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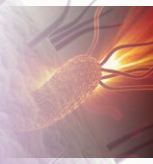


Applications Notebook

Antibiotic Analysis using HPLC/ECD

AG Notebook 217_050_04

Design: MGO-studio, Maarssen, NL
Illustrations: Antec Scientific

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS Aminoglycosides Analyzer. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this notebook are subject to change without further notice.

The author and publisher acknowledge their duty to provide as accurate a publication as possible. Nevertheless, they cannot be held liable for any possible inaccuracies in this publication.

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Part II: Macrolide Antibiotics

Your theory is crazy, but it's not crazy enough to be true.

Niels Bohr

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

Macrolide antibiotics
Azithromycin
Azaerythromycin
Clarithromycin
Erythromycin
Roxithromycin

Amikacin and Kanamycin in Bulk Drugs

- **United States Pharmacopeia USP30–NF25 used as basis for this application**
- **FlexCell with exchangeable gold electrode**
- **Analysis of main substituent and impurities**

Summary

The United States Pharmacopeia (USP) has two monographs describing the analysis of both Kanamycin [5] and Amikacin [6] using LC-PAD. The ALEXYS Kanamycin and Amikacin Analyzer is a dedicated LC solution for the analysis of both antibiotics which matches the USP requirements for peak resolution, tailing and reproducibility. In this note typical results obtained with the Analyzer are shown to demonstrate its performance.

Introduction

Kanamycin and amikacin are closely related, water soluble, broad spectrum aminoglycoside antibiotics. Kanamycin is obtained from Streptomyces kanamyceticus. Amikacin is synthesised by acylation of an amino group of kanamycin A with L-(-)-g- amino-α- hydroxybutyric acid (LHABA). Both antibiotics can be analysed using ionexchange chromatography in combination with pulsed amperometric detection [1-4].

Figure 1: ALEXYS Kanamycin and Amikacin Analyzer

Table 1

Conditions	
HPLC	ALEXYS Kanamicin and Amikacin Analyzer
Temperature	32 °C for separation and detection
Flow rate	0.5 mL/min
Flow cell	FlexCell™ with Au WE and Ag/AgCL REF
ADF	0.5 Hz
Range	2 µA/V

Results

USP requirements

The results listed in the table below are based on an average of ten 20 µL injections of a mixture of 8 mg/L Kanamycin and 20 mg/L Amikacin in water.

Table 2

USP system suitability requirement		
Parameter	USP criteria	Result
Peak resolution	> 3	> 5
Tailing factor	< 2	< 1.6
Reproducibility, area (%RSD)	< 3	1.5% (n=10)

For both Kanamycin and Amikacin an RSD smaller then 1.5% in area was achieved for 10 replicate injections (USP requires <3%). Peak resolution between amikacin and kanamycin was >5 (better than 3 is required for USP). The peak tailing factor for both components was better then 1.6 (USP requires smaller than 2).

Linearity of kanamycin was investigated in the range of 1.6 - 8 mg/L. Linearity of amikacin was investigated in the range of 4 - 20 mg/L. In all cases correlation coefficients were better than 0.998 for peak areas and peak heights.

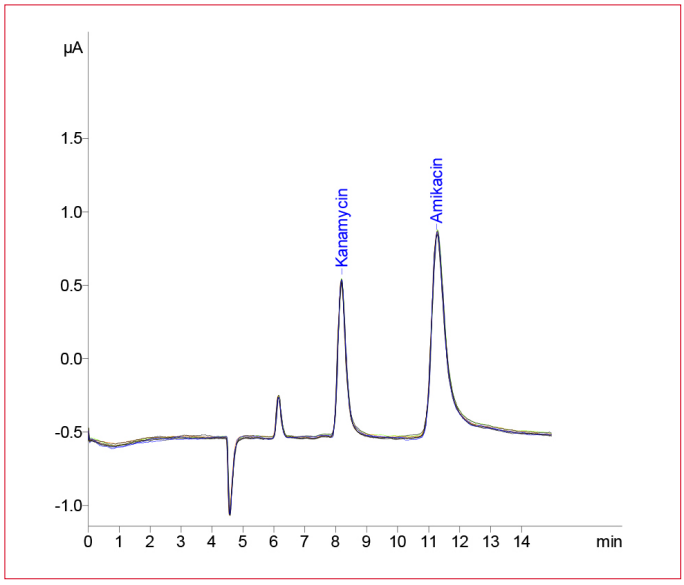


Figure 2: Overlay of 10 injections of 20 µL of 8 mg/L Kanamycin and 20 mg/L Amikacin in water.

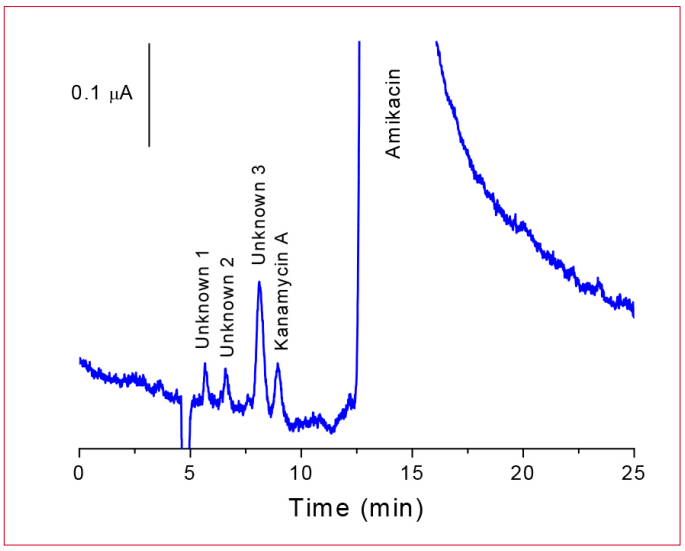


Figure 3: Impurities in a solution of 200 mg/L Amikacin.

Ordering information

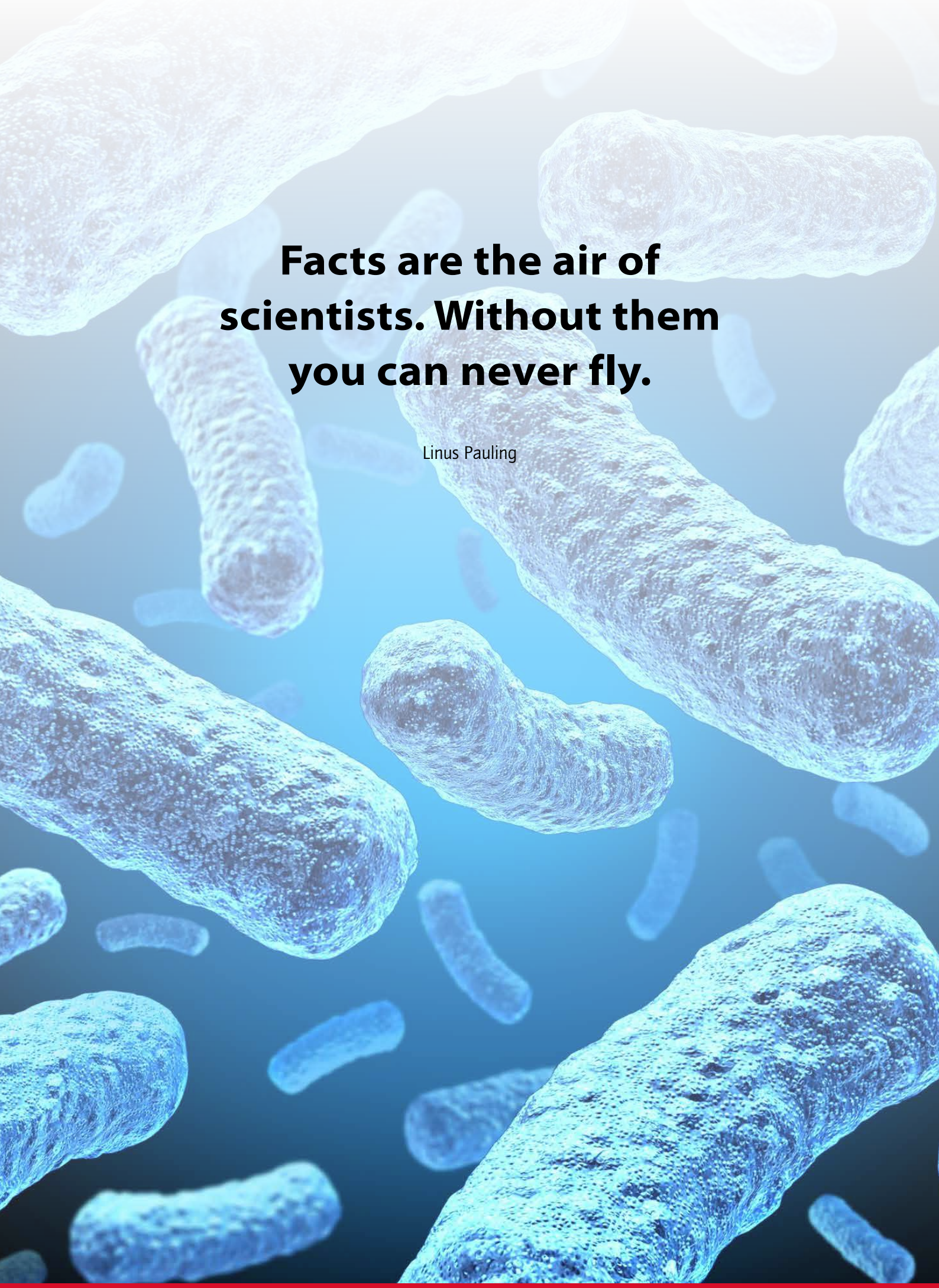
180.0058C	ALEXYS Kanamycin and Amikacin Analyzer, including column, flow cell, and kit
250.1080	ALC-525 anion exchange column, 250x4.6mm, 7um
250.1082	ALC guard column starter kit

References

1. E. Adams, J. Dalle, E. De Bie, I. De Smedt, E. Roets, J. Hoogmartens, *Analysis of kanamycin sulfate by liquid chromatography with pulsed electrochemical detection,* J. Chromatogr. A, 766 (1997) 133-139.
2. E. Adams , G. Van Vaerenbergh, E. Roets, J. Hoogmartens, *Analysis of amikacin by liquid chromatography with pulsed electrochemical detection,* J. Chromatogr. A, 819 (1998) 93-97
3. David A. Stead, *Current methodologies for the analysis of aminoglycosides*, J. Chromatogr. B, 747 (2000) 69-93
4. W.R. LaCourse, *Pulsed Electrochemical Detection in High Performance Liquid Chromatography.*, John Wiley & Sons, New York, 1ed,1997.
5. United States Pharmacopeia (USP), *Kanamycin Sulfate,* USP30-NF25 Page 2434
6. United States Pharmacopeia (USP), *Amikacin Sulfate,* USP30-NF25 Page 1372

Conclusion

The ALEXYS Kanamycin and Amikacin Analyzer provides a sensitive and reliable solution for the analysis of Kanamycin and Amikacin bulk drugs. It matches the USP requirements for peak resolution, tailing and reproducibility.



Facts are the air of scientists. Without them you can never fly.

Linus Pauling

Application Note
Aminoglycoside Antibiotics

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

Gentamicin Sulphate According to EP and USP Method

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

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.

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].

Figure 1: ALEXYS Aminoglycosides Analyzer.

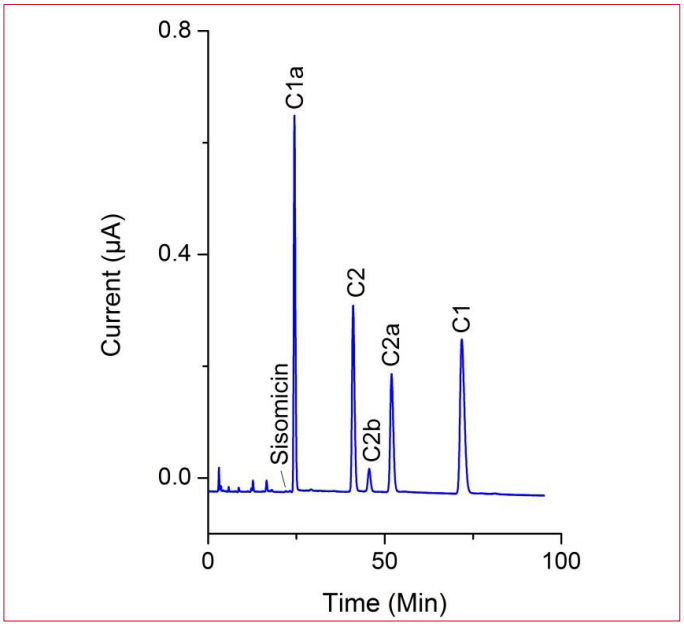


Figure 2: 20 µL injection of a 200 µg/mLGentamicin sample in mobile phase (Test solution (b) as described in EP and USP monograph).

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*.

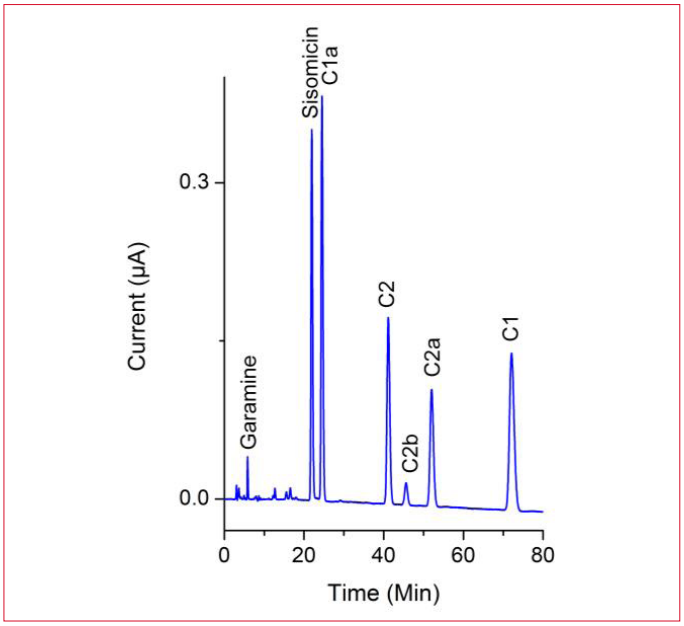


Figure 3: 20 µL injection 20 µg/mL Sisomicin sulphate CRS with 100 µg/mL Gentamicin sample in mobile phase (Reference solution (d) as described in EP monograph).

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).*

Results

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).

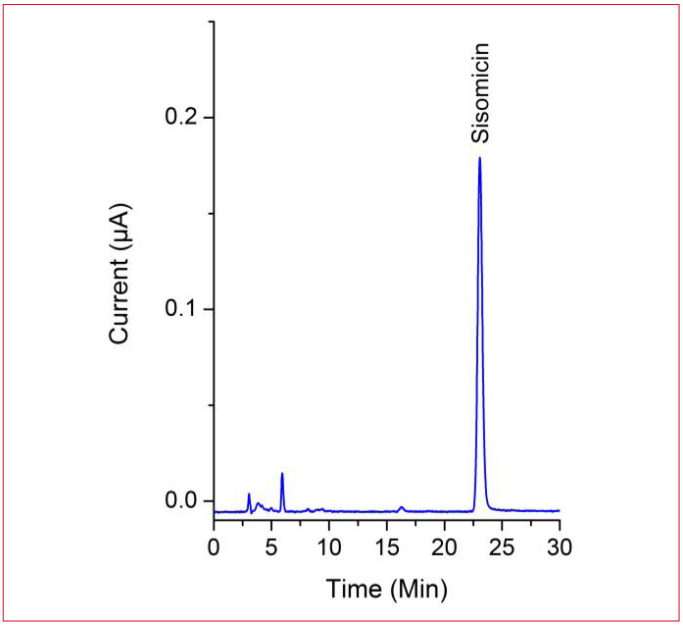


Figure 4: 20 µL injection 10 µg/mL Sisomicin sulphate CRS in mobile phase.

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 and 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).

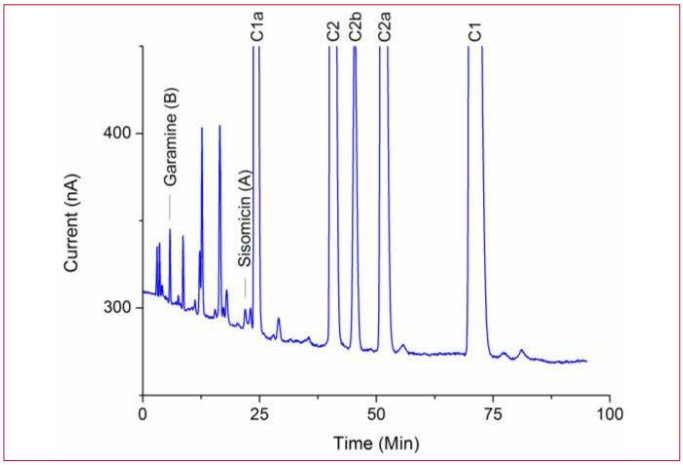


Figure 5: 20 µL injection of test solution (a) for the impurity quantification (1 mg/mLGentamicin sample in mobile phase).

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

Test and 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

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 7

Reagents and 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

Ordering information

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

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

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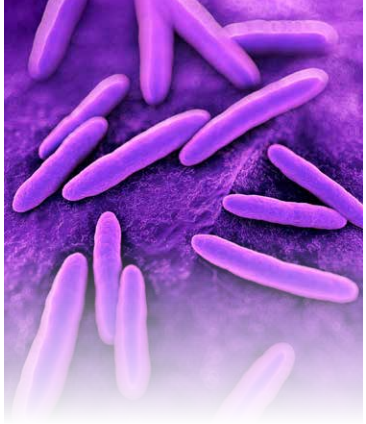
Application Note
Aminoglycoside Antibiotics



The most reliable LC-EC applications for Antibiotics analysis

Aminoglycosides
Amikacin
Framycetin Sulphate
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Spectinomycin
Tobramycin

Macrolide antibiotics
Azithromycin
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Clarithromycin
Erythromycin
Roxithromycin

Gentamicin Sulphate in Pharmaceutical Preparations

- **European Pharmacopoeia 6.0 (2008) used as basis for this application**
- **Analysis of main substituent and impurities**
- **Reproducible & robust**

Summary

In the European Pharmacopoeia 6.0 (2008) the use of a reversed-phase polymeric column is prescribed for this application [1]. In literature it is shown that such a column may result in very wide and tailing peaks [3]. We have confirmed this and found much better separation using a C18 silica-based column.

In this application note typical results obtained with the ALEXYS® gentamicin Analyzer based on a C18 column are reported, demonstrating its performance for the analysis of gentamicin.

Introduction

Like neomycin and tobramycin, gentamicin belongs to the group of aminoglycoside antibiotics. It is manufactured by a fermentation process and 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 [2].

Figure 1: ALEXYS Aminoglycosides Analyzer.

Method

The ALEXYS system equipped with a second pump for the post-column addition of NaOH was used. The mobile phase was prepared as described in the EP monograph [1]: 60g/L Na_2SO_4 (water free), 1.75 g/L octane sulphonic acid, sodium salt, 3 mL/L tetrahydrofuran (THF), 50 mL/L 0.2 M KH_2PO_4 (pH = 3). The flow rate was 1.5 mL/min. A 0.76 mol/L NaOH solution (prepared from a 50 % stock solution) was added post-column with a flow rate of 0.6 mL/min, leading to a final pH of about 13. The cell current was about 2 µA with the PAD settings selected. Note: only use stabilized THF solvents in the mobile phase to assure low cell currents.

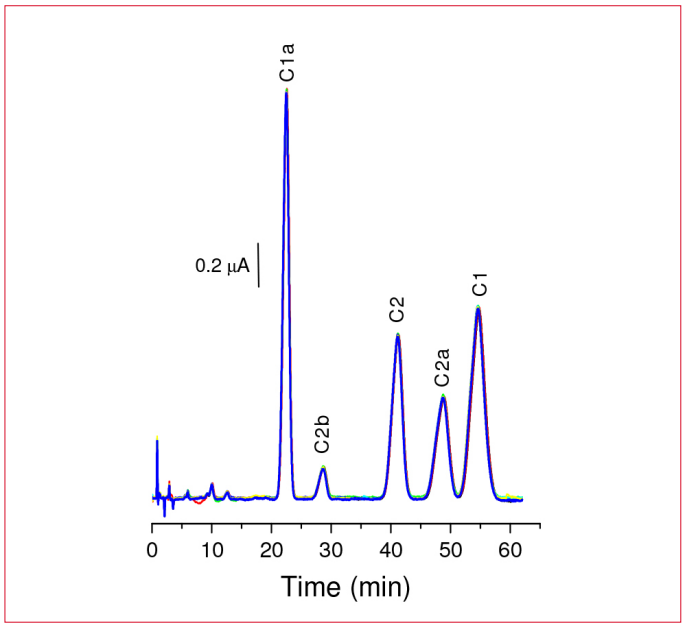


Figure 2: Gentamicin sample (400 µg/ml, 20 µl injected). Overlay of 7 chromatograms. Peak identities were derived from paper [2] and based on peak area percentages.

Table 1

Conditions	
HPLC	ALEXYS Gentamicin Analyzer
Temperature	45 °C for separation and detection
Flow rate	1.5 mL/min, post-column: 0.6 mL/min
Flow cell	FlexCell™ with Au WE and HyREF™
ADF	0.5 Hz
Range	10 µA/V

Results

Linearity & Repeatability

Linearity of gentamicin was investigated in the concentration range of 50 – 500 µg/mL. For all gentamicin derivatives the correlation coefficients were better than 0.998 for peak areas and peak heights. The relative standard deviation (RSD) in peak area for 10 replicate injections for gentamicin was ranging between 0.9 and 2.5% for gentamicin C1 and C2b, respectively. The RSD for the retention times was better then 0.2%. Peak resolution between gentamicin C2a and C1 was 1.6.

EP requirements

In the EP monographs for gentamicin Sulphate a system suitability requirement is specified for the *peak-to-valley ratio*. The peak-tovalley ratio is specified as Hp/Hv , where Hp = height above the baseline of the peak due to gentamicin C2a, and Hv = height above the baseline of the lowest point of the curve separating this peak from the peak due to gentamicin C2. the peak-to-valley ratio Hp/Hv > 2.0. In Table 1 this EP requirement is compared with the typical results obtained with the ALEXYS gentamicin Analyzer.

Table 2

EP system suitability requirement

Parameter	EP criteria	Result
peak-to-valley ratio Hp/Hv	> 2.0	100

It is evident from Fig. 2 that gentamicin C2 and C2a are well baseline separated and therefore the peak-to-peak ratio requirement is easily met by the gentamicin Analyzer.

References

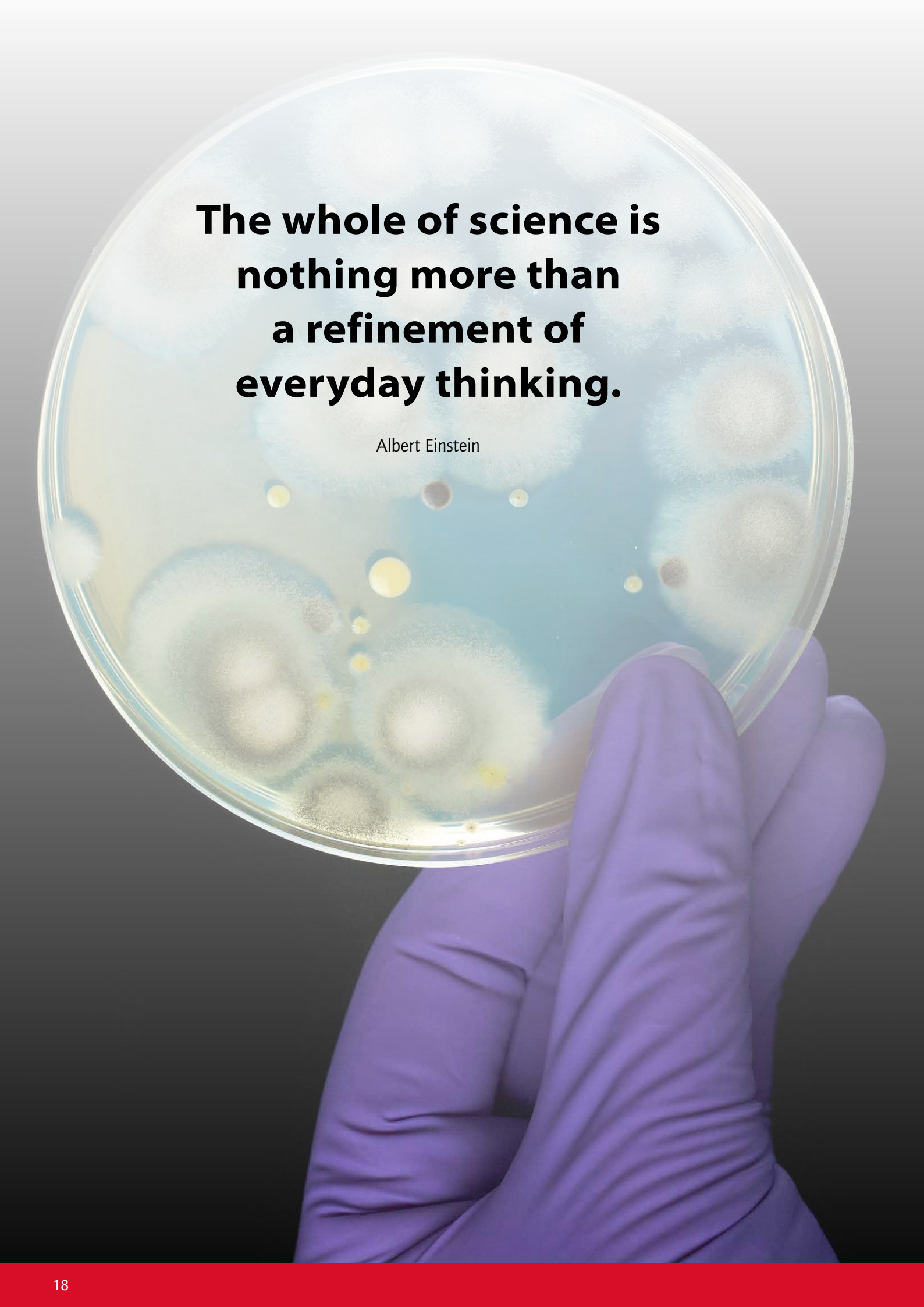
1. Gentamicin sulphate, *European Pharmacopoeia*, 6.0, (2008) 1965-1967
2. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", *John Wiley & Sons, New York*, 1ed,1997.
3. E. Adams, W. Roelants, R. De Paepe, E. Roets, J. Hoogmartens, *J. Pharm. Biomed. Anal.*, 18, 689-698 (1998).

Ordering information

180.0056C	ALEXYS Aminoglycosides Analyzer, including column, flow cell, and post-column addition kit
250.1068	ALA-510 C18 column, 100x4.6mm, 5um

Conclusion

The ALEXYS Gentamicin Analyzer provides a reliable solution for the routine analysis of gentamicin in pharmaceutical preparations. It meets the EP requirement for peak-to-valley ratio between gentamicin C2 and C2a.


The whole of science is nothing more than a refinement of everyday thinking.
Albert Einstein

Application Note
Aminoglycoside Antibiotics

The most reliable LC-EC applications for Antibiotics analysis

Aminoglycosides
Amikacin
Framycetin Sulphate
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- **FlexCell with exchangeable gold electrode**
- **Analysis of main substituent and impurities**
- **Reproducible & robust**

Summary

The European Pharmacopoeia (EP) has two monographs describing the analysis of Neomycin and Framycetin [4,5] using LC-PAD. The ALEXYS Aminoglycosides Analyzer is a dedicated LC solution for the analysis of Neomycin and Framycetin, which matches the EP requirements for peak resolution and signal-to-noise ratio of the principal peak (Neomycin B). In this application note typical results obtained with the Aminoglycosides Analyzer are reported demonstrating its performance for the analysis of impurities in bulk drugs.

Neomycin and Framycetin Sulphate in Bulk Drugs

Introduction

Neomycin is an antibiotic complex consisting of a mixture of the aminoglycosides Neomycin A, B and C, obtained from Streptomyces fradiae, were Neomycin B is the main constituent. It is a widely-used broad spectrum water-soluble antibiotic useful primarily in infections involving aerobic bacteria. It is available as skin ointment (e.g., creams, gels, lotions, etc.) and eye drops. Framycetin (also known as Neomycin B sulphate) is an aminoglycoside antibiotic similar to Neomycin and commonly sold under the brand name Soframycin. Impurities in neomycin and framycetin preparations are analyzed using reversed phase HPLC, with post-column NaOH addition and pulsed amperometric detection (LC-PAD) [1-3].

Figure 1: ALEXYS Aminoglycosides Analyzer.

Method

The Aminoglycosides Analyzer is applied for the analysis of several aminoglycosides including Neomycin, Tobramycin and Spectinomycin. The Analyzer is equipped with a second pump for the post-column addition of 0.5M NaOH to facilitate PAD detection of the aminoglycosides [2,3]. For post-column mixing a low dead volume Tee connector was used and a PEEK mixing coil with a volume of 375 µL between the Tee and the flow cell.

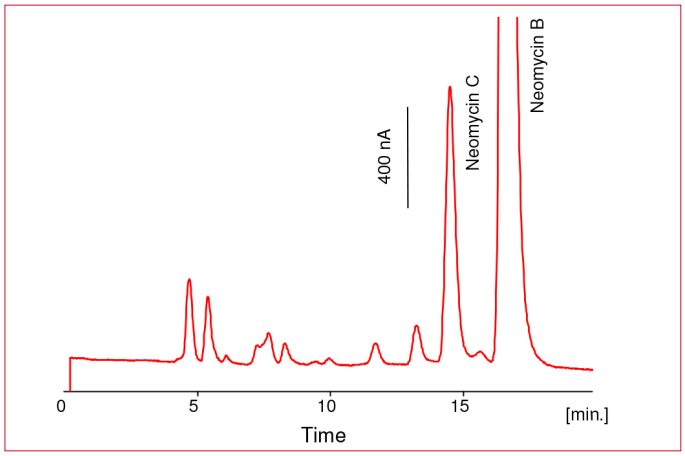


Figure 2: Chromatogram of a 0.5 mg/mL solution of commercial Neomycin sulphate formulation, 10 µL injected. Neomycin B is the main constituent and neomycin C the main impurity.

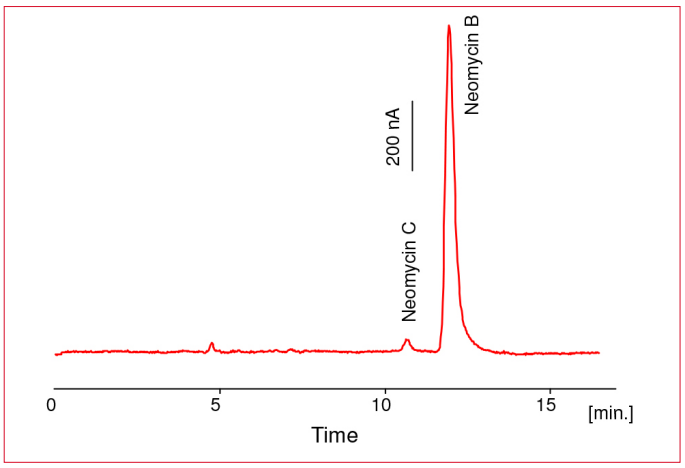


Figure 3: Example chromatogram of a 75 µg/mL Framycetin sulphate reference standard solution (EP BP190 –F67029), 10 µL injected (Note that chromatogram 1b was recorded with slightly different LC conditions as 1a).

The mobile phase was prepared as described in the European Pharmacopoeia monographs [4,5]. The optimal mobile phase consisted of 2% trifluoroacetic acid (20 mL/L) and 8 mL/L of a commercial 50% carbonate-free NaOH solution.

Results

According to the EP the pH may be changed to optimize the resolution between Neomycin C and the principal peak (Neomycin B) if necessary. The effect of pH on the separation of Neomycine was investigated with the aminoglycosides Analyzer by varying the amount of 50% NaOH solution in the mobile phase.

In Fig. 3 two chromatograms are shown recorded with a mobile phase with 6 mL/L 50% NaOH (blue curve, pH 1.18) and 8 mL/L 50% NaOH (red curve, pH 1.21), respectively. The retention time for Neomycin C and B shifted significant with increasing pH, and a change in resolution of 1.7 to 2.3 was observed. It is evident from that the pH of the mobile phase is an effective parameter to optimize the LC separation of the aminoglycosides and its impurities.

Table 1

Conditions	
HPLC	ALEXYS Aminoglycosides Analyzer
Oven temperature	32 °C (column and detection)
Flow rate	0.7 mL/min, 0.5 mL/min post column
Flow cell	FlexCell™ with Au WE and HyREF™
ADF™	0.5 Hz
Range	10 µA/V

EP criteria

In the EP monographs for Neomycin and Framycetin two system suitability requirements are specified for peak resolution and signal-to-noise ratio of the principal peak. In Table 2 the criteria of the EP are compared with the typical results obtained with the ALEXYS Aminoglycosides Analyzer.

An example chromatogram of reference solution (c) for the calculation of the signal-to-noise ratio of Neomycin B is shown in Fig. 5. The EP requirements for both peak resolution and S/N ratio are met with the aminoglycosides Analyzer.

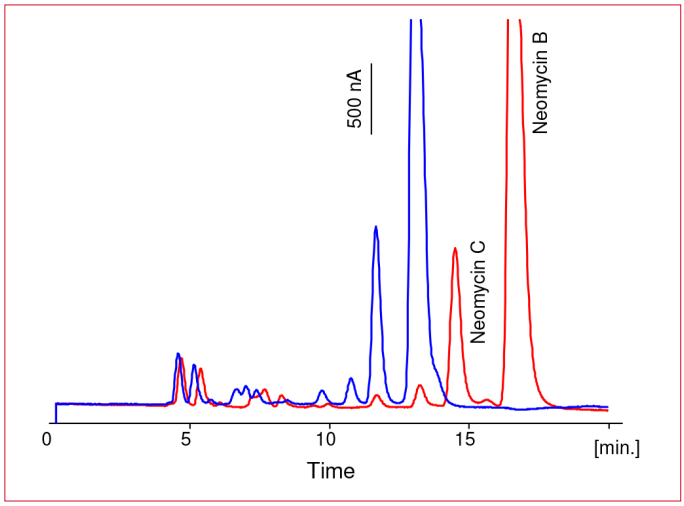


Figure 4: Effect of pH on the separation. Blue: mobile phase with 6 mL/L 50% NaOH (pH 1.18), Red: mobile phase with 8 mL/L 50% NaOH (pH 1.21).

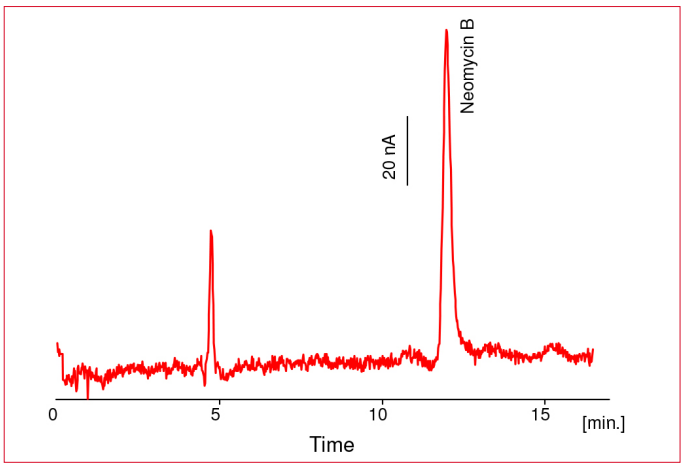


Figure 5: EP system suitability: chromatogram of a 10 µL injection of 5 µg/mL framycetin (reference solution c) for signal-to-noise ratio calculation (Neomycin B). Actual S/N = 25.

The C_{LOD} for Neomycin B is approximately 0.6 µg/mL. The C_{LOD} defined as the concentration that gives a signal that is three times the peak-to-peak noise.

Table 2

EP system suitability requirement		
Parameter	EP criteria	Result
Peak resolution	> 2	2.3
S/N principle peak	> 10	25

Repeatability

The repeatability of the method was evaluated by executing 11 repetitive injections (10 µL) of a 0.5 mg/mL Framycetin and 0.5 mg/mL Neomycin solution. The relative standard deviation (RSD%) for retention time, peak area and height are listed in table II.

Table 3

Peak table			
	%RSD tR	%RSD H	%RSD A
Neomycin			
Neomycin B	0.11	1.06	0.62
Neomycin C	0.08	1.42	2.42
Framycetin			
Neomycin B	0.04	2.48	1.92
Neomycin C	0.10	1.06	1.75

For Neomycin B and C, RSD's smaller then 2.5% (n=10) were found for both peak area and peak height.

References

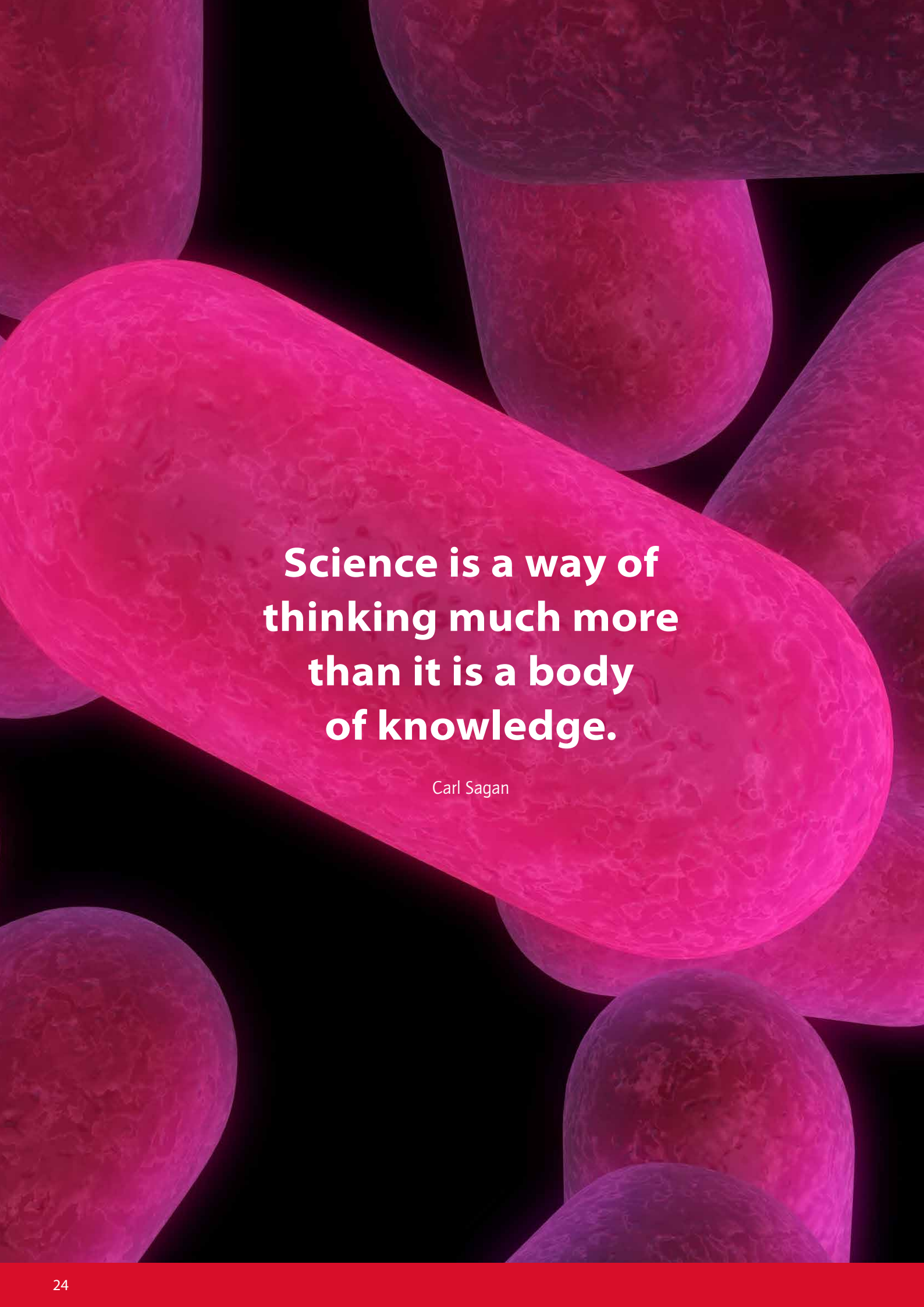
1. David A. Stead, "Current methodologies for the analysis of aminoglycosides", J. Chromatogr. B, 747 (2000) 69–93
2. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", John Wiley & Sons, New York, 1ed,1997.
3. E. Adams, R. Schepers, E. Roets, J. Hoogmartens, "Determination of neomycin sulfate by liquid chromatography with pulsed electrochemical detection", J. Chromatogr. A, 741 (1996) 233 - 240
4. "Neomycin sulphate", European Pharmacopoeia, 6.0, (2008) 2487-3489
5. "Framycetin sulphate", European Pharmacopoeia, 6.0, (2008) 1947-1949

Ordering information

180.0050C	ALEXYS Aminoglycosides Analyzer, including column, flow cell, and post-column addition kit
250.1070	ALA-525 C18 column, 250x4.6mm, 5um

Conclusion

The ALEXYS® Aminoglycosides Analyzer provides a sensitive and reliable solution for the analysis of impurities in Neomycin and Framycetin bulk drugs. It meets the EP requirements for peak resolution and signal-to-noise ratio.


Science is a way of thinking much more than it is a body of knowledge.
Carl Sagan

Application Note
Aminoglycoside Antibiotics

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 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

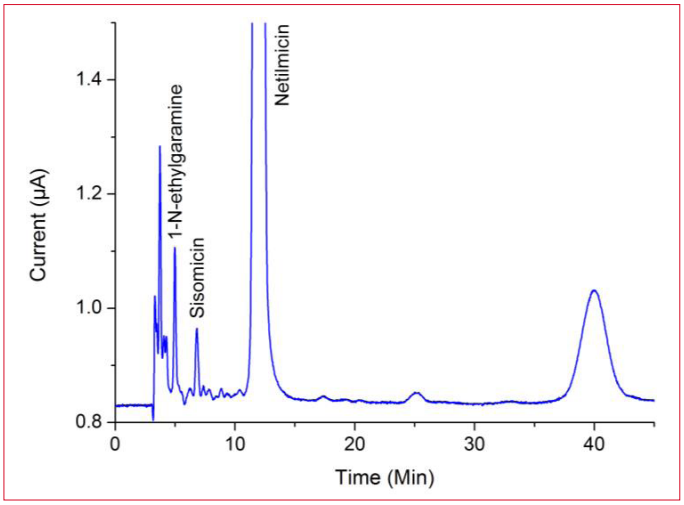


Figure 2: 20 µL injection of a 1 mg/mL Netilmicin sample in mobile phase (Test solution (a) as described in the EP monograph).

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 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 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	VT-03™ with Au WE, stainless steel AE and Ag/AgCl RE, spacer 120 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
Range	20 µA
I-cell	ca. 2.5 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 (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-ethyl garamine (impurity B) in the recorded chromatograms of the sample solutions were identified using the chromatogram of reference solution (d).

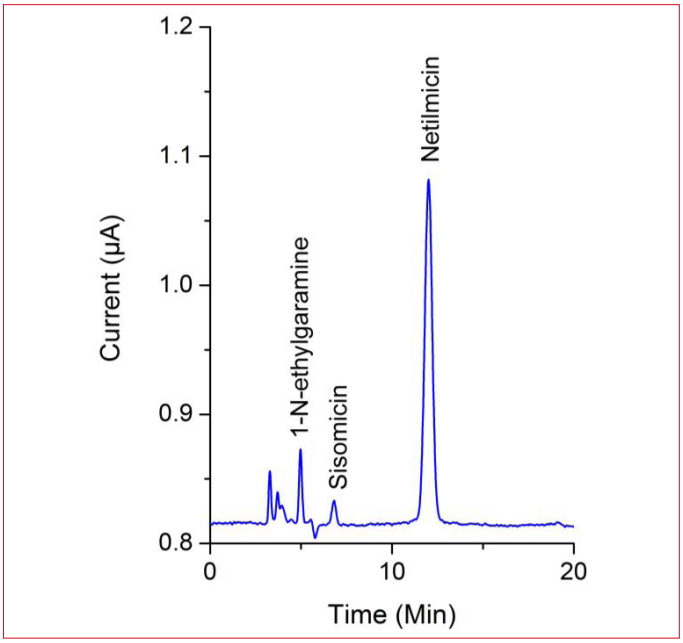


Figure 3: 20 µL injection of a standard consisting of 10 µg/mL Netilmicin sulphate CRS, 10 µg/ml Sisomicin sulphate CRS and 8.2µg/ml 1-N-ethyl garamine sulphate CRS in mobile phase (Reference solution (d) as described in EP monograph).

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 requirement 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:* Signal-to-Noise ratio: minimum 10 for the principal peak in the chromatogram obtained with the test solution (b).

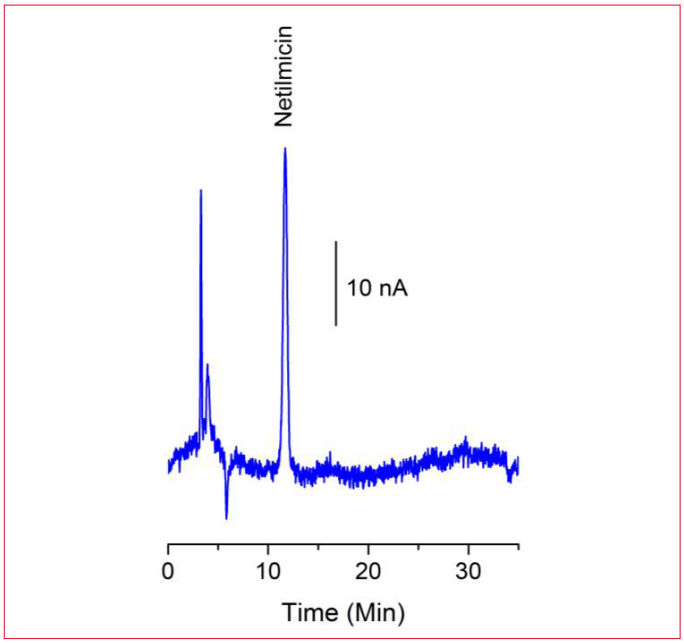


Figure 4: 20 µL injection of 1 µg/mL Netilmicin sample in MP (test solution (b) as described in EP monograph).

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.

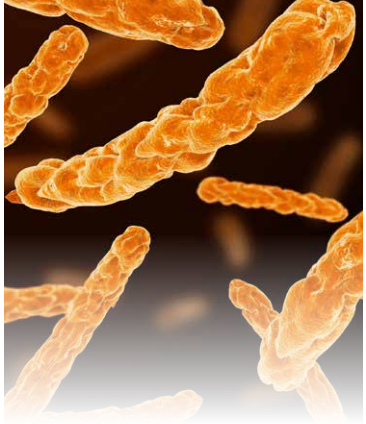
References

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, 8um

Application Note
Aminoglycoside Antibiotics



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

Spectinomycin and Lincomycin

- **FlexCell with exchangeable gold electrode**
- **Analysis of main substituent and impurities**
- **Reproducible & robust**

Summary

In this note a method is described for the simultaneous analysis of spectinomycin and lincomycin using the ALEXYS Spectinomycin, Lincomycin Analyzer. The method is based on reversed phase chromatography in combination with a step gradient. Detection is accomplished using post-column addition of sodium hydroxide in combination with pulsed amperometric detection (PAD)[2].

Introduction

Lincomycin and spectinomycin are aminoglycoside antibiotics that are mainly used for veterinary purposes. They are often added as a mixture to the drinking water of poultry to prevent respiratory infections. The simultaneous analysis of both components in formulations is complicated by the large difference in chromatographic retention behaviour [1].

Figure 1: ALEXYS Aminoglycosides Analyzer for Spectinomycin

Method

Table 1

Conditions	
HPLC	ALEXYS 'Lincomycin, spectinomycin Analyzer' (part no. 180.0059A)
Flow rate	0.4 mL/min; post-column: 0.2 mL/min
Sample	20 µl injection
Temperature	35 °C for column, mixing and flow cell
Flow cell	FlexCell™ with Au WE and HyREF™
Range	50 µA/V
Icell	About 7 µA

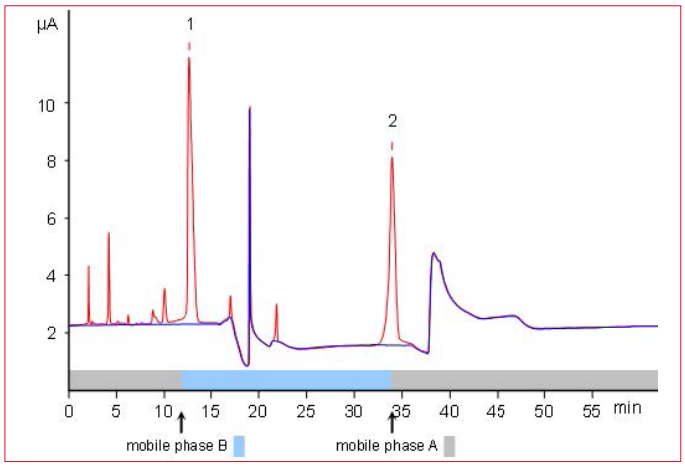


Figure 2: Overlay of baseline (blue) and chromatogram (red) of 100 mg/L spectinomycin (1) and lincomycin (2) dissolved in mobile phase A. The black scheme represents the step gradient program.

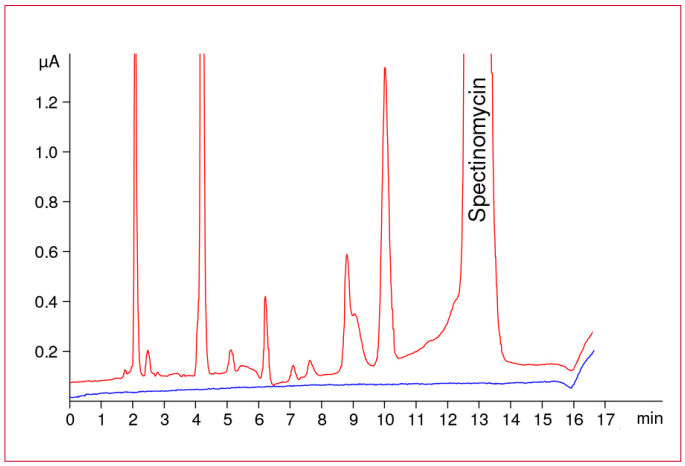
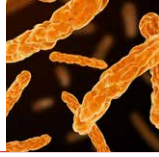


Figure 3: Detail of first 15 minutes of Fig. 2 showing spectinomycin impurities.



The chromatography is based on a step gradient elution using two mobile phases that differ in the ionic strength and THF concentration. This results in the chromatogram shown in Figure 2. A lag time of about 4 minutes can be observed when comparing the changes in the baseline and the step gradient pattern.

The composition of mobile phase A is chosen so that spectinomycin and the early eluting impurities are separated within 15 minutes (Figure 3). To speed up the elution of lincomycin, mobile phase B is applied after 12 minutes. After the elution of lincomy- cin, the system is allowed to stabilise for 26 minutes in mobile phase A.

The retention times are significantly affected by the concentration of THF in the mobile phase in the range of 0-2% (Fig. 2). A THF concentration higher than 2% should not be used, as this results in mobile phase precipitation (milky white colour) that clogs the system. It is also important to use stabilised THF to assure low cell currents.

Lincomycin which has a protonated amine function at pH 3, is retained by ion pairing with OSA. Retention time of lincomycin is therefore not only affected by THF but also by ionic strength. The apolar spectinomycin is primarily affected by THF only.

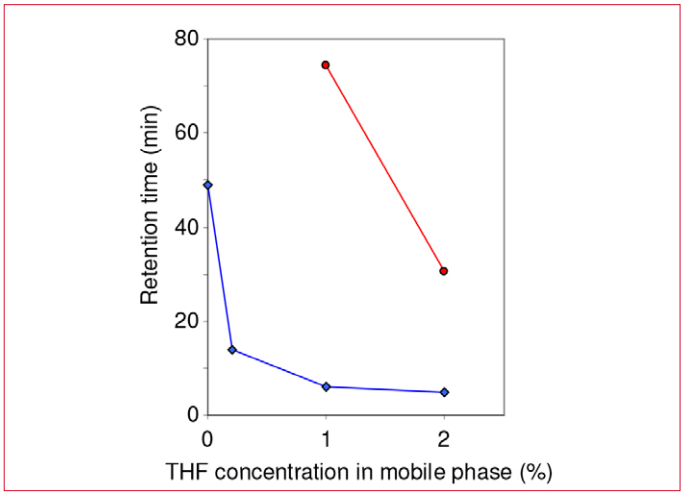


Figure 4: Effect of THF concentration in mobile phase A on retention time of spectinomycin (blue) and lincomycin (red).

Before starting a sample queue the ion pair LC system must be equilibrated by running a few blank chromatograms. The stabilisation takes about 3 hours, during which the gradient should run 3 times. This can be observed from Fig. 3 (a comparable pattern was observed for spectinomycin), where the analyses were started after prolonged stabilisation of the system in mobile phase B.

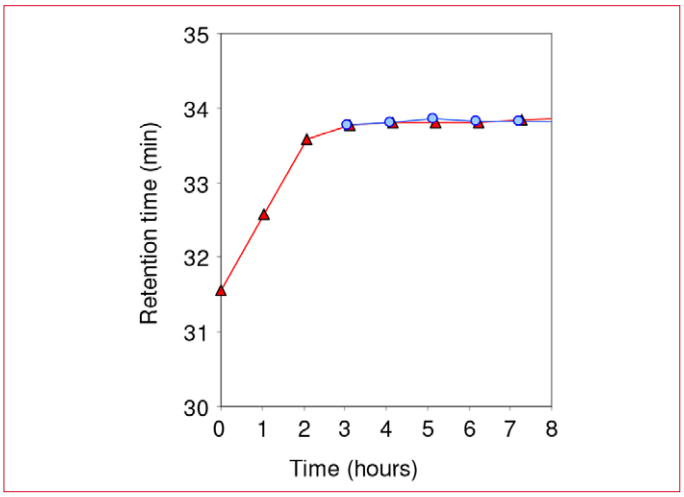


Figure 5: Retention time of lincomycin. The first 3 runs show a strong shift in retention time due to equilibration of the LC system (red). If a sequence is started with 3 blank injections the performance is greatly improved (blue).

Results

Linearity

In the concentration range of 10-50 mg/L the correlation coefficient with peak area is 0.999 or better for spectinomycin as well as lincomycin (Fig. 6).

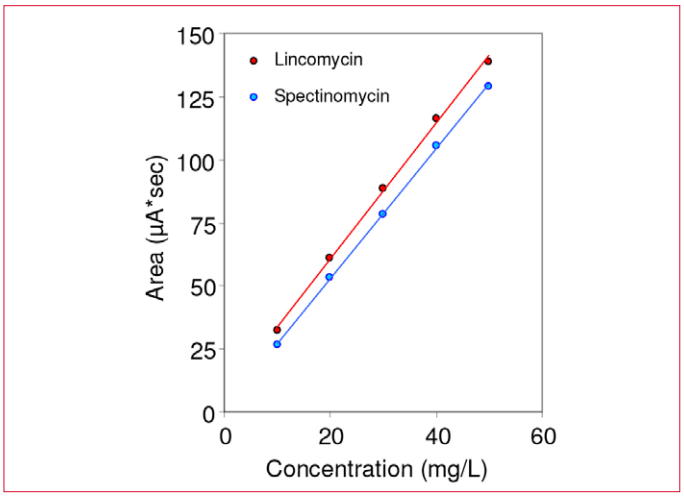


Figure 6: Calibration plot with linear regression lines for spectinomycin and lincomycin.

Intra-day reproducibility

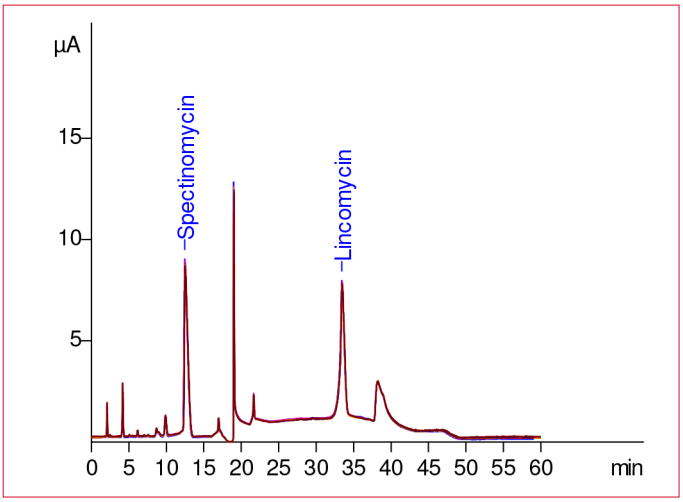


Figure 7: Overlay of 6 chromatograms of 100 mg/L spectinomycin and lincomycin in mobile phase A.

Table 2

Reproducibility of chromatograms shown in Fig. 7

Parameter	*Ret. time %RSD*	*Height %RSD*	*Area*
spectinomycin	Height	2.0	%RSD
lincomycin	Area	1.3	2.0

A representative overlay of 6 consecutively measured chromatograms is given in Fig. 5 with RSD values given in Table 2. Reproducibility (n=6) of 2% RSD or better was observed for peak area of spectinomycin and lincomycin on all days. This RSD value is better than the system requirements for the comparable isocratic analysis of spectinomycin , according to the European Pharmacopoeia [3], which is 3% RSD (n=6) or better. Reproducibility of 0.2 % or better was observed for retention times.

Polishing the gold working electrode

As the gold working electrode is consumed during pulsed amperometric detection, the cell volume increases, which leads to lower signals over time. To restore the signal and cell volume, the gold electrode should be polished to the original flat surface. A special polishing kit for gold working electrodes has been developed. It consists of three disks with decreasing abrasive (30 – 1 micron) [5].

The 30 µm disk is used to remove the surface indentation if present. The intermediate disk removes most of the roughness from the fist step, and the 1 µm diamond polishing step will restore the surface to a mirror-like shine. After this procedure the system needs 10 h of stabilisation time while running the gradient program (Fig. 6), after which the signal is reproducible with an intraday RSD of 2% or better (see above).

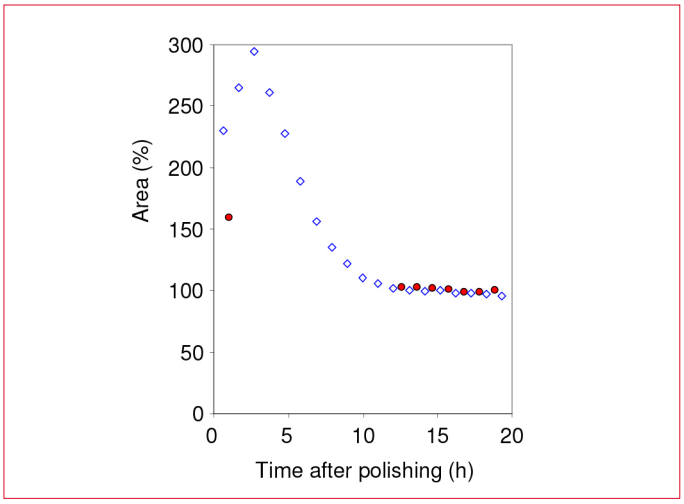
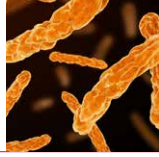


Figure 8: . Relative peak area of lincomycin after having polished the gold working electrode. Blue squares represent a series of continuous analyses, and red circles represent a series of analyses where blank gradient traces were run for 10 hours between the first and subsequent injection.



Inter-day reproducibility

The inter-day repeatability was measured in relation to the polishing procedure (see above) that is required to clean the gold electrode once in 2 weeks. Average area data from 4 different days are presented in Table 3. Variations from flow cell polishing (day 1 and 12) and a stand by time of a day (day 11) are shown in this table.

Variation in peak area or peak height (intra-day repeatability) is better than 6% RSD. Table 3 shows the average (n=6) peak height and peak area of 100 mg/L spectinomycin and lincomycin in mobile phase A on different days.

The measurements on day 1 and 12 were done 10-12 h after polishing (P) the gold WE disk. The measurement of day 10 were done after letting the system stabilise for 3 hours after a system shut down of a day. Until day 11 continuous measurements were done followed by polishing on day 12.

Table 3

Interday reproducibility

		Height, µA		Area, µA*sec	
Day	*Events*	*Spec.*	*Linco.*	*Spec.*	*Linco.*
1	P+10h	8.4	6.0	298	255
10	Start+3h	9.6	6.7	290	264
11	-	8.6	6.7	263	272
12	P+12h	9.1	6.4	293	275
intra day average		9	6	286	267
%RSD		5.9	5.7	5.5	3.5

References

1. J. Szúnyog, E. Adams, K. Liekens, E. Roets, J. Hoogmartens, *Journal of Pharmaceutical and Biomedical Analysis* 29:213-220 (2002)
2. W. R. LaCourse, *Pulsed Electrochemical Detection in High-Performance Liquid Chromatography, Wilet,* New York, 1997
3. "Spectinomycin Dihydrochloride Pentahydrate", European Pharmacopoeia, 6.0, (2008) 2947-2949
4. V. P. Hanko, W. R. LaCourse, C. O. Dasenbrock, J. S. Rohrer, *Drug Development Research* 53:268-280 (2001).
5. Antec Leyden, *Flattening & Polishing kit for metal WE: User Guide,* part number 250.7010

Ordering information

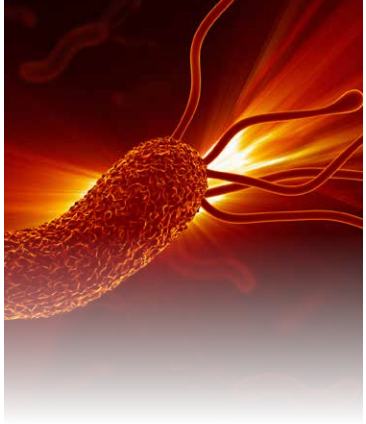
180.0059C	ALEXYS 'Lincomycin, spectinomycin Analyzer'
250.1125	ALF-315 C18 column, 150x3.0mm, 3um

Conclusion

The ALEXYS Lincomycin, spectinomycin Analyzer is a robust and reliable solution for the routine analysis of spectinomycin, lincomycin and its impurities.


The science of today
is the technology
of tomorrow.
Edward Teller

Application Note
Aminoglycoside Antibiotics



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

Spectinomycin in Pharmaceutical Preparations

- **European Pharmacopoeia 6.0 (2008) used as basis for this application.**
- **FlexCell with exchangeable gold electrode**
- **Analysis of main substituent and impurities**
- **Reproducible & robust**

Summary

The ALEXYS® Aminoglycosides Analyzer is a dedicated LC solution for the analysis of Spectinomycin, which matches the EP requirements for peak resolution and repeatability of the principal peak. The European Pharmacopoeia, 6.0, (2008), 2947-2949 was used as a basis to set-up this method. In this application note typical results obtained with the Aminoglycosides Analyzer are reported demonstrating its performance for the analysis of impurities in Spectinomycin bulk drugs.

Introduction

Spectinomycin is an aminoglycoside- like antibiotic produced by Streptomyces spectabilis. In solution, spectinomycin will undergo a ring opening and closing of the hemiketal function, resulting in an equilibrium mixture of four possible anomers. Hydrolysis with acid produces actinamine and in basic solutions actinospectinoic acid (ASA) is formed. Important fermentation impurities are dihydrospectinomycin and dihydroxyspectinomycin [1, 2].Because of the presence of glycoside groups in Spectinomycin and by-products, LC with pulsed amperometric detection (PAD) has been applied for analysis [3]. Conditions are to a large extent in correspondence with the EP requirements [4,5].

Figure 1: ALEXYS Aminoglycosides Analyzer for Spectinomycin.

Method

Solutions and standards are prepared as described in the EP method [4,5]. Assay validation was done with special attention to EP requirements.

Table 1

Conditions	
HPLC	ALEXYS Aminoglycosides Analyzer (part no.180.0050A)
Flow rate	1 mL/min, post-column: 0.5 mL/min
Flow cell	FlexCell™ with Au WE and HyREF™
Temperature	35 DC for separation and detection
Range	2 µA/V
ADF	0.5 Hz
I-cell	about 2 µA

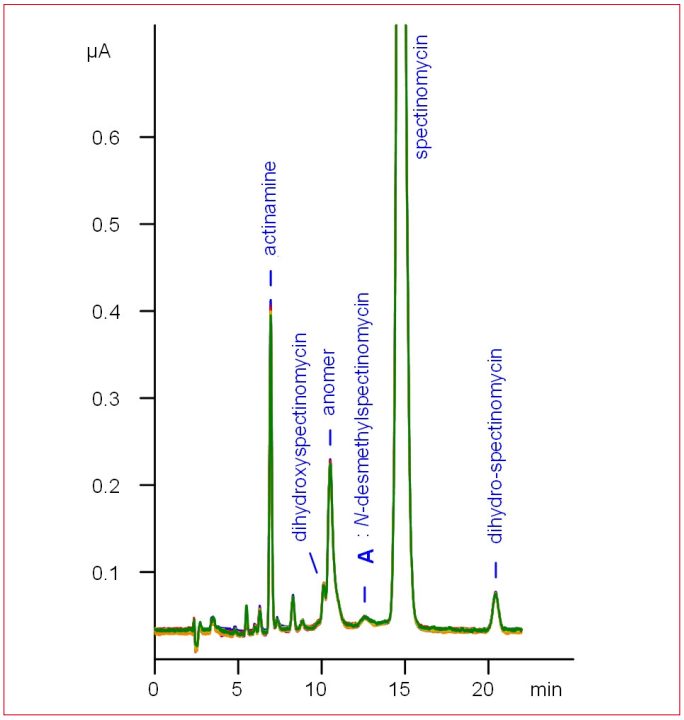


Figure 2: Overlay (n=6) of 20 µL injections of 80 mg/L Spectinomy- cin-HCl in mobile phase (diluted from 800 mg/L Spectinomycin in water, standing time 68 h).

Results

In the EP monographs for Spectinomycin [4.5] two system suitability requirements are specified:

[1] Peak resolution: between impurity E and the principle peak (Spectinomycin), R > 1.5.
[2] Repeatability: maximum RSD(%) for the principle peak, n=6 injections, RSD > 3%.

In Table 2 the criteria of the EP are compared with the typical results obtained with the ALEXYS Aminoglycosides Analyzer.

Table 2

EP system suitability requirement		
Parameter	EP criteria	Result
RSD of principal peak	< 3.0 %	1.0 %
Resolution, peak 'A'	> 1.5	2.5

Table 2. Performance ALEXYS aminoglycosides Analyzer versus EP system suitability requirements.

It is evident from table I that the EP requirements for both peak resolution and repeatability are met by the ALEXYS aminoglyco- sides Analyzer.

References

1. J. Szunyog, E. Adams, K. Liekens, E. Roets, J. Hoogmartens, *Journal of Pharmaceutical and Biomedical Analysis,* 29 (2002) 213–22.
2. D. Debremaeker, E. Adams, E. Nadal, B. Van Hove, E. Roets, J. Hoogmartens, *Journal of Chromatography A*, 953 (2002) 123–132
3. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", *John Wiley & Sons, New York,* 1ed,1997.
4. "Spectinomycin Dihydrochloride Pentahydrate", *European Pharmacopoeia*, 6.0, (2008) 2947-2949
5. "Spectinomycin Sulphate Tetrahydrate for veterinary use", *European Pharmacopoeia*, 6.0, (2008) 2949 -2951

Ordering information

180.0050C	ALEXYS Aminoglycosides Analyzer, including column, flow cell, and post-column addition kit
250.1070	ALA-525 C18 column, 250x4.6mm, 5um

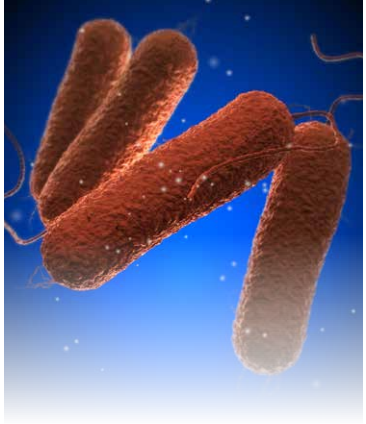
Conclusion

The ALEXYS Aminoglycosides Analyzer provides a reliable solution for the routine analysis of Spectinomycin in Pharmaceutical Preparations. It meets the EP system suitability requirement for resolution and repeatability.

Research is what I'm doing when I don't know what I'm doing.

Wernher von Braun

Application Note
Aminoglycoside Antibiotics



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

Tobramycin According to EP Method

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

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.

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].

Figure 1: ALEXYS Aminoglycosides Analyzer.

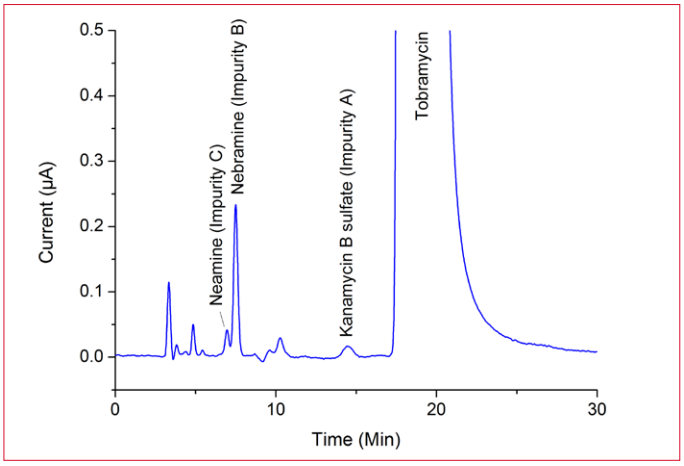


Figure 2: . 20 µL injection of a 1 mg/mL Tobramycin sample in mobile phase (Test solution (a) as described in the EP monograph).

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.

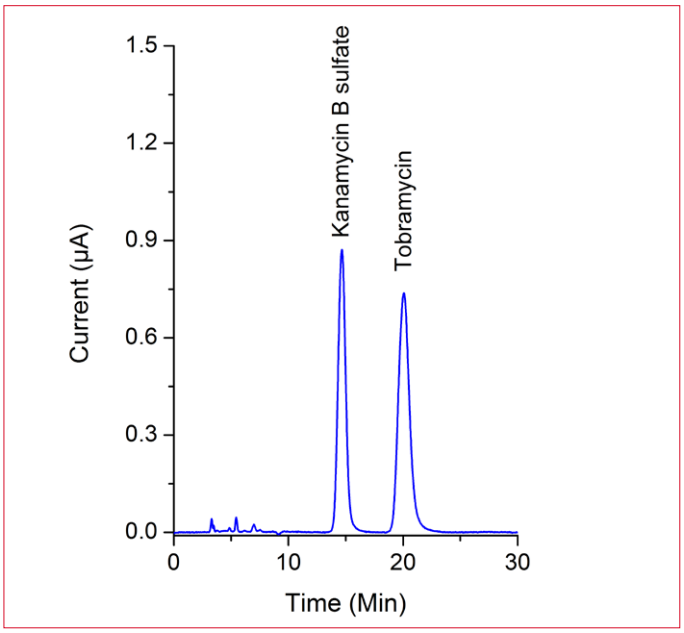


Figure 3: 20 µL injection of a standard consisting of 0.05 mg/mL kanamycin B and 0.05 mg/mL Tobramycin CRS in mobile phase (Reference solution (d) as described in EP monograph).

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.

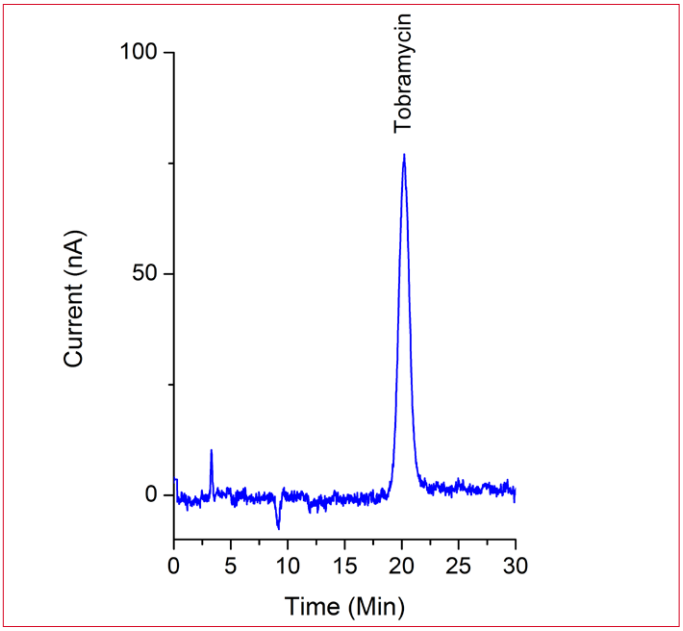


Figure 4: 20 µL injection of 2.5 µg/mL Tobramycin CRS in mobile phase (reference solution (b) as described in EP monograph).

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 and 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.

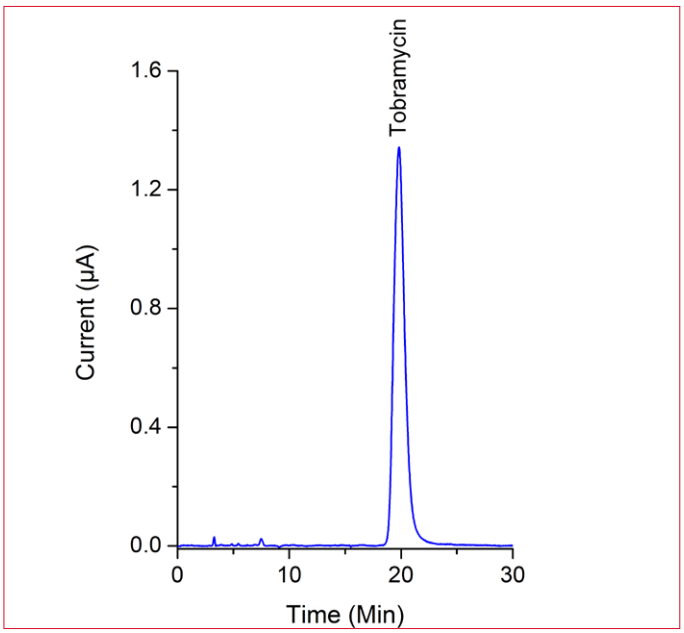


Figure 5: 20 µL injection of 100 µg/mL Tobramycin sample solution in mobile phase (sample solution (b) as described in EP monograph) for the Tobramycin assay analysis.

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.

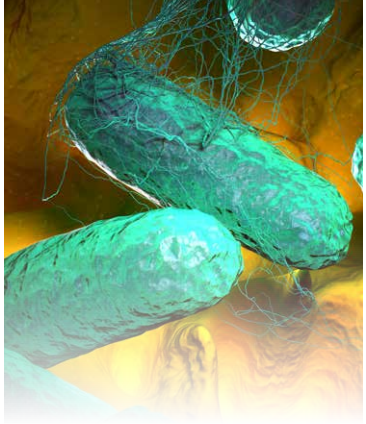
References

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

Ordering information

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

Application Note
Aminoglycoside Antibiotics



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Macrolide antibiotics
Azithromycin
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Clarithromycin
Erythromycin
Roxithromycin

Tobramycin in Pharmaceutical Preparations

- **European Pharmacopoeia 6.0 (2008) used as basis for this application**
- **FlexCell with exchangeable gold electrode**
- **Analysis of main substituent and impurities**
- **Reproducible & robust**

Summary

The European Pharmacopoeia describes a method for the analysis of Tobramycin and its impurities based on LC-PAD [3]. The ALEXYS Aminoglycosides Analyzer is a dedicated solution for the analysis of aminoglycoside antibiotics using a silica-based C18 column.

In this application note results are shown for the analysis of Tobramycin and its impurities using the ALEXYS Aminoglycosides Analyzer.

Tobramycin in Pharmaceutical Preparations

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 by-products.

The analysis of the byproduct 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].

Figure 1: ALEXYS Aminoglycosides Analyzer.

Method

The Aminoglycosides Analyzer (see figure 1) is a versatile solution, it contains all LC hardware and the analytical column for the analysis of several aminoglycosides including Neomycin, Tobramycin and Spectinomycin. The Analyzer is equipped with a second pump for the post-column addition of NaOH. Addition of NaOH is necessary to make the mobile phase strongly alkaline (pH > 12) , in order to allow PAD detection of the aminoglycosides using an Au electrode [2]. The mobile phase was prepared as described in the European Pharmacopoeia monograph [3].

Table 1

LC-EC Conditions	
HPLC	ALEXYS Aminoglycoside Analyzer (part no. 180.0050A)
Flow rate	1 mL/min, post-column: 0.6 mL/min
Cell	FlexCell™ with Au WE and HyREF™
Sample	20 µL
Mobile phase	52 g/L Na2SO4, 1.5 g/L OSA, 3 mL/L THF, 10 mmol/L KH2PO4, pH 3
Addition	0.76 moL/L NaOH post column
Temperature	45 °C for column, mixing and flow cell
E-cell	E1, E2, E3: 0.1, 0.75, -0.15 V ts, t1, t2, t3: 0.1, 0.32, 0.2, 0.4 s
I-cell	ca. 2 µA

Results

In an example chromatogram is shown of a 20 µL injection of 100 µg/mL Tobramycin dissolved in mobile phase. The chromatogram is zoomed in on the baseline to show the impurities (Kanamycin B, Neamine). The impurity Kanamycin B and Tobramycin are sufficiently separated with a resolution of 3.07.

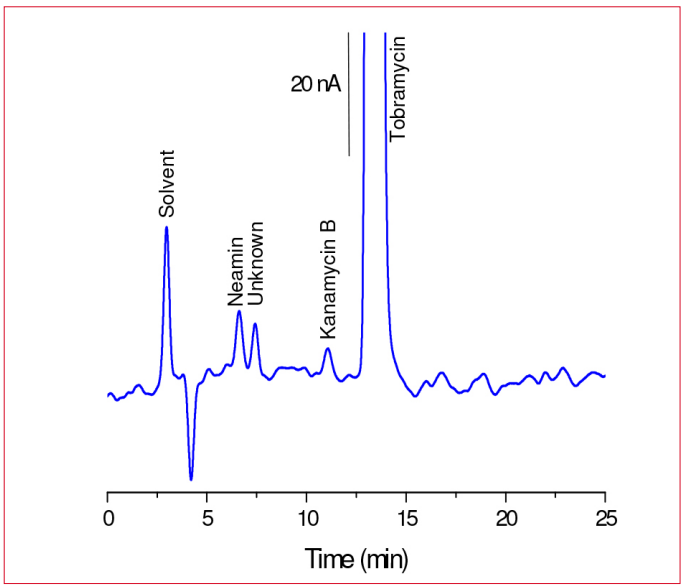

Figure 2: Tobramycin sample (100 µg/mL, 20 µl injected). The peak height of the Tobramycin peak is 2.32 µA. Impurities as percentage % of the main peak are: Neamin 0.38%, unknown 0.29%, Kanamycin B 0.19%.

The EP requires a resolution > 3. In the EP monograph the concentration of octane sulphonic acid in the mobile phase is designated as a variable to optimize the resolution between Kanamycin B and the principal peak (Tobramycin). This parameter can be used to increase the resolution if necessary.

Another EP system suitability requirement is the signal-to-noise ratio of the principle peak of a 20 µL injection of reference standard B (2.5 µg/mLTobramycin CRS solution). The S/N ratio of the principle peak in that case should be larger then 10. The signal-to-noise ratio for the principle peak using the ALEXYS aminoglycosides Analyzer was estimated to be around 15 under the specified conditions (estimation based on the noise and peak height of the principle peak in the chromatogram shown in figure 2, the value for peak height was divided by 40 to reflect a concentration of 2.5 µg/mL Tobramycin).

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. "Tobramycin", European Pharmacopoeia, 6.0, (2008) 3085-3086

Ordering information

180.0050C	ALEXYS Aminoglycosides Analyzer, including column, flow cell, and post-column addition kit
250.1070	ALA-525 C18 column, 250x4.6mm, 5um

Conclusion

The ALEXYS® Aminoglycosides Analyzer is a suitable solution for the analysis of Tobramycin and its impurities in bulk drugs.

If the facts don't fit the theory, change the facts.

Albert Einstein

Application Note
Antibiotics

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

Azithromycin, Erythromycin and other Macrolide Antibiotics

- **Method based on USP 30-NF25, May 2007**
- **Analysis of main substituent and impurities**
- **Column USP L49 stationary phase**

Summary

In this note the USP monograph USP 30-NF25 issued in May 2007 has been used as a basis for the development of a method for the analysis of macrolide antibiotics, using the ALEXYS Macrolide Antibiotics analyzer with a dual flow cell configuration and a column with USP L49 stationary phase.

With this LC system macrolide antibiotics such as Clarithromycin, Erythromycin, Roxithromycin, and Azithromycin were analyzed.

Introduction

The macrolides are a group of antibiotics whose activity stems from the presence of a macrolide ring, a large macrocyclic lactone ring to which one or more deoxy sugars may be attached. Macrolide antibiotics are used to treat infections caused by Gram positive bacteria, Streptococcus pneumoniae, and Haemophilus influenzae infections such as respiratory tract and soft tissue infections. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and, therefore, macrolides are a common substitute for patients with a penicillin allergy.

Azithromycin (Azi) is a semisynthetic macrolide antibiotic chemically related to erythromycin and clarithromycin [1-4]. It is effective against a wide variety of bacteria organisms, such as Hemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus, and many others. Azithromycin is used to treat bacterial infections such as bronchitis; pneumonia; sexually transmitted diseases (STD); and infections of the ears, lungs, skin, and throat.

Figure 1: ALEXYS Macrolide Antibiotics Analyzer.

Method & results

The USP method for Azi uses two flow cells in series, cell 1 for screening and cell 2 for detection of the analytes. The first flow cell is a Reactor cell, the second is the VT-03 cell.

Stock solutions of 220 µM Azaerythromycin (Aza) and 440 µM Azi were prepared in 100% acetonitrile. The final standards were obtained by diluting the stock solutions with mobile phase.

Table 1

Conditions	
HPLC	ALEXYS Azithromycin Analyzer
Temperature	35 °C for separation and detection
Flow rate	1.0 mL/min
Flow cell	1 Flexcell™, GC with Hy-REF™
Flow cell	2 VT-03, 2 mm GC sb REF, 50 µm spacer
ADF	0.5 Hz
Range	100 nA/V

Some conditions differ slightly from the USP method.

Hydrodynamic voltammogram

The USP method requires a working potential of 0.70 ± 0.05 V for cell 1 and 0.82 ± 0.05 V for cell 2. As can be seen in the voltammogram this working potential is not optimal for cell 2. Therefore a working potential of 0.87 V for cell 2 has been used in all experiments, which is the best possible setting within the USP specifications. The working potential of cell 1 was set to 0.65 V.

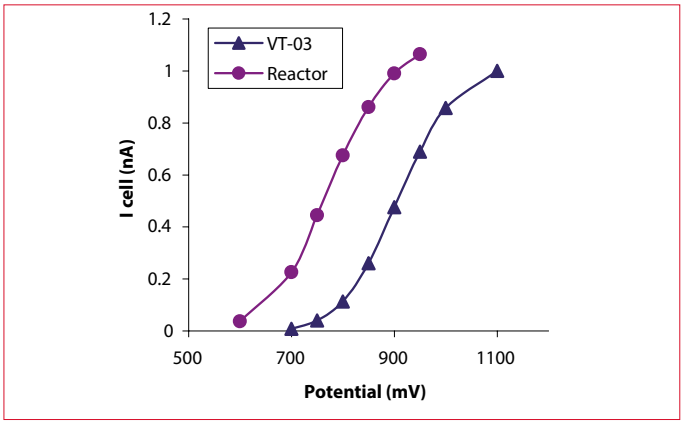

Figure 2: Hydrodynamic voltammogram for Azi on cell 1(dots) and 2 (triangles).

USP criteria

The USP method has a number of method specifications for selectivity, reproducibility, peak asymmetry, plate number and relative retention time. In Table 2 the criteria of the USP are compared with the LC performance obtained with the system. The chromatographic resolution is determined by injecting a standard solution of 4.5 µM Aza and 4.4 µM Azi. Resolution of Azi and Aza is better than 4 (Fig. 3). The relative retention time of Aza vs. Azi is 0.7.

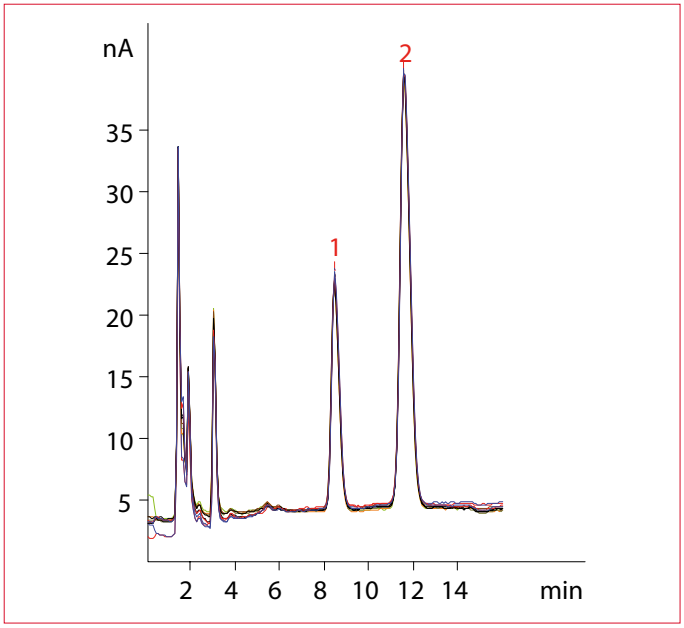

Figure 3: Overlay of repeatability study of 4.4 µM Aza (1) and 4.4 µM Azi (2). Signal cell 2.

The repeatability has been studied for 10 replicate injections of 4.4 µM of Azi and Aza. The relative standard deviation in peak areas was < 1% (Fig. 3).

Table 2

USP system suitability requirement			
Parameter	USP criteria	Aza	Azi
Retention time (min)	-	8.4	11.6
Rel. retention time	0.7 and 1	0.7	1
%RSD area (4.4 µM)	< 2.0%	0.9 %	0.9 %
Theoretical Plates	> 1000	2725	3020
Resolution	> 2.5		4.2
Tailing factor	0.9 to 1.5	1.4	1.3

Some conditions differ slightly from the USP method.

A small shift in retention time was found when using the mobile phase for more than one day. In a period of 5 days this shift is about 30 seconds. This is probably due to a drop in pH of mobile phase. Over the same period of time the pH dropped from 11.0 to 10.8. It should be noted that a 15 mmole/L phosphate buffer at pH 11.0 is not optimal. It is therefore advised to prepare fresh mobile phase every day.

The asymmetry (A) of the Azi peak was calculated using A = 0.5 * [width]/[width of left-half of peak] measured at 5% of peak height. According to the USP the asymmetry (tailing factor) should be a value between 0.9 and 1.5. We found values between 1 and 1.4. The USP requires a column efficiency of more than 1000 plates for Azi and Aza. A column efficiency of more than 2500 plates was found for both substances.

Linearity is measured by constructing a calibration line in the-concentration range 0.1 – 40 µM Azi (not shown). The regression line Y= a + bX is given by Y = 127 [± 59] + 212 [± 3] X. Correlation coefficient r is 0.9992.

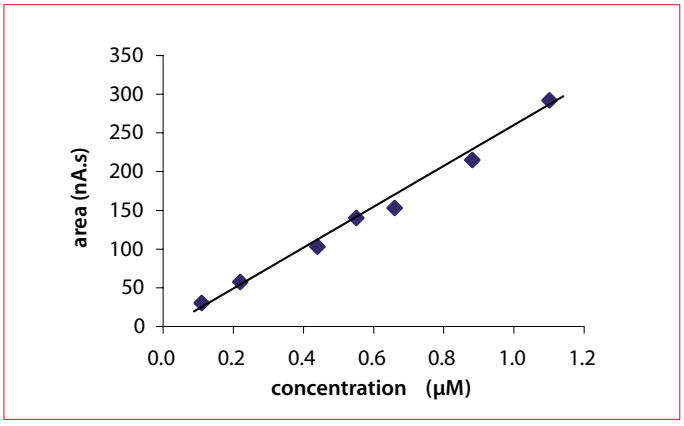

Figure 4: Regression line for 0.1 – 1.1 µM Azi.

For calculation of detection limit a regression line in the concentration range 0.1 – 1.1 µM has been used (Fig. 4). The detection limit calculated as 3 Syx/b is 0.1 µM [5]. Calculation of detection limit as 3 times the peak-to-peak noise of the baseline, results in a detection limit of about 40 nmol/L (Fig. 5). Note that the detection limit is negatively affected by non-optimised working potential, required by USP.

Clarithromycin, Roxithromycin and Erythromycin

A mixture of several macrolides was analysed to illustrate the chromatographic performance of the method. All components are separated with at least a resolution of 2.5.

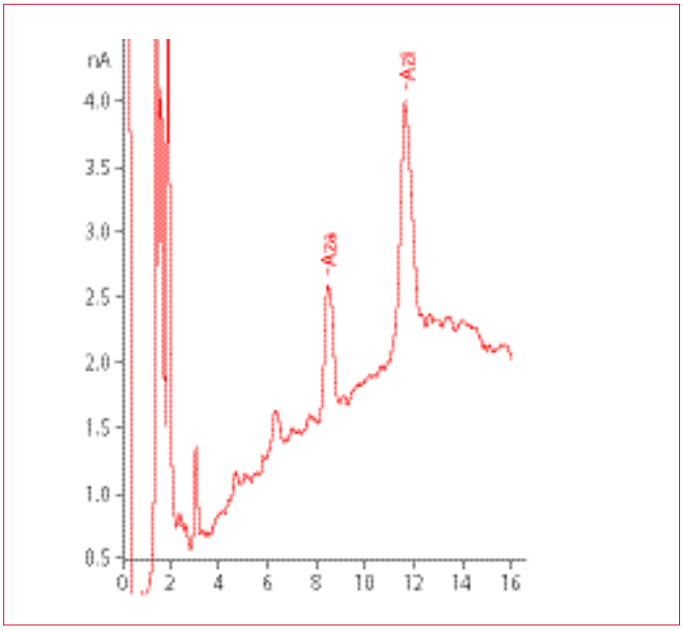

Figure 5: Analysis of 200nM Aza and Azi.

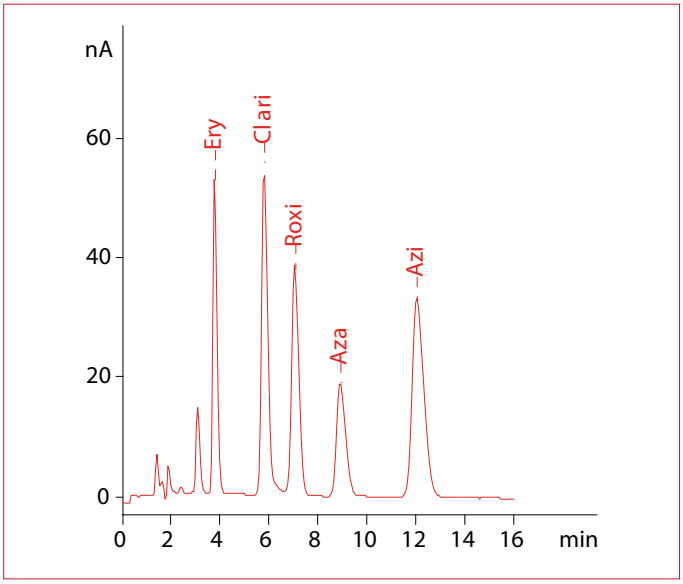

Figure 6: Analysis of 4.5 µM Clarithromycin, Erythromycin, Roxithromycin, Aza and Azi. Signal cell 2.

Examples of pharmaceutical formulations

Below a few examples are shown of the analysis of pharmaceutical formulations.

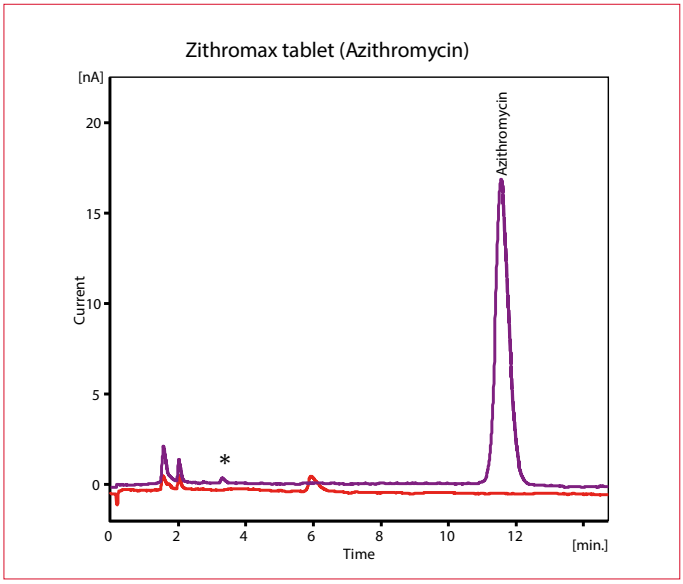

Figure 7: Overlay of chromatograms from blank and 10 µM Azithromycin in mobile phase, based on extraction of a Zithromax tablet. The peak marked with a * is an additional peaks to the main peak that is not present in the blank.

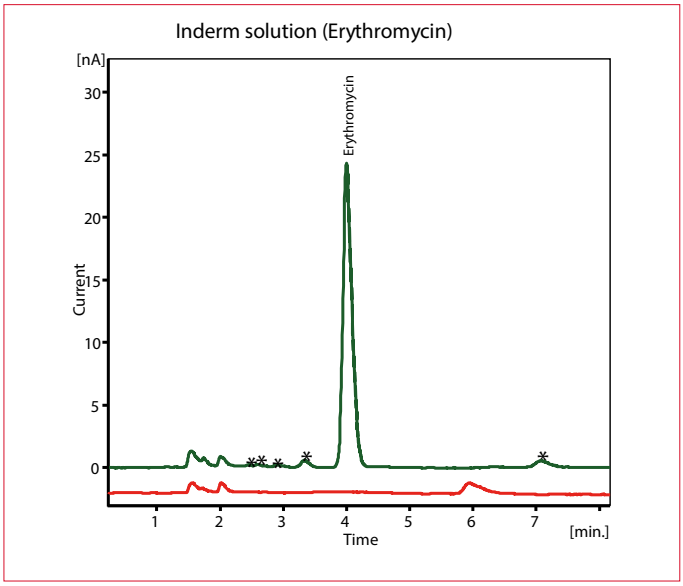

Figure 8: Overlay of chromatograms from blank and 10 µM Erythromycin in mobile phase, based on dilution of 'Inderm' solution. The peaks marked with a * are additional peaks to the main peak that are not present in the blank.

References

1. USP-NF: page No. 185. *Pharmacopeia form: Volume No.23* page No. 3407
2. J. Sastre Toraño, H.-J. Guchelaar; *Journal of Chromatography* B, 720 (1998) 89-97
3. Chieko Taninaka, Hisakazu Ohtani, Erika Hanada, Hajime Kotaki, Hitoshi Sato, Tatsuji Iga, *Journal of Chromatography B,* 738 (2000) 405–411
4. H. Toreson, B-M. Eriksson, *J. Chromatogr.* B 673 (1995) 81-89
5. JN Miller and JC Miller; *Statistics and Chemometrics for Analytical Chemistry,* 5th ed, 2005, Pearson Education, Harlow, England. (ISBN: 0130228885).

Ordering information

180.0086E	ALEXYS Azithromycin Analyzer, USP
250.1126	ALG-515, 150 X 4.6 mm, 3 µm
250.1128	ALG guard column inserts, 3pk
250.1130	ALG guard column holder

Conclusion

A method has been developed for the analysis of Azithromycin, based on the USP method from 2007. The method and results are in compliance with the USP requirements.

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