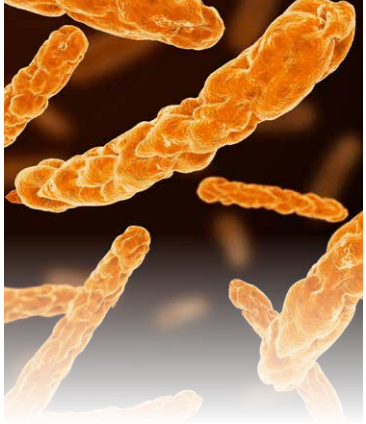
Application Note
Aminoglycoside Antibiotics



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'
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

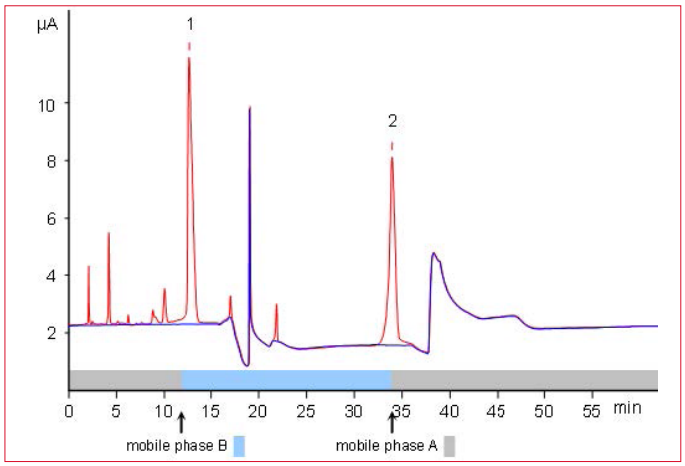


Figure 2: Overlay of baseline (blue) and chromatogram (red) of 100 mg/L spectinomycin (1) and lincomycin (2) dissolved in mobile phase A. The black scheme represents the step gradient program.

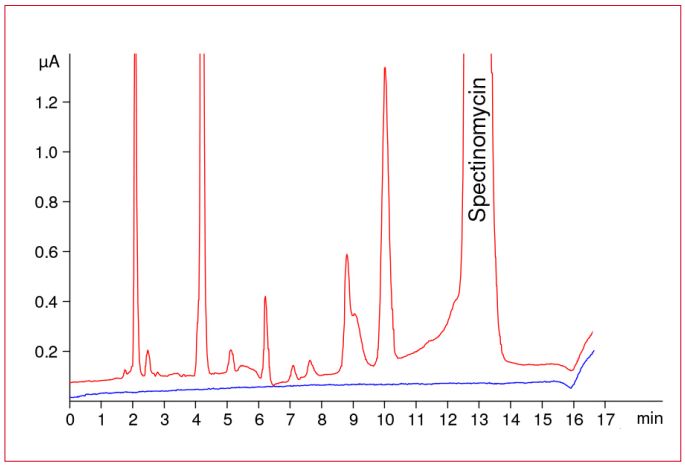
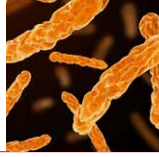


Figure 3: Detail of first 15 minutes of Fig. 2 showing spectinomycin impurities.



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.

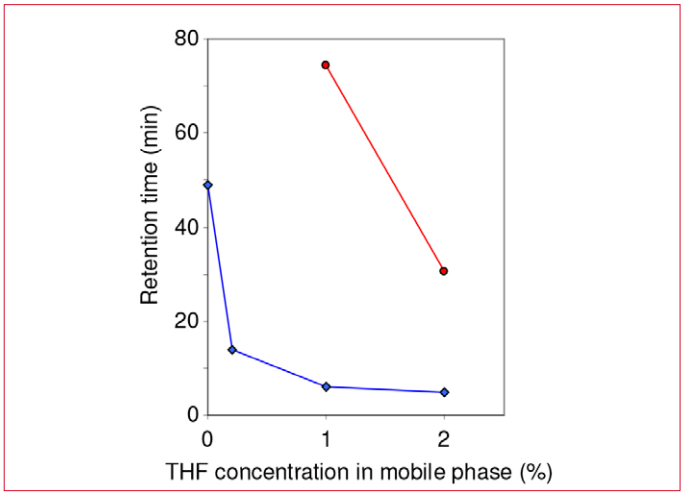


Figure 4: Effect of THF concentration in mobile phase A on retention time of spectinomycin (blue) and lincomycin (red).

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.

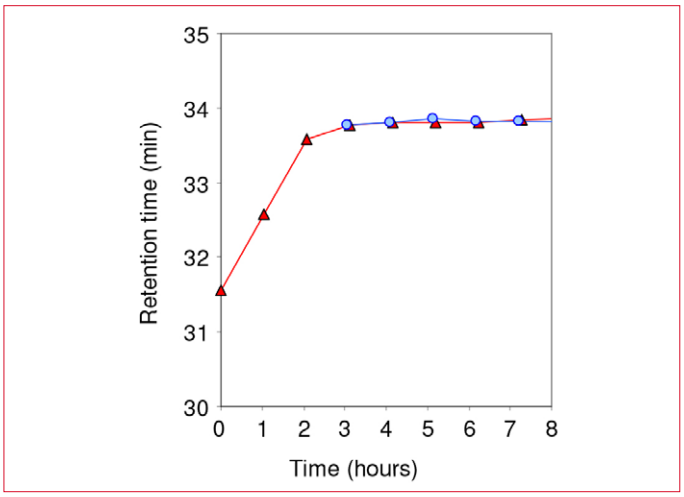


Figure 5: Retention time of lincomycin. The first 3 runs show a strong shift in retention time due to equilibration of the LC system (red). If a sequence is started with 3 blank injections the performance is greatly improved (blue).

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).

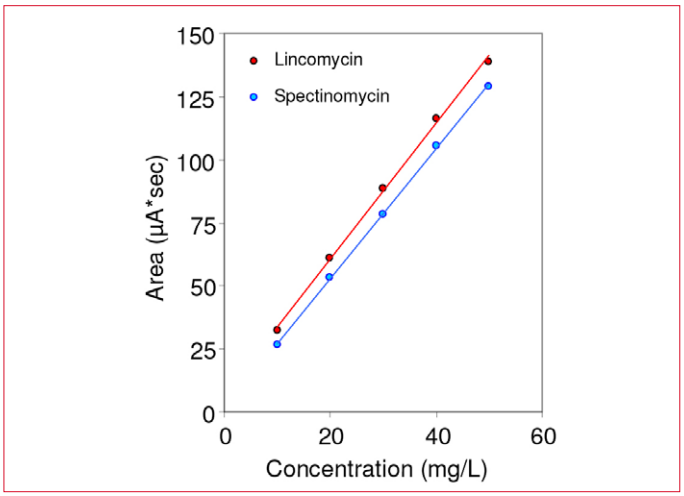


Figure 6: Calibration plot with linear regression lines for spectinomycin and lincomycin.

Intra-day reproducibility

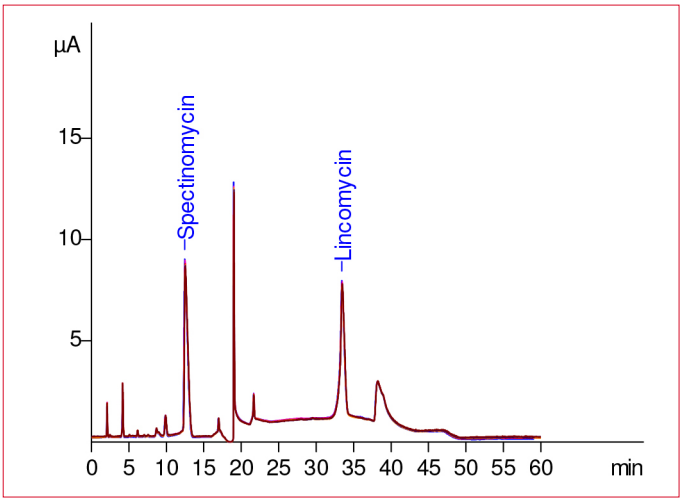


Figure 7: Overlay of 6 chromatograms of 100 mg/L spectinomycin and lincomycin in mobile phase A.

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).

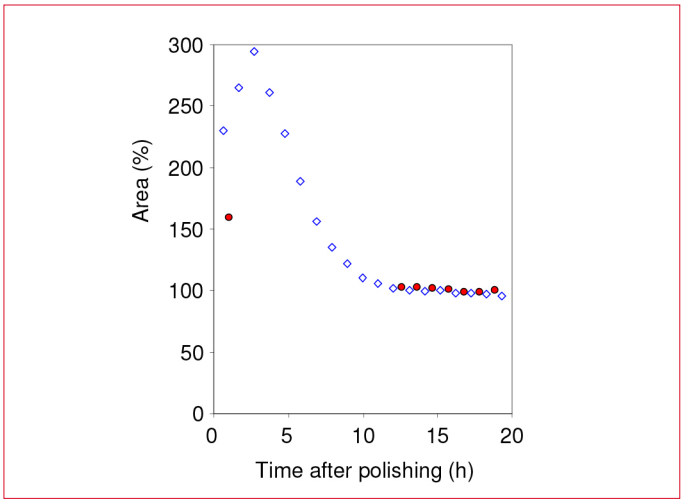
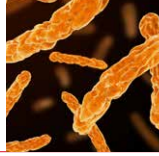


Figure 8: . Relative peak area of lincomycin after having polished the gold working electrode. Blue squares represent a series of continuous analyses, and red circles represent a series of analyses where blank gradient traces were run for 10 hours between the first and subsequent injection.



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.

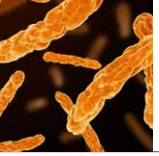
Table 3

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

Conclusion

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



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

180.0059W	Aminoglycoside Analyzer including Flowcell
250.1125	ALF-315 C18 column, 150x3.0mm, 3um

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