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Short communication

An interlaboratory study on the suitability of a gradient LC-UV method as a compendial method for the determination of erythromycin and its related substances

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ABSTRACT

A liquid chromatographic (LC) method for the analysis of erythromycin and related substances has been adapted from an isocratic method developed by Chepkwony et al. (2001) [1]. The suitability of the method for general application as a compendial (pharmacopoeia) method has been assessed by means of an interlaboratory (collaborative) study. The method involves LC separation on a XTerra C18 column kept at 65 °C and UV detection at 210 nm. Five laboratories, located in Europe and the United States (US), participated in the study. Four erythromycin samples were tested. The main components (erythromycin A (EA), erythromycin B (EB), erythromycin C (EC)) and the impurities were determined. The analysis of variance was carried out on the results of the five laboratories to evaluate the between-laboratory consistencies and the laboratory-sample interaction. The estimates for the repeatability and reproducibility of the method, expressed as relative standard deviation (RSD) of the result of the determination of EA, were calculated to be 0.8% and 1.4% respectively. It is concluded that the method examined is a good replacement for the methods currently described in the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopoeia (USP), especially for its enhanced selectivity.

1. Introduction

Erythromycin is a mixture of macrolide antibiotics produced by *Streptomyces erythreus* during fermentation [1,2]. In this process several related substances can be formed [3], those specified in the European Pharmacopoeia (Ph. Eur.) are: EB, EC, erythromycin F (EF), erythromycin E (EE), N-demethylerythromycin A (NdMeEA), anhydroerythromycin A (AEA), erythromycin A N-oxide (EANO), pseudoerythromycin A enol ether (PsEAEN) and erythromycin A enol ether (EAEN). Some of these impurities can also arise from degradation. In an acidic medium EAEN and AEA are formed [4], while PsEAEN is formed in an alkaline medium [5]. The assay method described in the Ph.Eur. involves the determination of the sum of the contents of EA, EB and EC. The structures of EA and its specified related substances are shown in Fig. 1. Several liquid chromatographic (LC) methods for the separation of erythromycin have been published. An isocratic LC method for the analysis of EA and related substances on a XTerra C18 column has been described previously [1]. Dehouck et al. already reported that the XTerra RP18 silica stationary phase showed better selectivity and efficiency than other columns used for the separation of erythromycin [4]. The method exhibits improved selectivity compared to the method currently prescribed by the Ph. Eur. [6] and the USP [7]. However, the total analysis time is quite long. In order to accelerate the elution, a gradient step has been added to the method and the modified method has been further investigated in a collaborative study to check its robustness and suitability to replace the current Ph. Eur. and USP method.

2. Experimental

2.1. Apparatus and columns

The protocol prescribed the use of a gradient method at a flow rate of 1.0 ml/min. A XTerra RP C18 column, 250 mm × 4.6 mm i.d.,

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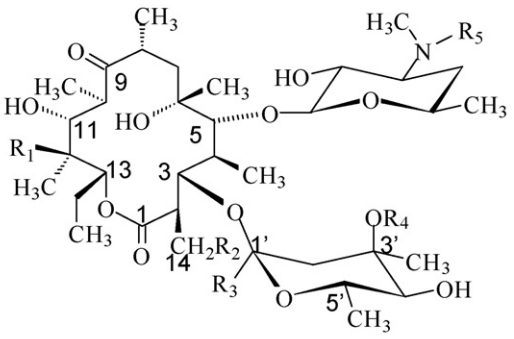
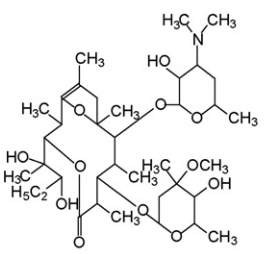
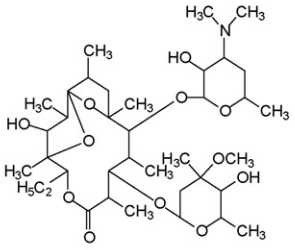


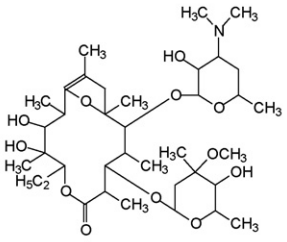
	R_1	R_2	R_3	R_4	R_5
Erythromycin A (EA)	OH	H	H	CH_3	CH_3
Erythromycin B (EB)	H	H	H	CH_3	CH_3
Erythromycin C (EC)	OH	H	H	H	CH_3
Erythromycin E (EE)	OH	-O-		CH_3	CH_3
Erythromycin F (EF)	OH	OH	H	CH_3	CH_3
***N*-demethylerythromycin A (NdMeEA)**	OH	H	H	CH_3	H



Pseudoerythromycin A enol ether (PsEAEN)



Anhydroerythromycin A (AEA)



Erythromycin A enol ether (EAEN)

Fig. 1. Chemical structures of erythromycin A and specified related substances.

3.5 μm (Waters Corporation, Milford, Massachusetts, USA), kept at 65 °C in a water bath or hot air oven, was used as stationary phase by all laboratories. The volume injected was 100 μl and the detection wavelength was set at 210 nm. The autosampler was set at 4 °C in order to maximize the stability of the solutions.

2.2. Mobile phase

Two mobile phases consisting of acetonitrile–0.2 M K_2HPO_4, pH 7.0–water were prepared: mobile phase A (35:5:60, v/v) and mobile phase B (50:5:45, v/v). The gradient programme used was: (0 till x): 100% A; (x till ($x+2$)): 100–0% A; (($x+2$) till ($x+9$)): 0% A (($x+9$) till ($x+10$)): 0–100% A; (($x+10$) till ($x+20$)): 100% A; where x is the retention time (min) of EB.

2.3. Samples, chemicals and solvents

Three erythromycin samples, each having different purity levels, were of commercial origin. Sample 4 was the proposed erythromycin chemical reference substance (CRS) 3 of the Ph. Eur. The reference substances employed were: EA Ph. Eur. CRS (purity: 96.7%), EB Ph. Eur. CRS (purity: 99.0%) and EC Ph. Eur. CRS (purity: 97.6%). The in-house prepared erythromycin for peak identification CRS (reference solution (d)) contained EA, EC, EB and the specified impurities: EF, NdMeEA, EE, AEA, PsEAEN and EAEN. All solvents and reagents complied with Ph. Eur. requirements [8].

2.4. Preparation of test and reference solutions

All samples were dissolved in a mixture of methanol–0.066 M K_2HPO_4, pH 8.0 (2:3, v/v).

The test solutions concentration was 4.0 mg/ml. Three reference solutions were used for the determination of EA and its impurities:

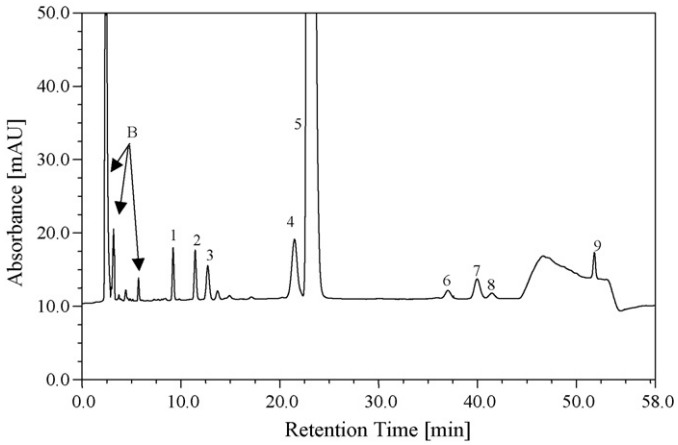


Fig. 2. Typical chromatogram of sample solution S3. B = blank peaks; 1 = EF; 2 = NdMeEA; 3 = EC; 4 = EE; 5 = EA; 6 = AEA; 7 = EB; 8 = PsEAEN; 9 = EAEN.

Table 1
General information on equipment and method performance results.

Laboratory	Column heating device	System suitability test						
		S_{EA}	R_s (NdMeEA–EC)	R_s (PsEAEN–EB)	P/V (EE–EA)	S/N_{EA}	Repeatability (%)[a]	t_R (EB)
1	Oven	1.8	2.4	1.6	2.8	54	0.17	34
2	Oven	1.0	1.8	1.5	12	348	0.55	38
3	Oven	1.6	2.8	1.5	26	154	0.05	39
4	IB	1.1	1.8	1.7	7	141	0.67	34
5	IB	1.9	2.8	1.4	2.2	135	0.65	40

IB = immersion bath; S_{EA} = symmetry factor of EA; R_s = resolution; P/V = peak-to-valley ratio; S/N_{EA} = signal-to-noise ratio of EA (reference solution (c)).
[a] Repeatability of the peak area of EA expressed as % relative standard deviation (RSD); t_R (EB) = retention time of EB.

the content of EA was determined against reference solution (a), containing 4.0 mg/ml EA CRS, the content of EB and EC against reference solution (b), containing 0.2 mg/ml EB CRS and 0.2 mg/ml EC CRS. Reference solution (c), a 1% (v/v) dilution of reference solution (a), was used for the content determination of the impurities. Both PsEAEN and EAEN show a higher UV absorbance than EA. This was taken into account in the calculations: the ratio between the responses of EA and PsEAEN and the ratio between the responses of EA and EAEN were both 0.08. AEA shows a lower UV absorbance than EA. The ratio between the responses of EA and AEA was 2. Reference solution (d) was used for system suitability testing (SST), for the identification of the impurities and for the adjustment of the gradient programme. Reference solution (c) was used to test the sensitivity of the detector by determination of the signal-to-noise ratio (S/N), which was calculated according to the Ph. Eur. specification [9].

2.5. Set-up of the study

The five laboratories enrolled were from authority, university and pharmaceutical industry and were located in Europe and the United States. Each laboratory tested the four erythromycin samples in duplicate under repeatability conditions. Each replicate consisted of an individual preparation of a sample solution which was injected three times. The content of EA, EB and EC, of the specified impurities: EF, NdMeEA, EE, AEA, PsEAEN and EAEN and of all unspecified impurities was determined using the results obtained with reference solutions (a), (b) and (c). The identification of the peaks was made by means of a typical chromatogram of reference solution (d) that was delivered to all participating laboratories along with the CRS.

3. Results and discussion

A typical chromatogram of sample S3 is shown in Fig. 2. The content of EA and all identified substances was calculated.

3.1. System suitability check and qualitative responses

The laboratories were required to equilibrate their LC system with the mobile phase before analysis. Chromatographic characteristics were calculated according to Chapter 2.2.29 "liquid chromatography" of the Ph. Eur. [9]. Table 1 includes information on the column, the conditions used and the results of the method performance tests carried out by each laboratory. Only when the prescribed requirements were met, the other solutions were prepared and analysed according to the order prescribed in the protocol. The symmetry factor S was calculated for the EA peak. The results varied between 1.0 and 1.9, i.e. outside the general range of 0.8–1.5 prescribed by the Ph. Eur. [9]. The resolution factor R_s was calculated for the peak pairs NdMeEA–EC and EB-PsEAEN. In the protocol a limit of 1.2 for both was set. If necessary, the acetonitrile content in the mobile phase and/or the gradient programme had to be adapted in order to obtain the required separation. All laboratories satisfied to the resolution requirements. The peak-to-valley ratio (P/V) of the peaks corresponding to EE and EA was also calculated. The values ranged from 2.2 to 26. Lab 3 tested the system suitability also at the end of the study. The values for the resolution did not change significantly, however the P/V ratio decreased from 26 to 10. The selectivity of the LC system was also evaluated by running reference solution (d), containing EA, EC, EB and the specified impurities: EF, NdMeEA, EE, AEA, PsEAEN and EAEN. All laboratories obtained separations similar to that shown in Fig. 2.

The signal-to-noise ratio (S/N) values varied between 54 and 348, corresponding to limits of quantification of 0.2% and 0.03% respectively. Therefore a disregard limit of 0.2% was applied in the determination of the impurities. This is higher than the disregard limit of 0.06% prescribed in the current monograph of the Ph. Eur. [6], but this lower value could not be attained. When checking the disregard limit (S/N = 10) prescribed in the method of the current monograph a value of 0.5% instead of 0.06% was obtained. With the here proposed method, performed in the same laboratory and with the same apparatus, a disregard limit of 0.07% was obtained.

The repeatability of injection was tested by injecting reference solution (a) six times and the relative standard deviation (RSD) of the peak area of EA complied with the general limit of 0.85%, prescribed in Chapter 2.2.46 of the Ph. Eur. [9].

3.2. Quantitative responses

The content of EA in the four samples was determined by comparing the peak area corrected for sample mass with the peak area corrected for sample mass and purity of EA in reference solution (a). Table 2 shows the mean values for EA. The content of EC and EB in the four samples was determined by comparing the corresponding area (corrected for sample mass) with the area (corrected for sample mass and purity) of EC or EB in reference solution (b). The content of all other impurities was expressed in terms of EA using reference solution (c). Table 3 shows the mean of mean values for the minor components EB and EC and for the impurities. The specified impurities EF, NdMeEA, EC and EE were found to be present in all four samples, while impurities EAEN and PsEAEN were found

Table 2
Mean values (%, m/m) for erythromycin A.

Laboratory	Samples			
	S1	S2	S3	S4
1	88.8 (0.8)	88.7 (0.5)	89.8 (1.0)	97.6 (0.6)
2	90.8 (0.4)	90.9 (0.3)	91.6 (0.5)	100.3 (0.5)
3	92.2 (0.4)	91.5 (0.5)	92.5 (0.7)	100.2 (0.5)
4	89.4 (1.7)	90.0 (0.4)	90.3 (0.6)	97.6 (0.6)
5	91.0 (1.2)	89.6 (0.4)	90.6 (0.4)	98.8 (2.4)
Mean of means	90.5	90.1	91.0	99.2
RSD %	1.5	1.2	1.2	1.3

RSD values (%, $n = 6$) within the laboratories are given in parentheses.

Table 3
Mean of mean values (%) for related substances.

Sample	EF	NdMeEA	EC	EE	AEA[a]	EB	Sum of other impurities <1%
S1	1.02 (3)	0.76 (24)	1.80 (11)	2.02 (8)	0.46 (21)	1.16 (3)	1.04 (41)
S2	0.48 (3)	0.87 (7)	3.27 (2)	0.71 (16)	0.30 (22)	1.03 (3)	0.61 (22)
S3	0.60 (2)	0.73 (7)	0.69 (4)	2.22 (8)	0.48 (15)	0.97 (4)	0.22[b]
S4	0.51 (6)	0.70 (18)	0.27 (48)	0.30 (32)	0.36 (23)	–[c]	0.45 (29)

RSD (%) between the five laboratories are given in parentheses. Content of impurities was calculated based on a disregard limit of 0.20%. The last column shows the sum of all other detectable peaks above the disregard limit.

[a] Not detected in Lab 1, samples S1, S2, S3 and S4.

[b] Only ≥0.20% in Lab 4.

[c] Not detected in Lab 1 and ≤0.20% for Lab 2, 3, 4 and 5.

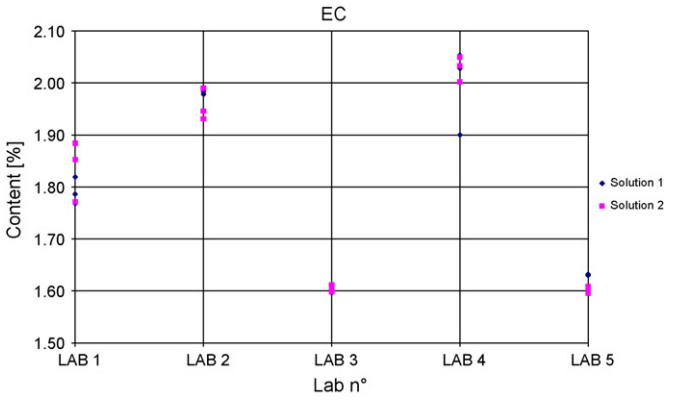


Fig. 3. The contents (%) of six injections of EC (two solutions, three injections each).

below the disregard limit in all four samples. AEA was not detected by Lab 1 in any of the four samples and EB was not detected by the same laboratory in sample S4. The other detectable impurities were all below 1.0% and those above the disregard limit of 0.2% were summed and the values for each sample are given in the last column of Table 3. Analysis of variance (ANOVA) was performed on the calculated contents of EA and its related substances for the five collaborating laboratories. The results of the ANOVA test showed that the between-laboratory variance was significant at the 5% level. The variation within the laboratories was significantly lower. This is illustrated in Fig. 3, where the content (%) of EC of six analyses is plotted for the five different laboratories. As can be seen, the within-laboratory variation is much smaller than the between-laboratory variation. This explains why ANOVA indicates a significant between-laboratory variance. From a pharmaceutical point of view, these statistically significant differences might not be relevant in daily practice since all impurities fall well within the prescribed limits. The estimates for the repeatability and reproducibility, expressed as RSD, were 0.8% and 1.4% respectively [10]. These values are similar to the values calculated for the actual Ph. Eur. method as reported by Paesen et al. [11].

Several causes can be considered for the higher variation between the laboratories. First, the integration of each impurity peak depends on the resolution of the peaks. If the peaks are not baseline separated, different integration methods of neighboring peaks may lead to different results. On the other hand, possible differences in humidity between laboratories could result in differences in moisture absorption during sampling and explain part of the between-laboratory variation. Also, for each of the four samples when comparing three injections of the same solution, the results for AEA showed a response increase of 0.1–0.16% over 36 h, which were needed to carry out the study. Notwithstanding the fact that the autosampler was set at 4 °C, as prescribed in the protocol, this increase was observed in all the collaborating laboratories except for Lab 1, where AEA was not detected in any of the four samples.

The enol structures are detected more easily due to their higher absorbance. This explains why a correction factor of 0.08 is used for EAEN and PsEAEN. For EAEN the sensitivity is even higher because it elutes during the gradient, which makes the peak sharper. However, the content of these enol ethers was in all cases below the disregard limit of 0.2%, even before correction.

4. Conclusions

All laboratories achieved a good selectivity allowing the determination of EA and its related substances. Due to small within-laboratory variance, observed for EA and its related substances, ANOVA showed a significant between-laboratory variance. The laboratory-sample interaction variance was not significant. The estimates for the repeatability and reproducibility, expressed as RSD, were 0.8% and 1.4% respectively. Based on the present study it can be concluded that the method examined is suitable to replace the existing official Ph. Eur. and USP methods.

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