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Interlaboratory study of the analysis of benzylpenicillin by liquid chromatography

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Abstract

A liquid chromatography method for analysis of benzylpenicillin was examined in a collaborative study involving seven laboratories. The method comprised an isocratic part, which is used in the assay. The isocratic part corresponds to the assay method for benzylpenicillin used by a manufacturer. When the isocratic part is combined with gradient elution, the method is suitable for purity control. Five samples of benzylpenicillin (sodium and potassium salts) were analysed. The main component and the impurities were determined. An analysis of variance proved the 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 (S.D.) of the result of the determination of benzylpenicillin, were calculated to be 0.71 and 0.80, respectively. © 1998 Elsevier Science B.V.

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1. Introduction

The selectivity of seven isocratic liquid chromatography (LC) methods which were reported for the assay of benzylpenicillin had been previously examined [1]. It was observed that the method using a mobile phase of 0.05 *M* phosphate buffer (pH 3.5)–methanol (64:36) consistently gave good selectivity on different stationary phases. It had also been suggested that phenylacetic acid may be used in the resolution test. Based on this method, a gradient elution method had been developed, which was shown to be suitable as a related substances test. Indeed, it was possible to elute more strongly retained impurities by increasing the methanol content of the mobile phase from 36% to 50% with a

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linear gradient over 20 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 benzylpenicillin was examined by seven laboratories.

2. Experimental

2.1. Samples and reagents

Five benzylpenicillin samples including one sodium salt (1) and four potassium salts (2, 3, 4 and 5) of different origin were made available by the European Pharmacopoeia (Strasbourg, France). Sample 1 was used as the reference substance to which a content of 96.3% was assigned for the purpose of this study. Phenylacetic acid was used in the resolution test.

Solvents and reagents were of Ph. Eur. quality [2]. Two mobile phases were used. Mobile phase A was a mixture of 0.5 *M* phosphate buffer (pH 3.5)–methanol–water (10:30:60, v/v/v) and mobile phase B was a mixture of the same components with a ratio (10:50:40, v/v/v).

Samples were prepared with water at the following concentrations, for assay: 50 mg/50 ml, for purity testing: 80 mg/20 ml. The resolution test solution was prepared by dissolving 10 mg of phenylacetic acid CRS and 10 mg of benzylpenicillin CRS (sample 1) in 50 ml of water.

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, an injector with a loop of about 20 μl, except for laboratory 2 using a 8-μl loop for the assay, a UV detector set at 225 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× 0.46 cm I.D. except for laboratory 5 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 laboratory 2 where a temperature of 50°C was used for the assay.

For the assay isocratic elution was used with a mobile phase ratio A:B of 70:30. The composition of the isocratic mobile phase was adapted in order to have a capacity factor of 4.0 to 6.0 for benzylpenicillin and a resolution between benzylpenicillin and phenylacetic acid of at least 6.0. For the related substances test, isocratic elution combined with gradient elution was performed as follows: after isocratic elution of the benzylpenicillin peak with a

Table 1
General information on columns and method performance

L	Stationary phase[a]	Amount (%) of methanol in mobile phase	k' PG	S PG	N PG	R_s PG-PH	Repeatability PG (N=6)			Linearity r PG	Intercept I(%)
							Peak area R.S.D. %	Retention time (min)			
								Mean	R.S.D. %		
1	Hypersil C_{18}[b]	36	4.8	1.3	7110	10	0.12	14.1	0.07	0.9999	1.8
2	Nucleosil C_{18}[c]	36	5.5	0.8	8000	9.5	0.39	11.1	0.14	0.9996	1.6
3	Hypersil C_{18}	38	5.4	1.5	6850	9.9	0.18	17.7	0.45	0.9998	0.6
4	Supelcosil C_{18}	36	5.4	2.1	5350	9.2	0.25	15.6	0.14	0.9996	1.2
5	Hypersil C_{18}	36	4.4	1.5	7290	7.5	0.17	10.7	0.10	0.9999	1.1
6	Hypersil C_{18}	38	4.3	1.1	11 770	8.3	0.22	12.7	0.15	0.9999	6.3
7	Hypersil C_{18}	36	6.0	1.2	7980	11	0.25	18.9	1.09	0.9995	1.3

L=laboratory; k'=capacity factor; S=symmetry factor; N=theoretical plate number; R_s=resolution; PG=benzylpenicillin; PH=phenylacetic acid; R.S.D.=relative standard deviation; r=coefficient of correlation for PG 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 50°C.

Table 2
Individual values (%, m/m) for the content of benzylpenicillin

Laboratory	Samples											
	S2			S3			S4			S5		
1	99.9	100.0	99.7	98.6	98.2	98.1	100.4	100.2	99.6	98.4	98.3	97.7
2	99.3	99.5	99.4	98.3	97.8	98.3	100.2	99.8	99.4	97.7	97.7	97.8
3	100.5	101.6	100.4	99.0	99.4	99.5	97.1	98.9	99.0	98.8	99.3	97.0
4	99.4	99.4	99.8	98.3	97.9	98.7	100.2	100.3	100.3	97.7	97.7	97.8
5	99.8	100.0	100.4	99.9	99.9	98.7	100.1	100.2	100.7	98.6	97.7	98.2
6	99.7	99.6	99.5	99.0	99.0	98.1	99.8	100.6	97.4	96.0	97.7	100.2
7	99.1	99.1	99.4	97.7	97.6	99.7	100.1	98.8	99.7	97.4	97.2	97.4

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 20 min, this ratio was held for 15 min, then the column was equilibrated with a mobile phase ratio A:B of 70:30 during 15 min.

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 are shown in Table 1. The calculation of the chromatographic parameters was carried out according to the Ph. Eur. [3]. The capacity factor k', the symmetry factor S, and the theoretical plate number N were calculated for the benzylpenicillin peak. After six consecutive injections of sample 1, the relative standard deviation was calculated for the area of the benzylpenicillin peak and for its retention time. The coefficient of correlation r was calculated for a calibration curve determined at concentrations corresponding to 70, 100 and 130% of the amount prescribed for the assay. The total number of analyses was 14. The intercept values were calculated as a percentage of the area corresponding to 100%. Laboratory 6 reported a high value for the intercept, which was probably due to overloading of the detector.

Samples were analysed three times, using independently prepared solutions. Individual results for the main component, expressed as % (m/m) benzylpenicillin sodium and potassium salt, are listed in Table 2. Results for sample 1 are not reported since this was used as the reference substance. Means and R.S.D. 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 the procedures described in Refs. [4,5] by Youden and Steiner, and Wernimont. The individual results were first examined for outliers by using Dixon's criterion [5].

Table 3
Mean values (%, m/m) for the content of benzylpenicillin

Laboratory	Sample			
	S2	S3	S4	S5
1	99.9 (0.5)	98.3 (0.3)	100.1 (0.4)	98.1 (0.4)
2	99.4 (0.1)	98.2 (0.3)	99.8 (0.4)	97.7 (0.1)
3	100.8 (0.7)	99.3 (0.3)	98.3 (1.1)	98.4 (1.2)
4	99.5 (0.2)	98.3 (0.4)	100.2 (0.1)	97.7 (0.1)
5	100.1 (0.3)	99.5 (0.7)	100.3 (0.3)	98.2 (0.4)
6	99.6 (0.1)	98.7 (0.5)	99.2 (1.7)	97.9 (2.1)
7	99.2 (0.2)	98.3 (1.2)	99.5 (0.7)	97.4 (0.2)
Mean of means	99.8 (0.5)	98.7 (0.5)	99.6 (0.7)	97.9 (0.3)

R.S.D. 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)	6.58	6	1.10	L/LS=1.32 *F* 0.95(6,18)=2.66
Laboratory–sample interaction (LS)	14.99	18	0.83	LS/S=1.63 *F* 0.95(18,56)=1.79
Between replicates (S)	28.27	56	0.51	

The lower value (98.7%) obtained for sample 3 in laboratory 5 was calculated to be an outlier, but given that only three values were available, all results were retained. Moreover, Table 3 shows that the so-called outlier is the value closest to the grand mean for sample 3. Cochran's test was performed for outlying mean values [6]. The test value for the standard deviations in laboratory 6 for sample 5 was 0.707, while the critical value (1%) is 0.664 ($N=3$, $P=7$). So the mean value in laboratory 6 for sample 5 is an outlier. Nevertheless all results were used for further calculation. The ranked mean values were examined for outlying laboratories [4]. No laboratory was excluded.

An analysis of variance was carried out in order to investigate for precision estimates or significant laboratory–sample interaction [5]. The results are listed in Table 4. There is no significance between laboratory variance at the 5% level. The laboratory–sample interaction variance is also not significant at the 5% level. Estimates of the repeatability of the LC method and of the reproducibility were calculated [4]. The standard deviations thus obtained were 0.71 and 0.80, respectively. Both values are quite low and very satisfactory for a LC method.

After performance of the gradient elution for the injection of water and each sample, the content of impurities was calculated by comparison with a 1:100 dilution of the sample 1 solution and also blank injection. 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 Fig. 1.

One laboratory (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 the sum of impurities for each sample and the mean for six laboratories. After each value the total number of impurities is reported in parentheses. For benzylpenicillin potassium samples 2–5, no single impurity exceeded a value of 0.21% (m/m). The results show that the sum of impurities may vary

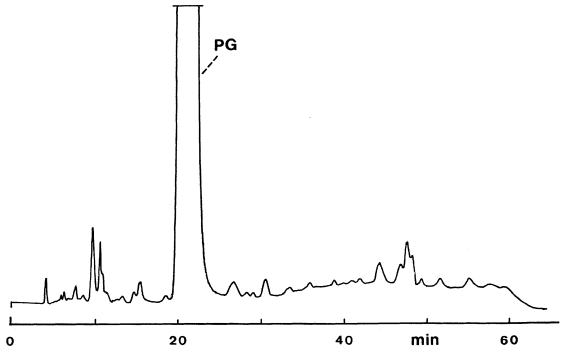

Fig. 1. Typical chromatogram of sample 1, obtained in laboratory 2.

Table 5
Sum of impurities (%, m/m) for each sample

Laboratory	Sample			
	S2	S3	S4	S5
1	0.1 (1)	0.3 (4)	0.1 (1)	0.2 (3)
2	0.1 (1)	0.6 (5)	0.1 (1)	0.6 (6)
3	0.1 (1)	0.4 (3)	0.00	0.1 (2)
4	0.1 (1)	0.5 (5)	0.1 (1)	0.6 (6)
5	0.1 (1)	0.6 (6)	0.1 (2)	0.5 (5)
7	0.2 (2)	0.6 (7)	0.1 (1)	0.1 (1)
Mean	0.1	0.5	0.1	0.4

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

Table 6
Composition (%, m/m) of the benzylpenicillin samples

Sample	LC: benzylpenillin	LC: impurities	Loss on drying[a]	Total
2	99.8	0.1	0.5	100.4
3	98.7	0.5	0.1	99.3
4	99.6	0.1	ND	99.7
5	97.9	0.4	0.2	98.5

ND=not determined due to lack of sample.
[a] the loss on drying was determined only in laboratory 7.

considerably. This is due to the fact that peaks below 0.05% were disregarded and that borderline values around 0.05% may be interpreted differently by laboratories. This will not cause a problem with samples containing more considerable amounts of impurities.

Using LC results for the main component and impurities together with the results for the loss on drying, obtained according to the Ph. Eur. [7], Table 6 reports the total composition for samples 2–5. The loss on drying of samples 2, 3 and 5 was determined in laboratory 7 only, because of lack of sample. The total values show that LC combined with loss on drying accounts for about 98.5% or more of the total mass of these samples.

4. 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 benzylpenicillin but also for the purity testing.

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