

# A simple and rapid high-performance liquid chromatographic method for determining tobramycin in pharmaceutical formulations by direct UV detection

## Abstract

**Background:** Tobramycin, an aminoglycoside antibiotic, is a polar pharmaceutical compound which lacks a UV absorbing chromophore. Due to the absence of a UV absorbing chromophore and high polar nature of this antibiotic, the analysis of such compounds becomes a major challenge. **Objective:** To overcome these problems, a novel method for the determination aminoglycoside tobramycin was developed and validated based on reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detector. **Materials and Methods:** An isocratic mobile phase consists of buffer 0.05 M diammonium hydrogen phosphate, pH adjusted to 10.0 using tetramethyl ammonium hydroxide. Chromatography was carried out at 25°C on a Purosphere RP-8e, 250 mm x 4.6 mm, 5µm. The detection was carried out using variable wavelength UV-Vis detector set at 210 nm. The compounds were eluted isocratically at a steady flow rate of 1.0 mL/min. **Result and Discussion:** Tobramycin retention time was about 9.0 min with an asymmetry factor of 1.4. A logarithmic calibration curve was obtained from 0.47 to 0.71 mg/mL ( $r > 0.9998$ ). Within-day %RSD was 0.29 ( $n = 6$ , 0.60 mg/mL) and between-day %RSD was 0.54. Specificity/ selectivity experiments revealed the absence of interference from excipients, recovery from spiked samples was between 99.0–100.0 percent. **Conclusions:** A HPLC method based on UV detection has been developed and validated for determination of tobramycin from ophthalmic solution. The method is simple, rapid, specific, accurate (error 0.80%), precise (RSD <2.0%) and linear ( $r^2=0.9998$ ). The described method is suitable for routine analysis and quality control of ophthalmic solution containing tobramycin.

**Key words:** Amino glycoside, derivatization, isocratic, reverse phase chromatography, tobramycin

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## INTRODUCTION

Tobramycin is an amino glycoside antibiotic used to treat various types of bacterial infections, particularly Gram-negative infections. It works by binding to a site on the bacterial 30S and 50S ribosome, preventing formation of the 70S complex. As a result, mRNA cannot be translated into protein and cell death ensue. Tobramycin is preferred over gentamicin for *Pseudomonas aeruginosa* pneumonia due to better lung penetration and bactericidal activity.<sup>[1,2]</sup>

Like all amino glycosides, tobramycin does not pass through the gastrointestinal tract, so for systemic use it can only be given intravenously or intramuscularly.<sup>[1]</sup> A sterile tobramycin ophthalmic solution (eye-drops) with a tobramycin concentration of 0.3% is available in the market.

United states Pharmacopoeia 2008 (USP 31) have described the procedure for assay of raw material and tobramycin ophthalmic solution by high-performance liquid chromatography using derivatization with 2,4-Dinitrofluorobenzene and tris (hydroxymethyl aminomethane) reagent, mixture of buffer (2 gm of

tris (hydroxymethyl aminomethane) and 20 ml of 1N sulfuric acid) and acetonitrile in the ratio 40:60 v/v as the mobile phase at a flow rate of 1.2 ml/min, and a column (3.9 × 300 mm) that contains L1 packing, with detector wavelength set at 365 nm.<sup>[3]</sup> 2, 4-Dinitrofluorobenzene and tris (hydroxyl methyl aminomethane) reagent are stable for 5 days and 4 hours respectively. Derivatization is carried out at 60°C constant temperature.

In comparison with derivatization, simple reverse-phase chromatographic methods have the advantages of reducing analysis time, enhancing sensitivity and flexibility, and lowering the cost of the instruments and maintenance. The biggest disadvantage of derivatization has been lack of stability. The reaction products are not stable and have a short half-life possibly because of a spontaneous intermolecular rearrangement. Another disadvantage of derivatization is that it reacts with only few functional groups.<sup>[4]</sup>

The literature survey shows that several methods like HPLC with evaporative light scattering detection, electrochemical detection, LC/MS, HPLC UV-Vis have been reported for the determination of tobramycin with derivatization.<sup>[5-20]</sup> These reported methods and the USP method are not rapid for assay of tobramycin in ophthalmic solutions. All the reported and official methods are complex, insensitive, and risky. However, as per bibliographical revisions performed, no analytical method has been reported for direct (without derivatization) determination of tobramycin by high-performance liquid chromatography.

The present study was aimed at developing a simple, specific, accurate, and precise HPLC method for determination of tobramycin in commercially available pharmaceutical formulations such as ophthalmic solution, injection, suspensions, and in-house prepared ophthalmic suspension, based on direct UV- detection, in which a 100% buffer was used as a mobile phase to determine the compounds for use in routine quality control applications associated with these ingredients.

The proposed method for the determination of tobramycin in pharmaceutical formulations by HPLC UV detectors is first of its kind without involving derivatization. This information could be very useful for many of the pharmaceutical industries for the determination of this compound and for those who do not have the costly instruments, selective detectors such as a refractive index detector.

## MATERIALS AND METHODS

### Instrumentation

Integrated high-performance liquid chromatographic systems LC-2010AHT from Shimadzu Corporation (Chromatographic and Spectrophotometric Division, Kyoto, Japan) consisted of a binary gradient system, high-speed auto-sampler, column oven, and UV-Vis detector. A Purospher RP-8e, 250 mm × 4.6 mm, 5µm analytical column from Merck, Germany, was used as a stationary phase. Chromatograms were recorded and integrated on PC installed with LC solution chromatographic software, version 1.22 SP1 (Shimadzu, Kyoto, Japan).

### Reference substances, reagents, and chemicals

Working standard of tobramycin was obtained from Chongqing daxin pharmaceuticals laboratories Ltd., China. Diammonium hydrogen phosphate and tetramethyl ammonium hydroxide were purchased from Panreac (Barcelona) Espana. Distilled water was obtained from a Milli-Q system, Millipore, Milford, MA, USA. All the chemicals and reagents were of analytical or reagent grade. Reference standards of tobramycin were obtained from United States Pharmacopoeia Convention, Rockville, MD, USA. Ophthalmic formulations containing tobramycin were developed and manufactured in our research and development laboratory.

### Chromatographic conditions

The isocratic mobile phase consisted of 0.05 M diammonium hydrogen phosphate, pH adjusted to 10.0 ± 0.05 using tetramethyl ammonium hydroxide (25% solution in water). The mobile phase was filtered and degassed through a membrane filter of 0.45 µm porosity under vacuum. A Purosphere RP-8e, 250 mm × 4.6 mm, 5 µm analytical column was used as a stationary phase. A constant flow rate of 1.0 ml/min was employed throughout the analysis. A variable UV-Vis detector was set at 210 nm. All pertinent analyses were made at 25°C and volume of solution injected on to the column was 50 µl. The mobile phase was used as diluent for standard and sample preparations.

### Samples

Test samples were ophthalmic solutions prepared in-house and purchased from the local market with composition 3.0 mg/ml of tobramycin. Other test samples used were accelerated stability samples with similar composition.

## Solution preparation

### Tobramycin standard solution

Tobramycin *standard* solutions were prepared by transferring accurately about 66.0 mg of tobramycin working standard equivalent to 60.0 mg of anhydrous tobramycin to a 100 ml volumetric flask. About 70 ml of diluent was added and sonicated for few minutes to solubilize tobramycin. The solution was diluted to volume with the diluent and mixed. The solution was filtered through a 0.45 µm membrane filter and 50 µl was injected.

### Estimation from formulations

Five bottles of ophthalmic suspension, containing tobramycin 3.0 mg/ml, were shaken gently and transferred to a glass beaker and mixed. About 5.0 gm of ophthalmic suspension was weighed accurately into a 25 ml volumetric flask, about 10 ml of diluent was added, shaken to disperse the sample, and diluted to volume with diluent and mixed. This solution theoretically contains 0.60 mg/ml of tobramycin. The solution was filtered through a 0.45 µm membrane filter and 50 µl was injected directly on to the column.

### Quantitation

Peak areas were recorded for all peaks. Peak areas were taken into account to quantitate the label amount in milligram per ml of ophthalmic suspension by using the following formula:

$$\text{Tobramycin mg/ml} = R_u/R_s \times C/100 \times 25/W \times 1/L \times P \times D$$

where  $R_u$  is the peak area obtained from tobramycin in the investigation solution;  $R_s$  is the peak area obtained from tobramycin in the standard solution;  $C$  is the weight (mg) of tobramycin working standard taken to prepare the standard solution;  $W$  is the weight (g) of the test sample;  $P$  is purity of tobramycin working standard,  $L$  is the labeled amount of tobramycin in mg/ml of ophthalmic suspension, and  $D$  is the density of the ophthalmic suspension.

## RESULTS AND DISCUSSION

### Chromatography

Our method development started with the search for the suitable column and mobile phase.

A chromatographic system comprising 0.02 M formic acid:acetonitrile (50: 50 v/v), as a mobile phase at a constant flow rate of 1.0 ml/min, silica column, 250 mm × 4.0 mm, 5µm analytical column as a stationary phase,

and detector wavelength at 205 nm resulted in no peak elutions even after 60 minutes of run time. The mobile phase consisting of a 0.02 M aqueous potassium ammonium phosphate buffer and acetonitrile in the ratio 50:50, v/v, was tried in isocratic conditions on the Spherisorb ODS-1, 250 mm × 4.6 mm, 5µm to obtain symmetrical peak shapes and clear separation of the signal peaks from the solvent front peaks.

Upon investigation of the two chromatographic systems containing 0.05 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide, and 0.1 M potassium dihydrogen phosphate, pH adjusted to 6.9 using a potassium hydroxide solution as the mobile phase at a constant flow rate of 1.0 ml/min, BioSep SEC-S2000, 300 mm × 7.8 mm and a Purosphere RP-8e, 250 mm × 4.6 mm, 5µm analytical column as a stationary phase and detector wavelength at 205 nm resulted in peak elution at 3.2 minutes and 9.7 minutes respectively, the first investigation resulted in tobramycin peak eluted very close to negative peak (peak from diluent) and the second resulted in a tailing factor as high (>3) as shown Figure 1.

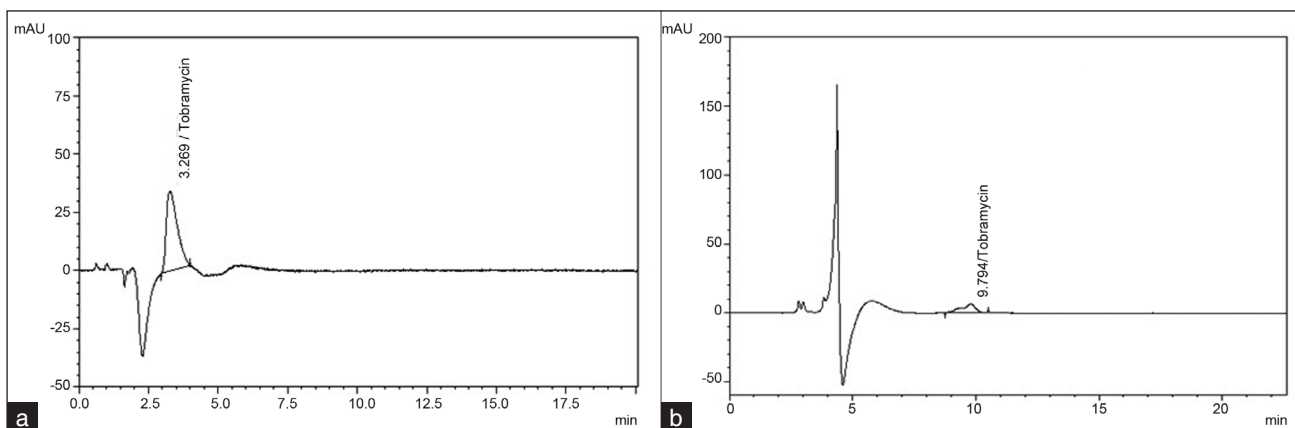
Further, to develop a suitable and robust LC method for the determination of tobramycin by UV detection, different mobile phases and columns were employed [Table 1] to achieve the best signal response and retention time.

Finally, the mobile phase consisting of water: 0.05 M diammonium hydrogen phosphate, pH adjusted to 10.0 ± 0.05 using tetra methyl ammonium hydroxide (25% solution in water) at a constant flow rate of 1.0 ml/min and detector wavelength set at 210 nm, using a Purosphere RP-8e, 250 mm × 4.6 mm, 5µm column, was found to be appropriate, allowing well signal response of tobramycin.

### Optimization of HPLC

The pH of the mobile phase can affect the analyte retention time as well as the detection sensitivity. Figure 2 shows the result of detection response (peak area), efficiency (shown as plate number  $N/c$  column) and capacity factor of tobramycin at different pHs. The optimal pH 10.0 ± 0.05 was chosen for the determination of tobramycin.

Concentration of the buffer is another factor that can alter the ion-pair formation. Figure 3 shows the capacity factor and detection response (peak area) as the concentration of the buffer varied. Response was minimal when less than 0.05 M diammonium hydrogen phosphate was used. This may be due



**Figure 1:** (a) Chromatogram of tobramycin showing first investigation. Chromatographic column: BioSep SEC-S2000, 300 mm × 7.8 mm, mobile phase: 0.05 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide, flow rate 1 ml/min. Detector wavelength: 205 nm. (b) Chromatogram of tobramycin showing second investigation. Chromatographic column: Purosphere RP-8e, 250 mm × 4.6 mm, 5 μm, mobile phase: 0.05 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide, flow rate 1 ml/min. Detector wavelength: 205 nm

**Table 1: Employed mobile phases, columns and elution time during the investigation of tobramycin**

Column	Mobile phase	Elution time
Silica column, 250 mm × 4.0 mm, 5 μm	0.02 M formic acid: acetonitrile (50: 50 v/v)	*No peak eluted
Spherisorb ODS-1, 250 mm × 4.6 mm, 5 μm	0.02 M ammonium phosphate: acetonitrile, (50: 50 v/v)	*No peak eluted
BioSep SEC-S2000, 300 mm × 7.8 mm.	0.05 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide.	**14 minutes
Purosphere RP-8e, 250 mm × 4.6 mm, 5 μm	0.1 M potassium dihydrogen phosphate, pH adjusted to 6.9 using potassium hydroxide solution	***4.3 minutes
BioSep SEC S2000, 300 mm × 7.8 mm	0.05 M sodium acetate: acetonitrile (50: 50 v/v)	*No peak eluted
Waters, Spherisorb ODS-1, 250 mm × 4.6 mm, 5 μm	0.05 M boric acid: acetonitrile (50: 50 v/v)	*No peak eluted
Waters, Spherisorb ODS-1, 250 mm × 4.6 mm, 5 μm	0.1% aqueous glacial acetic acid: acetonitrile, (50: 50 v/v)	*No peak eluted
Alltech allsphere, ODS-2, 250 mm × 4.6 mm, 10 μm	4.8% aqueous protriptylene hydrochloride: acetonitrile (50: 50 v/v)	*No peak eluted
BDS, Hypersil C18, 50 mm × 4.6 mm, 5 μm	2.4% aqueous protriptylene hydrochloride: acetonitrile (50: 50 v/v)	*No peak eluted
Waters, Spherisorb ODS-1, 250 mm × 4.6 mm, 5 μm	0.05 M aqueous diammonium hydrogen phosphate: acetonitrile, (50: 50 v/v)	*No peak eluted
Supelcosil LC-8, 250 mm × 4.6 mm, 5 μm	0.01 M sodium dihydrogen phosphate: acetonitrile (950: 50 v/v) pH adjusted to 6.9 using potassium hydroxide	*No peak eluted
Purosphere RP-8e, 250 mm × 4.6 mm, 5 μm	0.05 M diammonium hydrogen phosphate, pH adjusted to 10 using tetramethyl ammonium hydroxide	8.9 minutes

\*No peak tobramycin eluted after 60 minutes., \*\*Tobramycin peak eluted very close to negative peak (peak from diluents), \*\*\*Peak shape was not acceptable, tailing factor high.

to highly aqueous environment that is unfavorable for ion pairing. Therefore, pH 10.0 and 0.05 M diammonium hydrogen phosphate was chosen for estimation of tobramycin. Typical chromatogram of the test solution is shown in Figure 3.

### Method validation

The test method for the determination of tobramycin was validated to include the essential demands of International Conference on Harmonization (ICH) guidelines.<sup>[21]</sup> Parameters like specificity, linearity, accuracy, precision, range, robustness, and system suitability were examined.

### Specificity

No interferences were observed due to the obvious presence of excipients and mobile phase.

### Linearity

Peak areas versus concentration in milligram per milliliter were plotted for tobramycin at the concentration range between 80% and 120% of the target level. Tobramycin showed linearity between 0.47 and 0.71 mg/ml with a correlation coefficient ( $r^2$ ) of 0.9998.

### Accuracy

Accuracy of the proposed HPLC determination was evaluated from the assay results of the components.



Accuracy was done by performing the assay of samples and calculated the peak area responses of different samples by the component recovery method.

### Stock solution

A Stock solution was prepared by dissolving accurately weighed portions of about 666 mg of tobramycin in portions of the mobile phase and diluted to produce 100 ml solution.

An appropriate portion of the stock solution was spiked into a blank placebo matrix to produce concentrations of 80, 100, and 120 of the target level. Mean recovery of spiked samples was 99.45 % for tobramycin [Table 2].

### Precision

Instrumental precision was determined by six replicate determinations of standard solution and the relative standard deviation was 0.29 for tobramycin.

Method precision or intra-assay precision was performed by preparing six different samples

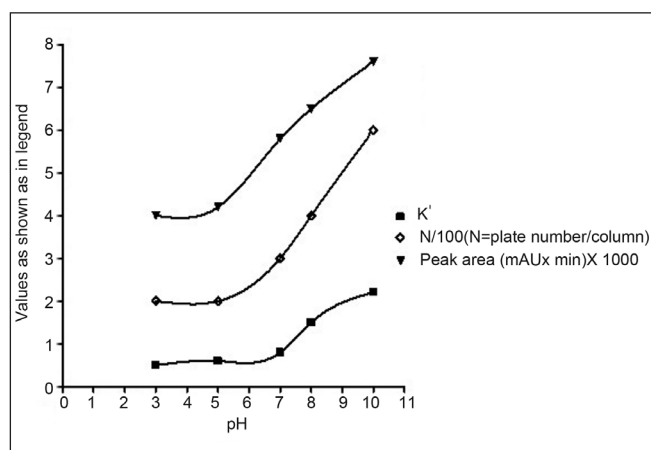
involving different weighing. Each solution was injected in triplicate under same conditions and the mean value of peak area response for each solution was taken. Corrections in area were made for each weight that had been taken to prepare six sample solutions and relative standard deviation of the contents of tobramycin the six sample solutions was calculated. Relative standard deviation was 0.29 for tobramycin.

Intermediate precision was performed by analyzing the samples by two different analysts employing different instruments. The standard solution and six different samples at 100% target level were prepared by each analyst. The relative standard deviation obtained from 12 assay results by two analysts was 0.42 for tobramycin.

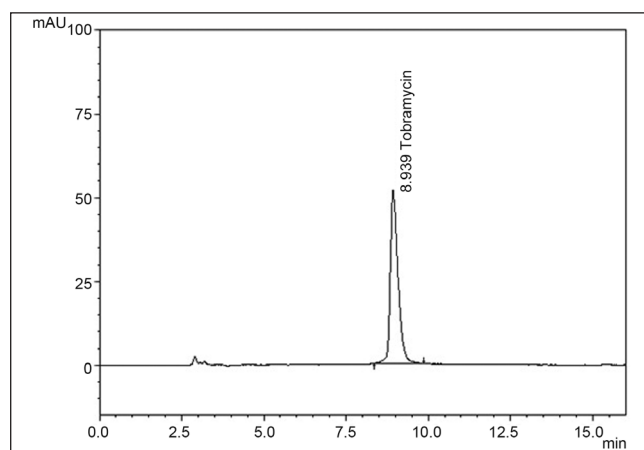
### Robustness

Robustness of the proposed method was performed by keeping chromatographic conditions constant with the following deliberate variations:

- (i) change in the column oven temperature
- (ii) change in flow rate from 1.0 ml/min to 1.2 ml/min.



**Figure 2:** Effect of pH on (peak area) and efficiency (shown as plate number N/column) and capacity factor of tobramycin column: Purosphere RP-8e, 250 × 4.6 mm, 5 $\mu$ m, mobile phase: 0.05 M diammonium hydrogen phosphate, pH adjusted to 10 using tetramethyl ammonium hydroxide



**Figure 3:** A typical chromatogram of test sample by proposed methods column: Purosphere RP-8e, 250 × 4.6 mm, 5 $\mu$ m, mobile phase: 0.05 M diammonium hydrogen phosphate, pH adjusted to 10.0 using tetramethyl ammonium hydroxide, flow rate 1 ml/min,  $\lambda$  set at 210 nm

**Table 2: Accuracy data (analyte recovery): Tobramycin**

	Amount std. added (mg/ml)	Amount std. recovered (mg/ml)	Determined (% of target level)	Recovered (%)	Bias (%)
1	0.4727	0.4687	79.32	99.15	-0.85
	0.4729	0.4696	79.44	99.29	-0.75
	0.4726	0.4678	79.19	98.98	-1.02
2	0.5909	0.5903	99.91	99.91	-0.09
	0.5911	0.5903	99.87	99.87	-0.13
	0.5908	0.5888	99.68	99.68	-0.32
3	0.7090	0.7054	119.39	99.49	-0.51
	0.7093	0.7051	119.28	99.40	-0.60
	0.7089	0.7042	119.20	99.30	-0.70

**Table 3: Comparison of system suitability parameters: Tobramycin**

Parameters	Proposed method	Change in buffer composition -5%	Change in flow rate +5%
Retention time	8.93	8.88	8.50
Peak asymmetry	1.43	1.32	1.36
Theoretical plates	6566.38	6490.63	6023.84
Capacity factor	1.98	1.88	1.92

**Table 4: Application of the developed HPLC method to the determination of tobramycin in dosage forms**

Test sample	Concentration	Assay <sup>b</sup> %
Ophthalmic solution	0.3 %	99.87
Injection	40 mg/ml after reconstitution	99.68
Suspension	Tobramycin 0.3% and dexamethasone 0.1%	100.02
In-house <sup>a</sup> (suspension)	Tobramycin 0.3% and dexamethasone 0.1%	101.23
Stress sample <sup>a</sup> (suspension)	Tobramycin 0.3% and dexamethasone 0.1%	100.05

<sup>a</sup>Formulation was prepared in the research and development lab. <sup>b</sup>Each value is the average of two determination

The standard solution was injected six times in replicate for each minor change. System suitability parameters like peak asymmetry, theoretical plates, capacity factor, and relative standard deviation were recorded for each peak and found to be within acceptable limits.

Six test samples at the target concentration level were prepared and analyzed for each change. The percentage difference in assay and relative standard deviations was calculated during each change and found to be  $\pm 0.5\%$  and less than 1.0, respectively. It was noted that slight addition of solvents in the mobile phase affects the method and does not produce results of similar system suitability, as in the proposed method it resulted in poor peak response or no elution of tobramycin peak.

#### System suitability

System suitability tests were performed to chromatograms obtained from standard and test solutions to check parameters such as column efficiency, peak asymmetry, and capacity factor of tobramycin peaks. Results obtained from six replicate injections of standard solution as per the proposed method are summarized in Table 3.

#### Application of the proposed method

In-house prepared samples, innovator samples, and samples stored at accelerated stability conditions (40°C/25%RH) were evaluated for assay of tobramycin. The method gave reproducible results of assay for all the samples tested for tobramycin. The excipients in the formulation and the probable degradation products of tobramycin as a result of accelerated storage did not interfere with the estimation of the component. The assay of the test samples (RT and accelerated) and innovator samples are summarized in Table 4.

## CONCLUSIONS

A HPLC method based on UV detection has been developed and validated for determination of tobramycin from an ophthalmic solution. The method is simple, rapid, specific, accurate (error 0.80%), precise (RSD <2.0%), and linear ( $r^2=0.9998$ ). The described method is suitable for routine analysis and quality control of the ophthalmic solution and injection containing tobramycin.

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