

# Determination of gentamicin and related impurities in gentamicin sulfate using simple eluents

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## Keywords

Dionex IonPac AmG-3 $\mu$ m C18 column, aminoglycoside, Dionex ICS 5000+ system, PAD, electrochemical detection, drug substance, antibiotic, USP, EP, Dionex ICS-6000 system

## Goal

To simplify and speed up the determination of gentamicin composition and impurities using a Thermo Scientific™ Dionex™ IonPac™ AmG-3 $\mu$ m C18 column

## Introduction

The analysis of gentamicin sulfate in pharmaceutical formulations based on an ion-pairing HPLC-PAD method using a C18 silica-based column is described in United States Pharmacopeia (USP) and The European Pharmacopeia (EP) monographs.<sup>1-3</sup> Application Note 72647<sup>4</sup> has demonstrated that the USP Gentamicin Sulfate monograph Content of Gentamicins method and the USP in-process revision Gentamicin Sulfate monograph method for organic impurities could be successfully executed with a Thermo Scientific™ Dionex™ IonPac™ AmG-3 $\mu$ m C18 column using either the 4- or 3-potential carbohydrate waveform. The separation, linearity, reproducibility, and sensitivity were found to meet or exceed the current USP performance requirements.

The eluent of the USP and EP monograph methods contains trifluoroacetic acid, pentafluoropropionic acid, and acetonitrile. Eluent (mobile phase) pH is adjusted to 2.6 with sodium hydroxide to avoid silica-bonded phase hydrolysis when exposed to lower pH conditions. The Dionex IonPac AmG-3 $\mu$ m C18 columns are specifically designed for ion-pairing reversed-phase analysis of various aminoglycoside antibiotics with superior resistance towards acidic conditions.<sup>5</sup> Therefore, an aqueous TFA solution can be used as the eluent without adjusting its pH to a higher value.

In this application update, the eluent in USP/EP monograph is modified in two ways, with each modification used to make a new method. Method A uses 100 mM TFA as the eluent. Method B is similar to Method A, but it includes 2% acetonitrile to accelerate the analysis. Because sodium hydroxide was not added into the eluents, the pH is lower than in the USP/EP monograph method. Therefore, both methods use 0.76 M NaOH as the post-column agent instead of 0.5 M NaOH used in the USP/EP monograph to achieve a similar pH for detection. The system suitability of each method was evaluated and compared with the monograph performance requirements. Two samples were analyzed. The percentage of gentamicin C major components results were compared with USP acceptance criteria. Impurity results were compared with the acceptance criteria of the EP Gentamicin Sulfate monograph and USP Gentamicin Sulfate in-process revision monograph.

## 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+ system or the Thermo Scientific™ Dionex™ ICS-6000 system using a Thermo Scientific™ Dionex™ AXP pump to add the post-column reagent.

The procedure for system preparation and setup can be found in Thermo Scientific Application Note 72647<sup>4</sup> with support from specific product manuals.<sup>5-8</sup>

### 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)
- Sodium hydroxide (w/w) 50% (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-3µm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3µm C18 Separation, 4 × 150 mm (P/N 302693)
Eluent:	Method A: 100 mM TFA Method B: 100 mM TFA (98%) + acetonitrile (2%)
Flow Rate:	0.8 mL/min*
Column Temperature:	35 °C
Injection Volume:	20 µL (Full loop)
Auto Sampler Temperature:	5 °C
Reference Electrode:	Ag/AgCl
Working Electrode:	Conventional electrode gold, 3 mm diameter with a 5-mil gasket
Post-column Reagent:	0.76 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:	Method A: 65 min Method B: 25 min

\*The USP monograph describes the column as follows: Type – L1 (i.e. C18) size 250 mm, ID 4.6 mm; 5 µm packing L1. The diameter of the Dionex IonPac AmG-3µm 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

#### Method A, 100 mM TFA

To prepare 2 L of eluent, add 15.3 mL of trifluoroacetic acid into a glass 2 L volumetric flask containing approximately 1800 mL of degassed DI water. Immediately transfer this solution to a glass eluent bottle and blanket it with nitrogen at 5 to 8 psi.

#### Method B, 100 mM TFA (98%) + acetonitrile (2%)

Mix 100 mM TFA and acetonitrile at a ratio of 98:2 using IC pump channels A and B. Alternatively, the eluent could be premixed and one channel used (not tested).

### Post-column reagent (0.76 M NaOH)

To prepare 1 L of post-column reagent, add 40.0 mL 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 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.

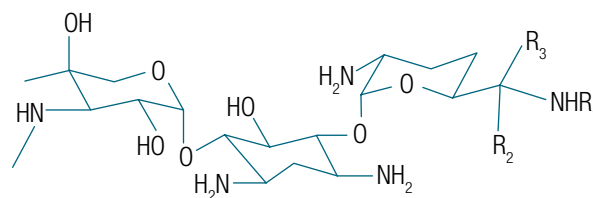
Note: Store all standards and samples in a refrigerator after preparation.

## Results and discussion

## System suitability

In the USP monograph for gentamicin sulfate (Figure 1), 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 the system suitability standard and 10 µg/mL sisomicin sulfate. Figure 2 shows this separation with a Dionex IonPac AmG-3µm C18 column set using the two methods. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated using both methods. TFA acts as the ion-pairing



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
C <sub>1a</sub>	H	H	H
C <sub>2</sub>	H	CH <sub>3</sub>	H
C <sub>2b</sub>	CH <sub>3</sub>	H	H
C <sub>2a</sub>	H	H	CH <sub>3</sub>
C <sub>1</sub>	CH <sub>3</sub>	CH <sub>3</sub>	H

**Figure 1. Structure of gentamicin**

agent and plays an important role in the gentamicin separation. Gentamicin separation is normally completed in 60 min when using 100 mM TFA as the eluent (Figure 2A). To accelerate the separation, 2% acetonitrile was added to the 100 mM TFA eluent, and this resulted in a separation that was less than 25 min (Figure 2B).

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

Eluent: A) 100 mM Trifluoroacetic acid,  
B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)

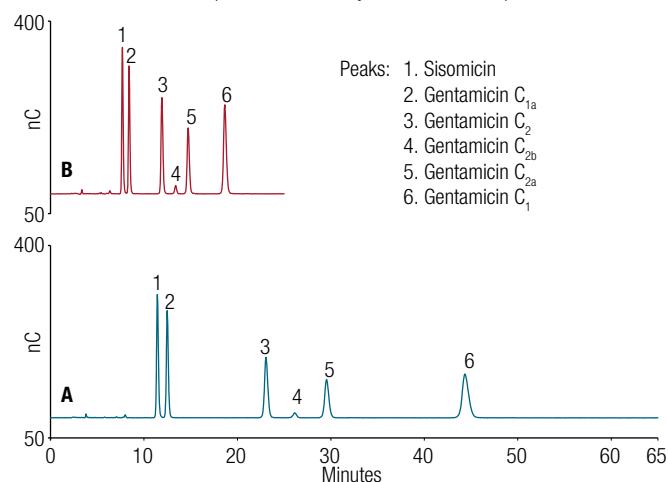
Inj. Volume: 20  $\mu$ L

Column Temp.: 35  $^{\circ}$ C

Flow Rate: 0.8 mL/min

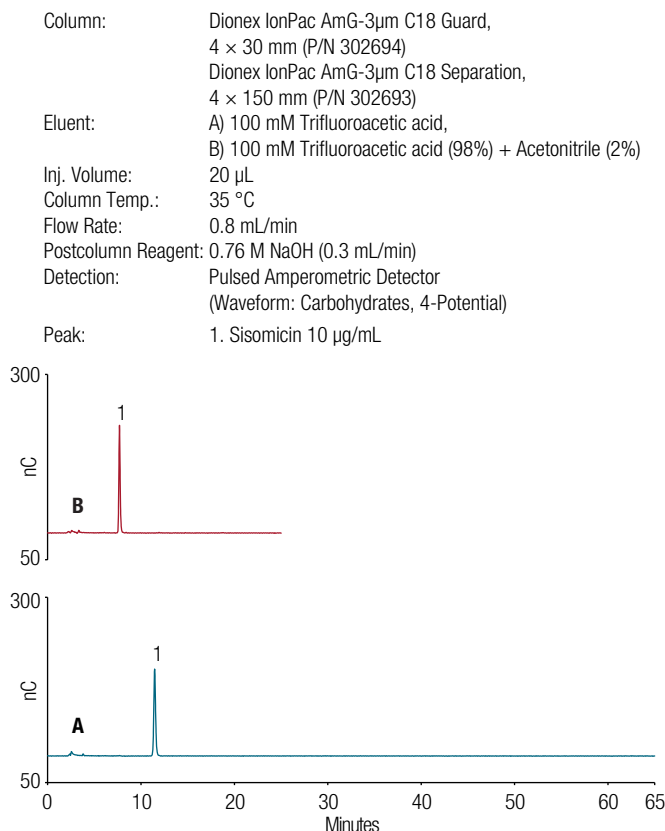
Postcolumn Reagent: 0.76 M NaOH (0.3 mL/min)

Detection: Pulsed Amperometric Detector  
(Waveform: Carbohydrates, 4-Potential)



**Figure 2. Separation of system suitability standard**

Figure 3 shows chromatograms of sisomicin sulfate using both methods. Sisomicin is detected with good sensitivity using either method.



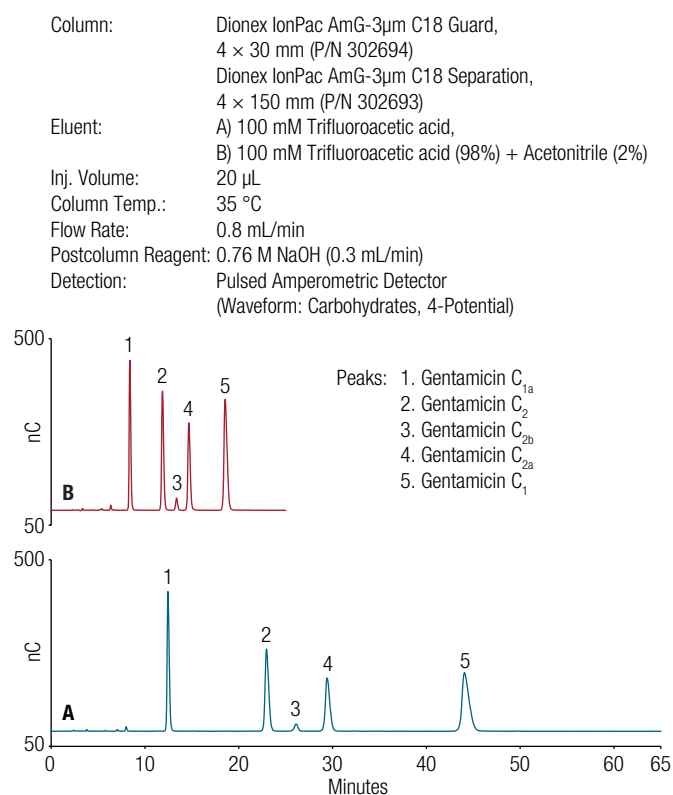
**Figure 3. Sisomicin (10  $\mu$ g/mL)**

Using either method the system suitability requirements are met for all parameters (Table 2). Peak resolution between C2 and C2b is 4.53 and 3.97 for Methods A and B, respectively, exceeding the USP and EP requirement of 1.5. Peak resolution between sisomicin and C1a is 2.85 and 2.63 for Method A and Method B, respectively, exceeding the EP requirement of 1.2. The S/N of 10  $\mu$ g/mL sisomicin sulfate is 248 and 242 for Methods A and B, respectively, easily exceeding the EP requirement of 20.

## Sample analysis

### Content of gentamicins analysis

Standard and sample solution (b) were used for content of gentamicins analysis. Figure 4 shows the separation of a USP gentamicin standard using both methods. The five gentamicin constituents were well separated from each other. Figure 5 shows the separation of gentamicin sample #1 (0.2 mg/mL) using both methods. A few impurities were detected and they were separated from the five gentamicin constituents. Figure 6 shows the separation of gentamicin sample #2 (0.2 mg/mL) using both methods, more than 20 impurities were observed and they were separated from the five gentamicin constituents.



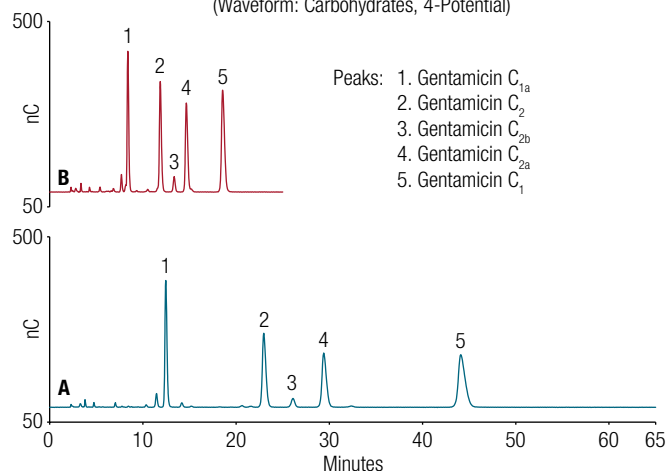
**Figure 4. Separation of a gentamicin USP reference standard (0.2 mg/mL) using Methods A and B**

**Table 2. System suitability**

Test	EP Criteria	Measured (Method A)	Measured (Method B)
Resolution between Sisomicin and C1a	>1.2	2.85	2.63
Resolution between C2 and C2b	>1.5*	4.53	3.97
S/N (Sisomicin 10 $\mu$ g/mL)	>20	248	242

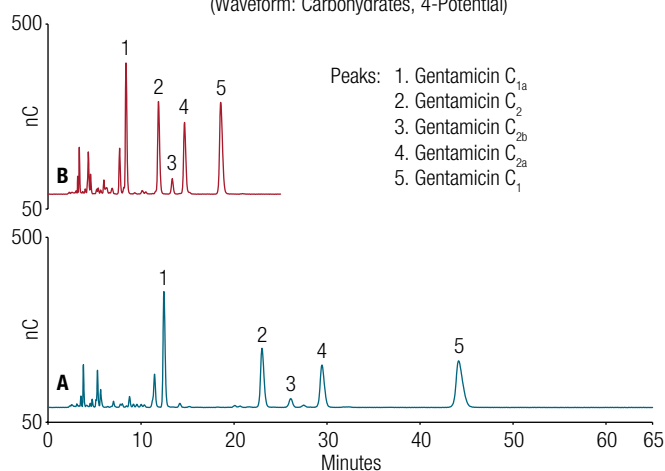
\*Also the USP criterion

Column: Dionex IonPac AmG-3 $\mu$ m C18 Guard,  
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Dionex IonPac AmG-3 $\mu$ m C18 Separation,  
4  $\times$  150 mm (P/N 302693)  
Eluent: A) 100 mM Trifluoroacetic acid,  
B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)  
Inj. Volume: 20  $\mu$ L  
Column Temp.: 35  $^{\circ}$ C  
Flow Rate: 0.8 mL/min  
Postcolumn Reagent: 0.76 M NaOH (0.3 mL/min)  
Detection: Pulsed Amperometric Detector  
(Waveform: Carbohydrates, 4-Potential)



**Figure 5. Separation of gentamicin sample #1 (0.2 mg/mL) using Methods A and B**

Column: Dionex IonPac AmG-3 $\mu$ m C18 Guard,  
4  $\times$  30 mm (P/N 302694)  
Dionex IonPac AmG-3 $\mu$ m C18 Separation,  
4  $\times$  150 mm (P/N 302693)  
Eluent: A) 100 mM Trifluoroacetic acid,  
B) 100 mM Trifluoroacetic acid (98%) + Acetonitrile (2%)  
Inj. Volume: 20  $\mu$ L  
Column Temp.: 35  $^{\circ}$ C  
Flow Rate: 0.8 mL/min  
Postcolumn Reagent: 0.76 M NaOH (0.3 mL/min)  
Detection: Pulsed Amperometric Detector  
(Waveform: Carbohydrates, 4-Potential)



**Figure 6. Separation of gentamicin sample #2 (0.2 mg/mL) using Methods A and B**

The relative percentage of each gentamicin derivative in the USP reference standard and the two samples were calculated using the peak areas obtained from the chromatograms shown in Figures 4, 5, and 6. 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 C1a, gentamicin C2, gentamicin C2a, gentamicin C2b, and gentamicin C1 from the sample solution

As shown in Tables 3 and 4, both samples met the USP acceptance criteria. The results agree with the results from the same samples reported in Application Note 72647.

#### **Percentage of impurities in gentamicin sulfate samples**

Sample solutions (a) were used for impurities analysis. Figures 7 and 8 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.



Table 3. Percentage of each gentamicin in gentamicin sulfate (Method A)

Test	C1a	C2	C2b	C2a	C1	C2+C2a	C2b+C1
USP Standard	23.3	23.2	2.1	18.6	32.7	41.8	34.9
Sample #1	22.6	22.7	2.9	20.7	31.2	43.3	34.1
Sample #2	24.3	21.0	3.4	18.8	32.5	39.8	35.9
USP Acceptance Criteria	10–35					25–55	25–50

Table 4. Percentage of each gentamicin in gentamicin sulfate (Method B)

Test	C1a	C2	C2b	C2a	C1	C2+C2a	C2b+C1
USP Standard	22.8	22.9	2.3	19.5	32.5	42.4	34.8
Sample #1	22.4	22.4	3.0	21.0	31.2	43.4	34.2
Sample #2	23.8	21.5	3.5	19.3	31.9	40.8	35.4
USP Acceptance Criteria	10–35					25–55	25–50

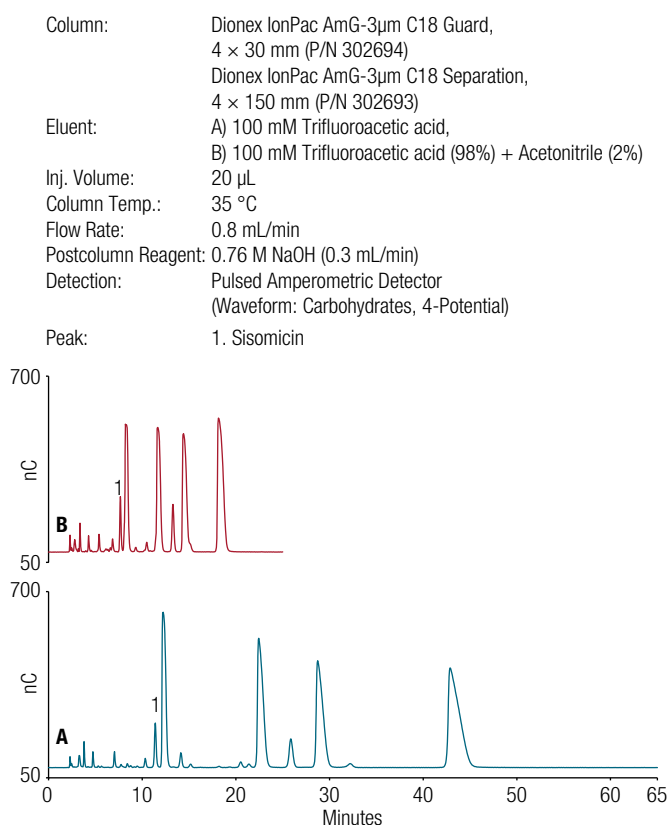


Figure 7. Separation of Gentamicin sample #1 (1 mg/mL) using Methods A and B

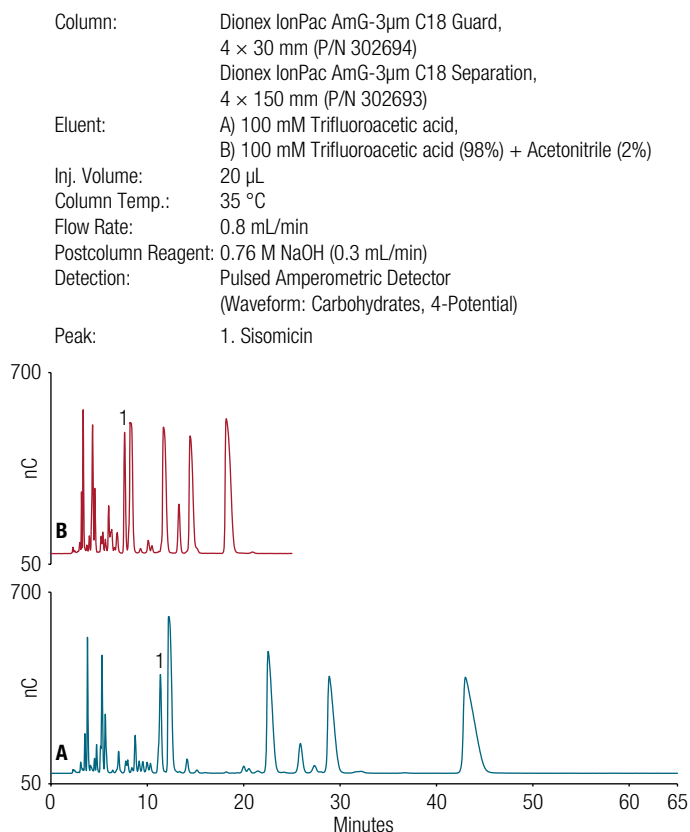


Figure 8. Separation of gentamicin sample #2 (1 mg/mL) using Methods A and B

EP Gentamicin Sulfate monograph and the USP in-process revision of the Gentamicin Sulfate monograph 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 7 and 8) and compared to the response of the principal impurity sisomicin obtained from the chromatogram of 10 µg/mL sisomicin sulfate (Figure 3).

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

$rU$  = Peak response of each individual 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)

Tables 5 and 6 show the percentage of sisomicin and total impurities of samples #1 and #2 using Methods A and B, respectively, and compare this 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 sisomicin and total impurities criteria. The results agree with the results for the same samples reported in Application Note 72647.

## Conclusion

This application update demonstrated that gentamicin sulfate and related impurities can be separated with a Dionex IonPac AmG-3µm C18 column using two modified methods. Method A is a simple eluent method (100 mM TFA). Method B is a fast method that involves the addition of 2% acetonitrile to the eluent to accelerate the separation 2.5 times without compromising resolution and column performance. The separation and sensitivity of both methods were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements.

**Table 5. Percentage of impurities in gentamicin sulfate (Method A)**

	Sisomicin	Any Other Individual Impurity	Total Impurities
Sample #1	1.29	<1.29	4.7
Sample #2	3.05	<2.73	15.5
EP Monograph/ USP In-process Revision Acceptance Criteria	3.0	3.0	10

**Table 6. Percentage of impurities in gentamicin sulfate (Method B)**

	Sisomicin	Any Other Individual Impurity	Total Impurities
Sample #1	1.29	<1.29	4.7
Sample #2	3.05	<2.73	15.5
EP Monograph/ USP In-process Revision Acceptance Criteria	3.0	3.0	10



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