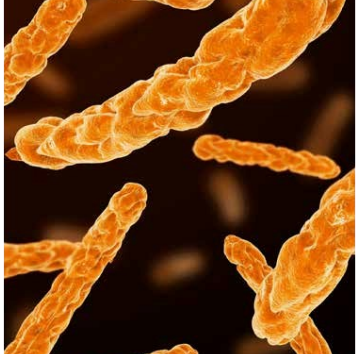
Application Note

Aminoglycoside Antibiotics



The most reliable LC-EC applications for Drugs & Pharmaceuticals analysis

Antipsychotic drugs
Clozapine
Olanzapine
Risperidone

PET imaging tracer
Fluorodeoxyglucose (FDG)
FDG impurities

Pharmaceuticals, API
Acetaminophen
Artemether
Artemisinin
Dihydro- artemisinin
Betadex sulfobutyl ether sodium
Etoposide
Epinephrine
Heparin
mesna BNP7787
8-OH-DPAT
Vincristine

Sulfides
Glutathione
Aminothiols
Disulfides

Aminoglycoside drugs
Amikacin
Framycetin sulphate
Gentamicin sulphate
Kanamycin
Netilmycin
Neomycin sulfate
Spectinomycin
Lincomycin
Tobramycin

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 analyzer based on the Antibiotics LC base system with post-column addition kit. The method is based on reversed phase chromatography with a step gradient. Detection is accomplished using post-column addition of sodium hydroxide in combination with pulsed amperometric detection (PAD).

Introduction

Lincomycin and spectinomycin (Fig. 1) 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 behavior [1].

In this note an LC-ECD method is presented for the simultaneous analysis of spectinomycin and lincomycin using the ALEXYS analyzer.

Method

Separation

The chromatography is based on a step gradient elution using two mobile phases that differ in ionic strength and THF concentration (Table 1).

The composition of mobile phase A is chosen so that spectinomycin and the early eluting impurities are separated within 15 minutes ((Fig. 2). Both components contain amine groups (which are protonated at pH 3) and their retention on a C18 column is facilitated by ion pairing with octane sulfonate.

To speed up the elution of lincomycin, mobile phase B is applied after 12 minutes. The retention time of lincomycin is affected by the change in THF (Fig. 3) but also by ionic strength (which affects the interaction with the ion pairing agent). We found that a THF concentration higher than 2% should not be used, as this results in mobile phase precipitation (it turns a milky white color), which will clog the system. It is also important to use stabilized THF to assure low cell currents. After the elution of lincomycin, the system is allowed to stabilize for 26 minutes in mobile phase A.

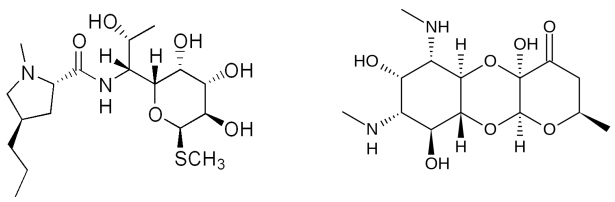


Figure 1: Structural formulas of lincomycin (l) and spectinomycin (r)

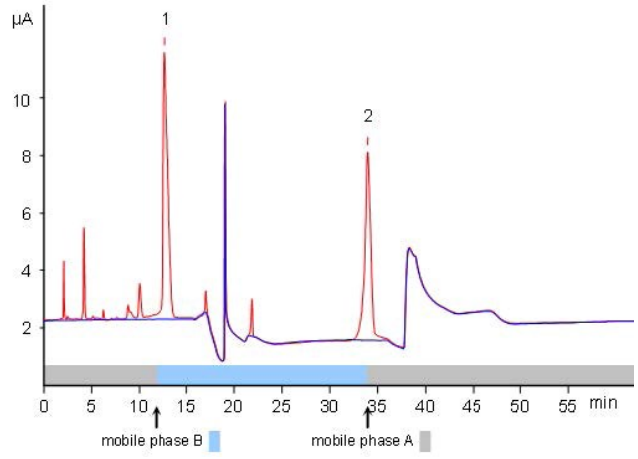


Figure 2: Analysis of 100 mg/L spectinomycin (1) and lincomycin (2) dissolved in mobile phase A (red trace) in an overlay with the analysis of a blank (blue trace). LC-ECD conditions as in Table 1. The bottom bar indicates the timing of

Table 1

LC-ECD conditions (EP)	
HPLC*	ALEXYS Antibiotics base system - Isocratic + Post-column kit EP
Column	Thermo Scientific™ Betabasic™ 18 HPLC Column 150x3.0mm, 3 µm
Mobile phase (MP)	**A**: 25 mM citric acid, 25 mM phosphoric acid, pH 3.0 (set with NaOH solution), 25 mg/L octanesulfonic acid sodium salt, 2 mL/L stabilized THF **B**: 100 mM citric acid, 100 mM phosphoric acid, pH 3.0 (set with NaOH solution), 25 mg/L octanesulfonic acid sodium salt, 20 mL/L stabilized THF
MP gradient	0 - 12 min: A; 12 - 34 min: B; 34 - 60 min: A
Flow rate	0.4 mL/mL
Post-column addition	1 M NaOH at 0.2 mL/min
Mixing coil volume	80 µL
Temperature	35 °C for separation, mixing and detection
$V_{injection}$	20 µL
Flow cell	FlexCell™ with Au and HyREF, 50 µm spacer
Potential waveform (3-step)	E1, E2, E3: +0.12, +0.7, -0.7 V t1, t2, t3, ts: 0.4, 0.2, 0.35 s, 80 ms
Range	50 µA/V
ADF	0.5 Hz
I-cell	About 7 µA

*) Note that the presented data are obtained with an older version of the ALEXYS system than shown in figure 9.

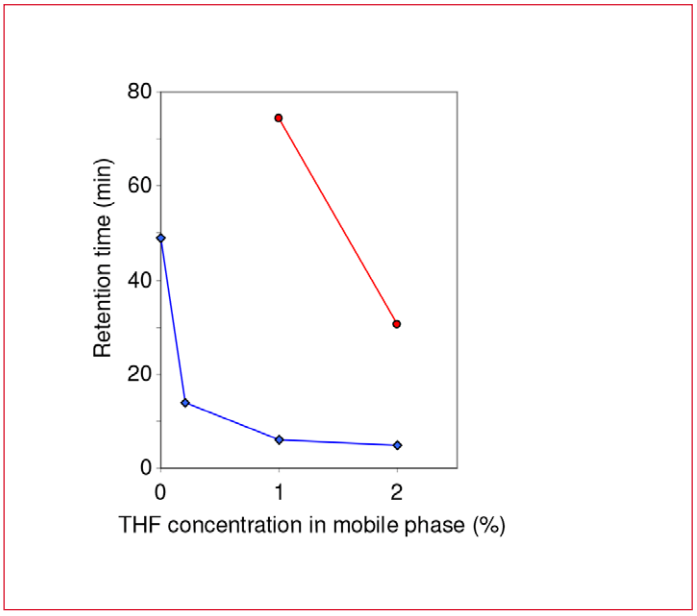


Figure 3: Effect of THF concentration in mobile phase on the retention time of spectinomycin (blue) and lincomycin (red) when running an isocratic separation.

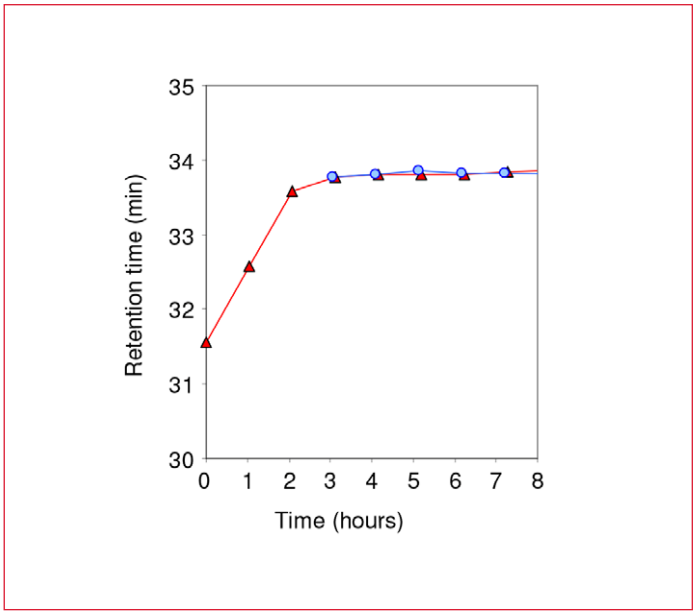


Figure 4: Retention time of lincomycin after prolonged equilibration with mobile phase B before t=0. The first 3 runs show a strong shift in retention time due to equilibration of the LC system (red). If a sequence is preceded by running the complete step-gradient 3 x, then the initial stability is greatly improved (blue).

Chromatographic equilibration procedure

Before starting a sequence of analyses, the ion pairing chromatographic conditions have to be equilibrated, otherwise retention will shift during the first 3 analyses (Fig. 4). The stabilization takes about 3 hours, during which the gradient should be run 3 times. A comparable pattern was observed for spectinomycin (not shown).

Detection

Electrochemical detection of lincomycin and spectinomycin requires a high pH (post-column NaOH addition with 2nd pump) and Pulsed Amperometric Detection (PAD) on a gold electrode. A 3-step pulse was applied (Table 1).

Flow cell maintenance

As the gold working electrode is slowly consumed during 3-step 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 regularly be flattened and polished to the original flat surface [5]. The FlexCell has a removable electrode and is easy to service. After polishing the gold electrode, the system needs 10 h of stabilization time while running the gradient program (Fig. 5) to reach good prolonged signal stability with an intraday RSD of 2% or better (see results section). We recommend to clean the gold electrode at least every 2 weeks when in continuous use.

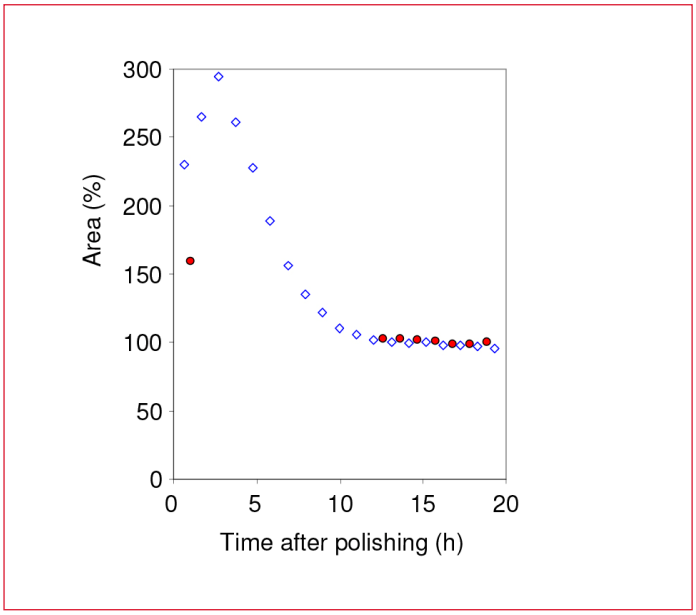


Figure 5: Relative peak area of lincomycin after having polished the gold working electrode. Blue squares represent a series of continuous analyses, and red circles represent a series of analyses where blank gradient traces were run for 10 hours between the first and subsequent injection.

Results

A typical chromatogram as obtained with the method is shown in Fig. 2. A lag time of about 4 minutes can be observed when comparing the changes in the baseline and the step gradient pattern. Spectinomycin and lincomycin are well separated from blank and baseline disturbances. The early eluting impurities from spectinomycin are separated within the first 15 minutes (Fig. 6).

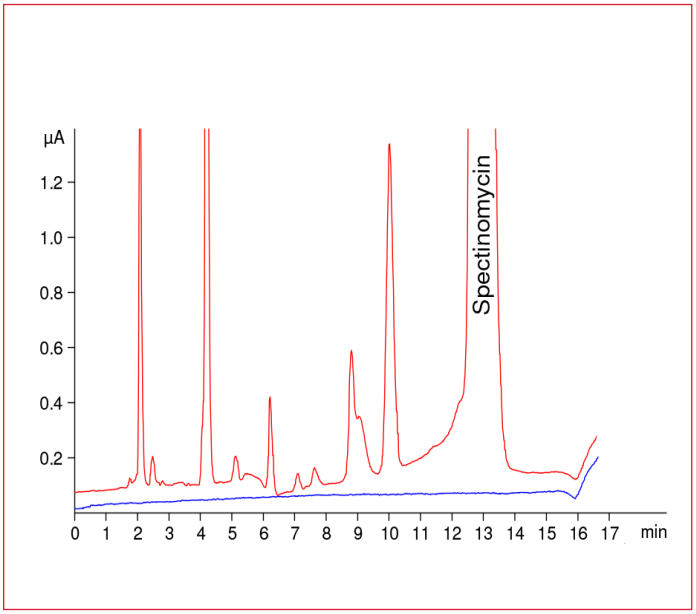


Figure 6: Detail of Fig. 2 showing the first 15 minutes where spectinomycin impurities elute.

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. 7).

Intra-day reproducibility

A representative overlay of 6 consecutively measured chromatograms is given in Fig. 8 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.

Table 2

Reproducibility (chromatograms in Fig. 8); n=6

Component	*RSD (%) retention time*	*RSD (%) height*	*RSD (%) area*
Spectinomycin	0.18	2.0	2.0
Lincomycin	0.08	1.3	2.0

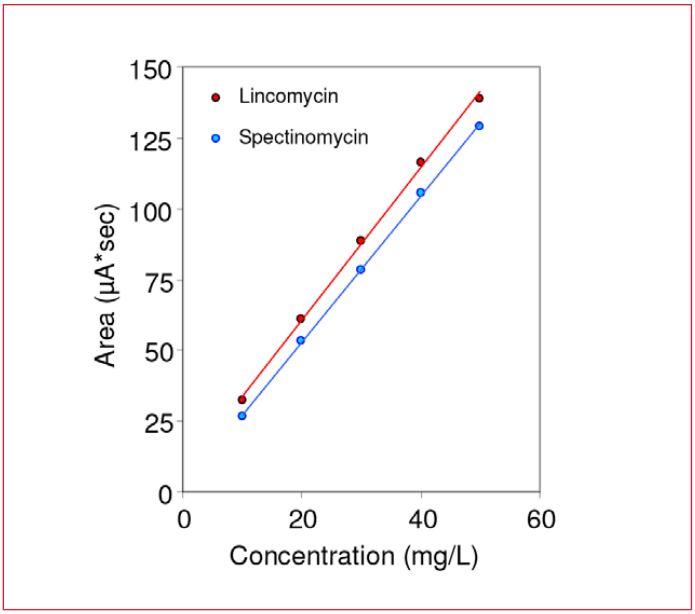


Figure 7: Calibration plot with linear regression lines for spectinomycin and lincomycin.

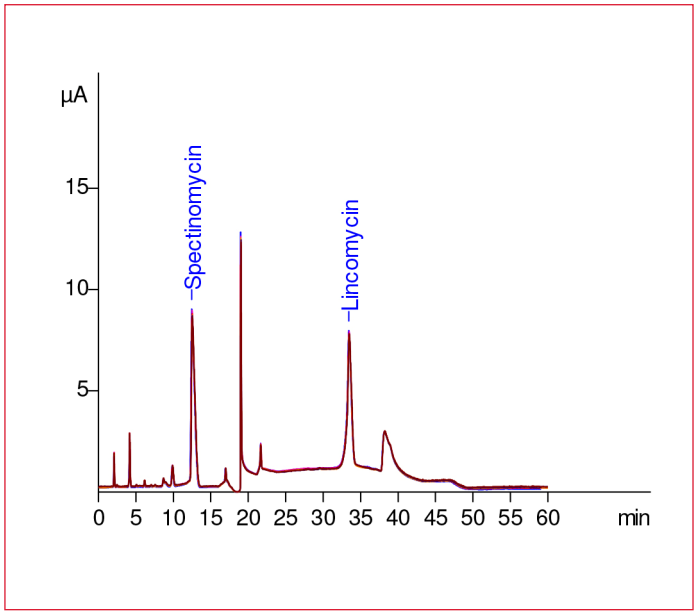
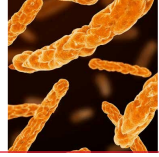


Figure 8: Overlay of 6 chromatograms of 100 mg/L spectinomycin and lincomycin in mobile phase A.



Inter-day reproducibility

The inter-day repeatability was measured in relation to the gold electrode maintenance procedure (see method section). Average peak response data from 4 different days are presented in Table 3. For each day, different events preceded the analysis of data: on day 1 and 12 the electrode was polished before analyses, on day 11 the analysis were done after having continuously run the gradients (stand-by mode), and on day 10 the system had been left to equilibrate for 3 hours (running gradients) after a system shut down of a day. Variation in peak area or peak height (intra-day repeatability) was better than 6% RSD.

Table 3

Interday reproducibility.
Average response (n=6) on different days, based on analyses of 100 mg/L spectinomycin and lincomycin in mobile phase A

		Peak height (µA)		*Peak area (µA*sec)*	
Day	*Events before analysis*	*Spec.*	*Linco.*	*Spec.*	*Linco.*
1	Polish + 10 h equilibration	8.4	6.0	298	255
10	Shut down + 3 h equilibration	9.6	6.7	290	264
11	Continuous analyses	8.6	6.7	263	272
12	Polish + 12 h equilibration	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 Scientific, Flattening & Polishing kit for metal WE: User Guide, part number 250.7010

Conclusion

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

Figure 9. The ALEXYS analyzer for Spectinomycin and Lincomycin, consisting of the ALEXYS Antibiotics base system - isocratic, post-column addition kit (NaOH) and FlexCell with gold working electrode, HyREF and stainless steel auxiliary electrode. The ALEXYS analyzer is fully controlled by DataApex™ Clarity™ software.

Ordering information

Recommended ALEXYS analyzer + parts	
180.0058W	ALEXYS Antibiotics base system - Isocratic
180.0605EP	Post Column Kit EP
102.4325EP	Flexcell Au HyREF with stainless steel AUX
250.1045	Flattening/polishing kit for metal WE
184.0209	Glass bottle assembly, 1L, Helium

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

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