Determination of spectinomycin and related impurities in spectinomycin dihydrochloride

Authors

Jingli Hu and Jeffrey Rohrer

Thermo Fisher Scientific
Sunnyvale, CA

Keywords

Dionex IonPac AmG-3µm C18 column, aminoglycoside, Dionex ICS-5000⁺ HPIC system, PAD, electrochemical detection, drug substance, antibiotic, ion-pair reversed-phase HPLC, EP, Dionex ICS-6000 HPIC system

Goal

To demonstrate that spectinomycin content can be determined and related impurities separated with a Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18 column using a simple eluent method (0.1 M TFA) compared to the eluent in the European Pharmacopeia (EP) monograph

Introduction

Spectinomycin is a broad-spectrum, water-soluble antibiotic belonging to the group of aminoglycoside antibiotics. It is valuable in the treatment of bacterial infections in human and animals. Spectinomycin is isolated from the fermentation broth of *Streptomyces spectabilis* and the isolate could contain biosynthetically related components including (4S)-dihydrospectinomycin, (4R)-dihydrospectinomycin, dihydroxyspectinomycin, and some degradation products. It is important to characterize and quantify the active pharmaceutical ingredient (API) and impurities in the drug substance to ensure its quality and safety.

The number of impurities makes the chromatographic analysis challenging. Detection of spectinomycin and related impurities is problematic because they lack good UV-absorbing chromophores. Ion-pair reversed-phase liquid chromatography is widely used to separate aminoglycosides by
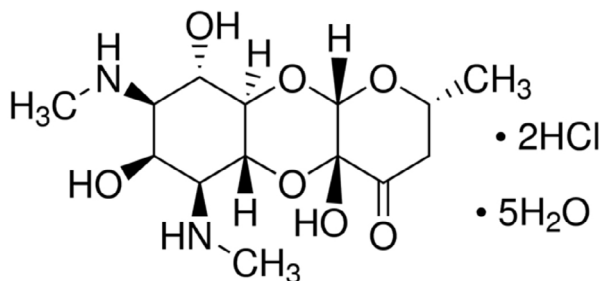
Figure 1. Structure of spectinomycin dihydrochloride pentahydrate

using volatile perfluorinated carboxylic acids, such as trifluoroacetic acid (TFA) and pentafluoropropionic acid (PFPA). This separation method has been combined with electrochemical detection.[1-3] 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 associated 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. Derivatization is typically not required for electrochemical detection, which simplifies the analysis. The analysis of spectinomycin dichloride in pharmaceutical formulations based on ion-pairing HPLC-PAD is described in the European Pharmacopoeia (EP).[4]

The eluent used in the EP monograph method contains oxalic acid, heptafluorobutyric acid, and acetonitrile. Eluent (mobile phase) pH is adjusted to 3.2 with sodium hydroxide to avoid silica-bonded phase hydrolysis when exposed to lower pH conditions. The Dionex IonPac AmG-3µm C18 columns are specifically designed for ion-pair reversed-phase analysis of various aminoglycoside antibiotics. The stationary phase is prepared through the covalent bonding of C18 ligands onto a polymer-encapsulated silica media, which ensures high stability when exposed to varied mobile phase conditions such as low pH, high temperature, different organic solvents, and highly aqueous solutions.[5] Therefore, an aqueous TFA solution can be used as the eluent without adjusting its pH to a higher value. In addition, the Dionex IonPac AmG-3µm column is packed in a PEEK column body rather than in a stainless-steel body. A stainless-steel column, as well as steel used in conventional HPLC systems, can release significant levels of metal, particularly when corrosive eluents are used. These metal ions can then interfere with the electrochemical detection.

In this application note, the eluent in the EP Spectinomycin Dichloride monograph was modified to 0.1 M TFA. In addition, here we apply a 4-potential waveform to detect spectinomycin, rather than the 3-potential waveform reported in the EP monograph. Compared to the 3-potential waveform, the 4-potential waveform minimizes electrode wear and improves long-term peak area reproducibility.[6] Key performance parameters were evaluated with a Dionex IonPac AmG-3µm C18 column including system suitability, separation, repeatability, linearity, and limits of detection. Two samples were analyzed. The percentage of spectinomycin in these samples was determined. Impurities were also determined and compared with EP Spectinomycin Dichloride monograph acceptance criteria.

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 sample tray cooling, 250 µL sample syringe (P/N 074306),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 048227)

  – Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.5

*This method can be run on a single Dionex ICS-5000+ HPIC system or single Thermo Scientific™ Dionex™ ICS-6000 HPIC 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[1] with support from specific product manuals.[7-8]

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)
- Helium, ultrahigh purity grade from Airgas

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 50% (w/w) (Fisher Scientific P/N SS254-500)
- United States Pharmacopeia (USP) Spectinomycin dihydrochloride reference standard (Sigma-Aldrich P/N 1618003)

Samples
Two spectinomycin dihydrochloride pentahydrate samples were purchased from Sigma-Aldrich.

- Sample #1: P/N S4014-5G Lot 122K0561
- Sample #2: P/N 22189-32-8 Lot 75F-06295

Preparation of solutions and reagents
Eluent (0.1 M TFA)
To prepare 2 L of eluent, add 22.8 g of trifluoroacetic acid to a 2 L glass volumetric flask containing approximately 1800 mL DI water and bring the volume to 2 L with DI water. Immediately transfer this solution to a glass eluent bottle, degas the eluent by sparging with helium gas for at least 10 min, and blanket it with helium at 6 to 8 psi.

Note: Weigh trifluoroacetic acid using a balance in a fume hood.

Chromatographic conditions

Columns:	Dionex IonPac AmG-3μm C18 Guard, 4 × 30 mm (P/N 302694) Dionex IonPac AmG-3μm C18 Analytical, 4 × 150 mm (P/N 302693)
Eluent:	0.1 M trifluoroacetic acid (TFA)
Flow Rate:	0.8 mL/min*
Column Temp.:	30 °C
Injection Volume:	20 μL (Full loop)
Autosampler Temp.:	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 with delivered by pump 2
Detection:	Pulsed Amperometric Detection (Electrochemical Detector)
Detection Compartment Temp.:	30 °C
Detection Waveform:	Gold, Carbohydrates, 4-Potential (Table 1)
System Backpressure:	~2500 psi
Run Time:	20 min

* The EP monograph describes the column as follows: Octadecylsilyl Silica Gel Columns, size 250 mm, ID 4.6 mm; 5-μm packing. The diameter of the Dionex IonPac AmG-3μm C18 column is 4 mm. Therefore, the flow rate was adjusted from 1 mL/min (EP monograph condition) to 0.8 mL/min.

Table 1. Carbohydrates, 4-potential waveform

Time (s)	Potential (V)	Integration
0	0.1	Off
0.2	0.1	On
0.4	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.5	-0.1	Off

Post-column reagent (0.76 M NaOH)
To prepare 1 L of post-column reagent, degas 954 g
DI water by sparging helium gas for at least 10 min in a
plastic eluent bottle and add 40 mL 50% (w/w) NaOH
into the eluent bottle. Immediately blanket it with helium
at 6 to 8 psi. Gently swirl the bottle to complete mixing.
Always maintain the eluents under 6 to 8 psi of helium to
reduce diffusion of atmospheric carbon dioxide. Prepare
new NaOH eluent if left un-blanketed for more than
30 min.

Note: It is very important to degas the eluent and post-
column reagent by sparging with helium gas rather than
ultrasonic agitation to avoid a slow decrease in peak area
response over time.

Standard solutions
Standards and samples are prepared as described in the
EP monograph.

Spectinomycin reference solution, 150 µg/mL
Dissolve 3 mg of USP Spectinomycin dihydrochloride
reference standard in 20 mL eluent. Prepare the solution
immediately before use to avoid formation of anomers.
Use this solution for the related impurities system
suitability test.

Spectinomycin reference solution, 80 µg/mL
Dissolve 40 mg of USP Spectinomycin dihydrochloride
reference standard in 50 mL DI water. Allow to stand for
no less than 15 h and not more than 72 h (formation of
anomers). Dilute 5 mL of this solution to 50 mL with the
eluent. Use this solution for the assay system suitability
test.

Sample preparation
Sample solution (a), 150 µg/mL
Dissolve 15 mg of spectinomycin dihydrochloride sample
in 100 mL of eluent. Prepare the solutions immediately
before use to avoid formation of anomers.

Sample solution (b), 1.5 µg/mL
Dilute 1 mL of sample solution (a) to 100 mL with eluent.

Sample solution (c), 80 µg/mL
Dissolve 40 mg of spectinomycin dihydrochloride sample
in 50 mL of DI water. Allow to stand for no less than 15 h
and not more than 72 h so that the equilibrium between
spectinomycin and its anomers is reached. Dilute 5 mL
of this solution to 50 mL with the eluent.

Sample solution (d), 40 µg/mL
Mix 5 mL of sample solution (c) with 5 mL eluent.

Use sample solutions (a) and (b) for related impurities
analysis. Use sample solutions (c) and (d) for the
spectinomycin assay.

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

Results and discussion
System suitability
In the Spectinomycin EP monograph, two system
suitability requirements are specified. These
requirements are that the resolution between impurity
E and spectinomycin is >1.5 for the related substances
reference solution (150 µg/mL) and the maximum relative
standard deviation (RSD) of 3% for the spectinomycin
peak area for six injections of the assay reference
solution (80 µg/mL).

Table 2 shows that the EP requirements for both peak
resolution and repeatability are met. Figure 2 shows
a separation of the system suitability solution using
a Dionex IonPac AmG-3µm C18 column set. The
impurities were tentatively identified using the relative
retention times listed in the EP monograph. Impurity E
and spectinomycin were well separated. Peak resolution
between impurity E and spectinomycin is 3.17, exceeding
the EP requirement of 1.5.

Table 2. System suitability

Test	EP criterion	Measured
Resolution between impurity E and spectinomycin	>1.5	3.17
Repeatability: Peak area RSD (%) of six injections	<3	0.36

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: 0.1 M TFA
Inj. Volume: 20 µL
Column Temp.:   30 °C
Flow Rate: 0.8 mL/min
Post-Column
Reagent: 0.76 M NaOH (0.3 mL/min)
Detection: Pulsed Amperometric Detector(Waveform: Carbohydrates, 4- Potential)
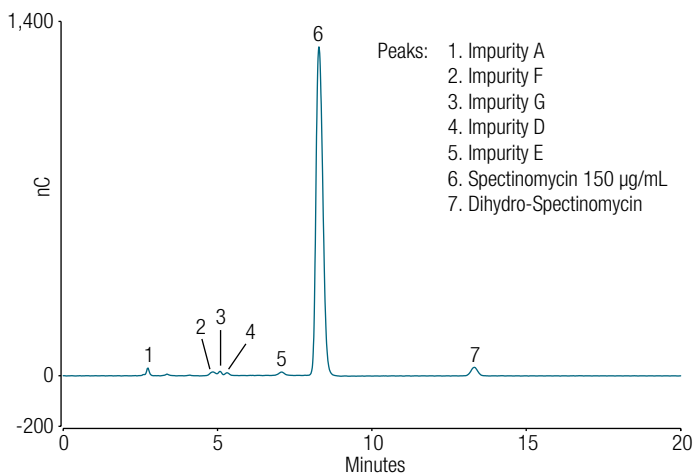

Peaks: 1. Impurity A
        2. Impurity F
        3. Impurity G
        4. Impurity D
        5. Impurity E
        6. Spectinomycin 150 µg/mL
        7. Dihydro-Spectinomycin

Figure 2. Separation of a system suitability standard (spectinomycin 150 µg/mL) using a Dionex IonPac AmG-3µm C18 column

Figure 3 shows an overlay of six replicate injections of a spectinomycin standard (80 µg/mL) from the repeatability analysis. As shown in Table 2, the RSD of peak area for six injections of spectinomycin standard is 0.36%, easily exceeding the EP requirement of 3%.
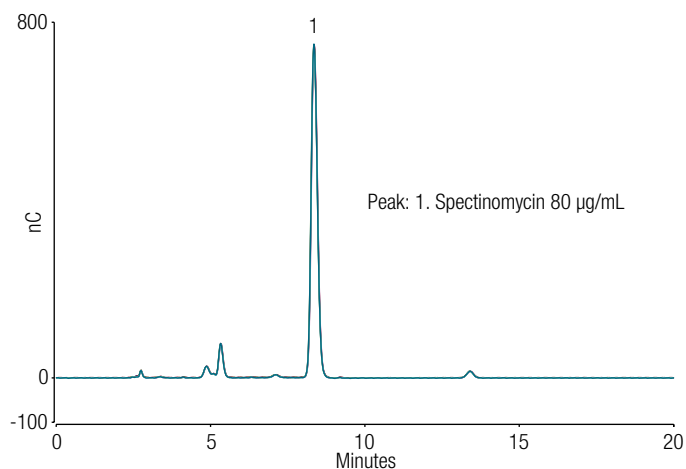

Peak: 1. Spectinomycin 80 µg/mL

Figure 3. Overlay of six injections of spectinomycin standard (80 µg/mL)

Linearity

The EP monograph uses a single concentration of 80 µg/mL for the assay. The linearity of spectinomycin electrochemical response to concentration was investigated in the concentration range of 1 to 80 µg/mL (1, 5, 10, 20, 30, 40, 50, 60, 70, 80 µg/mL). The calibration standards were prepared by serially diluting the 80 µg/mL standard solution with eluent. Figure 4 shows the calibration curve; the coefficient of determination (r^2) is 0.9999 using quadratic fitting and 0.9986 using linear fitting. The linearity in the concentration range of 1–40 µg/mL is better with $r^2 = 0.9996$ (Figure 5). This reveals that a sample concentration of 80 µg/mL is a little out of the linear response range and 40 µg/mL is a better concentration for sample analysis.
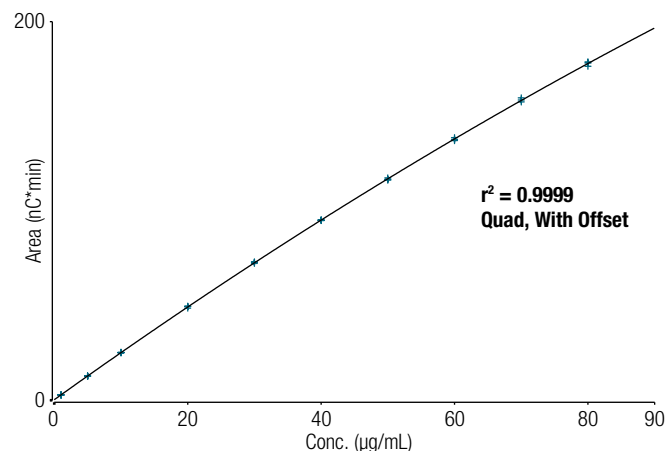

$r^2 = 0.9999$
Quad, With Offset

Figure 4. Calibration of spectinomycin 1–80 µg/mL (Quadratic fitting)

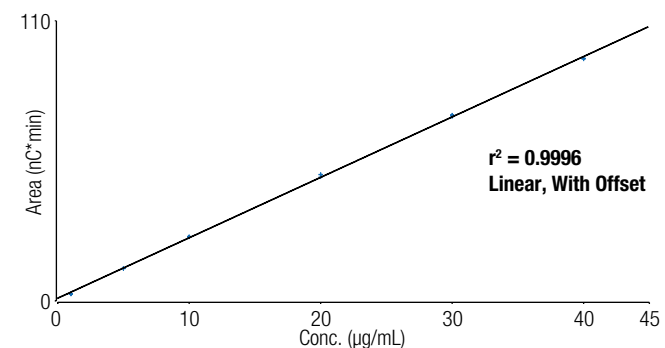$r^2 = 0.9996$
Linear, With Offset

Figure 5. Calibration of spectinomycin 1–40 µg/mL (Linear fitting)

Method limits of detection and quantification

The United States Pharmacopeia chapter on method validation specifies a S/N of 3 for the determination of the limit of detection (LOD) and a S/N of 10 for the determination of the limit of quantitation (LOQ).[9]

To determine the LOD and 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 spectinomycin peak. The LOD and LOQ were then calculated from the average peak height of three injections of spectinomycin (0.45 µg/mL). Table 3 summarizes the LOD and LOQ of spectinomycin in sample solution and in spectinomycin dichloride powder. Figure 6 shows the chromatogram of 1.5 µg/mL spectinomycin. Spectinomycin is sensitively detected.
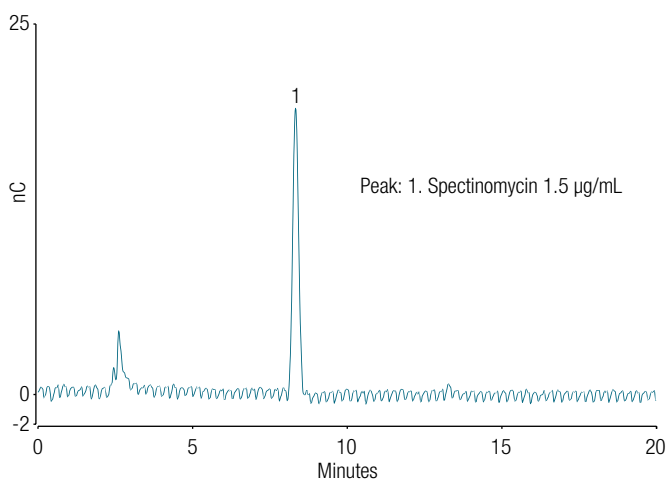

Figure 6. Spectinomycin standard (1.5 µg/mL)

Sample analysis
Spectinomycin assay

The linearity evaluation showed that the EP monograph assay concentration of 80 µg/mL is a little out of the linear range. In this study, spectinomycin concentrations of 80 µg/mL and 40 µg/mL were both used for the assay and the results compared (Table 4). Figure 7 shows the separation of spectinomycin sample #2 (40 µg/mL). A few impurities were detected and they were separated from spectinomycin.
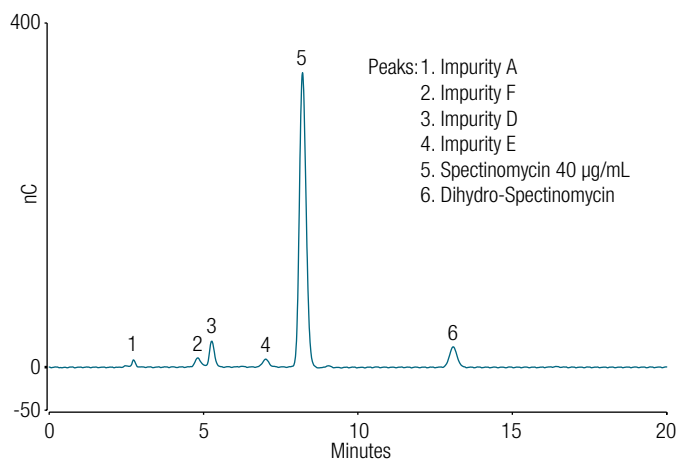

Figure 7. Separation of sample #2 (40 µg/mL) using a Dionex IonPac AmG-3µm C18 column

Table 4. Percentage of spectinomycin in sample

	40 µg/mL used for assay	80 µg/mL used for assay
Sample #1	100	101
Sample #2	99.7	100

Table 3. LOD and LOQ

Analyte	LOD (µg/mL) in sample solution	LOQ (µg/mL) in sample solution	LOD in spectinomycin dihydrochloride powder (µg/mg)	LOQ in spectinomycin dihydrochloride powder (µg/mg)
Spectinomycin	0.162	0.54	1.08	3.60

The percentage of spectinomycin in the spectinomycin sample was calculated as below:

% Spectinomycin in sample = (Rs/ Rd) × 100

Rs = Sum of spectinomycin and dihydro-spectinomycin peak area response (sample solution)

Rd = Sum of spectinomycin and dihydro-spectinomycin peak area response (standard solution)

Percentage of impurities in spectinomycin dihydrochloride samples

Sample solutions (a) and (b) were used for impurities analysis. Figure 8 shows the chromatogram of sample solution (a) of sample #2.
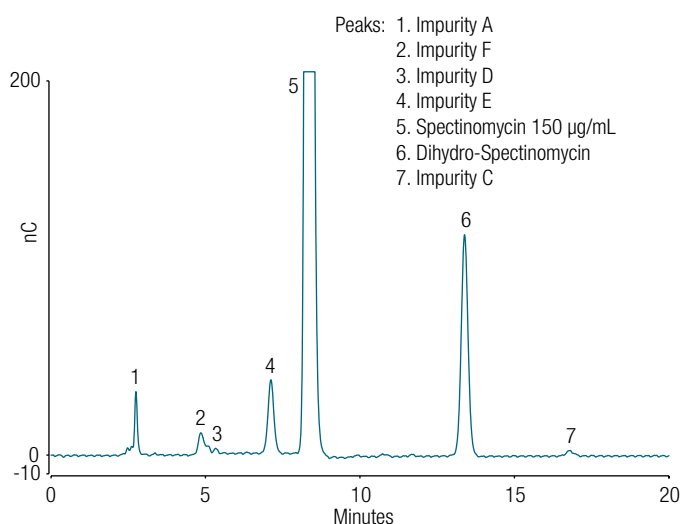
The EP monograph lists acceptance criteria for impurity levels in commercial samples. For that purpose, all impurities were calculated using the peak areas of impurities obtained from the chromatogram of the spectinomycin sample solution (a) and compared to the peak area of the spectinomycin obtained from the chromatogram of spectinomycin sample solution (b).

% of impurity in sample = ($r1/r2$)

$r1$ = peak response of each individually impurity from the 150 µg/mL sample solution (a)

$r2$ = peak response of spectinomycin from the 1.5 µg/mL sample solution (c)

Table 5 shows the percentage of individual and total impurities of standard and sample and compared with the EP acceptance criteria. The standard and the two samples pass the impurity criteria.

Peaks: 1. Impurity A
2. Impurity F
3. Impurity D
4. Impurity E
5. Spectinomycin 150 µg/mL
6. Dihydro-Spectinomycin
7. Impurity C

Figure 8. Separation of spectinomycin sample #2 (150 µg/mL) using a Dionex IonPac AmG-3µm C18 column

Table 5. Percentage of impurity in spectinomycin dihydrochloride

Test	A	C	F	G	D	E	Any other individual impurity	Total impurities
USP Standard	0.253	ND	0.243	0.594	0.546	0.730	<0.272	2.98
Sample #1	0.138	ND	0.849	0.491	0.977	0.638	<0.143	3.09
Sample #2	0.521	0.311	0.263	ND	0.175	2.059	<0.182	3.51
EP monograph Acceptance Criterion	1	1	1	1	4	4	1	6

thermo scientific

Conclusion

This application note demonstrates that spectinomycin dihydrochloride and related impurities can be separated with a Dionex IonPac AmG-3µm C18 column using a simple eluent method (0.1 M TFA) compared to the method in the EP monograph. The separation, repeatability, and sensitivity of this method were found to meet or exceed the current EP Spectinomycin Dihydrochloride monograph performance requirements. This method is reliable and can be used for the routine evaluation of spectinomycin samples.

References

1. Thermo Scientific Application Note 72647: Determination of gentamicin and related impurities in gentamicin sulfate. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-72647-ic-impurities-gentamicin-sulfate-an72647-en.pdf

2. Thermo Scientific Application Update 72648: Determination of gentamicin and related impurities in gentamicin sulfate using simple eluents. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/au-72648-ic-gentamicin-sulfate-simple-eluents-au72648-en.pdf

3. Thermo Scientific Application Note 72792: Determination of etimicin and related impurities in etimicin sulfate. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-72792-ic-etimicin-in-etimicin-sulfate-an72792-en.pdf

4. Spectinomycin Dihydrochloride Pentahydrate, European Pharmacopeia (EP) 2008, Page 2947-2949.

5. Thermo Scientific Dionex IonPac AmG-3µm C18 Columns Product Manual P/N 065728, May 2017. https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-065728-IC-IonPac-AmG-3um-C18-Man065728-EN.pdf

6. Thermo Scientific Technical Note 21: Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/TN-21-Optimal-Settings-Pulsed-Amperometric-Detection-Carbohydrates-ED40-TN70670-EN.pdf

7. Thermo Scientific Dionex ICS-5000+ Ion Chromatography System Operator's Manual P/N 065446-02, December 2014. https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-065446-IC-ICS-5000-Operators-Manual.pdf

8. Thermo Scientific Electrochemical Detection User's Compendium, P/N 065340-02, April 2013. https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-065340-Electrochemical-Detection-Man065340-EN.pdf

9. United States Pharmacopeia 40 The National Formulary. 35 General Chapter <1225>, Validation of Compendial Methods, U.S. Pharmacopeial Convention, Inc., Rockville, MD, 2018.

10. United States Pharmacopeia General Chapter <621> Chromatography, in USP National Formulary (NF): USP 40, 2018.

Find out more at **thermofisher.com/PharmaIC**

Thermo Fisher
SCIENTIFIC