Application Note

Macrolide 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

Azithromycin
According to USP method

- **US Pharmacopoeia 41 NF 36 (2018)**
- **Azithromycin oral suspension, capsules and injections**
- **Impurity analysis (ECD)**
- **Assay of active pharmaceutical ingredient (ECD and UV)**

Summary

Azithromycin is a semi-synthetic macrolide antibiotic, chemically related to erythromycin and clarithromycin [1-3]. It is effective against a wide variety of (Gram positive) bacteria. Azithromycin is used to treat bacterial infections of the ears, skin, lungs, throat and some sexually transmitted diseases (STD's). To determine the contents of active pharmaceutical ingredient (API) in azithromycin formulations, the US Pharmacopoeia (USP) published assays based on liquid chromatography in combination with electrochemical and UV detection [4]. For the impurity analysis of azithromycin formulations LC-ECD is the method of choice.

The assay and impurity analysis of azithromycin formulations (injectable, oral suspensions and capsules) was evaluated on an Antec ALEXYS analyzer using the LC- ECD and UV methods described in the official 2018 USP 41—NF36 monograph [4]. In this application note typical results obtained with the ALEXYS® Analyzer are reported, demonstrating its suitability for the routine analysis of azithromycin in various formulations.

Introduction

Azithromycin is available in an injectable formulation as well as formulations for oral administration (capsules, tables, powder for preparation of a suspension), and each formulation has to be shown to meet the acceptance criteria described in dedicated USP monographs [4] to guarantee quality for markets that have to adhere to USP regulations. Part of all the tests is a quantification assay which is based on HPLC separation, followed by either UV detection or electrochemical detection (ECD). Azithromycin has both a UV-absorbing chromophore, and an amine moiety that is electrochemically detectable after HPLC separation. In addition to the 'Assay', the injectable azithromycin formulation requires a check for a list of various impurities, which should not exceed the given limit levels. This method is based on ECD.

Each method includes a set of specific system suitability parameters that have to be within specification before applying the method to real samples. Some monographs prescribe a check for separation performance, whereas other monographs set specific criteria for repeatability, or a combination of the two.

Figure 1: *ALEXYS Azithromycin Analyzer with optional UVD 2.1L UV detector.*

The ALEXYS Azithromycin Analyzer is a dedicated LC-ECD system to perform the assay and impurities analysis of azithromycin using a chromatographic system as described in the USP monographs. The system can be expanded with an optional UV detector to run the assay specified in the monograph for 'azithromycin for injection'. This application note shows typical results for the system suitability tests when using the ALEXYS system with both EC and UV detector.

ALEXYS Azithromycin Analyzer

The ALEXYS Azithromycin Analyzer is a dedicated LC system consisting of the AS 6.1L autosampler with sample cooling option, the P6.1L pump, DECADE Elite electrochemical detector (ECD) and the optional UVD 2.1L UV detector. Where the USP monograph specifies the use of ECD, it states the application of a 'dual series glassy carbon electrode' in 'oxidative screen mode'. In principle, one electrode would suffice to detect azithromycin and the related impurities, but to match the prescription of the USP monograph, the DECADE Elite is equipped with two flow cells. The first GC electrode is in a low-dead-volume FlexCell with a maintenance free HyREF reference electrode right after the column. The second electrode is covered by a 2 mm GC SenCell with a saltbridge reference electrode, and this is the cell that is actually used for detection. A short piece of tubing interconnects both flow cells.

Voltammogram

The ECD settings given in the USP monographs are a combination of working potential and background current with only a narrow window for adjustments. The setting for the working potential at the detection electrode should be between 770—870 mV. A voltammogram was constructed around these values, showing the highest response is at 870 mV (Figure 2). However, the limits for background current is 70-100 nA (assay) or 70-120 nA (impurities analysis) and the example given in Figure 2 shows that a sub-optimal working potential has to be applied to meet this specification.

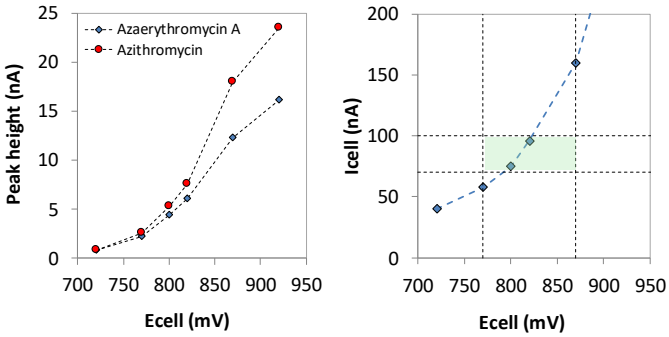


Figure 2: *Hydrodynamic voltammogram of azithromycin and azaerythromycin A at a GC sb SenCell (left panel) and the response of the background current (Ic) to the various working potentials (right panel). The green area in the right plot indicates the allowed settings as given in the USP monographs for Azithromycin in capsules or oral suspension ('Assay'). Results based on the analysis of the 'System suitability solution'.*

Chemicals

The mobile phase was prepared according each separate tested monograph. We found that meeting the specification for background current requires the use of highly pure LC-MS grade acetonitrile: some lower grade acetonitrile options that we tested were associated with higher (out of spec) background currents. The test solutions ('System suitability solution' and 'Standard solution') were also prepared according the monograph, using USP reference standards.

The buffering capacity of phosphate is not optimal at the prescribed pH, and the pH needs careful adjustment not to overshoot. It is also important to set the pH after adding the acetonitrile, as setting the pH before mixing results in a higher pH value after mixing and a higher (out of spec) background current.

Electrode maintenance

To obtain a stabile background current and response, our advise is to polish the working electrodes, refill the reference electrode with the appropriate solutions (see flow cell user manuals) and let the background current stabilize at least 1 hour. Working potential can then be adjusted to get the background current within spec if necessary.

Azithromycin for Oral Suspension — Assay
Azithromycin for Capsules — Assay

The method settings for the assay of Azithromycin in capsules and Azithromycin in oral suspension are identical.

Table 1

Conditions

HPLC	ALEXYS Azithromycin analyzer
Column	ZirChrom-PBD 4.6 x 150 mm, 3 µm (ZirChrom), USP L49
Mobile phase	[29% v/v acetonitrile; 71% v/v 2.7 g/L KH_2PO_4 in DI water], adjusted to pH 11.0 with KOH solution
Flow rate	1.5 mL/min
Temperature	35 °C for separation and detection
$V_{injection}$	50 µL
Flow cell 1 (screen)	FlexCell™ with GC and HyREF, spacer 50 µm
Ecell 1	650 mV
Flow cell 2 (detection)	2 mm GC SenCell™ with saltbridge, AST setting 2
Ecell 2	800 mV
Range	20 nA
ADF	0.02 Hz
I-cell (detection)	Ca. 85 nA

The methods prescribe LC separation on columns with packing L29 or L49. We applied a ZirChrom PBD 4.6 x 150 mm, 3 µm (packing L49) for the evaluation tests. Table 1 shows the conditions as actually applied (which is based on the USP monograph).

When starting up this analysis, we advise to let the system stabilize for at least 5 hours, while analysing the standard solution, until the RSD (n=5) is in spec.

Table 2

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Resolution between azaerythromycin A and azithromycin (*system suitability solution*)	> 2.5	7.4
Column efficiency (*standard solution*)	> 1000	7651
Tailing factor (*standard solution*)	0.9—1.5	1.0
Relative standard deviation (*standard solution*)	< 2.0%	1.4%

The system suitability requirements are met for all parameters (Table 2).

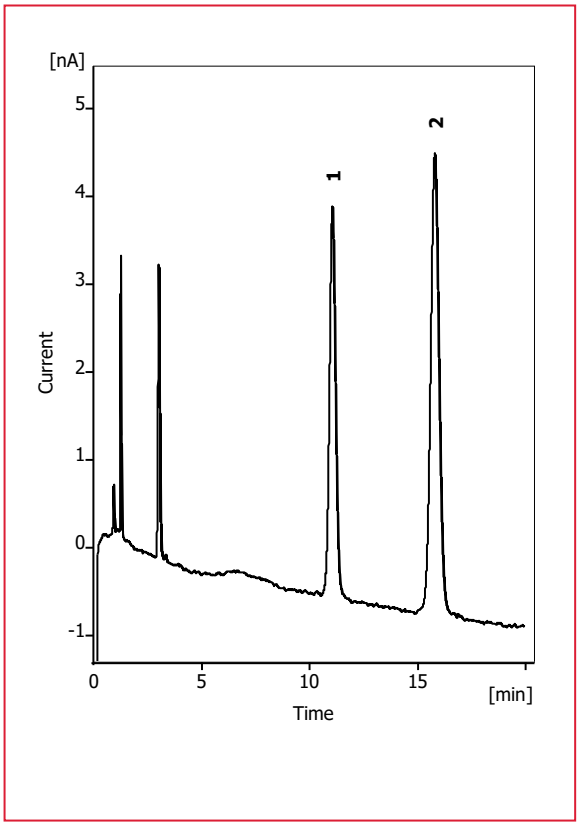


Figure 3: *Chromatogram from 4.4 µM USP azaerythromycin A RS (1) and 4.4 µM azithromycin (2) in mobile phase (system suitability solution, as described in the USP monograph for 'azithromycin in capsules' and 'azithromycin for oral suspension', section 'assay'), 25 µL injection.*

Azithromycin for Injection — Impurities: Limit of azithromycin N-oxide (and others)

This method prescribes LC separation on a column with packing L49. We applied a ZirChrom PBD 4.6x15cm, 3 µm for the evaluation tests. Table 3 shows the conditions as actually applied (which is based on the USP monograph).

The system suitability test for this method is based on a check for peak shape and repeatability (n=6). As the analysis time for each chromatogram is close to 1.5 hours, starting this analysis up is most efficient overnight: the stabilisation time is about 2-3 runs before the results start to be repeatable within spec so we advice to program a sample queue with at least 8 repeated analyses of the standard solution before running real samples. RSD values should then be calculated based on the last 6 chromatograms.

Table 3

Conditions

HPLC	ALEXYS Azithromycin analyzer
Column	ZirChrom-PBD 4.6 x 150 mm, 3 µm (ZirChrom), USP L49
Mobile phase	[23% v/v acetonitrile; 77% v/v 3.5 g/L K_2HPO_4] in DI water, adjusted to pH 10.55 with KOH solution
Flow rate	1 mL/min
Temperature	35 °C for separation and detection
$V_{injection}$	50 µL
Flow cell 1 (screen)	FlexCell™ with GC and HyREF, spacer 50 µm
Ecell 1	650 mV
Flow cell 2 (detection)	2 mm GC SenCell™ with saltbridge, AST setting 2
Ecell 2	870 mV
Range	20 nA
ADF	0.02 Hz
I-cell (detection)	Ca. 105 nA

The system suitability requirements are met for all parameters (Table 4).

Table 4

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Tailing factor (*standard solution*)		
azithromycin	< 2.0	1.1
N-demethylazthromycin	< 2.6	2.0
Relative standard deviation (*standard solution*)		
azithromycin N-oxide	< 10%	5.8%
desosaminylazithromycin	< 10%	3.9%
N-demethylazithromycin	< 10%	5.9%
azithromycin	< 10%	2.7%

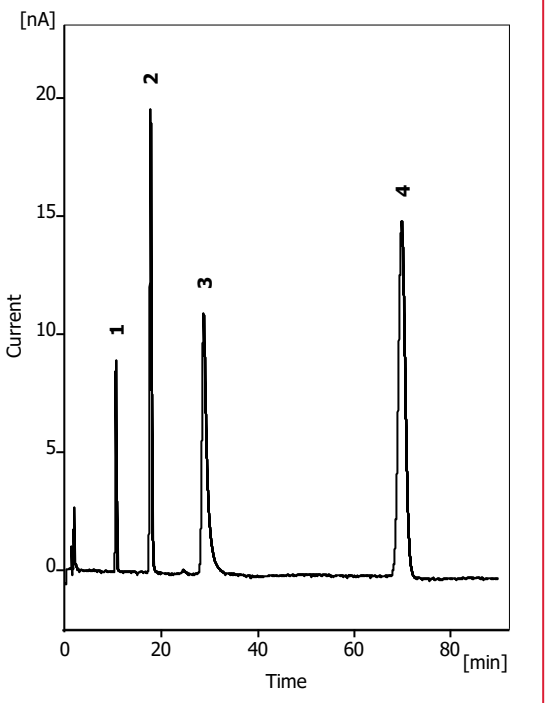


Figure 4: *Chromatogram from 1 µg/mL USP azithromycin N-oxide RS (1), 0.9 µg/mL USP desosaminylazithromycin RS, (2), 3.2 µg/mL USP N-demethylazithromycin RS (3) and 3.2 µg/mL USP azithromycin RS (4) in mobile phase ('standard solution', as described in the USP monograph for 'azithromycin for injection', section 'Impurities', limit of azithromycin N-oxide and others), 50 µL injection.*

Limit of Quantitation (LOQ)

As the reporting level for impurities is 0.05% relative to the level of azithromycin (info in the monograph), the LOQ (SN ratio =10) of this method should be at least 0.05% relative to the concentration of azithromycin in the sample solution (0.3 mg/mL), which is 0.15 µg/mL.

The analysis of a standard mix with each component at a concentration of 0.15 µg/mL resulted in peak SN ratios higher than 10 for each of the tested impurities (Figure 5). This means that the system not only meets the system suitability requirements, but also that it is sensitive enough to be used for the impurities analysis.

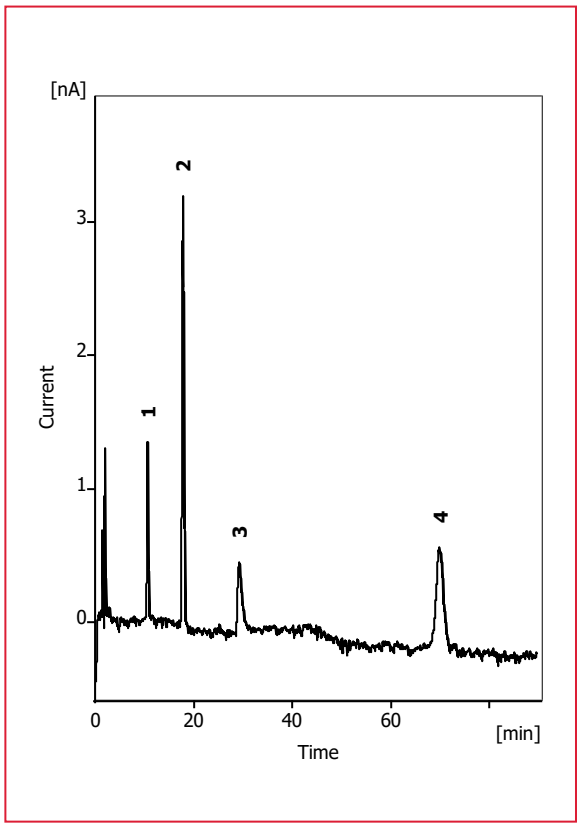


Figure 5: *Chromatogram from 0.15 µg/mL USP azithromycin N-oxide RS, USP desosaminylazithromycin RS, USP N-demethylazithromycin RS and USP azithromycin RS in mobile phase, 50 µL injection.*

Azithromycin for Injection — Impurities: Limit of aminoazithromycin (and others)

This method prescribes LC separation using a guard and analytical column with packing L67. We applied the 4.6 x 10 mm and 4.6 x 250 mm Shodex Asahipak ODP-50 with 5 µm particles. This method also states the use of 40 °C as the temperature for the HPLC column, but higher temperatures were also found to be associated with higher background currents. Therefore, a temperature of 35 °C is applied, which is still in range of the allowed adjustments as long as the system suitability requirements are met [5]. Table 3 shows the conditions as actually applied (which is based on the USP monograph).

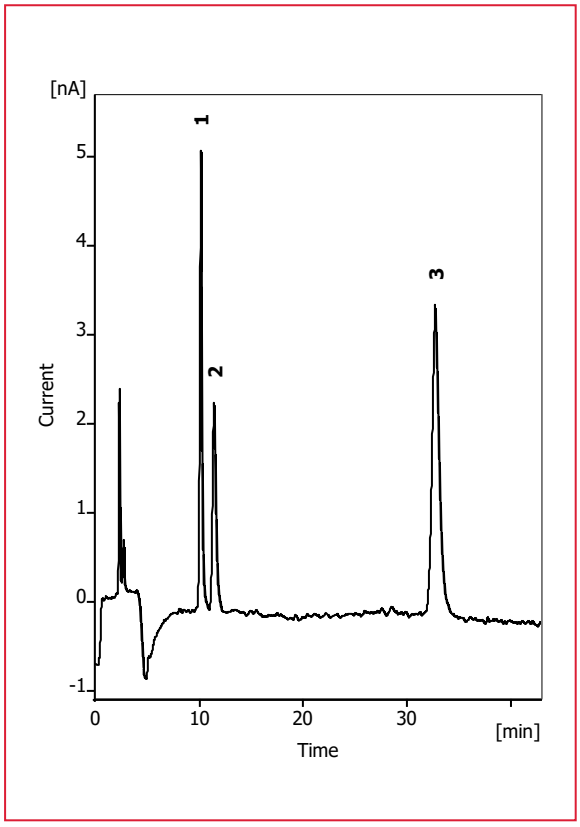
The system suitability test for this method is checking the chromatographic performance and repeatability (n=5). As the analysis time for each chromatogram is more than 1 hour, starting this analysis up is most efficient overnight: the stabilisation time is at least 4 hours before the results start to be repeatable. Also be aware that some of the impurities have elution times much later than that of the main peak. We advise to program a sample queue with at least 8 repeated analyses of the standard solution before running real samples. RSD values should then be calculated based on the last 5 chromatograms.

Table 5

Conditions	
HPLC	ALEXYS Azithromycin analyzer
Column	Asahipak ODP-50G 4A guard column, 4.6 x 10 mm Asahipak ODP-50 4E, 4.6 x 250 mm ID, 5 µm, USP L67
Mobile phase	[46% v/v acetonitrile; 54% v/v 3.5 g/L K_2HPO_4] in DI water, adjusted to pH 11.0 with KOH solution
Flow rate	1 mL/min
Temperature	35 °C for separation and detection
$V_{injection}$	25 µL
Flow cell 1 (screen)	FlexCell™ with GC and HyREF, spacer 50 µm
Ecell 1	650 mV
Flow cell 2 (detection)	2 mm GC SenCell™ with saltbridge, AST setting 2
Ecell 2	870 mV
Range	20 nA
ADF	0.01 Hz
I-cell (detection)	Ca. 105 nA

Table 6

USP system suitability requirements		
Parameter	*USP criteria*	*Measured*
Resolution between desoaminylazithromycin and N-demethylazithromycin (*standard solution*)	>1.5	2.3
Tailing factor (*standard solution*) azithromycin	<1.5	1.2
Relative standard deviation (*standard solution*) azithromycin	<5%	1.4%



The system suitability requirements are met for all parameters (Table 4).

Figure 6: *Chromatogram from 1.8 µg/mL USP desosaminyl-azithromycin RS (1), 4.2 µg/mL USP N-demethylazithromycin RS (2) and 6 µg/mL USP azithromycin RS (3) in 46% acetonitrile ('standard solution', as described in the USP monograph for 'azithromycin for injection', section 'impurities', limit of aminoazithromycin and others), 25 µL injection.*

Limit of Quantitation (LOQ)

As the reporting level for impurities is 0.05% relative to the level of azithromycin [4], the LOQ (SN ratio =10) of this method should be at least 0.05% relative to the concentration of azithromycin in the sample solution (0.6 mg/mL), which is 0.3 µg/mL.

The analysis of a standard mix with each component at a concentration of 0.3 µg/mL resulted in peak SN ratios higher than 10 for each of the tested impurities (Figure 7). This means that the system not only meets the system suitability requirements, but also that it is sensitive enough to be used for this impurities analysis.

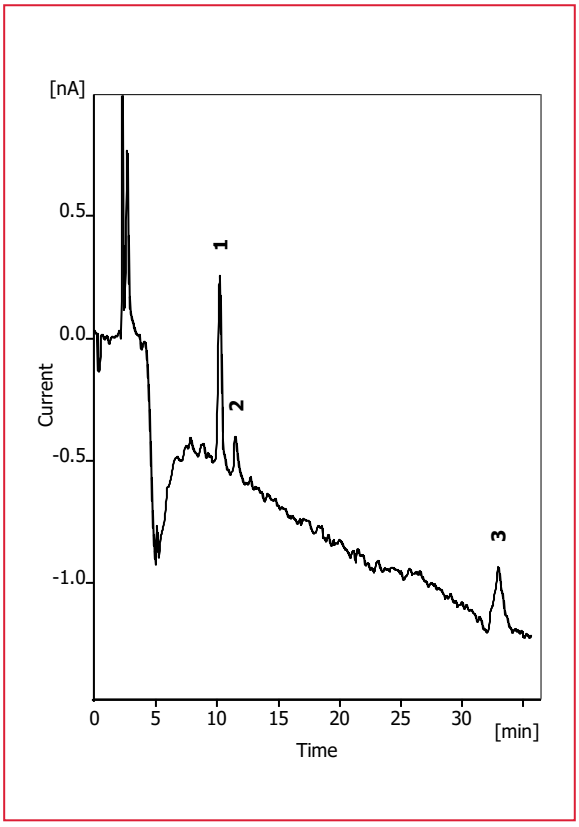


Figure 7: *Chromatogram from 0.3 µg/mL USP desosaminylazithromycin RS (1), USP N-demethylazithromycin RS (2) and USP azithromycin RS (3) in 46% acetonitrile (25 µL injection).*

Azithromycin for injection — Assay

The assay of azithromycin for injections as described in the USP 41 monograph is based on isocratic separation on an USP L67 phase (polymeric RP-C18 column) , followed by UV detection at a wavelength of λ = 215 nm. The LC conditions of the assay are listed in table 7.

Table 7

Conditions	
HPLC	ALEXYS Azithromycin analyzer + UVD 2.1L UV detector
Column	Shodex™ Asahipak-50 4D, 4.6 x 150 mm ID, 5 µm, USP L67
Mobile phase	52:48 (v/v) acetonitrile and 6.7 g/L KH_2PO_4 in DI water, adjusted to pH 11.0 ± 0.1 with KOH solution
Flow rate	1 mL/min
Temperature	40 °C for separation (column in DECADE Elite oven compartment)
$V_{injection}$	15 µL (partial loopfill)
Flow cell	10 mm optical path length, 1/16", 10 µL volume (pn A4061XB)
Wavelength	215 nm
Filter	1.0 sec (time constant)

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. In figure 8 an example chromatogram is shown obtained with the system suitability solution. The relative retention time for azaerythromycin A is 0.68, which corresponds to the value indicated in the monograph.

In the USP monograph for azithromycin for injection the following system suitability requirement are specified for the assay:

- *Resolution:* not less than 2.5 between the azaerythromycin A and azithromycin peak obtained with the SST solution.
- Tailing factor: not less than 0.9 and not more than 1.5 for the azithromycin peak obtained with the standard solution
- Relative standard deviation: Not more than 2% (n=5) for the azithromycin peak area of the peak obtained with the standard solution (see figure 9).

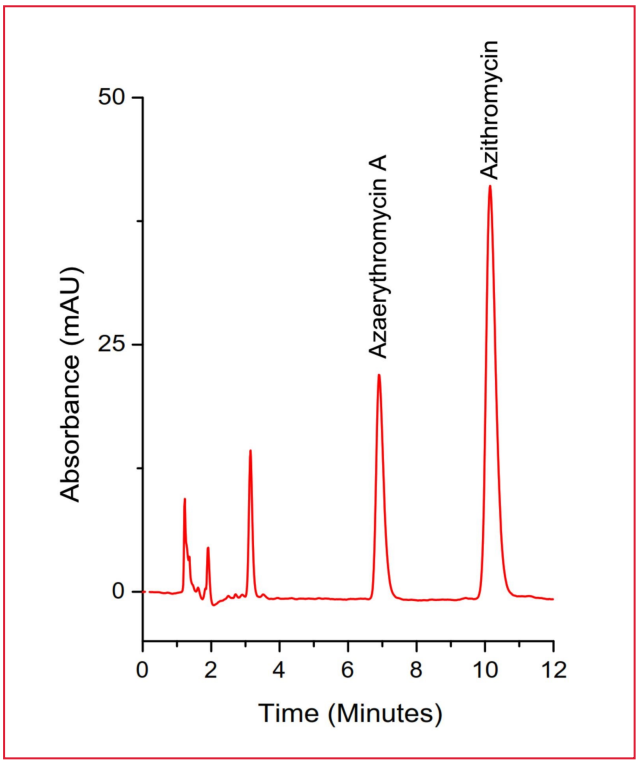


Fig. 8. *Example chromatogram of an 15 µL injection of the SST solution consisting of 1 mg/mL azaerythromycin and 1 mg/mL azithromycin in acetonitrile and DI water 52:48 (v/v).*

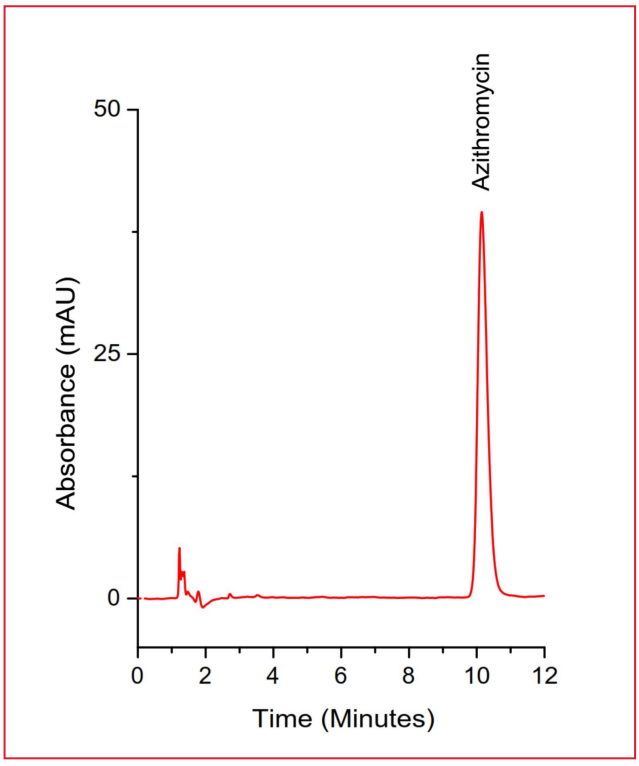


Fig. 9. *Example chromatogram of an 15 µL injection of the standard solution of 1 mg/mL azithromycin in acetonitrile and DI water 52:48 (v/v).*

In table 8 the results are shown based on chromatograms recorded with the SST and standard solution. The system suitability requirements are met for all parameters.

Table 8

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Resolution between azaerythromycin A and azithromycin	> 2.5	6.8
Tailing factor of azithromycin	0.9—1.5	1.3
Relative standard deviation (peak area) of azithromycin (*n=5*)	< 2.0%	0.8%

References

1. J. Sastre Toraño, H.-J. Guchelaar; *Journal of Chromatography B*, 720 (1998) 89-97
2. C. Taninaka, H. Ohtani, E. Hanada, H. Kotaki, H. Sato, T. Iga, *Journal of Chromatography B,* 738 (2000) 405–411
3. H. Toreson, B-M. Eriksson, *Journal of Chromatography B,* 673 (1995) 81-89
4. Official Monographs / Azithromycin, *United States Pharmacopoeia (USP)* **41**, (2018)
5. General Chapter <621> “Chromatography” in United States Pharmacopeia 41 National Formulary 35 (USP 40-NF 35), pp. 508-520.

Conclusion

The ALEXYS Azithromycin analyzer provides a suitable solution for the analysis of active pharmaceutical ingredient & impurities in commercial azithromycin formulations following the official methods described in the USP 41 monographs. If required, the system can be equipped with an optional UV detector enabling the possibility to run both the ECD as well as the UV methods in the monographs on the ALEXYS system.

Ordering information

180.0086W	ALEXYS Azithromycin Analyzer, including DECADE Elite DCC electrochemical detector and flow-cells
Azithromycin for Oral Suspension/Capsules—Assay **Azithromycin for injection— Impurities:** **Limit of azithromycin N-oxide (and others)**	
#ZR03-1546*	ZirChrom®-PBD 4.6x150 mm, 3 µm (ZirChrom), USP L49
Azithromycin for injection— Impurities: **Limit of aminoazithromycin (and others)**	
ODP-50G 4A** ODP-50 4E**	Shodex™ Asahipak ODP-50G 4A guard column, 4.6 x 10 mm Shodex™ Asahipak ODP-50 4E, 4.6 x 250 mm ID, 5 µm, USP L67
Azithromycin for injection— Assay	
187.ADA01XA#	UVD2.1L Detector with deuterium lamp, without flow cell
187.A4061XB#	Flow cell cartridge 10 mm path length, 10 µL, SST
ODP-50 4D**	Shodex™ Asahipak-50 4D, 4.6 x 150 mm ID, 5 µm, USP L67

#) UV detector and optical flow cell required for assay of Azithromycin for injection.
*) Manufactured and sold by ZirChrom Separations Inc., USA
**) Manufactured and sold under the brand name Shodex™ by Showa Denko K.K, Japan.

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