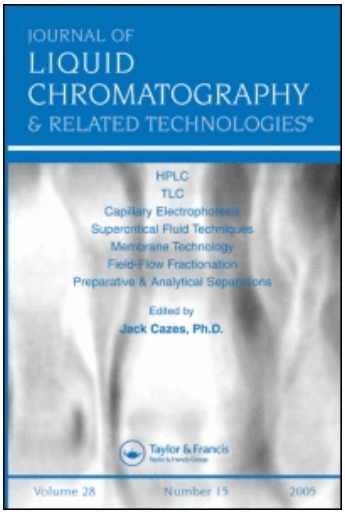

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COLLABORATIVE STUDY OF THE ANALYSIS OF ERYTHROMYCIN BY LIQUID CHROMATOGRAPHY ON WIDE-PORE POLY(STYRENE-DIVINYLBENZENE)

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ABSTRACT

A previously established method for analysis of erythromycin and related substances by liquid chromatography using a wide-pore poly(styrene-divinylbenzene) stationary phase was examined in a multicentre study involving 6 laboratories and a total of 7 columns. Three erythromycin samples were analysed. The main component and the impurities were determined. An analysis of variance treating each column as a different laboratory, showed absence of consistent laboratory bias and of laboratory-sample interaction. Estimates for the repeata-

bility and reproducibility of the method, expressed as relative standard deviation (RSD) of the result of the determination of erythromycin A, were calculated to be 1.1 % and 1.3 % respectively.

INTRODUCTION

Erythromycin is a mixture of macrolide antibiotics produced by fermentation, of which erythromycin A (EA) is the main component. During fermentation several related substances can be formed : erythromycin B (EB), EC, ED, EF, EE, N-demethylerythromycin A (dMeEA), anhydroerythromycin A (AEA), erythromycin A N-oxide (EANO), pseudoerythromycin A enol ether (psEAEN) and erythromycin A enol ether (EAEN). In acidic medium EAEN and AEA are formed[1], while psEAEN and pseudoerythromycin A hemiketal (psEAHK) are formed in alkaline medium [2]. Structures of these compounds were shown elsewhere [3].

A liquid chromatographic (LC) method for analysis of erythromycin A and related substances on poly(styrene-divinylbenzene) (1000 Å) has been described previously [3]. The suitability of this method for general application as a pharmacopoeial method was examined in this study, which was carried out on seven columns, in six laboratories, using three samples.

EXPERIMENTAL

Apparatus and Columns

The protocol prescribed the use of a pump for isocratic delivery of the mobile phase at a flow rate of 2.0 ml/min. The equipment further consisted of a fixed loop injector with a loop of 100 µl, a column heating

device maintained at 70 °C (a water bath was preferable), a UV detector set at 215 nm and an integrator allowing peak area measurements. The collaborators were asked to use 25 x 0.46 cm i.d. columns, packed with poly(styrene-divinylbenzene) (PSDVB), 5 to 10 μm, 1000 Å. Laboratory packed columns were packed with PLRP-S, 8 μm, 1000 Å from Polymer Laboratories, Church Stretton, Shropshire, UK. Prepacked columns were purchased from different distributors of Polymer Laboratories.

Mobile phase

Liquid chromatographic grade or distilled 2-methyl-2-propanol (165 ml) and LC grade acetonitrile (30 ml) were added to a mixture of 50 ml of 0.2 M potassium phosphate buffer pH 9.0 and 755 ml distilled water. The mobile phase was degassed by ultrasonication or by another suitable method. The collaborators were asked to adjust, if necessary, the 2-methyl-2-propanol content in order to reach the requirements for resolution.

Samples, Chemicals and Solvents

The reference sample (E-R) used was the European Pharmacopoeia Chemical Reference Substance (Ph. Eur. CRS) Batch n° 3, to which a content of 89.0 % m/m EA was assigned [3]. It should be noted that the official potency of the Ph. Eur. CRS is expressed in IU/mg. A reference substance of dMeEA was available, which was used to determine the resolution between dMeEA and EA. Reference samples for the related substances were not used. The content of related impurities was expressed in terms of EA and calculated with reference to a 1 % dilution of the

E-R reference solution. The samples to be examined (E-S1, E-S2, E-S3) were of commercial origin. Chemicals complied with Ph. Eur. requirements [4].

A mixture (1:1) of methanol and 0.2 M potassium phosphate buffer pH 7.0 was used to dissolve the samples. For quantitative analysis solutions were prepared containing 4.0 mg/ml of erythromycin. If an autosampler was used, cooling of the sample vials to 5 °C had to be employed.

RESULTS AND DISCUSSION

Equipment and Method Performance

In all, seven columns were used in 6 laboratories. A typical chromatogram is shown in Fig. 1. Table 1 includes information regarding columns, conditions used and results of performance checks carried out by each laboratory. One laboratory used home-packed columns, the others used commercially purchased columns. In laboratory 6 a new column as well as a column used previously were tested. The amount of 2-methyl-2-propanol was practically the same in all laboratories. The flow rate prescribed in the protocol was 2.0 ml/min. Two collaborators used 1.0 ml/min. Laboratory 2 did so because of baseline noise problems encountered at a higher flow rate. The relation between flow-rate of heated mobile phase and baseline noise has been discussed elsewhere [5]. This noise is generally dependent on the type of detector and is probably due to poor heat-exchange efficiency. This may be solved by using another detector or by using longer tubing between column and detector or by reducing the flow rate. Laboratory 3 obtained high backpressure at 2.0 ml/min as well as high base-line noise and therefore

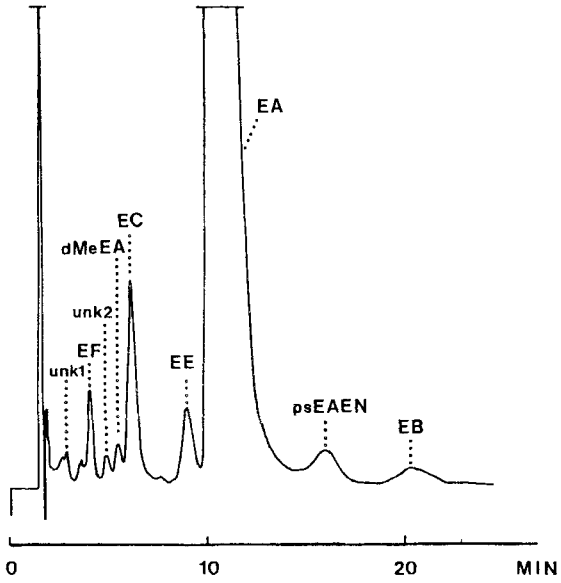

Figure 1. Typical chromatogram of sample E-S1, obtained in laboratory 5.

decided to use 1.0 ml/min. Probably this high backpressure was due to local technical problems, since the same laboratory also reported backpressure problems in another collaborative study, using the same column. In addition, no other participants mentioned a backpressure problem. Backpressure in the conditions prescribed was normally about 130 bar. The sensitivity was also checked. In this test the ratio was calculated of the peak height of EA on injection of a solution containing 0.02 EA mg/ml to the noise level. All laboratories complied with the required minimal ratio of 3.

Chromatographic characteristics were calculated according to the monograph "liquid chromatography" of the

TABLE 1
General Information on Equipment and Method Performance

Laboratory	Stationary phase PLRP-S 1000 Å, 5 μm	% v/v 2-methyl-2-propanol in mobile phase	Column heating device	Flow rate (ml/min)	Sensitivity	S	R_s	Repeatability (n=5) Peak area RSD %		Retention time EA		Linearity r EA
						EA	dMeEA-EA	EA	EE	Mean	RSD%	
1	prepacked	16.5	immersion bath	2	20	1.4	7.2	0.3	2.2	12.53	0.4	0.9965
2	prepacked	17.4	oven	1	6.6	1.75	7.8	0.4	5.6	24.63	0.9	0.9910
3	prepacked	16.9	oven	1	3.6	1.2	4.4	0.3	6.3	24.00	0.1	1.000
4	prepacked	16.5	immersion bath	2	14	1.0	6.3	4.1	10.6	9.07	1.5	0.9998
5	prepacked	17.5	oven	2	6.0	1.7	5.8	0.2	3.0	10.58	0.15	0.9985
6a	home-packed	16.5	immersion bath	2	5.2	1.9	7.4	0.85	7.0	11.9	0.1	0.9980
6b	home-packed	16.5	immersion bath	2	5.0	2.4	6.4	0.9	5.2	12.43	0.2	0.9912

S = symmetry factor; R_s = resolution; RSD = relative standard deviation; r = coefficient of correlation.
a = new column; b = used column.

Ph. Eur. [6]. The symmetry factor S was calculated for the EA peak. The results varied between 1.0 and 2.4, the latter value being obtained with a column that had been intensively used for 4 months. The home-packed columns in laboratory 6 gave the highest values.

The resolution R_s was calculated for the pair dMeEA and EA. In the protocol a limit of 5.5 was put on this resolution. Collaborators were asked to adjust the 2-methyl-2-propanol content of the mobile phase in order to improve the resolution. Laboratory 3 did not reach this resolution limit but continued the study without contacting the organizing laboratory. This laboratory was using a hot air oven to heat the column. Problems which can be encountered with a hot air oven have been discussed in detail elsewhere [7]. Inadequate heating of the mobile phase, as can occur in a hot air oven, causes loss of resolution. The problem may be overcome by using an immersion bath, assuring faster heat-exchange. Laboratory 1, facing the same problem of resolution using a hot-air oven, replaced the hot-air oven by an immersion bath and the required resolution was achieved. Indeed, it is believed that insufficient heating of the mobile phase entering the column causes a temperature gradient inside the column, which reduces the resolution. Laboratory 5, also facing the same resolution problem, contacted the organizing laboratory and after advice was given, the resolution was improved by preheating of the mobile phase. Another laboratory, also using a hot air oven, reported unability to maintain adequate temperature control at 2.0 ml/min. and interrupted the study. A waterbath was not available in this laboratory. When the column, used in this laboratory, was checked in the organizing laboratory, normal results were obtained. This laboratory has been excluded as a participating laboratory.

TABLE 2
Individual Results (% m/m) for Erythromycin A

Laboratory	Sample											
	E-S1				E-S2				E-S3			
1	85.19	85.03	87.06	88.13	89.82	90.10	92.36	91.38	85.52	84.85	83.71	85.82
2	88.35	87.31	86.68	85.54	91.03	89.39	91.62	89.26	83.69	84.56	84.69	82.92
3	81.69	84.03	92.94	87.70	85.96	89.88	89.09	90.81	75.27	84.83	86.97	83.83
4	88.26	89.55	86.35	88.99	91.41	91.22	90.02	91.65	85.48	88.10	85.99	87.46
5	88.86	86.61	86.79	87.85	93.45	90.31	90.63	90.47	85.58	84.66	83.85	83.87
6a	86.04	86.24	86.31	86.85	90.25	90.24	89.78	89.86	82.74	83.87	83.39	85.45
6b	86.41	88.39	87.22	85.90	90.30	92.77	90.89	90.38	83.54	83.87	83.78	83.69

a = new colum; b = used column

The repeatability, expressed as the relative standard deviation (RSD, %) was calculated for five consecutive injections of the same solution of E-R. The required RSD values for the peak area of EA and EE were ≤ 2 % and ≤ 25 % respectively. Laboratory 4 reported higher RSD values, which might be partly due to the mode of integration of the EE and EA peaks. Indeed, on the chromatograms of laboratory 4 it was observed that the small EE peak, which was eluted immediately before the EA peak and the EA peak itself were both integrated in the "baseline-baseline" mode, thus cutting off the major part of the EE peak and a small part of the EA peak. Better integration modes are "baseline-valley" for the EE peak and "tailing peak" for the EA peak, so the peaks are completely integrated in a more repeatable way. The repeatability of the retention time is a measure of the quality of the pump system used, while the linearity is a measure of the quality of the detection system. The correlation coefficient r was calculated for a regression curve determined over the range 350-450 μg of E-R injected, corresponding to 88 %-112 % of the prescribed served : the repeatability in Table 3 was much better than that in Table 1. It should be emphasized that in spite of the reported technical problems with the heating, the flow rate and the integration of the peaks, the means of means have RSD values which are remarkably low. These values are similar to those obtained in comparable collaborative studies on tetracyclines [8, 9]. This is an indication for the method being robust, although technically not easy to perform.

Table 4 shows means of mean values for the impurities. The content of impurities is expressed in terms of EA. Variation on the content of the impurities eluted before the main peak is rather high, which can be explained by the complex composition of the samples and

TABLE 3
Mean Values (% m/m) for Erythromycin A

Laboratory	Samples		
	E-S1	E-S2	E-S3
1	86.4 (1.7)	90.9 (1.3)	85.0 (1.1)
2	87.0 (1.4)	90.3 (1.3)	84.2 (1.0)
3	86.6 (5.6)	88.9 (2.4)	82.7 (6.2)
4	88.3 (1.6)	91.1 (0.8)	86.8 (1.4)
5	87.5 (1.2)	91.2 (1.6)	84.5 (1.0)
6a	86.4 (0.4)	90.0 (0.3)	83.9 (1.4)
6b	87.0 (1.2)	91.1 (1.3)	83.7 (0.2)
Mean of means	87.0	90.5	84.8
RSD %	0.8	0.9	1.3

Relative standard deviations (RSD %) are given in parentheses.

also by the poor specific absorbance combined with the rather low content of most of the impurities. As expected, RSD values improve as the content of the impurity increases, as for e.g. EC.

The amount of EE reported by laboratory 4 was about five times less than that found by the other laboratories. This can be explained by the "baseline-baseline" integration mode used in this laboratory, already mentioned above. This explains the high RSD value for EE in Table 4. psEAEN was not detected by laboratory 3 in samples E-S1 and E-S3. The same laboratory was not able to integrate EB in sample E-S2. This is in concordance with the larger noise obtained by this

TABLE 4
Mean of Mean Values (%) for Related Subsances

Sample	UNK1	EF	UNK2[a]	dMeEA[b]	EC	EE	psEAEN[c]	EB[d]
E-S1	0.25 (60)	0.8 (43)	ND	0.3 (37)	3.6 (20)	1.3 (42)	1.0 (34)	0.9 (35)
E-S2	ND	0.4 (61)	0.4 (30)	0.3 (41)	1.7 (18)	1.2 (41)	0.4 (20)	0.7 (22)
E-S3	ND	0.5 (11)	ND	0.4 (35)	1.8 (16)	1.1 (44)	0.3 (45)	4.0 (17)
E-R	ND	0.5 (62)	0.3 (40)	0.3 (38)	1.6 (21)	1.2 (46)	0.4 (97)	0.6 (49)

ND = not detected. RSD (%) are given in parentheses.
[a] not separated from EF in lab 3; [b] not separated from EC in lab 3, sample E-S3; [c] not detected in lab 3, samples E-S1 and E-S3; [d] not properly integrated in lab 3, sample E-S2.

laboratory (Table 1) and with the lower sensitivity due to the lower flow rate. Laboratory 2 which also operated at a flow rate of 1.0 ml/ml had no detection problems, probably due to the lower noise (Table 1). Concerning the results for psEAEN, it should be emphasized that the specific absorbance of enol ether structures is more than 10 times higher than for other erythromycins. This means amount to be analysed.

Analysis of Samples

Samples were analysed four times, using four separately prepared solutions. Individual results for the main compound, expressed as % m/m EA, relative to the EA content assigned to E-R, are reported in Table 2. Means and RSD values for EA are given in Table 3. In laboratory 3 the variation between replicates was higher than in other laboratories. It is not clear why, in this laboratory, the RSD values for the samples were much higher than that obtained for the repeatability test in Table 1. In laboratory 4 the opposite situation was ob- that values for psEAEN, expressed as EA are greatly overestimated.

From the above-mentioned technical problems it might be deduced that this LC method should be performed at a lower flow rate. Although this would eliminate most of the technical problems and improve the quality of the separation it should not be forgotten that this would not only increase the retention time but also decrease the sensitivity of the method especially towards the peaks eluted after EA. Laboratory 3, working at 1 ml/min, had some difficulties with the detection of EB. EB is an antibiotically active component and the determination of its content might be required as part of the assay.

Analysis of Variance

In order to further analyse the results obtained for the main component, a number of statistical calculations was performed following described procedures [10,11]. To facilitate these calculations the two columns used in laboratory 6 (6a and 6b) were considered as two separate laboratories. The results were first examined for outliers. The means were ranked for outlying laboratories [11]. The ranked mean values were also examined for outlying mean values by using Dixon's criterion [11]. Following the calculations of these statistical parameters laboratory 3 was close to the limits leading to elimination. This was also the laboratory showing much variation on the replicate results for EA (see Table 3). An analysis of variance was carried out to search for consistent laboratory bias or significant laboratory-sample interaction [10]. The analysis of variance was first performed using the results of all the collaborating laboratories (see Table 5). The variation between replicates was not homogeneous when the results of all the laboratories were included. In Table 5 it is seen that the variance between replicates (3.57) is high and that the between laboratory variation is significant at the 1 % level. Estimates of the repeatability (within laboratory variance) and of the reproducibility (between laboratory variance) were calculated [10]. The RSD values were 1.89 and 1.90 respectively. This can be explained by the fact that the variation was mainly due to the replicate error, so that the reproducibility was the same as the repeatability. After elimination of laboratory 3 using the test for homogeneity of the variation between replicates (ref. 10, p. 77), the variance was homogeneous. An analysis of variance was carried out using the results of the 6 remaining laboratories (Table

TABLE 5
Analysis of Variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
7 laboratories				
Between labs (L)	48.20	6	8.03	L/LS = 5.28 F_{99} (6,12) = 4.82
Laboratory-sample interaction (LS)	18.27	12	1.52	
Between replicates (S)	224.64	63	3.57	LS/L = 0.43 F_{99} (12,63) < 2.50 > 2.34
6 laboratories				
Between labs (L)	27.73	5	5.55	L/LS = 3.99 F_{99} (5,10) = 5.64 F_{95} (5,10) = 3.33
Laboratory-sample interaction (LS)	13.90	10	1.39	LS/L = 1.25 F_{99} (10,54) < 2.08 > 1.99
Between replicates (S)	59.99	54	1.11	

5). There was no significant between laboratory variance at the 1 % level, but there was at the 5 % level. The laboratory-sample interaction variance was not significant. Estimates for the repeatability and the reproducibility, expressed in RSD, were 1.1 % and 1.3 % respectively. These values are very satisfactory for a chromatographic method, using detection at 215 nm.

CONCLUSION

It can be concluded that the LC method is suitable for purity control and assay of erythromycin. However, to operate the system at 2.0 ml/min, more technical skills are required than at 1.0 ml/min, when the analysis time is longer and the sensitivity may be lower.

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