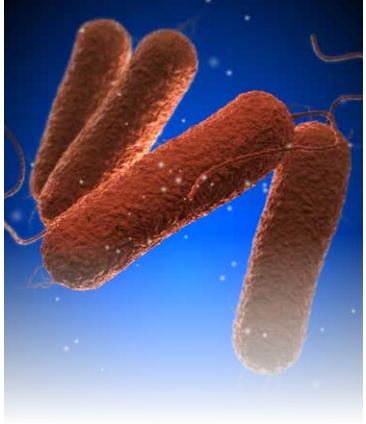
Application Note

Aminoglycosides Antibiotics



Pharmaceutical & Biotech analysis

Aminoglycosides
- Amikacin
- Framycetin Sulphate
- Gentamicin Sulphate
- Kanamycin Sulphate
- Lincomycin
- Neomycin
- Spectinomycin
- Tobramycin

PET imaging tracer
- FDG

Macrolide antibiotics
- Azithromycin
- Azaerythromycin
- Clarithromycin
- Erythromycin
- Roxithromycin

Bioanalysis of pharmaceutics
- Artemisinin
- Dihydro-artemisinin
- Artemether
- Etoposide
- 8-OH-DPAT
- mesna BNP7787
- Vincristine

Tobramycin according to EP Method

- **European Pharmacopoeia 8.1 (2014)**
- **Analysis of composition and impurities**
- **Reproducible & robust**

Introduction

Tobramycin belongs to the group of the aminoglycoside antibiotics. Like the other aminoglycosides, it binds to bacterial ribosomes and causes non-functional proteins to accumulate within the cell leading to cell death. It is often effective against bacterial strains that prove resistant to other aminoglycosides like gentamicin. The production is mainly achieved by fermentation resulting in several minor byproducts.

The analysis of the by-product contribution in bulk tobramycin and preparations is important as to insight in stability, quality control and authenticity. A number of qualitative and quantitative methods has been published so far [1] but the focus is mainly on tobramycin and not on the by-products. Because of the presence of sugar groups in both tobramycin and by-products LC with pulsed amperometric detection (PAD) is a highly selective and sensitive analytical tool [2, 3]. The analysis of Tobramycin in pharmaceutical formulations based on HPLC-PAD is described in the European Pharmacopoeia [4].

Summary

The Tobramycin analysis in pharmaceutical preparations was evaluated on an Antec ALEXYS LC-EC analyzer, using the exact method and conditions described in the official 2014 EP monograph (8.1).

In this application note typical results obtained with the ALEXYS® aminoglycosides analyzer are reported, demonstrating its performance for the routine analysis of Tobramycin in pharmaceutical preparations.

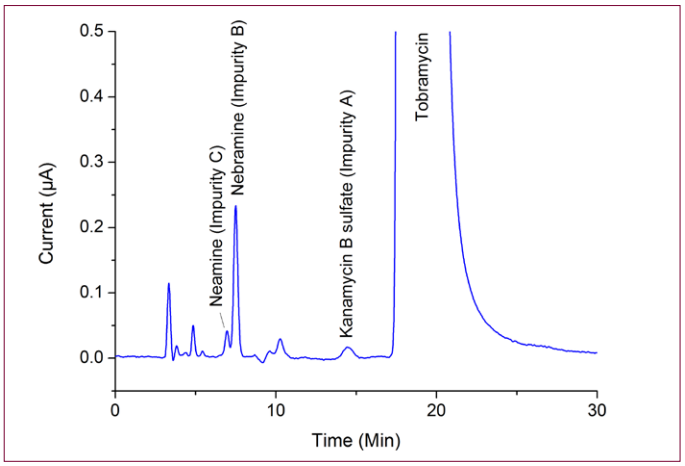


Figure 1: . 20 µL injection of a 1 mg/mL Tobramycin sample in mobile phase (Test solution (a) as described in the EP monograph).

Method

The European Pharmacopoeia method is based on separation of Tobramycin over a polymeric reversed phase column followed by post-column addition of NaOH and pulsed electrochemical detection. In the monographs the use of the following column type is described for the separation of Tobramycin: size 250 mm, ID 4.6 mm, styrene-divinylbenzene copolymer stationary phase with 100 nm pores and a particle size of 8 µm. The Agilent PLRP-S 1000Å 8 µm, 250 x 4.6 mm column which matches this criteria was chosen for the method evaluation.

For the detection of Tobramycin and its impurities PAD is mandatory using an Au working electrode (WE), Ag/AgCl reference electrode (RE) and stainless steel auxiliary electrode (AE). The Antec VT-03 electrochemical flow cell matches these requirements and was used in this evaluation. Note that both column and flow cell are not per se the optimal choice for separation & detection but were chosen to fore fill the EP assay.

An alternative approach for the analysis of Tobramycin based on a silica-based C18 column and a FlexCell is described in reference [5].

Table 1

LC-EC Conditions	
HPLC	ALEXYS aminoglycoside Analyzer with post-column addition kit (375 µL mixing coil)
Column	4.6 mm ID x 25 cm, 8µm, packing styrene-divinylbenzene copolymer with a pore size of 100 nm
Mobile phase	52 g/L of anhydrous sodium sulfate, 1.9 g/L of sodium octane sulfonate, 3mL/L tetrahydrofuran, 50mL/L 0.2M potassium dihydrogen phosphate previously adjusted to pH3.0 with a 10% solution of phosphoric acid.
Post-column reagent	20 g/L NaOH (carbonate-free)
Flow rate	1.0 mL/min, post-column: 0.3 mL/min
Vinjection	20 µL
Temperature	55°C for separation, mixing and detection
Flow cell	VT-03™ with Au WE, stainless steel AE and Ag/AgCl RE, spacer 100 µm
Potential waveform	E1, E2, E3: +0.05, +0.75, -0.15 V ts, t1, t2, t3: 0.2, 0.4, 0.2, 0.4 s
I-cell	ca. 1.8 µA
ADF	0.5 Hz
Range	10 µA

The ALEXYS LC-EC Analyzer was equipped with a second pump for the post-column addition of 20 g/L NaOH (carbonate-free). Mixing of the post-column reagent was achieved using a 375 µL PEEK mixing coil. The mobile phase was prepared as described in the EP monographs (Table 1). The concentration sodium oc-tane sulfonate was adjusted to 1.9 g/L to optimize the separation. Note: only use stabilized THF (stabilized with butylhydroxytoluene) in the mobile phase to assure low background cell currents. A 3 step waveform was applied with the following settings E1 = +0.05 V, E2 = +0.75 V, E3 = -0.15 V, t1 = 0.4 s, t2 = 0.15 s, t3 = 0.45 and ts = 300ms. The cell current was typical about 1.8 µA with these PAD settings.

The temperature for separation and detection was set to 55°C. The tray-cooling of the autosampler was set to 4°C to keep the sample vials cooled during execution of the analysis sequence.

Results

The peaks of Tobramycin, Kanamycin B (impurity A), Nebramine (impurity B) and Neamine (impurity C) were identified using the chromatogram of reference solution (d) and test solution (a) shown in figure 1 and 2, respectively.

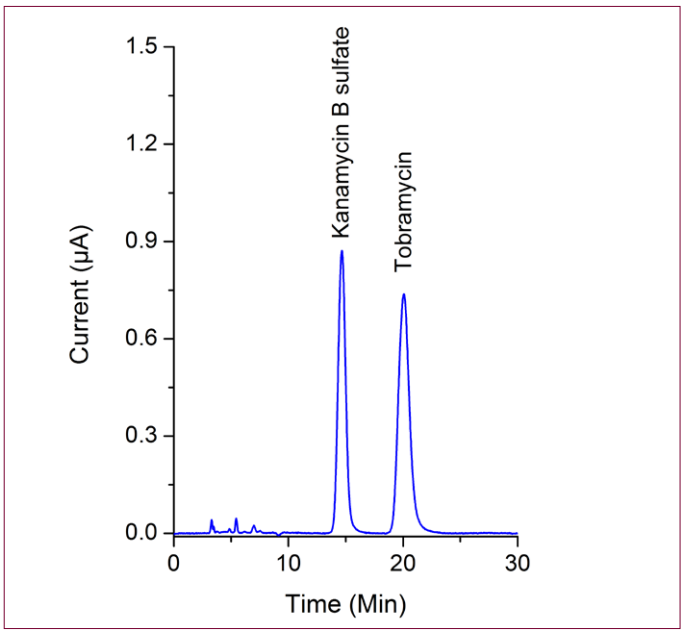


Figure 2: 20 µL injection of a standard consisting of 0.05 mg/mL kanamycin B and 0.05 mg/mL Tobramycin CRS in mobile phase (Reference solution (d) as described in EP monograph).

Table 2

Retention Time		
Component	*Retention (min)*	*Relative Retention**
Kanamycin B sulfate (Impurity A)	14.5	0.79
Nebramine (Impurity B)	7.5	0.41
Neamine (Impurity C)	7.0	0.38
Tobramycin	18.2	1

*) Relative retention time (RRT) with reference to Tobramycin (18.2 min).

System Suitability

In the EP monographs for Tobramycin the following system suitability requirement are specified:

- *Resolution:* minimum 3.0 between Kanamycin B (impurity A) and Tobramycin in the chromatogram obtained with reference solution (d), see figure 2.
- *Signal-to-Noise ratio:* minimum 10 for the principal peak in the chromatogram obtained with reference solution (b), see figure 3.

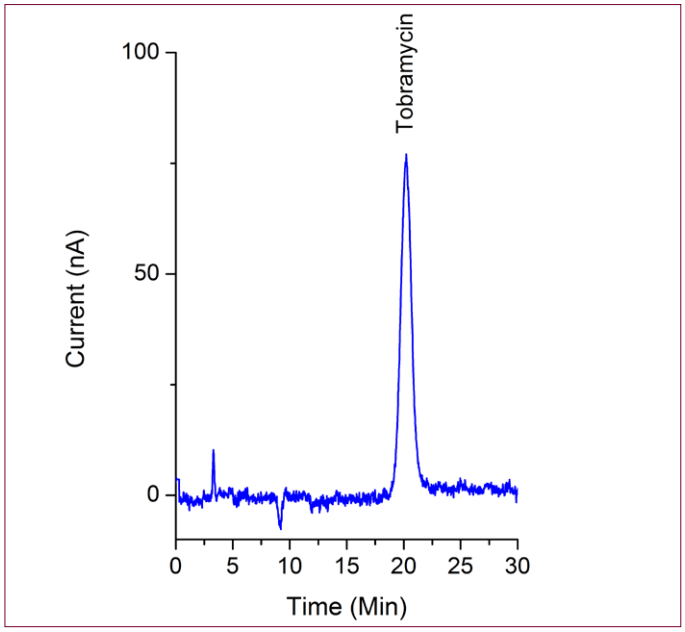


Figure 3: 20 µL injection of 2.5 µg/mL Tobramycin CRS in mobile phase (reference solution (b) as described in EP monograph).

Table 3

EP System Suitability Requirement		
Parameter	*EP criteria*	*Measured*
Resolution between Impurity A and Tobramycin	> 3.0	3.5
Signal-to-Noise ratio (Tobramycin)	> 10	17

The system suitability requirements are met for both parameters (table 3).

Linearity & Repeatability

The linearity of Tobramycin and Kanamycin B (impurity A) was investigated in the concentration range of 10 - 50 µg/mL. For both components the correlation coefficients were better than 0.999 for peak areas. The relative standard deviation (RSD) in peak area for Tobramycin was determined for 8 replicate injections of test solution (b), which is a 0.1 mg/mL Tobramycin sample solution in mobile phase (see figure 4). The RSD was 0.7 % for the Tobra-mycin peak area.

Sample Analysis

A commercial Tobramycin sample (CUD 621uA2B) was analyzed to determine the composition and related substances (impurities) using the acceptance criteria described in the EP monograph.

Assay

To determine the content (%) of Tobramycin in the sample the response of a 100 µg/mL Tobramycin sample solution (sample solution (b)) is compared to a 100 µg/mL Tobramycin CRS stand-ard (reference solution (e)) and the contents calculated. See figure 4 and table 4 below.

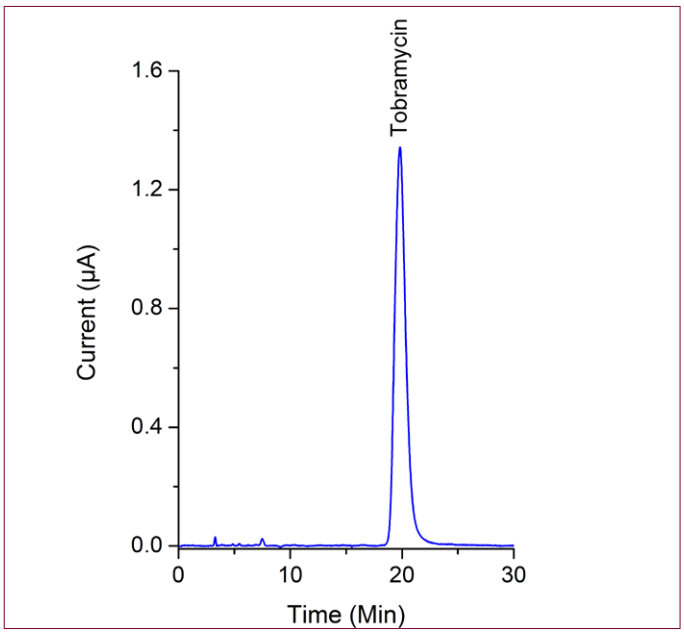


Figure 4: 20 µL injection of 100 µg/mL Tobramycin sample solution in mobile phase (sample solution (b) as described in EP monograph) for the Tobramycin assay analysis.

Table 4

Assay		
Sample	*EP criteria %*	*Measured**
Sample CUD 621uA2B	97-102	99.1

*) calculated on non-anhydrous sample

The contents was within the specified limits of the EP monograph.

Impurity analysis

To determine the impurity level in the sample, the responses of the impurity peaks of a test solution (a) containing a 1 mg/mL Tobramycin sample in mobile phase were compared to the response of the principle peak of reference solution (c). The chromatogram of test solution (a) is shown in figure 1.

Table 5

Impurity analysis Tobramycin sample CUD 621uA2B			
Impurity	*RRT**	*Peak Area (nA.s)*	*Discard#*
2	0.21	280	Y
3	0.24	58	Y
4	0.27	562	Y
5	0.30	103	Y
Neamine	0.38	663	Y
Nebramine	0.41	4327	N
8	0.53	137	Y
9	0.57	594	Y
Kanamycin sulphate B	0.80	843	Y

*) Relative retention time (RRT) with reference to Tobramycin (18.2 min).
#) Discard limit: any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent) shown in figure 3.

The EP acceptance criteria for the amount of impurities are:

- *Any impurity:* Not more than twice the area of the Tobramycin peak in the chromatogram obtained with reference solution (c), and not more than 1 such peak having an area more than the area of the Tobramycin peak obtained with reference solution (c).
- *Total impurities:* Not more than 3x the peak area of the Tobramycin peak in the chromatogram obtained with reference solution (c).
- *Discard limit:* Impurities with peak areas smaller than the peak area of the principle peak (Tobramycin) in the chromatogram of reference solution (b) can be discarded.

Table 6

Results Impurity Analysis Tobramycin Sample			
Impurity	*RRT*	*Relative Peak Area**	*EP criteria*
Nebramine (impurity B)	0.41	0.84	< 2
Total impurities*	-	0.84	< 3

*) The relative peak area of the impurity is calculated in the following way: Relative peak area = Peak area of the impurity divided by the peak area of the Tobramycin peak in the chromatogram obtained with reference solution (c).

In table 5 the peak responses (peak area in nA.s) are listed for all impurities found. Only the impurities with a response larger than the discard limit are taken into account in the calculation of the relative amount of impurities as specified under the limits section in the EP monograph. The results are shown in table 6.

The analyzed sample is in compliance with the acceptance criteria for both the contents and the impurity limits as set by the EP for Tobramycin and its impurities.

Conclusion

The ALEXYS Aminoglycosides Analyzer provides a suitable solution for the analysis of the composition & impurities in Tobramycin following the official method of the EP.

References

1. David A. Stead, *"Current methodologies for the analysis of aminoglycosides"*, J. Chromatogr. B, 747 (2000) 69–93
2. W.R. LaCourse, *"Pulsed Electrochemical Detection in High Performance Liquid Chromatography"*, John Wiley & Sons, New York, 1ed, 1997.
3. J. Szunyog, E. Adams, E. Roets, J.Hoogmartens, 23, J. Pharm. Biomed. Anal., (2000) 891-896
4. Tobramycin, *European Pharmacopoeia (EP)*, 8.1, (2014) 3434 -3436
5. *Tobramycin in pharmaceutical preparations,* Antec application note, 217_014

Figure 5: ALEXYS Aminoglycosides Analyzer.

PART NUMBERS AND CONFIGURATIONS

180.0056C	ALEXYS Aminoglycosides analyzer, including column, flow cell, and post-column addition kit
250.1075	PLRP-S 1000 Å, 250x4.6mm, 8um

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The application was developed with the European Pharmacopoeia, 6.0, (2008) as a basis and conditions may vary slightly from the EP method. The actual performance may be affected by factors beyond Antec Leyden's control. Specifications mentioned in this application note are subject to change without further notice.

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