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Development of an enhanced separation of erythromycin and its related substances by liquid chromatography

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Abstract

A new HPLC-UV method for the determination of the impurity profile of erythromycin is developed. In contrast to the liquid chromatography described in the European Pharmacopoeia the analysis could be performed at a temperature of 25 °C. Erythromycin samples were analysed on an endcapped RP phase with cyanopropyl groups on the surface using gradient elution with 32 mM potassium phosphate buffer pH 8.0 and acetonitrile/methanol (75:25). The aforementioned method shows clear improvements compared to the actual method of the European Pharmacopoeia, which is less selective and sensitive.

Keywords: HPLC; Erythromycin; European Pharmacopoeia

1. Introduction

Aminoglycoside, macrolide and polypeptide antibiotics are often complex mixtures of structurally related substances because of their biotechnological production. Depending on the fermentation conditions and purification procedures they are formed of one or a couple of main components accompanied by structurally similar minor components and impurities, which can be degradation products and by-products of the manufacturing process.

The macrolide antibiotic erythromycin A consists of a polyhydroxylactone skeleton connected to two sugars. The aglycone part of all erythromycin molecules, the erythronolide, is a 14-membered lactone ring. Depending on the type of erythromycin this lactone ring is substituted via 4-position with a cladinose in case of erythromycin A, erythromycin B, erythromycin E, erythromycin F, *N*-demethylerythromycin A, erythromycin A N-oxide and with a mycarose in case of erythromycin C and erythromycin D. All erythromycin molecules contain the aminosugar D-desosamine which is β-glycosidically linked to the 6-position of the lactone ring.

Erythromycin is composed of a main component, i.e. erythromycin A, and the minor components, i.e. EB, EC, ED, EE, EF, *N*-demethylerythromycin A (NdMeEA) and erythromycin A N-oxide (EANO) [1]. The European Pharmacopoeia 5.0 (Ph. Eur.) [2] defines the content of erythromycin as the sum of erythromycin A, B and C. The content of EB and EC is limited to 5% and the content of any other related substances to 3%.

Furthermore, acidic degradation products such as anhydroerythromycin A (AEA) and erythromycin A enol ether (EAEN) [3,4] and basic degradation products such as pseudoerythromycin A enol ether (PsEAEN) [5] can occur in varying amounts in erythromycin samples (see Fig. 1).

The current high performance liquid chromatography (HPLC) method [6] in the Ph. Eur. 5 utilizes a styrene–divinylbenzene copolymer as stationary phase at a column temperature of 70 °C. This method fraught with problems, e.g. solutions of erythromycin are instable at temperature above 60 °C [7]. Hence, it may be possible to detect degradation products that arise during analysis. The peak shape is insufficient for quantitative analysis of the minor components. Furthermore, some column manufacturers give a temperature limit for the polystyrene divinylbenzene copolymer of 60 °C [8] or 80 °C [9]; however one manufacturer declines a maximum

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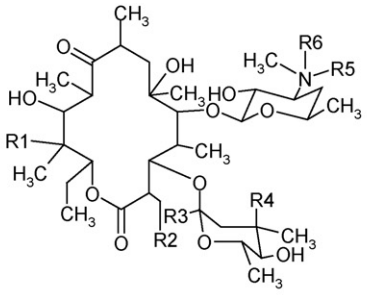
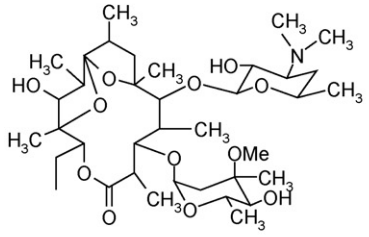
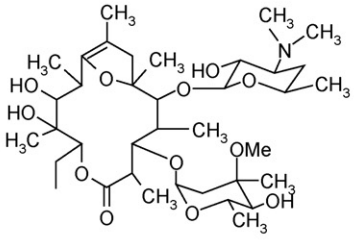


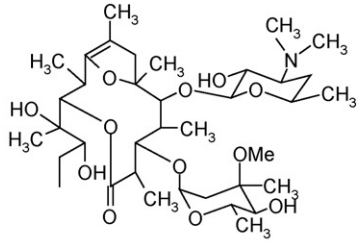
	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A	OH	H	H	OCH_3	CH_3	-
Erythromycin B	H	H	H	OCH_3	CH_3	-
Erythromycin C	OH	H	H	OH	CH_3	-
Erythromycin D	H	H	H	OH	CH_3	-
Erythromycin E	OH	-O-		OCH_3	CH_3	-
Erythromycin F	OH	OH	H	OCH_3	CH_3	-
N-demethylerythromycin A	OH	H	H	OCH_3	H	-
Erythromycin A N-oxide	OH	H	H	OCH_3	CH_3	O



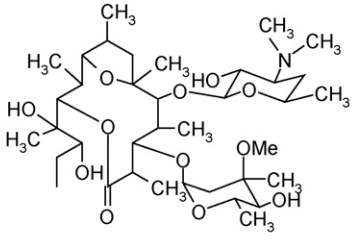
Anhydroerythromycin A



Erythromycin A enol ether



Pseudoerythromycin A enol ether



Pseudoerythromycin A hemiketal

Fig. 1. Structural formulae of erythromycin A, related substances and degradation products.

temperature of 150 °C [10]. So it would be difficult to find an appropriate column.

Beside the method described in the Ph. Eur. 5 ample work has been described for the analysis of erythromycin and related substances by HPLC. All of them exhibit some drawbacks, either the use of ion pair reagent in the mobile phase [11–13] which often leads to peak tailing, column switching [13,14], a pre-column derivatization procedure [15] or a three-step gradient of mobile phase which leads to baseline drift [16] or high temperature during separation [17]. Thus, we tried to develop a facile HPLC utilizing a direct determination of erythromycin which is of high robustness and selectivity.

2. Experimental

2.1. Chemicals and samples

All chemicals and reagents were of p.a. or HPLC grade. Dipotassium hydrogen phosphate was purchased from Sigma–Aldrich (Seelze, Germany), phosphoric acid 85% from Merck (Darmstadt, Germany), methanol and acetonitrile from FSA Laboratory Supplies (Loughborough, UK). A Milli-Q®-water purification system (Millipore Corporation, Bedford, MA, USA) was used throughout all procedures involving water. Erythromycin A, B, C and *N*-demethylerythromycin A CRS were obtained from the EDQM (Strasbourg, France) and anhy-

droerythromycin A, erythromycin A enolether and pseudoerythromycin A enolether were obtained from Ercros (Fyse, Spain).

A couple of commercial samples were provided from the Federal Institute for Drugs and Medical Devices (BfArM, Bonn, Germany) which contained the typical amounts of the impurities EA, EB, EC, ED, EE, EF and EANO as well as the degradation products. These samples were used as standards.

All samples were dissolved in a mixture of acetonitrile and water in a ratio of 60:40 to get a concentration of 20 mg/ml.

2.2. Apparatus

The liquid chromatography for erythromycin was performed on an Agilent System 1100 LC (Böblingen, Germany) consisting of a vacuum degasser, a binary pumping system forming a high pressure gradient by a static mixer (delay volume of 600–900 μl), an autosampler, a thermostated column compartment, an UV–visible diode array detector and a LC 3D ChemStation equipped with HP Kayak XM600 and 3DSoftware (Version 8.04).

2.3. Chromatography

As a stationary phase a Nucleodur CN-RP column (5 μm, 250 mm × 4.0 mm i.d.) (Macherey-Nagel, Düren, Germany) was used.

Gradient elution was applied using (A) 32 mM potassium phosphate buffer by dissolving 5.57 g dipotassium hydrogen phosphate in 1000 ml water adjusted with concentrated phosphoric acid to pH 8.0 and a mixture (B) of acetonitrile/methanol (75/25). Gradient was run with 33% B from 0 to 28 min and 33–45% B from 28 to 60 min, postrun with 33% B for 10 min.

After injection of 20 μl of the sample solution the HPLC system was operated at a flow rate of 1.0 ml/min. The column temperature was set at 25 °C and the detection wavelength at 215 nm.

3. Results and discussion

3.1. Development of the LC conditions

Because of the instability of erythromycin in acidic and alkaline solutions [18,19] samples were dissolved in a mixture of acetonitrile/water due to its stability in this media up to 168 h [20].

The character of the stationary phase is an important parameter that affects the selectivity in LC. Thus, different RP column materials like an acid/base deactivated C18 phase (Nucleosil-AB), a C12 phase with trimethylsilane endcapping (Max RP), a C18 phase with polar endcapping (Luna RP), and an endcapped nitrile phase (Nucleodur CN-RP) were tested for the method development in order to obtain the optimal separation.

The molarity of the phosphate buffer was examined in a range of 16–32 mM. At higher concentrations the buffer precipitates in the HPLC system using gradient elution because of the concentration of the amount of the organic solvent in the mobile phase. It was seen that an increasing buffer molarity improved the peak shape significantly and therefore, the highest possible buffer molarity of 32 mM was used in further development of the method.

In the next step, the influence of pH of the buffer in the mobile phase was tested. Below pH 7.0 the resolution of the components decreases rapidly with loss of selectivity. At pH 7.0 the components EC and NdMeEA as well as AEA, EA and EB, PsEAEN are not sufficiently separated using the described stationary phases. At pH 9 NdMeEA as well as AEA coelute with the main component. Therefore pH 8.0 was selected for further investigations because the separation was found to be the best.

Applying these conditions on a Nucleosil-AB column as well as on a Luna column NdMeEA coelutes with EC and the peak tailing of EA is not satisfactory. The application of a Max RP column decreases the resolution of the components. The best results could be achieved on a Nucleodur CN-RP column. Separation of EC and NdMeEA was optimized by adding increasing amounts of methanol to the mobile phase B. In order to enhance the resolution between PsEAEN and EB a gradient characterized by an increasing amount of acetonitrile/methanol was applied.

Finally, the best separation of all components of erythromycin was obtained on a Nucleodur CN-RP column (5 μm, 250 mm × 4.0 mm i.d.) using 32 mM potassium phosphate buffer at pH 8.0 as mobile phase A and acetonitrile/methanol (75:25) as mobile phase B. The gradient was run with 33% B from 0 to 28 min and 33–45% B from 28 to 60 min using a flow rate of 1.0 ml/min. A typical chromatogram of a spiked commercial sample is shown in Fig. 2.

3.2. Validation of the related substances

The method was validated according to ICH guidelines with respect to a limit test of the related substances of erythromycin [21]. The selectivity was verified by spiking a commercial erythromycin sample with related substances. With the aforementioned method all related substances could be baseline separated from the main component and from each other. Seven-point

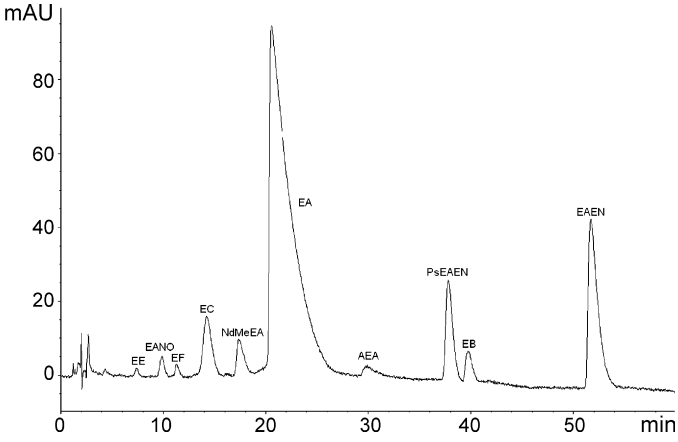


Fig. 2. Typical chromatogram of a spiked erythromycin sample. Stationary phase: Nucleodur CN-RP (5 μm, 250 mm × 4.0 mm i.d.), mobile phase: (A) 32 mM potassium phosphate pH 8.0, (B) acetonitrile/methanol (75:25), sample concentration: 20 mg/ml, gradient elution: 28 min 33% B, 28–60 min 33–45% B, flow rate: 1.0 ml/min, injection volume: 20 μl.

Table 1
Validation data of linearity and precision

Linearity	Range (mg/ml)	0.003–0.72
	Intercept	2.91
	Slope	0.04
Precision of the method ($n = 6$)	Standard deviation	0.39
	R.S.D. (%)	0.13
Run-to-run precision ($n = 6$)	R.S.D. (%)	0.20
Interday precision ($n = 6$)	Standard deviation	0.46
	R.S.D. (%)	0.15

The interday day precision was checked within 2 days.

calibration graphs ($r^2 > 0.998$) were employed in a range of 0.003–0.72 mg/ml for a solution of EA. The calibration standards were measured three times randomly. The precision of the method was determined by double measurements of six solutions containing 0.5% EA. The standard deviation was found to amount to 0.39. Run-to-run precision was controlled by six injections of a solution containing 0.5% EA. For checking the interday precision six solutions containing 0.5% EA were measured within 2 days. The precision data as well as further validation data summarized in Table 1 are satisfactory in all cases.

Next the robustness of the method was tested by varying critical parameters like column temperature, pH, flow rate and composition of the mobile phase considering the resolution of the peaks of an erythromycin solution containing 1 mg/ml in each case of EB, EC, NdMeEA, AEA EAEN and PsEAEN. Varying the column temperature (24, 25, 26 °C) as well as variation of the pH (7.9, 8.0, 8.1) of the mobile phase has no influence on the resolution of the peaks. In the case of varying the composition of the mobile phase, e.g. the variation of the amount of mobile phase B in the isocratic part of the method (31% B, 33% B, 35% B), the resolution of EC and NdMeEA decreases in a way that a baseline separation could not be guaranteed indicating that the method is not robust against the composition of the mobile phase. The same effect was observed by varying the flow rate (0.9, 1.0, 1.1 ml/min). The data of robustness with respect to resolution R_s are summarized in Table 2 and displayed in Fig. 3.

Hence, the method is not robust against variation of the composition of the mobile phase and the flow rate [22]. Therefore, a system suitability test has to be performed. Since the resolution between EC and NdMeEA is a critical parameter the method is only valid if the resolution is higher than 1.5.

The limits of detection (LODs) and limits of quantification (LOQs) of the related substances were estimated by means of the baseline noise method. The baseline noise was evaluated

Table 2
Robustness of the method based on the variation of the R_s factor between two adjacent peaks

Variation conditions	R_s EC/NdMeEA	Relative variation (%)	R_s NdMeEA/EA	Relative variation (%)	R_s EA/AEA	Relative variation (%)
Standard conditions	1.71	–	1.29	–	2.43	–
Flow rate						
0.9	1.97	15.20	1.37	6.20	2.50	2.88
1.1	1.22	−28.65	1.21	−6.20	2.36	−2.88
pH						
7.9	1.69	−1.17	1.28	−0.78	2.42	−0.41
8.1	1.74	1.75	1.30	0.78	2.48	2.06
Temperature						
24 °C	1.81	5.85	1.38	6.89	2.58	6.17
26 °C	1.65	−3.51	1.22	−5.43	2.35	−3.29
Mobile phase						
31% B	2.07	21.05	1.31	1.55	5.29	5.79
35% B	1.24	−27.49	1.22	−5.43	4.63	−1.24

Variation conditions	R_s AEA/PsEAEN	Relative variation (%)	R_s PsEAEN/EB	Relative variable (%)	R_s EB/EAEN	Relative variation (%)
Standard conditions	4.99	–	1.89	–	3.71	–
Flow rate						
0.9	5.22	4.60	1.98	4.76	3.90	5.12
1.1	4.69	−6.01	1.77	−6.35	3.59	−3.23
pH						
7.9	4.82	3.41	1.88	−0.53	3.70	−0.27
8.1	5.18	−3.81	1.95	3.17	3.74	0.81
Temperature						
24 °C	5.23	4.81	1.98	4.76	3.97	7.01
26 °C	4.91	−1.60	1.81	−4.23	3.60	−2.96
Mobile phase						
31% B	5.29	6.01	1.97	4.23	3.90	5.12
35% B	4.63	−7.21	1.79	−5.29	3.54	−4.58

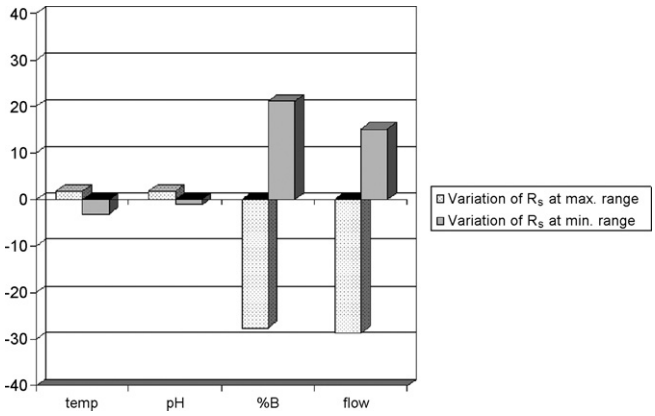


Fig. 3. Relative variation of the resolution factor (R_s). The bars are marked with temp. for temperature, % B for the composition of the mobile phase in the isocratic part and flow for flow rate.

by recording the detector response before and after the peak. The LODs for each compound determined as signal-to-noise ratios (S/N) higher than three and the LOQs for each compound determined as signal-to-noise ratios (S/N) higher than 10 data are shown in Table 3. The calculated limits of detection allow a limitation of all related substances less than stated in the EP namely in a range of 1%.

Table 3
Limit of detection of related substances and resolution factors of adjacent peaks in the order of increasing retention time

Substance	Resolution factor	LOD (μg/ml)	LOQ (μg/ml)
EC	–	4.43	130.77
NdMeEA	1.66	12.89	216.57
EA	1.20	–	–
AEA	1.86	11.91	404.54
PsEAEN	3.62	0.02	11.47
EB	1.91	2.79	188.96
EAEN	3.69	0.04	4.19

4. Quantification of some commercial lots

Quantification was performed using the normalization method in consideration of all occurring peaks except solvent peaks [23]. The content of a component was calculated as the percentage of the total area of the sum of all peaks.

By the use of a reference solution of EA CRS the content of impurities were calculated as described in the Ph. Eur. Using

Table 4
Quantification of some commercial samples of erythromycin base using the normalization method

	B13 (%)	B16 (%)	B19 (%)	B30 (%)	B34 (%)	B36 (%)	B42 (%)	B46 (%)	EP 5.0 (%)
EA	95.17	95.06	94.35	95.87	94.79	94.77	97.16	94.87	
EB	2.28	0.59	0.26	2.05	0.45	3.29	0.30	2.36	≤5.0
EC	1.64	1.69	2.84	1.39	3.61	1.62	1.70	1.82	≤5.0
Sum of the contents of EA, EB, EC	99.09	97.34	97.45	99.31	98.85	99.67	99.16	99.05	93.0–102.0
EE	0.36	0.32	0.75	0.36	0.44	0.23	0.24	0.21	≤3.0
EF	0.28	0.83	0.42	0.22	BLOQ	BLOQ	BLOQ	0.20	≤3.0
NdMeEA	0.22	0.76	0.57	BLOQ	0.26	BLOQ	0.09	0.21	≤3.0
EANO	BLOQ	0.66	0.80	BLOQ	0.36	BLOQ	0.49	BLOQ	≤3.0
AEA	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	≤3.0
EAEN	BLOQ	BLOQ	BLOQ	0.11	BLOQ	0.10	BLOQ	0.29	≤3.0
PsEAEN	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	≤3.0
Total impurity	0.86	2.57	2.54	0.69	1.06	0.33	0.82	0.91	≤7.0

Sample concentration: 20 mg/ml dissolved in a mixture of acetonitrile–water (60/40). Total impurity declares the sum of all related substances except EB and EC; BLOQ: below limit of quantification; disregard limit: 0.06%; correction factor for EAEN and PsEAEN: 0.07.

Table 5
Quantification of some commercial samples of erythromycin base using a reference solution of EA CRS as accomplished in the European Pharmacopoeia

	B13 (%)	B16 (%)	B19 (%)	B30 (%)	B34 (%)	B36 (%)	B42 (%)	B46 (%)	EP 5.0 (%)
EA	94.05	98.32	94.98	96.80	94.49	93.36	93.20	94.54	
EB	2.88	0.99	0.36	2.48	0.67	3.42	0.45	3.19	≤5.0
EC	1.62	1.97	2.85	1.58	3.98	1.79	1.77	1.91	≤5.0
Sum of the contents of EA, EB, EC	98.55	101.28	98.20	100.86	99.14	98.57	95.42	99.64	93.0–102.0
EE	0.35	0.46	0.72	0.52	0.51	0.30	0.31	0.26	≤3.0
EF	0.44	0.89	0.40	0.32	BLOQ	BLOQ	BLOQ	0.28	≤3.0
NdMeEA	0.27	0.70	0.70	BLOQ	0.36	BLOQ	0.12	0.27	≤3.0
EANO	BLOQ	0.98	0.92	BLOQ	0.51	BLOQ	0.64	BLOQ	≤3.0
AEA	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	≤3.0
EAEN	BLOQ	0.07	BLOQ	0.15	BLOQ	0.13	BLOQ	0.38	≤3.0
PsEAEN	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	≤3.0
Total impurity	1.06	3.03	2.74	0.99	1.38	0.43	1.07	1.19	≤7.0

Total impurity declares the sum of all related substances except EB and EC. Sample concentration: 20 mg/ml dissolved in a mixture of acetonitrile–water (60/40).

chromatograms obtained with this diluted reference solution the percentage of impurities were calculated (see Table 5). Compared to the results obtained with the normalization method the content of each impurity does not differ much; hence it is possible to utilize normalization method for the calculation of the content.

The content of EAEN and PsEAEN were corrected by a correction factor determined as 0.07.

Results of some lots collected from the European and American market are shown in Tables 4 and 5. Chepkwony et al. [17] describe the appearance of impurity ED. Since the impurity was not available as a reference compound we checked by means of a LC/MS-method whether ED is present in the lots collected from the markets. However, no ED could be detected.

Comparing the results in considerations of the LODs with contents given in the Ph. Eur. 5.0 the aforementioned method is more sensitive as the method described in the Ph. Eur. because the limit of detection of the components lies under the stated disregard limit. As the content of any other related substances does not exceed the limit of 0.9% (in more than 100 batches studied, data not shown) it would be useful to decrease the defined limit to 1.0%. Furthermore, no additional unknown impurities could also be detected in the batches studied.

5. Conclusion

In this study a method for the direct determination of the components and the impurity profile of erythromycin is described. It is able to separate and quantify all related substances with an acceptable precision. Since these lots were analysed using LC/MS the content of ED does not exceed 0.01%. So no ED could be detected. In consideration of the LODs of the related substances the method is more suitable as the described method in the Ph. Eur. to limit all impurities to 1%.

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