Application Note

Sulfur-Containing Antibiotics

The most reliable LC-EC applications for Antibiotics analysis

Aminoglycosides
Amikacin
Framycetin Sulphate
Gentamicin Sulphate
Kanamycin Sulphate
Lincomycin
Neomycin
Netilmicin
Spectinomycin
Tobramycin

Macrolide antibiotics
Azithromycin
Azaerythromycin
Clarithromycin
Erythromycin
Roxithromycin

Sulfur-containing antibiotics
Sulfamethoxazole
Trimethoprim

Sulfamethoxazole and Trimethoprim

- **Sulfur-containing antibiotics**
- **Multi-step Pulsed Amperometric Detection (MPAD)**
- **FlexCell with Au working electrode**

Summary

Sulfamethoxazole (SMX) and trimethoprim (TMP) are antibiotics which are often used as a mixture in pharmaceutical tablet formulations such as Co-trimoxazole or Bactrim [1]. These tablets contain both SMX and TMP, often in a 5:1 ratio, and are used for the treatment of bacterial infections, such as pneumonia, bronchitis, ear or intestine infections [2]. The SMX-TMP combination is listed on the WHO Model List of Essential medicines as a first-choice treatment for urinary tract infections [3]. SMX is part of the group of sulphonamides or sulfa drugs with a sulphonamide functional group, TMP is an aminopyrimidine antibacterial agent and does not contain sulfur.

Sulphonamide antibiotics, containing not fully oxidized sulfur, can be detected electrochemically with Pulsed Amperometric Detection on a gold working electrode [4,5]. The method is based on the application of a multi-step waveform consisting of a series of linear cyclic scans between two potentials [5] allowing sensitive detection of sulfur-containing compounds. This application note describes the analysis of sulfamethoxazole and trimethoprim in pharmaceutical tablets based on a HPLC-PAD method using the Antec ALEXYS HPLC-PAD Analyzer.

Introduction

Sulfur-containing antibiotics are a group of compounds with antibacterial properties and which contains at least one sulfur group. Examples of sulfur-containing antibiotics are ampicillin, penicillin, lincomycin and sulfamethoxazole.

Sulfamethoxazole is a sulfonamide bacteriostatic antibiotic, this means that the antibiotic functions via inhibition of bacterial protein synthesis. By inhibition of the dihydropteroate synthase, the formation of dihydropteroic acid is prevented which leads to a stop of bacterial growth due to a lack of folic acid. Sulfamethoxazole, abbreviated as SMZ or SMX, is often found in pharmaceutical products together with trimethoprim (TMP) [8]. Trimethoprim is a non-sulfur containing antibiotic with antiprotozoal properties that blocks the production of tetrahydropholic acid and is usually used in a 1:5 ratio with SMX [9]. This formulation is sold under different trade names, such as Co-trimoxazole, Bactrim and Septra [1]. Co-trimoxazole is used to treat bacterial infections, such as pneumonia, bronchitis and urinary tract, ear or intestine infections [2].

Sulfamethoxazole and trimethoprim can be quantified using for example HPLC-UV or HPLC-MS/MS [4,6]. However, due to the lack of a strong chromophore in SMX the sensitivity of UV detection is poor. HPLC-MS/MS allows sensitive direct detection of antibiotics, but it requires more expensive analysis equipment.

Fig. 1. *ALEXYS Antibiotics base system. Note: the displayed pump in this particular configuration is an P 6.1L quaternary LPG version.*

A more cost-effective alternative for MS/MS detection is Pulsed Amperometric Detection [5,7].

This application note describes a method for the sensitive quantification of sulfamethoxazole and trimethoprim with the ALEXYS HPLC-PAD Analyzer (figure 1) using the new Multi-step Pulsed Amperometric Detection (MPAD) mode. The option to program multi-step waveforms up to 30 time points is available for the DECADE Elite from FW version 1.09 and onwards. This new measurement mode can be used when the Elite is controlled by software. The MPAD mode is supported in DECADE Elite control drivers of the following Chromatography Data Systems:

- DataApex Clarity™ CDS (8.3*)
- Thermo Scientific™ Chromeleon™ CDS (7.5 SR5)
- Agilent OpenLAB™ CDS (2.3.0)
- Agilent OpenLAB Chemstation™ (C.01.09)

*) Between parentheses the required CDS version (or newer).

Method

The analysis was performed using a ALEXYS Antibiotics base system, consisting of a P6.1L pump, AS6.1L autosampler, ET 210 eluent tray and DECADE Elite electrochemical detector. For detection, a FlexCell was used equipped with a gold working electrode and HyREF (Pd/hydrogen) reference electrode. The system was controlled via a PC using the Clarity Chromatography Data System software (version 8.3). The LC conditions are listed in table 1.

Table 1

Conditions	
HPLC	ALEXYS Antibiotics base system - Isocratic
Detector	DECADE Elite EC detector
Column	Thermo Scientific ™ Betabasic™ 18, 50 x 3.0mm ID, 3µm
Mobile phase	150 mM sodium acetate, pH 3.75, 11% acetonitrile Mobile phase sparged and kept under an inert atmos-phere with helium 5.0 with the ET210 eluent tray.
Flow rate	0.5 mL/min
Injection volume	5 µL
Temperature	35 °C for separation and detection
Flow cell	FlexCell™ with Au WE and HyREF (Pd) RE, 50 µm spacer
Potential waveform	See table 2
Range	50 µA
I-cell	ca. 4 µA
ADF	0.1 Hz

Separation

The separation of SMX and TMP is performed on a C18 3 µm reversed phase column (50 x 3 mm) under acid conditions using 150 mM sodium acetate (pH 3.75) as eluent. To decrease the run time, 11% acetonitrile was added to the mobile phase.

It is important to keep the mobile phase free of O_2, for this reason the mobile phase is kept under an inert atmosphere of helium 5.0. For the preparation of 2 L of mobile phase, 17.1 mL glacial acetic acid was added to 580 mL deionized water. Using approximately 2 mL of 50% carbonate-free sodium hydroxide solution, the pH was set to 3.75 and deionized water was added to create a 600 mL 500 mM sodium acetate (pH 3.75) solution. In a 2 L polypropene copolymer (PPCO) bottle, 1180 mL water and 220 mL acetonitrile were added to the 500 mM sodium acetate and after mixing, the mixture was degassed in an ultrasonic bath for 15 minutes. Subsequently the mixture was sparging with helium for another 15 minutes and the mobile was blanketed with Helium during analysis (approximately 0.3 bar Helium pressure) using the ET 210 eluent tray.

Detection

For the pulsed amperometric detection of sulfur-containing antibiotics, the Antec FlexCell™ is used. This flow cell has a thin-layer design and consists of a Au working electrode (WE), HyREF (Pd/hydrogen) reference electrode (RE) and carbon-loaded PTFE auxiliary electrode (AE).

The MPAD waveform for detection of sulfur-containing antibiotics is shown in figure 2 and is derived from reference [10].

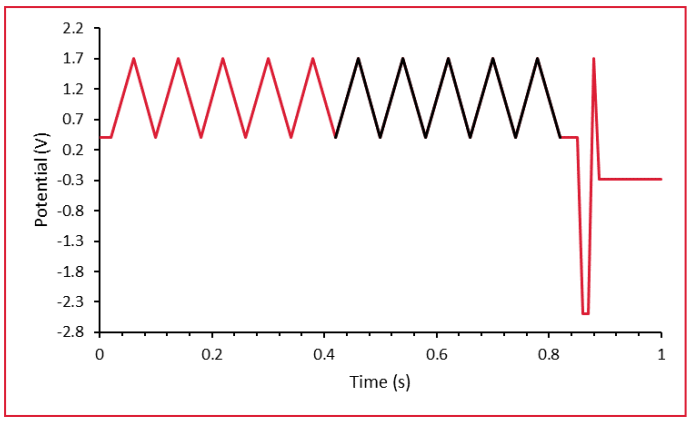

Fig. 2. *The MPAD waveform for the detection of sulfur-containing antibiotics.. The part of the curve shown in black is the sampling period, the time period in which the current signal is measured.*

Sulfur-containing antibiotics are detected via an oxide-catalyzed mechanism, the so-called mode II PAD detection as described by LaCourse [11]. During a pulse cycle the analyte is first adsorbed onto the Au electrode surface prior to detection. At the applied detection potential, surface-stabilized oxidation of the analyte occurs alongside with the formation of surface oxides. The formation of the surface oxides is important in this process. The labile surface oxide intermediates facilitate the transfer of oxygen moieties to the analyte. The formation of surface oxides together with residual oxidized analyte will prevent re-adsorption of new analyte molecules. Therefore, a reductive cleaning step is required at the end of every pulse cycle to obtain a bare and reactive Au surface again.

Table 2

PAD waveform (Pulse mode -2)

Time (s)	*Potential (V)*	*Data sampling*
0.00	0.40	
0.02	0.40	
0.06	1.70	
0.10	0.40	
0.14	1.70	
0.18	0.40	
0.22	1.70	
0.26	0.40	
0.30	1.70	
0.34	0.40	
0.38	1.70	
0.42	0.40	Start
0.46	1.70	
0.50	0.40	
0.54	1.70	
0.58	0.40	
0.62	1.70	
0.66	0.40	
0.70	1.70	
0.74	0.40	
0.78	1.70	
0.82	0.40	End
0.85	0.40	
0.86	-2.50	
0.87	-2.50	
0.88	1.70	
0.89	-0.28	
1.00	-0.28	

For the formation of surface oxides relatively large anodic potentials are required. As a result the majority of the large current signal (background) originates from the surface oxide formation, rather than oxidation of the analyte itself.

In order to minimize the background current and achieve a stable baseline, potential scans are executed during the pulse cycle instead of applying a static detection potential. By scanning the potential, the resulting current signal of oxide formation is rejected (subtracted by the reductive current), yielding effectively only the current of the analyte. The waveform for detection of sulfur-containing antibiotics consists of a sequence of 10 scans followed by a cleaning pulse. During the 5 last scans of the pulse cycle the current is measured. The first 5 scans in the pulse cycle are applied for stabilization.

Preparation of standards and samples

Standards: 1 g/L stock standards of SMX and TMP in 70% (v/v) methanol/water were prepared by dissolving 0.5 g antibiotic in 50 mL 70% (v/v) methanol. Further dilutions were made in 70% (v/v) methanol.

Sample preparation: Half a Co-trimoxazole Aurobindo 480 mg tablet containing 400 mg SMX and 80 mg TMP was placed in a 50-mL volumetric flask and was brought to volume using 70% (v/v) methanol. After 20 minutes of sonication, the sample was centrifugated at 3000 x g and the supernatant collected, to remove the insoluble excipients of the tablet. Using 70% (v/v) methanol, 200-, 400– and 800– fold dilutions of the sample were made of which 5 µL was injected.

Results

In figure 3 an example chromatogram is shown of a 5 µL injection of a standard mixture consisting of 10 mg/L SMX and TMP in 70% methanol. Within 8 minutes both sulfamethoxazole and trimethoprim are eluted. The compounds are well separated with a resolution of 6.5. Both peaks show tailing which is typical for this type of compounds using PAD detection under the specified conditions [4,10].

Linearity, Repeatability and LOD

The linearity of the response of SMX and TMP was investigated in the concentration range of 0.5—40 mg/L, which corresponds with a molar concentration range of 2-156 µM and 2—136 µM for SMX and TMP, respectively. For both antibiotics, linearity was excellent with correlation coefficients better than 0.999 (figure 4).

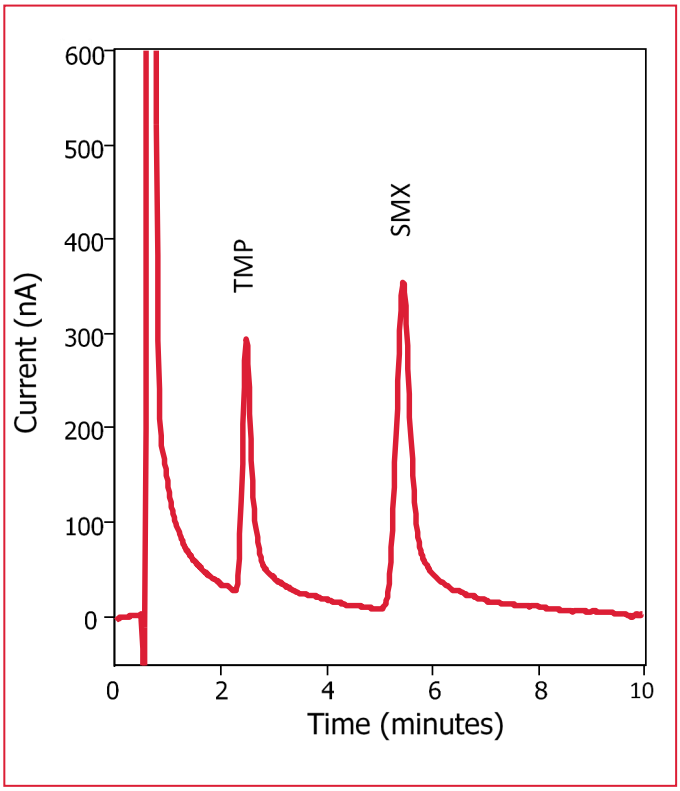

Fig. 3. *5 µL injection of a standard mixture containing 10 mg/L sulfamethoxazole and trimethoprim in 70% methanol.*

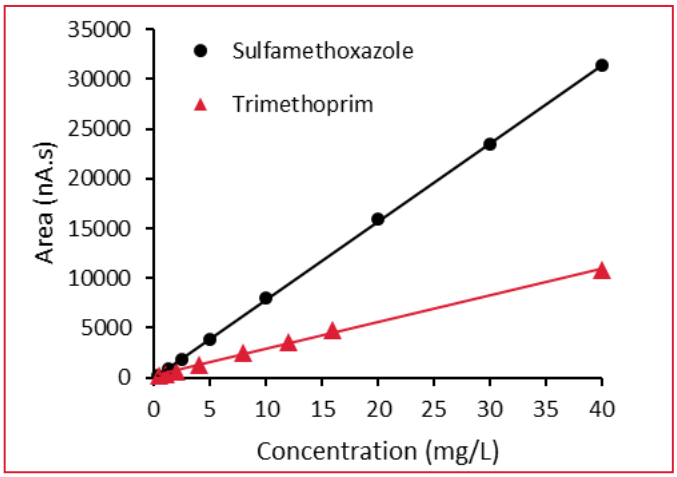

Fig. 4. *Calibration curve of sulfamethoxazole and trimethoprim in the concentration range 0.5—40 mg/L. (R> 0.999).*

The relative standard deviation (RSD) of the retention times and peak areas were determined for 10 replicate injections of a 1 and 10 mg/L standard mixture of SMX and TMP. The results are shown in table 3. RSD's for retention time were < 0.35%. For the peak areas the RSD's were < 2% and < 3% for the 1 and 10 mg/L standard, respectively. These data demonstrate that with this method reproducible analysis of SMX and TMP can be achieved.

Table 3

Repeatability of 5 µL injections of a 1 and 10 mg/L sulfamethoxazole & trimethoprim standard in 70% MeOH (n=10)

	RSD's (%) 1 mg/L		RSD's (%) 10 mg/L	
Compound	t_R	*Area*	t_R	*Area*
SMX	0.15	2.41	0.14	1.43
TMP	0.22	2.73	0.33	1.00

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on the response of the calibration standard with the lowest concentration, which is 0.2 mg/L. The results are shown in table 4. The LOD was calculated as the analyte response corresponding to 3x the ASTM noise. The LOQ was calculated as the analyte response corresponding to 10x the ASTM noise. The noise was calculated based on a 3 minute section of the baseline noise close to the compounds of interest (6 segments of 0.5 minutes).

Table 4

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

	LOD			LOQ
Compound	*µg/L (ppb)*	*nmol/L*	*pg injected*	*µg/L (ppb)*
SMX	48	190	240	160
TMP	79	272	395	263

The Limit of Quantitation found for SMX and TMP are 160 and 263 µg/L, respectively. These LOQ's are more than sufficient to reliably quantify the SMX and TMP content in co-trimoxazole tablets. Pharmaceutical tablet formulations typically contain 400mg/80 mg or 800mg/160mg SMX/TMP per tablet.

Sample analysis

As an example, a Co-trimoxazole Aurobindo 480 mg tablet was analyzed using the HPLC-PAD method. 280 mg of a tablet was dissolved in 50 mL 70% (v/v) methanol and worked-up using the sample preparation procedure described on the previous page. The sample was 200x diluted in 70% (v/v) methanol prior to injection. The resulting chromatogram is shown in figure 6 together with an overlay of a sulfamethoxazole and trimethoprim standard and a blank injection of 70% (v/v) methanol.

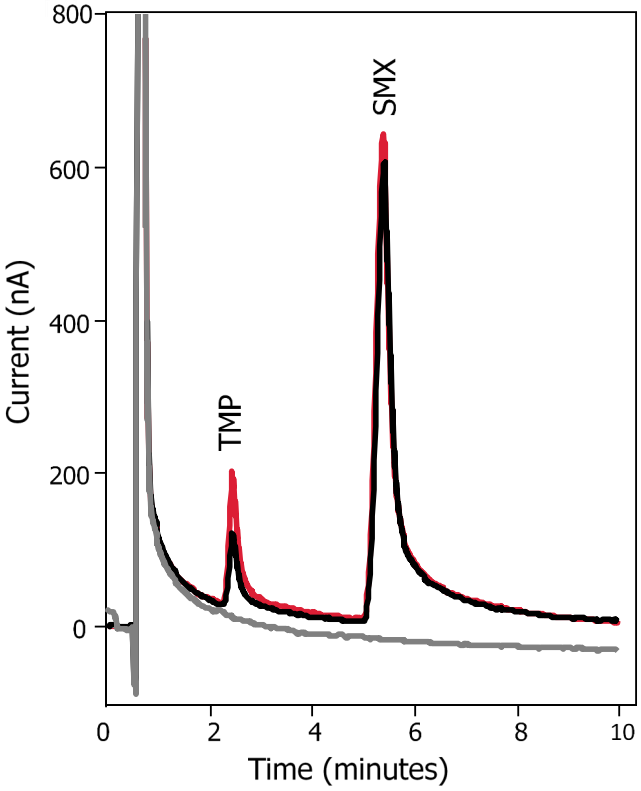

Fig. 6. *Overlay of a 5 µL injection of a blank (grey), 8 mg/L TMP and 20 mg/L standard (red) and a Co-Trimoxazole sample (black).*

Table 5

Contents of antibiotics in a Aurobindo Co-Trimoxazol tablet

Compound	*Amount per tablet (mg)*
SMX	84
TMP	422

The calculated amounts of 84 mg trimethoprim and 422 mg sulfamethoxazole in the tablet corresponds to approximately 105% of the amount of API on the package label.

References

1. Drugs.com, Bactrim, https://www.drugs.com/pro/bactrim.htmL
2. Medline Plus, Co-trimoxazole, https://medlineplus.gov/druginfo/meds/a684026.htmL
3. L. Roth et. al., "Monographs for medicines on WHO's Model List of Essential Medicines", Bulletin of the World Health Organization. (2018), 96 (6), p378–385.
4. Dionex/Thermo Scientific, Determination of Sulfur-Containing Antibiotics Using Integrated Pulsed Amperometric Detection (IPAD), Application note 132
5. D.C. Johnson, W.R. LaCourse, Liquid Chromatography with Pulsed Electrochemical Detection at Gold and Platinum Electrodes, Anal. Chem, 1990, 62, p589A-597A
6. D. Gonzalez et al., Simultaneous determination of trimethoprim and sulfamethoxazole in dried plasma and urine spots, Bioanalysis, (2015), 7(9), p1137-1149
7. W.R. LaCourse, G.S. Owens, Pulsed Electrochemical Detection of Thio-Compounds Following Micro chromatographic Separations, Anal. Chim. Acta, (1995), 307, p301-319
8. Pubchem, Sulfamethoxazole, https://pubchem.ncbi.nlm.nih.gov/compound/Sulfamethoxazole
9. Pubchem, Trimethoprim, https://pubchem.ncbi.nlm.nih.gov/compound/trimethoprim
10. V.P. Hanko et al. Determination of Sulfur-Containing Antibiotics Using High-Performance Liquid Chromatography With Integrated Pulsed Amperometric Detection, Drug Development Research, (2001), 53, p268—280
11. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997

Conclusion

The ALEXYS HPLC-PAD analyzer based on the DECADE Elite detector with FlexCell (Au WE, HyREF) offers a simple and cost-effective solution for the sensitive analysis of sulfur-containing antibiotics. To demonstrate the performance of the system the amount of sulfamethoxazole and trimethoprim in co-trimoxazole tablets was successfully quantified.

The new MPAD mode in the DECADE Elite enables detection using advanced multistep waveforms consisting of repetetive potential scans suitable for sulfur-containing aliphatic compounds.

Ordering information

180.0058W	ALEXYS Antibiotics base system - Isocratic
102.4325	FlexCell Au HyREF
184.0205	PPCO bottle assembly, 2L, Helium
250.1045	Flattening/polishing kit for metal WE

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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