

Determination of gentamicin and related impurities in gentamicin sulfate

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Keywords

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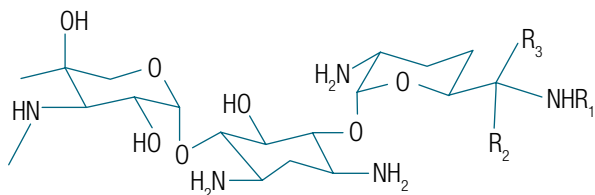
Goal

To validate the gentamicin sulfate United States Pharmacopeia (USP) monograph method for gentamicin composition and impurities using a Thermo Scientific™ Dionex™ IonPac™ AmG-3 μ m C18 column

Introduction

Gentamicin is a broad spectrum water-soluble antibiotic belonging to the group of aminoglycoside antibiotics. It is valuable in the treatment of serious infections caused by gram-negative bacteria. Gentamicin is manufactured by a fermentation process and consists of a mixture of related gentamicin components. The main constituents are gentamicin C1, C1a, C2, C2a, and C2b. (Figure 1). Other related substances, such as sisomicin, garamine, gentamicin B1, and 2-deoxystreptamine are formed in small amounts during the manufacturing process.

All aminoglycosides have a narrow therapeutic range and their use is limited because of potential renal and otovestibular toxicity. The small difference between the effective and toxic concentrations call for monitoring of the given aminoglycoside levels to ensure optimal therapy and to minimize the risk of a toxic side effect, particularly in patients with renal failure.¹ It is essential to characterize a drug substance's purity by identifying and



| | 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 |

Figure 1. Structure of gentamicin

quantifying the impurities, which ensures drug safety and efficacy. Gentamicin components differ in their antimicrobial potencies and toxicity in animals. It has also been reported that there is a wide variation in the major component ratio between different gentamicin products. Thus, this suggests the need to routinely investigate and control the ratio of major components of gentamicin C, as well as related substances in these commercial products.

The number of impurities and components makes the chromatographic analysis challenging. Detection of the different components of gentamicin is problematic because of the lack of a good UV-absorbing chromophore. Ion-pairing reversed-phase liquid chromatography is widely used to separate aminoglycosides by using volatile perfluorinated carboxylic acids, such as trifluoroacetic acid (TFA) and pentafluoropropionic acid, and this separation method has been paired with electrochemical detection. Pulsed amperometric detection (PAD), a powerful detection technique with a broad linear range and very low detection limits, is ideally suited for detecting aminoglycoside antibiotics and their impurities. Electrochemical detection has advantages relative to other techniques in that an oxidation potential can be selected for specific analytes while other compounds remain undetected, and derivatization is not required for detection, which simplifies the analysis. The analysis of gentamicin sulfate in pharmaceutical formulations based on ion-pairing HPLC-PAD is described in the U.S. and European Pharmacopoeias.^{2,3}

The Dionex IonPac AmG-3 μ m C18 columns are specifically designed for ion-pairing reversed-phase separation of various aminoglycoside antibiotics. The stationary phase is prepared through the covalent bonding of C18 ligands onto a polymer-encapsulated silica media, which ensures ultra-stability when exposed to various mobile phase conditions such as low pH, high temperature, different organic solvents, and highly aqueous solutions.⁴ The Dionex IonPac AmG-3 μ m column is packed in a PEEK column body rather than stainless steel. A stainless steel column can release significant levels of metal contamination, particularly when corrosive eluents are used. Metal ions can interfere with electrochemical detection.

Here we apply a 4-potential waveform to detect gentamicin components, rather than the 3-potential waveform reported in the USP Gentamicin Sulfate monograph for the Content of Gentamicins test.² Compared to the 3-potential waveform, the 4-potential waveform minimizes electrode wear and dramatically improves long-term peak area reproducibility.⁵ The European Pharmacopeia (EP) Gentamicin Sulfate monograph describes organic impurity analysis and acceptance criteria in commercial samples. There is also an in-process revision for the USP Gentamicin Sulfate monograph that includes the addition of an organic impurities test that shares most of the conditions of the Content of Gentamicins test.

In this application note, the gentamicin sulfate analysis in the USP monograph was evaluated with a Dionex IonPac AmG-3 μ m C18 column using a 4-potential waveform for electrochemical detection of carbohydrates. Other than the waveform, the method and conditions were exactly as described in the USP Gentamicin Sulfate monograph. Key performance parameters were evaluated including system suitability separation, linearity, limits of detection, and precision. Two samples were analyzed. The percentage of gentamicin C major components results were compared with USP acceptance criteria. Impurity results were compared with EP Gentamicin Sulfate monograph and USP Gentamicin Sulfate in-process revision monograph's acceptance criteria.⁶ We also compared results of the two analyses using the 4- and 3-potential waveforms.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system including*:
 - Dionex ICS-5000+ DP Pump module
 - Dionex ICS-5000+ DC Detector/Chromatography module with ED Electrochemical Detector
 - Dionex AS-AP Autosampler with 250 µL sample syringe (P/N 074306) and 1200 µL buffer line (P/N 074989) and 1.5 mL vial trays (P/N 074936).
- Dionex™ ICS-5000+ ED Electrochemical Detector Cell (P/N 072044)
- ED conventional working electrode, gold, 3 mm (P/N 063723) with 5 mil gasket (P/N 063550)
- Reference electrode pH, Ag/AgCl (P/N 061879)
- Knitted reaction coil, 375 µL, unpotted (P/N 043700)
- Three-way manifold (P/N 48227)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.5

*This method can be run on a single Dionex ICS-5000+ or Thermo Scientific™ Dionex™ ICS-6000 system using a Thermo Scientific™ Dionex™ AXP pump to add the post-column reagent.

Consumables

- Glass autosampler vials 1.5 mL with slit septum (P/N 055427)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Nitrogen ultrahigh purity

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Trifluoroacetic acid (Fisher Scientific P/N PI28901)
- Pentafluoropropanoic acid (Sigma-Aldrich® P/N 245917-50G)
- Sodium hydroxide 50% (w/w) (Fisher Scientific P/N SS254-500)
- Acetonitrile (Fisher Scientific P/N A955-4)
- USP Gentamicin Sulfate Reference standard (Sigma-Aldrich P/N 1289003-200MG)
- USP Sisomicin Sulfate Reference standard (Sigma-Aldrich P/N 1612801-500MG)

Samples

Two gentamicin samples were purchased from Sigma-Aldrich. Sample #1 claims to meet all USP specifications and sample #2 does not make that claim.

Chromatographic conditions

| | |
|------------------------------------|--|
| Columns: | Dionex IonPac AmG-3 μ m C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3 μ m C18 Separation, 4 × 150 mm (P/N 302693) |
| Eluent: | 7 mL/L trifluoroacetic acid, 250 μ L/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile |
| Flow Rate: | 0.8 mL/min* |
| Column Temp.: | 35 °C |
| Injection Volume: | 20 μ L (Full loop) |
| Autosampler Temperature: | 5 °C |
| Reference Electrode: | Ag/AgCl |
| Working Electrode: | Conventional electrode gold, 3 mm diameter with a 5-mil gasket |
| Post-column Reagent | 0.5 M NaOH |
| Post-column Reagent Flow Rate: | 0.3 mL/min delivered by pump 2 |
| Detection: | Pulsed Amperometric Detector (Electrochemical Detector) |
| Detection Compartment Temperature: | 35 °C |
| Detection Waveform: | Gold, Carbohydrates, 4-Potential (Table 1) |
| System Backpressure: | ~2600 psi |
| Run Time: | 65 min |

* The USP monograph describes the column as follows: Type – L1 (i.e. C18) size 250 mm, ID 4.6 mm; 5- μ m packing L1. The diameter of the IonPac AmG-3 μ m C18 column is 4 mm. Therefore, the flow rate was adjusted from 1 mL/min (USP monograph condition) to 0.8 mL/min.

Table 1. Carbohydrates, 4-potential waveform

| Time (s) | Voltage (V) | Integration |
|----------|-------------|-------------|
| 0 | 0.1 | Off |
| 0.20 | 0.1 | On |
| 0.40 | 0.1 | Off |
| 0.41 | -2.0 | Off |
| 0.42 | -2.0 | Off |
| 0.43 | 0.6 | Off |
| 0.44 | -0.1 | Off |
| 0.50 | -0.1 | Off |

Preparation of solutions and reagents

Eluent

To prepare 2 L, add 14 mL of trifluoroacetic acid, 500 μ L of pentafluoropropanoic acid, and approximately 9.4 mL of 50% (w/w) NaOH into a glass 2 L volumetric flask containing approximately 1800 mL of degassed DI water. The pH of the solution should be around 2.6; if not, adjust the amount of 50% (w/w) NaOH to achieve 2.6. Add 30 mL of acetonitrile and bring the volume to 2 L with degassed DI water. Immediately transfer this solution to a glass eluent bottle and blanket it with nitrogen at 5 to 8 psi.

Post-column reagent (0.5 M NaOH)

To prepare 1 L of post-column reagent, weigh 40.0 g of 50% (w/w) NaOH into a plastic 1 L volumetric flask containing approximately 800 mL of degassed DI water. Briefly stir this solution (15–30 s) and then bring to volume. Immediately transfer this solution to the plastic eluent bottle on the HPAE-PAD system and blanket it with nitrogen at 5 to 8 psi. Gently swirl the bottle to complete mixing. Always maintain the eluents under 5 to 8 psi of nitrogen to reduce diffusion of atmospheric carbon dioxide. Prepare new NaOH eluent if left unblanketed for more than 30 min.

Stock standard solutions

Gentamicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade gentamicin sulfate in 25 mL of eluent.

Sisomicin sulfate stock, 1 mg/mL

Dissolve 25 mg of USP grade sisomicin sulfate in 25 mL of eluent.

Working standard solutions

Gentamicin sulfate standard, 0.2 mg/mL

Dilute 5 mL of gentamicin sulfate stock to 25 mL with eluent.

Sisomicin standard, 10 µg/mL

Dilute 1 mL of sisomicin sulfate standard stock to 100 mL with eluent.

System suitability solution, (100 µg/mL USP Gentamicin Sulfate RS and 20 µg/mL of USP Sisomicin Sulfate RS in eluent)

To 5 mL of gentamicin sulfate stock standard, add 1 mL of sisomicin sulfate stock standard, and dilute to 50 mL with eluent.

Sample preparation

Sample solution (a), 1 mg/mL

Dissolve 25 mg of sample in 25 mL of eluent. Use this sample preparation for impurity analysis.

Sample solution (b), 0.2 mg/mL

Dilute 5 mL of sample solution (a) to 25 mL with eluent. Use this sample preparation for the Content of Gentamicins analysis.

Notes: Store all standards and samples in a refrigerator after preparation.

System preparation and setup

A Dionex ICS 5000+ dual system has two pumps. Use the first pump to deliver eluent and the second pump to deliver post-column reagent. Connect extra tubing to the second pump outlet to achieve ~2000 psi pressure for lowering baseline noise.

The post-column addition of NaOH solution will require installation of a knitted reaction coil after the column but before the detector. Install a PEEK mixing tee (P/N 048227) after the column and use the second pump of the DP to deliver the post-column solution to the tee. Direct the third port on the tee to the reaction coil, followed by the electrochemical detector cell.

Rinse the cell body, working electrode, and gasket thoroughly with DI water and dry with a lab wipe. Caution: Do not touch the working electrode gold surface with any paper products as this can contaminate the working electrode. Assemble the cell following the Dionex ICS 5000+ operator manual⁷ and Dionex ED User's Compendium for Electrochemical Detection⁸ by first installing the working electrode gasket flat against cell body. Avoid any wrinkles in the gasket, as this will cause a poor fit and subsequent leaks and poor detection. Install the conventional working electrode with the metal face down over the gasket. Install the yoke block by squeezing the tabs and sliding it on the cell body. Align the yoke block parallel to the cell body and rotate the yoke block knob clockwise until you hear three "clicks". Install the cell into the ED module and connect the yellow cable to the yellow port.

To calibrate the pH-Ag/AgCl reference electrode, install the reference electrode blue cable into the black port. Immerse the reference electrode in pH 7 buffer to at least mid-level of the electrode. Select the "pH Calibration" button on the ED Panel and follow the instructions to calibrate the electrode including using pH 10 buffer. After calibration is complete, rinse the buffer solution off the electrode with DI water, and gently, but firmly, screw in or rotate the reference electrode clockwise into the reference electrode port of the electrochemical cell until the reference electrode is finger-tight. For best results, replace the reference electrode after six months of use.

While running the ED cell, bubbles may be trapped in the cell. Air bubbles in the cell can cause spikes in the baseline. To prevent air from becoming trapped in the cell, increase the backpressure on the cell by connecting backpressure tubing to the cell outlet. The backpressure limit for the ED cell is 690 kPa (100 psi). Do not exceed this limit. Six feet of black (0.01" i.d.) PEEK tubing at the cell outlet can generate 30–40 psi backpressure, which can prevent bubble formation.

Condition the column using the eluent at 0.8 mL/min for 20 min before connecting the column to detector.

After selecting the waveform, confirm flow is passing through the cell and turn the cell voltage to the ON position.

A layer of contamination may occasionally build up on the gold working electrode of the amperometry cell. When this occurs, the electrode must be polished to restore performance. Indications that the working electrode needs to be polished are visible electrode discoloration or a decrease in peak area response. The procedure for polishing the working electrode can be found in the product manual.⁹

When the system is idle for short periods (1–2 weeks), the pump should be left at a reduced flow rate of 0.05 mL/min to achieve rapid startup. When the system must be shut down for a period of several weeks, the pump and electrochemical cell may be simply turned off. For shutdown periods exceeding several weeks, all plumbing lines should be resealed, and the reference electrode should be removed from the electrochemical cell and stored in saturated KCl.

Results and discussion

System suitability

In the USP monograph for gentamicin sulfate, the system suitability requirements specify resolution between gentamicin C2 and gentamicin C2b as >1.5. The EP gentamicin sulfate monograph includes two additional requirements: Signal-to-noise ratio (S/N) > 20 for 10 µg/mL sisomicin and resolution > 1.2 between sisomicin and Gentamicin C1a.

The system suitability was evaluated using the chromatograms of a system suitability standard and 10 µg/mL sisomicin sulfate. Figure 2 shows the first chromatogram using a Dionex IonPac AmG-3µm C18 column set. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated. Figure 3 shows the chromatogram of sisomicin sulfate. Sisomicin is sensitively detected.

Column: Dionex IonPac AmG-3µm C18 Guard,
4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation,
4 × 150 mm (P/N 302693)
Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)

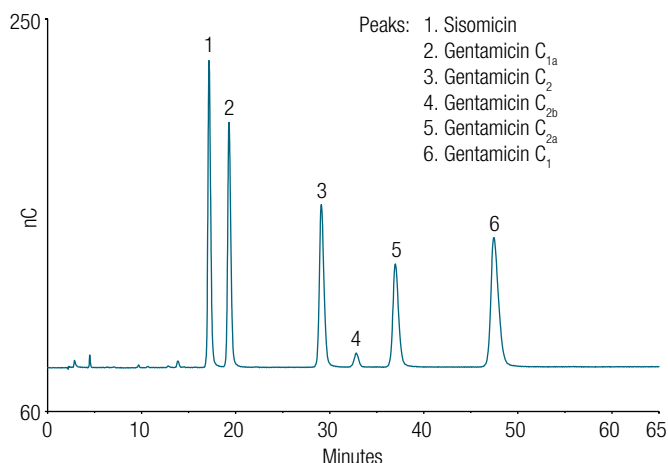


Figure 2. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG C18 column

Column: Dionex IonPac AmG-3µm C18 Guard,
4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation,
4 × 150 mm (P/N 302693)
Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)
Peak: 1. Sisomicin

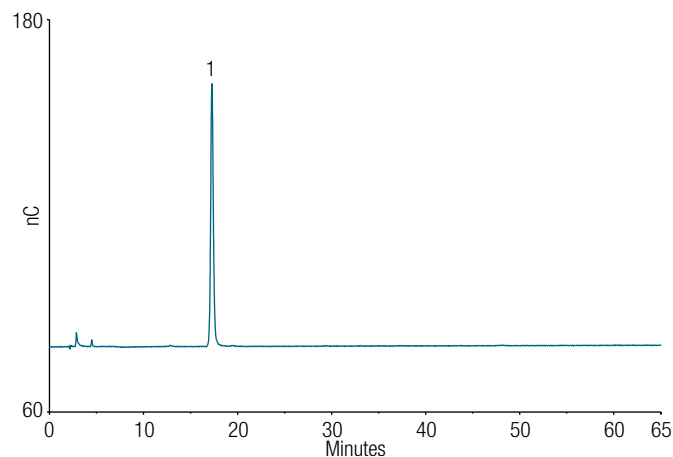


Figure 3. Sisomicin Sulfate USP standard (10 µg/mL)

The system suitability requirements are met for all parameters (Table 2). Peak resolution between C2 and C2b is 4.2, exceeding the USP and EP requirement of 1.5. Peak resolution between sisomicin and C1a is 3.75, exceeding the EP requirement of 1.2. The S/N of 10 µg/mL sisomicin sulfate is 233, easily exceeding the EP requirement of 20.

Table 2. System suitability using the 4-potential carbohydrate waveform

| Test | EP Criteria | Measured |
|--------------------------------------|-------------|----------|
| Resolution between Sisomicin and C1a | >1.2 | 3.75 |
| Resolution between C2 and C2b | >1.5* | 4.20 |
| S/N (Sisomicin 10 µg/mL) | >20 | 233 |

*Also the USP criterion

Linearity

The linearity of gentamicin electrochemical response was investigated in the concentration range of 10 to 200 µg/mL (10, 25, 50, 100, 200 µg/mL). For all gentamicin derivatives, the coefficients of determination were better than 0.997. Figure 4 shows the calibration curve using C1 peak area; the coefficient of determination is 0.9991. This reveals that a sample concentration of 200 µg/mL is within the response linear range and can be used for analysis.

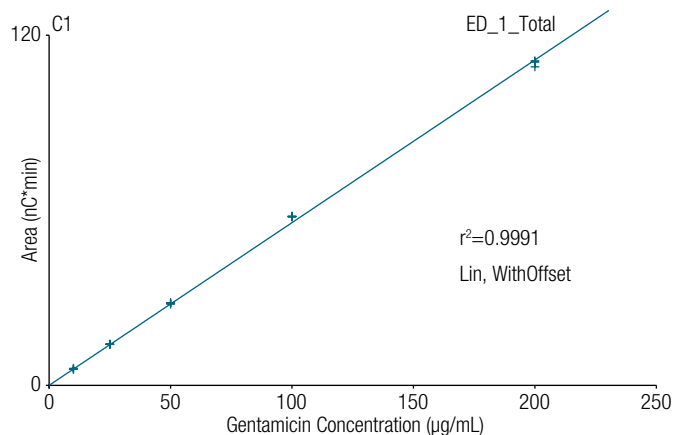


Figure 4. Calibration of gentamicin (C1 peak area)

Method limits of detection and quantification

The USP method for validation specifies a S/N of 3 for the determination of the limit of detection and a S/N of 10 for the determination of the limit of quantitation (LOQ).¹⁰

To determine the limit of detection (LOD) and limit of quantification (LOQ), the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1 min segment of the baseline where no peaks elute but close to the peak of interest. The LOD and LOQ were then calculated from the average peak height of three injections of sisomicin sulfate (0.2 µg/mL).

Table 3 summarizes the LOD and LOQ of sisomicin in sample solution and in gentamicin sulfate powder.

Table 3. LOD and LOQ

| Analyte | LOD (µg/mL) in Sample Solution | LOQ (µg/mL) in Sample Solution | LOD in Gentamicin Sulfate Powder (µg/g) | LOQ in Gentamicin Sulfate Powder (µg/g) |
|-----------|--------------------------------|--------------------------------|---|---|
| Sisomicin | 0.173 | 0.577 | 173 | 577 |

Method precision

Method precision performance was evaluated with five replicate injections of gentamicin sample #2 (0.2 mg/mL).

Figure 5 shows an overlay of the chromatograms from the precision analysis.

Column: Dionex IonPac AmG-3 μ m C18 Guard,
4 \times 30 mm (P/N 302694)
Dionex IonPac AmG-3 μ m C18 Separation,
4 \times 150 mm (P/N 302693)
Eluent: 7 mL/L trifluoroacetic acid, 250 μ L/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
Inj. Volume: 20 μ L
Column Temp.: 35 $^{\circ}$ C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)

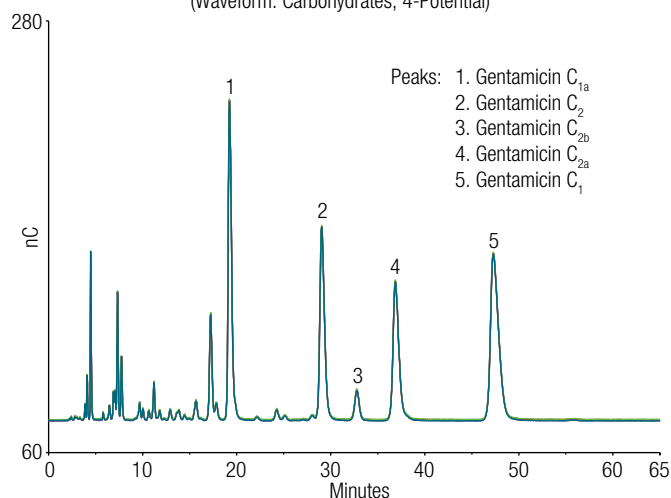


Figure 5. Overlay of five injections of sample #2 (0.2 mg/mL)

As shown in Table 4, the relative standard deviation (RSD) for 5 injections of sample #2 ranged between 0.1 and 0.5%.

Sample analysis

Content of gentamicins analysis

Standard and sample solution (b) were used for content of gentamicins analysis. Figure 6 shows the separation of a USP gentamicin standard. The five gentamicin constituents were well separated. Figure 7 shows the separation of gentamicin sample #1 (0.2 mg/mL); a few impurities were detected and they were separated from the five gentamicin constituents. Figure 8 shows the separation of gentamicin sample #2 (0.2 mg/mL); more than 20 impurities were observed and they were separated from the five gentamicin constituents.

Table 4. Peak area precision of five injections of sample #2, 0.2 mg/mL

| Injection | C1a | C2 | C2b | C2a | C1 |
|-----------|-------|-------|-------|-------|-------|
| 1 | 69.1 | 57.8 | 8.96 | 53.2 | 86.5 |
| 2 | 69.3 | 58.2 | 9.07 | 53.4 | 86.2 |
| 3 | 69.4 | 58.1 | 9.04 | 53.5 | 86.5 |
| 4 | 69.3 | 58.3 | 9.02 | 53.0 | 86.4 |
| 5 | 69.3 | 58.3 | 9.01 | 53.5 | 86.2 |
| RSD | 0.17% | 0.34% | 0.45% | 0.36% | 0.15% |

The relative percentage of each gentamicin constituent in the USP reference standard and the two samples was calculated using the peak areas obtained from the chromatograms shown in Figures 6, 7, and 8. The calculation method is shown below:

$$\text{Result} = (rU/rT) \times 100$$

rU = Peak area response corresponding to the particular gentamicin from the sample solution

rT = Sum of all peak area response of gentamicin C_{1a}, gentamicin C₂, gentamicin C_{2a}, gentamicin C_{2b}, and gentamicin C₁ from the sample solution.

Column: Dionex IonPac AmG-3 μ m C18 Guard,
4 \times 30 mm (P/N 302694)
Dionex IonPac AmG-3 μ m C18 Separation,
4 \times 150 mm (P/N 302693)

Eluent: 7 mL/L trifluoroacetic acid, 250 μ L/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile

Inj. Volume: 20 μ L
Column Temp.: 35 $^{\circ}$ C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)

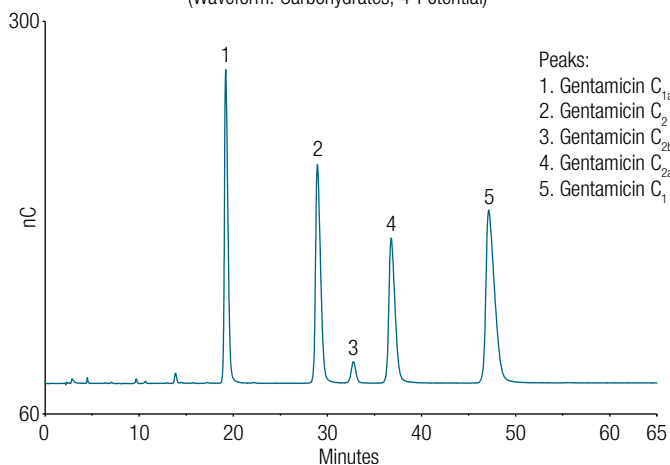


Figure 6. Separation of a gentamicin sulfate USP reference standard (0.2 mg/mL) using a Dionex IonPac AmG C18 column

Column: Dionex IonPac AmG-3 μ m C18 Guard,
4 \times 30 mm (P/N 302694)
Dionex IonPac AmG-3 μ m C18 Separation,
4 \times 150 mm (P/N 302693)

Eluent: 7 mL/L trifluoroacetic acid, 250 μ L/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile

Inj. Volume: 20 μ L
Column Temp.: 35 $^{\circ}$ C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)

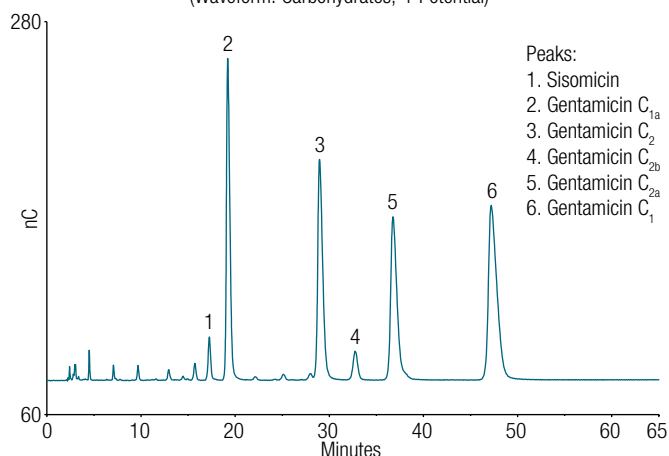


Figure 7. Separation of gentamicin sample #1 (0.2 mg/mL) using a Dionex IonPac AmG C18 column

Column: Dionex IonPac AmG-3 μ m C18 Guard,
4 \times 30 mm (P/N 302694)
Dionex IonPac AmG-3 μ m C18 Separation,
4 \times 150 mm (P/N 302693)

Eluent: 7 mL/L trifluoroacetic acid, 250 μ L/L pentafluoropropanoic acid,
adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile

Inj. Volume: 20 μ L
Column Temp.: 35 $^{\circ}$ C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector
(Waveform: Carbohydrates, 4-Potential)

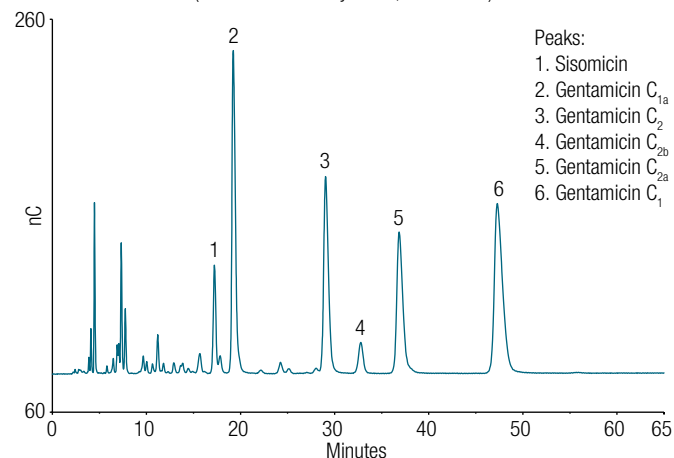


Figure 8. Separation of gentamicin sample #2 (0.2 mg/mL) using a Dionex IonPac AmG C18 column

As shown in Table 5, both samples met the USP acceptance criteria for the Content of Gentamicins test.

Table 5. Percentage of each gentamicin in gentamicin sulfate

| Test | C1a | C2 | C2b | C2a | C1 | C2+C2a | C2b+C1 |
|-------------------------|-------|------|-----|------|------|--------|--------|
| USP Standard | 23.3 | 23.3 | 2.1 | 19.1 | 32.2 | 42.4 | 34.3 |
| Sample #1 | 22.7 | 22.6 | 2.8 | 21.3 | 30.6 | 43.9 | 33.3 |
| Sample #2 | 25.1 | 21.0 | 3.3 | 19.4 | 31.2 | 40.4 | 34.5 |
| USP Acceptance Criteria | 10–35 | | | | | 25–55 | 25–50 |

Percentage of impurities in gentamicin sulfate samples

Sample solutions (a) were used for impurities analysis. Figures 9 and 10 show the chromatograms of samples #1 and #2, respectively. The five times greater concentration of these samples compared to the samples used for the Content of Gentamicins analysis allows the impurity peaks to be more easily observed.

The EP Gentamicin Sulfate monograph and the USP in-process revision of the Gentamicin Sulfate monographs describe acceptance criteria for impurity levels in commercial samples. For that purpose, all impurities were calculated using the peak areas obtained from the chromatogram of the sample solutions (Figures 9 and 10) and compared to the response of the principle impurity sisomicin obtained from the chromatogram of sisomicin sulfate 10 µg/mL (Figure 3).

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
 Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
 Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
 Inj. Volume: 20 µL
 Column Temp.: 35 °C
 Flow Rate: 0.8 mL/min
 Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
 Detection: Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
 Peak: 1. Sisomicin

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
 Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
 Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
 Inj. Volume: 20 µL
 Column Temp.: 35 °C
 Flow Rate: 0.8 mL/min
 Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
 Detection: Pulsed Amperometric Detector (Waveform: Carbohydrates, 4-Potential)
 Peak: 1. Sisomicin

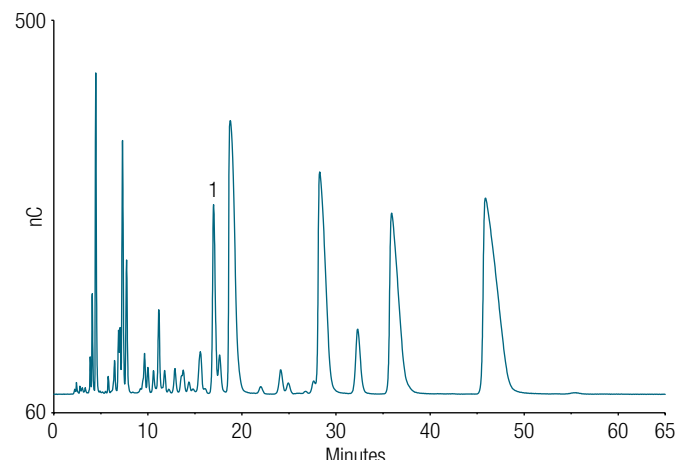
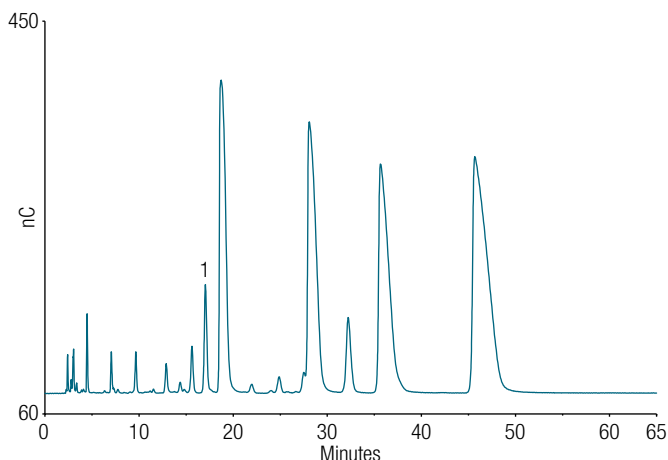


Figure 9. Separation of gentamicin sample #1 (1 mg/mL) using a Dionex IonPac AmG C18 column

Figure 10. Separation of gentamicin sample #2 (1 mg/mL) using a Dionex IonPac AmG C18 column

$$\text{Result} = (rU/rs) \times (Cs/Cu) \times 100$$

rU = Peak response of each individually impurity from the 1 mg/mL sample solution

rs = Peak response of sisomicin from the 10 µg/mL standard solution

Cs = Concentration of USP Sisomicin Sulfate RS in the standard solution (mg/mL)

Cu = Concentration of Gentamicin Sulfate in the sample solution (mg/mL)

Table 6 shows the percentage of sisomicin and total impurities of samples #1 and #2 and compared with the USP acceptance criteria. Sample #1 met all USP impurity acceptance criteria as was claimed in its product description. Sample #2 did not pass the USP total impurities criteria.

Waveform comparison

The analysis of the gentamicin was evaluated using the 3-potential carbohydrate waveform that is in the USP and EP Gentamicin Sulfate monographs (Table 7). Figure 11 shows the separation of a system suitability standard using the 3-potential waveform. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated.

Table 6. Percentage of impurity in gentamicin sulfate

| | Sisomicin | Any Other Individual Impurity | Total Impurities |
|--|-----------|-------------------------------|------------------|
| Sample #1 | 1.31 | <1.31 | 4.1 |
| Sample #2 | 2.64 | <2.64 | 14.1 |
| EP monograph/USP in process revision Acceptance Criteria | 3.0 | 3.0 | 10 |

Table 7. Three-potential waveform (USP monograph method)

| Time (s) | Voltage (V) | Integration |
|----------|-------------|-------------|
| 0 | 0.05 | Off |
| 0.1 | 0.05 | On |
| 0.4 | 0.05 | Off |
| 0.41 | 0.75 | Off |
| 0.55 | 0.75 | Off |
| 0.56 | -0.15 | Off |
| 1.00 | -0.15 | Off |

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
 Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
 Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile
 Inj. Volume: 20 µL
 Column Temp.: 35 °C
 Flow Rate: 0.8 mL/min
 Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
 Detection: Pulsed Amperometric Detector (Waveform: 3-Potential)

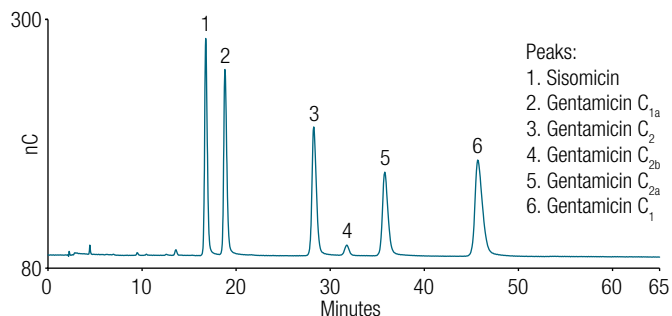


Figure 11. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG C18 column with the 3-potential waveform

Figure 12 shows 10 µg/mL sisomicin with the 3-potential waveform. The system suitability requirements are met for all parameters (Table 8). Figure 13 shows the chromatogram of sample #1 (0.2 mg/mL) using the 3-potential waveform. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin in the sample were well separated and the results using this waveform were equivalent with the results using the 4-potential waveform.

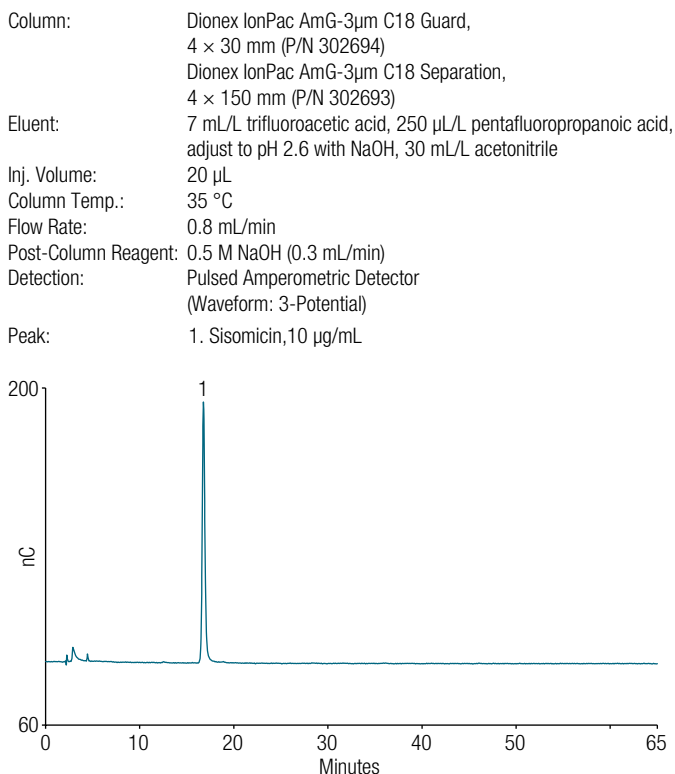


Figure 12. Sisomicin sulfate USP Reference standard (10 µg/mL) using a Dionex IonPac AmG C18 column with the 3-potential waveform

Table 8. System suitability using the 3-potential waveform (USP monograph) waveform

| Test | EP Criteria | Measured |
|--------------------------------------|-------------|----------|
| Resolution between Sisomicin and C1a | >1.2 | 3.72 |
| Resolution between C2 and C2b | >1.5* | 4.05 |
| S/N (Sisomicin 10 µg/mL) | >20 | 304 |

*Also the USP criterion

Column: Dionex IonPac AmG-3µm C18 Guard, 4 × 30 mm (P/N 302694)
Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)

Eluent: 7 mL/L trifluoroacetic acid, 250 µL/L pentafluoropropanoic acid, adjust to pH 2.6 with NaOH, 30 mL/L acetonitrile

Inj. Volume: 20 µL
Column Temp.: 35 °C
Flow Rate: 0.8 mL/min
Post-Column Reagent: 0.5 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector (Waveform: 3-Potential)

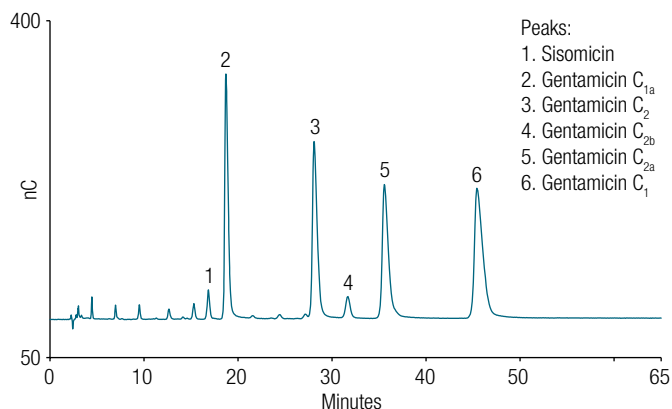


Figure 13. Separation of gentamicin sample #1 (0.2 mg/mL) using a IonPac AmG C18 column with the 3-potential waveform

All the gentamicin congeners evaluated had higher responses using the 3-potential waveform than the 4-potential carbohydrate waveform. However, as discussed in Technical Note 21⁵, the 4-potential waveform differs from the 3-potential waveform in that it uses a negative rather than positive potential for electrode cleaning. Therefore, electrode wear is greatly minimized and long-term reproducibility is improved. Overall, in our opinion, the 4-potential waveform is a better choice for this application.

Conclusions

This application note demonstrated that the USP Gentamicin Sulfate monograph Content of Gentamicins method and the USP in-process revision Gentamicin Sulfate monograph method for organic impurities method could be successfully executed with a Dionex IonPac AmG-3 μ m C18 column using either the 4-potential carbohydrate waveform or the 3-potential waveform described in the USP and EP monographs. The separation, linearity, reproducibility, and sensitivity were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements. This method is reliable and can be used for the routine monitoring of gentamicin.

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