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Interlaboratory study of the analysis of ampicillin by liquid chromatography[1]

Yongxin Zhu[a], M.L. Moreno[b], E. Porqueras[b], E. Bourke[c], A. Bruzzi[c], M. Aletrari[d], P. Kanari[d], D. Partasidou[d], J. Nienhuis[e], W. Ferigo[f], J.L. Robert[f], J.H. McB. Miller[g], J.M. Spieser[g], E. Roets[a], J. Hoogmartens[a,*]

[a]*Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, K.U. Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium*
[b]*Centro National De Farmacobiologia, Madrid, Spain*
[c]*National Drugs Advisory Board, Dublin, Ireland*
[d]*State General Laboratory, Nicosia, Cyprus*
[e]*Rijksinstituut voor Geneesmiddelenonderzoek, Leiden, Netherlands*
[f]*Laboratoire National de Santé, Luxembourg, Luxembourg*
[g]*European Pharmacopoeia, Strasbourg, France*

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Abstract

A liquid chromatographic method for the analysis of ampicillin was examined in a collaborative study involving seven laboratories. The method included an isocratic part, which is used in the assay. The isocratic part is similar to the assay method for ampicillin of the US Pharmacopeia XXIII Revision. When the isocratic part is combined with gradient elution, the method is suitable for purity control. Six samples of ampicillin (anhydrous, trihydrate and sodium salt) with varying purity were analysed. The main component and related substances were determined. An analysis of variance proved the absence of consistent laboratory bias. The laboratory–sample interaction was significant. Estimates of the repeatability and reproducibility of the method, expressed as standard deviations of the result of the determination of ampicillin, were calculated to be about 0.9 and 1.1 respectively.

Keywords: Ampicillin; Liquid chromatography; Interlaboratory study

1. Introduction

Previously, the selectivity of four isocratic liquid chromatographic (LC) methods which were described for the assay of ampicillin was examined [1]. It was observed that the USP assay

* Corresponding author.

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method [2] with acetic acid (1.0 M)– potassium dihydrogenphosphate (1.0 M)–acetonitrile–water (1:10:80:909, v/v/v/v) as the mobile phase reproducibly gave good selectivity on different stationary phases. It was also suggested that cefradine may be used in the resolution test instead of caffeine, which was prescribed in the USP method. Based on the adapted USP method, a gradient elution method was 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 to 40% with linear gradient elution over 30 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 ampicillin was examined in seven laboratories. A sample of anhydrous ampicillin proposed as a 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.

2. Experimental

2.1. Samples and reagents

Six ampicillin samples, including two anhydrous samples (1 and 2), two trihydrates (3 and 4) and two sodium salts (5 and 6), of different origin were used. Sample 1 was the proposed CRS to which a provisional content of 98% was assigned for the purpose of this study. An anhydrous ampicillin was chosen as the proposed CRS since this is the most stable form. The two sodium salts were manufactured by different procedures. Sample 6 was obtained by freeze-drying and sample 5 was obtained by crystallization. Cefradine 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 acetic acid (2 M)–potassium dihydrogenphosphate (0.2 M)–acetonitrile–water (0.5:50:50: up to 1000, v/v/v/v) and mobile phase B was a mixture of the same components in different proportions (0.5:50:400: up to 1000, v/v/v/v). The pH of the mobile phases was not adjusted.

Samples were prepared with mobile phase A as the solvent at the following concentrations: for assay, anhydrous ampicillin 27, ampicillin trihydrate 31 and ampicillin sodium 29 mg per 50 ml; and for purity testing, anhydrous ampicillin 27, ampicillin trihydrate 31 and ampicillin sodium 29 mg per 10 ml. The resolution test solution was prepared by mixing 5.0 ml of cefradine CRS solution (2 mg per 50 ml) with 5.0 ml of ampicillin solution (sample 1, 27 mg per 50 ml).

2.2. Materials and methods

The equipment consisted of a solvent-delivery system capable of developing gradient elution with a flow rate of 1.0 ml min^{-1}, a fixed-loop injector with a loop of about 50 μl, a UV detector set at 254 nm and an integrator allowing peak-area measurements. Different brands of C_{18} stationary phases of 5 μm particle size were used and all columns measured 25 × 0.46 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 and all other columns were prepacked. The column temperature was ambient.

For the assay, isocratic elution was used with a mobile phase ratio A:B of 85:15. The composition of the isocratic mobile phase was adjusted in order to have a capacity factor of 1.7–2.6 for ampicillin and a resolution between ampicillin and cefradine of at least 3. For the related substances test, isocratic elution combined with gradient elution was used as follows: after isocratic elution of the ampicillin peak with a mobile phase ratio A:B as used in the assay, linear gradient elution was started to reach a mobile phase ratio A:B of 0:100 over a period of 30 min; this ratio was held for 15 min, then the column was equilibrated with a mobile phase ratio A:B of 85:15 for 15 min.

Table 1
General information on columns and method performance[a]

L	C	Stationary phase	P	Amount of acetonitrile in mobile phase (% v/v)	k' (AM)	S (AM)	n (AM)	R_s (AM–CE)	Repeatability (AM) ($n = 6$)			
									Peak area RSD (%)	Retention time (min)		Linearity, r (AM)
										Mean	RSD (%)	
1	a	Hypersil C_{18}[b]	5	10.3	2.1	0.68	2130	5.8	0.83	9.6	0.24	0.9991
2	b	Hypersil C_{18}	5	12.0	2.1	0.64	2680	6.0	0.27	7.2	0.42	0.9998
3	c	Nucleosil C_{18}	5	10.3	2.2	0.75	5560	7.2	0.22	9.2	0.29	0.9999
4	d	Nucleosil C_{18}	5	10.3	2.5	1.0	6940	7.4	0.90	10.0	2.15	0.9993
5	e	Spherisorb ODS-2	5	10.3	2.1	0.59	3520	5.9	0.38	7.2	0.07	0.9995
6	f	Nucleosil C_{18}	5	10.3	1.7	0.66	7580	9.0	0.34	11.9	0.80	0.9999
7	g	Kromasil C_{18}	5	10.3	2.6	0.75	4250	7.8	0.18	6.4	0.45	0.9960

[a] L = laboratory; C = column; P = particle size (μm); k' = capacity factor; S = symmetry factor; n = theoretical plate number; R_s = resolution; AM = ampicillin; CE = cefradine; RSD = relative standard deviation; r = correlation coefficient for AM in the range 70–130%
[b] Laboratory-packed column; all other columns were prepacked

Table 2
Individual values (% w/w) for the content of ampicillin

Laboratory	Sample														
	S2			S3			S4			S5			S6		
1	96.77	95.98	97.10	82.64	82.82	83.22	83.41	82.11	83.74	96.03	95.94	96.60	89.15	89.87	89.82
2	96.22	97.85	96.99	83.52	83.76	83.29	82.68	82.79	82.35	94.94	96.53	96.45	88.85	90.57	90.04
3	96.45	96.09	96.68	82.98	82.39	83.06	83.19	82.65	83.60	96.47	96.02	95.78	92.12	91.40	89.27
4	93.97	93.58	94.25	84.45	81.65	81.69	83.06	82.07	82.64	94.02	95.60	95.81	90.17	90.36	88.56
5	97.27	96.97	96.72	80.96	81.74	81.77	81.81	82.63	79.62	91.81	95.66	94.75	87.57	90.72	88.50
6	97.20	96.93	96.17	84.40	84.02	82.79	83.78	84.24	82.57	92.97	95.32	93.64	90.76	92.05	90.85
7	95.38	96.66	97.23	84.35	83.22	83.79	82.45	83.49	83.47	96.54	95.73	95.80	90.40	90.50	90.76

Table 3
Mean values (% w/w) for the content of ampicillin[a]

Laboratory	Column	Sample				
		S2	S3	S4	S5	S6
1	a	96.59 (0.6)	82.89 (0.4)	83.09 (1.0)	96.19 (0.4)	89.61 (0.4)
2	b	97.02 (0.8)	83.52 (0.3)	82.61 (0.3)	95.97 (0.9)	89.82 (1.0)
3	c	96.41 (0.3)	82.81 (0.4)	83.15 (0.6)	96.09 (0.4)	90.93 (1.6)
4	d	93.93 (0.4)	82.60 (1.9)	82.59 (0.6)	95.14 (1.0)	89.70 (1.1)
5	e	96.99 (0.3)	81.49 (0.6)	81.35 (1.9)	94.07 (2.1)	88.93 (1.8)
6	f	96.77 (0.5)	83.74 (1.0)	83.53 (1.0)	93.98 (1.3)	91.22 (0.8)
7	g	96.42 (1.0)	83.79 (0.7)	83.14 (0.7)	96.02 (0.5)	90.55 (0.2)
Mean of means		96.30 (1.1)	82.97 (1.0)	82.78 (0.8)	95.35 (1.0)	90.11 (0.9)

[a] RSD values (%) are given in parentheses.

Table 4
Analysis of variance

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between laboratories (L)	29.73	6	4.95	L/LS = 2.92 F 0.95(6,24) = 2.51 F 0.99(6,24) = 3.74
Laboratory–sample interaction (LS)	40.79	24	1.70	LS/S = 2.25 F 0.99(24,70) = 2.07
Between replicates (S)	52.82	70	0.75	

3. Results and discussion

One column was used in each of the seven laboratories. General information about the columns, composition of the isocratic mobile phase and results of performance checks carried out by each laboratory is given in Table 1. The calculation of the chromatographic parameters was carried out according to the Ph. Eur. [4]. the capacity factor, k', symmetry factor, S, and theoretical plate number, n, were calculated for the ampicillin peak. After six consecutive injections the relative standard deviation (RSD) was calculated for the area of the ampicillin peak and for its retention time. The correlation coefficient r was calculated for a calibration curve determined in the range corresponding to 70–130% of the amount prescribed for the assay. The intercept was not significant compared with the standard error estimate ($S_{y,x}$) except in laboratory 2, where the intercept was 2.3% of the area compared with 100%, and in laboratory 6, where the intercept was 3.5% of the area compared with 100%.

Samples were analysed three times, using independently prepared solutions. Individual results for the main component, expressed as % (w/w) ampicillin or sodium ampicillin, 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 vlaues by using Dixon's criterion [5]. No laboratory was excluded. For one mean (laboratory 4, sample 2) the limit was slightly exceeded at the 1% level. Nevertheless, all the results were used in further calculations.

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 significant

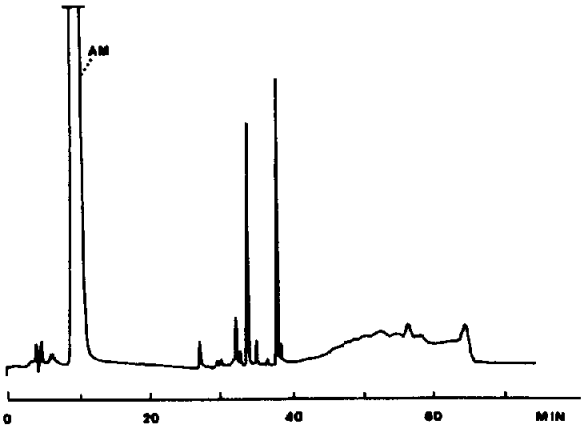

Fig. 1. Typical chromatogram of sample 6. AM = ampicillin.

between–laboratory variance at the 1% level but at the 5% level the limit is exceeded. The laboratory–sample interaction variance is significant at the 1% level. This means that the analytical method will show greater variation when carried out by different laboratories than within one laboratory, but no consistent laboratory bias exists. Estimates of the repeatability of the LC method (within-laboratory variance) and of the reproducibility (between-laboratory variance) were calculated [5]. The standard deviations thus obtained were 0.9 and 1.1, respectively. Compared with the content of ampicillin, both values are low and satisfactory for a LC method.

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% were not reported. A typical chromatogram is shown in Fig. 1.

Table 5
Sum of impurities for each sample[a]

Laboratory	Sample					
	1	2	3	4	5	6
1	1.49 (5)	2.58 (5)	1.82 (5)	1.76 (6)	2.27 (9)	7.48 (10)
2	1.35 (5)	2.56 (5)	2.09 (6)	1.60 (9)	2.01 (12)	7.88 (12)
5	1.34 (3)	2.47 (3)	1.74 (2)	1.27 (5)	1.54 (6)	10.05 (10)
6	1.60 (4)	2.89 (7)	2.16 (6)	2.40 (12)	2.09 (10)	8.51 (12)
7	1.40 (4)	2.34 (3)	1.88 (5)	1.65 (6)	1.70 (7)	8.39 (10)
Mean	1.44	2.57	1.94	1.74	1.92	8.46
RSD (%)	7.5	7.9	9.1	24	15	11

[a] The results from five laboratories were used. The number of impurities is given in parentheses.

Table 6
Composition of the ampicillin sample[a]

Sample	LC: ampicillin	LC: impurities	Water content[b]	Total
1	98.28	1.44	0.28	100.0
2	96.57	2.57	0.59	99.73
3	83.21	1.94	13.44	98.59
4	83.02	1.74	13.13	97.89
5	95.62	1.92	0.66	98.20
6	90.37	8.46	0.58	99.41

[a] The content of 98.28% is assigned to sample 1.
[b] Water was determined by Karl Fischer titration; for samples 2–6 the water content was determined only in laboratory 7.

Two laboratories (3 and 4) had problems with the integration of small peaks on the slope of the gradient; the area was clearly overestimated and therefore their results were rejected. 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 RSD for each peak separately. Table 5 reports for five laboratories the sum of impurities for each sample and the mean. After each value the total number of impurities is given in parentheses. For ampicillin (anhydrous and trihydrate), no single impurity exceeded a value of 2.0% (w/w). For ampicillin sodium sample 5, no single impurity exceeded a value of 1.0% (w/w), but for the freeze-dried sample 6, one single impurity, corresponding to the ampicillin dimer, reached a value of 3.9% (w/w), another reached a value of 4.0% (w/w), whereas the others did not exceed 0.9% (w/w).

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 98.28% for the content of the proposed CRS. With this value, the contents of samples 2–6 were calculated from 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 explains about 98% or more of the total mass of these samples. The water content of samples 2–6 was determined in one laboratory only, owing to lack of sample.

4. Conclusion

The LC method is suitable not only for the assay of ampicillin but also for the related substances test.

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