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

Gentamicin Sulphate according to EP & USP Method

- **European Pharmacopoeia 8.1 (2014)**
- **U.S. Pharmacopeia 37-NF32 (2014)**
- **Analysis of composition and impurities**
- **Reproducible & robust**

Introduction

Gentamicin is a broad spectrum water-soluble antibiotic belonging to the group of aminoglycosides. It is manufactured by a fermentation process and consists of a mixture of related gentamicin components. The main constituents are gentamicin C1, C1a, C2 and C2a. Usually also other minor aminoglycosides are found in a pharmaceutical gentamicin preparation. The number of impurities and components possible makes the chromatographic analysis not quite straightforward.

Because of the presence of a sugar moiety in these analytes the selectivity and inherent sensitivity of pulsed amperometric detection (PAD) is a very attractive approach [1]. The analysis of Gentamicin sulphate in pharmaceutical formulations based on HPLC-PAD is described in the European and U.S Pharmacopoeia [2,3].

Summary

The Gentamicin sulphate analysis in pharmaceutical preparations was evaluated on an Antec ALEXYS LC-EC analyzer, using the exact method and conditions described in the official 2014 USP monograph (37-NF32) and EP monograph (8.0).

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

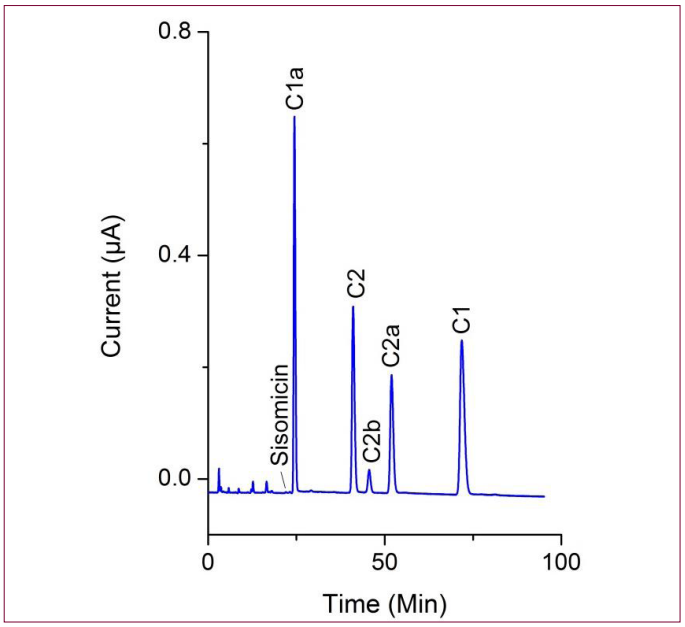

Figure 1: 20 µL injection of a 200 µg/mLGentamicin sample in mobile phase (Test solution (b) as described in EP and USP monograph).

Method

The method & conditions for separation and detection described in the 2014 EP and USP monograph are almost identical. The monographs differ slightly with respect to system suitability requirements and acceptance criteria for pharmaceutical formulation. In addition, the EP also describes the analysis of related substances (impurities).

In the monographs the use of the following column type is described for the separation of Gentamicin: size 250 mm, ID 4.6 mm, octadecylsilyl silca gel stationary phase (packing L1) and particle size 5 µm. The Phenomenex Luna 5 µ C18(2), 250 x 4.6 mm column which matches this criteria was chosen for the method evaluation.

For the detection of Gentamicin 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 USP and EP assay. An alternative approach for the analysis of Gentamicin with significantly shorter analysis time is described in reference [5].

Table 1

LC-EC Conditions	
HPLC	ALEXYS Gentamicin Analyzer with post-column addition kit (375 µL mixing coil)
Column	4.6 mm ID x 25 cm, 5µm packing L1
Mobile phase	7 mL/L Trifluoroacetic acid, 250 µL/L Pentafluoropropanoic acid, 4 mL/L 12.5M NaOH (carbonate-free) adjusted to pH 2.6, 15.5 mL/L Acetonitrile
Flow rate	1.0 mL/min, post-column: 0.3 mL/min
$V_{injection}$	20 µL
Temperature	35°C for separation, mixing and detection
Flow cell	VT-03™ with Au WE, stainless steel AE and Ag/AgCl Salt bridge RE, spacer 50 µm
Potential waveform	E1, E2, E3: +0.05,+0.75, -0.15 V ts, t1, t2, t3: 0.3, 0.4, 0.15, 0.45 s
I-cell	ca. 0.5 µA
ADF	0.5 Hz

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 & USP monographs (Table 1). The concentration Acetonitrile was slightly adjusted to 15.5 mL/L to optimize the separation. 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 0.5 µA with these PAD settings.

The peaks of the Gentamicin main constituents and impurities (A: Sisomicin; B: Garamine) in the recorded chromatogram were identified using the chromatogram supplied with the standard *Gentamicin for peak identification CRS*.

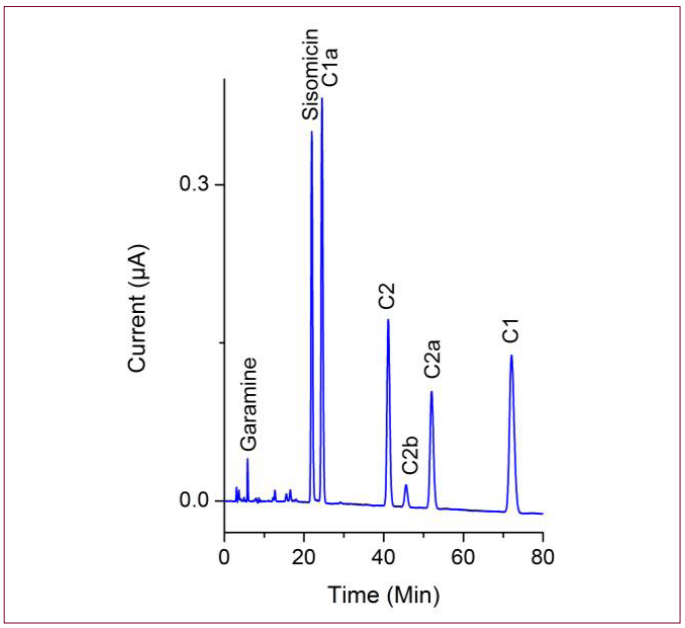

Figure 2: 20 µL injection 20 µg/mL Sisomicin sulphate CRS with 100 µg/mL Gentamicin sample in mobile phase (Reference solution (d) as described in EP monograph).

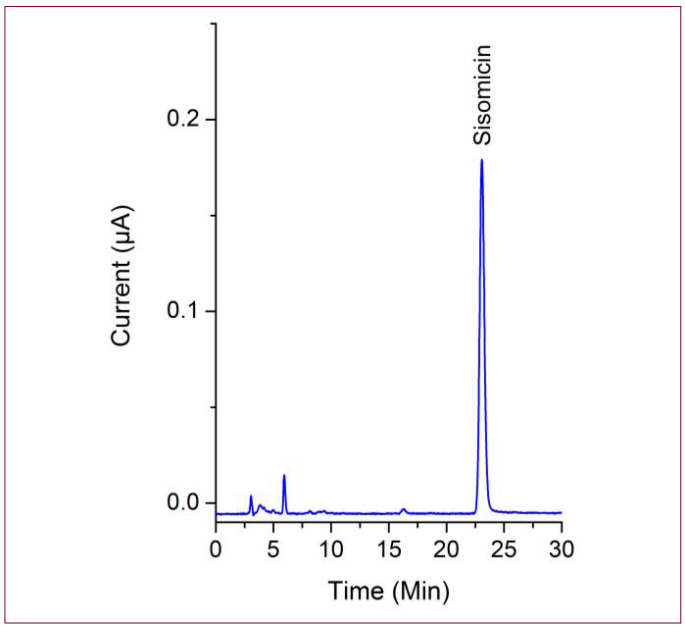

Figure 3: 20 µL injection 10 µg/mL Sisomicin sulphate CRS in mobile phase.

Table 2

Retention Time of Gentamicin Main Constituents		
Component	*Retention time (min)*	*Relative Retention**
Garamine (Impurity B)	5.8	0.27
Sisomicin (Impurity A)	22.0	1.0
Gentamicin C1a	24.5	1.1
Gentamicin C2	41.1	1.9
Gentamicin C2b	45.6	2.1
Gentamicin C2a	52.0	2.4
Gentamicin C1	72.1	3.3

**) Relative retention time with reference to impurity A (22 min).*

System Suitability

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

- *Resolution:* minimum 1.2 between impurity A and Gentamicin C1a and 1.5 between Gentamicin C2 and C2b in chromatogram obtained with reference solution (d).
- *Signal-to-Noise ratio:* minimum 20 for the principal peak in the chromatogram obtained with the reference solution (c).

The system suitability was evaluated using the chromatograms of reference solution (c) and (d), see figure 3 and 2 respectively.

Table 3

EP System Suitability Requirement		
Parameter	*EP criteria*	*Measured*
Resolution between Impurity A & C1a	> 1.2	3.2
Resolution between C2 and C2b*	> 1.5	3.4
Signal-to-Noise ratio (Impurity A)	> 20	323

**) USP requirement: resolution between C2 and C2.*

The system suitability requirements are met for all parameters (table 3). Note: in the USP monograph the only system requirement is that the resolution between C2 and C2b is met (> 1.5).

Linearity & Repeatability

The linearity of gentamicin was investigated in the concentration range of 25 – 200 µg/mL. For all gentamicin derivatives the correlation coefficients were better than 0.997 for peak areas. The relative standard deviation (RSD) in peak area for a triplicate injection of test solution (b) was ranging between 0.3 – 0.6% for C1, C1a, C2 and C2a. Only for C2b, with its relatively low peak height, the RSD was slightly higher (1.1%). The LOD (S/N ratio of 3) for Impurity A was 9 ng/mL.

Sample Analysis

For a commercial sample the composition and related substances were analyzed and evaluated using the EP and USP acceptance criteria. The relative percentage of each gentamicin derivative in the commercial formulation was calculated using the peak area obtained from the chromatogram of test solution (b) shown in figure 1. The sum of all peak areas (C1a, C2 C2a, C2b and C1) corresponds to 100%.

Note that the calculation of the composition for the EP and USP slightly differ. In the EP the sum of C2, C2a and C2b is used; in the USP monograph the sum of C2 + C2a and the sum of C2b + C1. The results are shown in table 4; it is evident that the evaluated commercial sample met the acceptance criteria of both the EP and USP.

Table 4

EP System Suitability Requirement				
Peak	*EP**		*USP**	
	Limits (%)	Calculated (%)	Limits (%)	Calculated (%)
C1a	10-30	28	10-35	28
C2	35-55	41	25-55	38
C2a				
C2b			25-50	34
C1	25-45	31		

**) The calculation of the composition for EP and USP slightly differ. In the EP the sum of C2, C2a and C2b is used; in the USP monograph the sum of C2 + C2a and the sum of C2b + C1.*

In addition, the EP monograph also describes acceptance criteria for impurity levels in commercial samples. For that purpose all impurities are quantified and compared to the response of the principal peak (Impurity A) obtained from the chromatogram of reference solution (c).

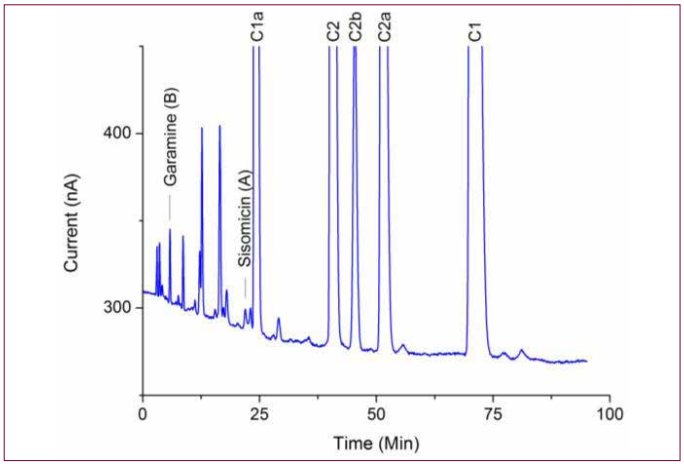

Figure 4: 20 µL injection of test solution (a) for the impurity quantification (1 mg/mLGentamicin sample in mobile phase).

The relative peak areas of all impurities in the commercial sample are listed in table 5.

Table 5

Impurity Analysis		
Impurity	*RT (min)*	*Relative Peak Area**
1	3.1	0.07
2	3.6	0.07
3	4.2	0.02
Garamine (Impurity B)	5.8	0.11
5	7.6	0.02
6	8.7	0.11
7	11.2	0.02
8	12.2	0.13
9	12.7	0.39
10	15.5	0.03
11	16.5	0.50
12	17.3	0.04
13	18.0	0.11
Sisomicin (Impurity A)	22.0	0.08
15	23.1	0.08
17	28.0	0.02
18	29.1	0.09
22	55.6	0.07
24	77.2	0.07
25	81.1	0.11
Total	-	2.11

**) Relative Peak Area of the impurities are calculated in the following way: Relative peak area = Area of the impurity divided by the peak area of the principle peak in the chromatogram obtained with reference solution (c).*

The EP acceptance criteria for the amount of impurities are:

- *Impurity A, B (and any other impurity)*: Not more than 3x the peak area of sisomicin peak in the chromatogram of reference solution (c).
- *Total impurities:* Not more than 10x the peak area of sisomicin peak in the chromatogram of reference solution (c).
- *Discard limit:* Impurities with peak areas smaller than 0.5x the peak area of sisomicin peak in the chromatogram of reference solution (c) can be discarded.

The commercial sample met all impurity acceptance criteria. In fact the response of the majority of all impurities in the sample was under the discard limit of 0.5.

Table 6

LC-EC Conditions	
HPLC	ALEXYS Gentamicin Analyzer with post- column addition kit (375 µL mixing coil)
Column	4.6 mm ID x 25 cm, 5µm packing L1
Mobile phase	7 mL/L Trifluoroacetic acid, 250 µL/L Pentafluoropropanoic acid, 4 mL/L 12.5M NaOH (carbonate-free) adjusted to pH 2.6, 15.5 mL/L Acetonitrile
Flow rate	1.0 mL/min, post-column: 0.3 mL/min
$V_{injection}$	20 µL
Temperature	35°C for separation, mixing and detection
Flow cell	VT-03™ with Au WE, stainless steel AE and Ag/AgCl RE, spacer 50 µm
Potential waveform	E1, E2, E3: +0.05,+0.75, -0.15 V ts, t1, t2, t3: 0.3, 0.4, 0.15, 0.45 s
I-cell	ca. 0.5 µA
ADF	0.5 Hz

Table 7

Test & Reference solutions EP	
Sample solution (a)	1 mg/mL Gentamicin sample in MP
Sample Solution (b)	0.2 mg/mL Gentamicin sample in MP
Reference Solution (a)	0.2 mg/mL Gentamicin for peak identification CRS in MP
Reference Solution (b)	1 mg/mL Sisomicin CRS in MP
Reference Solution (c)	10 µg/mL Sisomicin CRS in MP
Reference Solution (d)	20 µg/mL Sisomicin CRS with 100 µg/mL Gentamicin sample in MP

Table 8

Retention Time		
Component	*Retention time (min)*	*Relative Retention*
Garamine (Impurity B)	5.8	0.27
Sisomicin (Impurity A)	22.0	1.0
Gentamicin C1a	24.5	1.1
Gentamicin C2	41.1	1.9
Gentamicin C2b	45.6	2.1
Gentamicin C2a	52.0	2.4
Gentamicin C1	72.1	3.3

Table 9

EP system suitability requirements		
Parameter	*EP criteria*	*Measured*
Resolution between Impurity A & C1a	> 1.2	3.2
Resolution between C2 and C2b	> 1.5	3.4
Signal-to-Noise ratio (Impurity A)	> 20	323

Table 10

Relative composition of Commercial Gentamicin Sample (K64)				
Peak	*EP**		*USP**	
	Limits (%)	Calculated (%)	Limits (%)	Calculated (%)
C1a	10-30	28	10-35	28
C2	35-55	41	25-55	38
C2a				
C2b			25-50	34
C1	25-45	31		

**) The calculation of the composition for EP and USP slightly differ. In the EP the sum of C2, C2a and C2b is used; in the USP monograph the sum of C2 + C2a and the sum of C2b + C1.*

Conclusion

The ALEXYS Aminoglycosides Analyzer provides a reliable solution for the analysis of the composition & impurities in commercial Gentamicin Preparations following the official methods of the EP and USP.

Table 11

Impurity Analysis		
Impurity	*RT (min)*	*Relative Peak Area**
1	3.1	0.07
2	3.6	0.07
3	4.2	0.02
Garamine (Impurity B)	5.8	0.11
5	7.6	0.02
6	8.7	0.11
7	11.2	0.02
8	12.2	0.13
9	12.7	0.39
10	15.5	0.03
11	16.5	0.50
12	17.3	0.04
13	18.0	0.11
Sisomicin (Impurity A)	22.0	0.08
15	23.1	0.08
17	28.0	0.02
18	29.1	0.09
22	55.6	0.07
24	77.2	0.07
25	81.1	0.11
Total	-	2.11

*) Relative Peak Area of the impurities are calculated in the following way: Relative peak area = Area of the impurity divided by the peak area of the principle peak in the chroma- togram obtained with reference solution (c).

Table 12

Reagents & Standards	
NaOH 50%, carbonate-free	Boom Chemicals, pn 80011912
Trifluoroacetic acid, HPLC grade	Fischer Scientific, pn T/3258/PB05
Pentafluorpropionic acid, 97%	Acros Chemicals, pn 416920500
Acetonitrile, HPLC grade	Acros Chemicals, pn 268270025
Deionized Water. >18 MΩ-cm	Barnstead, Easy pure II
Gentamicin sulfate CRS, 16500 IU/vial	EP, pn G0200000, batch 8.1
Gentamicin for peak identification CRS*	EP, pn Y0001363, batch 1.0
Sisomicin sulphate CRS, 77.7%	EP, pn S0660000, batch 2.1

*)Gentamicin for peak identification CRS; not injected, reference chroma to- gram for peak identification downloaded from the following location: http://crs.pheur.org/db/4DCGI/View=Y0001363

References

1. W.R. LaCourse, *"Pulsed Electrochemical Detection in High Performance Liquid Chromatography"*, John Wiley & Sons, New York, 1ed,1997.
2. Gentamicin sulphate, *European Pharmacopoeia (EP)*, 8.1, (2014) 2326 -2382
3. Gentamicin sulphate, *United States Pharmacopoeia* (USP), USP37-NF32, 3138-3139
4. V. Manyanga, K. Kreft, B. Divjak, J. Hoogmartens, E. Adams, *J. Chromatogr. A,* 1189, 347-354 (2008).
5. *Gentamicin Sulphate in pharmaceutical formulations,* Antec application note, 217_013

Figure 5: ALEXYS Aminoglycosides Analyzer.

PART NUMBERS AND CONFIGURATIONS

180.0056C	ALEXYS Aminoglycosides analyzer, including column, flow cell, and post-column addition kit
250.1070B	ALA-525 column, 250x4.6mm, 5um C8

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.