Application Note

Macrolide 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

Erythromycin ointments According to USP method

- **US Pharmacopoeia 43 NF 38 (2020)**
- **Topical and ophthalmic ointments**
- **Contents of Active Pharmaceutical Ingredients (API)**
- **ALEXYS analyzer with FlexCell**

Summary

Erythromycin is a macrolide antibiotic first isolated in 1952 from the bacteria *Saccharopolyspora erythraea* and is used for a variety of bacterial infections and acne [1]. For treatment of eye infections commercial erythromycin formulations are available as ophthalmic ointments. For the treatment of inflammatory acne topical ointments, gels or lotions are typically applied. To determine the contents of the API in formulations the US Pharmacopoeia published an assay for Erythromycin in ointments based on High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) [2].

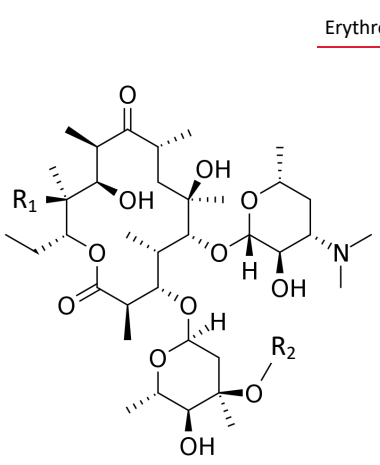
The assay for erythromycin in ointments was evaluated on an Antec ALEXYS Analyzer, using the exact method and conditions described in the official 2020 USP 43—NF38 monograph [3,4]. In this application note typical results obtained with the ALEXYS® Analyzer are reported, demonstrating its excellent performance for the routine analysis of erythromycin in pharmaceutical ointments.

Introduction

Erythromycin (figure 1) is an antibiotic in the class of antibiotics known as macrolide antibiotics, which also includes azithromycin and clarithromycin. Macrolides consist of a large macrocyclic (14-, 15- or 16-membered) lactone ring with one or more deoxy sugars [5]. They are useful in treating respiratory, skin, soft tissue, sexually transmitted, *H. pylori* and atypical mycobacterial infections. For treatment of eye infections commercial erythromycin formulations are available as ophthalmic ointments. For the treatment of inflammatory acne topical ointments, gels or lotions are typically applied. The erythromycin API used in formulations is typically composed of several related compounds, such as erythromycin A, B and C. Erythromycin A, the main constituent, has been found to have the most antibacterial activity, followed by erythromycin B. Erythromycins C has only half the activity of A.

The US Pharmacopoeia published an assay to determine the contents of the API in erythromycin ointments based on High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). In this assay the contents of the API is determined by quantification of all active ingredients (erythromycin A, B and C) and compared with the contents labelled on the pharmaceutical formulation for evaluation.

In this application note, the assay for erythromycin in ointments was evaluated with an ALEXYS Analyzer (figure 6), using the exact method and conditions as described in the official 2018 USP 41—NF36 monograph [3,4].



Erythromycin	R_1	R_2
A	OH	CH_3
B	H	CH_3
C	OH	H

Figure 1: structural formula of erythromycin A, B and C

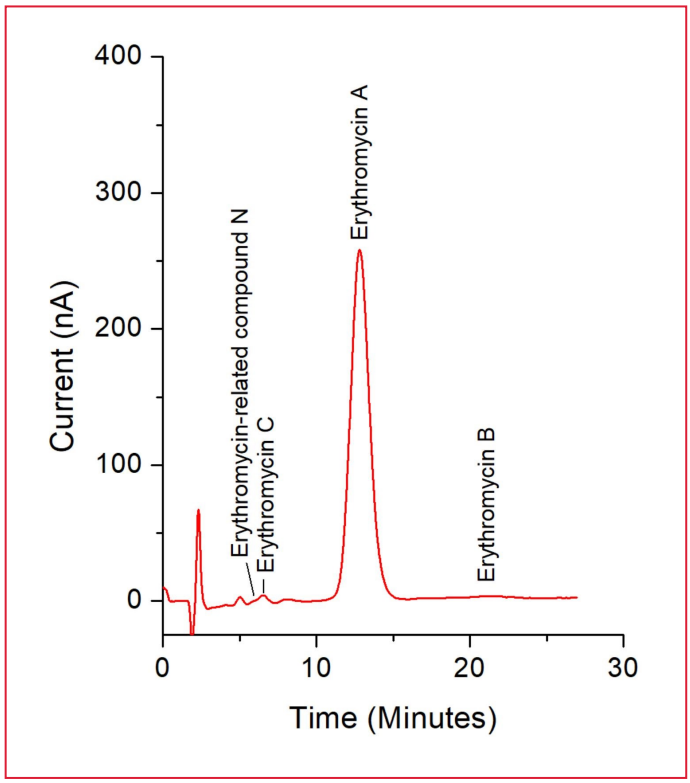


Figure 2: 10 µL injection of a worked up sample of a commercial erythromycin topical lotion (erythromycin content according to label: 10 mg/g).

Method

The assay described in the USP monograph is based on anion-exchange chromatography using isocratic separation, in combination with electrochemical detection of the analytes on a glassy carbon working electrode. The method details are listed in table 1 on the next page.

For the assay a column with USP L50 packing is required. The L50 phase is described as:

Multifunction resin with reverse-phase retention and strong anion-exchange functionalities. The resin consists of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, particles 3 to 15 µm in diameter, and a surface are of not less than 350 m^2 per g. Substrate is coated with quaternary ammonium functionalized latex particles consisting of styrene cross-linked with divinylbenzene.

A column matching this description was chosen for the method evaluation (Table 1).

Table 1

Conditions	
HPLC	ALEXYS Antibiotics base system - gradient
Column	Thermo Scientific™ OmniPac™ PAX-500 column, 50 x 4.0 + 250 x 4.0 mm ID, 8 µm (USP L50)
Solution A	Mixture of acetonitrile and water (90:10). Blanketed with Helium to keep it CO_2 free
Solution B	0.04 mg/mL of sodium hydroxide in water (carbonate-free)
Mobile phase	Solution A : Solution B, ratio 56:44%
Flow rate	1.0 mL/min (Isocratic)
$V_{injection}$	10 µL
Temperature	40 °C for separation and detection
Flow cell	FlexCell™ with GC WE and HyREF (Pd/H_2) RE, spacer: 130 µm
Potential waveform	E1, E2 : +0.9, -0.9V, ts, t1, t2: 0.1, 0.5, 0.1 s
Range	1 µA
I-cell	ca. 2 µA
ADF	0.01 Hz

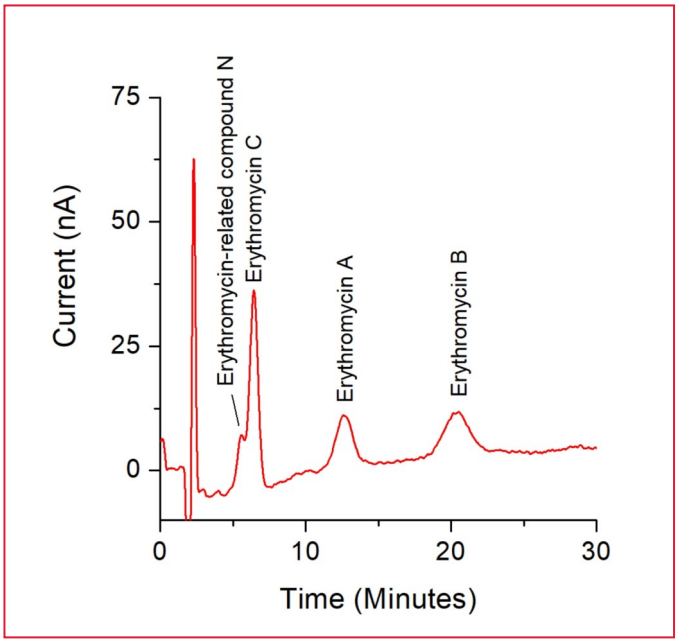


Figure 3: 10 µL injection of a standard consisting of 26.4 µg/mL erythromycin A , 32.6 µg/mL erythromycin B and C and 200 µg/mL erythromycin-related compound N in diluent (SST solution as described in the USP).

The mobile phase was prepared as described in the USP monograph (Table 1). Special care was taken to proportion the two solutions A and B into the LC system, using an LPG quaternary gradient pump, rather than preparing the mobile phase in a bottle by mixing the two solutions prior to introduction in the LC system. It is not advised by the column manufacturer to add the acetonitrile directly to the hydroxide solution [6]. The major concern is that at sufficiently high concentrations of acetonitrile in hydroxide, the acetonitrile will hydrolyze, thus forming acetate and ammonia. Another concern to be aware of is the limited solubility of hydroxide in acetonitrile.

A 2-step potential waveform was applied with the following settings E1 = +0.90 V, E2 = -0.90 V, t1 = 0.5 s, t2 = 0.1 s and ts = 100 ms. The cell current was typically about 2 µA with these PAD-mode settings. The short negative step of –0.90V is applied to clean and avoid contamination of the glassy carbon electrode surface. Both FlexCell (thin layer cell) and SenCell (wall-jet design) were tested during the method evaluation, but only the FlexCell gave sufficient response under the applied method conditions. A special 130 µm spacer was used in combination with the FlexCell.

All standards necessary to run the assay (SST, standard solution 1 and standard solution 2) were prepared as described in the monograph. The diluent for the standards is a mixture of methanol:water 50:50 v%. See table 2 for details about the chemicals and standards used in this evaluation.

Table 2

Reagents and standards	
Description	*Supplier, part number*
NaOH 50% in aq. solution Reag. Ph. Eur. 1081406 carbonate-free	VWR, pn 87938.290
Acetonitrile, Optima™ LC/MS Grade	Fisher Chemical, pn A955212
Deionized Water. >18 MΩ-cm, TOC<10 ppb	Barnstead, Easy pure II
n-Hexane 95+%, for HPLC	Acros , pn 232100010
Methanol, Ultra gradient HPLC grade 'Baker HPLC analyzed	J.T. Baker, pn 8402
Erythromycin (1 g)	Sigma Aldrich PHR1039-1G
Erythromycin B (100 mg)	USP Catalog # 1242010, 100 mg
Erythromycin C (50 mg)	USP Catalog # 1242021, 50 mg
Erythromycin Related Compound N (N-Demethylerythromycin A) (50 mg)	USP Catalog # 1242032, 50 mg

System suitability

In figure 3 an example chromatogram is shown of a 10 µL injection of a system suitability standard consisting of 26.4 µg/mL erythromycin A, 32.6 µg/mL erythromycin B and C and 200 µg/mL erythromycin-related compound N in diluent. The relative retention time for all four compounds correspond to the values indicated in the monograph (table 3).

In the USP monographs for erythromycin ointments, the following system suitability requirement are specified:

- *Resolution:* minimum 0.6 between Erythromycin-related compound N and erythromycin C; minimum 2.5 between erythromycin C and erythromycin B; minimum 2.5 between erythromycin A and erythromycin, obtained with the SST solution (see figure 3).
- Tailing: maximum 2.0 for erythromycin A, obtained with Standard solution 1 (see figure 4).
- Relative standard deviation: maximum 3% for erythromycin A, obtained with Standard solution 1 (see figure 4).

Table 4 shows the results based on chromatograms recorded with Standard solution 1 and the SST solution. The system suitability requirements are met for all parameters. It is evident from table 4 that the RSD's for erythromycin B and erythromycin C are significantly higher than for erythromycin A, because they are present in much lower concentrations in the sample. Moreover, the higher RSD's for erythromycin C are also the result of the close elution of erythromycin-related compound N, which makes quantification more difficult. Note that this is inherent to the separation method and conditions described by the USP, and reflected by the poor SST separation criteria (R_s > 0.6 between erythromycin-related compound N and erythromycin C).

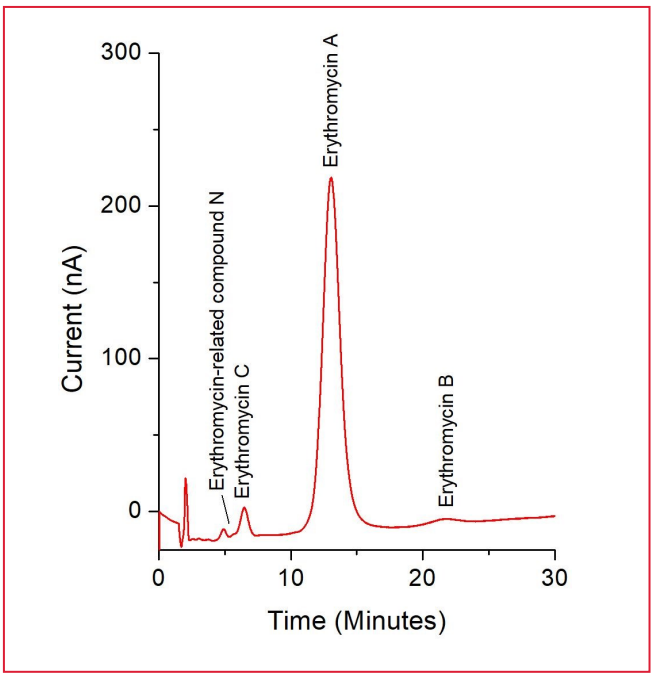


Figure 4: 10 µL injection of 0.66 mg/mL erythromycin in diluent (Standard solution 1 as described in USP monograph).

Table 3

Retention Time of erythromycin and related substances

Component	*Retention (min)*	*Relative Retention**
Erythromycin-related compound N	5.6	0.4
Erythromycin C	6.4	0.5
Erythromycin A	12.6	1.0
Erythromycin B	20.5	1.6

*) Relative retention time (RRT) with reference to erythromycin A (12.6 min).

Table 4

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Resolution, R_s		
R_s, Related compound N* - erythromycin C	> 0.6	0.8
R_s, Related compound C - erythromycin A	> 2.5	3.6
R_s, Related compound A - erythromycin B	> 2.5	2.9
Tailing factor, T_f		
T_f, erythromycin C	< 2	1.0
T_f, erythromycin A	< 2	1.1
T_f, erythromycin B	< 2	1.0
Relative Standard Deviation, RSD (%), n=6#		
$RSD_{Peak\ Area}$, erythromycin C	< 3%	2.8
$RSD_{Peak\ Area}$, erythromycin A	< 3%	0.4
$RSD_{Peak\ Area}$, erythromycin B	< 3%	1.9

*) Erythromycin-related compound N (N-Demethylerythromycin A).
#) Reported values are the average of multiple consecutive sets of 6 injections of standard solution 1.

For the stabile analysis of erythromycin it is absolutely essential that both eluents (solution A and B) are kept under a Helium atmosphere to prevent the formation of carbonate in the mobile phase due to dissolved CO_2 in alkaline media. Carbonate ions present in the mobile phase will interact with the stationary phase of an anion-exchange column, affect the selectivity, and lead to instable retention behavior (shifting retention times of all analytes). Proper conditioning of the column at installation, and regular regeneration of the column by procedures specified by the manufacturer [6] are important to maintain good separation performance of the column.

The LOD's for all compounds of interest were estimated using the SST solution. The detection limits found for erythromycin A, B and C were in the range of 0.5 - 2 µg/mL.

Sample analysis

A commercial 1% erythromycin topical lotion, was analyzed as an example of a relevant pharmaceutical product. The contents of erythromycin stated on the product label: 10 mg erythromycin/gram lotion. The erythromycin contents was verified by quantification of all active ingredients (erythromycin A, B and C) using the acceptance criteria described in the USP monograph.

Sample preparation consisted of the following steps:

- 6 gram of product, corresponding to a nominal amount of 60 mg API was transferred in a separation funnel filled with 50 mL hexane and shaken to dissolve.
- 4 separate portions of 20 mL diluent were used to extract the API from the hexane solution.
- The four fractions were collected in a 100 mL volumetric flask and brought to volume with diluent to 100 mL.
- 5 mL of the solution was filtered over a 0.2 µm Sartorius syringe filter.
- 10 µL of the filtered solution was injected in the LC system and analyzed.

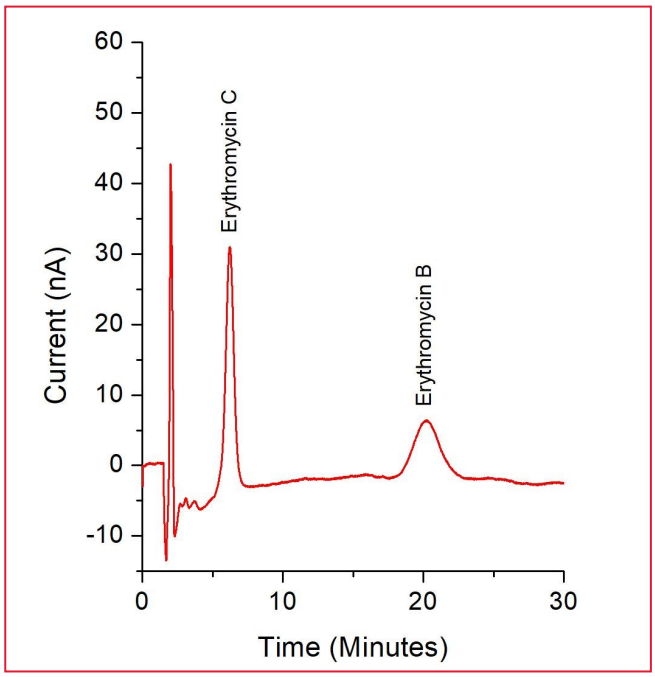


Figure 5: 10 µL injection of 34 µg/mL erythromycin B and 34 µg/mL erythromycin C in diluent (Standard solution 2 as described in USP monograph).

Figure 2 shows an example chromatogram obtained with the injected sample solution. It is evident from the chromatogram that erythromycin A is the main constituent in the lotion and erythromycin B and C are present in small amounts. Therefore, standard solution 2, containing a relatively low concentration of 34 µg/mL erythromycin B and C (figure 5), is used for their quantification. Standard solution 1 (figure 4) is used for the quantification of erythromycin A.

The percentage of erythromycin A relative to the labelled amount of erythromycin in the portion of the sample is calculated with this formula:

$$\text{Erythromycin A (\%)} = (r_u/r_s) \times (C_s/C_u) \times P \times 100$$

Where:

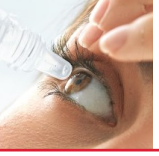
r_u = Peak area erythromycin A from the sample solution

r_s = Peak area erythromycin A from standard solution 1

C_s = Concentration of API in standard solution 1 (mg/mL)

C_u =Nominal concentration of API in sample solution (mg/mL)

P = potency of erythromycin A in standard (mg/mg)



The potency of the standard can be obtained from the data sheet or certificate of analysis of the standard. The relative percentages of erythromycin B and C are calculated in an identical way, only in this case the peak areas of standard solution 2 are used (r_s values) and the potency values from the corresponding erythromycin B and C standards. For details about the calculations refer to the official USP monographs [3,4].

The percentage relative to the labelled amount of erythromycin in the product is calculated by summing the percentages of erythromycin A, B and C. The USP acceptance criteria for percentage of the labelled amount of erythromycin is 90 - 120%. Table 4 shows the result for the analyzed sample.

Table 4

Percentage of API relative to the labelled amount

Component	*Percentage (%)**	*USP criteria*	*Result*
Erythromycin A	98.4		
Erythromycin B	0.7		
Erythromycin C	0.7		
Total	99.8	90—120%	Passed

*) Reported values are based on measurements of duplicate worked up samples. The relative standard deviation was ± 0.4% for the reported total, so 99.8 ± 0.4%

The commercial 1% erythromycin topical lotion met the acceptance criteria of the USP with respect to the amount of API in the product. The quantified amount of erythromycin in the product was in close correspondence with the labelled amount on the package

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References

1. Erythromycin, Wikipedia, https://en.wikipedia.org/wiki Erythromycin
2. W.R. LaCourse, *"Pulsed Electrochemical Detection in High Performance Liquid Chromatography"*, John Wiley & Sons, New York, 1ed,1997.
3. Erythromycin Ointment, *The United States Pharmacopoeia 43th ed., National Formulary 38th ed.*, United States Pharmacopeial Convention, Rockville MD, (2020),
4. Erythromycin Ophthalmic Ointment, The United States Pharmacopoeia 43th ed., National Formulary 38th ed., United States Pharmacopeial Convention, Rockville MD, (2020)
5. Macrolide, Wikipedia, https://en.wikipedia.org/wiki/ Macrolide
6. PAX-500 column product manual, Thermo Scientific, Document No. 034217, revision 9 (2003)

Conclusion

The ALEXYS analyzer for erythromycin based on the DECADE Elite detector in combination with the FlexCell with glassy carbon working electrode offers a suitable solution for the assay of erythromycin ointments as described in the USP 43 - NF38 monographs. The evaluation data in this application note demonstrate that all USP system suitability requirements were met and an example of a commercial pharmaceutical product could be successfully analyzed.

Figure 6: The ALEXYS analyzer for Erythromycin, consisting of the ALEXYS Antibiotics base system - Gradient, and dedicated flow cell and bottles. The base system consists of a LPG quaternary gradient P6.1L pump with integrated degasser an AS6.1L autosampler, an ET 210 Eluent tray for helium blanketing, and the DECADE Elite electrochemical detector. 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

Detector only	
176.0035A	DECADE Elite SCC electrochemical detector
102.4305	FlexCell GC HyREF
102.2218	Spacer for F/R cell, 130 µm
Recommended ALEXYS analyzer	
180.0056W	ALEXYS Antibiotics base system - Gradient
102.4305	Flexcell GC HyREF
102.2218	Spacer for F/R cell, 130 µm
184.0209	Glass bottle assembly, 1L, Helium
184.0205	PPCO bottle assembly, 2L, Helium

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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