



The most reliable LC-EC
applications for
Antibiotics analysis

Aminoglycosides

Amikacin
Framycetin Sulphate
Gentamicin Sulphate
Kanamycin Sulphate
Lincomycin
Neomycin
Spectinomycin
Tobramycin

Macrolide antibiotics

Azithromycin
Azaerythromycin
Clarithromycin
Erythromycin
Roxithromycin

Netilmicin Sulphate According to EP Method

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

Summary

The Netilmicin sulphate analysis 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 Netilmicin sulphate in pharmaceutical preparations.

Introduction

Netilmicin is a semi-synthetic aminoglycoside antibiotic synthesized by alkylation of sisomicin (1-N-ethyl derivative). It is an effective antibiotics used in the treatment against a wide range of gram-positive and gram-negative bacteria. Netilmicin is available as injectable and ophthalmic pharmaceutical preparations.

In Netilmicin, besides sisomicin also low concentrations of other components are present, formed during the synthesis. Such as the 2'-N-ethyl & 6'-N-ethyl derivatives of sisomicin (alkylation products) and 1-N-ethylgaramine (hydrolysis product).

UV detection is not suitable for the detection of low levels of related substance of Netilmicin because it has only a weak UV chromophore. However, due to the presence of a sugar moiety in these analytes, pulsed amperometric detection (PAD) can be successfully utilized [1-3]. The analysis of Netilmicin sulphate in pharmaceutical formulations based on HPLC-PAD is described in the European Pharmacopoeia [4].



Figure 2: ALEXYS Kanamycin and Amikacin Analyzer

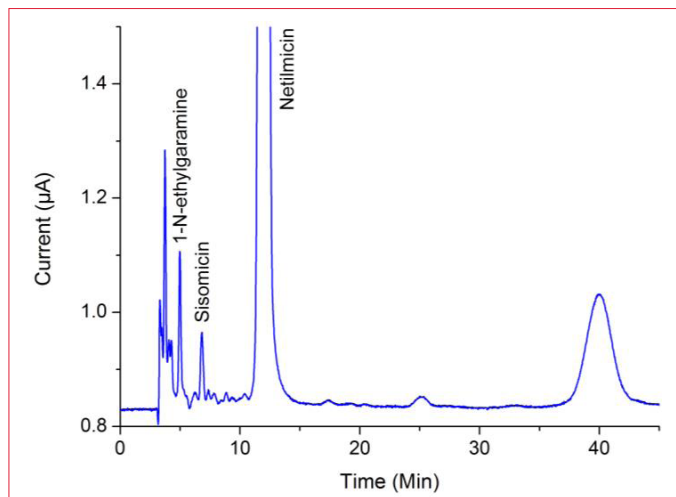


Figure 2: 20 µL injection of a 1 mg/mL Netilmicin sample in mobile phase (Test solution (a) as described in the EP monograph).

Method

In the monographs the use of the following column type is described for the separation of Netilmicin: 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 Netilmicin PAD is mandatory using an Au working electrode (WE), Ag/AgCl reference electrode (RE) and stainless steel auxiliary electrode (AE). The Antec 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 requirements. An alternative approach based on a silica-based C18 column for the analysis of Netilmicin is described in reference [3].



Table 1

LC-EC conditions	
HPLC	ALEXYS Aminoglycosides Analyzer with post-column addition kit (375 µL mixing coil)
Column	4.6 mm ID x 25 cm, packing styrene-divinylbenzene copolymer with a pore size of 100 nm, particle size 8µm
Mobile phase	35 g/L of anhydrous sodium sulfate, 2.0 g/L of sodium octane sulphate, 10ml/L tetrahydrofuran, 50ml/L 0.2M potassium dihydrogen phosphate previously adjusted to pH3.0 with a 22.5g/L solution of phosphoric acid.
Reagent	20 g/L sodium hydroxide (carbonate-free)
Flow rate	1.0 mL/min, post-column: 0.3 mL/min
Vinjection	20 µL
Temperature	50°C for separation, mixing and detection
Flow cell	Sencell* with Au WE, stainless steel AE and Ag/AgCl RE, AST 3
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
Range	20 µA
I-cell	ca. 2.5 µA
ADF	0.5 Hz

*Original: VT-03™ with Au WE, st. steel AE and Ag/AgCl RE, spacer 120 µm

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 (Table 1). The concentration sodium octane sulphate was adjusted to 2 g/L to optimize the separation. Note: only use stabilized THF (stabilized with butylhydroxytoluene) in the mobile phase to assure low 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 [1,4]. The cell current was typical about 2.5 µA with these PAD settings.

Results

The peaks of Netilmicin, Sisomicin (impurity A) and 1-N-ethylgaramine (impurity B) in the recorded chromatograms of the sample solutions were identified using the chromatogram of reference solution (d).

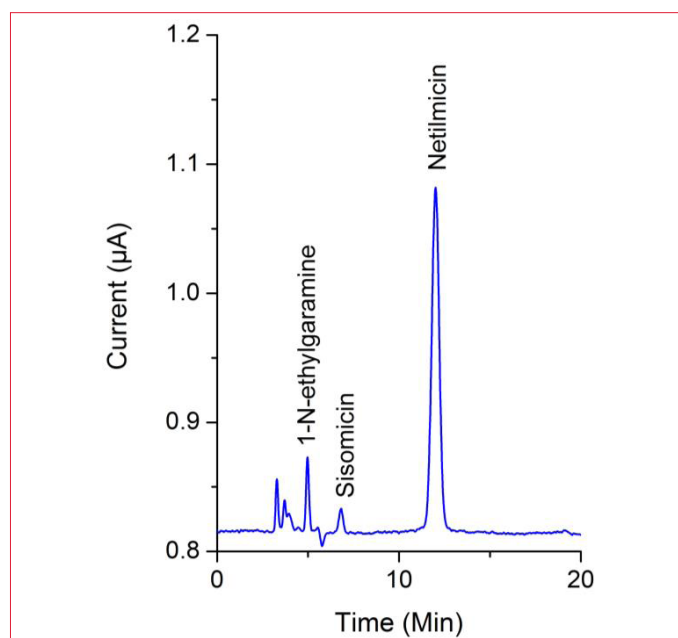


Figure 3: 20 µL injection of a standard consisting of 10 µg/mL Netilmicin sulphate CRS, 10 µg/ml Sisomicin sulphate CRS and 8.2µg/ml 1-N-ethylgaramine sulphate CRS in mobile phase (Reference solution (d) as described in EP monograph).

Table 2

Retention time of Netilmicin and related substances		
Component	Retention (min)	Relative Retention*
1-N-ethylgaramine (Impurity B)	5.0	0.41
Sisomicin (Impurity A)	6.8	0.57
Netilmicin	12.0	1.0

*) Relative retention time (RRT) with reference to Netilmicin (12 min).

System suitability

In the EP monographs for Netilmicin sulphate the following system suitability requirements are specified:

- **Resolution:** minimum 2.0 between 1-N-ethylgaramine (impurity B) and sisomicin (impurity A); minimum 3.0 between sisomicin (impurity A) and Netilmicin in chromatogram obtained with reference solution (d).
- **Signal-to-Noise ratio:** minimum 10 for the principal peak in the chromatogram obtained with the test solution (b).

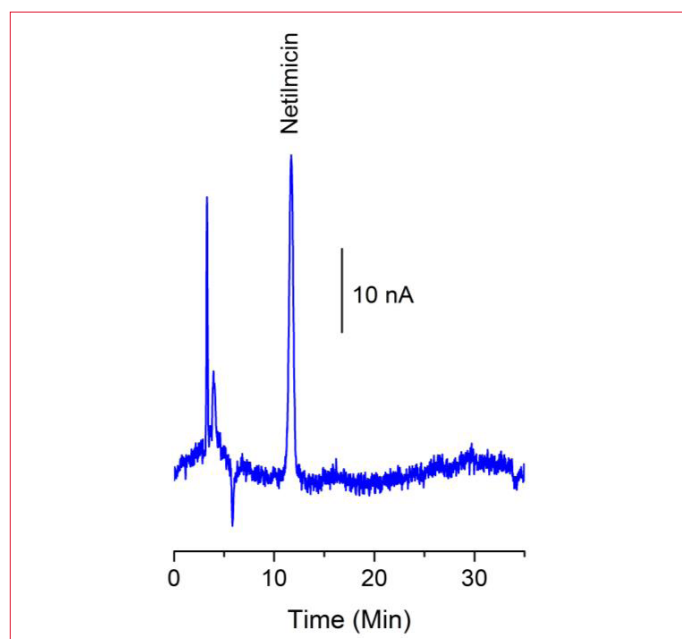


Figure 4: 20 µL injection of 1 µg/mL Netilmicin sample in MP (test solution (b) as described in EP monograph).

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

Table 3

EP system suitability requirement		
Parameter	EP criteria	Measured
Resolution between Impurity B & A	> 2.0	4.5
Resolution between impurity A and Netilmicin	> 3.0	8.0
Signal-to-Noise ratio (Netilmicin)	> 10	15.3

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

Linearity and repeatability

The linearity of Netilmicin and the impurities A and B were investigated in the concentration range of 10 – 30 µg/mL. For all components the correlation coefficients were better than 0.997 for peak areas. The relative standard deviation (RSD) in peak area was determined for 6 replicate injections of reference solution (d), see figure 2 and table 4. The RSD was < 2% for the impurities and 0.6% for the Netilmicin peak.

Table 4

Repeatability (n=6)		
Component	RSD Area* (%)	Measured
1-N-Ethylgaramine (Impurity B)	1.2	4.5
Sisomicin (Impurity A)	1.9	8.0
Netilmicin	0.6	15.3

*) RSD's based on 6 repetitive injections of reference solution (d).

Sample analysis

An unknown Netilmicin sample (K62) was analyzed to determine the composition and related substances (impurities) using the acceptance criteria described in the EP monograph. For that purpose all relevant impurities were quantified in test solution (a) and compared to the response of the corresponding peaks obtained from the chromatogram of reference solution (d). The chromatograms of test solution (a) and reference solution (d) are shown in figure 1 and 2, respectively.



Table 5

Impurity analysis Netilmicin sample (K62)			
Impurity	RRT*	Peak Area (nA.s)	Discard [#]
2	0.31	3336	N
3	0.34	455	Y
4	0.36	683	N
1-N-ethylgaramine	0.41	2857	N
6	0.52	190	Y
Sisomicin	0.57	1838	N
8	0.61	257	Y
9	0.65	332	Y
10	0.74	229	Y
11	0.86	159	Y
Netilmicin	1	407419	-
13	1.45	295	Y
14	2.08	1252	N
15	3.33	30274	N

*) Relative retention time (RRT) with reference to Netilmicin (12 min). #) Discard limit: any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) shown in figure 3.

The EP acceptance criteria for the amount of impurities are:

- **Impurity A:** Not more than the peak area of the sisomicin peak (second peak) in the chromatogram of reference solution (d).
- **Impurity B:** Not more than the peak area of the 1-N-ethylgaramine peak (first peak) in the chromatogram of reference solution (d).
- **Any other impurities:** Not more than the peak area of the Netilmicin peak (third peak) in the chromatogram of reference solution (d).
- **Total of other impurities:** Not more than 2x the peak area of the Netilmicin peak (third peak) in the chromatogram of reference solution (d).
- **Discard limit:** Impurities with peak areas smaller than the peak area of the principle peak (Netilmicin) in the chromatogram of test solution (b) can be discarded.

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

Table 6

Results impurity analysis Netilmicin sample (K62)			
Impurity	RRT	Relative Peak Area*	EP criteria
2	0.31	0.4	< 1
4	0.36	0.1	< 1
1-N-ethylgaramine	0.41	4.4	< 1
Sisomicin	0.57	6.2	< 1
14	2.08	0.2	< 1
15	3.33	3.9	< 1
Total of other impurities	-	4.6	< 2

*) The relative peak areas of the impurities are calculated in the following way: Relative peak area = Peak area of the impurity divided by the peak area of the corresponding peak in the chromatogram obtained with reference solution (d). For the unknown impurities the Netilmicin peak (third peak) is taken as the reference (see limits section in the EP monograph).

The analyzed sample did not comply with the acceptance criteria for the impurity limits as set by the EP for impurity A, B and an unknown impurity with a relative retention time of 3.33. The total of other impurities was calculated by taking the sum of the relative peak areas of impurity 2,4,14 and 15 in the sample. The amount of total other impurities also exceeded the EP acceptance criteria.

Conclusion

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

References

1. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", John Wiley & Sons, New York, 1ed,1997.
2. E. Adams, D. Peulings, M. Rafiee, E. Roets, J. Hoogmartens, *J. Chromatogr. A*, 812, 151-157 (1998).
3. V. Manyanga, J. Hoogmartens, E. Adams, *J. Sep. Sci.*, 33, 1897-1903 (2010).
4. Netilmicin sulfate, *European Pharmacopoeia (EP)*, 8.1, (2014) 2837 -2839

Ordering information

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

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