DIONEX

Application Note 267

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Analysis of the Aminoglycoside Antibiotics Kanamycin and Amikacin Matches USP Requirements

INTRODUCTION

Kanamycin and amikacin are broad-spectrum aminoglycoside antibiotics that are closely related (Figure 1). Kanamycin, used to treat a wide variety of serious gram-negative-bacterial infections, is purified from fermentation of *Streptomyces kanamyceticus* and is usually formulated as a sulfate in both oral and intravenous forms. Kanamycin, like many other aminoglycosides, can have oto- and nephrotoxic side effects, so the patient needs to be closely monitored after kanamycin administration.[1] The main component of purified kanamycin is kanamycin A, and the minor structurally related constituents are kanamycin B, C, and D (Figure 1).

Amikacin is commonly administered parenterally for the treatment of gram-negative infections resistant to kanamycin, gentamicin, or tobramycin. Compared to other aminoglycosides, the amikacin molecule has fewer sites susceptible to enzymatic reaction. Amikacin is synthesized by acylation of the amino group of kanamycin A with L-(-)-γ-amino-α-hydroxybutyric acid (L-HABA). As a result, kanamycin A and L-HABA are expected impurities in commercial amikacin samples.

The purity of these antibiotics must be determined and must meet specified criteria before clinical use. The detection of aminoglycosides is not straightforward because they do not have a significant UV-absorbing chromophore. Paper chromatography, ion-exclusion chromatography, gas-liquid chromatography after silylation, and reversed-phase LC with derivatization have been reported for the analysis of kanamycin purity.[2]

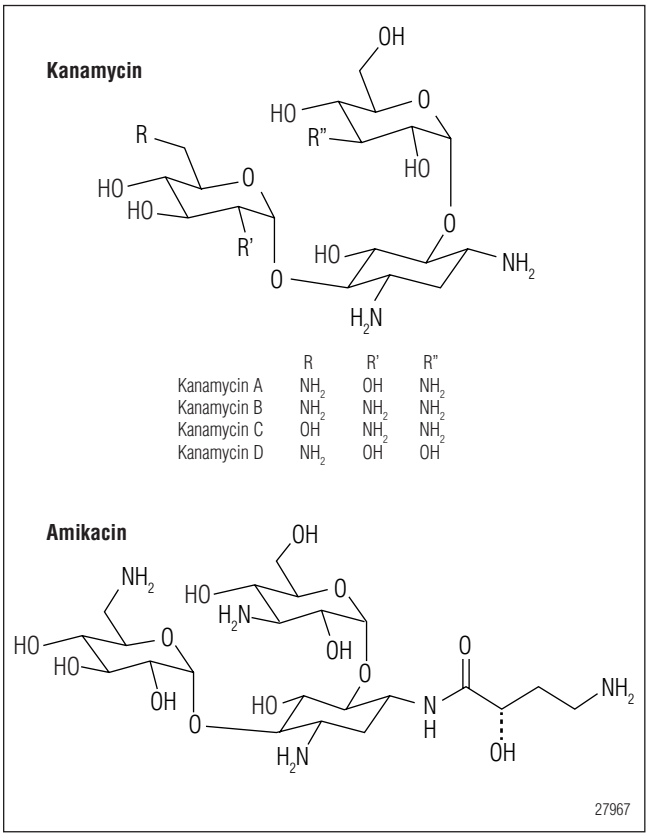

Figure 1. Chemical structures of kanamycin A, B, C, D, and amikacin.

Detection methods using pre- or postcolumn chemical derivatization have been used for amikacin.[3] However, these techniques are time consuming and require considerable sample preparation.

Aminoglycosides can be oxidized and detected by amperometry, a robust detection technique with a broad linear range and low detection limits.[4] High-performance anion-exchange chromatography in combination with pulsed amperometric detection (HPAE-PAD) provides a sensitive and reliable analytical method for aminoglycoside antibiotics.[5-7] The United States Pharmacopeia (USP) monographs for kanamycin and amikacin drug substances both use HPAE-PAD for assay.[8, 9] The same assay methods are also used for the assay of kanamycin and amikacin drug products.[10]

The work shown here evaluates the HPAE-PAD assay method described in the USP monographs for kanamycin and amikacin[8,9] and describes a revised HPAE-PAD method using a CarboPac® MA1 column and disposable Au-on-polytetrafluoroethylene (PTFE) working electrodes for the analysis of kanamycin and amikacin. Key parameters evaluated are precision, linearity, and resolution. The revised method meets or exceeds the USP requirements for peak resolution, tailing (also referred to as peak asymmetry), and precision. The use of disposable electrodes provides the benefits of shorter equilibration time and greater electrode-to-electrode reproducibility. Compared to other disposable Au electrodes, the Au-on-PTFE electrodes have longer lifetimes and can operate at higher hydroxide concentrations. The described method provides good sensitivity, high sample throughput, and retention time reproducibility for kanamycin and amikacin.

EQUIPMENT

Dionex ICS-3000 or -5000 system including:

- Gradient or Isocratic Pump
- DC Detector/Chromatography Module
- 20 µL Injection loop
- Electrochemical Detector (P/N 061718)
- Disposable Au-on-PTFE Working Electrode (P/N 066480, package of 6)
- Ag/AgCl Reference Electrode (P/N 061879)
- 2 mil PTFE gaskets (P/N 060141)
- AS Autosampler
- Chromeleon® Chromatography Data System (CDS) software
- Eluent Organizer, including 2 L plastic bottles and pressure regulator
- Polypropylene injection vials with caps (0.3 mL vial kit, Dionex P/N 055428)
- Nalgene® 125 mL HDPE narrow mouth bottles (VWR P/N 16057-062)
- Nalgene 250 mL HDPE narrow mouth bottles (VWR P/N 16057-109)
- Nalgene 250 mL 0.2 µm nylon filter units (VWR P/N 28199-371)
- Nalgene 1000 mL 0.2 µm nylon filter units (VWR P/N 28198-514)

REAGENTS AND STANDARDS

Reagents

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better, filtered through a 0.2 µm filter immediately before use

Standards

Kanamycin sulfate (Sigma-Aldrich Chemical Co. Cat # K4000)

Kanamycin sulfate reference standard (USP Cat # 1355006)

Amikacin disulfate salt (Sigma-Aldrich Chemical Co. Cat # A1774)

Amikacin reference standard (USP Cat # 1019508)

Sodium hydroxide 50% (w/w) (Fisher Scientific Cat # SS254-500)

Carbohydrate Waveform		
Time (s)	**Potential (V)**	**Integration**
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	-2.0	
0.42	-2.0	
0.43	+0.6	
0.44	-0.1	
0.50	-0.1	

CONDITIONS

Method

Columns:	CarboPac MA1 Analytical, 4 × 250 mm (P/N 044066) CarboPac MA1 Guard, 4 × 50 mm (P/N 044067)
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL (full loop)
Temperature:	30 °C
Back Pressure:	1500 psi
Eluent:	115 mM NaOH
Detection:	PAD (Au)
Background:	30–70 nC

Reference Electrode

Mode:	Ag/AgCl mode
Noise:	30 pC

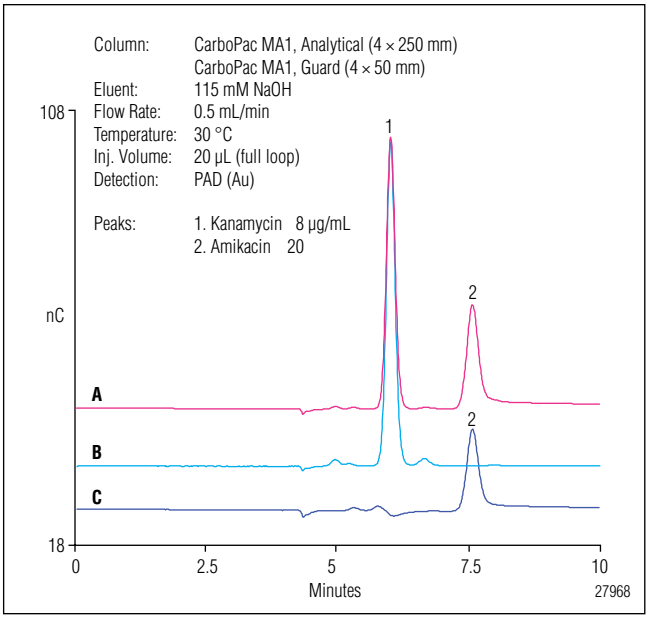

Figure 2. Typical chromatograms of (A) resolution solution (kanamycin 0.008 mg/mL and amikacin 0.02 mg/mL), (B) commercial kanamycin A sulfate sample, and (C) commercial amikacin sample.

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

115 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ–cm) containing as little dissolved carbon dioxide as possible. Biological contamination must be absent. Obtain source water using a water purification system consisting of filters manufactured without electrochemically active substances (e.g., glycerol). Prior filtration through 0.2 µm porosity nylon under vacuum is recommended to remove particulates and reduce dissolved air. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the column, causing a loss of chromatographic resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. Sodium hydroxide (50% w/w) is much lower in carbonate and is the recommended source for sodium hydroxide.

Dilute 5.9 mL of a 50% (w/w) sodium hydroxide into 994.1 mL of thoroughly degassed water to yield a 115 mM sodium hydroxide solution. Maintain the eluents under 34 to 55 kPa (5 to 8 psi) of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and minimize microbial contamination.[11]

Stock and Sample Preparation

Place solid kanamycin and amikacin reference standards in plastic vials and dissolve in deionized water to 0.16 mg/mL and 0.2 mg/mL stock standards, respectively. Further dilute the stock solutions to 0.008 mg/mL (kanamycin sulfate) and 0.02 mg/mL (amikacin) with DI water. Maintain the solutions frozen at -40 °C until needed. Prepare the research grade kanamycin and amikacin samples similarly.

RESULTS AND DISCUSSION

Separation

Figure 2 shows the separation of kanamycin and amikacin on the CarboPac MA1 column. The relative retention times are 1 for kanamycin and 1.3 for amikacin. Peak resolution between kanamycin and amikacin is >4, exceeding the USP requirement of 3. The asymmetry for both kanamycin and amikacin is 1.1 (USP requires <2). The total analysis time is 10 min, providing high sample throughput. A small baseline dip is seen at ~6 min and it co-elutes with kanamycin. However, at the concentrations tested, the contribution of the dip is insignificant (<3%).

Ruggedness

The variance due to different columns was tested by comparing results from columns from two different lots. Columns from different lots gave similar results. In addition, other waveforms (USP and *AAA-Direct*) were evaluated. The waveform reported for assay methods for kanamycin and amikacin in their USP monographs gave similar results. However, the USP waveform is not recommended for the disposable Au electrodes because the electrode will fail in less than 24 h. The *AAA-Direct* waveform also provides similar results (data not shown).

Linear Range

Linearity was investigated in the range of 2 to 16 µg/mL for kanamycin and 4 to 40 µg/mL for amikacin. The highest concentration investigated for both the aminoglycosides was twice the concentration used in the USP assay method. The correlation coefficient was 0.9993 for kanamycin and 0.9991 for amikacin (Table 1).

Precision

The RSD for retention time was 0.16 for kanamycin and 0.07 for amikacin for nine replicate injections (USP requirements <0.3%). The between-day precision was 0.01 for kanamycin and 0.07 for amikacin. The intra-day peak area precisions were 0.99 for kanamycin and 1.2 for amikacin (Table 1). The between-day peak area precisions were 1.3 and 2.3 for kanamycin and amikacin, respectively. The high precision suggests that this method can be used to analyze relatively pure aminoglycoside antibiotics like kanamycin and amikacin without column regeneration.

Product Degradation

Degradation of kanamycin and amikacin may occur during manufacturing, formulation, shipping, and storage. Degradation is monitored to evaluate the potency and quality of the active pharmaceutical ingredient. Pharmaceutical product stability is studied by exposing the product to acidic or basic conditions. Elevated temperatures are used to accelerate these studies.

Table 1. Calibrations for Precisions and Precisions for Kanamycin Sulfate and Amikacin (n = 9 injections)

Analyte	Range (µg/L)	Corr. Coeff. (r^2)	RT (min)	RT Precision (RSD)	Peak Area (nC*min)	Peak Area Precision (RSD)
Kanamycin Sulfate	2–16	0.9993	6.00	0.16	11.11	0.99
Amikacin	4–40	0.9991	7.6	0.07	5.35	1.2

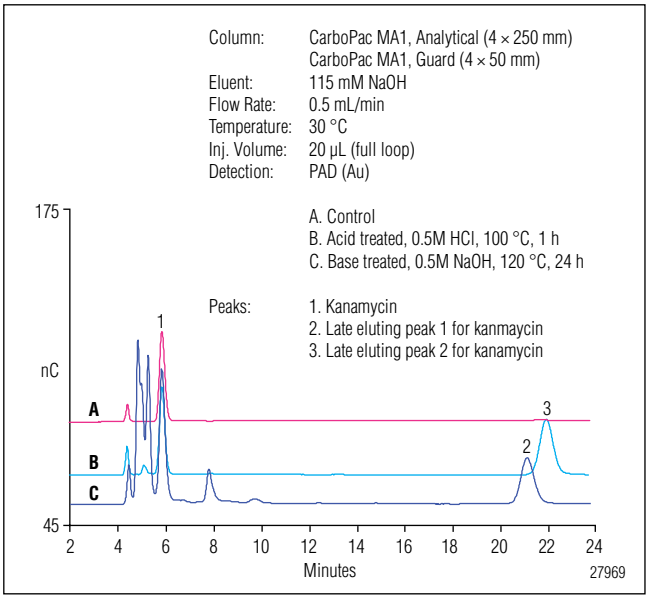

Figure 3. An accelerated stability study of kanamycin using (B) forced acid and (C) base degradation of kanamycin.

Kanamycin and amikacin were treated with high acid (0.5 M HCl) at 100 °C for 1 h, or base (0.5 M NaOH) at 120 °C for 24 h and 2 h, respectively. The samples were adjusted to neutral pH prior to analysis. Figure 3 shows the degradation products for kanamycin, and their relative amounts under acidic and basic conditions. Most of the degradation products elute within 10 min. Interestingly, there are late-eluting peaks at ~21 (Figure 3, peak 2) and 22 min (Figure 3, peak 3) in samples degraded under basic and acidic conditions, respectively. Similar late-eluting thermal decomposition product has been reported for the aminoglycoside drug streptomycin.[12] The identity of the late-eluting peak is not known, but the long retention time may interfere with subsequent injections if a shorter run time is used.

Figure 4 shows the degradation product for amikacin under acidic and basic conditions. First, there is a peak eluting at 6 min (same retention time as kanamycin, peak 1 in Figure 4). This suggests that under basic conditions amikacin loses its acetylated group, resulting in a kanamycin-like molecule. Second, no intact amikacin is detected in samples exposed to basic conditions. Thirdly, similar to kanamycin, amikacin also exhibits late-eluting peaks at ~20.5 (Figure 4, peak 3) and 22 min (Figure 4, peak 4) under basic and acidic degradation conditions, respectively. Peak 4 (Figure 4) for amikacin can be similar to peak 3 (Figure 3) in kanamycin, suggesting that the late-eluting species formed in both under acid-degradation conditions are similar. Additionally, the resolution of the closely eluting peaks can be improved if a lower column temperature (20 °C) is used (data not shown). These results show the capability of the HPAE-PAD method to be used in stability assays for these aminoglycoside antibiotics.

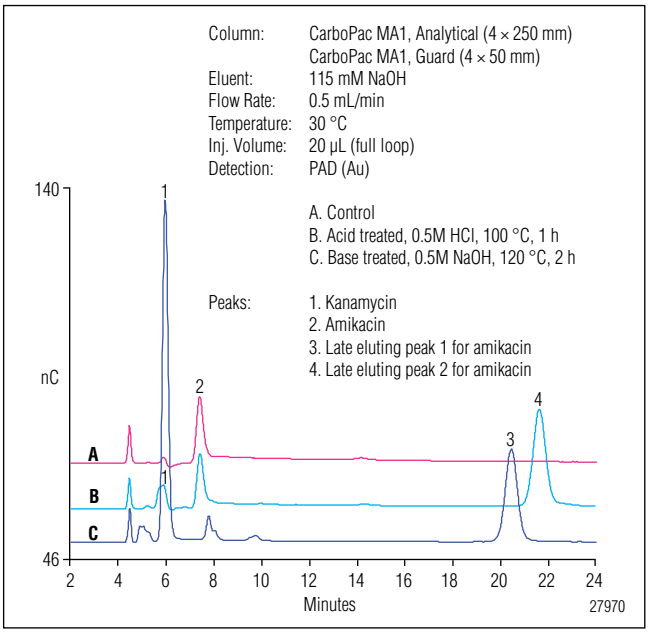

Figure 4. An accelerated stability study of amikacin using (B) forced acid and (C) base degradation of amikacin.

Accuracy

To evaluate accuracy, recoveries were determined in acid-degraded samples spiked with kanamycin and amikacin. The acid-degraded samples were adjusted to neutral pH prior to being spiked. Recovery was 80% for kanamycin and 86% for amikacin, suggesting that the method is accurate.

CONCLUSION

This work describes an HPAE-PAD method for the analysis of two closely related aminoglycoside antibiotics, kanamycin A and amikacin. The method uses the CarboPac MA1 column with hydroxide eluent. The disposable gold working electrode provides consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility. The method is shown to be accurate and reliable and meets the USP requirements for peak resolution, tailing (asymmetry), and reproducibility.

SUPPLIERS

Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275, U.S.A. Tel: 800.766.7000

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A. Tel: 800-932-5000.

Sigma-Aldrich Chemical Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A. Tel: 800-558-9160.

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