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

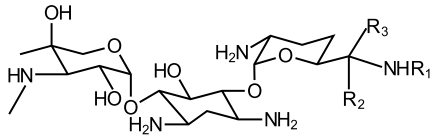
Xuefei Sun and Xiaodong Liu
Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words
Acclaim AmG C18, aminoglycoside, gentamicin, IP-RPLC, pH stable

Goal
To describe a high-performance separation of gentamicin congeners and their 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 specially designed C18 bonded silica media that has superior resistance towards acidic conditions, such as the 100 mM trifluoroacetic acid (TFA) aqueous solution used in this work. Five gentamicin congeners (C_1, C_{1a}, C_2, C_{2a}, and C_{2b}) are well separated and more than 20 minor components are detected. The use of a small percentage of organic solvent or operation at elevated temperature enables faster separation without compromising the resolution and column performance.

Introduction
Gentamicin is a widely used broad-spectrum aminoglycoside antibiotic to treat the infections caused by aerobic, Gram-negative bacteria in humans and animals. It is produced by the fermentation process of *Micromonospora purpurea* and consists of a mixture of related gentamicin components. Its active pharmaceutical ingredient (API) includes four major components: gentamicin C_1, gentamicin C_{1a}, gentamicin C_2, and gentamicin C_{2a} and a minor compound gentamicin C_{2b} (Figure 1). Besides the major APIs, some related substances, impurities and degradation products are also formed in small amount during fermentation.[1-3] It is essential to characterize the pharmaceutical API purity by identifying and quantifying the impurities, which ensures the drug safety and quality. However, this is challenging for biosynthetic products like gentamicin.

Liquid chromatography (LC) is a powerful technique to analyze complicated mixtures. However, gentamicin and its related substances are very hydrophilic compounds with multiple positive charges and limited solubility in many organic solvents, which makes conventional RPLC and HILIC separations challenging. Currently, ion-pairing reversed-phase liquid chromatography (IP-RPLC) is widely utilized to analyze aminoglycosides by using volatile perfluorinated carboxylic acids, such as trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA), as pairing ions in the mobile phase. This helps to retain the aminoglycosides on the column and improve the separation.[1-3]



	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.

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Due to the lack of a suitable chromophore, aminoglycosides and their related compounds cannot be detected by UV or fluorescence detection without extensive derivatization. Therefore, alternatives such as corona charged aerosol detectors (CAD),[2] evaporative light scattering detectors (ELSD), mass spectrometers (MS)[1,3] and electrochemical detectors (e.g. PAD)[4,5] are frequently used to detect these compounds. As described in the European and U.S. Pharmacopoeia, the analysis of gentamicin is based on an HPLC-PAD method using a C18 silica based column.[4,5] The mobile phase contains TFA, HFBA, and acetonitrile, and its pH is adjusted to 2.6 by sodium hydroxide (NaOH) to avoid the silica bonded phase hydrolysis when exposed to lower pH conditions.

Here, an easy and rugged method that uses an Acclaim AmG C18 column and 100 mM TFA aqueous solution as the mobile phase is presented to separate gentamicin congeners and related substances. The Acclaim AmG C18 column is packed with a specifically designed C18 bonded silica media with superior resistance towards acidic conditions. TFA aqueous solution can be used as the mobile phase for most aminoglycoside analysis without adjusting its pH or adding organic solvent. Five gentamicin congeners are well separated and more than 20 minor components are also detected using CAD detection. To accelerate the analysis, a small amount of organic solvent can be added in the TFA solution through isocratic or gradient elution. Fast analysis can also be achieved by operating at elevated temperature without compromising the resolution and column performance.

Experimental

Consumables

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Trifluoroacetic acid [TFA, $C_2HF_3O_2$] (Fisher Scientific P/N PI-28901)
- Acetonitrile [MeCN, C_2H_3N] (Fisher Scientific P/N A955-1)
- Gentamicin sulfate (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)

HPLC 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 (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:	Gentamicin sulfate, 1 mg/mL

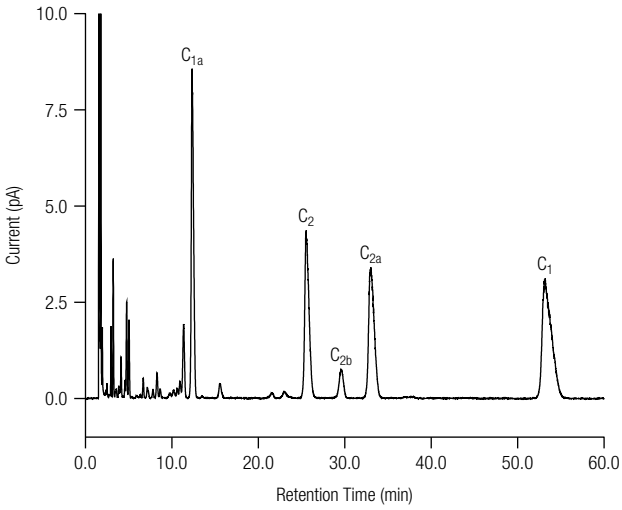
Software

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

Results and Discussion

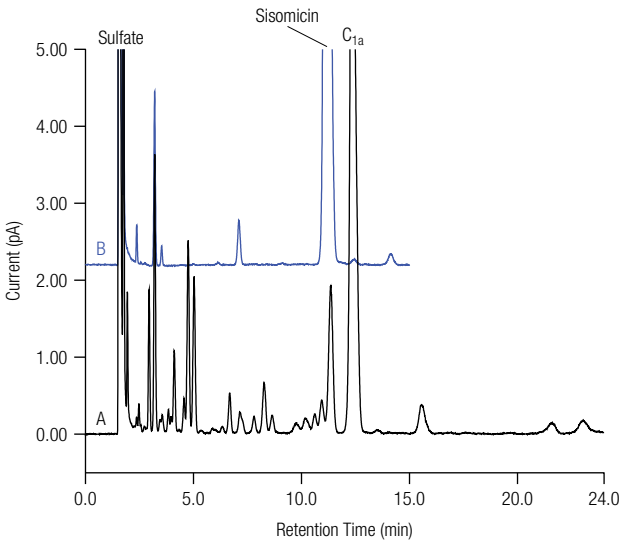
The IP-RPLC technique using an Acclaim AmG C18 column retains the gentamicin components and then separates them with appropriate selectivity. Figure 2 shows the isocratic separation of gentamicin sulfate sample using the Acclaim AmG C18 column, a simple mobile phase (100 mM TFA), and CAD detection. The five congeners (C_1, C_{1a}, C_2, C_{2a}, and C_{2b}) were well separated. The resolutions between the isomers (C_2, C_{2a}, and C_{2b}) were all greater than 3. In addition, more than 20 impurity or gentamicin-related substances were observed. Figure 3 shows the impurity peaks eluted before gentamicin C_2; a sisomicin standard is included as a comparison to help to identify the impurities. The resolution between sisomicin and gentamicin C_{1a} peaks is 2.3, which meets the European Pharmacopeia criteria (>1.2).

TFA acts as the ion-pairing agent and plays an important role in the gentamicin separation. Figure 4 illustrates the analysis of gentamicin using different concentrations of TFA as the mobile phase. With the increase in TFA concentration, the retention time of each individual component increased significantly and the resolution between peaks also improved. For example, the resolution between sisomicin and gentamicin C_{1a} increased from 1.32 at 40 mM TFA to 2.5 at 100 mM TFA. The resolution between gentamicin C_2 and C_{2b} increased from 3.5 at 40 mM TFA to 4.4 at 100 mM TFA. Meanwhile, the gentamicin C_{2b} peak moved close to the center between the C_2 and C_{2a} peaks. Therefore, the recommended mobile phase to obtain appropriate resolution, especially between the minor peaks, is 100 mM TFA.



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 °C
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	Gentamicin (1 mg/mL)

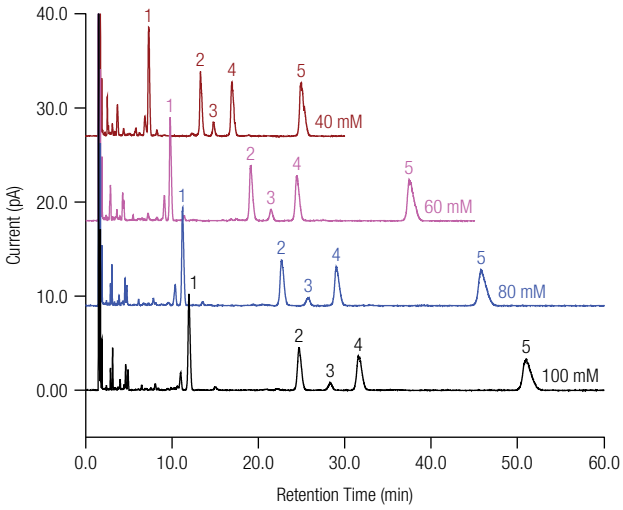
Figure 2. Analysis of gentamicin using an Acclaim AmG C18 column.



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 °C
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	A. Gentamicin (1 mg/mL) B. Sisomicin (0.2 mg/mL)

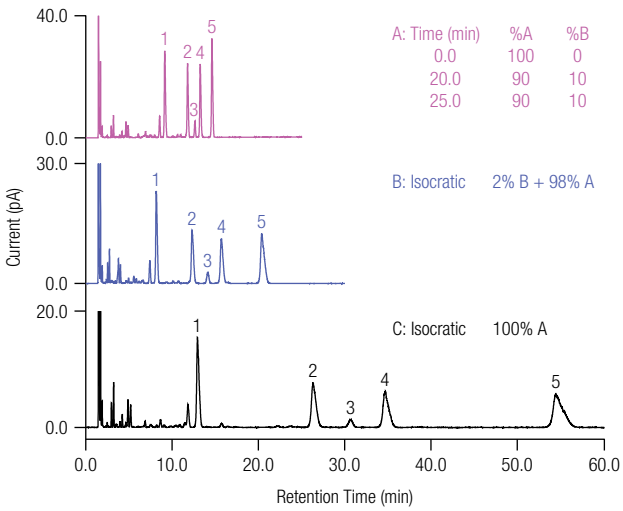
Figure 3. Gentamicin related substances detected in gentamicin sample.

Gentamicin analysis is normally completed in 60 minutes when using 100 mM TFA as the mobile phase. To accelerate the separation, a small percentage of organic solvent can be added to the mobile phase. Figure 5 shows the effect of the organic solvent in mobile phase on the gentamicin separation. When 2% acetonitrile was added to the 100 mM TFA as the mobile phase for isocratic separation, the analysis was completed in less than 25 min (Figure 5B). When a gradient elution was applied (with slope of 0.5% acetonitrile per min), the separation was carried out in less than 15 minutes (Figure 5A). When compared with the isocratic separations (Figures 5B and 5C), narrower and more symmetrical peaks were achieved. The resolution between the sisomicin and gentamicin C_{1a} peaks in three cases shown in Figure 5 were almost the same (2.55–2.58). However, the resolution between gentamicin C_2 and C_{2b} changed from 3.65 (Figure 5A) to 3.96 (Figure 5B), and then to 4.51 (Figure 5C).



Column:	**Acclaim AmG C18**, 3 µm
Dimension:	4.6 × 150 mm
Mobile Phase:	40 mM - 100 mM TFA
Flow Rate:	1 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	Gentamicin (1 mg/mL)
Peak ID:	1. C_{1a} 2. C_2 3. C_{2b} 4. C_{2a} 5. C_1

Figure 4. Separation of gentamicin using different TFA concentration mobile phases.



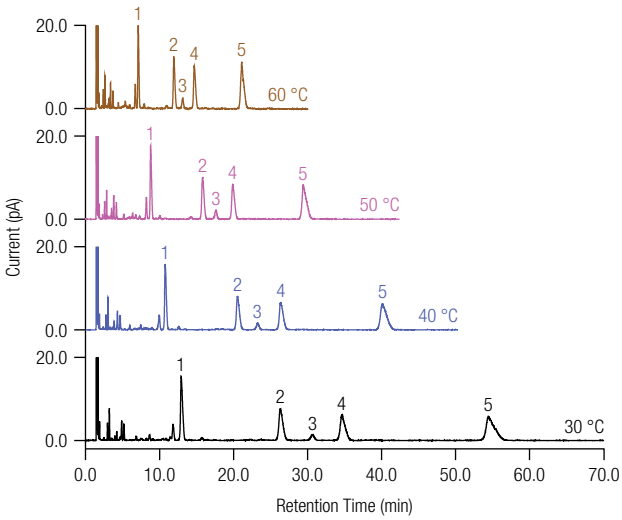
Column:	**Acclaim AmG C18**, 3µm
Dimension:	4.6 × 150 mm
Mobile Phase A:	100 mM TFA
Mobile Phase B:	Acetonitrile
Flow Rate:	1 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	Gentamicin (1 mg/mL)
Peak ID:	1. C_{1a} 2. C_2 3. C_{2b} 4. C_{2a} 5. C_1

Figure 5. Investigation of organic solvent effect on gentamicin separation.

Another method to obtain faster separation is to perform the analysis at elevated temperature. Figure 6 shows the separation of gentamicin at different temperatures. With the increase in temperature, the analysis time was shortened but the resolution decreased. For example, the separation was completed in less than 22 min at 60 °C. The resolution between sisomicin and gentamicin C_{1a} decreased from 2.58 (30 °C) to 1.59 (60 °C), and the resolution between gentamicin C2 and C2b changed from 4.51 (30 °C) to 3.16 (60 °C), but all major components and impurities were still completely resolved.

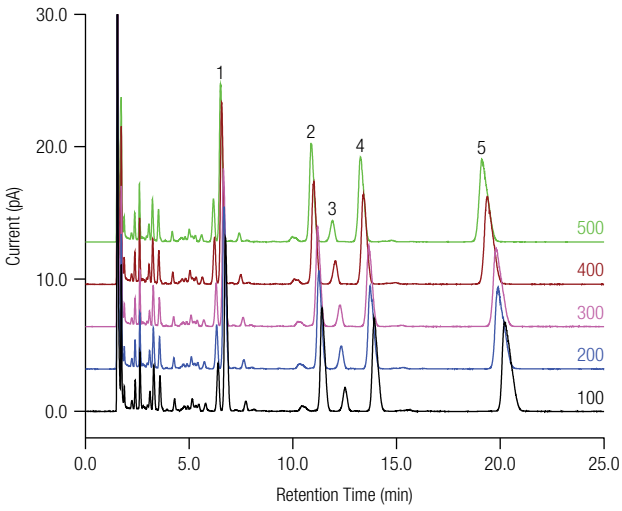
The challenge for separation at higher temperatures is the stationary phase stability under such harsh conditions.

The Acclaim AmG C18 column is specially designed to tolerate harsh conditions, such as low pH and high temperature conditions. Figure 7 illustrates 500 continuous runs of gentamicin at 60 °C using 100 mM TFA as the mobile phase. During more than 200 hours exposure to pH ~1 solution at 60 °C, the column maintained consistent separations. The retention of gentamicin C1 decreased by less than 10%, indicating that Acclaim AmG C18 columns can be used for hundreds of samples under these harsh conditions and that significantly more would be expected for injections at lower temperatures in 100 mM TFA.



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
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	Gentamicin (1 mg/mL)
Peak ID:	1. C_{1a} 2. C_2 3. C_{2b} 4. C_{2a} 5. C_1

Figure 6. Separation of gentamicin at different temperatures.



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.:	60 °C
Detection:	Corona Veo RS (Filter = 5.0 s; Evaporation Temp = 35 °C; Data Rate = 5 Hz; Power Function = 1.00)
Sample:	Gentamicin (1 mg/mL)
Peak ID:	1. C_{1a} 2. C_2 3. C_{2b} 4. C_{2a} 5. C_1

Figure 7. Ruggedness test of an Acclaim AmG C18 column.

Conclusion

- The Acclaim AmG C18 column separates gentamicin sulfate and its related substances using a simple, rugged, and reproducible method.
- Small amounts of organic solvent in the mobile phase accelerates the separation of gentamicin sulfate congeners.
- An elevated temperature allows an accelerated separation of gentamicin sulfate congeners.
- The Acclaim AmG C18 column is fully compatible with applications requiring low pH and elevated temperature.

References

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Useful Links

AppsLab Library

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

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