Application Note

Drugs & Pharmaceuticals

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 Sulfate
Gentamicin Sulfate
Kanamycin
Netilmicin
Neomycin sulfate
Spectinomycin
Lincomycin
Tobramycin
Streptomycin

Amikacin Sulfate - EP Method

- **European Pharmacopeia 10.0 (2020)**
- **ALEXYS analyzer with EC and UV detector**
- **Amikacin Sulfate injections**
- **Contents of active pharmaceutical ingredient (UV)**
- **Impurities analysis (ECD)**

Summary

Amikacin is a semi-synthetic antibiotic used in the treatment of for example bacterial infections caused by *Pseudomonas aeruginosa* and *Escherichia coli* [1,2]. It is derived from Kanamycin A and during synthesis, multiple side products can be formed. These impurities present in the pharmaceutical formulation should not exceed a certain limit and are quantified using HPLC with electrochemical detection as described in the Amikacin monograph in the European Pharmacopeia 10.0 (EP) [3]. The contents of Active Pharmaceutical Ingredients (API) in the EP monograph is determined by an assay based on UV detection.

Both the assay and impurity analysis of Amikacin Sulfate were evaluated on an Antec ALEXYS® Analyzer, using the exact methods and conditions described in the official EP 10.0 monograph. In this application note typical results obtained with the ALEXYS® Analyzer are reported, demonstrating its excellent performance for the routine analysis of Amikacin Sulfate formulations.

Amikacin Sulfate - EP method

Introduction

Aminoglycosides are a group of antibiotics used since the 1940s for the treatment of a wide variety of bacterial infections. Due to antibiotic resistance new semi-synthetic aminoglycosides were developed in the 70s, such as Netilmicin and Amikacin. Amikacin is a semi-synthetic aminoglycoside antibiotic derived from Kanamycin A which is effective against a broad spectrum of gram-negative bacteria, including Pseudomonas, E. coli, Enterobacter and Acinetobacter. It is most widely used for treatment of severe infections involving multidrug-resistant bacteria. Aminoglycosides are poorly absorbed from the gastrointestinal tract and therefore are given parenterally, via intramuscular or intravenous injection, or topically, via application to the skin.

Amikacin is synthesized by acylation with the l-(-)-γ-amino-α-hydroxybutyryl side chain at the C-1 amino group of the deoxystreptamine moiety of Kanamycin A [4]. During this process, many different side products can be formed. Examples of such impurities are 3-HABA Kanamycin A (impurity A), 1,3-Di -HABA Kanamycin A (impurity B), Kanamycin (impurity D), 1,6'-Di-HABA Kanamycin A (impurity F) and Amikacin B (impurity H). These impurities (so-called related substances) present in pharmaceutical formulation should not exceed a certain limit and are quantified using HPLC with electrochemical detection as described in the Amikacin sulfate monograph in the European Pharmacopeia. The contents of Active Pharmaceutical Ingredient (API) in Amikacin formulations is determined by an assay based on UV detection [3]. In this application note, both the impurity analysis and assay for Amikacin Sulfate as defined in the EP 10.0 (2020) are evaluated, using the Antec ALEXYS analyzer.

ALEXYS analyzer for Amikacin

The ALEXYS analyzer for Amikacin consists of an AS6.1L autosampler, a P6.1L pump quaternary LPG gradient pump, ET 210 eluent tray, DECADE Elite electrochemical detector and a FlexCell with Gold (Au) working electrode (WE). See figure **6** on the last page. A second P6.1L isocratic pump is used for post-column addition of NaOH solution prior to electrochemical detection. DataApex™ Clarity™ Chromatography Data System (CDS) software is used for instrument control and data-acquisition. The ET 210 eluent tray is equipped with a helium delivery system which facilitates sparging and blanketing of all LC mobile phases with an inert gas atmosphere to avoid the introduction of CO_2 in the solutions.

Table 1

LC-ECD conditions (EP)	
HPLC	ALEXYS Antibiotics base system - gradient (incl. the DECADE Elite electrochemical detector) + flow cell and post-column kit EP
Column	Discovery® C18 HPLC Column, 250 x 4.6 mm, 5 µm
Mobile phase A	1.8 g/L sodium octanesulfonate, 20 g/L anhydrous sodium sulfate, 2 % tetrahydrofuran *stabilized* and 5 % 0.2 M potassium dihydrogen phosphate adjusted to pH 3.0 in deionized water.
Mobile phase B	1.8 g/L sodium octanesulfonate, 28 g/L anhydrous sodium sulfate, 2 % tetrahydrofuran *stabilized* and 5 % 0.2 M potassium dihydrogen phosphate adjusted to pH 3.0 in deionized water.
Post column	500 mM sodium hydroxide
Flow rate	Separation: 1 mL/min Post column addition: 0.3 mL/min
Temperature	40°C for separation and detection
Back pressure	Approximately 95 bar (separation), 45 bar (post-column)
$V_{injection}$	20 µL
Flow cell*	FlexCell with stainless steel auxiliary electrode, gold working electrode, HyREF reference electrode and 130 µm spacing
Potential waveform (3-step)	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/V
ADF	0.1 Hz
I-cell	About 1.5 µA

*) Original work was performed with cell with saltbridge reference electrode, Antec advises to use a maintenance-free HyREF palladium electrode.

To run the assay specified in the EP monograph for Amikacin the system was expanded with an optional UVD2.1L UV detector.

Amikacin Sulfate - Related substances (ECD)

The EP method for the quantification of impurities in Amikacin Sulfate is based on gradient separation on a C18 column under acidic conditions followed by post-column addition of a NaOH solution and pulsed amperometric detection (PAD) on a Au working electrode [5]. In Table 1 the conditions for separation and detection are listed.

Separation

In the EP monograph the use of the following column type is specified for the separation of Amikacin and impurities: length 0.25 m, diameter 4.6 mm and end-capped octadecylsilyl silica gel as stationary phase with 5 µm particle size. The Supelco Discovery® C18, 250 x 4.6 mm, 5 µm column which matches these requirements was therefore chosen for the method evaluation.

Table 2

Gradient program

Time (min)	*A (%)*	*B (%)*	*Description*
0	100	0	Separation and detection
3	100	0	
38	30	70	
38.1	0	100	
68	0	100	
68.1	100	0	Re-equilibration
78	100	0	

Amikacin and impurities are separated using a potassium phosphate buffer with sodium sulfate at pH 3.0 and octane sulphonic acid salt (OSA) as ion pairing agent. 2% v/v of tetrahydrofuran (THF) was added as organic modifier. The sodium sulfate concentration is varied during the run from 20 g/L to 28 g/L, see gradient program in table 2. It is very important to use THF stabilized with butylated hydroxytoluene (BHT) as inhibitor. This will prevent the formation of electrochemically active peroxides in the THF solvent . The use of un-stabilized THF may lead to high background currents, wandering baselines and poor sensitivity. The EP monograph allows adjustment of the tetrahydrofuran concentration to optimize the separation between impurity B and Amikacin if required.

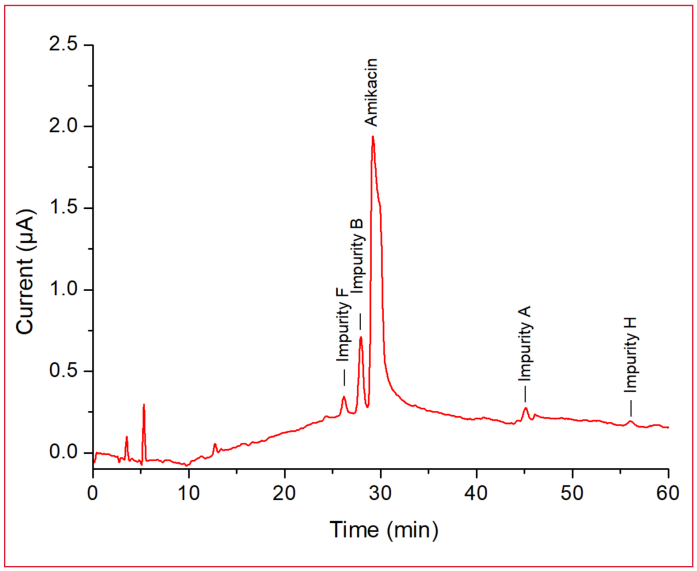

Figure 1: Chromatogram of an 20 µL injection of reference solution C consisting of 0.5 mg/mL Amikacin for system suitability CRS (containing impurity A, B, F and H) in mobile phase A. Note the poor peak shape of the main constituent Amikacin due to column overloading effect.

Table 3

Relative retention times

Compound	*RT (min)*	*RRT (measured)*	*RRT (EP)*
Impurity I	4.20	0.14	0.13
Impurity F	25.77	0.89	0.92
Impurity B	27.53	0.95	0.95
Amikacin	28.97	1.00	1.00
Impurity A	44.57	1.54	1.62
Impurity H	55.27	1.91	1.95

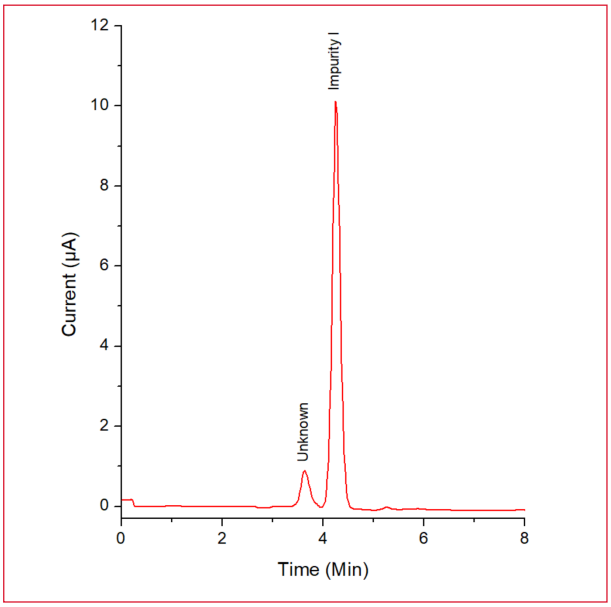

Figure 2: Chromatogram of an 20 µL injection of reference solution D consisting of 3.3 µg/mL Impurity I CRS in mobile phase A.

The retention times of Amikacin and impurities (A, B, F H and I) are determined using the reference solutions C and D. Example chromatograms are shown in figure 1 and 2. The relative retention times (RRT) found are listed in table 3. The RRT's with reference to Amikacin (28,97 min) are in good correspondence with the values specified in the EP monograph.

Table 4

EP system suitability requirements

Parameter	*EP criteria*	*Measured*
H_{peak}, Impurity B,	-	480 nA
H_{valley}, Impurity B/Amikacin	-	50 nA
Peak-to-valley ratio	>5	9.6

In the EP monograph the following system suitability requirement is specified:

- Peak-to-valley ratio (H_p/H_v): minimum 5

Where H_p = peak height above the baseline of impurity B and H_v the valley above the baseline between impurity B and Amikacin It is evident from table 4 that with a H_p/H_v ratio of 9.6 the EP system suitability requirements are met.

The maximum allowed level of impurities (limits of the related substances) in pharmaceutical formulations following the EP are:

- Impurities A,B,F,H,I: maximum 0.5% for each impurity
- Other impurities: maximum 0.5% for each impurity
- Total: maximum 1.5%
- Reporting threshold: 0.1%

The reporting threshold represents the lowest concentration of an impurity which should be quantified in samples. The Limit of Quantitation (LOQ) of the method, defined as LOQ = 10x (S/N), should therefore be equal or better than the reporting threshold for accurate quantification. In the EP monograph a test solution containing 0.66 mg/mL Amikacin sulfate is used to determine the impurity levels. An impurity threshold of 0.1% in that case represents a concentration of 0.66 µg/mL. To determine the LOQ of the method a standard solution containing 5.1 µg/mL Amikacin sulfate CRS in mobile phase was analyzed (see figure 3 and table 5). The calculated LOQ was 0.32 µg/mL, which is a factor two below the reporting threshold.

Table 5

LOQ

Parameter	*EP criteria*	*Measured*
Concentration Amikacin sulfate (µg/mL)	-	5.1
Height (nA)	-	208
Noise (nA)*	-	1.3
LOQ (µg/mL)	0.66	0.32

*) A Savitsky-Golay post-run filter was applied in Clarity CDS, using a filter window of 20 points. The noise was calculated using the ASTM method with a segment size of 0.5 min in the time window from 13—28 minutes in the chromatogram.

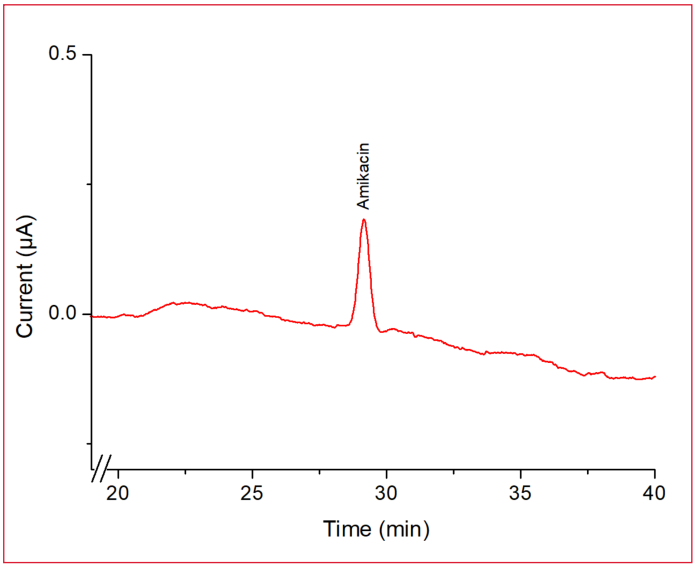

Figure 3: Zoomed chromatogram of an 20 µL injection of a reference solution consisting of 5.1 µg/mL of Amikacin sulfate CRS in mobile phase A.

A commercial formulation of 100 mg/2 mL of Amikacin sulfate for parenteral use (injectable) in aqueous solution was analyzed as an example. The chromatogram of the test solution is show in figure 5. The commercial sample was old and almost 6 years passed its expiration date (08/2015) at the moment of analysis. The chromatogram of the test solution is show in figure 4. The test solution was prepared by dilution of an Amikacin Sulfate sample containing 100 mg Amikacin in 2 mL aqueous solution to a concentration of 0.66 mg/mL in mobile phase A. In case of a sample in powder form, dissolve 33 mg of the substance in 50 mL mobile phase A to prepare the test solution.

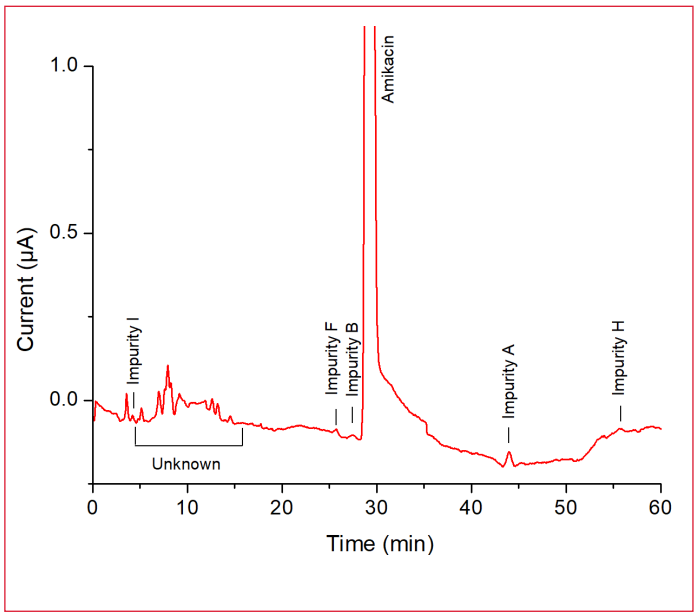

Figure 4: Chromatogram of an 20 µL injection of a test solution of 0.66 mg/mL Amikacin sulfate for parenteral use (injectable) in mobile phase A.

Table 6

Impurity analysis of amikacin Sulfate sample

Peak	*RRT**	*contents (%)#*	*Within limit*
Unknown 1	0.12	0.13 %	Y
Impurity I	0.14	**	Y
Unknown 2	0.17	**	Y
Unknown 3	0.18	**	Y
Unknown 4	0.24	0.18 %	Y
Unknown 5	0.27	0.66 %	N
Unknown 6	0.32	0.24 %	Y
Unknown 7	0.43	**	Y
Unknown 8	0.45	**	Y
Unknown 9	0.50	**	Y
Impurity F	0.89	**	Y
Impurity B	0.95	**	Y
Amikacin	1.00	**	Y
Impurity A	1.52	0.20 %	Y
Impurity H	1.92	**	Y
Total		1.41 %	Y

*) Relative retention time (RRT) with reference to Amikacin.
**) Below reporting threshold of 0.1%.
#) The response of Amikacin from reference solution B used for quantification of the impurity levels

The results of the sample analysis are shown in table 6 om the next page. It is evident that although the total amount of impurities is below the limit of 1.5% ,the sample is rejected based on the maximum amount of the individual impurities. The impurity at a RTT of 0.27 (unknown 5) is above the limit of 0.5% (0.66 %). The most probable case is sample degradation.

Amikacin sulfate—Assay (UV)

The assay of amikacin sulfate in the EP 10.0 monograph is based on isocratic separation on the same type of C18 column as specified for the impurity analysis. Therefore, also a Supelco Discovery® C18, 250 x 4.6 mm, 5 µm column was used for the evaluation of the assay on the ALEXYS analyzer. The LC-UV conditions for the assay are listed in table 7. Amikacin and impurities are separated using a potassium phosphate buffer with sodium sulfate at pH 3.0 and octane sulphonic acid (OSA) as ion pairing agent. 6% v/v of acetonitrile was added as organic modifier. The concentration of acetonitrile (ACN) in the mobile phase is a critical factor for the peak shape of Amikacin peak. At concentrations above 7% v/v ACN peak fronting was observed and below 5.8% peak splitting and peak tailing. That

Table 7

Conditions

HPLC	ALEXYS Antibiotics base system - gradient (incl. the DECADE Elite electrochemical detector) + UVD 2.1L UV detector
Column	Discovery® C18 HPLC Column, 250 x 4.6 mm, 5 µm
Mobile phase	1.8 g/L sodium octane sulfonate, 20 g/L anhydrous sodium sulfate, 6 % acetonitrile and 5 %v/v 0.2 M potassium dihydrogen phosphate adjusted to pH 3.0 in deionized water.
Flow rate	1 mL/min
Temperature	40°C for separation (column in DECADE Elite oven compartment)
$V_{injection}$	20 µL
Flow cell	10 mm optical path length, 1/16", 10 µL volume (pn A4061XB)
Wavelength	200 nm
Filter	1.0 sec (time constant)

peak splitting might occur is also explicitly mentioned in the monograph at the system suitability requirements (symmetry factor). The column is kept at 40°C in the oven compartment of the DECADE Elite electrochemical detector, so no additional column thermostat is required in the system. UV detection was performed at a wavelength of λ = 200 nm using the UVD2.1L single wavelength UV detector.

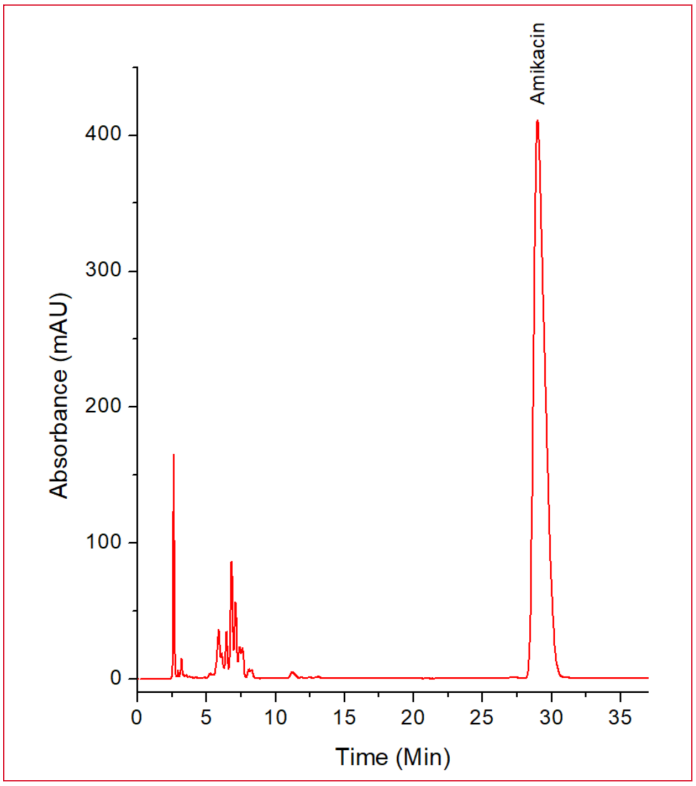

Figure 5: Chromatogram of an 20 µL injection of a test solution of 5 mg/mL Amikacin sulfate for parenteral use (injectable) in mobile phase.

A reference solution of 5 mg/mL of Amikacin CRS in mobile phase was prepared and injected 6 times (Chromatograms not shown) to check the system suitability requirements. The run in the monograph is specified as 1.3 times the retention time of Amikacin with a retention time for Amikacin of about 30 min. Amikacin eluted at a retention time of 28.7 min under the specified LC conditions on the ALEXYs analyzer, so the run time was set to 37 minutes in the Clarity method file.

The following system suitability requirements are specified for the assay based on the chromatograms obtained with the reference solution:

- *Symmetry factor:* maximum 1.5 for the peak of Amikacin.
- *Repeatability:* maximum relative standard deviation of 1.5% for the peak area of Amikacin for 6 subsequent injections.

In table 8 the results are shown based on chromatograms recorded with the reference standard. It is evident that the system suitability requirements are met for both parameters.

Table 8

EP system suitability requirements for the amikacin assay

Parameter	*EP criteria*	*Measured*
Symmetry factor for the amikacin peak	≤ 1.5	1.4
Relative standard deviation (peak area) for amikacin (n=6)	≤ 1.5%	0.3

A commercial formulation of 500 mg/2 mL of Amikacin sulfate for parenteral use (injectable) in aqueous solution was analyzed as an example. The chromatogram of the test solution is show in figure 5. The commercial sample was old and more than 8 years passed its expiration date of 06/2013 at the moment of analysis. The calculated amount of API based on the EP assay, 494 ± 4 mg/2 mL, was slightly lower than the labelled amount on the package.

References

1. Amikacin, Wikipedia, https://en.wikipedia.org/wiki/Amikacin
2. Aminoglycoside, Wikipedia, https://en.wikipedia.org/wiki/Aminoglycoside
3. Amikacin, Amikacin sulfate, European Pharmacopoeia (EP), 10.0 (2020), 1813 - 1818
4. M.S. Ramirez , M.E. Tolmasky, Amikacin: Uses, Resistance, and Prospects for Inhibition, Molecules, 22 (2017), 2267
5. W.R. LaCourse, “Pulsed Electrochemical Detection in High Performance Liquid Chromatography”, John Wiley & Sons, New York, 1ed, 1997.

Conclusion

The ALEXYS analyzer for amikacin sulfate provides a suitable solution for the analysis of pharmaceutical amikacin sulfate formulations following the official methods described in the EP 10.0 monograph. If required, the system can be equipped with an optional UV detector . This enables the possibility to run both the monograph for related substances (ECD) as well as the assay (UV) on the ALEXYS LC system.

Figure 6: The ALEXYS analyzer for Amikacin EP, consisting of the ALEXYS Antibiotics base system - gradient, a dedicated flow cell, post-column kit and bottles. The base system consists of an AS6.1L autosampler, P6.1L pump quaternary LPG gradient pump, ET 210 Eluent tray for helium blanketing, and the DECADE Elite electrochemical detector. The post-column kit EP (not shown in figure) contains a P6.1L pump with solvent selection valve and dual channel degasser for post-column addition of NaOH. The system is delivered with DataApex™ Clarity™ Chromatography Data System (CDS) software. Optionally, control drivers are available to operate the system in Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR 5 and up).

Ordering information

ALEXYS analyzer for Amikacin impurity analysis (ECD)	
180.0056W	ALEXYS Antibiotics base system - Gradient
180.0605EP	Post Column Kit EP
102.4325EP	Flexcell Au HyREF, SS AUX
184.0209	Glass bottle assembly, 1L, Helium
102.2218	Spacer for F/R cell, 130 µm
250.1045	flattening/polishing kit for metal WE
504971*,#	Discovery® C18 HPLC Column, 250 x 4.6 mm, 5 µm
Add-on parts for the Amikacin assay analysis (UV)#	
187.ADA01XA	UVD2.1L Detector with deuterium lamp, without flow cell
187.A4061XB	Flow cell cartridge 10 mm, 10 µl, 1/16", SST (UVD2.1L)

*) Column are manufactured and sold by Merck Supelco (Merck KGaA, Darmstadt, Germany).
#) For both the assay and amikacin impurity analysis described in the EP monograph a Discovery® C18 HPLC Column is used.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 71 5813333