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ORIGINAL ARTICLE

A Novel Sensitive Method for Quantitative Determination of Tobramycin by Spectrophotometer using Diphenylamine

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Author`s Contribution
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ABSTRACT

Objective: Tobramycin, which is an aminoglycoside antibiotic containing two aminoglycosidic units with the central scaffold of six membered cyclohexane ring. The -OH groups and -NH2 absorb electromagnetic radiation near lower wave length. Therefore, basicity, hydrophilicity and lack of a UV absorbing chromophore makes it challenging for detection by UV- visible detector of HPLC and spectrophotometer. Therefore, our study aims to present a simple and sensitive UV method for estimation of Tobramycin (TOB) in bulk and pharmaceutical formulations.

Method: For this purpose, we prepared derivative of tobramycin, diphenylamine acid was used as catalyst under high temperature during this experiment. Under drastic conditions the glycosidic linkage opened and amino sugar unit becomes free (OHLA). The diphenylamine reacts with amino sugar unit resulting in formation of enamine, which is a colored complex and absorbs EMR.

Result: The Tobramycin product showed absorbance at 635 nm with molar absorptivity 9.4 x 10^{-5}mole^{-1}. ml^{-1}. cm^{-1}. Optimization conditions for the derivatization were also investigated by HPLC. The procedure was valid because it did not show change in absorbance up to 7days. The percentage of recovery of TOB was 98.0 – 103% with SD of less than 1% for both standard and samples.

Conclusion: Thus, a simple, sensitive and efficient method was developed and validated for bulk and pharmaceutical analysis of Tobramycin.

INTRODUCTION

The chemical name of Tobramycin is (2s,3R,5S,6R)-4-AMINO-2-{(1S,2S,3R,4S,6R)-4,6-diamino-3-{(2R,5S,6R)-3-amino-6-(amino methyl)-5-hydroxan-2-yl}-2-hydroxycyclohexyl) oxy-6-{hydroxymethyl)oxane-3,5-diol.9 (Figure **1**). It is a potent aminoglycoside antibiotic, having extended spectrum of activity against *S. aureus,* Enterobacteriaceae, and *Pseudomonas aeruginosa* [1].

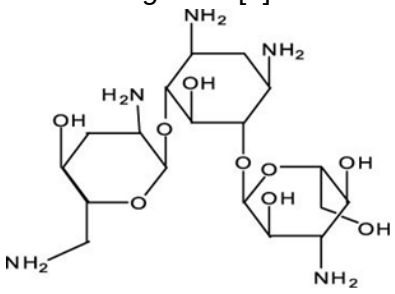


Figure 1. Tobramycin.

TOB acts by inhibiting synthesis of protein in bacterial cells as other aminoglycosides which is related binding to bacterial ribosomes and its 30-s and 50-s subunits [2, 3]. In vitro studies have demonstrated TOB is active against susceptible strains of following microorganisms; Staphylococci, including *S. aureus* and *S. epidermidis* (coagulase-positive and coagulase-negative) including penicillin resistant strains. It has also been found to be active against isolates resistant to other antibiotics and effectively used in patients with underlying illnesses suffering from community-acquired lower respiratory tract infection (LRTI) [4]. Dosing frequency of once daily dosage is routinely found to be efficacious [5].

Topical ocular preparation of TOB is commonly administered for corneal and conjunctival bacterial infections with safe and effective outcomes [6-8]. In addition, intermittent administration of inhaled tobramycin in conjunction with standard therapy for cystic fibrosis improves pulmonary function, decreases the density of *P. aeruginosa* in expectorated sputum, and reduces the need for intravenous antipseudomonal antibiotics and hospitalization [9]. TOB also has less side effects than other aminoglycosides. Clinical data reveals it produces less nephrotoxicity and auditory toxicity [10].

TOB lacks chromogenic groups due to which it cannot be quantified directly through HPLC or Spectrophotometer. However, Research studies demonstrate procedure for the high-performance liquid chromatographic determination of tobramycin in serum and pharmaceuticals using pre-column derivatization and HPLC with direct UV detection [11-13] and capillary electrophoresis analysis followed by subsequent chromatographic analysis on a RP column within direct UV detection [14].

The analysis of TOB have also been carried out through simple LC/MS method for quantitative analysis of tobramycin in pharmaceutical formulations [15].

EXPERIMENTAL

Materials and reagents

For experiment, the materials DPA-Diphenylamine (99% assay) from Merck, HCl acid (37% assay) from J.K Enterprises, glacial acetic acid (99.8 – 100% assay) from sigma Aldrich, N-Hexane (95% assay) from Riedel de han, chloroform (99.5% assay) from Dae-Jung, methanol (99.5% assay) and tobramycin USP standards were purchased.

Other materials used included volumetric flask, stirrer, beakers, pipette and measuring cylinder and Test tubes of 200×18 mm long were used for heating samples in autoclave. All glass ware materials used were of Analytical grade.

INSTRUMENTATION

The present method was performed using 1cm cells of quartz on double beam spectrophotometer UV-Vis 1601 (Shimadzu corporation, Japan).

Preparation of standard solution

Accurately weighed 75 mg of Tobramycin USP reference standard was transferred to a 25ml volumetric flask and water was added to dissolve it. Finally, water was added up to mark to make up the volume.

Preparation of DPA reagent

Accurately weighed 2gm of DPA was taken and transferred into a conical flask, add 90ml of glacial acetic acid and 45ml of HCl which was covered with stopper. The mixture was shaken till complete dissolution.

Analytical method for determination of TOB

A quantity of 1ml (3mg/ml) aqueous solution of TOB was taken in 200x18 mm test tubes. Minimum 3 tubes containing required amount

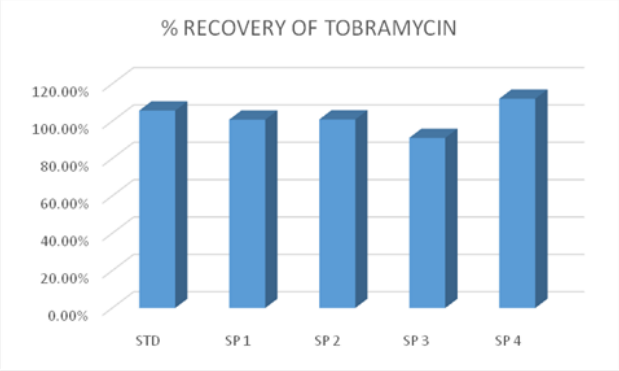
of sample was followed by addition of 5ml DPA solution. The solution was heated for 30 minutes at temperature of 100 – 105 °C in an Autoclave. After cooling, an absorbance of colored solution at 635 nm was observed using reagent as blank.

Optimization Parameters of Method

Table 1: Optimization parameters.

S. No	Parameters	Observations
01	λ max	635 nm
02	Bears law limits	500 mcg to 3 mg
03	Absorptivity	9.4 x 10^{-5}mol/ml/cm
04	Slope	0.45
05	LOQ	500 mcg --- 3 mg
06	Standard deviation	0.181

Tobramycin available in eye drops and in the form of ointment. The recovery of TOB in four different pharmaceutical preparations was also analyzed through UV under same procedure which is represented as under.



Validation of proposed method

The proposed method was validated according to USP guidelines with respect to specificity, Linearity, accuracy, precision, Robustness and Ruggedness [16] (16).

Linearity

The linearity was determined by correlation coefficient of r^2 = 0.997 indicating good linearity, as shown in table of optimization parameters of method.

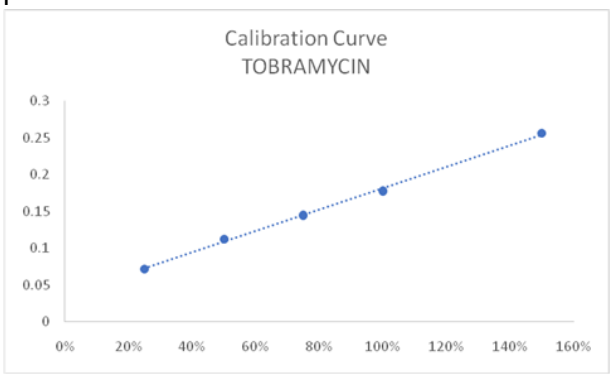


Figure 2. Tobramycin Calibration Curve

Table 2: Regression equation.

Drug	Regression equation	r^2value
Tobramycin	y = 0.001x + 0.039	0.997

Accuracy

Accuracy was checked at different concentration levels within the specified range. There replicate measurements were recorded at each concentration level. The results were recorded as percent recovery as shown in Table **3**.

Table 3: Accuracy.

Sample No	TOB Concentration %	% Recovery
1	50%	50.12
2	75%	76.1
3	100%	100.1

Precision

Precision was checked at three concentration levels. Three replicate measurements were recorded at each concentration level. The results were summarized in Table **4**. The calculated % RSD was below 2% Indicating excellent precision of the proposed procedure at both levels of reproducibility and repeatability.

Table 4: Precision.

Parameters	% Recovery	Mean value	± S.D	± RSD
Intraday Precision		102.4%	± 2%	±1.96%
1	102.4%			
2	104.5%			
3	100.4%			
Interday Precision		101.52%	±1.53%	± 1.51 %
1	99.98%			
2	103.4%			
3	101.18%			

Robustness

Robustness of the procedure was assessed by evaluating the influence of small variation in experimental variables on the analytical performance of the method. DPA volume was varied and reaction time was also altered.

In these experiments, one experimental parameter was changed while the other parameter was kept constant, and the recovery percentage was calculated each time (Table **5**). The small variation in any of the variables did not affects the results. This gives indication for the reliability of the proposed method during routine work.

Table 5: Robustness.

S.No	PARAMETRS	DPA (ml)	DPA (ml)	%RSD
1	DPA Volume	5.5ml	4.5 ml	± 0.74
2	TOB concentration	3 mg	3mg	
3	% Recovery	96.6%	97.10%	

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of normal test conditions. To examine ruggedness of the procedure, the analysis was done using different assay temperatures, and the results were evaluated as shown in Table **6**.

Table 6: Ruggedness.

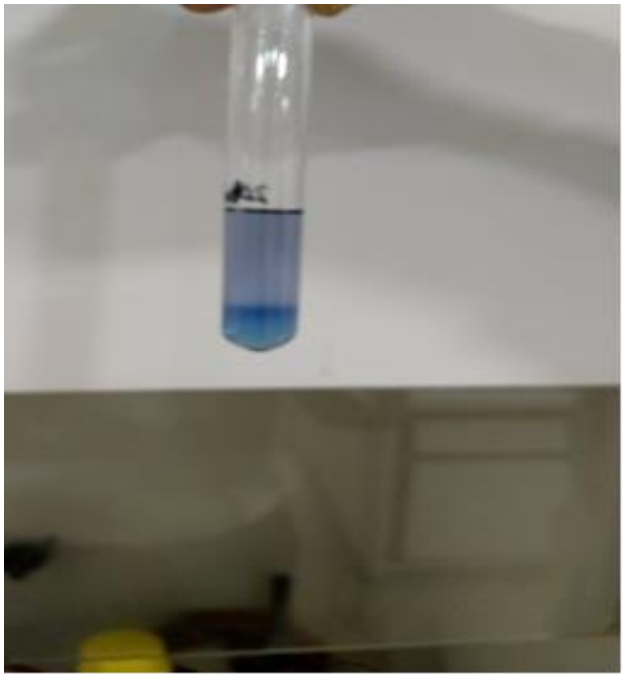
Temperature	% Recovery	% RSD
15 ºC	97.5%	1.03 %
25 ºC	96.85%	
40 ºC	98.5%	

Stability Studies

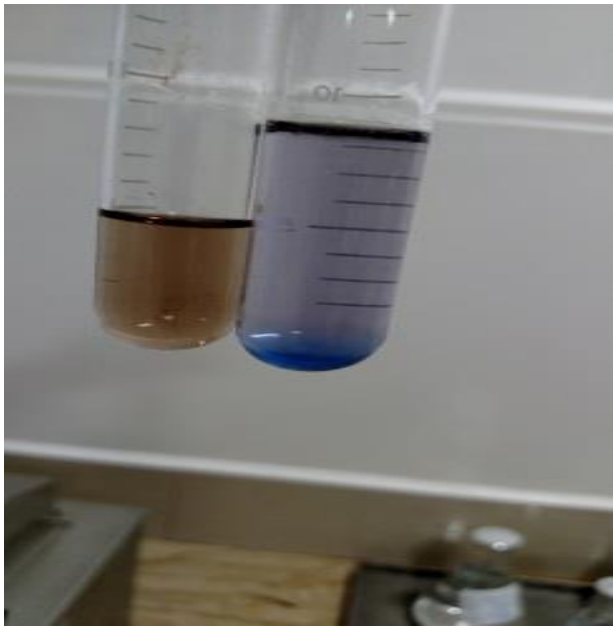
Additionally, stress testing was carried out to determine stability of testing method. The drug was subjected to altered temperature range, acidic effect, light effect and effect of change of solvent system.

Temperature and Time

For determination of temperature effect, a sample is heated in autoclave at a range of varies temperature 30 - 100 °C for 10 to 15 minutes. It was observed that with passage of time at higher temperature deep coloration appeared.



Blue color is the indication of enamine formation.



Brown color indicates the decomposition of enamine complex due to overheating.

Acid Affect

The Diphenylamine reagent is prepared in 4 different combinations: HCl/CH_3COOH, H_2SO_4/CH_3COOH, $HCLO_4/CH_3COOH$ solution. The most effective reagent for reaction is HCl/CH3COOH combination.

It was observed that the Trichloro acetic acid addition in test solution of tobramycin after heating for 15 minutes then addition of DPA reagent and heat again according of procedure also enhanced the coloration.

Light Affect

Diphenylamine reagent is light sensitive; it must keep in dark and 28 ºC temperature. Otherwise, Standards and samples were found to lower Optical densities / OD values.

Solvent Effect

To check the effect of different solvents on the test the required amount of 1ml of Tobramycin was taken in each test tube and volume was makeup to 25ml with solvents given below.

Table 7: Solvent effect on stability.

S. No.	Solvents	Observation	Solubility
1	Methanol	No change	Soluble but color become fade
2	Water	Precipitation and color change	Not soluble
3	Hexane	No color change	Not Soluble divide mixture into phases
4	Chloroform	No color change	Completely soluble
5	Acetonitrile	Soluble	Completely soluble and also stable in it.

RESULTS AND DISCUSSIONS

The aminoglycoside contains amino glucose units which is hydrolyzed at high temperature in the presence of Strong Acidic Medium, the sugar unit reacts with Diphenylamine and form

a blue colored complex. The color deepness is directly proportional to the concentration of Tobramycin. The color intensity is measured at 635nm. The results were observed to be accurate, precise and linear.

CONCLUSION

In this test method DPA reagent (Dech Reagent) forms colored complex through a reaction with the part of amino glucose unit of tobramycin. The acid is used for the hydrolysis of amino glucose unit and DPA reacts with aldehyde group and forms Enamine.

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