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Identification of tobramycin impurities for quality control process monitoring using high-performance anion-exchange chromatography with integrated pulsed amperometric detection

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ABSTRACT

Commercial-scale fermentation for tobramycin manufacture is carried out with *Streptomyces tenebrarius*. Impurity profiling during various phases of pharmaceutical production is important for evaluating the effectiveness of a processing step and meeting regulatory requirements. High-performance anion-exchange (HPAE) chromatography with integrated pulsed amperometric detection (HPAE-IPAD) is a highly sensitive method used to assay tobramycin and to assess purity, but no prior publications demonstrated the capability of this technique to monitor purity at various stages of production at either the typical concentrations or in the typical matrices of a manufacturing process. In addition, the identities of the impurity peaks observed in commercial sources of tobramycin when assayed by using HPAE-IPAD are mainly unknown. Regulatory agencies generally require these impurities to be characterized when found above certain limits, and when present at higher levels require toxicological studies. In this paper, we analyze tobramycin samples using HPAE-IPAD at different stages of production and show the impurity profile and concentration changes through the manufacturing process. We successfully identified nearly all the impurity peaks found in commercially available tobramycin, based on known degradation pathways deduced from extreme pH forced degradation studies, which we experimentally reproduced, and based on previously known related substances found in *S. tenebrarius* fermentation broth. In crude and final tobramycin products, we identified the peaks for neamine, kanamycin B, nebramine, kanosamine, 2-deoxystreptamine. We tentatively identified deoxystreptamine–kanosaminide in crude and final products, and kanamycin A, carbamoyl-kanamycin B and carbamoyl-tobramycin in down stream process intermediates of a *S. tenebrarius* fermentation culture. Results presented in this paper support the effective use of the HPAE-IPAD method for in-process impurity profiling of tobramycin, and as a stability-indicating technique after product purification.

1. Introduction

Tobramycin is a water-soluble aminoglycoside antibiotic purified from the fermentation of the actinomycete *Streptomyces tenebrarius* and is used in a variety of pharmaceutical applications [1,2]. Kanamycin B, nebramine, and neamine (also known as neomycin A) are three known impurities of tobramycin recognized in the European Pharmacopoeia (EP) [3] that result from either incomplete purification of the drug, or from its degradation. Additionally, carbamoyl-kanamycin B, carbamoyl-tobramycin, kanamycin A, deoxystreptamine, deoxystreptamine–kanosaminide, and kanosamine are potential impurities [4,5]. The chemical structures of tobramycin, process intermediates, and major degradation products are shown in Fig. 1.

Due to the lack of commercial availability of most tobramycin impurities, we were previously only able to identify two of the

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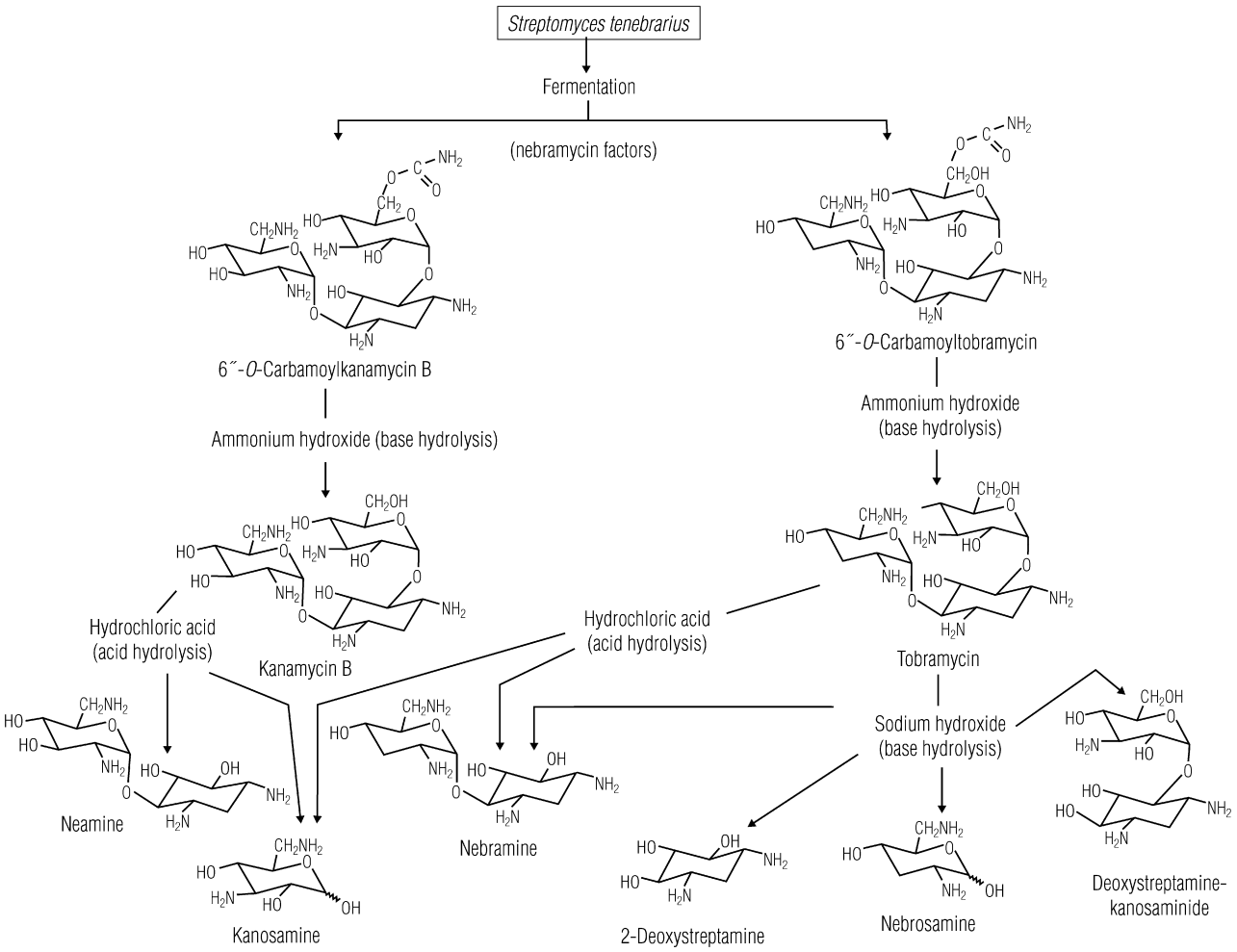

Fig. 1. The chemical structures of tobramycin, process intermediates, and degradation products.

six major peaks typically observed in commercially available tobramycin [6]. Generally, regulatory authorities [7–9] require pharmaceutical firms to identify any impurity in the finished product. An impurity whose content is >0.10% should be identified, and evaluated for biological safety when >0.15%. No reporting is required for impurities <0.05%. These levels can vary depending on the toxicity of the impurity, whether it is a degradation product, and the target daily dosage [9]. Our previous publication [6] that analyzed commercial tobramycin showed levels of some impurity peaks that exceeded the threshold required for their identification, and we therefore considered it important to know the identity of these impurity peaks. Following our initial publication, we attempted to analyze impurity peak fractions that we collected from injections of 1 mM solutions of tobramycin using mass spectroscopy (MS), but were unsuccessful due to the low concentrations of the collected analytes relative to the sample's high salt content. We considered using a carbohydrate membrane desalter [10] coupled to MS, but the low recovery of small amino sugars from this device was expected to render this approach ineffective.

In this publication, we duplicated the hydrolysis conditions of Brandl and Gu [4]. They found that tobramycin degrades to nebramine and kanosamine in acidic solution, and that it degrades to nebramine, deoxystreptamine and deoxystreptamine–kanosaminide in basic solution. The hydrolysis conditions used by Brandl and Gu yield many of the same degradation products expected during long-term storage. In their publication, they used a refractive index detection method and chemical-derivatization techniques; less sensitive and indirect detection methods, respectively. For each hydrolysis condition, we correlated the appearance of High-performance anion-exchange (HPAE) chromatography peaks using the sensitive and direct detection provided by integrated pulsed amperometric detection (IPAD) with the known degradation products to deduce peak identities. These results also support the stability-indicating capability of the HPAE-IPAD technique. In addition, we show the application of this technique to monitor tobramycin impurity levels in samples collected at different stages of tobramycin production. We present data showing that HPAE-IPAD is suitable for the evaluation of tobramycin purity during the manufacturing process, and provide the identity of the impurities observed during this process, including the final material.

2. Experimental

2.1. Standards and samples

Tobramycin (Sigma–Aldrich Chemical Co., Cat# T40014), kanamycin B (Sigma–Aldrich Chemical Co., Cat# B5264), and neamine (International Chemical Reference Substances, World Health Organization; Cat# 9930354) standards were prepared as described previously [6]. Commercial-scale fermentation was carried out with *S. tenebrarius*. The crude nebramycin complex was

recovered from the fermentation broth. After hydrolysis of the nebramycin complex with 3N ammonium hydroxide, tobramycin was isolated using ion-exchange columns, and then crystallized in alcohol. In-process materials were sampled at various different stages, including the finished product. Samples of finished products from several different batches were also collected. Samples were dried, weighed, and reconstituted to 10 mg/mL, and then diluted to 5 μg/mL for analysis. Forced degradation studies under extreme pH were performed according to the method of Brandl and Gu [4] using 1.0 mL of the finished tobramycin batch at 5.0 mg/mL, containing either 0.5 M NaOH (base hydrolysis, 120 °C, 24 h, sealed polypropylene vial), or containing 0.5 M HCl (acid hydrolysis, 100 °C, 1 h, sealed borosilicate glass vial). Reactions were terminated with the pH-neutralizing addition of 0.50 mL of 500 mM HCl or NaOH, respectively, yielding 1.5 mL of tobramycin hydrolysates at 3.33 mg/mL in 0.33 M NaCl. To ensure accurate results, the tobramycin standards and hydrolysate samples were dissolved to a final 10-μg/mL concentration in water (333-fold dilution) for analysis using polypropylene injection vials and other labware (e.g., pipettes, pipette tips, sample vials) during all stages to prevent adsorptive losses. Blank hydrolysates were prepared identically to the tobramycin hydrolysates, but without the antibiotic. Non-hydrolyzed blanks were similarly prepared, but without the heat-treatment; and tobramycin controls (at 10 μg/mL) were prepared by addition of NaCl to yield a 1-mM concentration, the equivalent salt concentration after a 333-fold dilution of the hydrolysates.

2.2. Chromatography

The chromatography system consisted of Dionex GP50 gradient pump with degas option and GM-4 gradient mixer, EG50 Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide) and CR-ATC, EG40/50 vacuum degas conversion kit, ED50A Electrochemical Detector, AS50 Autosampler, AS50TC Thermal Compartment, and Chromeleon® Chromatography Workstation (Dionex Corporation, Sunnyvale, CA, USA). Tobramycin and its impurities were separated with a CarboPac® PA1 (4 mm × 250 mm, Dionex Corporation) anion-exchange column with its guard (4 mm × 50 mm; USP designation L46). The electrochemical waveform was +0.13 V from 0.00 to 0.04 s, +0.33 V from 0.05 to 0.21 s, +0.55 V from 0.22 to 0.46 s, +0.33 V from 0.47 to 0.56 s, −1.67 V from 0.57 to 0.58 s, +0.93 V at 0.59 s, and +0.13 V at 0.60 s, using the combination Ag/AgCl/pH reference electrode with the instrument set in the pH mode and with current integrated between 0.21 and 0.56 s for detection. We used *AAA-Direct*™-Certified disposable gold working electrodes (replaced every 7 days) [11]. Tobramycin and its impurities were separated using 2.00 mM KOH, produced by the eluent generator, at a flow rate of 0.50 mL/min and a column temperature of 30 °C. This method used a 20-μL injection and had a run time of 35 min. The column set was washed once a week with 100 mM KOH for 60 min and then re-equilibrated to 2.00 mM for 2 h to achieve the best reproducibility of retention times.

3. Results and discussion

3.1. Fermentation and purification process assessment

Commercial-scale fermentation for production of tobramycin uses *S. tenebrarius*. At the end of the fermentation, prior to purification, a "fermentation" sample was drawn for analysis. In addition to the culture medium, this fermentation broth consists of a nebramycin complex, which includes primarily carbamoyl-kanamycin B and carbamoyl-tobramycin, with only trace levels of kanamycin B and tobramycin. HPAE-IPAD analysis of this sample (Fig. 2, chromatogram A) confirms the presence of only trace levels of kanamycin B and tobramycin. The fermentation broth chromatogram also revealed six major peaks (having an area percentage >0.05% of the total eluting peak area) and four minor peaks (each having percentages <0.05% of the total), instead of three major factors described by Stark et al. [12]. We also found that neamine (neomycin A, peak 9, a degradation component of kanamycin B) is present at a relatively low concentration in this fermentation broth. We identified one of the minor peaks on the tail of neamine as kanamycin A (peak 10) based on the retention time of a known standard. To the best of our knowledge, the presence of trace amounts of kanamycin A in these fermentation broths has not previously been reported.

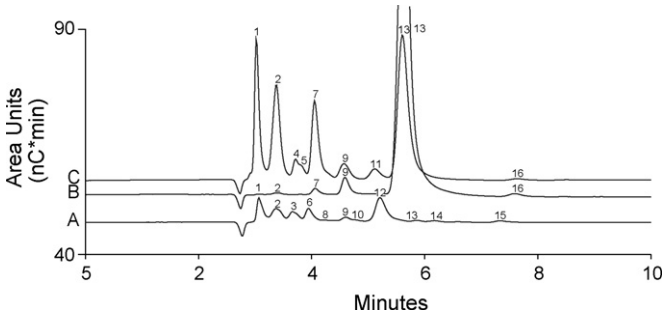

Fig. 2. The comparison of tobramycin process intermediates using HPAE-IPAD with eluent generation, CarboPac PA1 (column #1); 20 μL injections of 5 μg solid/mL. (A) *Streptomyces tenebrarius* fermentation culture, (C) ammonium hydroxide hydrolyzed fermentation culture, (B) crude (partially purified) tobramycin from the ammonium hydroxide hydrolyzed fermentation culture. 1, 3, 4, 5, 8, 14, 15: unknown identity; 2: 2-deoxy-streptamine; 6: carbamoyl-kanamycin B (tentative identification); 7: kanamycin B; 9: neamine (neomycin A); 10: kanamycin A; 11: deoxystreptamine–kanosaminide (tentative identification); 12: carbamoyl-tobramycin (tentative identification); 13: tobramycin; 16: nebramine.

After hydrolysis of the concentrated broth with 3N ammonium hydroxide, which is used in the manufacture of tobramycin, carbamoyl-kanamycin B and carbamoyl-tobramycin known to exist in this sample are converted to kanamycin B, and tobramycin, respectively. Chromatography of this sample shows the appearance of kanamycin B and tobramycin peaks (Fig. 2, chromatogram C, peaks 7 and 13, respectively). For this sample, there were a total of seven major peaks (peaks 1, 2, 4, 7, 9, 11, and 13), which included tobramycin (peak 13), kanamycin B (peak 7), and neamine (peak 9), and 2 minor peaks (peaks 5 and 16).

Purification of the ammonium hydroxide hydrolyzed fermentation broth concentrate by ion-exchange chromatography yields an enriched crude tobramycin product. In this sample we found a significant reduction in the number and amount of impurities (Fig. 2, chromatogram B) when compared to the chromatograms of the fermentation broth and its hydrolyzed concentrate. In this crude product, we observed a total of three major peaks, including tobramycin, kanamycin B, and neamine, and three minor peaks. The two earlier eluting impurities observed in the fermentation broth (and remaining in the hydrolysate), having retention times of 3.0–3.1 (peak 1) and 3.3–3.5 min (peak 2), were almost completely removed by the ion-exchange purification step. The impurity peak at 5.2–5.3 min (peak 11) was also effectively removed during this purification, but the impurity peak at 7.4–7.8 min (peak 16) following tobramycin was not.

We also compared the crude hydrolyzed tobramycin concentrate to two batches of finished product, the result of further crystallization steps (Fig. 3). The chromatograms of the two finished product batches showed significantly lower amounts of the

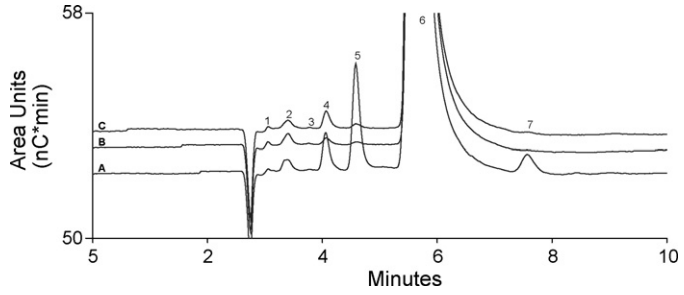

Fig. 3. The comparison of a crude (partially purified) tobramycin batch with two finished batches of tobramycin using HPAE-IPAD with eluent generation, CarboPac PA1 (column #1). (A) Crude batch C060417, the fermentation broth following hydrolysis with ammonium hydroxide, isolation using ion-exchange chromatography, and enrichment by crystallization; (B) finished batch U0511028, and (C) finished batch U0603016 following additional crystallization enrichment steps. The reduction in impurities for the two finished batches compared to the crude sample shows the effectiveness of crystallization for purification. The two batches of finished product show variability in the amount of kanamycin B removed (peak 4), and nebramine (peak 7) during this purification step. 1: unknown identity; 2: 2-deoxy-streptamine; 3: carbamoyl-kanamycin B (tentative identification); 4: kanamycin B; 5: neamine (neomycin A); 6: tobramycin; 7: nebramine.

detected impurity peaks compared to the crude material, and showed differences in the amounts of the impurity peaks. The calculated percentage of the six impurities, expressed as tobramycin peak area equivalents, is presented in Table 1. Peak 2 (see Section 3.2 for identity) was the highest percentage impurity in the finished tobramycin production batch U0511028 (0.30%), while peak 4 (kanamycin B) was the highest in batch U0603016 (0.43%). Both percentages exceeded the 0.15% qualification threshold level recommended in the ICH guidelines for impurities in drug substances [8,9], a threshold where establishing biological safety is required. For the six impurity peaks, only unknown peaks 3 and 7 were found to be consistently below the 0.05% threshold reporting level generally recommended in the ICH guidelines established for new drug substances [8,9].

Based on the chromatograms of in-process materials collected at different manufacturing stages, and the separation of tobramycin from its related substances, this chromatographic method can accurately assess the quality of the tobramycin preparation at different stages of its production and therefore has high process quality-indicating capability. Analysis of these finished commercial batches of tobramycin obtained from Livzon New North River Pharmaceutical Company exhibited nearly identical impurity profiles to material obtained from different commercial sources (USP and Sigma–Aldrich) that we presented in a prior publication [6]. These samples differed only by their relative proportion of impurities, suggesting that this method can be used to assess product quality from multiple manufacturers.

3.2. Impurity peak identification

The in depth study of the chromatograms of the unhydrolyzed fermentation and ammonium hydroxide hydrolyzed samples (Fig. 2) reveals the peak eluting at 3.9 min (peak 6, chromatogram A) in the unhydrolyzed sample disappears after hydrolysis, and a new peak at 4.1 min (peak 7, chromatogram C) appears that has the same retention time as kanamycin B. This suggests that peak 6 (Fig. 2) is carbamoyl-kanamycin B. Similarly, the peak at 5.2 min (peak 12, chromatogram A) is presumed to be carbamoyl-tobramycin and is converted to tobramycin at 5.6 min (peak 13, chromatogram C). Because neither carbamoyl-kanamycin B nor carbamoyl-tobramycin is commercially available, we were unable to confirm these identifications at this time. Confirmation of

Table 1
Identification and percent peak impurities in a crude and two finished batches of tobramycin

Impurity identity	Percent impurity[a]										
	Unknown	2-Deoxy-streptamine	Carbamoyl-kanamycin B[b]	Kanamycin B	Neomycin A	Kanamycin A	Carbamoyl-tobramycin[b]	Deoxystreptamine –kanosamide[b]	Nebramine	Kanosamine	Total
Impurity #[c]	1[d]	2	3	4	5	ND	ND	ND	7	ND	
Retention time (min)	3.0–3.1	3.3–3.5	3.7–3.9	4.0–4.2	4.5–4.6	4.7–4.9	5.1–5.3	5.4–5.5	7.4–7.8	26.5–27.5	
Finished tobramycin production batch U0511028											
Mean	0.069%	0.300%	0.015%	0.153%	0.079%	ND	ND	ND	0.007%	ND	0.624%
S.D.	0.005%	0.022%	0.004%	0.023%	0.005%				0.004%		
Finished tobramycin production batch U0603016											
Mean	0.059%	0.216%	0.016%	0.428%	0.112%	ND	ND	ND	0.032%	ND	0.863%
S.D.	0.017%	0.016%	0.008%	0.015%	0.005%				0.002%		
Crude tobramycin production batch C060417											
Mean	0.065%	0.324%	0.015%	0.918%	2.749%	ND	ND	ND	0.779%	ND	4.851%
S.D.	0.004%	0.027%	0.005%	0.016%	0.039%				0.007%		

[a] Percent impurity based on percent peak area relative to tobramycin peak area.
[b] Peak identity not confirmed.
[c] The impurity peak number, as it appears in Fig. 3.
[d] Peak 1, unkown identity, but probably a mixture of substances. Peak 1 may also be found in water blanks where the injection vials are unwashed prior to use.
ND, not detected in the crude or finished production samples, but detected in fermentation or hydrolyzed samples.

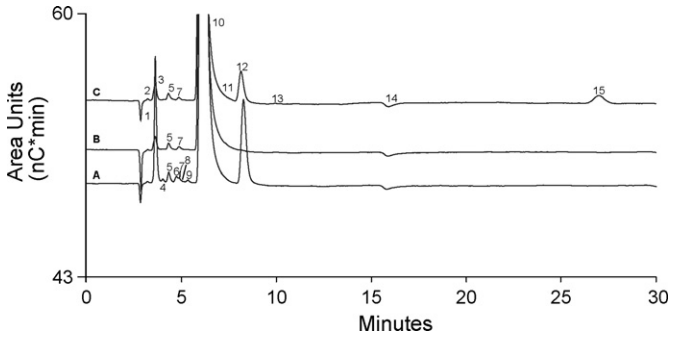

Fig. 4. Comparison of finished tobramycin batch U0511028 with the same material hydrolyzed in base and in acid, pH neutralized, and diluted to 10 μg/mL. (A) Base-hydrolyzed tobramycin (500 mM NaOH, 120 °C, 24 h); (B) tobramycin (unhydrolyzed); (C) acid-hydrolyzed tobramycin (500 mM HCl, 100 °C, 1 h). 1: void; 2, 6, 8, 11, 13: unknown identity; 3: 2-deoxy-streptamine; 4: carbamoyl-kanamycin B (tentative identification); 5: kanamycin B, 7: neamine (neomycin A); 9: deoxystreptamine kanosaminide, 10: tobramycin; 12: nebramine, 14: first baseline dip; 15: kanosamine. *Note*: chromatograms in Figs. 1 and 2 were obtained using a different column (CarboPac PA1 column #1) than in this figure and Fig. 5 (column #2), so the retention times varied slightly.

the peak identities for these compounds would be possible if carbamoyl-kanamycin B and carbamoyl-tobramycin were synthesized and analyzed under these conditions.

The only structural difference between tobramycin and kanamycin B is an additional hydroxyl group in kanamycin B, and the additional hydroxyl group results in less retention. Therefore, we believe that the removal of the same hydroxyl group from neamine, would cause the resulting nebramine to elute later. Furthermore, because tobramycin and kanamycin B have similar degradation pathways; neamine is a degradant of kanamycin B and elutes after kanamycin B, we expect that nebramine, a degradant of tobramycin at the same stage of degradation as the kanamycin B to neamine degradation, to elute later than tobramycin. To confirm this, we performed forced degradations on a finished commercial batch of tobramycin using either extreme high or low pH at elevated temperatures. We analyzed these samples using a different CarboPac PA1 column than what was used for Figs. 2 and 3, and Table 1, and found the retention times were slightly longer. Tobramycin eluted at 6.3 min on the first column (Fig. 3, peak 6), and at 6.5 min (Figs. 4 and 5, peak 10) on the second column (3% increase). The impurity peak following tobramycin increased from 7.6 to 8.4 min (12% increase). Because we were able to obtain the same chromatographic profile for both columns, after re-injecting samples we were able to confidently determine the new retention times for all the previously observed peaks. The acid hydrolysis of finished tobramycin yielded two major peaks, one at 8.4 min and another at 27 min on column #2 (Fig. 4, chromatogram C, peaks 12 and 15, respectively). Brandl and Gu [4] found that tobramycin degrades to nebramine and kanosamine in acidic solution. We also observed that base hydrolysis of finished tobramycin yielded two major peaks, one at 3.8 min and another at 8.4 min (Fig. 4, chromatogram A, peaks 3 and 12, respectively). Brandl and Gu [4] found that tobramycin degrades to nebramine, 2-deoxystreptamine and deoxystreptamine–kanosaminide in basic solution. Because nebramine was common to both degradation pathways, we concluded the peak at 8.4 min (peak 12, chromatograms A and C) was nebramine, eluting after tobramycin as expected.

Peak 2 in the finished tobramycin product (retention time 3.3–3.5 min, Table 1), which also exists in the fermentation broth, is also a base-induced degradant of tobramycin. We identify this peak as 2-deoxystreptamine because it is the only known factor in the *S. tenebrarius* fermentation broth [5] that is also a degradant of tobramycin and that has not been previously identified [4]. Furthermore, 2-deoxystreptamine contains two amine-functional groups that are expected to have electrochemical response using the detection waveform selected for this method.

The acid hydrolysis of tobramycin yields two degradation products, nebramine and kanosamine. Because only two major degradation peaks were observed, and one was identified as nebramine, we believe the remaining peak at 27 min (Fig. 4, chromatogram C, peak 15) is kanosamine.

As previously discussed, base hydrolysis of tobramycin should yield three degradation products, nebramine, 2-deoxystreptamine and deoxystreptamine–kanosaminide, but only two major degradation peaks were observed. Both nebramine and 2-deoxystreptamine peaks were previously identified as discussed above. After close inspection of the base-hydrolyzed chromatogram (Fig. 4, chromatogram A), we found one new minor peak (peak 9) and two minor peaks with increased peak areas (peaks 4 and 7) compared with the starting material (Fig. 4, chromatogram B). Peaks 4 and 7 were also found in the chromatogram of the hydrolysis blank (heated NaOH without tobramycin, Fig. 5, chromatogram A), while peak 9 was absent. A peak with the same retention time as peak 9 was also observed in the ammonium hydroxide

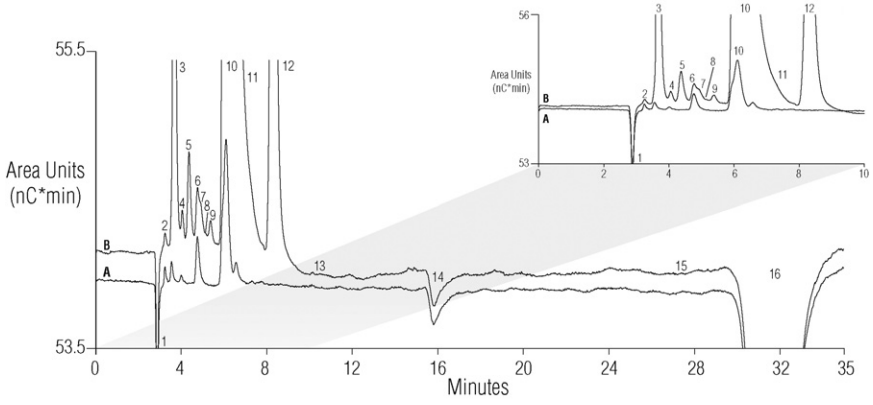

Fig. 5. The comparison of base hydrolyzed 10 μg/mL tobramycin (finished batch U0511028) (chromatogram B) with blank hydrolysis control (chromatogram A) to explain the deoxystreptamine–kanosaminide position. 1: void, 2, 4, 6, 8, 11, 13: unknown identity, 3: 2-deoxy-streptamine, 5: kanamycin B, 7: neamine (neomycin A), 9: deoxystreptamine kanosaminide, 10: tobramycin, 12: nebramine, 14: first baseline dip, 15: kanosamine, 16: second baseline dip. Peaks observed in the blank hydrolysate (chromatogram A) result from trace vial or reagent contaminants produced during base hydrolysis, except peak 10 (chromatogram A) is a carryover tobramycin peak from a previous injection. *Note*: chromatograms in Figs. 1 and 2 were obtained using a different column (CarboPac PA1 column #1) than in this figure and Fig. 4 (column #2), so the retention times varied slightly.

hydrolysate of fermentation broth (Fig. 2, chromatogram C, peak 11) before purification. Therefore, we tentatively assign peak 9 (Figs. 4 and 5), and the peak 11 (Fig. 2) with retention time 5.4 min to be deoxystreptamine–kanosaminide.

Impurity peak 1 (Figs. 2 and 3), resolved from the column void volume by about 0.6 min, was not identified. Our experience with injections of water blanks, with and without pre-rinsing sample injection vials and other labware prior to their use has indicated that this early peak may be reduced or eliminated. We have also determined that at lower eluent concentrations this peak can partially resolve into several peaks (results not shown), indicating it is a mixture of substances, some of which are not derived from the sample. Although we included peak 1 in Table 1, and its percent peak area exceeded 0.05%, we are not convinced its measure is meaningful for quality assessments, and further studies relating this peak area to a meaningful substance that elutes with peak 1 would be required before adopting specifications for this peak.

4. Conclusion

Based on comparison of chromatograms for tobramycin materials collected during different stages in the manufacturing process, we conclude that this chromatographic method can accurately assess the quality of tobramycin during in-process production. Our analysis of these finished commercial batches of tobramycin exhibited nearly identical impurity profiles using material obtained from three different commercial sources, but found they differed in their relative proportion. Combining results for the chromatographic peaks found in fermentation samples, and tobramycin material hydrolyzed at both high and low extreme pH, we identified peaks for kanamycin B, neamine, nebramine, kanosamine, and 2-deoxystreptamine; all impurities of crude and finished tobramycin. We also tentatively identified peaks for kanamycin A, deoxystreptamine–kanosaminide, carbamoyl-kanamycin B, and carbamoyl-tobramycin; impurities of *S. tenebrarius* fermentation broths and their hydrolyzed derivatives.

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