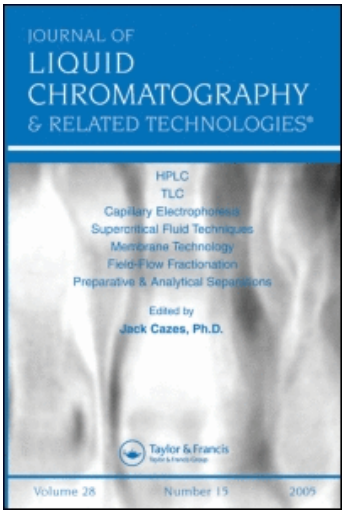

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Zhu Yongxin[a]; E. Roets[a]; A. Bruzzi[b]; A. J. W. Smelt[c]; C. Van der Vlies[c]; P. Betto[d]; M. L. Moreno[e]; E. Porqueras[e]; H. H. Wendebourg[f]; K. Sinivuo[g]; D. De Kaste[h]; A. Mayrhofer[i]; N. Graby[j]; G. Kister[a]; J. H. McB Miller[k]; C. J. Nap[k]; J. M. Spieser[k]; J. Hoogmartens[a]

[a] Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, Leuven, Belgium [b] National Drugs Advisory Board, Dublin, Ireland [c] Gist-Brocades, Delft, The Netherlands [d] Istituto Superiore Di Sanita, Rome, Italy [e] Centra Nacional De Farmacobiologia, Madrid, Spain [f] Staatliches Lebensmitteluntersuchungsamt, Oldenburg, Germany [g] National Agency for Medicines, Helsinki, Finland [h] Rijksinstituut voor Geneesmiddelenonderzoek, Leiden, The Netherlands [i] National Institute for Quality Control of Drugs, Vienna, Austria [j] Agence du Médicament, Montpellier, France [k] European Pharmacopoeia, Strasbourg, France

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INTERLABORATORY STUDY OF THE ANALYSIS OF AMOXICILLIN BY LIQUID CHROMATOGRAPHY

Zhu Yongxin,[1] E. Roets,[1] A. Bruzzi,[2] A. J. W. Smelt,[3] C. Van der Vlies,[3] P. Betto,[4] M. L. Moreno,[5] E. Porqueras,[5] H. H. Wendebourg,[6] K. Sinivuo,[7] D. De Kaste,[8] A. Mayrhofer,[9] N. Graby,[10] G. Kister,[1] J. H. McB. Miller,[11] C. J. Nap,[11] J. M. Spieser,[11] J. Hoogmartens[1]*

[1] Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, K. U. Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium, [2] National Drugs Advisory Board, Dublin, Ireland, [3] Gist-Brocades, Delft, The Netherlands, [4] Istituto Superiore Di Sanita, Rome, Italy, [5] Centro Nacional De Farmacobiologia, Madrid, Spain, [6] Staatliches Lebensmitteluntersuchungsamt, Oldenburg, Germany, [7] National Agency for Medicines, Helsinki, Finland, [8] Rijksinstituut voor Geneesmiddelenonderzoek, Leiden, The Netherlands, [9] National Institute for Quality Control of Drugs, Vienna, Austria, [10] Agence du Médicament, Montpellier, France, [11] European Pharmacopoeia, Strasbourg, France

ABSTRACT

A liquid chromatography method for analysis of amoxicillin was examined in a collaborative study involving 11 laboratories. The method comprised an isocratic part, which is used in the assay. The isocratic part is similar to the assay method for amoxicillin of the United States Pharmacopeia 23. When the isocratic part is combined with gradient elution, the method is suitable for purity control. Five samples of amoxicillin (trihydrate and sodium salt) with varying purity were analysed.

The main component and the impurities were determined. An analysis of variance proved absence of consistent laboratory bias. The laboratory-sample interaction was not significant. Estimates for the repeatability and reproducibility of the method, expressed as standard deviations (SD) of the result of the determination of amoxicillin, were calculated to be 1.1 and 1.3 respectively.

INTRODUCTION

The selectivity of five isocratic liquid chromatography (LC) methods which were described for the assay of amoxicillin had been previously examined.[1] It was observed that the USP assay method[2] using a mobile phase of 0.05 M phosphate buffer pH 5.0-acetonitrile (96:4) consistently gave good selectivity on different stationary phases. It had also been suggested that cefadroxil may be used in the resolution test. Based on the adapted USP method, a gradient elution method had been developed, which was shown to be suitable as a related substances test.[1] Indeed, it was possible to elute oligomers and other more strongly retained impurities by increasing the acetonitrile content of the mobile phase from 4% to 20% with a linear gradient elution over 25 min which started immediately after the elution of the main peak. In this interlaboratory study, the applicability of the method for assay and purity testing of amoxicillin was examined by 11 laboratories. A sample of amoxicillin trihydrate, proposed as Chemical Reference Substance (CRS) to the European Pharmacopoeia (Ph. Eur.), was incorporated in the study as one of the samples. The results of this interlaboratory study will be used not only to examine the suitability of the method but also to assign a content to the CRS.

EXPERIMENTAL

Samples and Reagents

Five amoxicillin samples including three trihydrates (1, 2 and 3) and two sodium salts (4 and 5) of different origin were used. Sample 1 was the proposed CRS to which a provisional content of 85.0 % was assigned for the purpose of this study. Cefadroxil CRS of the Ph. Eur. was used in the resolution test. Solvents and reagents were of Ph. Eur. quality.[3] Two mobile phases were used. Mobile phase A was a mixture of 0.05 M phosphate buffer pH 5.0-actonitrile (99:1, v/v) and mobile phase B was a mixture of the same components with a ratio (80:20, v/v).

Table 1

General Information on Columns and Method Performance

L	Stat'y Ph.	Amount (%) Acetonitrile in Mobile Phase	k' AMO	S AMO	n AMO	Rs AMO-CE	Peak Area RSD%	Ret. Time (min) Mean	Ret. Time (min) RSD%	Linearity r AMO	Intercept I (%)
1	Hypersil C18[b]	2.5	2.5	1.2	6680	9.0	0.38	6.6	0.32	0.9999	1.0
2	Nucleosil C18	2.5	2.0	1.0	8270	7.1	0.09	8.9	0.30	0.9998	2.7
3	Hypersil C19	2.5	2.7	1.5	10680	9.1	0.13	7.5	0.59	0.9997	1.6
4	Lichrospher C18	2.5	1.3	0.9	8570	6.9	0.40	6.8	2.40	0.9978	3.3
5	Hypersil C18	2.5	2.1	1.1	12500	13	0.50	7.4	0.51	0.9995	1.1
6	Nucleosil C18[c]	2.5	1.5	1.1	4340	3.0	0.10	5.3	0.10	0.9999	1.1
7	Nucleosil C18	2.5	2.0	1.0	11530	8.8	0.47	6.7	0.16	0.9999	0.4
8	Hypersil C18	2.5	1.2	0.9	8650	11	0.04	7.5	0.60	0.9999	2.3
9	Supelcosil C18[c]	2.9	1.3	1.6	7490	5.1	0.67	6.0	0.25	0.9978	0.1
10	Kromasil C18	2.5	1.2	1.0	7920	10	0.54	7.7	0.26	0.9993	6.5
11	Nucleosil C18	2.9	1.0	1.0	7680	5.5	0.26	8.6	0.43	0.9998	4.0

L = laboratory; k' = capacity factor; S = symmetry factor; n = theoretical plate number; Rs = resolution; AMO = amoxicillin; CE = cefadroxil; RSD = relative standard deviation; r = coefficient of correlation for AMO in the range 70-130 %; I = value of intercept in % of the area corresponding to 100%.

[a] Particle size for all columns is 5 μm.

[b] Laboratory-packed column, all other columns were prepacked.

[c] Column temperature at 40°C.

Samples were prepared with mobile phase A as the solvent at the following concentrations, for assay: 30 mg/50 mL, for purity testing: 30 mg /20 mL. The resolution test solution was prepared by mixing 5.0 mL of cefadroxil CRS solution (4 mg/50 mL) with 5.0 mL of amoxicillin solution (sample 1, 30 mg/50 mL).

Materials and Methods

The equipment consisted of a solvent delivery system capable to develop gradient elution with a flow rate of 1.0 mL min^{-1}, a injector with a loop of about 50 µl, except for laboratories 6 and 7 using a 25 µl loop, a UV detector set at 254 nm and an integrator allowing peak area measurements. Different brands of C_{18} stationary phases with 5 µm particle size were used, all columns measured 25 x 0.46 cm i.d. except for laboratory 7 using a column with 0.40 cm i.d. The laboratories were free to chose the brand. Table 1 lists the columns used. The column used in laboratory 1 was laboratory-packed, all other columns were prepacked. The column temperature was ambient, except in laboratories 6 and 9 where a temperature of 40 °C was used.

For the assay isocratic elution was used with a mobile phase ratio A:B of 92:8. The composition of the isocratic mobile phase was adapted in order to have a capacity factor of 1.2 to 2.7 for amoxicillin and a resolution between amoxicillin and cefadroxil of at least 3.0. For the related substances test isocratic elution combined with gradient elution was performed as follows: after isocratic elution of the amoxicillin peak with a mobile phase ratio A:B as used in the assay, a linear gradient elution was started to reach a mobile phase ratio A:B of 0:100 over a period of 25 min, this ratio was held for 15 min, then the column was equilibrated with a mobile phase ratio A:B of 92:8 during 15 min.

RESULTS AND DISCUSSION

One column was used in each of the eleven laboratories. General information about the columns, composition of isocratic mobile phase composition and results of performance checks carried out by each laboratory is shown in Table 1. The calculation of the chromatographic parameters was carried out according to the Ph. Eur.[4] The capacity factor k', the symmetry factor S, and the theoretical plate number n were calculated for the amoxicillin peak. After 6 consecutive injections the relative standard deviation was calculated for the area of the amoxicillin peak and for its retention time.

Table 2

Individual Values (%, m/m) for the Content of Amoxicillin

Laboratory	Samples											
	S2			S3			S4			S5		
1	85.46	85.39	84.46	80.78	81.79	82.29	97.13	96.65	97.35	95.81	95.89	96.26
2	83.30	84.44	83.94	80.72	81.42	81.58	94.30	95.22	94.99	93.47	96.03	95.32
3	84.86	85.41	85.10	81.32	82.83	82.23	96.99	94.96	95.46	94.37	96.11	94.49
4	84.65	83.66	85.05	81.07	80.03	86.03	97.51	92.80	90.14	91.18	93.96	91.43
5	83.66	84.34	83.06	80.42	79.70	80.07	95.35	93.75	93.69	93.67	94.34	93.34
6	85.2	84.1	84.0	80.9	80.5	80.0	95.7	95.8	90.0	95.6	95.7	93.8
7	85.2	85.3	85.3	79.9	80.4	81.4	96.8	96.0	95.1	95.8	94.6	95.5
8	84.53	83.84	84.11	80.92	80.57	81.07	94.65	94.85	95.03	94.25	94.37	94.30
9	85.77	84.28	84.01	82.39	81.60	80.55	96.02	95.17	95.12	95.28	95.00	94.99
10	85.58	85.32	84.38	80.48	80.52	79.55	96.21	96.46	94.22	95.17	96.42	94.13
11	84.52	85.04	84.41	80.98	80.97	91.49	95.39	94.86	94.90	95.56	94.74	95.08

Table 3

Mean Values (%, m/m) for the Content of Amoxicillin

Laboratory	Sample S2	S3	S4	S5
1	85.10 (0.6)	81.62 (0.9)	97.04 (0.4)	95.99 (0.3)
2	83.89 (0.7)	81.24 (0.6)	94.84 (0.5)	94.94 (1.4)
3	85.12 (0.3)	82.13 (0.9)	95.80 (1.1)	94.99 (1.0)
4	84.45 (0.8)	82.57 (4.3)	93.48 (4.0)	92.19 (1.7)
5	83.69 (0.8)	80.06 (0.4)	94.26 (1.0)	93.78 (0.5)
6	84.43 (0.8)	80.47 (0.6)	94.13 (3.0)	95.03 (1.1)
7	85.27 (0.1)	80.57 (0.8)	95.97 (0.9)	95.30 (0.6)
8	84.16 (0.4)	80.85 (0.3)	94.84 (0.2)	94.31 (0.1)
9	84.69 (1.1)	81.51 (1.1)	95.44 (0.5)	95.09 (0.2)
10	85.09 (0.7)	80.18 (0.7)	95.63 (1.3)	95.24 (1.2)
11	84.52 (0.4)	81.15 (0.4)	95.05 (0.3)	95.13 (0.4)
Mean of means	84.59 (0.6)	81.12 (1.0)	95.13 (1.0)	94.73 (1.1)

RSD values (%) are given in parentheses.

The coefficient of correlation r was calculated for a calibration curve determined in the range corresponding to 70-130 % of the amount prescribed for the assay. The intercept values were calculated as a per cent of the area corresponding to 100 %.

Samples were analysed three times, using independently prepared solutions. Individual results for the main component, expressed as % (m/m) amoxicillin or sodium amoxicillin, are listed in Table 2. Results for sample 1 are not reported since this was used as the reference substance. Means and RSD values are given in Table 3.

In order to analyse further the results obtained for the main component, a number of statistical calculations were performed following described procedures.[5,6] The ranked mean values were examined for outlying laboratories and also for outlying mean values by using Dixon's criterion.[5] No laboratory was excluded. For one mean (laboratory 4, sample 5) the limit (0.68) was slightly exceeded (0.69) at the 1 % level. Nevertheless, all the results were used in further calculations.

Table 4

Analysis of Variance

Source of Variance	Sum of Square	Degree of Freedom	Mean Square	Variance Ratio
Between laboratories (L)	40.07	10	4.01	L/LS = 2.60 F0.95(10,30 = 2.16 F0.99(10,30) = 2.98
Laboratory-sample interaction (LS)	46.34	30	1.54	LS/S = 1.22 F0.95(30,88) = 1.59
Between replicates (S)	111.43	88	1.27	

An analysis of variance was carried out in order to investigate for consistent laboratory bias or significant laboratory-sample interaction.[6] The results are listed in Table 4. There is no significance between laboratory variance at the 1 % level, so no consistent laboratory bias exists. The laboratory-sample interaction variance is not significant. Estimates of the repeatability of the LC method (within laboratory variance) and of the reproducibility (between laboratory variance) were calculated.[5] The standard deviation thus obtained was 1.1 and 1.3 respectively. Compared to the content of amoxicillin, both values are quite low and satisfactory for a LC method. This method will show greater variation when carried out by different laboratories than within one laboratory, but no consistent laboratory bias exists.

After performance of the gradient elution for each sample, the content of impurities was calculated by comparison with a 1:100 dilution of the reference solution. Participants were asked to report the retention time and percentage amount of each impurity. Impurities smaller than 0.05 percent were not reported. A typical chromatogram is shown in Figure 1.

Two laboratories (3 and 6) did not report results for the impurities. It was also observed that the retention times of the impurities varied with the different laboratory conditions and therefore it was not possible to identify all the corresponding peaks and to calculate a mean value and relative standard deviation for each peak separately. Table 5 reports for nine laboratories the sum of impurities for each sample and the mean. After each value the total number

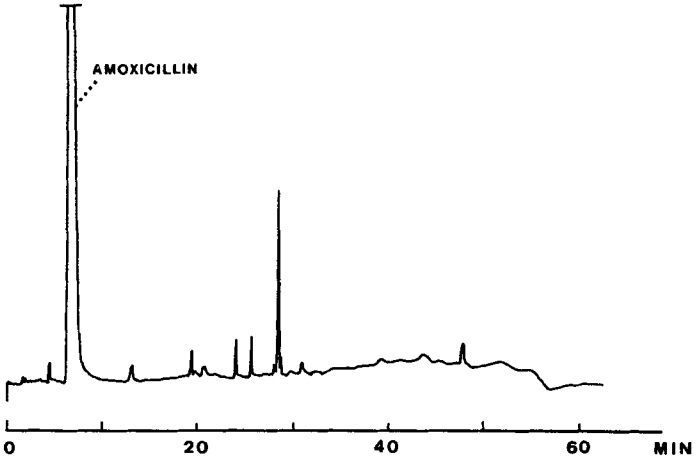

Figure 1. Typical chromatogram of sample 5, obtained in laboratory 1.

Table 5

Sum of Impurities (% m/m) for Each Sample

Laboratory	Sample S1	S2	S3	S4	S5
1	0.51 (6)	0.87 (8)	2.31 (14)	0.93 (5)	1.25 (8)
2	0.66 (8)	0.96 (10)	2.18 (14)	1.12 (6)	1.19 (6)
4	0.41 (4)	0.94 (7)	2.16 (10)	1.39 (6)	1.72 (6)
5	0.51 (5)	0.83 (7)	2.16 (12)	1.10 (5)	1.22 (5)
7	0.64 (7)	0.95 (11)	2.24 (11)	1.13 (7)	1.31 (7)
8	0.53 (8)	1.08 (9)	2.13 (13)	1.61 (9)	1.75 (7)
9	0.39 (2)	1.06 (8)	1.61 (8)	0.73 (2)	0.90 (4)
10	0.94 (8)	1.28 (10)	2.38 (12)	1.25 (5)	1.36 (6)
11	0.70 (6)	1.04 (9)	1.97 (11)	0.97 (6)	1.86 (7)
Mean	0.59	1.00	2.13	1.14	1.39
RSD%	29	13	10	23	22

The results from 9 laboratories were used. The number of impurities is given in parentheses.

Table 6

Composition (%, m/m) of the Amoxicillin Samples

Sample	LC: Amoxicillin	LC: Impurities	Water Content*	Total
1	86.14	0.59	13.27	100.0
2	85.72	1.00	13.10	99.82
3	82.21	2.13	12.94	97.28
4	96.41	1.14	0.60	98.15
5	96.00	1.39	0.78	98.17

The content of 86.14% is assigned to sample 1.
* Water was determined by Karl Fischer titration, for samples 2-5 the water content was determined only in laboratory 11.

of impurities is reported in parentheses. For amoxicillin trihydrate samples 1-3, no single impurity exceeded a value of 0.6% (m/m). For amoxicillin sodium samples 4 and 5, no single impurity exceeded a value of 0.7 % (m/m).

Using these results and results for the water content, obtained by Karl Fischer titration following the Ph. Eur.,[7] it was possible to calculate a value of 86.14 for the content of the proposed CRS. With this value the content of samples 2-5 was calculated from the LC results. Table 6 reports these results together with the LC results for impurities and water content. The total values show that LC combined with water content accounts for about 97.3 % or more of the total mass of these samples. The water content of samples 2-5 was determined in laboratory 11 only, due to lack of sample.

CONCLUSION

It can be concluded that the LC method shows a reproducible selectivity on different C_{18} columns and that the method is suitable not only for the assay of amoxicillin but also for the related substances test.

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