Application Note

Aminoglycosides

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

- **Method according to EP 8.1 (2014) and 9.2 (2017)**
- **Analysis of 'Related substances'**
- **Results well within limits of System Suitability tests**
- **Reproducible and robust**

Summary

The analysis of Netilmicin sulphate according to the method of the European Pharmacopoeia was evaluated on an Antec ALEXYS® analyzer, using the exact method and conditions described in the official EP monograph. In 2017 the EP published a modified LC method as a supplement of the EP 9.2, with improved separation of netilmicin and related substances in comparison with the method given in 8.1. Additionally, the EP 9.2 updated the impurity limits of Netilmicin sulphate based on current market quality. The updated method is evaluated using the ALEXYS® Aminoglycosides Analyzer and both sets of data are shown in this application note.

In this application note typical results as obtained with the ALEXYS® 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 formulations, besides sisomicin also low concentrations of other components can be present that were formed during synthesis, such as 1-N-ethylgaramine (hydrolysis product), 2'-N-ethyl- and 6'-N-ethyl-derivatives of sisomicin (alkylation products). The European Pharmacopoeia (EP) sets limits to these 'related substances' and prescribes an ion-pairing HPLC-ECD method for analysis [1, 2].
In 2017 the EP published a modified method as a supplement of the EP 9.2, with improved separation performance. The method given in EP 8.1 was based on the application of a polymer-based HPLC column with 8 µm particles, and with the update this was changed to a silica based C_{18} column with 5 µm particles and some slight modifications in the composition of the mobile phase. All other analytical parameters were kept the same.The presence of a sugar moiety in netilmicin and the related substances makes pulsed amperometric detection (PAD) a suitable detection method [3-6]. The EP method for analysis of netilmicin sulphate prescribes the use of PAD with an electrochemical flow cell consisting of an Au working electrode (WE), Ag/AgCl reference electrode (REF) and stainless steel auxiliary electrode (AUX). This is not the most user-friendly combination of electrodes and conditions as it requires regular maintenance; nevertheless, in this application note we are showing the good performance using the ALEXYS LC-ECD system with flow cells that are conform the EP description.

Figure 1: ALEXYS analyzer for analysis of Netilmicin.

LC system

The ALEXYS analyzer for Netilmicin is a dedicated LC-ECD system to perform the analysis of the related substances of Netilmicin using a liquid chromatographic system conform the description in the EP monograph. The analyzer consists of the ALEXYS Antibiotics base system - isocratic, post-column addition kit (NaOH) and DECADE Elite with FlexCell with gold working electrode. The system is dedicated to run analysis which needs post-column reagent addition: it has a second pump running NaOH solution, which is necessary to increase the pH for PADdetection. The mixing of the post-column reagent with the column effluent takes place in the 375 µL PEEK mixing coil before the flow cell. This application note shows typical results for the system suitability tests when using the ALEXYS system. The first part of this application note shows the results that are conform the EP 9.2 (2017) method, followed by results as obtained for the previous EP 8.1 (2014) method.

Method - EP 9.2 monograph

The EP 9.2 method prescribes LC separation on a silica based C18 column with 5 µm particle size. We applied a Zorbax sb-C18 column for the evaluation tests. The reference solutions were prepared according to the monograph using EP reference standards. Table 1 shows all the conditions as actually applied (conform the EP monograph).

Table 1

LC-EC Conditions EP 9.2

HPLC*	ALEXYS Antibiotics base system - Isocratic + Post Column Kit EP
Column	Zorbax sb-C18 (250 X 4.6 mm ID, 5µm particle size)
Mobile phase	20 g/L of anhydrous sodium sulfate, 0.3 g/L of sodium octane sulfonate, 20 ml/L tetrahydrofuran stabilized, 50 ml/L 0.2 M potassium dihydrogen phosphate previously adjusted to pH 3.0 with a 22.5g/L solution of phosphoric acid.
Post-column addition	20 g/L sodium hydroxide (carbonate-free)
Flow rate	1.0 mL/min; 0.3 mL/min post column addition
Temperature	50 °C for separation, mixing and detection
$V_{injection}$	20 µL
Flow cell**	SenCell™ with Au WE, stainless steel AUX and Ag/AgCl REF. AST position: 3
Potential waveform	E1, E2, E3: +0.05, +0.75, -0.15 V ts, t1, t2, t3: 0.2, 0.4, 0.2, 0.4 s
Range	20 µA
ADF	0.5 Hz
I-cell	About 1.5 µA

*) Note - the presented data are obtained with an older version of the ALEXYS LC system than shown in fig 1. **) Original work was performed with a VT-03, recommended cell: FlexCell with Au WE, HyREF and stainless steel AUX.

The chromatogram of a sample of netilmicin for analysis of the related substances will by definition show a very large peak of netilmicin and only low levels of the related substances. Signal overloading (distorted peak) for netilmicin can be minimized by applying the highest AST position on the flow cell, while also keeping good sensitivity for the related substances.

In case of poor resolution, the EP 9.2 method allows for an adjustment of the concentration of sodium octanesulfonate in the mobile phase. In such cases or when significant changes in retention time occur it may be helpful to regenerate/clean the column. It is also important to use the *stabilized* THF (with butylhydroxytoluene) in the mobile phase to assure a low background current.

Results - EP 9.2 monograph

Peak identification

The peaks of sisomicin (impurity A), 1-N-ethylgaramine (impurity B) and netilmicin are identified based on a chromatogram of 'reference solution (d)' (Figure 2). Impurities E and F are identified using the chromatogram of 'reference solution (e)'.

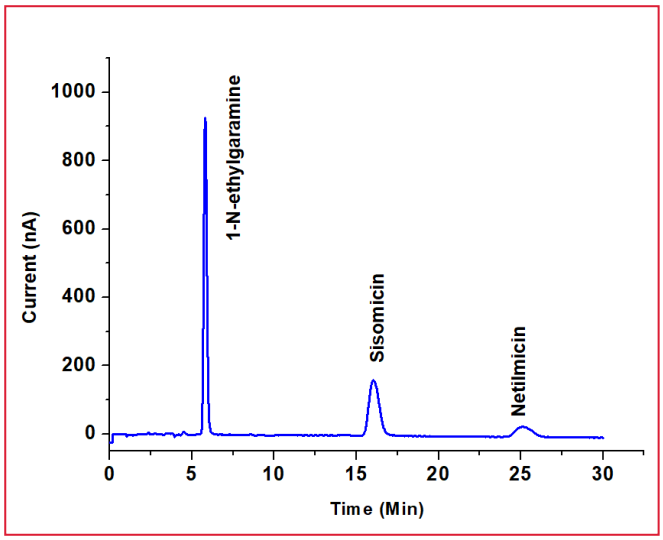

Figure 2: 20 µL injection of 'reference solution (d)' (as described in EP 9.2) consisting of 6 µg/mL Netilmicin sulfate for LC assay CRS, 20 µg/mL Sisomicin sulfate CRS and 20 µg/mL 1-N-ethylgaramine sulfate CRS in mobile phase.

The relative retention times (RRT) of the impurities given in the EP monograph are used as a guideline to identify the peaks (Table 2).

Table 2

Retention times and relative retention times (RRT) of Netilmicin and related substances (method EP 9.2)

Component	*Retention (min)*	*RRT* (measured)*	*RTT* (EP)*
1-N-ethylgaramine (Impurity B)	5.7	0.2	0.4
Sisomicin (Impurity A)	15.7	0.6	0.7
Netilmicin	24.5	1.0	1.0
Impurity E	47.8	1.9	1.9
Impurity F	51	2.1	2.1

*) Relative retention time (RRT) to Netilmicin.

System suitability test

Before analysing samples with the EP method, it should be checked if the system gives enough resolution among peaks based on a chromatogram of 'reference solution (d)' (Figure 2).

The system suitability criteria are met (Table 3).

Table 3

System suitability criteria (method EP 9.2)

Parameter	*EP criteria*	*Measured*
Resolution between impurity A and impurity B	≥ 10	12.9
Resolution between impurity A and netilmicin	≥ 6.0	6.1

*) Based on chromatogram from 'reference solution (d)'

Linearity and repeatability

The response linearity of netilmicin and impurities A and B were measured in the concentration range 10 - 100 µg/mL and resulted in correlation coefficients >0.999. The repeatability was <2% RSD based on 'reference solution (d)' (Table 4).

Table 4

Repeatability, based on 6 injections reference solution (d)'

Component	*RSD area (%)*
1-N-Ethylgaramine (Impurity B)	1.2
Sisomicin (Impurity A)	1.3
Netilmicin	1.9

Sample analysis

As an example, a further unspecified netilmicin sulphate sample was analyzed according to the EP method and compared against the acceptance criteria. The sample was processed into 'test solution' (Figure 3) and impurity levels were quantified based on comparison with the chromatogram of 'reference solution (d)' (Figure 2).

The (updated) EP limits for the amount of impurities are:

- Impurity A, B, E and F: for each impurity maximum 1.0%
- Any other impurities: for each impurity maximum 0.3%
- Total of impurities: maximum of 3.0 %
- Reporting threshold: 0.1%

The results for the impurity levels of the sample were compared against the limits as set by the EP (Table 5) and it shows that this sample did not pass the test for the impurity limits. Impurity A, B, E and F were within limits, but two unknown impurities showed too high levels and the total impurities also exceeded the limits as set by the EP.

Table 5

Impurity analysis of netilmicin sulphate sample

Peak	*RRT**	*% content*	*Within limit*
1	0.14	0.26%	Y
2	0.16	0.60%	**N**
3	0.19	0.11%	Y
4	0.22	**	Y
5	0.24	0.39%	**N**
6	0.28	0.12%	Y
1-N-ethylgaramine (imp. B)	0.30	0.21%	Y
8	0.39	**	Y
9	0.47	0.23%	Y
10	0.60	**	Y
11	0.70	0.12%	Y
12	0.72	0.14%	-
13	0.78	0.23%	Y
14	0.90	0.26%	Y
Netilmicin	1.00	-	-
Impurity E	2.38	0.54%	Y
Impurity F	2.63	0.32%	Y
Total		3.52	**N**

*) Relative retention time (RRT) to netilmicin.
**) Below reporting threshold limit of 0.1%

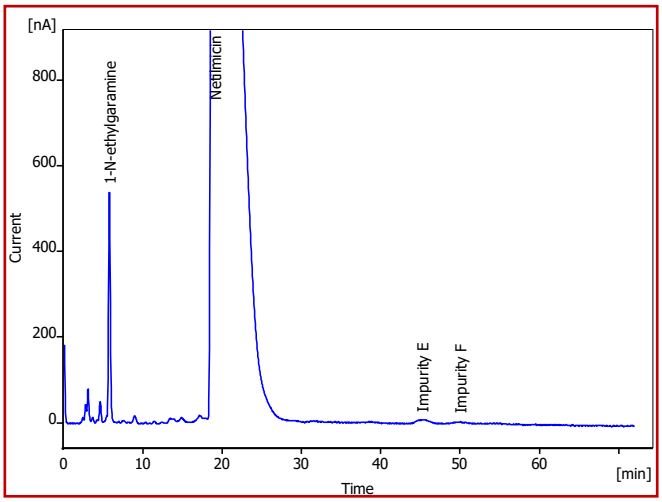

Figure 3: chromatogram of 20 µL injection of 2 mg/mL netilmicin sulphate sample prepared in mobile phase ('test solution' prepared as described in EP monograph 9.2).

Alternative flow cells

The use of a saltbridge as a reference electrode when running a 3-step PAD-mode under alkaline conditions is by design going to result in the accumulation of dissolved gold. To assure reproducible results, the salt bridge has to be refilled with fresh salt solution and cotton on a regular basis. This is an easy procedure, but it costs time and effort.

In cases where deviations from the EP method are allowed, we would advise the use of the maintenance-free HyREF reference electrode for detection of aminoglycosides in PAD-mode on a gold working electrode.

Another specific part of the method that requires regular maintenance is the gold working electrode. This gets consumed slowly by the 3-step PAD mode as prescribed by the EP. We normally advise the FlexCell with easy serviceable working electrode in such cases; however, this flow cell type has an AUX electrode of conductive polymer, which not conform the EP description (AUX of stainless steel). The SenCell flow cell Au with stainless steel AUX has a fixed electrode and will need more regular service at factory (we advise to have at least one spare cell to minimize downtime).

A comparison between the response of a SenCell with saltbridge and HyREF resulted in comparable peak responses (tested with 'reference solution (d)'). The comparison between the response of a SenCell (AST 3) and a FlexCell (120 um spacer) both fitted with HyREF showed a somewhat higher peak response for the FlexCell, but comparable signal-to-noise ratios. This means that the alternative Au HyREF FlexCell can be used without compromising the results when a deviation from the EP method is allowed.

Method - EP 8.1 monograph

The older EP 8.1 method prescribed LC separation on a styrene-divinylbenzene copolymer stationary phase with particle size of 8 µm. The Agilent PLRP-S 1000Å 8 µm, 250 x 4.6 mm column was selected for the method evaluation. At the time of testing, the Antec VT-03 electrochemical flow cell was selected (which has been succeeded by the SenCell in the meantime).

Table 6 shows all the conditions as actually applied (conform the EP monograph).

Table 6

LC-EC conditions EP 8.1

HPLC	ALEXYS Antibiotics base system - Isocratic + Post Column Kit EP
Column	Agilent PLRP-S 1000Å 8 µm, 250 x 4.6 mm
Mobile phase	35 g/L of anhydrous sodium sulfate, 2.0 g/L of sodium octane sulfonate, 10 ml/L tetrahydrofuran stabilized, 50 ml/L 0.2 M potassium dihydrogen phosphate previously adjusted to pH 3.0 with a 22.5 g/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
$V_{injection}$	20 µL
Temperature	50 °C for separation, mixing and detection
Flow cell	VT-03™ with Au WE, stainless steel AUX and Ag/AgCl REF, 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 mobile phase was prepared as described in the EP monograph, but with a small adjustment to the mobile phase to optimize the separation: the concentration sodium octane sulphonate was increased from 0.5 g/L to 2 g/L.

Note: it is important to use the *stabilized* THF (with butylhydroxytoluene) in the mobile phase to assure a low background current.

Results - EP 8.1 monograph

Peak identification

The peaks of sisomicin (impurity A), 1-N-ethylgaramine (impurity B) and netilmicin are identified based the chromatogram of 'reference solution (d)' (Figure 4).

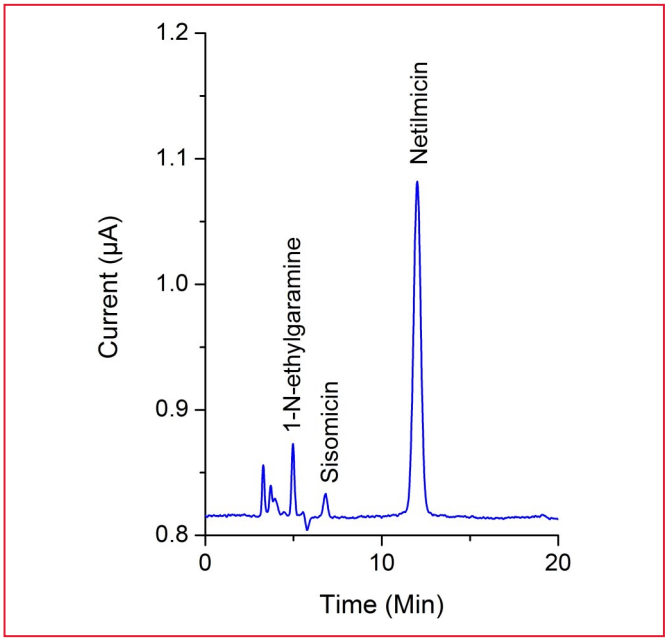

Figure 4: 20 µL injection of 'reference solution (d)' as described in the EP monograph, 8.1, consisting of 10 µg/mL Netilmicin sulphate CRS, 10 µg/ml Sisomicin sulphate CRS and 8.2 µg/ml 1-N-ethyl garamine sulphate CRS in mobile phase.

Table 7

Retention time of Netilmicin and related substances

Component	*Retention (min)*	*RRT**
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) to Netilmicin.

System suitability test

Before analysing samples with the EP method, it should be checked if the system meets the system suitability requirements. The tests described in the EP 8.1 method check for resolution and sensitivity, based on a chromatogram of 'reference solution (d)' (Figure 4) and 'test solution (b)' (Figure 5). The system suitability criteria are met (Table 8).

Table 8

System suitability criteria (method EP 8.1)

Parameter	*EP criteria*	*Measured*
Resolution between Impurity A and B*	≥ 2.0	4.5
Resolution between Impurity A and netilmicin*	≥ 3.0	8.0
SN ratio of principal peak**	>10	15.3

*) Based on chromatogram from 'reference solution (d)'

**) Based on chromatogram from 'test solution (b)'

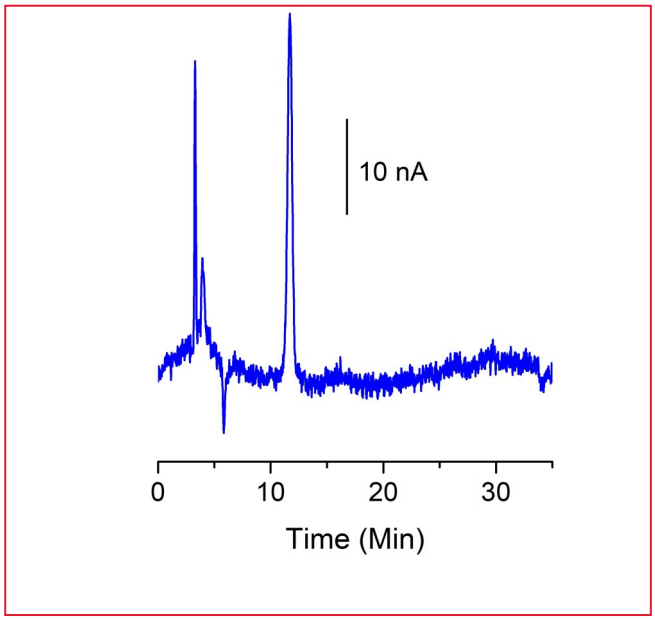

Figure 5: 20 µL injection of 1 µg/mL Netilmicin sulfate in mobile phase ('test solution (b)' as described in EP monograph 8.1).

Linearity and repeatability

The response linearity of netilmicin and impurities A and B were measured in the concentration range 10 - 30 µg/mL and resulted in correlation coefficients >0.997 (peak area). The repeatability was <2% RSD for the impurities and 0.6% for netilmicin, based on 6 replicate injections of 'reference solution (d)' (Table 9).

Table 9

Repeatability, based on 6 injections reference solution (d)'

Component	*RSD Area* (%)*
1-N-Ethylgaramine (Impurity B)	1.2
Sisomicin (Impurity A)	1.9
Netilmicin	0.6

Sample analysis

A further unspecified Netilmicin sample 'K62' was analyzed according the EP method and compared against the acceptance criteria. The sample was processed into 'test solution (a)' (Figure 6) and impurity levels were quantified based on comparison with the chromatogram of 'reference solution (d)' (Figure 4).

The peak areas of all impurities in the Netilmicin sample are listed in Table 10. The table also shows if a peak was discarded for the impurity analysis.

Table 10

Analysis of netilmicin sample 'K62'

Peak	*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) to Netilmicin.
**) Discard limit: any peak with an area less than that of the principal peak in the chromatogram obtained with 'reference solution (b)' (Figure 5).

The EP acceptance criteria for the amount of impurities are:

- Impurity A <1%: not more than the peak area of the sisomicin peak in the chromatogram of 'reference solution (d)'.
- Impurity B <1%: not more than the peak area of the 1-N-ethylgaramine peak in the chromatogram of 'reference solution (d)'.
- Any other impurities <1%: Not more than the peak area of the netilmicin peak in the chromatogram of 'reference solution (d)'.
- Total of other impurities <2%: Not more than 2x the peak area of the netilmicin peak in the chromatogram of 'reference solution (d)'.
- Discard limit 0.1%: Impurities with peak areas smaller than the peak area of the netilmicin peak in the chromatogram of 'test solution (b)'.

The impurity levels of sample 'K62' are shown in Table 11, and it shows that this sample did not comply with the acceptance criteria for the impurity limits as set by the EP. Impurity A, B and an unknown impurity with a relative retention time of 3.33 (peak '15') showed too high levels. The total amount of 'other impurities' (sum of the relative areas of peak 2, 4, 14 and 15) also exceeded the EP acceptance limits.

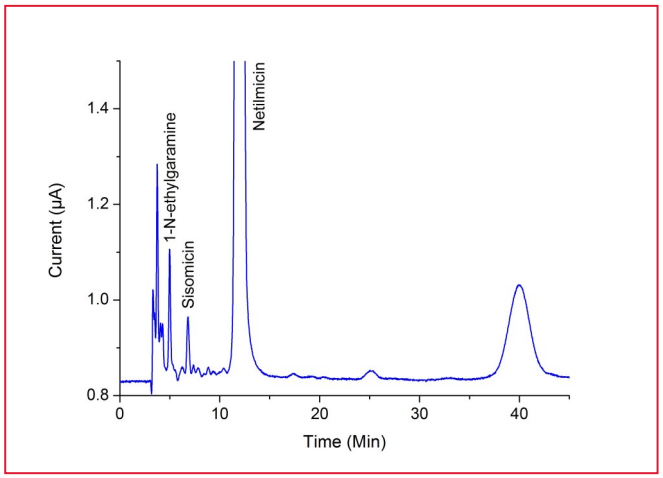

Figure 6: 20 µL injection of 1 mg/mL netilmicin sample prepared in mobile phase ('test solution (a)' as described in EP monograph 8.1).

Table 11

Impurity analysis of netilmicin sample 'K62'

Peak	*RRT*	*Relative 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.

Conclusion

The ALEXYS Analyzer for Netilmicin provides a suitable solution for the analysis of the related substances in commercial Netilmicin formulations following the official method of the EP 9.2 (and also for previous version 8.1).

References

1. Netilmicin sulfate, *European Pharmacopoeia (EP)*, **8.1**, (2014) 2837 -2839
2. Netilmicin sulfate, European Pharmacopoeia (EP), **9.2**, (2017) supplement, 4579
3. T.M Bayless, et al. , Lactase Non-persistence and Lactose Intolerance, Curr Gastroenterol Rep, **19** (2017) , 23.
4. W.R. LaCourse, *"Pulsed Electrochemical Detection in High Performance Liquid Chromatography"*, John Wiley & Sons, New York, 1ed,1997.
5. E. Adams, D. Peulings, M. Rafiee, E. Roets, J. Hoogmartens, *J. Chromatogr. A*, **812**, 151-157 (1998).
6. V. Manyanga, J. Hoogmartens, E. Adams, *J. Sep. Sci.*, **33**, 1897-1903 (2010).

Ordering information

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
Column EP 8.1 (2014)	
PL1512-5802*	PLRP-S 1000 Å, 250x4.6mm, 8um
Column EP 9.2 (2017)	
880975-902*	Agilent Zorbax Stabile Bond C18, 250 X 4.6 mm ID , 5µm

*) Manufactured and sold by Agilent Technologies, Santa Clara, US.

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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