DIONEX

Application Update 167

Determination of Tobramycin in Crude and In-Process Production Samples During Manufacturing Using HPAE-IPAD

INTRODUCTION

Tobramycin is purified from the products of an actinomycete *Streptomyces tenebrarius* fermentation. These fermentation broths typically consist of cell culture media, a very complex sample matrix that includes salts. The product of microbial fermentation is carbamoyl-tobramycin, which is converted to tobramycin during manufacture using ammonium hydroxide hydrolysis. Tobramycin is further purified by extraction in alcohol, crystallization, and then separation by large scale (e.g., 1000 L columns) ion-exchange chromatography.

Application Note 61 (AN 61) describes a method for determination of tobramycin and its impurities in commercially available finished products (standards) using Reagent-Free™ high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAE-IPAD). Detection using IPAD is sensitive and direct, while eluent generation provides the benefits of high reproducibility and ease-of-use.[1] The tobramycin samples assayed in AN 61 are highly purified, and do not have appreciable amounts of salt or other sample components that can interfere with the assay. High salt content in tobramycin samples can shorten retention times and broaden peaks. This update describes how tobramycin and its typical impurities can be determined in manufacturing process matrices using the same HPAE-IPAD method described in AN 61. The same method is used to assay tobramycin that is intentionally degraded, demonstrating its suitability as a stability indicating method.

EQUIPMENT

Dionex ICS-3000 ion chromatography system consisting of:

- Gradient or Isocratic Pump, with vacuum degas option and GM-4 gradient mixer (P/N 049135)
- Electrochemical Detector with *AAA-Direct*™ Certified (Au) Disposable Electrodes (P/N 060082, package of 6; 060140, package of 24) and combination pH/Ag/AgCl Reference Electrode (P/N 044198 for ED40 and ED50, or 061879 for ICS-3000 systems)
- EG Eluent Generator with KOH eluent generator cartridge (EluGen® II Hydroxide; P/N 053921)
- Vacuum Degas Conversion Kit (P/N 055431 for older systems or 063353 for current systems)
- CR-ATC, Continuously Regenerated Anion Trap Column (P/N 060477)
- Autosampler with 20 µL injection loop
- Eluent Organizer, including four 2 L plastic bottles and pressure regulator
- Chromeleon® Chromatography Data System
- Helium; 4.5 grade, 99.995%, < 5 ppm oxygen (Praxair)
- Filter unit, 0.2 µm nylon (Nalgene® 90 mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter.
- Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)
- Polypropylene injection vials with caps (0.3 mL vial kit, Dionex P/N 055428)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Standards

Tobramycin (Sigma-Aldrich Chemical Co.; Cat. # T-4014)

Neamine (also known as neomycin A hydrochloride; International Chemical Reference Substances; World Health Organization; Cat. # 9930354)

Kanamycin A (Sigma-Aldrich Chemical Co.; Cat# K-1637)

Kanamycin B (also known as bekanamycin sulfate; Sigma-Aldrich Chemical Co.; Cat# B-5264)

CONDITIONS

Method

Columns:	CarboPac® PA1 Analytical, 4 × 250 mm (P/N 035391) CarboPac PA1 Guard, 4 × 50 mm (P/N 043096)
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL (full loop)
Temperature:	30 °C
Typical Operating Backpressure:	2460–2590 psi (with restrictor tubing installed between the degas apparatus and the injector)
Eluent Generation Method:	2.00 mM KOH; isocratic, 15 min run time or longer as needed
Detection:	Integrated pulsed amperometry
Background:	33–96 nC
Reference Electrode Mode:	pH
Waveform:	*AAA-Direct* waveform

Time (s)	Potential (V)	Integration
0.00	+0.13	
0.04	+0.13	
0.05	+0.33	
0.21	+0.33	Begin
0.22	+0.55	
0.46	+0.55	
0.47	+0.33	
0.56	+0.33	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

It is essential to use high-quality water of high resistivity (18 MΩ-cm) containing as little dissolved carbon dioxide as possible. Biological contamination should be absent. Source water must be obtained using a water purification system consisting of filters manufactured without electrochemically active surfactants (e.g., glycerol). Prior filtration through 0.2 µm porosity nylon under vacuum is recommended to remove

particulates and reduce dissolved air. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of helium or high-purity nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and opportunistic microorganisms.

Standards

Solid tobramycin, kanamycin B, kanamycin A, and neamine standards were placed in plastic vials and dissolved in deionized water to a 10 mg/mL concentration and diluted for use as described in AN 61.[1]

Note: Tobramycin, and to a lesser extent kanamycin B, kanamycin A, and neamine, when dissolved in water adsorbs to glass surfaces. Significant losses due to adsorption occur at dilute concentrations. Polypropylene injection vials and other labware must be used to ensure accurate results.

Samples

All samples were generously provided by Dr. Harry H. Liu, Ph.D. of Crick Pharma, Inc. (Cambridge, MA, 02140, USA) and Chengmin Zheng, Shaorong Zhang, Xiaojie Liu, and Xinping Tang of Livzon New North River Pharmaceutical Co., Ltd. (Qingyuan, Guangdong, China). For each of the following samples received, 100 mg of solid was reconstituted in 10 mL of water, and then serially diluted with water to 5 µg/mL for HPAE-IPAD analysis. Moisture and salt contents were unknown for each sample received, and therefore no corrections were applied for potency. No additional drying was performed prior to the reconstitution of each of the four samples below.

1. Fermentation broth—20 mL of *Streptomyces tenebrarius* fermentation broth in growth media, vacuum dried.
2. Hydrolyzed fermentation broth—15 mL *Streptomyces tenebrarius* fermentation broth hydrolyzed with 6 M ammonium hydroxide and vacuum dried.
3. Crude tobramycin—Tobramycin isolated from hydrolyzed fermentation broth, partially purified by ion-exchange chromatography and crystallization in alcohol, vacuum dried.
4. Finished tobramycin—Tobramycin purified with additional alcoholic crystallization steps, vacuum dried. Two separate batches were provided.

RESULTS AND DISCUSSION

The manufacture of tobramycin starts with the fermentation of a strain of the actinomycete *Streptomyces tenebrarius* in a proprietary growth media optimized for production of the nebramycin complex. Besides the microorganisms, this fermentation broth is a complex matrix typically consisting of one or more carbon and nitrogen sources, essential metabolic precursors, co-factors, and various salts required for cell growth, metabolic intermediates, waste products, and the desired nebramycin complex consisting of carbamoyl-tobramycin, among other related substances.

Free tobramycin is not produced by *Streptomyces tenebrarius,* and therefore alkaline hydrolysis of the fermentation broth is required to chemically convert the carbamoyl-tobramycin to tobramycin. Hydrolysis also converts other related substances present in the broth, such as carbamoyl-kanamycin B, another nebramycin complex ingredient produced by the microorganism. The 3 N ammonium hydroxide used for alkaline hydrolysis is removed during evaporation to dryness under vacuum.

This dry residue is a very impure form of tobramycin. Initial isolation of tobramycin from impurities is accomplished using both ion-exchange chromatography and crystallization in alcohol. The tobramycin material produced from this process is known as crude tobramycin. Further purification is accomplished using additional ion-exchange and crystallization steps, and leads to a highly purified finished tobramycin that complies with USP and other standard-setting organizations' purity criteria.

Finished products chemically degrade by different pathways. Under acidic or basic conditions, tobramycin degrades into smaller subunits that retain electrochemical activity, but still lack a good chromophore. These degradation products have been previously described.[2-4]

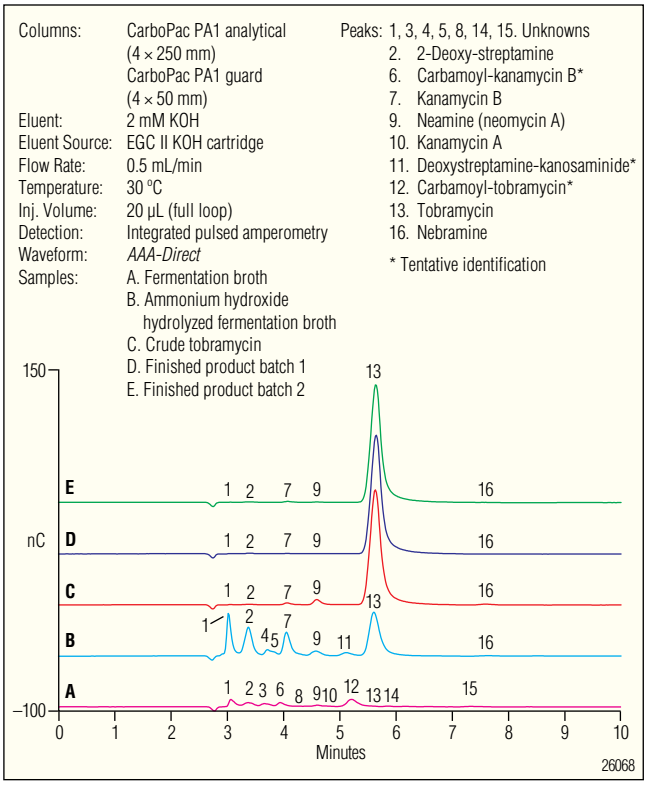

Figure 1. Determination of tobramycin and impurities in manufacturing process intermediates and final products.

Fermentation Broth

The culture medium towards the end of the fermentation process, just prior to harvest, should consist of carbamoyl-kanamycin B and carbamoyl-tobramycin, with only trace levels of kanamycin B and tobramycin.[4] The HPAE-IPAD analysis of this sample (Figure 1, Trace A) confirms the presence of only trace levels of kanamycin B (peak 7) and tobramycin (peak 13). The tentative identification of the carbamoyl-kanamycin B and carbamoyl-tobramycin peaks were previously described as peaks 6 and 12, respectively.[2] We found a total of 10 peaks in this chromatogram, more than the three factors described by Stark et al.[4] Among these we found neamine (neomycin A, peak 9, a degradation component of kanamycin B), and kanamycin A (peak 10). This chromatogram showed no interferences, shifts in retention times, or peak broadening that would occur if these samples were not suited for HPAE-IPAD analysis. This method successfully analyzes fermentation broths used for the manufacture of tobramycin, and the same or similar method should be suitable for assaying the fermentation broths of other aminoglycoside antibiotics.

Base-Hydrolyzed Broth Concentrate

Hydrolysis of the concentrated broth used in the manufacture of tobramycin with 3 N ammonium hydroxide converts carbamoyl-kanamycin B and carbamoyl-tobramycin to kanamycin B and tobramycin, respectively. Chromatography of this sample shows the appearance of kanamycin B and tobramycin peaks (Figure 1, Trace B, peaks 7 and 13, respectively), and seven other peaks (peaks 1, 2, 4, 5, 9, 11, and 16). Because salt was not removed in this manufacturing step, this sample represents a potential challenge for this method to determine tobramycin. No retention time shifts or peak broadening was observed, demonstrating the suitability of HPAE-IPAD for this analysis.

Crude Tobramycin

Crude tobramycin, the result of ion-exchange chromatography and crystallization techniques for the isolation of tobramycin, is expected to contain fewer and lower amounts of the impurities found in the hydrolyzed fermentation broth. Figure 1, Trace C shows the separation of tobramycin from these impurities in this crude sample, and clearly shows a purer product.

Finished Tobramycin (Final Product)

The finished product, the result of further ion-exchange and crystallization steps, is expected to be more purified than the crude material. The chromatograms for two finished product batches (Figure 1, Traces D and E) both showed significantly lower amounts of the detected impurity peaks compared to the crude material (Figure 1, Trace C). The calculated percentage for the sum of the six impurities, expressed as tobramycin peak area equivalents, ranged from 0.6 to 0.9% for the two finished batches, while the sum of the six impurity peaks in the crude material was 4.9%. These results again demonstrate the capability of this method to determine tobramyin in process intermediates and final products, and to evaluate the quality of the product throughout the process. The method's sensitivity allows these samples to be diluted so that other sample ingredients (e.g., salt) do not interfere. The chromatograms of these finished batches are similar to the final product, as evaluated in AN 61.[1] This indicates that the industrial processes involved in manufacturing tobramycin are probably similar, and that the results presented in this update, for this process, would be applicable to other manufacturers.

Product Degradation

Degradation of tobramycin may occur during storage, or if it is subjected to a new process as part of a new pharmaceutical formulation. Degradation should be monitored to assess the potency and quality of the drug product. Two common tests used for monitoring the stability of pharmaceutical products involve exposure to acidic or basic conditions. Elevated temperatures are used to accelerate these studies.

Finished tobramycin (batch #1) was treated with high concentrations (0.5 M) of hydrochloric acid and sodium hydroxide at 100 °C for 1 h and 120 °C for 24 h, respectively.[2] Figure 2 shows the degradation products formed from this study, and the loss of tobramycin peak area (Figure 2, Trace A), and the gain in peak areas for its known degradation products (Figure 2, Traces B and C).[2,3,5] These results show the capability of HPAE-IPAD for use in a stability indicating assay for tobramycin, and for evaluating the drug for its associated degradation products.

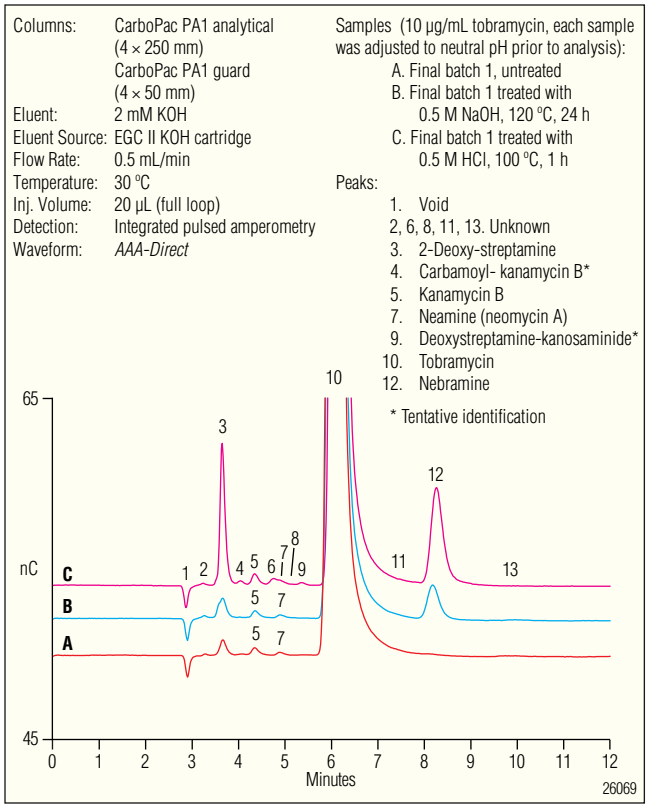

Figure 2. An accelerated stability study using forced acid and base decomposition of finished tobramycin (10 µg/mL).

CONCLUSION

Based on comparison of chromatograms for tobramycin materials collected during different stages in the manufacturing process, this chromatographic method can accurately assess the quality of tobramycin during in-process production. This was demonstrated by the progressive decreases in the amount of impurities found for the process samples moving forward through the manufacturing process and analyzed by HPAE-IPAD. The two finished commercial batches of tobramycin exhibited the highest level of purity, and the trace levels of the impurities showed nearly identical profiles, although some differences in the relative proportions of the impurity peaks were observed. These profiles also closely resembled that obtained from a different commercial source (presented in AN 61), suggesting the manufacturing process for tobramycin is similar throughout the industry. Forced degradation of tobramycin under both high and low pH conditions demonstrated that this method can be used as a stability indicating assay.

REFERENCES

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