of gentamicin compounds in drug products. The separation uses an ion-pairing reagent gradient of heptafluorobutyric and trifluoroacetic acids in 5% acetonitrile in water. By maintaining a constant organic content of the mobile phase and adjusting the ion-pairing reagent, the charged aerosol detector response is consistent throughout the gradient, and baseline drift due to the gradient are minimized. The individual gentamicin compounds as well as the impurity sisomicin are eluted with good resolution.

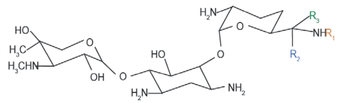
Results: Method precision, as RSD, for individual sample preparations ranged between 0.77–4.86 for triplicate injections. The determined amounts in the samples were consistent with the label claim for gentamicin present in the products. Recoveries for gentamicin sulfate, added to samples after extraction, ranged from 80–113%, indicating consistent response and the absence of interfering matrix compounds eluting with the gentamicin peaks. Between-day analysis precisions for the assay are within 10%, showing good precision.

Conclusions:
A method has been developed to separate the five gentamicin sulfate congeners and sisomicin, a common impurity. The method was used to quantify gentamicin sulfate in ointments, solutions, and creams with a total analysis time of 15 min using a gradient of ion-pairing reagents. This assay provides a method for gentamicin product analysis which is fast, reproducible, and avoids either pre- or postcolumn sample derivatization.

Introduction

Aminoglycoside antibiotics are proven human and veterinary antibiotics that have broad-spectrum activity, particularly against gram negative bacteria.[1] Many of these antibiotics are manufactured by microbial culture (fermentation) processes. These processes can produce a mixture of antibiotic compounds. One example antibiotic manufactured by fermentation is gentamicin, which is produced by Micromonospora echinospora (Micromonospora purpurea). Gentamicin sulfate is a mixture of four major compounds: gentamicins C_1, C_{1a}, C_2, and C_{2a}. In addition, gentamicin C_{2b} is commonly present as a minor component. These gentamicins, as well as fermentation impurities and degradation products, are structurally similar and do not possess chromophores, making chromatographic separation and subsequent detection challenging.

FIGURE 1. Gentamicin C structures.



Gentamicin	R_1	R_2	R_3
C_{1a}	H	H	H
C_2	H	CH_3	H
C_{2b}	CH_3	H	H
C_{2a}	H	H	CH_3
C_1	CH_3	CH_3	H

Methods

Sample preparation was performed by extraction as previously described.[2]

Liquid Chromatography

- Thermo Scientific Dionex UltiMate RSLC system consisting of:
  - SRD-3600 Integrated Solvent and Degasser Rack
  - HPG-3400RS Binary Rapid Separation Pump with Solvent Selector Valves
  - WPS-3000TRS Rapid Separation Well Plate Sampler, Thermostatted
  - TCC-3000RS Thermostatted Column Compartment
- Thermo Scientific Dionex Corona ultra RS Charged Aerosol Detector

Fraction Collection for Analyte Identification

- UltiMate™ 3000 WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection

Conditions

Column:	Thermo Scientific Acclaim RSLC PolarAdvantage II (PA2) 2.2 µm, 2.1 × 100 mm
Mobile Phase A:	0.025:5:95 heptafluorobutyric acid:acetonitrile:DI water
Mobile Phase B:	0.3:5:95 trifluoroacetic acid:acetonitrile:DI water
Gradient:	From 0–3 min 99–90% mobile phase A (1–10% B) From 3–8 min 90–0% mobile phase A (10–100% B) From 8–11 min 100% mobile phase B 4 min of equilibration at 99% mobile phase A before injection
Flow Rate:	0.45 mL/min
Temperature:	15 ºC
Inj. Volume:	1.0 µL
Detection:	Charged aerosol detector, low filter, 60 Hz data collection rate, nebulizer temperature, 15 °C

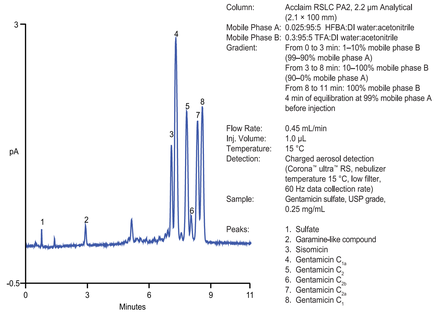
Mass Spectrometry (MS)
Thermo Scientific Q Exactive benchtop LC-MS/MS mass spectrometer with high resolution accurate mass (HR/AM) detection using Thermo Scientific Xcalibur 2.2 SP1 with Foundation 2.0 SP1 and Q Exactive™ Orbitrap™ MS instrument control software 2.0 SP1. The MS resolution was set at 70,000, which generates accurate measurement of protonated molecular and fragment ions with deviation usually less than 2 parts-per-million (ppm).

Chromatographic Data Analysis
Thermo Scientific Dionex Chromeleon 7 Chromatography Data System (CDS) software was used for chromatographic data collection and processing. Chromeleon™ 6.8 CDS software was used for instrument control during fraction collection of gentamicin components.

Results

Separations

FIGURE 2. Separation of gentamicin sulfate on the Acclaim™ RSLC PA2, 2.2 µm Analytical (2.1 × 100 mm).



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)
Sample: Gentamicin sulfate, USP grade, 0.25 mg/mL

Peaks:
1. Sulfate
2. Garamine-like compound
3. Sisomicin
4. Gentamicin C_{1a}
5. Gentamicin C_2
6. Gentamicin C_{2b}
7. Gentamicin C_{2a}
8. Gentamicin C_1

Peak Assignments

Peak assignments were made by comparing the relative peak areas with the certificate of analysis provided with the product combined with the MS analysis. For MS identification, individual peaks were collected after separation on the Acclaim RSLC PA2 column using a Dionex UltiMate 3000 WPS-3000TBFC, lyophilized to remove the trifluoroacetic and heptafluorobutyric acids, and reconstituted in 25% acetonitrile with 1% formic acid in DI water (resulting estimated sample concentrations ranged between 0.5–4.6 µg/mL).

Identity of collected fractions were confirmed by matching measured accurate molecular mass and characteristic fragments.[3–4] Gentamicins C_{1a}, C_2, C_{2b}, C_{2a}, and C_1, 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 retention similar to an ethyl-garamine standard and comparison to published literature using a similar method.[5]

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_{1a}	7.33	0.05	0.169	0.64
Gentamicin C_2	7.86	0.06	0.108	1.13
Gentamicin C_{2b}	8.08	0.08	0.011	3.69
Gentamicin C_{2a}	8.38	0.05	0.096	1.22
Gentamicin C_1	8.62	0.40	0.114	0.14
Gentamicin Group	N/A	N/A	0.550	0.72

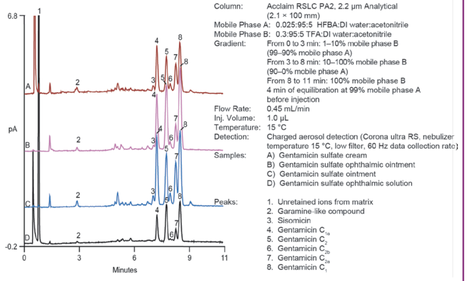
Linearity, Precision, Limits of Quantification

Retention time precision was excellent, indicating a stable method. Additionally, peak area precision was very good, with lower precision for the compounds at lowest abundance (Table 1).

Linearity of response for the gentamicin complex was investigated between 0.025–0.75 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 0.9972.

Using the peak height for gentamicin C1, the estimated limit of quantification (LOQ), based on a signal-to-noise ratio of 10, was 0.01 mg/mL (10 ng injected on column) of gentamicin sulfate. Using the peak height of the least prevalent congener, gentamicin C_{2b}, the estimated LOQ, based on a signal/noise ratio of 10, was 0.075 mg/mL (75 ng injected on column) of gentamicin sulfate.

FIGURE 3. Separation of gentamicin in A) topical cream extract, B) ophthalmic ointment extract, C) topical ointment extract, and D) diluted ophthalmic solution (25% signal offset).



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_{1a}
5. Gentamicin C_2
6. Gentamicin C_{2b}
7. Gentamicin C_{2a}
8. Gentamicin C_1

Sample Analysis Chromatography (Figure 3)

Four samples (two ointments, one cream, and one ophthalmic solution) were assayed for gentamicin content. Salts from the saline matrix of the ophthalmic solution were weakly retained and did not interfere with quantification of gentamicin. Interferences from the cream and ointment bases were not observed.

TABLE 2. Gentamicin determination precision, recovery, and results for analysis of four sample types; triplicate assays were performed each day for three days.

Sample	Measured Gentamicin (mg/mL)	Intraday Precision (RSD) N = 3	Between-Day Assay Precision (RSD)	Label Claim (w/w %)	Determined Amount (w/w %)	Amount Spiked (mg/mL)	Recovery (%)
Ointment	0.12 ±0.001	7.3	8.1	0.1	0.13	0.080	103
	0.14 ±0.007				0.11	0.080	111
	0.13 ±0.003				0.12	0.080	113
Ophthalmic Ointment	0.13 ±0.003	0.9	6.5	0.3	0.28	0.080	111
	0.13 ±0.001				0.28	0.080	92
	0.13 ±0.001				0.28	0.080	80
Cream	0.087 ±0.002	9.8	9.9	0.1	0.10	0.010	101
	0.087 ±0.001				0.10	0.010	98
	0.073 ±0.001				0.11	0.010	98
Ophthalmic Solution	0.051 ±0.001	4.2	6.7	0.3	0.29	0.034	112
	0.052 ±0.002				0.31	0.034	91
	0.055 ±0.001				0.31	0.034	93

Sample Analysis Results (Table 2)

Both intraday (three samples) and between-day (three days) analysis precisions were within 10%. Accuracy was evaluated both by comparison to the label claim and by measured recovery of added gentamicin sulfate standard. The assay results for analyzed samples were within 90–135% of the label claims, as is specified by USP monographs for the products.[6–8] As a further test of accuracy, recoveries of gentamicin spiked into the samples ranged between 80–113%.

Conclusion

- This assay provides a fast and accurate analysis of gentamicin containing drug products without sample derivatization.
- The ion-pairing gradient allows separation of smaller impurities with minimal baseline disturbance from the gradient.
- The use of <0.3% TFA in the mobile phase increases ruggedness, prolonging column life and minimizing detector maintenance.

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