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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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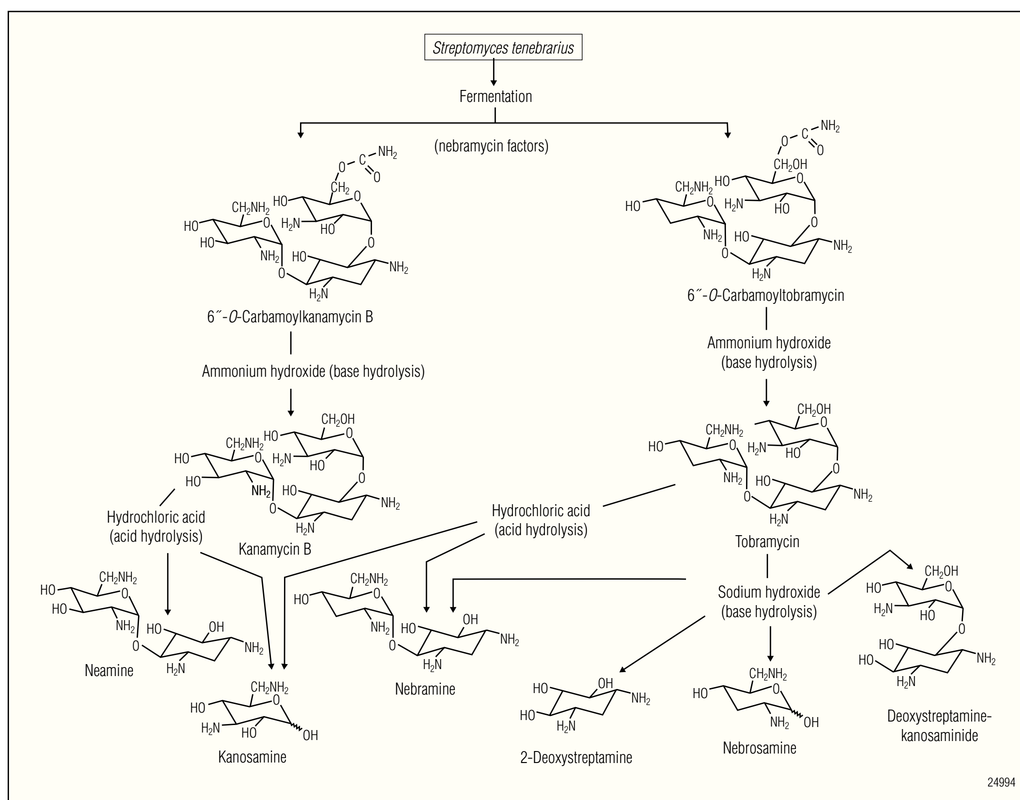

Figure 1. The chemical structures of tobramycin, process intermediates, and degradation products.

INTRODUCTION

Commercial-scale fermentation to manufacture tobramycin is carried out with *Streptomyces tenebrarius.* Impurity profiling during pharmaceutical production is important for evaluating the effectiveness of a processing step and meeting regulatory requirements. High-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAE-IPAD) is a highly sensitive method used to assay tobramycin and to assess purity. Currently, no published work evaluates 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. In this study, 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 identify 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 reproduce, and on previously known related substances found in *S. tenebrarius* fermentation broth (Figure 1). In crude and final tobramycin products, we identify the peaks for neamine, kanamycin B, nebramine, kanosamine, 2-deoxystreptamine. We tentatively identify 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. Our results support the use of the HPAE-IPAD method (Figure 2) for effective in-process impurity profiling of tobramycin, and as a stability-indicating technique after product purification.

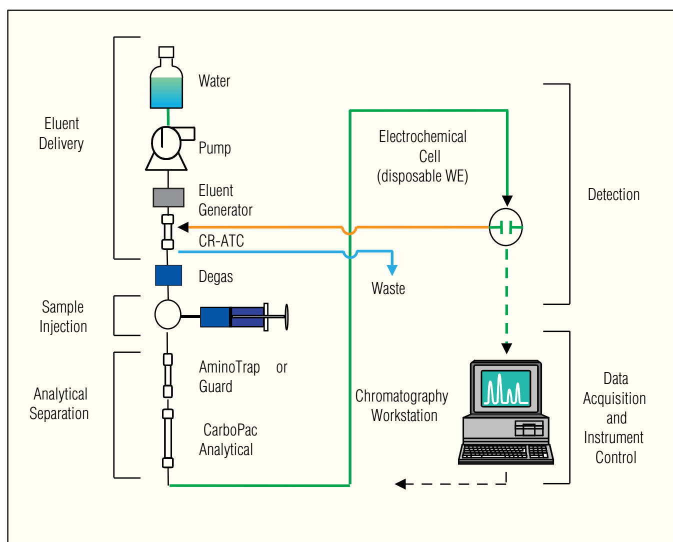

Figure 2. The Reagent-Free™ IC (RFIC) HPAE-IPAD system used for aminoglycoside determinations, consisting of the biocompatible non-corrosive IC, CarboPac PA1 column set, eluent generator (EluGen KOH), Continuously Regenerating Anion Trap Column (CR-ATC), and electrochemical detector.

EXPERIMENTAL

Equipment

The chromatography system consisted of Dionex instruments:

Chromeleon® Chromatography Workstation

Gradient pump with degas option and GM-4 gradient mixer

Eluent Generator with EGC II KOH eluent generator cartridge (EluGen® II Hydroxide)

CR-ATC

Vacuum degas conversion kit

Autosampler

Thermal Compartment

Electrochemical Detector

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 s to 0.46 s, +0.33 V from 0.47 s to 0.56 s, -1.67 V from 0.57 s 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.

Chromatography

Columns:	CarboPac® PA1 Analytical Column, 4 × 250 mm CarboPac Guard Column, 4 × 50 mm
Flow Rate:	0.50 mL/min
Temperature:	30 °C.
Injection Volume:	20 µL (full loop injection mode).
Reference Electrode Mode:	pH
Eluent:	2.00 mM KOH (by eluent generation)
Run Time:	35 min

RESULTS AND DISCUSSION

Fermentation and purification process assessment

Commercial-scale fermentation broth for production of tobramycin using *S. tenebrarius* contains culture medium, and a nebramycin complex, consists primarily of carbamoyl-kanamycin B, carbamoyl-tobramycin, and trace levels of kanamycin B and tobramycin (Figure 1). HPAE-IPAD analysis of this sample (Figure 3, chromatogram A) confirms the presence of only trace levels of kanamycin B and tobramycin. This chromatogram also revealed six major peaks and four minor peaks, instead of three major factors described by Stark et al.[1] We found that neamine (neomycin A, peak 9, a degradation component of kanamycin B) is present at a relatively low concentration in this fermentation broth (Figure 1). 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.

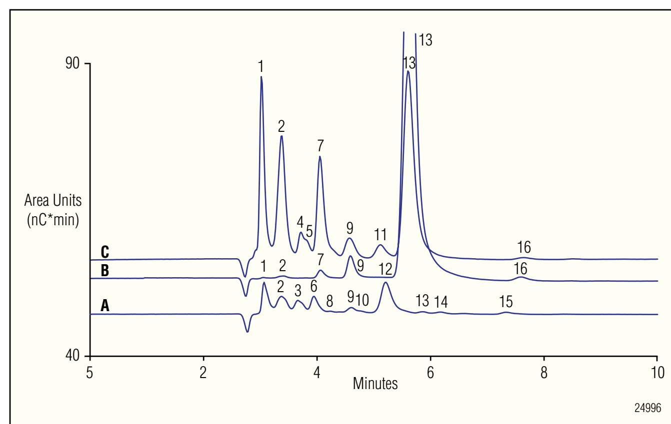

Figure 3. The comparison of tobramycin process intermediates using HPAE-IPAD with eluent generation, CarboPac PA1 (column #1); 20 µL injections of 5 µg solid/mL. (A) Streptomyces tenebrarius *fermentation culture, (C) ammonium hydroxide hydrolyzed fermentation culture, (B) crude (partially purified) tobramycin from the ammonium hydroxide hydrolyzed fermentation culture. Peaks: 1, 3– 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 3 N ammonium hydroxide, which is used in the manufacture of tobramycin, the carbamoyl-kanamycin B and carbamoyl-tobramycin are converted to kanamycin B, and tobramycin, respectively (Figure 1). Chromatography of this sample shows the appearance of kanamycin B and tobramycin peaks (Figure 3, chromatogram C, peaks 7 and 13, respectively). For this sample, we observed a total of 7 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 yielded an enriched crude tobramycin product. In this sample we observed a significant reduction in the number and amount of impurities (Figure 3, chromatogram B) when compared to the chromatograms of the fermentation broth (chromatogram A) and its hydrolyzed concentrate (chromatogram C). In this crude product, we observed a total of four major peaks, including tobramycin, kanamycin B, and neamine, and three minor peaks.

We also compared the crude hydrolyzed tobramycin concentrate to two batches of finished product, the result of further crystallization steps (Figure 4). The chromatograms of the two finished product batches showed significantly lower amounts of the detected impurity peaks compared to the crude material, and showed differences in the amounts of the impurity peaks.

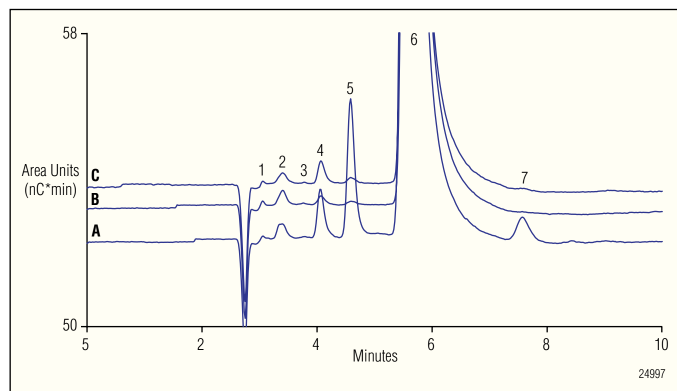

Figure 4. The comparison of a crude (partially purified) tobramycin batch with two finished batches of tobramycin using HPAE-IPAD with eluent generation, CarboPac PA1 (column #1). (A) Crude batch C060417, the fermentation broth following hydrolysis with ammonium hydroxide, isolation using ion-exchange chromatography, and enrichment by crystallization; (B) finished batch U0511028, and (C) finished batch U0603016 following additional crystallization enrichment steps. The 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.

Table 1 lists the calculated percentage of the six major impurities, expressed as tobramycin peak area equivalents. Peak 2 was the highest percentage impurity in the finished tobramycin production batch U0511028 (0.30%), while peak 4 (kanamycin B) was highest in batch U0603016 (0.43%). For the six impurity peaks detected in the finished batch, only unknown peaks 3 and 7 were found to be consistently below 0.05%.

Table 1

	Percent Impurity†										
Impurity Identity	**Unknown**	**2-Deoxy-streptamine**	**Carbamoyl-kanamycin B****	**Kanamycin B**	**Neomycin A**	**Kanamycin A**	**Carbamoyl-tomramycin****	**Deoxystreptamine-kanosamide****	**Nebramine**	**Kanosamine**	
Impurity # ‡	1*	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	**Total**
Finished Tobramycin Production Batch U0511028											
Mean	0.069%	0.300%	0.015%	0.153%	0.079%	ND	ND	ND	0.007%	ND	**0.624%**
SD	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%**
SD	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%**
SD	0.004%	0.027%	0.005%	0.016%	0.039%				0.007%		

*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.
**Peak identity not confirmed.
†Percent impurity based on percent peak area relative to tobramycin peak area.
‡The impurity number is the peak number as it appears in Figure 4.
ND, not detected in the crude or finished production samples, but detected in fermentation or hydrolyzed samples

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) and a prior publication.[2] These samples differed only by their relative proportion of impurities, suggesting that this method can be used to assess product quality from multiple manufacturers.

Impurity peak identification

A review of the chromatograms of the unhydrolyzed fermentation and ammonium hydroxide hydrolyzed samples (Figure 3) 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 (Figure 3) 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 are commercially available, we are unable to confirm these identifications at this time.

The only structural difference between tobramycin and kanamycin B is an additional hydroxyl group in kanamycin B (Figure 1), 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 (Figure 1); 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. Brandl and Gu found that tobramycin degrades to nebramine and kanosamine in acidic solution[3], and we observed two major peaks, one at 8.4 min and another at 27 min on column #2 (Figure 6, chromatogram C, peaks 12 and 15, respectively). Brandl and Gu also found that tobramycin degrades to nebramine, 2-deoxystreptamine and deoxystreptamine-kanosaminide in basic solution[3], and we observed two major peaks, one at 3.8 min and another at 8.4 min (Figure 5, chromatogram A, peaks 3 and 12, respectively). Because nebramine was common to both degradation pathways (Figure 1), we concluded the peak at 8.4 min (peak 12, chromatograms A and C) was nebramine, eluting after tobramycin as expected.

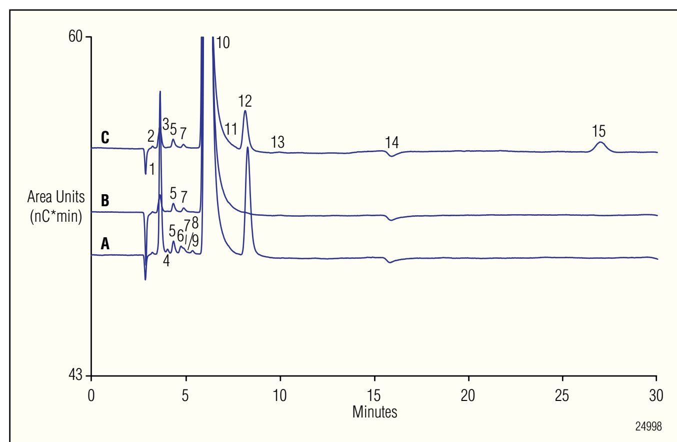

Figure 5. Comparison of finished tobramycin batch U0511028 with the same material hydrolyzed in base and in acid, pH neutralized, and diluted to 10 µg/mL. (A) base-hydrolyzed tobramycin (500 mM NaOH, 120 °C, 24 h); (B) Tobramycin (unhydrolyzed); (C) acid-hydrolyzed tobramycin (500 mM HCl, 100 °C, 1 h). 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 Figures 3 and 4 were obtained using a different column (CarboPac PA1 column #1) than in Figure 5 and 6 (column #2), so the retention times varied slightly.

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[4] that is also a degradant of tobramycin and that has not been previously identified.[3] 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 (Figure 1). Because only two major degradation peaks were observed, and one was identified as nebramine, we believe the remaining peak at 27 min (Figure 5, chromatogram C, peak 15) is kanosamine.

Base hydrolysis of tobramycin should yield three degradation products, nebramine, 2-deoxystreptamine and deoxystreptamine-kanosaminide (Figure 1), 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 (Figure 5, 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 (Figure 5, chromatogram B). Peaks 4 and 7 were also found in the chromatogram of the hydrolysis blank (heated NaOH without tobramycin, Figure 6, chromatogram A), while peak 9 was absent. A peak with the same retention time as peak 9 was also observed in the ammonium hydroxide hydrolysate of fermentation broth (Figure 3, chromatogram C, peak 11) before purification. Therefore, we tentatively assign peak 9 (Figures 5 and 6), and the peak 11 (Figure 3) with retention time 5.4 min to be deoxystreptamine-kanosaminide.

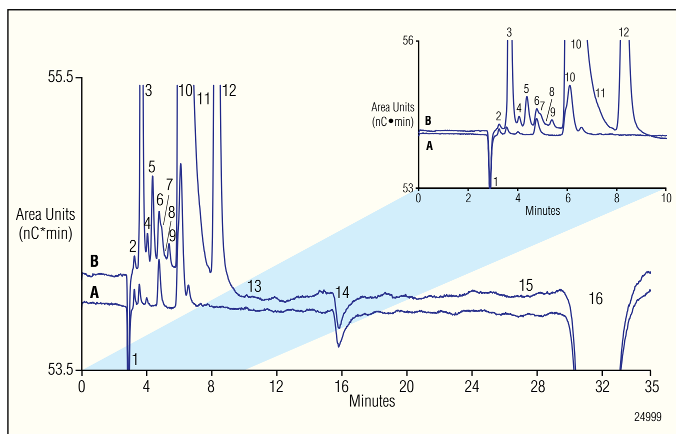

Figure 6. The comparison of base hydrolyzed 10 µg/mL tobramycin (finished batch U0511028) (chromatogram B) with blank hydrolysis control (chromatogram A) to explain the deoxystreptamine-kanosaminide position. 1) void; 2, 4, 6, 8, 11, 13) unknown identity, 3) 2-deoxy-streptamine, 5) kanamycin B, 7) neamine (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 Figures 3 and 4 were obtained using a different column (CarboPac PA1 column #1) than in Figure 5 and 6 (column #2), so the retention times varied slightly.

Impurity peak 1 (Figures 3 and 4), resolved from the column void volume by about 0.6 min, was not identified. This early peak is sometimes reduced or eliminated, based on our experience with injections of water blanks, with and without prerinsing sample injection vials and other labware prior to their use. We 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.

CONCLUSION

- HPAE-IPAD can accurately assess tobramycin in-process production quality and in combination with standards and forced degradation studies be used to identify impurities.
- Kanamycin B, kanamycin A, and neamine peaks were identified using commercially available standards.
- Nebramine, kanosamine, and 2-deoxystreptamine peaks were identified using acid and base degradation studies combined with previously published degradation pathways.
- Carbamoyl-kanamycin B, and carbamoyl-tobramycin peaks were tentatively identified in fermentation broth using a base degradation study combined with previously published compositions of *S. tenebrarius* cultured broth. We differentiated between these two peaks based on their predicted chromatographic elution order on the CarboPac PA1 column.
- Deoxystreptamine-kanosamide peak was tentatively identified by process of elimination.
- These compounds were observed in *S. tenebrarius* fermentation broth, and/or crude and finished tobramycin products, and/or the degradation products of high and low extreme pH treatments.

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