Analysis of Spectinomycin Using a pH Stable Specialty Column for Aminoglycoside Antibiotics Separation

Xuefei Sun and Xiaodong Liu
Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
Acclaim AmG C18, aminoglycoside, spectinomycin, IP-RPLC, HFBA

Goal
To describe a high-performance separation of spectinomycin and its biosynthetically related compounds using ion-pairing reversed-phase liquid chromatography (IP-RPLC). The separation is performed on a Thermo Scientific™ Acclaim™ AmG C18 column, packed with a novel C18 bonded silica phase specially designed for separation of aminoglycoside antibiotics under acidic conditions. In these separations pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) are used in the mobile phase to enhance the retention and optimize the separation of all detected components.

Introduction
Spectinomycin is a broad-spectrum aminocyclitol antibiotic used to treat bacterial infections in human and animals. Spectinomycin is isolated from the fermentation broth of Streptomyces spectabilis, and it could contain a number of biosynthetically related components including (4S)-dihydrospectinomycin, (4R)-dihydrospectinomycin, dihydroxyspectinomycin, and some degradation products.[1] It is important to characterize and quantify the active pharmaceutical ingredient (API) and impurities in the drug product to ensure its quality and safety. As one of the most powerful separation techniques, liquid chromatography is widely used to analyze the aminoglycoside antibiotics quantitatively and qualitatively. Because aminoglycosides and related substances are very hydrophilic and positively charged, it is difficult to retain them on the column when using conventional reversed-phase LC (RPLC). A HILIC approach is also challenging for this analysis due to limited solubility of the aminoglycosides in organic solvents. To address these challenges, ion-pairing reversed phase LC (IP-RPLC) methods have been developed to analyze aminoglycosides using various ion-pairing agents in the mobile phase.[2-5] Usually volatile perfluorinated carboxylic acids, such as trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA) are employed to enhance the retention of aminoglycosides on the reversed-phase columns, adjust the selectivity, and, therefore, improve the resolution.

Another challenge for aminoglycosides analysis is obtaining high sensitivity, which ensures detecting both APIs and the low level impurities. Due to the lack of suitable chromophores, aminoglycoside antibiotics and their related substances cannot be directly monitored by UV adsorption. Therefore, corona charged aerosol detectors (CAD),[2] evaporative light scattering detectors (ELSD),[3-4] mass spectrometers (MS)[5], and electrochemical detectors (ECD) are usually used to detect these compounds, avoiding the cumbersome pre-column or post-column derivatization process.

Here, a high-performance separation of spectinomycin and its related substances is presented using an Acclaim AmG C18 column and CAD detection. The Acclaim AmG C18 column is a specialty column designed for aminoglycoside analysis and is packed with a polymer encapsulated silica media covalently bonded with C18 functionality. The stationary phase has excellent stability

Thermo SCIENTIFIC

under extreme conditions, such as low pH and high temperature. An aqueous solution of TFA is used as the mobile phase for spectinomycin analysis without adjusting its pH or adding organic solvent. PFPA and HFBA can also be utilized in combination with the TFA solution to enhance the retention and selectivity. Excepting the principal peak and chloride ion peak, at least eight minor components are well separated and detected in a spectinomysin sample.

Experimental

Consumables

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Trifluoroacetic acid acid [TFA, $C_2HF_3O_2$] (Fisher Scientific P/N PI-28901)
- Pentafluoropropionic acid [PFPA, $C_3HF_5O_2$] (Purchased from a reputable supplier)
- Heptafluorobutyric acid [HFBA, $C_4HF_7O_2$] (Purchased from a reputable supplier)
- Spectinomycin dihydrochloride (Purchased from a reputable supplier)

Recommended Sample Handling Equipment

- Thermo Scientific™ Virtuoso™ vial, clear, 2 mL kit, pre-slit T/S septa (P/N 60180-VT405)
- Virtuoso Vial Identification System (P/N 60180-VT100)

Columns

- Acclaim AmG C18, 3 μm, 4.6 × 150 mm (P/N 088757)
- Acclaim AmG C18, 3 μm, 3.0 × 150 mm (P/N 088755)

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC System equipped with:
	SRD-3600 Solvent Racks with Degasser (P/N 5035.9230)
	DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
	WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	Thermo Scientific™ Dionex™ Corona™ Veo™ RS Charged Aerosol Detector (P/N 5081.0020)
Mobile Phase	100 mM TFA (aqueous)
Flow Rate	1 mL/min for 4.6 mm i.d. column 0.425 mL/min for 3.0 mm i.d. column
Column Temperature	30 °C
Detector	Corona Veo RS Charged Aerosol Detector (CAD) (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Injection Volume	5 μL for 4.6 mm i.d. column 2 μL for 3.0 mm i.d. column
Samples	Spectinomycin dihydrochloride, 1 mg/mL

Software

The Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

Spectinomycin is an aminocyclitol antibiotic belonging to the aminoglycoside family. Figure 1 illustrates the spectinomycin structure. It can undergo a ring opening and closing of the hemiketal function in solution and thus produces an equilibrium mixture of several possible anomers. In addition, it can be hydrolyzed under acidic conditions to generate actinamine. Therefore, there are a number of spectinomycin-related compounds and degradation products in the spectinomycin sample. It is challenging to resolve all of them due to their structural similarity.
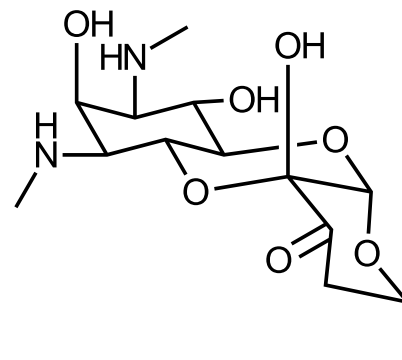

Figure 1. Structure of spectinomycin.

Figure 2 illustrates a typical isocratic separation of spectinomycin dihydrochloride using an Acclaim AmG C18 column, 100 mM TFA as the mobile phase, and CAD detection. In addition to the principal peak (spectinomycin) and chloride ion peak, eight minor peaks have been detected as spectinomycin-related substances and/or impurities. The spectinomycin peak is completely isolated from these impurity peaks. Most of the impurities are also totally resolved except peaks 2 and 3. This method is very convenient and reproducible because the mobile phase (100 mM TFA, pH ~1) is quite simple compared with most reported methods. The Acclaim AmG C18 stationary phase has a superior resistance towards the acidic environment and, therefore, unlike most conventional C18 columns, it is not necessary to adjust the mobile phase pH. Figure 3 shows six consecutive analyses of a spectinomycin sample. The separation was reproducible with an RSD for the principal peak area of 1.6% and no measurable change in retention time.
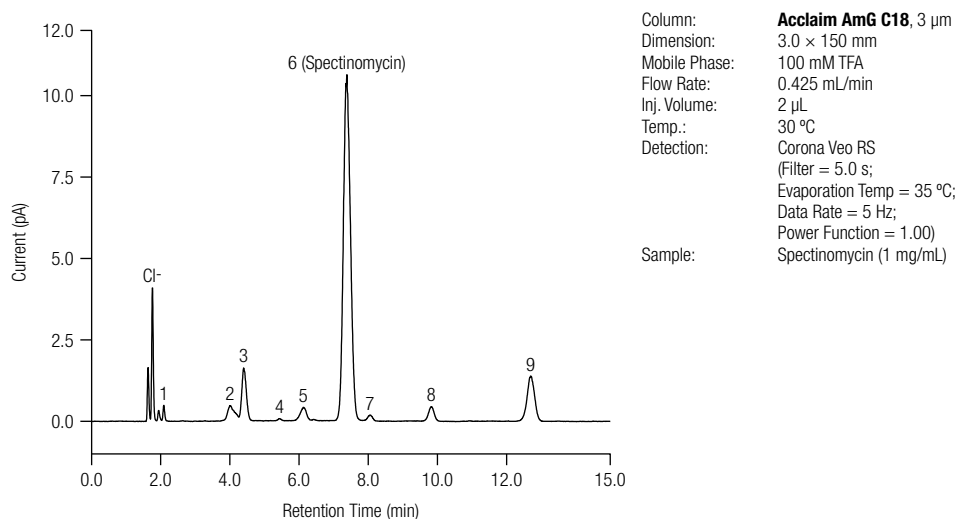
Column: **Acclaim AmG C18**, 3 μm
Dimension: 3.0 × 150 mm
Mobile Phase: 100 mM TFA
Flow Rate: 0.425 mL/min
Inj. Volume: 2 μL
Temp.: 30 ℃
Detection: Corona Veo RS
(Filter = 5.0 s;
Evaporation Temp = 35 ℃;
Data Rate = 5 Hz;
Power Function = 1.00)
Sample: Spectinomycin (1 mg/mL)

Figure 2. Separation of spectinomycin.

Column: **Acclaim AmG C18**, 3 μm
Dimension: 4.6 × 150 mm
Mobile Phase: 100 mM TFA
Flow Rate: 1 mL/min
Inj. Volume: 5 μL
Temp.: 30 ℃
Detection: Corona Veo RS
(Filter = 5.0 s;
Evaporation Temp = 50 ℃;
Data Rate = 5 Hz;
Power Function = 1.00)
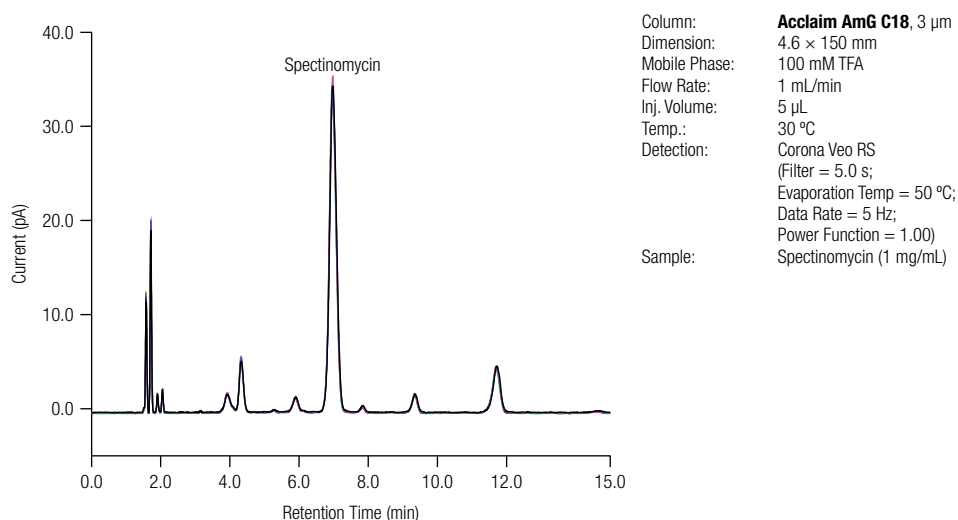Sample: Spectinomycin (1 mg/mL)

Figure 3. Overlay of 6 spectinomycin separations using an Acclaim AmG C18 column.

Improved resolution of the critical pair, peaks 2 and 3, was achieved by addition of PFPA or HFBA to the mobile phase. These stronger ion-pairing reagents not only help to retain spectinomycin, but also adjust the selectivity between the related compounds. Figure 4 illustrates the separation of spectinomycin using different concentrations of HFBA (0–5 mM) combined with TFA as the mobile phase. When the HFBA concentration increased from 0 to 5 mM, the retention time of some peaks changed significantly. Figure 5 shows the relationship between the capacity factor of some peaks and the HFBA content in mobile phase. Adding HFBA will increase the retention for all peaks and change the selectivity and resolution.

Figure 6 indicates the resolution change of two peak pairs with different HFBA content in the mobile phase. The resolution between the critical peak pair of peaks 2 and 3 increased from 1.25 to 1.85 and the resolution between spectinomycin and peak 5 increased from 3.6 to 7.8. PFPA has a similar effect on the separation as HFBA. Figure 7 shows the separation of spectinomycin using 10 mM PFPA combined with 50 mM TFA as the mobile phase, with all detected peaks completely separated.
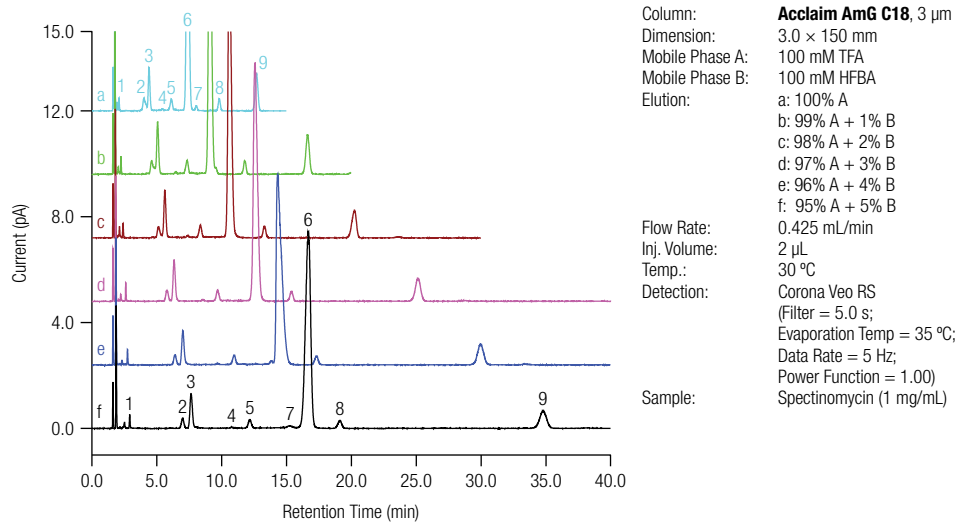
Column:	**Acclaim AmG C18**, 3 µm
Dimension:	3.0 × 150 mm
Mobile Phase A:	100 mM TFA
Mobile Phase B:	100 mM HFBA
Elution:	a: 100% A
	b: 99% A + 1% B
	c: 98% A + 2% B
	d: 97% A + 3% B
	e: 96% A + 4% B
	f: 95% A + 5% B
Flow Rate:	0.425 mL/min
Inj. Volume:	2 µL
Temp.:	30 ºC
Detection:	Corona Veo RS
	(Filter = 5.0 s;
	Evaporation Temp = 35 ºC;
	Data Rate = 5 Hz;
	Power Function = 1.00)
Sample:	Spectinomycin (1 mg/mL)

Figure 4. Separations of spectinomycin using different contents of HFBA in mobile phase.
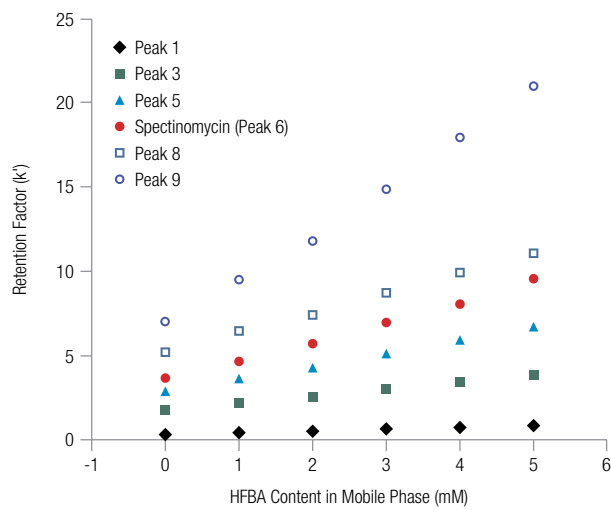

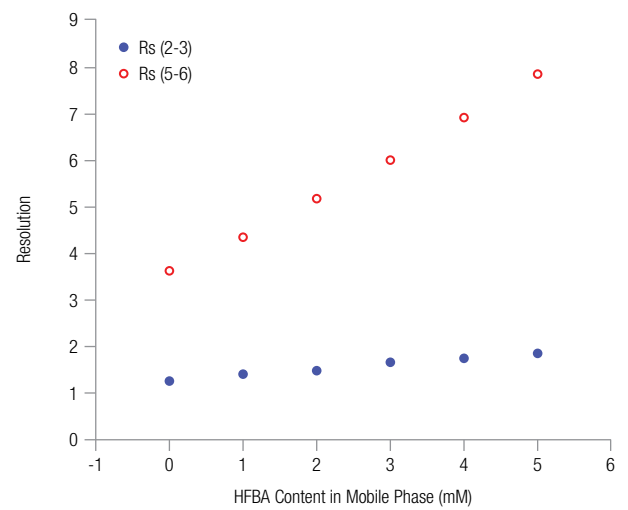Figure 5. HFBA effect on retention of spectinomycin and its related substances.

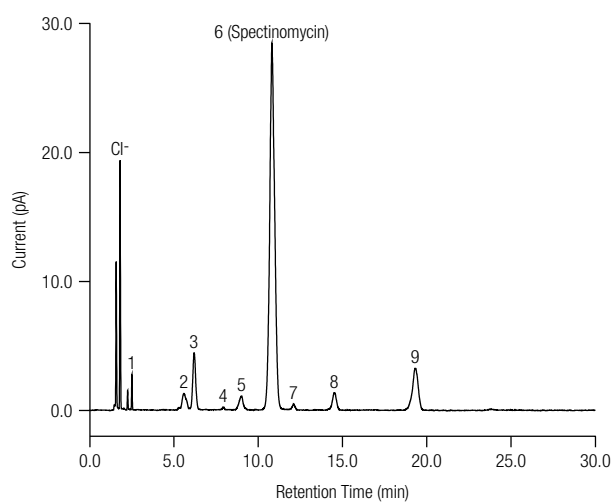Figure 6. HFBA effect on resolution of spectinomycin separation.

Figure 7. Separation of spectinomycin with PFPA in the mobile phase.

Column:	Acclaim AmG C18, 3 µm
Dimension:	4.6 × 150 mm
Mobile Phase:	10 mM PFPA + 50 mM TFA
Flow Rate:	1 mL/min
Inj. Volume:	5 µL
Temp.:	30 ºC
Detection:	Corona Veo RS
	(Filter = 5.0 s;
	Evaporation Temp = 35 ºC;
	Data Rate = 5 Hz;
	Power Function = 1.00)
Sample:	Spectinomycin (1 mg/mL)

Conclusion

- The Acclaim AmG C18 column separates spectinomycin and its related substances using a simple, rugged, and reproducible method.

- The addition of PFPA and HFBA to the mobile phase improves retention, selectivity, and resolution.

References

1. Spectinomycin dihydrochloride pentahydrate, *European Pharmacopoeia (EP)*, 2008, 2947-2949.

2. Stypulkowska, K.; Blazewicz, A.; Brudzikowska, A.; Warowna-Grzeskiewicz, M.; Sarna, K.; Fijalek, Z. Development of high performance liquid chromatography methods with charged aerosol detection for the determination of lincomycin, spectinomycin and its impurities in pharmaceutical products, *J. Pharm. Biomed. Anal.* **2015**, *112*, 8-14.

3. Zhou, J.; Zhang, L.; Wang, Y.; Yan, C. HPLC-ELSD analysis of spectinomycin dihydrochloride and its impurities, *J. Sep. Sci.* **2011**, *34*, 1811-1819.

4. Wang, Y.; Wang, M.J.; Li, J.; Yao, S.C.; Xue, J.; Zou, W.B.; Hu, C.Q. Determination of spectinomycin and related substances by HPLC coupled with evaporative light scattering detection, *Acta Chromatographica*, **2015**, *27*, 93-109.

5. Wang, Y.; Wang, M.; Li, J.; Yao, S.; Xue, J.; Zou, W.; Hu, C. Characterization of impurities in commercial spectinomycin by liquid chromatography with electrospray ionization tandem mass spectrometry, *J. Antibiot.* **2014**, *67*, 511-518.

Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library: https://appslab.thermoscientific.com/

For Research Use Only. Not for use in diagnostic procedures

To find a local representative, visit:
www.thermoscientific.com/columns

Thermo SCIENTIFIC

A Thermo Fisher Scientific Brand