

Evaluation and Improvement of the USP Assay Methods for the Aminoglycoside Antibiotics Kanamycin and Amikacin

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INTRODUCTION

Kanamycin and amikacin are broad spectrum aminoglycoside antibiotics that are closely related (Figure 1, Table 1). Kanamycin, used to treat a wide variety of serious gram-negative-bacterium infections, is purified from fermentation of *Streptomyces kanamyceticus* and is usually formulated as a sulfate in both oral and intravenous forms. The main component of purified kanamycin is kanamycin A, and the minor structurally related constituents are kanamycin B, C, and D.

Amikacin is commonly administered parenterally for the treatment of gram-negative infections resistant to kanamycin, gentamycin, or tobramycin, as the amikacin molecule has fewer sites susceptible to enzymatic reaction compared to other aminoglycosides. 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 meet specified criteria before clinical use. The detection of aminoglycosides is not straightforward, as 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.¹ Detection methods using pre- or postcolumn chemical derivatization have been used for amikacin.² 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.³ High-performance anion-exchange chromatography in combination with pulsed amperometric detection (HPAE-PAD) has been shown to provide a sensitive and reliable analytical method for aminoglycoside antibiotics.⁴⁻⁶ The United States Pharmacopeia (USP) monographs for kanamycin and amikacin drug substances both use HPAE-PAD for assay.^{7, 8} The same assay methods are also used for the assay of kanamycin and amikacin drug products.⁹

This presentation evaluates the HPAE-PAD-based assay method described in the USP monographs for kanamycin and amikacin^{5,6} but with modernization to include a disposable gold on polytetrafluoroethylene (PTFE) working electrode and improved electrochemical conditions (waveform). Key parameters evaluated are precision, linearity, and resolution. The 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 and high sample throughput and retention time reproducibility for kanamycin and amikacin, two closely related aminoglycoside antibiotics.

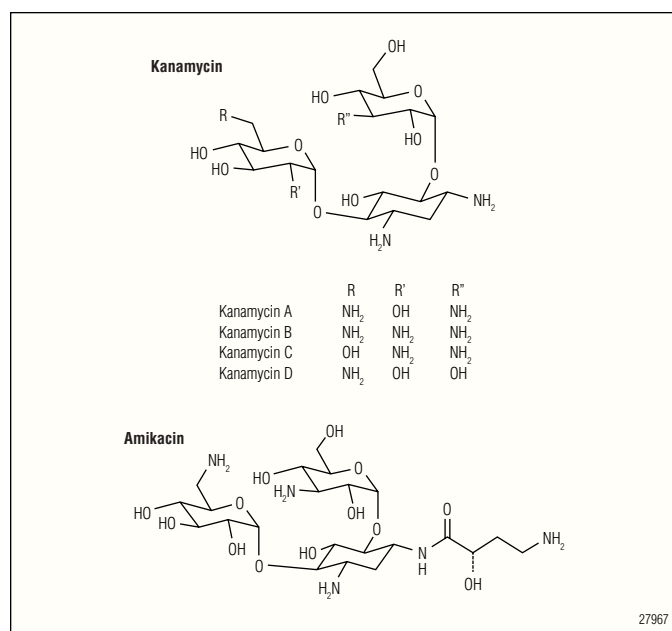


Figure 1. Chemical structures of kanamycin A, B, C, D, and amikacin.

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Table 1. List of Aminoglycosides, Their Origin, and Discovery

Name	Genera	Year Discovered
Streptomycin	<i>Streptomyces griseus</i>	1944
Neomycin	<i>Streptomyces fradiae</i>	1949
Kanamycin	<i>Streptomyces kanamyceticus</i>	1957
Paromomycin	<i>Streptomyces fradiae</i>	1959
Gentamicin	<i>Micromonospora purpurea</i>	1963
Tobramycin	<i>Streptomyces tenebrarius</i>	1968
Amikacin	<i>Streptomyces kanamyceticus</i>	1972
Netilmicin	<i>Micromonospora inyoensis</i>	1975
Spectinomycin	<i>Streptomyces spectabilis</i>	1962
Sisomicin	<i>Micromonospora inyoensis</i>	1970
Dibekacin	<i>Streptomyces kanamyceticus</i>	1971
Isepamicin	<i>Micromonospora purpurea</i>	1978

Conditions

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
Background: 30–70 nC
Reference Electrode: Ag/AgCl
Noise: 30 pC

RESULTS

Separation

Figure 2 shows the separation of kanamycin and amikacin using the CarboPac MA1 column. The relative retention times are 1.0 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 which co-elutes with kanamycin. However, at the concentrations tested, the contribution of the dip is insignificant (<3%).

EXPERIMENTAL DETAILS

Equipment

Dionex ICS-3000 system including:

Gradient or Isocratic Pump

DC Detector/Chromatography Module

20 µL Injection loop

Electrochemical Detector (P/N 061718)

Carbohydrate PTFE Disposable Au Working

Electrodes (P/N 066480, package of 6)

Ag/AgCl Reference Electrode (P/N 061879)

3 mm PTFE gaskets (P/N 63537)

AS Autosampler

Chromleon® Chromatography Data System (CDS) software

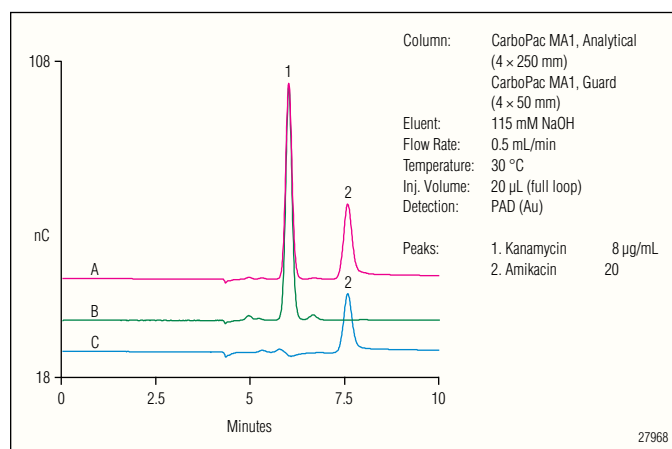


Figure 2. Typical chromatograms of (A) resolution solution (kanamycin 0.008 mg/mL and amikacin 0.02 mg/mL) (B) commercial kanamycin sulfate sample (C) commercial amikacin sample.

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, Table 2 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. The *AAA-Direct* waveform also provides similar results.

Table 2. Waveform Used for This Study vs USP Waveform

Waveform for This Study		USP Waveform		
Time (Seconds)	Potential (V)	Time (Seconds)	Potential (V)	Integration
0.00	+0.01	0.00	+0.04	
0.20	+0.01	0.30	+0.04	Begins
0.40	+0.01	0.50	+0.04	Ends
0.41	-2.0	0.51	+0.80	
0.42	-2.0	0.70	+0.80	
0.43	+0.06	0.71	-0.80	
0.44	-0.01	0.90	-0.80	
0.50	-0.01			

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 3).

Table 3. Linear Range and Precisions for Kanamycin 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	2–16	0.9993	6.00	0.16	11.11	0.99
Amikacin	4–40	0.9991	7.56	0.07	5.35	1.2

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. 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.

Monitoring 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. 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.

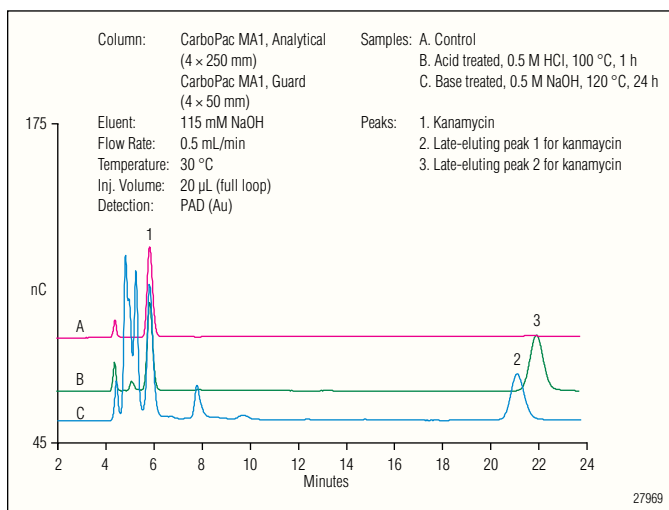


Figure 3. An accelerated stability study using (B) forced acid and (C) base degradation of kanamycin.

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.¹⁰ 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. Third, 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 column temperature of 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.

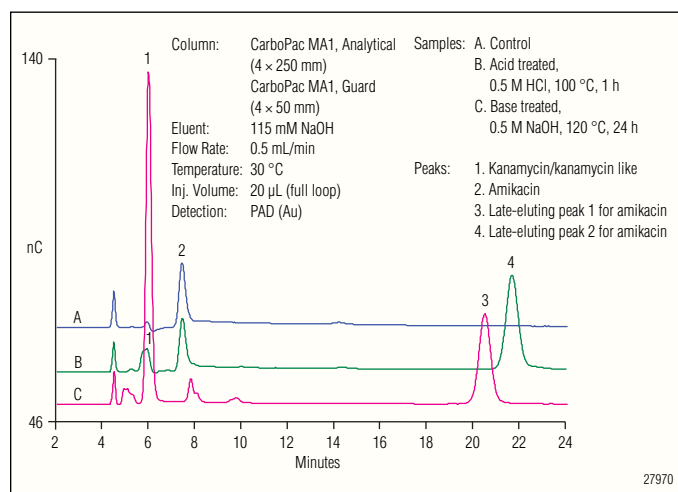


Figure 4. An accelerated stability study using (B) forced acid and (C) base degradation of amikacin.

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.

SUMMARY

Parameter	USP Specification	Current Method Result
Resolution	3	>4
Tailing Factor	<2	1.1
RT RSD	<0.3	0.16
Linear Range mg/L	Not specified	Kanamycin: 2–16 Amikacin: 4–40

- Matches and exceeds all USP requirements
- High sample throughput (run time: 10 min)
- Method applicable for stability assays
- Au on PTFE disposable electrodes suited for this application, provides consistently high detector response assuring greater instrument-to-instrument and lab-to-lab reproducibility

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