



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

Streptomycin Sulfate According to USP

- **Meets all requirements of U.S. Pharmacopeia 38-NF33, 2015**
- **ALEXYS Aminoglycoside Analyzer with AEC**
- **Flow cell with Au working electrode and stainless steel AUX**
- **Reproducible and robust**

Summary

The Streptomycin sulphate analysis was evaluated using the exact method and conditions described in the official 2015 USP monograph [5]. In this application note typical results obtained with the ALEXYS[®] LC-EC aminoglycosides analyzer are reported, demonstrating its performance for the assay of Streptomycin sulphate in commercial pharmaceutical preparations.



Streptomycin Sulfate According to USP

Introduction

Streptomycin was the first aminoglycoside antibiotic described in 1944 by Waksman et al [1] and is produced by microbial fermentation of the actinobacterium *Streptomyces griseus*. It was shown to inhibit the growth of aerobic gram-positive and gram-negative bacteria as well as the tubercle bacilli, and was in fact the first effective treatment for tuberculosis. Besides its common use for clinical treatment in humans it is also utilized as veterinary drugs and crop-protection agent. Like other aminoglycosides, streptomycin is potentially oto- and nephrotoxic. Streptomycin and its derivatives can be analyzed using ion-pair reversed phase liquid chromatography (RP-LC) in combination with UV detection at 195 nm or 205 nm [2,3], but this method had limitations. It requires high concentrations of the compounds to be detected by UV absorbance due to the absence of a good chromophore. Pulsed Amperometric Detection (PAD) is a better choice. Streptomycin and its impurities have a molecular structure which contain functional groups which can be oxidized and detected by PAD with superior selectivity and sensitivity [4]. The U.S. Pharmacopeia (USP) describes a compendial method to assay streptomycin sulfate using HPLC-PAD [5].



Figure 1: ALEXYS Aminoglycoside Analyzer for Streptomycin.

Method

The USP (38-NF33) method for streptomycin sulfate is based on isocratic separation using an anion exchange column and alkaline mobile phase (pH = 12.8) followed by PAD. One of the thermal degradation products of streptomycin (induced by heating the standard solution for 1 hour at 75°C) is used to check the system suitability of the assay.

Separation

In the monograph the use of the following column type is described for the separation of Streptomycin: size 250 x 4 mm ID analytical column containing a L46 packing, which is defined

as Polystyrene/divinylbenzene substrate agglomerated with quaternary amine functionalized latex beads, about 9 µm to 11 µm in diameter. The CarboPac PA1, 250 x 4.0 mm ID analytical column which has a L46 packing was chosen for the method evaluation. The analytical column was used in combination with a CarboPac PA1 guard column with the dimensions 50 x 4.0 mm.

Table 1

LC-EC Conditions	
HPLC	ALEXYS LC-EC Analyzer with low-pressure mobile phase selector
Column	CarboPac PA1 250 x 4 mm ID analytical column + CarboPac PA1 50 x 4 mm ID guard column (USP column packing L46)
Mobile phase	70mM sodium hydroxide (analysis), 200mM sodium hydroxide (column clean-up).
Flow rate	0.5 mL/min
Vinjection	20 µL
Temperature	30°C for separation & detection
Flow cell	VT-03™ with Au WE, stainless steel AE and Ag/AgCl RE, spacer 50 µm
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	ca. 0.3 µA
ADF	0.5 Hz
Range	2 µA

The analysis is based on a step-gradient, see table 2. A column clean-up/regeneration step after isocratic elution is necessary to remove late eluting (thermal) degradation products present in the Streptomycin products and standards. These degradation products may result from storage or chemical degradation during manufacture. Especially the heat-treated system suitability standard exhibits a large late eluting impurity response. Under isocratic conditions (70 mM NaOH) this impurity elutes after 160 minutes under isocratic conditions.

The ALEXYS LC-EC Analyzer was equipped with only one LC pump and a Vici electrically-actuated low pressure (LP) valve in the pump LP suction line to switch between mobile phase (70 mM NaOH) and the mobile phase for column clean-up (200 mM NaOH). The eluents were carefully prepared manually using a commercial 50% NaOH solution, carbonate-free. The diluent was deionized Water (resistivity >18 MΩ-cm) which was sonicated and sparged with Helium 5.0 prior to use. The appropriate amount of NaOH was carefully pipetted into the diluent to minimize the introduction of carbonate in the solution. The bottles with mobile phase and column



clean-up solution were blanketed with Helium during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis.

Table 2

Step-gradient program		
Time (min)	Mobile phase	Description
0 - 22	70 mM NaOH	Isocratic elution & detection
22 - 40	200 mM NaOH	Column clean-up/regeneration
40 - 85	70 mM NaOH	Re-equilibration to starting conditions

Detection

For the detection of Streptomycin using PAD a flow cell is required with 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. A 4-step potential waveform is used as described in the USP monograph to detect streptomycin and its impurities on the Au working electrode, see Table 1 and Figure 2 below.

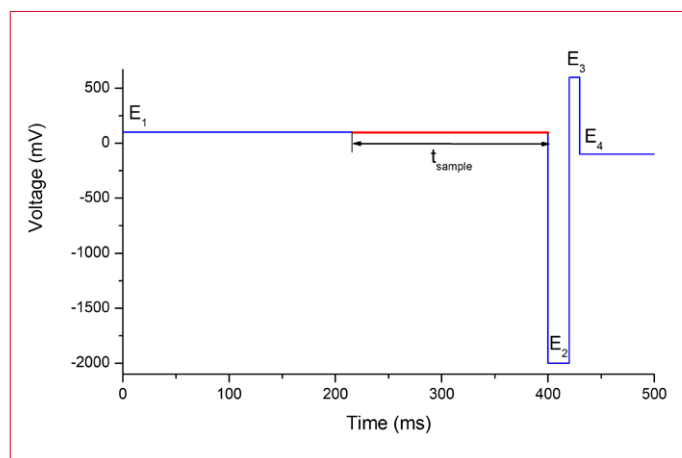


Figure 2: Applied PAD waveform as described in the USP monograph.

The temperature for separation and detection was set to 30°C. The cell current was typical about 0.3 μ A with these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has been claimed to have as benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [6]. Note that an ALEXYS LC-EC system with the new DECADE Elite electrochemical detector is required for PAD detection using a 4-step potential waveform.

Sample preparation

Standard preparation: 15 mg of USP Streptomycin Sulfate RS was accurately weighted and dissolved in 50 mL of water in a volumetric flask (sonicated for 1 minute and mixed). The obtained solution was subsequently 10x diluted using a 100 mL volumetric flask to obtain a final concentration of 0.03 mg/mL.

System suitability solution: 10 mL of the above mentioned standard was heated to 75°C for 1 hour and cooled down to ambient prior to use.

Assay preparation: 30 mg of a commercial Streptomycin Sulfate sample (Sigma-Aldrich, pn S6501, batch SLBD3728V) was transferred to a 100 mL volumetric flask and diluted with water to volume (sonicated for 1 minute and mixed). 10 mL was subsequently transferred to a second 100 mL volumetric flask and diluted to volume with water, resulting in a final concentration of 0.03 mg/mL.

Loss on drying

Prior to use the water contents of the commercial Streptomycin Sulfate sample was determined as specified in the monograph (loss on drying). An accurately weighted amount (around 100 mg) of Streptomycin sample was dried under vacuum (5 mmHg) at 60 °C for 3 hours. The dried sample was re-weighed and the loss on drying calculated. This analysis was performed in duplicate and an average weight loss of 3.4% was found. The measured weight loss of the sample was within the USP criteria (< 5.0%). The weight loss reported by the manufacturer of this specific sample in the certificate of analysis was 3.1%.

Results

System Suitability

In Figure 3 an overlay is shown of the chromatograms obtained with the USP standard solution (red curve) and the system suitability solution (blue curve). The retention times for the system suitability peak and Streptomycin were 6.55 and 13.08 minutes, respectively. It is evident from Figure 3 that the response of the main degradation product at 6.55 min increased significantly (more than 18 fold increase in peak height).



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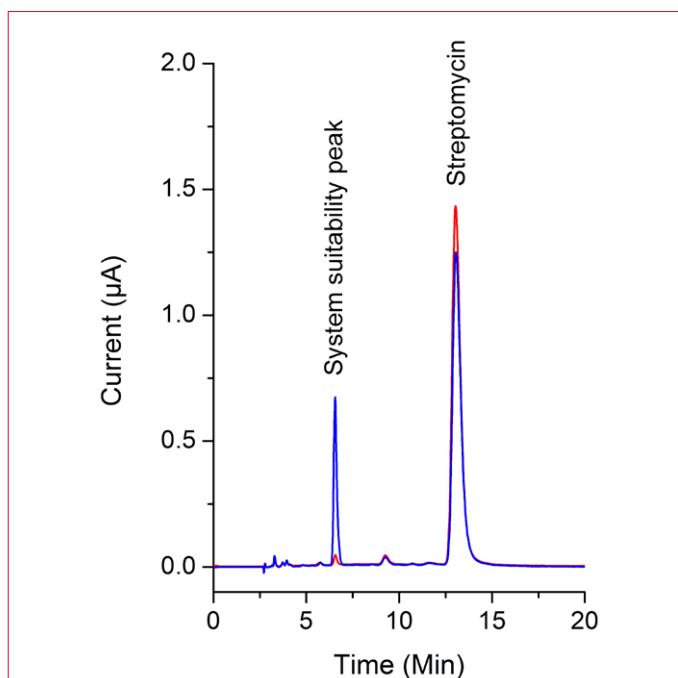


Figure 3: Chromatogram overlay of a system suitability solution (blue), and standard (red), both containing 30 µg/mL Streptomycin RS in water. Only the system suitability solution was heated for 1 hour at 75°C. The main degradation peak is designated 'system suitability peak' in the chromatogram.

In the USP monograph for Streptomycin Sulfate the following system suitability requirements are specified:

- **Relative retention time:** about 0.5 for the main degradation product and 1.0 for the Streptomycin peak in the chromatogram obtained with the system suitability solution.
- **Resolution:** not less than 3 between Streptomycin and main degradation product peak in the chromatogram obtained with the system suitability solution.
- **Column efficiency:** not less than 1000 theoretical plates for the Streptomycin peak in the chromatogram obtained with the standard solution.
- **Tailing factor:** not more than 2.0 for the Streptomycin peak in the chromatogram obtained with the standard solution.
- **Relative standard deviation:** not more than 5% (Area of Streptomycin peak) for replicate injections of the standard solution.

Table 3

USP system suitability requirement		
Parameter	USP criteria	Measured
Relative retention time (main degradation product)	0.5	0.5
Resolution (between main degradation product & Streptomycin)	> 3.0	11.3
Column efficiency (Streptomycin)	> 1000	3853
Tailing factor (Streptomycin)	< 2.0	1.5
RSD peak area, n=6 (Streptomycin)	< 5%	0.7%

The system suitability is evaluated using the chromatogram shown in Figure 3. The results are listed in table 3, it is evident that the system suitability requirements are met for all performance parameters.

Linearity, repeatability & LOD

The linearity for Streptomycin was investigated in the concentration range of 5 µg/mL – 40 µg/mL. In this concentration range the correlation coefficient for peak area was better than 0.995. The relative standard deviation (RSD) of the retention time, peak area and height were determined for 6 replicate injections of the USP Streptomycin Sulfate RS standard solution. The RSD's were 0.1%, 0.5% and 0.5%, respectively for the Streptomycin peak. The Limit of Detection (LOD) for Streptomycin, calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 30 segments of 0.5 min), was about 0.1 µmol/L (70 ng/mL).

Sample analysis

As an example a commercial sample was analyzed from Sigma Aldrich: S6501 Streptomycin sulfate salt (batch SLBD3728V). The sample is abbreviated as sample SLBD3728V from this point onwards. The chromatogram obtained from the sample solution is shown in Figure 4.

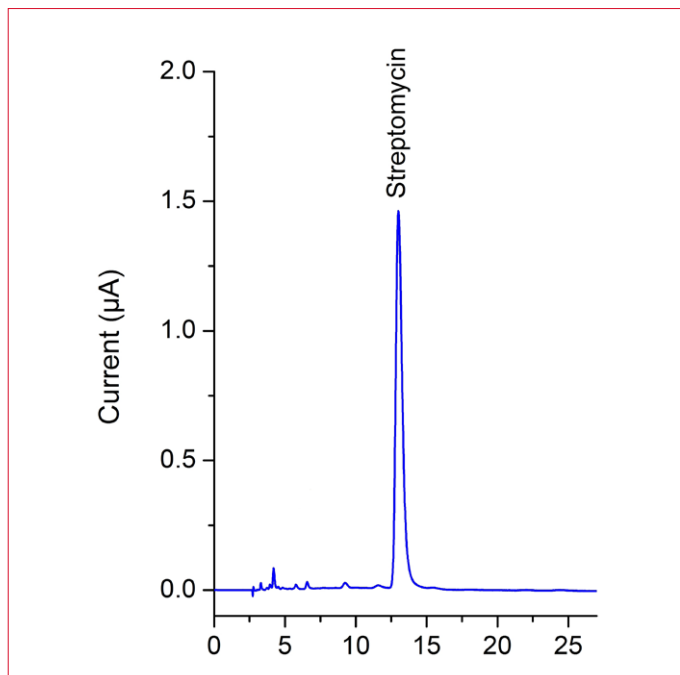


Figure 4: Analysis of Streptomycin sample SLBD3728V, 30 µg/mL in water.

The potency (contents) of the Streptomycin sulfate sample SLBD3728V, defined as the quantity of Streptomycin (C₂₁H₃₉N₇O₁₂) in µg per mg Streptomycin sulfate, is calculated as follows:

$$\text{Potency (in } \mu\text{g/mg)} = 1000 \times (C \times P / WU) \times (rU / rS)$$

Where:

C = Concentration in mg/mL of the USP Streptomycin Sulfate RS in the standard preparation.

P = Designated Streptomycin contents in µg per mg USP Streptomycin Sulfate RS.

Wu = Weight, in mg, of the Streptomycin sample taken to prepare the assay preparation.

rU = Streptomycin peak area obtain from the chromatogram of the assay preparation.

rS = Streptomycin peak area obtain from the chromatogram of the standard preparation.

The USP acceptance criteria for the Streptomycin potency in Streptomycin Sulfate is that the product should have a potency not less than 650 µg and not more than 850 µg of Streptomycin (C₂₁H₃₉N₇O₁₂) per mg. The result for sample SLBD3728V is listed in Table 4.

Table 4

Assay		
Sample	USP criteria µg/mg	Measured
SLBD3728V	650-850	717

The contents of Streptomycin in the analyzed Streptomycin sulfate sample was within the specified limits of the USP monograph.

Conclusion

The ALEXYS Aminoglycosides Analyzer offers a tailored solution to assay Streptomycin sulfate using the official method of the USP.



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References

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4. W.R. LaCourse, *Pulsed Electrochemical Detection in High Performance Liquid Chromatography*, John Wiley & Sons, New York, 1ed, 1997
5. *Streptomycin Sulfate*, The United States Pharmacopeia 38th ed., National Formulary 33th, United States Pharmacopeial Convention, Rockville MD, (2015), 5360
6. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, *Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform*, Anal. Chem, 70, (1998), 1496 - 1501

Ordering information

180.0051W	Aminoglycoside Analyzer including Flowcell
250.1076	CarboPac PA1, 250 x 4.0 mm ID
250.1077	CarboPac PA1 guard, 50 x 4.0 mm ID

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. 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.

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

