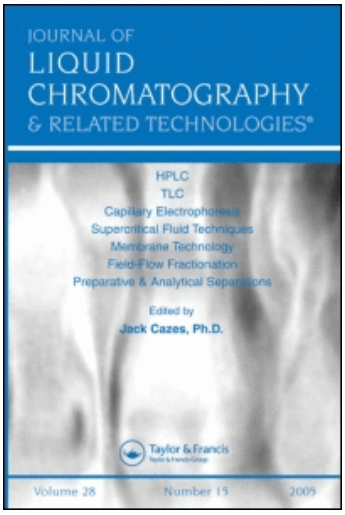

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Development and Validation of a Stability-Indicating HPLC Method for the Determination of Tobramycin and Its Related Substances in an Ophthalmic Suspension

H. Russ[a]; D. McCleary[a]; R. Katimy[a]; J. L. Montana[a]; R. B. Miller[a]; R. Krishnamoorthy[a]; C. W. Davis[a]
[a] Bausch and Lomb Pharmaceuticals, Inc, Research & Development, Parkway, Tampa

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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF TOBRAMYCIN AND ITS RELATED SUBSTANCES IN AN OPHTHALMIC SUSPENSION

H. Russ,[1,*,†] D. McCleary,[1] R. Katimy,[1] J. L. Montana,[1,‡]
R. B. Miller,[1,‡] R. Krishnamoorthy,[1] C. W. Davis[1,‡]

[1] Bausch and Lomb Pharmaceuticals, Inc.
Research & Development
8500 Hidden River Parkway
Tampa, FL 33637

ABSTRACT

An HPLC method was developed and subsequently validated for the quantitation of tobramycin and its related substances in an ophthalmic suspension. Current USP methodology for the assay of tobramycin involves pre-column derivatization with 2,4-dinitrofluorobenzene. The USP assay method was modified to encompass the determination of two known impurities (neamine and kanamycin) and a degradation product (nebramine). The method development involved evaluation of several factors including derivatization parameters (time, temperature, and acid concentration), mobile phase composition, column choice and temperature, wavelength evaluation, and response factors.

The optimized conditions for tobramycin and its related substances include derivatizing standards and samples for 20 minutes at 70°C with a 0.8 mM sulfuric acid. Chromatographic parameters include a mobile phase of acetonitrile / buffer (55/45; v/v) and a Nova-Pak C_{18}, 3.9 x 150 mm column, maintained under

ambient conditions. The wavelength of choice to maximize the detection of the related substances was 365 nm. Relative response factors for the impurities, neamine and kanamycin, and the degradation product, nebramine, were determined to be 1.00, 1.00, and 1.37 respectively.

The described method is linear, reproducible, accurate, and selective over a range of 0.1%-150% of its label claim (3 mg/mL). The method precision, relative standard deviation (RSD), among 6 independent samples was not more than 0.9% for the assay and not more than 19.7% for the related substances. The intermediate precision was 1.0% (n=18) for the assay and 15.5% (n=11) for the related substances. The mean absolute recovery of tobramycin between 25 - 150% label claim was 99.6% with an RSD of 1.2% (n=6), while between 0.1 - 10% label claim was 100.7% with an RSD of 9.1% (n=5). Selectivity was evaluated by subjecting the ophthalmic suspension to thermal, acidic, basic, oxidative, and UV stress conditions. No interference in the analysis of degradation products and impurities was observed. Consequently, the validated method for the determination of tobramycin and its related substances in the ophthalmic suspension is regarded as stability-indicating.

INTRODUCTION

Tobramycin is a topical antibiotic indicated in the treatment of eye infections. Like other aminoglycosides, the bactericidal activity of tobramycin is accomplished by specific inhibition of normal protein synthesis in susceptible bacteria. Tobramycin is one of the active ingredients in this ophthalmic suspension.

Current USP methodology was investigated and subsequently optimized to incorporate both the assay and the related substances.[1] The analysis of tobramycin has been performed by microbiological assay,[1] thin layer chromatography,[1] and HPLC[2-5] involving pre-column derivatization with 2,4-dinitrofluorobenzene. Another pre-column derivatization method was accomplished using o-phthalaldehyde and fluorescence detection.[6] These methods provide an adequate assay method of tobramycin. However, none of these methods were adequate enough to provide the ruggedness, selectivity, and stability-indicating capability that this method provides for tobramycin related substances.

This manuscript describes the development and validation of an isocratic reversed-phase HPLC method. The development required investigating derivatization parameters (time, temperature, and sulfuric acid concentration), mobile phase composition, column choice and temperature, wavelength, and response factors. The validated method is sensitive, accurate, reproducible, and stability indicating for the determination of tobramycin and its related substances in an ophthalmic suspension.

EXPERIMENTAL

Chemical & Reagents

The ophthalmic suspension was formulated at Bausch & Lomb Pharmaceuticals, Inc., Tampa, FL, USA. Tobramycin was a USP reference standard. 2,4-dinitrofluorobenzene, tris(hydroxymethyl)aminomethane and dimethylsulfoxide were purchased from Aldrich Chemical Company, Milwaukee, WI, USA. Dimethysulfoxide, reagent alcohol, sulfuric acid, sodium hydroxide, hydrochloric acid, HPLC grade water, and hydrogen peroxide were purchased from Fisher Chemical Company, Fairlawn, NJ, USA. HPLC grade acetonitrile was purchased from Burdick & Jackson, Muskegon, MI, USA. The water was deionized and distilled by the Milli-Q® Water System (Millipore Corporation, Bedford, MA, USA).

Apparatus

The chromatographic system consisted of an HPLC solvent pumping system, variable wavelength UV-visible detector set as 365 nm, variable volume injector, and Waters Millenium PDA software (Version 2.1) (Waters Associates, Milford, MA, USA) for integration.

A Waters Nova-Pak C_{18} column (3.9 x 150 mm) Waters Associates (Milford, MA, USA) was maintained at ambient temperature. The flow rate was approximately 1.5 mL/min with a typical operating pressure of 1500 psi.

Preparation of Solutions

Mobile phase

Dissolve 2.0 g of tris (hydroxymethyl)aminomethane in 960 mL water, add 20 mL 1N sulfuric acid and add 1200 mL of acetonitrile. Mix and filter through a 0.5 μm filter and degas.

Standard preparation

Accurately weigh Tobramycin, USP Reference Standard and dilute to volume with water to yield a concentration of 0.24 mg/mL. The solution needs to contain 10 mM sulfuric acid prior to derivatization.

Sample preparation

Accurately transfer the ophthalmic suspension (label claim 3 mg/mL) and dilute to volume with water to yield a concentration of 0.24 mg/mL. The solution needs to contain 10 mM sulfuric acid prior to derivatization.

Derivatization procedure

Into separate 50-mL volumetric flasks, transfer 4.0 mL of the standard preparation, 4.0 mL of the sample preparation, and 4.0 mL of water to serve as the blank. To each flask, add 10 mL of 2,4-dinitrofluorobenzene reagent (10 mg/mL in reagent alcohol), and 10 mL of tris (hydroxymethyl) aminomethane reagent (15 mg/mL in water/dimethylsulfoxide, 20/80; v/v) and mix. Place the stoppered flasks in a constant temperature bath at 70°C ± 2°C and heat for 20 minutes.

Remove the flasks from the bath, and allow the flasks to slightly cool for two (2) minutes. Add acetonitrile to about 2 mL below the 50 mL mark and swirl to mix the solution. Allow to cool to room temperature, then dilute to volume with acetonitrile.

System Suitability

The system suitability results were calculated according to the USP 23 <621> from typical chromatograms.[7] The instrument precision, as determined by six injections of the standard preparation, should provide a relative standard deviation of NMT 2.0. The tailing factor should not exceed 2.0 at 5% peak height. The resolution between tobramycin and nebramine should not be less than 2.0.

Specificity

The specificity of the method was studied through analysis of a control solution (unstressed ophthalmic suspension), a sample solution (stressed finished product ophthalmic suspension), and a stressed placebo solution (finished product without tobramycin). The finished product was subjected to thermal, acidic, basic, oxidative, and ultraviolet light environments for a set

period of time or until tobramycin degraded up to 30%, as determined by peak area percent. Four mL aliquots of the control solution, sample solution, and placebo solution were sealed in 50-mL volumetric flasks. The sample solution and placebo solution were exposed to various stress conditions. Thermal stressed samples were stored at 60°C. Acid stressed samples were achieved by adding 2.0 mL of 1N hydrochloric acid and heating at 60°C. Based stressed samples were achieved by adding 1.0 mL of 1N sodium hydroxide and heating at 60°C. Oxidative stressed samples were achieved by adding 1.0 mL of 3% hydrogen peroxide and heating at 60°C. Ultraviolet light stress samples were exposed to 254 and 365 nm.

Data Acquisition

The peak areas of tobramycin and its related substances were measured using Waters Millenium PDA software Version 2.1 (Waters Associates). The chromatographic data was automatically processed for peak area.

RESULTS AND DISCUSSION

Method Development

To obtain the best overall chromatographic conditions, the mobile phase was optimized by changing the composition of acetontrile and tris buffer. Furthermore, three different columns were evaluated; a Waters C_{18} μBondapak (3.9 x 300 mm, 10 μm, Waters Associates), a Beckman ODS Ultrasphere (4.6 x 250 mm, 5 μm, Beckman Instruments, Inc., Fullerton, CA), and a Waters C_{18} Novapak (3.9 x 150 mm, 4 μm, Waters Associates).

The optimal mobile phase composition was determined to be acetonitrile/tris buffer (55/45; v/v). The Waters C_{18} Nova-Pak column provided the best resolution with the shortest chromatographic run time and, consequently, was selected as the column of choice. Temperature studies of 25°C (ambient), 30°C, and 40°C had little effect on the assay of tobramycin and the related substances. Thus, an unthermostated condition is recommended for routine use. To determine the robustness of the derivatization process, samples were incubated at 60°, 65°, 70°, and 75°C and for various timed intervals ranging from 10 to 60 minutes (Table 1).

Therefore, over the ranges tested for time and temperature, there was no significant difference in recovery of tobramycin and its related substances. The greatest percent difference in the assay was 1.5% (excluding the 10 minute,

Table 1

Effect of Time and Temperature on the Determination of Tobramycin and its Related Substances

	Tobramycin Assay Results (n=16)			
	10 min.	**20 min**	**30 min**	**60 min**
60°C	95.9%	98.5%	98.4%	99.5%
65°C	98.2%	96.1%	98.4%	98.3%
70°C	97.0%	98.4%	97.1%	98.1%
75°C	97.3%	97.3%	97.4%	97.1%
	Related Substances Results at 60°C (n=12)			
	10 min	**20 min**	**30 min**	**60 min**
Neamine	0.01%	0.07%	0.04%	0.08%
Kanamycin	0.19%	0.22%	0.20%	0.22%
Nebramine	0.02%	0.04%	0.03%	0.04%
	Related Substances Results at 65°C (n=12)			
	10 min	**20 min**	**30 min**	**60 min**
Neamine	n/d	n/d	n/d	n/d
Kanamycin	0.19%	0.20%	0.25%	0.20%
Nebramine	0.06%	0.02%	0.05%	0.03%
	Related Substances Results at 70°C (n-12)			
	10 min	**20 min**	**30 min**	**60 min**
Neamine	n/d	n/d	n/d	n/d
Kanamycin	0.21%	0.22%	0.26%	0.23%
Nebramine	0.06%	0.03%	0.02%	0.05%
	Related Substances Results at 75°C (n=12)			
	10 min	**20 min**	**30 min**	**60 min**
Neamine	n/d	n/d	n/d	n/d
Kanamycin	0.20%	0.20%	0.20%	0.20%
Nebramine	0.03%	0.04%	0.04%	0.06%

n/d = not detected.

Table 2

Assay Precision of Tobramycin at 100% Label Claim*

Run	Individual Assay Values (%LC)	Average Assay (n=6) (%LC)	RSD (%)
	96.0		
1	95.8		
Chemist I	97.3	96.0	0.9
HPLC System 1	96.3		
R&D	96.2		
	94.6		
	97.5		
2	98.4		
Chemist I	98.3	96.9	0.3
HPLC System 2	98.0		
R&D	98.3		
	97.8		
	97.2		
3	96.3		
Chemist II	97.2	96.9	0.4
HPLC System 2	96.8		
R&D	96.7		
	97.1		
Intermediate Precision (n=18)		97.0	1.0

* Acceptance Criteria: RSD NMT 2.0%

60°C time period, which was most probably underderivatized). The 1.5% difference is within the acceptance criteria of 98.0 - 102.0%. Based on the data in Table 1, a temperature of 70°C and a time period of 20 minutes was selected for routine use.

Changes in the sulfuric acid concentrations were investigated to optimize the derivatization of tobramycin. A range of 0.03 mM to 10.4 mM sulfuric acid (final concentration) was examined. The concentration of tobramycin remained

Table 3

Precision of Tobramycin at 0.1% Label Claim (Related Substance Level)*

Run	Individual Assay Values (%LC)	Average Assay (n=6) (%LC)	RSD (%)
1	85.3		
Chemist I	53.7		
HPLC System 1	87.4	82.0	19.7%
R&D	89.7		
	94.0		
	63.5		
2	75.6		
Chemist I	87.4	78.6	12.4
HPLC System 2	85.2		
R&D	72.3		
	87.4		
Intermediate Precision (n=11)		80.1	15.5

* Acceptance Criteria: RSD NMT 25%

constant with a final concentration of at least 0.8 mM sulfuric acid. Thus, a final concentration of 0.8 mM sulfuric acid was recommended for the method. Typically, the use of a solution containing 10 mM sulfuric acid prior to derivatization when diluted during the course of sample preparation would result in a final concentration of 0.8 mM.

Wavelength Determination

Photo diode array detection was investigated from 335 to 375 nm. Although tobramycin has a greater area response at 355 nm, the related substances exhibit greater response at 365 nm. Therefore, a wavelength of 365 nm was selected.

Injection Volume Determination

Injection volume was investigated to determine its effect on detection at 0.1% label claim. Method development was performed with an injection volume of 20 μL, however, determination of a 0.1% label claim preparation was bordering on the limit of quantitation. Hence, a 30 μL injection was selected for validation because it can routinely quantitiate at the 0.1% level.

Relative Response Factor

Relative response factors (RRF) for neamine, kanamycin, and nebramine were determined in relation to tobramycin. If the RRF fell within 0.80 - 1.20, a factor of 1.00 was assigned, else the RRF would be specifically determined. Neamine and kanamycin resulted in a response factor of 1.00, while 1.37 was applied to nebramine.

VALIDATION

Precision

The precision (repeatability and intermediate precision) of the method was determined from one lot of finished product.

Repeatability

Six sample preparations were analyzed in a single session by Chemist I, with HPLC System 1. The RSD of the six results for the tobramycin assay was 0.9% (Limit not more than (NMT) 2.0%).

At 0.1% label claim (related substance level), five sample preparations were analyzed in a single session by Chemist I, with HPLC system 1. The RSD of the five results was 19.7% (RSD Limit NMT 25%).

Intermediate precision

Intermediate precision was evaluated using Chemist I, with HPLC System 2, to independently analyze another six sample preparations from the finished product and another six sample preparations on another day. The intermediate precision was 1.0% (n=18) for the assay and 15.5% (n=11) for the related substances.

Table 4

Accuracy of Tobramycin in Assay Range*

% Claim of Sample	Recovery (%) n= 6
25	97.6
50	98.9
75	99.4
100	100.3
125	100.7
150	100.6
Overall	99.6
% RSD	1.2

* Acceptance Criteria: ≥25% L.C.: 97.0% - 103%

Table 5

Accuracy of Tobramycin in Related Substances Range*

% Claim of Sample	Recovery (%) n= 6
0.1	102.6
0.25	115.2
0.5	99.5
1.0	91.6
10	94.4
Overall	100.7
% RSD	9.1

* Acceptance Critera: ≥10% L.C.: 75% -125%

The RSD limit for the assay level is NMT 2.0% and the limit for the related substances in NMT 25%. The average results are within the established limits as stated above. The low scatter in the data supports the high degrees of robustness for this analytical method (Tables 2 and 3).

Table 6

Specificity Results

Stress Condition	Peak Homogeneity Criteria: Angle≥ Threshold	Peak Homogenous (Yes/No)	Percent Degradation
Tobramycin USP Reference Std 25°C	0.03<1.02	Yes	N/A
Control 25°C	0.03<1.02	Yes	N/A
Thermal 60°C, 69 hours	0.03<1.02	Yes	3.4%
Acid 60°C, 69 hours	0.03<1.02	Yes	6.5%
Base 60°C, 69 hours	0.03<1.02	Yes	3.4%
Peroxide 60°C, 69 hours	0.03<1.02	Yes	22.1%
UV Light 69 hours	0.03<1.02	Yes	0.0%

Accuracy

The accuracy of the method was shown by analyzing placebo solutions spiked with known amounts of tobramycin. The accuracy met the acceptance criteria in both the assay and related substance ranges (Tables 4 and 5).

Specificity

Tobramycin ophthalmic suspension was stressed by thermal, acidic, basic, oxidative, and ultraviolet light for 69 hours. The results of the stress studies are

Table 7

Linearity*

% Label Claim	Acutal Concentration (μg/mL)	Average Peak Area Response (n=2)
150	26.476	4317493
125	22.063	3588450
100	17.651	2851067
75	13.238	2131329
50	8.825	1421275
25	4.413	701317
10	1.765	275305
1	0.177	28785
0.5	0.088	12977
0.25	0.044	613
0.1	0.017	1785

slope, m = 162748561
y-intercept, b = -7787.3
correlation coefficient, r = 1.000
% Bias = -0.2%

* Coefficient of correlation acceptance criteria: NLT 0.999; Bias

presented in Table VI. No interfering peaks at the retention time of tobramycin were observed in any of stressed samples. The control sample, stress samples and placebo samples were analyzed using an HPLC equipped with a Waters 996 photo-diode array system.

The tobramycin peak was determined to be homogenous since the purity angle ≤ the purity threshold (Table 6).

Linearity

A linear response in peak area for tobramycin over the range of 0.1% to 150% of the finished product label claim was observed. The correlation coefficient was 1.000 and the percent bias was -0.2% (Table 7).

Table 8

Stability of Solutions*

Preparation	Time (Hours)	% Assay	% Change
Standard	0	100.0	0.0
	24	994	0.6
	168	100.8	0.8
	192	101.7	1.7
Sample	0	96.6	0.0
	72	98.6	2.1

* Acceptance Criteria: Stable over the interval where the percent change from zero time is within ± 2%.

Table 9

Limit of Quantitation*

ID	Peak Areas	Avg. Area	RSD %	S/N	Avg. S/N
0.1% Label Claim Prep.	3630	3491	9.0	5.1	5.6
	3477			4.9	
	3996			4.7	
	3184			7.1	
	3136			5.8	
	3520			5.7	

* Acceptance Criteria: RSD NMT 10% and S/N NLT 5.

Range

The range of the method has been set at 0.1 to 150% tobramycin (3 mg/mL label claim) since the method has been shown to be precise, accurate, and linear within this region.

Stability of Solutions

The stability of analytical solutions was determined for the standard preparation and the sample preparation at room temperature. The standard

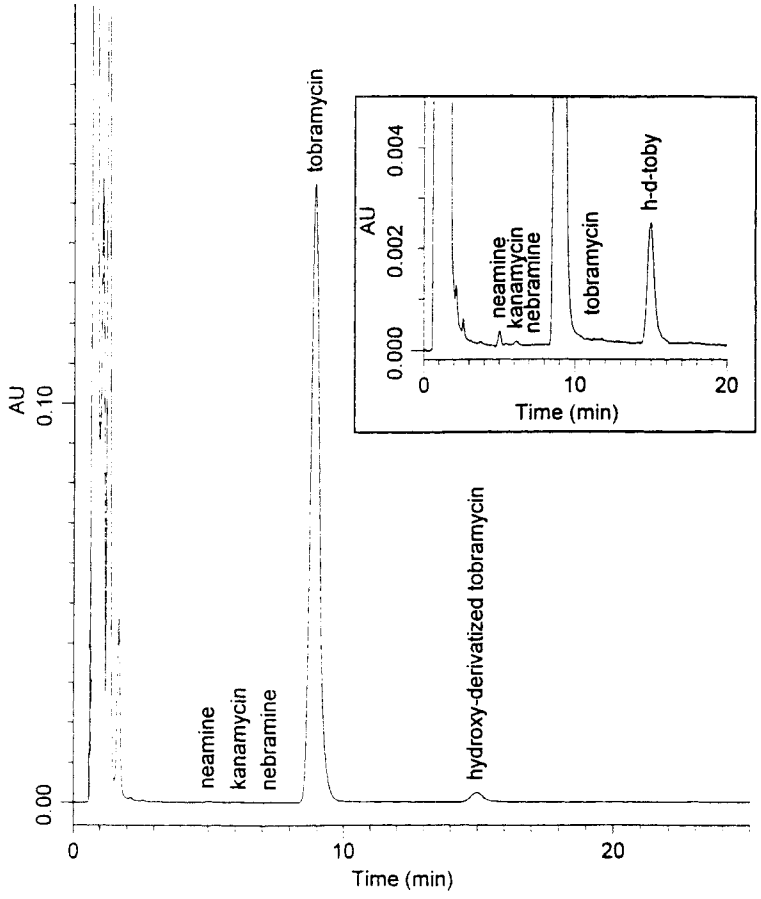

Figure 1. Standard preparation chromatograph.

preparation was analyzed at 0, 24, 168, and 192 hours. The sample preparations were analyzed at 0 and 72 hours. The data was evaluated for percent change from time zero. Since the % changes are within ± 2%, the standard and sample preparations are considered stable at room temperature for up to 192 and 72 hours, respectively (Table 8). Typical chromatograms obtained from a standard preparation and sample preparation are illustrated in Figures 1 and 2 respectively. The peak labelled hydroxy-derivatized tobramycin (h-d-toby) is a late eluting minor component of the derivatization reaction.

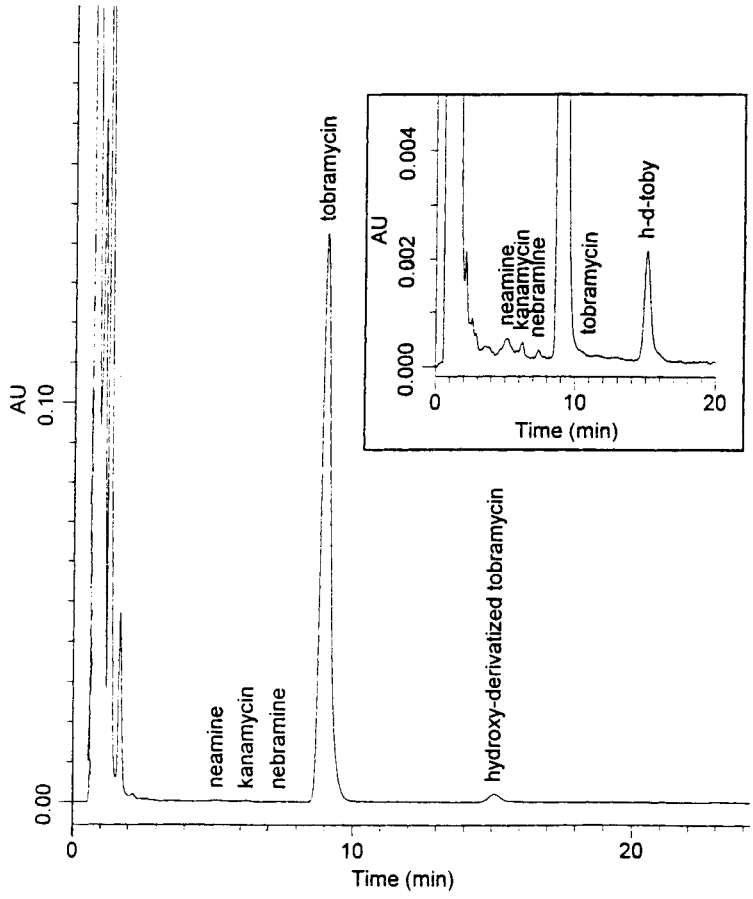

Figure 2. Sample preparation chromatograph.

Limit of Quantitation

The limit of quantitation was determined by using a precision (RSD) acceptance criteria of NMT 10% and a signal to noise ratio not less than (NLT) 5. A preparation of 0.1% label claim tobramycin in placebo exhibits an RSD of 9.0% (n=6) with a signal to noise ratio of 5.6 (Table 9).

CONCLUSION

The described isocratic HPLC method for the determination of tobramycin and its related substances has been evaluated for system suitability, linearity, precision, accuracy, stability of solutions, and specificity. The tobramycin peak response has been shown to be precise, accurate, and linear over the range of 0.1% - 150% (3 mg/mL label claim). Intermediate precision for the assays and related substances between chemists and chromatographic systems was demonstrated to be within 1.0% and 15.5%, respectively. The standard preparation is stable up to 192 hours. The sample preparation is stable up to 72 hours. Finally, the method has proven to be specific under a variety of stress conditions, while maintaining peak homogeneity. Consequently, the validated method for the determination of tobramycin and its related substance is regarded as stability-indicating.

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[‡] Present Address: MDS Tricon, 5415 W. Laurel Street, Tampa, FL 33607

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