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Interlaboratory study of a liquid chromatography method for erythromycin: determination of uncertainty

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Abstract

Erythromycin is a mixture of macrolide antibiotics produced by *Saccharopolyspora erythreas* during fermentation. A new method for the analysis of erythromycin by liquid chromatography has previously been developed. It makes use of an Astec C_{18} polymeric column. After validation in one laboratory, the method was now validated in an interlaboratory study. Validation studies are commonly used to test the fitness of the analytical method prior to its use for routine quality testing. The data derived in the interlaboratory study can be used to make an uncertainty statement as well. The relationship between validation and uncertainty statement is not clear for many analysts and there is a need to show how the existing data, derived during validation, can be used in practice. Eight laboratories participated in this interlaboratory study. The set-up allowed the determination of the repeatability variance, s_r^2, and the between-laboratory variance, s_L^2. Combination of s_r^2 and s_L^2 results in the reproducibility variance s_R^2. It has been shown how these data can be used in future by a single laboratory that wants to make an uncertainty statement concerning the same analysis.

Keywords: Interlaboratory study; Uncertainty; Reproducibility; Validation; Erythromycin

1. Introduction

Erythromycin is a mixture of macrolide antibiotics produced by *Saccharopolyspora erythreas* during

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fermentation. The main component of erythromycin is erythromycin A (EA) [1]. The following related substances are formed during the fermentation process: erythromycin B (EB), erythromycin C (EC), erythromycin D (ED), erythromycin E (EE), erythromycin F (EF) and *N*-demethylerythromycin A (NdMeEA). The structures of EA and its related substances are shown in Fig. 1. Under mild acidic conditions EA degrades to erythromycin A enol ether (EAEN) and anhydroerythromycin A (AEA) [2,3]. Pseudoerythromycin A enol ether (PsEAEN) and pseudoerythromycin A hemiketal (PsEAHK) are formed at (slightly) alkaline pH [4,5]. Erythromycin A N-oxide (EANO) and erythronolide B may also be found in bulk substance and commercial products. The content of erythromycin is calculated as the sum of EA, EB and EC. The content of EB and EC is limited to 5.0% each and that of any other related substance to 3.0% [6].

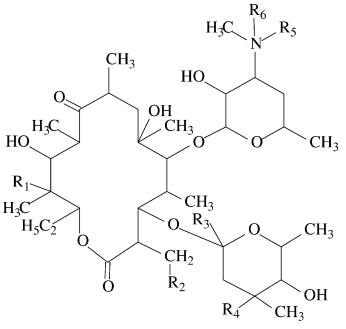
Two new liquid chromatography (LC) methods for the analysis of erythromycin have been developed recently [7,8]. Both showed clear improvements compared to the method currently prescribed by Ph. Eur. and USP [9]. After comparison of the methods, the one using an Astec C_{18} polymeric column was chosen for this interlaboratory study because of the good column stability [9]. This stationary phase consists of an octadecyl silane chemically bonded to a vinyl alcohol copolymer. The method was initially developed for the analysis of erythromycin in enteric coated tablets and was adapted for analysis of bulk substance [8,9]. It enables the separation of EANO, erythronolide B, EF, NdMeEA, EC, ED, EE, EA, AEA, PsEAEN, EB and EAEN. This LC method may be suitable to replace the existing official method.

Validation studies are commonly used to test the fitness of the analytical method prior to its use for routine testing [10]. Therefore the method was validated by one laboratory. This validation consisted of the evaluation of robustness, repeatability, linearity, limit of detection (LOD) and limit of quantitation (LOQ) [9]. To evaluate all method performance parameters, including the reproducibility, there is a need to perform an interlaboratory study. The data derived in the interlaboratory study, can also be used to make an uncertainty statement about measurement results. Uncertainty is defined in the EURACHEM Guide as ‘‘a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand’’ [10]. The uncertainty statement can be given as a standard deviation (or a given multiple of it) or as the width of a confidence interval. However, the relationship between the validation of a method and an uncertainty statement is not clear for many analysts. There is a need to show how the existing validation data can be applied in practice to estimate measurement uncertainty.

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same, homogeneous sample [11]. The precision may be considered at three levels: repeatability, intermediate precision and reproducibility [10–12]. The repeatability s_r expresses precision measured under as identical conditions as possible, i.e., over a short time, by the same analyst and on the same equipment. It is estimated normally within one laboratory, but usually can also be derived from an interlaboratory study set-up [12]. Intermediate precision, s_I, expresses within-laboratory variations: different days, different analysts, different equipment and/or different calibration. Finally the reproducibility, s_R, of an analytical method expresses its precision under the most diverse circumstances, including different laboratories, and thus has to be investigated in an interlaboratory study [12–14].

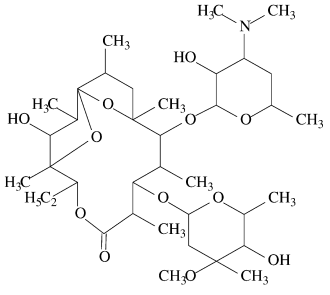
In order to estimate the precision of this new LC method on erythromycin, an interlaboratory study was carried out according to the principles of the ISO 5725-2 guide [12]. Eight laboratories participated in this study. The ISO 5725-2 recommends the participation of eight to 15 laboratories [12]. If less than eight laboratories participate in an interlaboratory study, the reproducibility and the repeatability will be estimated with less degrees of freedom, which has consequences on the confidence intervals and uncertainties estimated from it. The set-up of this study allowed one to estimate the repeatability variance, s_r^2, and the between-laboratory variance, s_L^2. Combination of s_r^2 and s_L^2 results in the reproducibility variance, s_R^2. This paper also shows how the results of the validation of the LC method can be used by a single laboratory to make an uncertainty statement concerning future results.

A



	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

B



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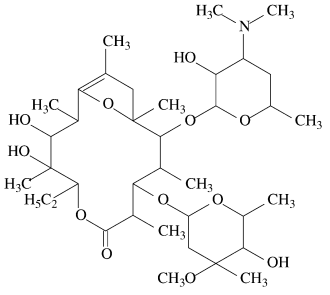

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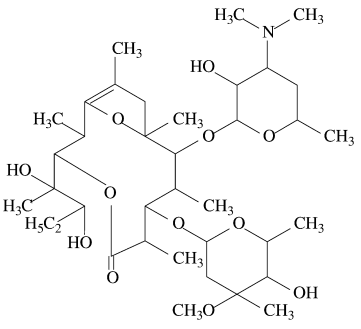

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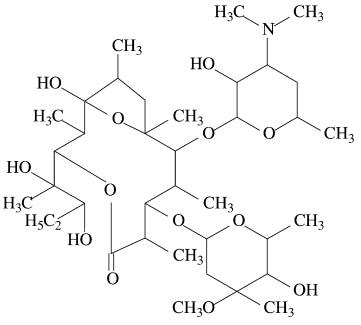

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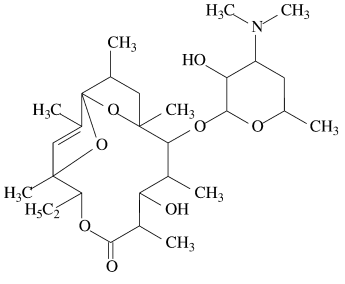

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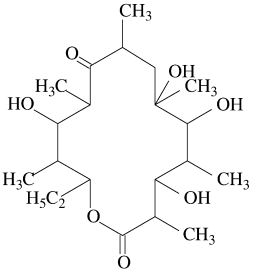

Fig. 1. Structures of (A) the erythromycins and some related substances, (B) anhydroerythromycin A, (C) erythromycin A enol ether and (D) pseudoerythromycin A enol ether, (E) pseudoerythromycin A hemiketal, (F) erythralosamine and (G) erythronolide B.

2. Experimental

2.1. Samples and chemicals

Four test samples of erythromycin, EA reference substance (purity: 96.7%), EB reference substance (97.9%), EC reference substance (97.7%) and NdMEA reference substance (96.4%) were provided by Abbott Labs. (North Chicago, IL, USA). The four test samples originated from different batches of drug substance. All solvents and reagents were of Ph. Eur. or USP quality.

2.2. Chromatographic conditions

An Astec C_{18} (250×4.6 mm I.D., 5 μm) polymeric column (Advanced Separations Technologies, Whippany, NJ, USA), kept at 50 °C in a waterbath or hot air oven, was used as stationary phase. As there is only one brand of this type of C_{18} polymeric column on the market, all the laboratories used the same column brand. Two mobile phases consisting of acetonitrile–0.2 *M* K_2HPO_4, pH 9.0–water were prepared: mobile phase A (30:6:64, v/v) and mobile phase B (50:6:44, v/v). They were used in a ratio of A–B (50:50). The flow-rate was 1.0 ml/min. The volume injected was 100 μl and the detection wavelength 215 nm. All samples were dissolved in a mixture of acetonitrile–0.066 *M* phosphate buffer, pH 8.0 (2:3, v/v). Test solutions contained 4.0 mg/ml of the test sample. Three reference solutions were used for the content determination of EA and its impurities: the content of EA was determined against reference solution A, containing 4.0 mg/ml of EA reference substance, the content of EB and EC against reference solution B, containing 0.2 mg/ml of EB reference substance and 0.2 mg/ml of EC reference substance. Reference solution C, a 3% (v/v) dilution of reference solution A, was used for the content determination of the other impurities. Both PsEAEN and EAEN show a higher UV absorbance response than EA. This was taken into account in the calculations: the ratio response EA/response PsEAEN was taken as 0.15 and the ratio response EA/response EAEN as 0.09. A reference solution containing 0.2 mg/ml of NdMEA, EC, and EB each and 0.16 mg/ml of EA was used for system suitability testing: the test was not valid unless the substances were eluted in the order NdMEA, EC, EA and EB and the resolution between the peaks corresponding to NdMeEA and EC was at least 1.5. If necessary, the concentration of acetonitrile in the mobile phase had to be adapted by varying the mobile phase ratio of A–B. All laboratories reached the prescribed resolution without adjusting the acetonitrile concentration. Thus, the mobile phase used by all laboratories consisted of acetonitrile–0.2 *M* K_2HPO_4, pH 9.0–water (40:6:54). The signal-to-noise ratio was determined using a 0.1% (v/v) dilution of reference solution A and had to be at least 10 [6]. If not, the detector had to be checked or replaced. The repeatability of the injection was tested by six injections of reference solution A, whereby the relative standard deviation of the peak area of EA was not allowed to exceed 0.85% [6]. If this repeatability was not achieved, the integration conditions and/or the equipment had to be checked.

2.3. Set-up of the study

Eight laboratories, three located in North America and five in Europe, participated in this study. Each laboratory analysed the four erythromycin samples in duplicate under repeatability conditions (Fig. 2). Each replicate consisted of an individual preparation of sample solution which was injected once. The contents of EA, EB and EC, of the identified impurities EANO, EF, NdMeEA, ED, EE, AEA, PsEAEN and EAEN and of all unidentified impurities were determined using the results obtained with the reference solutions A, B and C. Identification of the peaks was done by a typical chromatogram of a test sample delivered to all participating laboratories.

2.4. Statistical analysis of the results

The statistical analysis was performed according to ISO 5725-2. It started with a critical examination of the results in order to identify outliers or other problems. Both graphical and statistical tests were applied to evaluate the within-laboratory and between-laboratory consistencies.

A graphical consistency technique used to evaluate the within-laboratory variability is Mandel's *k* statistic:

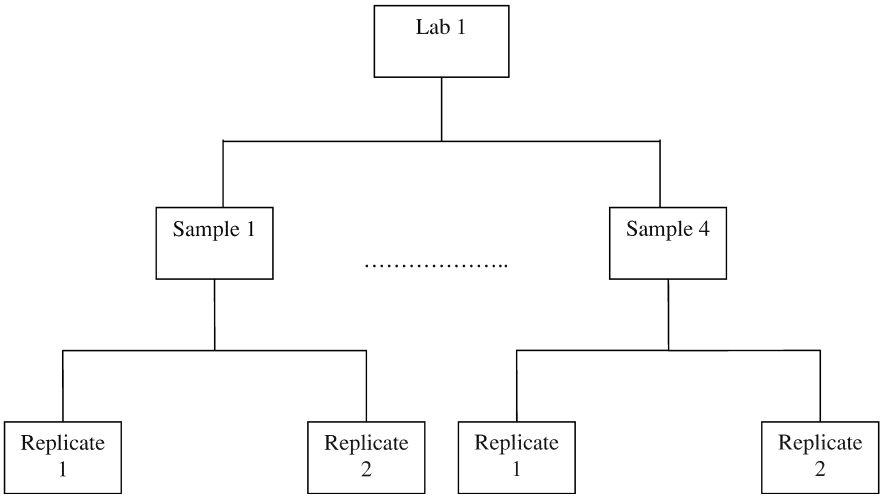

Fig. 2. Set-up of the interlaboratory study.

$$k_{ij} = \frac{s_{ij}\sqrt{p_j}}{\sqrt{\sum s_{ij}^2}} \quad (1)$$

with $i=1$ to p, p being the total number of laboratories and s_{ij} the standard deviation from n ($=2$) results for one sample within one laboratory and for substance j. These k_{ij} values are plotted. In order to determine problems in a certain laboratory or problems with a certain substance they were plotted ''grouped per laboratory'' or ''grouped per substance''. Fig. 3A and B show the plotted results for one of the samples and will be discussed later. Mandel's k was only used as a graphical consistency technique and not to decide on the removal of outliers.

To detect outliers or stragglers in within-laboratory variances, Cochran's test was applied as a numerical technique. Cochran's test is calculated as:

$$C = \frac{s_{\max}^2}{\sum_{i=1}^{p} s_i^2} \quad (2)$$

where $s_{\max}^2$ is the highest variance in the set and s_i^2 the variance from laboratory i. The criteria for outlier rejection were designated before the interlaboratory study was conducted and were taken from the ISO 5725-2 guideline [12]. Variances significant at $\alpha = 0.01$ were considered as outliers and were removed from the data set, while stragglers (values significant at $\alpha = 0.05$) were kept in for further calculations. Critical C values can be found in Ref. [12].

Mandel's h was used as a graphical consistency technique to test the between-laboratory variability. It is calculated as:

$$h_{ij} = \frac{\bar{y}_{ij} - \bar{\bar{y}}_j}{\sqrt{\frac{1}{(p_j - 1)}\sum_{i=1}^{p_j}(\bar{y}_{ij} - \bar{\bar{y}}_j)^2}} \quad (3)$$

with $i=1$ to p, p being the total number of laboratories, j the substance examined, $\bar{y}_{ij}$ the laboratory mean for substance j and $\bar{\bar{y}}_j$ the general mean of all laboratories for substance j. As for the Mandel's k statistic also these h_{ij} values are plotted. Again two figures were made: one shows the results ''grouped per laboratory'' and one ''grouped per substance''. Fig. 4A and B show the results for one of the samples and will be discussed later.

Grubbs' tests were used as numerical outlier techniques to evaluate the between-laboratory variability. Different Grubbs' tests were carried out: (i) for one outlying observation (G_1: smallest and G_p: largest value) and (ii) for two outlying observations ($G_{1,2}$: two smallest and $G_{p,p}$: two largest values). Equations used in the Grubbs tests for one outlier are:

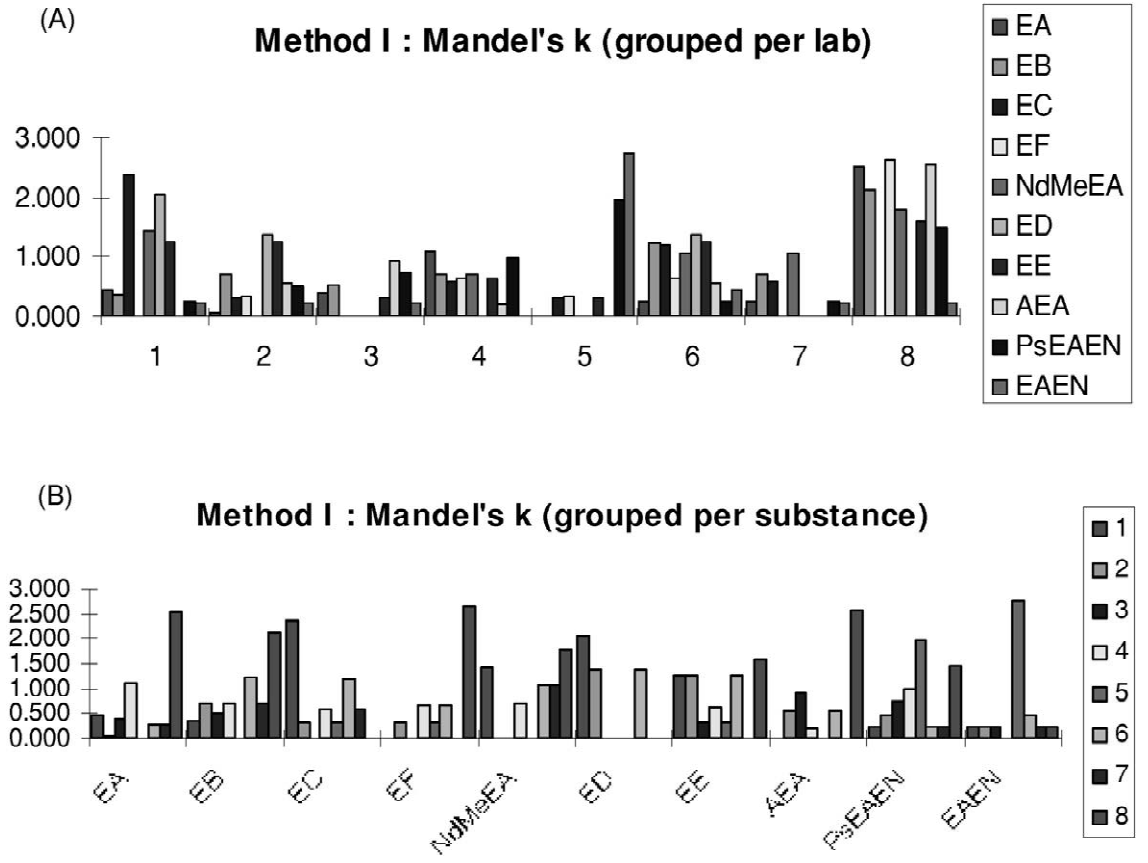

Fig. 3. Mandel's k statistic for a sample, used to examine within-laboratory consistency. (A) Grouped per laboratory, (B) grouped per substance.

$$G_1 = (\bar{x} - x_1)/s \quad (4)$$

and

$$G_p = (x_p - \bar{x})/s \quad (5)$$

with x_1 the smallest mean laboratory value, x_p the largest mean laboratory value, $\bar{x}$ the general mean value of all laboratories and s the standard deviation on all mean laboratory values. Equations used in the Grubbs' tests for two outliers are:

$$G_{1,2} = s^2_{1,2}/s^2_0 \quad (6)$$

with $s^2_{1,2} = \Sigma^p_{i=3}(x_i - \bar{x}_{1,2})^2$, $s^2_0 = \Sigma^p_{i=1}(x_i - \bar{x})^2$, x_i the result of laboratory i and $\bar{x}_{1,2}$ the mean of all laboratory results except the two smallest ones, and:

$$G_{p,p} = s^2_{p-1,p}/s^2_0 \quad (7)$$

with $s^2_{p-1,p} = \Sigma^{p-2}_{i=1}(x_i - \bar{x}_{p-1,p})^2$, $\bar{x}_{p-1,p} = \frac{1}{p-2}\Sigma^{p-2}_{i=1}x_i$ and $\bar{x}_{p-1,p}$ the mean of all laboratory results except the two largest ones.

Values should be smaller than the critical value for the Grubbs' tests for one outlying observation and larger than the critical value for the Grubbs' tests for two outlying observations. The critical values are reported in Ref. [12].

After removing the outlying values, a variance analysis was carried out. Repeatability, between-laboratory and reproducibility variances were estimated. Repeatability variance is calculated as:

$$s^2_{rj} = \frac{1}{2p}\sum_{i=1}^{p}(y_{ij1} - y_{ij2})^2 \quad (8)$$

with p = total number of laboratories, $i = 1$ to p, y_{ij1}

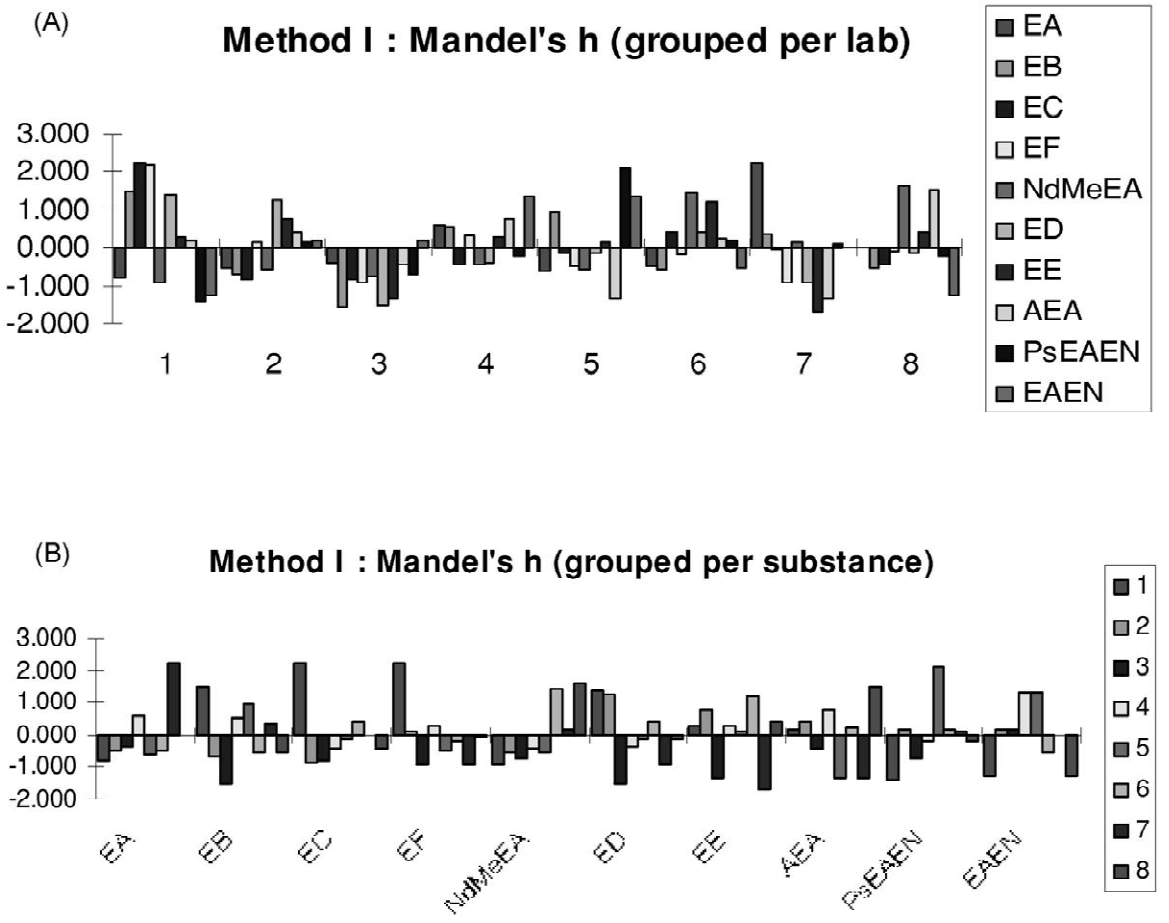

Fig. 4. Mandel's h statistic for a sample, used to examine between-laboratory consistency. (A) Grouped per laboratory, (B) grouped per substance.

the first and y_{ij2} the second replicate in laboratory i for substance j. Eq. (8) can only be used in the particular case where $n_{ij}=n=2$, which was the case in this study. The equation to calculate the between-laboratory variance is:

$$s^2_{Lj} = \frac{1}{p-1}\sum_{i=1}^{p}(\bar{y}_{ij} - \bar{\bar{y}}_j)^2 - \frac{s^2_{rj}}{2} \quad (9)$$

with p = total number of laboratories, $i=1$ to p, j = substance, $\bar{y}_{ij}$ the laboratory mean for substance j and $\bar{\bar{y}}_j$ the general mean of all laboratories for substance j. Also this equation can only be used if $n_{ij}=n=2$.

Reproducibility variance is calculated as the sum of repeatability variance and between-laboratory variance:

$$s^2_{Rj} = s^2_{rj} + s^2_{Lj} \quad (10)$$

If the different samples analysed show different contents for a given substance, the reproducibility standard deviations can be plotted as a function of the corresponding mean contents for that compound, in order to check whether a relationship between reproducibility standard deviation and content exists [12–14]. If the samples in a study show similar contents for a substance, this relationship can not be made and the reproducibility variances are pooled in order to achieve a general reproducibility variance for this method.

The standard uncertainty of an individual measurement x performed by a laboratory becomes:

$$u_x = \sqrt{s_{Rj}^2} = \sqrt{s_{rj}^2 + s_{Lj}^2} \quad (11)$$

When n measurements are carried out under repeatability conditions, the standard uncertainty $u_{\bar{x}}$ is calculated as:

$$u_{\bar{x}} = \sqrt{s_{rj}^2/n + s_{Lj}^2} \quad (12)$$

Imagine a company, analysing a sample n times under repeatability conditions in p sites. The standard uncertainty will then be calculated as:

$$u_{\bar{x}} = \sqrt{s_{rj}^2/(np) + s_{Lj}^2/p} \quad (13)$$

The expanded uncertainty U_x is used to provide an interval within which a large fraction of the distribution of the values, which could reasonably be attributed to the measurand, is expected. This expanded uncertainty, U_x, is obtained by multiplying the standard uncertainty by the two-sided tabulated t-value, $t_{\alpha/2}$, for the effective degrees of freedom. When the distributions concerned are normal, this $t_{\alpha/2}$ can be replaced by a coverage factor k of 2, giving an interval $x \pm U_x$ around x that contains approximately 95% of the distribution of values [10]. The expanded uncertainty is thus calculated as $U_x = ku_x = 2u_x$. The units of x, u_x and U_x are the same, being % content in this study.

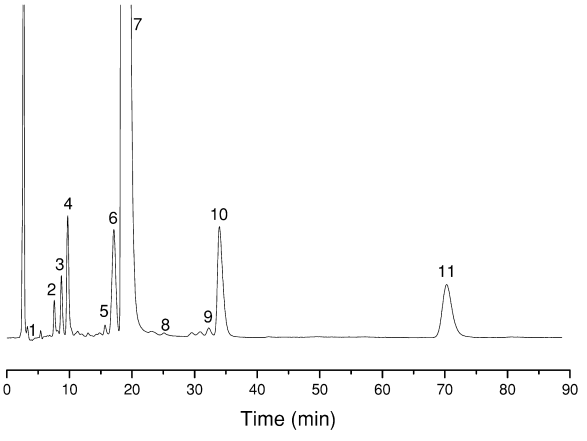

Fig. 5. Typical chromatogram of a sample. 1 = EANO (no peak in this sample), 2 = EF, 3 = NdMeEA, 4 = EC, 5 = ED, 6 = EE, 7 = EA, 8 = AEA, 9 = PsEAEN, 10 = EB, 11 = EAEN.

The above clearly shows the relationship between validation data and uncertainty statement. Validation data like repeatability variance, between-laboratory variance and reproducibility variance can be used to calculate the expanded uncertainty. This expanded uncertainty can than be used in future by other laboratories to make the uncertainty statement.

3. Results and discussion

A typical chromatogram of a test sample is shown in Fig. 5. No selectivity problems were mentioned nor observed by any laboratory. The contents of EA and all identified substances were calculated. Results are shown in Table 1. Because of between-laboratory differences due to problems with identification, no statistical evaluation of the unidentified substances was carried out. These were only present in minor concentrations ($<0.4\%$).

3.1. Determination of outliers and stragglers

After calculation of the laboratory means, the general grand means, variances and standard deviations of EA and identified substances, the outlying values were determined. These outlying values were removed and a variance analysis was carried out on the remaining results. The calculations for one of the samples will be discussed more in detail. Results for the other three samples were treated similarly.

Within-laboratory consistency was tested using Mandel's k and Cochran's test. Mandel's k results for the sample are shown in Fig. 3A and B. It can be observed (Fig. 3A) that several results of laboratory 8 show a large k value, suggesting a larger within-laboratory variance in this laboratory compared to the other laboratories. Also laboratories 1 and 5 show some large k values. Problems of within-laboratory uncertainty were not related to a given substance, as can be deduced from Fig. 3B. The results of the Cochran's test are shown in Table 2. As suggested by Mandel's k analysis, laboratory 8 shows a high within-laboratory variability compared to the other laboratories resulting in outlying values for EF and AEA, and a straggler for the main

Table 1
Original data for the sample discussed

Laboratory No.	EA	EB	EC	EF	NDMeEA	ED	EE	AEA	PsEAEN	EAEN
1	84.79	3.82	1.65	0.58	0.72	0.22	2.56	0.09	0.02	0.25
	84.46	3.80	1.57	0.58	0.68	0.19	2.52	0.09	0.021	0.24
2	85.06	3.58	1.06	0.41	0.72	0.19	2.58	0.09	0.044	0.31
	85.10	3.54	1.07	0.42	0.72	0.21	2.62	0.12	0.042	0.32
3	85.41	3.45	1.07	0.33	0.71	0.1	2.36	0.08	0.032	0.32
	85.13	3.48	1.07	0.33	0.71	0.1	2.35	0.03	0.029	0.31
4	86.39	3.68	1.13	0.42	0.72	0.14	2.53	0.13	0.04	0.37
	87.18	3.72	1.15	0.44	0.74	0.14	2.55	0.12	0.036	0.37
5	84.89	3.75	1.2	0.36	0.72	0.15	2.52	0	0.067	0.31
	84.89	3.75	1.19	0.37	0.72	0.15	2.53	0	0.075	0.43
6	85.03	3.61	1.31	0.38	0.83	0.16	2.63	0	0.11	0.27
	85.22	3.54	1.27	0.4	0.86	0.18	2.67	0	0.08	0.29
7	89.36	3.70	1.22	0.33	0.78	0.12	2.31	0	0.043	0.31
	89.17	3.66	1.2	0.33	0.75	0.12	2.31	0	0.042	0.3
8	84.96	3.52	1.14	0.44	0.83	0.15	2.53	0.1	0.035	025
	86.77	3.64	1.14	0.36	0.88	0.15	2.58	0.24	0.041	0.24

component EA. Also laboratory 5 had one outlying value for EAEN and laboratory 1 a straggler for EC. Stragglers were kept in the data set for further calculations, while outliers were removed.

After testing the within-laboratory consistency, the between-laboratory consistency is tested. Various patterns appear in the Mandel's *h* plots (Fig. 4A and B). Laboratories can have both positive and negative *h* values. Normal results should be spread randomly around zero. Remarkable is a pattern where all (or most) results for a laboratory are either positive or negative, as seen for laboratory 3 (Fig. 4A). The same pattern was observed for the other samples. It suggests the occurrence of a systematic error in laboratory 3. Although this does not necessarily lead to outlying values, the laboratory should be informed about this problem, and asked to check its equipment. No problems of between-laboratory variance, related to a certain compound were found, as can be deduced from Fig. 4B. The results of the Grubbs' tests are shown in Table 3. Only one outlying value, the result for EA of laboratory 7, was removed. Stragglers were found for laboratories 1, 6 and 8.

3.2. Variance analysis

After deleting the outliers, a variance analysis was carried out. Repeatability, between-laboratory and

Table 2
Results of the Cochran's test for the sample discussed

	EA	EB	EC	EF	NDMEEA	ED	EE	AEA	PSEAEN	EAEN
C	0.79	0.57	0.71	0.86	0.40	0.53	0.32	0.82	0.48	0.94
Outliers Critical value 0.79				Laboratory 8				Laboratory 8		Laboratory 5
Stragglers Critical value 0.68	Laboratory 8		Laboratory 1							

Table 3
Results of the Grubbs' tests for the sample discussed, (A) one outlying observation, (B) two outlying observations

	EA	EB	EC	EF	NDMEEA	ED	EE	AEA	PSEAEN	EAEN
A										
G_1	1.70	1.53	0.85	0.88	0.91	1.49	1.70	1.33	1.41	1.16
Critical values										
5%	2.13	2.13	2.13	2.02	2.13	2.13	2.13	2.02	2.13	2.02
1%	2.27	2.27	2.27	2.14	2.27	2.27	2.27	2.14	2.27	2.14
G_p	2.22	1.49	2.24	2.03	1.62	1.39	1.20	1.15	2.09	1.66
Critical values										
5%	2.13	2.13	2.13	2.02	2.13	2.13	2.13	2.02	2.13	2.02
1%	2.27	2.27	2.27	2.14	2.27	2.27	2.27	2.14	2.27	2.14
Outliers	Laboratory 7									
Stragglers			Laboratory 1	Laboratory 1						
B										
$G_{1,2}$	0.84	0.50	0.77	0.68	0.78	0.43	0.12	0.18	0.56	0.66
Critical values										
5%	0.11	0.11	0.11	0.07	0.11	0.11	0.11	0.07	0.11	0.11
1%	0.06	0.06	0.06	0.03	0.06	0.06	0.06	0.03	0.06	0.06
$G_{p,p}$	0.08	0.43	0.09	0.13	0.10	0.35	0.65	0.61	0.26	0.41
Critical values										
5%	0.07	0.11	0.11	0.07	0.11	0.11	0.11	0.07	011	0.11
1%	0.03	0.06	0.06	0.03	0.06	0.06	0.06	0.03	0.06	0.06
Stragglers			Laboratories 1, 6		Laboratories 6, 8					

reproducibility variance were calculated using Eqs. (8), (9) and (10), respectively. The results for the four samples are displayed in Table 4. The mean content for a given substance corresponds to the grand mean of all laboratories.

The four samples in this study showed similar contents for erythromycin and related substances and therefore these reproducibility variances were pooled. The pooled reproducibility variances for EA and all known impurities are shown in Table 5. They give an idea about the reproducibility of the method.

The ratio reproducibility variance to repeatability variance was also calculated. This ratio depended on the concentration of the substance in the sample. For the main peak (EA) the ratio is found to be 3.5, which, according to Boyer and Horwitz [15] theoretically might be expected [14,15]. For the impurities it can be noticed that the ratios are larger, as their concentrations in the sample are smaller. The relatively small ratios for PsEAEN and EAEN, although present in small amount and eluted late in the chromatogram, can be explained by their higher response factors, which lead to higher peak areas compared to other impurities, resulting in a smaller variability on their content determination.

A laboratory that applies this LC method to analyse erythromycin can use the information obtained from this interlaboratory study to make an uncertainty statement on the results. Therefore, the laboratory first has to prove that it is sufficiently proficient, i.e., the laboratory repeatability has to be similar to the repeatability obtained in this interlaboratory study. This can be evaluated by comparison of the repeatability variances by means of an F-test. If the repeatability is similar, the reproducibility standard deviation from the collaborative study can be used in the uncertainty statement. According to Eq. (11), the standard uncertainty of a single result x from a single laboratory for the content determination of EA becomes $u_x = 1.039$. The expanded uncertainty, using a coverage factor $k = 2$, is $U_x = 2.078$. The result x of this laboratory can than be written as $x \pm 2.1$.

If, in an individual laboratory, the content of EA is

Table 4
Results of the variance analysis, (A) for the sample discussed, (B), (C), (D) for the other samples

Variance	Substance									
	EA	EB	EC	EF	NDMEEA	ED	EE	AEA	PSEAEN	EAEN
A										
Repeatability	$2.95\cdot10^{-1}$	$1.59\cdot10^{-3}$	$5.63\cdot10^{-4}$	$7.14\cdot10^{-5}$	$3.94\cdot10^{-4}$	$1.06\cdot10^{-4}$	$4.94\cdot10^{-4}$	$3.14\cdot10^{-4}$	$8.25\cdot10^{-6}$	$6.43\cdot10^{-5}$
Between-laboratory	$3.84\cdot10^{-1}$	$1.06\cdot10^{2}$	$2.68\cdot10^{-2}$	$6.31\cdot10^{-3}$	$3.09\cdot10^{-3}$	$1.11\cdot10^{-3}$	$1.17\cdot10^{-2}$	$2.02\cdot10^{-3}$	$1.78\cdot10^{-4}$	$1.64\cdot10^{-3}$
Reproducibility	$6.78\cdot10^{-1}$	$1.22\cdot10^{-2}$	$2.74\cdot10^{-2}$	$6.38\cdot10^{-3}$	$3.49\cdot10^{-3}$	$1.21\cdot10^{-3}$	$1.22\cdot10^{-2}$	$2.33\cdot10^{-3}$	$1.87\cdot10^{-4}$	$1.71\cdot10^{-3}$
Mean content (%)	85.38	3.64	1.22	0.41	0.76	0.15	2.51	0.07	0.04	0.30
B										
Repeatability	$4.35\cdot10^{-1}$	$1.49\cdot10^{-3}$	$5.06\cdot10^{-4}$	$3.57\cdot10^{-5}$	$2.06\cdot10^{-4}$	$5.00\cdot10^{-5}$	$5.93\cdot10^{-4}$	$1.35\cdot10^{-3}$	$1.88\cdot10^{-5}$	$1.06\cdot10^{-4}$
Between-laboratory	$3.17\cdot10^{-1}$	$3.79\cdot10^{-2}$	$3.45\cdot10^{-2}$	$2.12\cdot10^{-3}$	$1.77\cdot10^{-3}$	$4.61\cdot10^{-4}$	$9.34\cdot10^{-3}$	$2.37\cdot10^{-3}$	$6.21\cdot10^{-5}$	$1.46\cdot10^{-3}$
Reproducibility	$7.52\cdot10^{-1}$	$3.94\cdot10^{-2}$	$3.50\cdot10^{-2}$	$2.15\cdot10^{-3}$	$1.98\cdot10^{-3}$	$5.11\cdot10^{-4}$	$9.93\cdot10^{-3}$	$3.72\cdot10^{-3}$	$8.09\cdot10^{-5}$	$1.56\cdot10^{-3}$
Mean content (%)	85.09	3.69	1.21	0.35	0.68	0.15	2.46	0.08	0.04	0.28
C										
Repeatability	$1.07\cdot10^{-1}$	$6.81\cdot10^{-4}$	$2.93\cdot10^{-4}$	$8.75\cdot10^{-5}$	$3.75\cdot10^{-5}$	$7.14\cdot10^{-6}$	$5.38\cdot10^{-4}$	$1.06\cdot10^{-4}$	$7.14\cdot10^{-6}$	$1.00\cdot10^{-4}$
Between-laboratory	1.17	$1.24\cdot10^{-2}$	$2.70\cdot10^{-3}$	$8.88\cdot10^{-3}$	$1.12\cdot10^{-2}$	$2.51\cdot10^{-3}$	$3.76\cdot10^{-2}$	$7.73\cdot10^{-3}$	$5.66\cdot10^{-5}$	$1.05\cdot10^{-3}$
Reproducibility	1.27	$1.31\cdot10^{-2}$	$2.99\cdot10^{-3}$	$8.97\cdot10^{-3}$	$1.12\cdot10^{-2}$	$2.52\cdot10^{-3}$	$3.81\cdot10^{-2}$	$7.84\cdot10^{-3}$	$6.38\cdot10^{-5}$	$1.15\cdot10^{-3}$
Mean content (%)	85.45	3.77	0.73	0.39	0.59	0.12	2.40	0.10	0.03	0.26
D										
Repeatability	$3.92\cdot10^{-1}$	$1.52\cdot10^{-3}$	$8.75\cdot10^{-4}$	$3.63\cdot10^{-4}$	$3.50\cdot10^{-4}$	$6.88\cdot10^{-4}$	$2.98\cdot10^{-3}$	$1.44\cdot10^{-4}$	$1.25\cdot10^{-5}$	$2.88\cdot10^{-4}$
Between-laboratory	1.14	$1.24\cdot10^{-2}$	$1.74\cdot10^{-3}$	$4.81\cdot10^{-3}$	$2.04\cdot10^{-3}$	$1.09\cdot10^{-3}$	$4.62\cdot10^{-3}$	$9.22\cdot10^{-3}$	$1.73\cdot10^{-4}$	$1.42\cdot10^{-3}$
Reproducibility	1.54	$1.39\cdot10^{-2}$	$2.61\cdot10^{-3}$	$5.17\cdot10^{-3}$	$2.39\cdot10^{-3}$	$1.77\cdot10^{-3}$	$7.61\cdot10^{-3}$	$9.37\cdot10^{-3}$	$1.86\cdot10^{-4}$	$1.71\cdot10^{-3}$
Mean content (%)	85.04	3.77	1.48	0.39	0.70	0.17	2.49	0.12	0.04	0.29

measured three times under repeatability conditions, the standard uncertainty on the mean result becomes, using Eq. (12):

$$u_{\bar{x}} = \sqrt{s^2_{r(EA)}/3 + s^2_{L(EA)}} = 0.934$$

The expanded uncertainty is $U_{\bar{x}} = 1.868$ and the mean result of the laboratory can be written as $\bar{x} \pm 1.9$. It is observed that analysing the sample three times instead of once does not improve the uncertainty considerably. In fact this is logical as the between-laboratory uncertainty contributes most to the total uncertainty. Nevertheless, the analysis should be done several times in order to prove that the laboratory repeatability is similar to the repeatability found in this interlaboratory study.

If a company has two sites, both analysing the sample three times under repeatability conditions, the

Table 5
Pooled variances from the four samples

Pooled variance	Substance									
	EA	EB	EC	EF	NDMEEA	ED	EE	AEA	PSEAEN	EAEN
Repeatability	$3.11\cdot10^{-1}$	$1.31\cdot10^{-3}$	$5.69\cdot10^{-4}$	$1.46\cdot10^{-4}$	$2.54\cdot10^{-4}$	$2.20\cdot10^{-4}$	$1.17\cdot10^{-3}$	$4.85\cdot10^{-4}$	$1.18\cdot10^{-5}$	$1.44\cdot10^{-4}$
Between-laboratory	$7.86\cdot10^{-1}$	$1.76\cdot10^{-2}$	$1.70\cdot10^{-2}$	$5.63\cdot10^{-3}$	$4.27\cdot10^{-3}$	$1.25\cdot10^{-3}$	$1.61\cdot10^{-2}$	$5.46\cdot10^{-3}$	$1.20\cdot10^{-4}$	$1.40\cdot10^{-3}$
Reproducibility	1.08	$1.89\cdot10^{-2}$	$1.75\cdot10^{-2}$	$5.78\cdot10^{-3}$	$4.53\cdot10^{-3}$	$1.47\cdot10^{-3}$	$1.72\cdot10^{-2}$	$5.94\cdot10^{-3}$	$1.32\cdot10^{-4}$	$1.54\cdot10^{-3}$
Mean content (%)	85.24	3.72	1.16	0.39	0.68	0.15	2.46	0.09	0.04	0.28
Ratio	3.5	14.4	30.8	39.6	17.8	6.7	14.7	12.3	11.1	10.7

Ratio = Reproducibility-to-repeatability variance ratio.

standard uncertainty on the content determination of EA becomes, using Eq. (13):

$$u_{\bar{x}} = \sqrt{s^2_{r(EA)}/6 + s^2_{L(EA)}/2} = 0.660$$

The expanded uncertainty will now be $U_{\bar{x}} = 1.320$ and the company can report the mean result with the corresponding uncertainty interval as $\bar{x} \pm 1.3$. In this case, an improvement of the uncertainty on the result is seen, because the between-laboratory uncertainty also decreased.

The standard and expanded uncertainties for the other substances can be calculated similarly. It should be noticed that the use of these standard uncertainties implies that the concentrations found are similar to the concentrations found in this interlaboratory study. If different concentrations for a given substance would have been examined in this interlaboratory study and a relationship was found between concentration and standard deviation, this relationship would allow to calculate the standard deviation for each concentration covered by this concentration range. Unfortunately, the composition of the samples examined in this study was not sufficiently different.

4. Conclusion

The reproducibility of a new LC method for the analysis of erythromycin was examined in an interlaboratory study. All laboratories achieved a good selectivity allowing the content determination of EA and all identified related substances. The variance analysis, carried out on these results, showed a good reproducibility of the method. The method is suitable to replace the existing official method. The results of the validation can also be used by any laboratory that wants to make an uncertainty statement for this method.

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