Determination of gentamicin and related impurities in gentamicin sulfate

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ABSTRACT

Purpose: To demonstrate that the USP Gentamicin Sulfate monograph Content of Gentamicins method and the USP in-process revision Gentamicin Sulfate monograph method for organic impurities method can be successfully executed with a Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18 column.

Methods: The analysis of gentamicin sulfate based on an ion-pairing HPLC-pulsed amperometric detection (PAD) method is described in the U.S. and European Pharmacopoeias (USP and EP). The eluent of the USP and EP monograph methods contains trifluoroacetic acid, pentafluoropropionic acid, and acetonitrile, and its pH is adjusted to 2.6 with sodium hydroxide to avoid silica-bonded phase hydrolysis when exposed to lower pH conditions. Dionex IonPac AmG-3µm C18 columns are specifically designed for superior resistance towards acidic conditions. Therefore, an aqueous TFA solution can be used as the eluent without adjusting its pH to a higher value. In addition to using the USP/EP method, we modified the eluent in the USP/EP monograph in two ways, with each modification used to make a new method. Method A is a simple eluent method that uses a 100 mM TFA eluent. Method B is a fast method that includes the addition of 2% acetonitrile to the eluent (final eluent concentration 98 mM TFA, 2% acetonitrile) to make the separation 2.5 times faster without compromising resolution and column performance.

Results: System suitability was evaluated as described in the USP Gentamicin Sulfate monograph using chromatograms of a system suitability standard and 10 µg/mL sisomicin sulfate. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated. Sisomicin is detected with good sensitivity. The separation, linearity, reproducibility, and sensitivity were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements. The separation and sensitivity of both modified methods were found to meet or exceed the current USP/EP Gentamicin Sulfate monograph performance requirements.

INTRODUCTION

Gentamicin is a broad-spectrum, water-soluble antibiotic belonging to the group of aminoglycoside antibiotics. The ratio of major components of gentamicin C and related substances needs to be routinely investigated and controlled in commercial products. The analysis of gentamicin sulfate based on an ion-pairing HPLC-pulsed amperometric detection (PAD) method is described in the U.S. and European Pharmacopoeias (USP and EP). The Dionex IonPac AmG-3µm column contains a silica-based packing material used for reversed-phase chromatography but is packed in a PEEK column body rather than stainless steel. This column was specifically designed for ion-pairing reversed-phase separations of aminoglycoside antibiotics and we used it to execute the methods in the USP and EP Gentamicin Sulfate monographs. Here we apply a four-potential waveform to detect gentamicin components, rather than the three-potential waveform reported in the USP Gentamicin Sulfate monograph for the Content of Gentamicins test. Compared to the three-potential waveform, the four-potential waveform minimizes electrode wear and dramatically improves long-term peak area reproducibility.

MATERIALS AND METHODS

Sample Preparation

Gentamicin Sulfate reference standard and sample were purchased from Sigma-Aldrich.

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.

Test Method(s)

Gentamicin sulfate analysis in the USP monograph was evaluated with a Dionex IonPac AmG-3µm C18 column using a four-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. We also compared results of the two analyses using the four- and three-potential waveforms. The eluent in USP/EP monograph is modified in two ways, each modification used to make a new method. Method A uses 100 mM TFA as the eluent. Method B used 98 mM TFA , 2 % acetonitrile to accelerate the analysis. The two modified methods were also evaluated.

Instrument

A Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC™ dual system was used. Figure 1 shows a flow diagram of the system configured for carbohydrate detection using electrochemical detection.

In PAD, using the four-potential waveform, the conventional working electrode is pulsed through the different potentials at set times, completing two cycles within one second (Figure 2). This waveform is optimized to provide a clean, stable gold layer in preparation for detection of the next eluting peak.

Figure 1. The Dionex ICS-5000⁺ HPIC system flow diagram configured for ED detection.

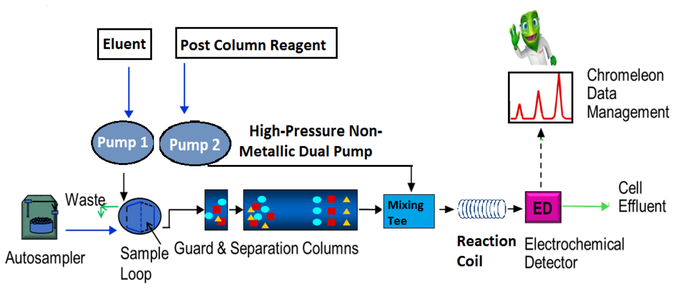
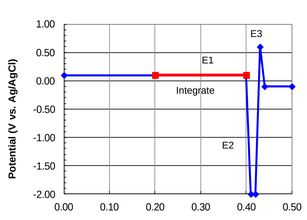


Figure 2. Four-potential carbohydrate waveform.



1. Detection potential (E1)
2. Duration time of E1 (t1)
3. Integration period (Integrate)
4. Reductive cleaning potential (E2)
5. Duration time of E2 (t2)
6. Oxidative cleaning potential (E3
7. Duration time of E3 (t3) (E4)
8. Pre-detection (oxide reduction) potential
9. Duration time of E4 (t4)

Data Analysis

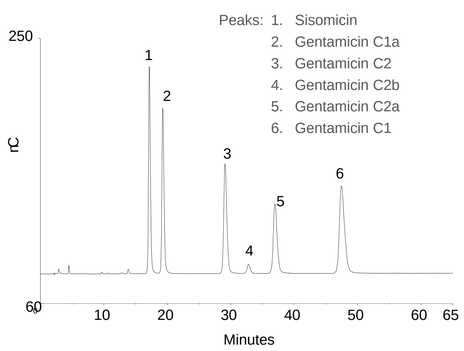
Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.

RESULTS

System suitability

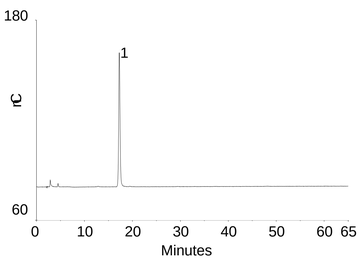
In the USP monograph for gentamicin sulfate, resolution >1.5 between gentamicin C2 and gentamicin C2b is specified in the system suitability requirements. The EP gentamicin sulfate monograph includes two additional requirements: Signal-to-noise ratio >20 for 10 µg/mL sisomicin and resolution >1.2 between sisomicin and gentamicin C1a. The system suitability was evaluated using the chromatograms of a system suitability standard and 10 µg/mL sisomicin sulfate. Figure 3 shows this separation using a Dionex IonPac AmG-3µm C18 column set. The five congeners (C1, C1a, C2, C2a, and C2b) and sisomicin were well separated. Figure 4 shows the chromatogram of sisomicin sulfate. Sisomicin is sensitively detected. The system suitability requirements are met for all parameters (Table 1). 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 signal-to-noise ratio of 10 µg/mL sisomicin sulfate is 233, easily exceeding the EP requirement of 20.

Figure 3. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG-3µm 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 Temperature:	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, four-potential)

Figure 4. Sisomicin USP standard (10 µg/mL)



Peak: 1. Sisomicin 10 µg/mL

Table 1. System suitability using the four-potential waveform.

Test	EP criteria	Measured
Resolution between sisomicin and C1a	>1.2	3.75
Resolution between C2 and C2b	>1.5	4.20
Signal to noise ratio (sisomicin 10 µg/mL)	>20	233

Linearity

The linearity of gentamicin was investigated in the concentration range of 10–200 µg/mL (10, 25, 50, 100, 200 µg/mL). For all gentamicin species, the coefficients of determination were better than 0.997. This reveals that a sample concentration of 200 µg/mL is within the response linear range and can be used for analysis.

Method precision

Method precision performance was evaluated with five replicate injections of gentamicin sample (0.2 mg/mL).The relative standard deviation (RSD) for five injections of sample #2 ranged between 0.1 to 0.5 %.

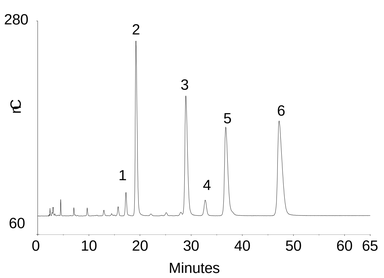
Method LOD and LOQ

The USP method for validation specifies a signal to noise (S/N) ratio of 3 for the determination of the LOD and a signal-to noise (S/N) ratio of 10 for the determination of the LOQ. The LOD and LOQ were then determined by injecting sisomicin sulfate (0.2 µg/mL). LOD in sample solution is 0.173 µg/mL and LOQ in sample solution is 0.577 µg/mL.

Sample analysis

Sample solution (b) was used for content of gentamicins analysis. Figure 5 shows the separation of gentamicin sample (0.2 mg/mL). A few impurities were detected and they were separated from the five gentamicin constituents.

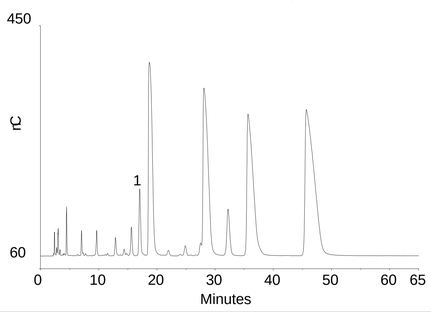
Figure 5. Separation of gentamicin sample (0.2 mg/mL) using a Dionex IonPac AmG-3µm C18 column.



Peaks: 1. Sisomicin
2. Gentamicin C1a
3. Gentamicin C2
4. Gentamicin C2b
5. Gentamicin C2a
6. Gentamicin C1

Sample solutions (a) were used for impurities analysis. Figures 6 shows the chromatograms of samples. 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.

Figure 6. Separation of gentamicin sample (1 mg/mL) using a Dionex IonPac AmG-3µm C18 column.

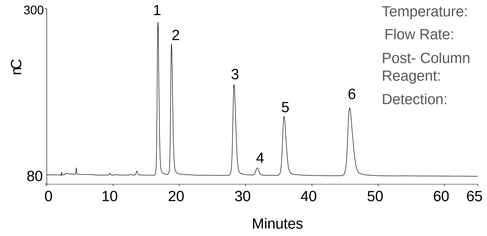


Peaks: 1. Sisomicin 13.1 µg/mL

Waveform comparison

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

Figure 7. Separation of a system suitability standard (gentamicin 100 µg/mL + sisomicin 20 µg/mL) using a Dionex IonPac AmG-3µm C18 column with the three-potential waveform.



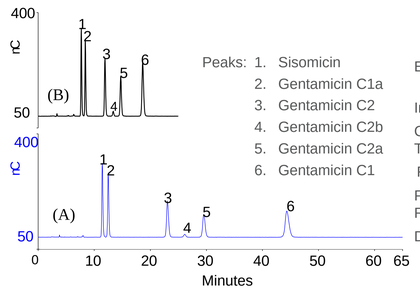
Peaks: 1. Sisomicin
2. Gentamicin C1a
3. Gentamicin C2
4. Gentamicin C2b
5. Gentamicin C2a
6. Gentamicin C1

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 Temperature:	35˚C
Flow Rate:	0.8 mL/min
Post- Column Reagent:	0.5 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: three-potential)

Modified methods

We modified the eluent in USP/EP monograph in two ways, with each modification used to make a new method. Method A is a simple eluent method that uses a 100 mM TFA eluent. Method B is a fast method that involves the addition of 2% acetonitrile to the eluent (Final eluent concentration 98 mM TFA, 2% acetonitrile) to make the separation 2.5 times faster without compromising resolution and column performance. The system suitability was evaluated using the chromatograms of the system suitability standard and 10 µg/mL sisomicin sulfate. Figure 6 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 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 8A). To accelerate the separation, 2% acetonitrile was added to the 100 mM TFA eluent (final eluent concentration 98 mM TFA, 2% acetonitrile), and this resulted in a separation that was less than 25 min (Figure 8B). Using either method the system suitability requirements are met for all parameters (Table 2).

Figure 8. Separation of a system suitability standard using method A and B.



Peaks: 1. Sisomicin
2. Gentamicin C1a
3. Gentamicin C2
4. Gentamicin C2b
5. Gentamicin C2a
6. Gentamicin C1

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:	A) 100 mM Trifluoroacetic acid, B) 98 mM Trifluoroacetic acid, acetonitrile 2%
Inj. Volume:	20 µL
Column Temperature:	35˚C
Flow Rate:	0.8 mL/min
Post- Column Reagent:	0.76 M NaOH (0.3 mL/min)
Detection:	Pulsed Amperometric Detector (Waveform: Carbohydrates, four-potential)

Table 2. System suitability using modified methods.

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
Signal to noise ratio (sisomicin 10 µg/mL)	>20	248	242

CONCLUSIONS

- This work 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-3µm 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/EP Gentamicin Sulfate monograph performance requirements. This method is reliable and can be used for the routine monitoring of gentamicin.
- 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 (final eluent concentration 98 mM TFA, 2% acetonitrile) to make the separation 2.5 times faster without compromising the 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.

REFERENCES

1. Thermo Scientific Technical Note 21: Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector.
2. Thermo Scientific Application Note 72647: Determination of gentamicin and related impurities in gentamicin sulfate.
3. Thermo Scientific Application Update 72648: Determination of gentamicin and related impurities in gentamicin sulfate using simple eluents.

TRADEMARKS/LICENSING

PO72770-EN 0718S