Evaluation of liquid chromatography methods for the analysis of benzylpenicillin and its related substances

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Abstract

Seven isocratic liquid chromatography (LC) methods have been examined for the separation of benzylpenicillin and its related substances on C_{18} or C_8, phenyl and poly(styrene–divinylbenzene) columns. The method proposed by a manufacturer, using a C_8 column and a mobile phase consisting of 0.05 M phosphate buffer, pH 3.5–methanol (62:38, v/v) gave the better selectivity. The amount of organic modifier was slightly adapted when it was used with different columns. Similar selectivity was obtained not only on C_8 materials but also on C_{18} packings. The selectivity on poly(styrene–divinylbenzene) was worse. A resolution test with phenylacetic acid was used as the method performance test. The robustness of this method was examined by applying a full factorial design to test the influence of content of organic modifier in the mobile phase, the pH of the mobile phase, the concentration of buffer in the mobile phase and the column temperature. The results show that the method is robust.

Keywords: Benzylpenicillin; Antibiotics

1. Introduction

Benzylpenicillin (Penicillin G; PG) is a natural penicillin produced by *Penicillin chrysogenum* with activity against Gram-positive bacteria, *Neisseria* and *Treponema pallidum*, the causative agent of syphilis. PG may contain precursors, side products from the biosynthesis and various degradation products, some of which are regarded as allergenic products. Fig. 1 shows the structures of PG and a number of related substances, which were available. Liquid chromatography (LC) has often been re-

ported for the analysis of PG [1–12]. However, the separation of PG and its related substances and the reproducibility of the selectivity on different columns have not been discussed sufficiently.

The aim of this study was to determine whether or not an existing assay method was sufficiently selective and robust and whether it could be adapted in order to be suitable as a related substances test. Seven isocratic LC methods were examined. Four of them are from manufacturers, one was described by Vadino et al. [1], one was described by Lipczynski [8] and the last is the USP 23 assay method of PG potassium injection [12]. Table 1 shows the LC conditions of the seven methods. Four methods use
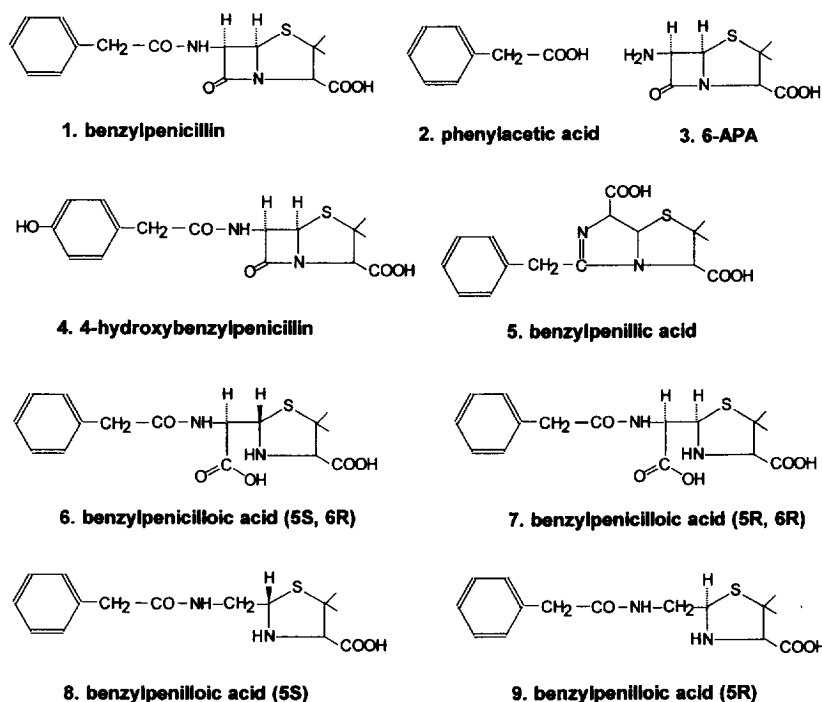
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Fig. 1. Structures of benzylpenicillin and its related substances.

Table 1
LC conditions for seven isocratic LC methods

Method and source	Mobile phase prescribed	Stationary phase prescribed[a]	Column temperature prescribed (°C)	Flow-rate (ml/min) prescribed[b]	Detection UV (nm) prescribed[c]	Column temperature used (°C)
1 Manufacturer 1	Phosphate buffer 0.02 M, pH 7.0–CH₃CN (85:15, v/v)	C_{18}, 5 μm	50	2	220	50
2 Vadino	Phosphate buffer 0.08 M, pH 4.15–CH₃CN (80:20, v/v)	C_{18}, <10 μm	ambient	0.75	254	30
3 Lipczynski	Phosphate buffer 0.05 M, pH 3.8–tetrahydrofuran (95:5, v/v)	C_{18}, 5 μm	25	1	230	30
4 USP	Phosphate buffer 0.08 M, pH 4.15–CH₃OH (55:45, v/v)	C_{18}, 5 μm	ambient	1	225	30
5 Manufacturer 2	Phosphate buffer 0.02 M, pH 6.5–CH₃CN (68:32, v/v)	C_8	not described	not described	258	30
6 Manufacturer 3	Phosphate buffer 0.02 M, pH 7.0–H₂O–CH₃CN (42:43:15, v/v/v)	Phenyl	ambient	1	228	30
7 Manufacturer 4	Phosphate buffer 0.05 M, pH 3.5–CH₃OH (62:38, v/v)	C_8	40	0.4	225	30 (C_{18}) 40 (C_8)

[a]Stationary phase used=stationary phase prescribed (method 7 also performed on C_{18} and polymer stationary phases).
[b]Flow-rate used, 1 ml/min.
[c]UV detection performed at 254 nm.

C_{18} stationary phases, two methods use C_8 stationary phases and one uses a phenyl stationary phase. In order to have more homogeneous conditions during the comparison, the described conditions for column temperature, flow-rate and detection wavelength were slightly adapted in our study, as shown in Table 1.

2. Experimental

2.1. Samples

PG is commercially available (Hoechst, Frankfurt, Germany). 6-Aminopenicillanic acid (6-APA, **2**) (Gist-Brocades, Delft, Netherlands) and phenylacetic acid (**3**) (Acros Chimica, Geel, Belgium) are the basic constituents of PG. 4-Hydroxybenzylpenicillin (**4**) can arise from the biosynthesis of PG. Related substance **4** was prepared by condensation of 4-hydroxyphenylacetic acid and 6-APA. The other related substances are decomposition products. Benzylpenillic acid (**5**) was prepared as described [13]. Benzylpenicilloic acid (5S, 6R) (**6**), benzylpenicilloic acid (5R, 6R) (**7**), benzylpenilloic acid (5S) (**8**) and benzylpenilloic acid (5R) (**9**) were prepared as described [14].

2.2. Solvents and reagents

Acetonitrile and tetrahydrofuran (HPLC grade) were from Rathburn (Walkerburn, UK). Methanol (Roland, Brussels, Belgium) was distilled before use. Phosphoric acid, potassium dihydrogen phosphate

and dipotassium hydrogen phosphate were from Acros Chimica. Water was distilled twice.

2.3. LC apparatus and columns

The equipment consisted of an L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa-N60 Valco injector (Houston, TX, USA), with a 20-μl loop, a Model L-4000 UV detector (Merck-Hitachi) and an integrator Model 3396 series II (Hewlett-Packard, Avondale, PA, USA). Most of the experiments were carried out in laboratory A (Leuven) using this equipment, but a number of experiments were repeated in laboratory B (Madrid), to examine for reproducibility. The equipment used in laboratory B was of similar quality. The columns (250×4.6 mm I.D.) used in this study are reported in Table 2. The column used in laboratory B is identified with an asterisk. Columns were immersed in a water bath at 30°C, unless stated otherwise.

2.4. Mobile phase and sample preparation

The mobile phases were prepared as described in Table 1. For some columns, the amount of organic modifier was slightly adapted in order to obtain a similar retention time for PG. The exact amount of organic modifier is reported in Table 3. In this study, water was used to dissolve the samples, and it was shown that a PG solution in water did not decompose significantly over a period of 20 h. For the selectivity study, the following concentrations were used: PG sodium salt, 2 mg/ml; related substances **2** and **5**, 0.2 mg/ml; **3**, **4**, **6** and **7**, 0.5 mg/ml; **8** and **9**, 0.25

Table 2
General information on columns

Columns	Stationary phases	Particle size (μm)
A, B[a]	Hypersil ODS (Shandon, Runcorn, UK)	5
C	Bio-Sil C_{18} (Bio-Rad, Nazareth, Belgium)	10
D	Spherisorb ODS-1 (Phase Separations, Queensferry, UK)	7
E	Zorbax C_8 (Du Pont, Wilmington, DE, USA)	5
F	Chromspher C_8 (Chrompack, Middelburg, Netherlands)	8
G	RoSil C_8 (Alltech, Deerfield, IL, USA)	10
H	Rsil Phenyl (Alltech, Deerfield, IL, USA)	10
I	PLRP-S (Polymer Laboratories, Church Stretton, UK)	7-9
J	PRP-1 (Hamilton, Reno, NV, USA)	8

[a]Column used in laboratory B.

Table 3
Concentrations of organic modifier for all methods and all columns

Column	Method						
	1	2	3	4	5	6	7
A	11	16	12	35			36
B[a]	13	17		35			38
C	7	16	10	28			30
D	6	12	7	26			26
E					8		38
F					12		26
G					14		30
H						15	
I							45.5
J							44

[a]Column used in laboratory B.

mg/ml. For the resolution test, the concentrations of PG and phenylacetic acid were 1 mg/ml each. For the robustness test, the concentrations of PG and related substances were 0.25 mg/ml each.

3. Results and discussion

3.1. Selectivity of the LC methods

During preliminary work, three different C_{18} stationary phases (A, C and D) were used for the selectivity study of methods 1, 2, 3 and 4 and three different C_8 stationary phases (E, F and G) were used for the selectivity study of methods 5 and 7 and one phenyl stationary phase (H) for method 6. Method 4 is similar to method 2, except that methanol is used as the organic modifier instead of acetonitrile. There is also a small difference in buffer concentration. The experiments for methods 1, 2, 4 and 7 were repeated on one C_{18} column in laboratory B. The concentration of the organic modifier in the mobile phase was adjusted for each column in order to obtain similar retention times for PG (see Table 3). For methods 1 and 2, methods 3 and 4 and methods 5 and 6, the results are shown in Figs. 2–4, respectively. It can be seen that PG is completely separated from its related substances with all the methods and on all the columns. In Figs. 2–4, it can be seen that for the same method, the sequence of elution of related substances can be different on different columns, which is common on silica

bonded reversed phases. After the preliminary work, it was clear that the methods from Vadino et al. ([1]; method 2), Lipczynski ([8]; method 3), the USP method ([12]; method 4) and method 7 gave the most satisfactory selectivity. Method 7 (see Fig. 5) was considered better than the others because the related substances are reasonably well separated from each other. The selectivity of method 3 is quite good also but it uses tetrahydrofuran as the organic modifier, which can lead to stability problems. Therefore, method 7 was retained for further work.

3.2. Further evaluation of method 7

3.2.1. Selectivity and resolution test

Method 7 was further examined with C_{18} columns and with poly(styrene–divinylbenzene) columns. The capacity factors are shown in Fig. 5. Columns A, B and C were used at 30°C instead of 40°C. The latter temperature was used for C_8 columns because it was prescribed by the original method. Columns I and J were used at 50°C. For polymer columns, higher temperatures are used to improve the efficiency. The elution pattern on the polymer columns is very different and the overall selectivity is worse. Therefore, these columns were not considered further. On all C_{18} and C_8 columns, PG is separated from its related substances. There is always a reasonable separation between the related substances on both C_8 and C_{18} columns. The elution pattern is not always the same. As a resolution test, or system suitability test, the separation between PG and phenylacetic acid (2) was chosen. Although the penilloic acids (8 and 9) are eluted closer to PG, 2 was chosen because 8 and 9 are not commercially available. Other commercial penicillins tested were eluted too far from PG. Table 4 shows general information on method performance using method 7. The different parameters were calculated according to the European Pharmacopoeia [15]. Because good results were obtained with C_{18} columns and because C_{18} columns are more frequently used than C_8 columns, we chose to continue with C_{18} columns. A typical LC chromatogram of PG and its related substances obtained according to method 7 on a Hypersil C_{18} column is shown in Fig. 6.

Method 1

$$\blacktriangledown PG \;\text{−}2\; \ast 3 \;\blacksquare 4 \;\times 5 \;\blacklozenge 6 \;\blacktriangle 7 \;\overline{X} 8 \;\bullet 9$$

Column

A

B*

C

D

0   2   4   6   8   10   k'

Method 2

$$\blacktriangledown PG \;\text{+}2\; \ast 3 \;\blacksquare 4 \;\times 5 \;\blacklozenge 6 \;\blacktriangle 7 \;\overline{X} 8 \;\bullet 9$$

Column

A

B*

C

D

0   2   4   6   8   k'

Fig. 2. Capacity factors of benzylpenicillin and its related substances according to methods 1 and 2.

3.2.2. Linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ)

Quantitative aspects of method 7 were examined using a Hypersil C_{18} column. For the linearity test, 12, 16, 20, 24 and 28 μg of PG were analysed. The 20 μg amount was injected six times, the other amounts three times. The following equation was obtained: $y=325.1x-127.6$, with $y=$peak area/1000 and $x=$amount injected (μg), with a correlation coefficient (r) of 0.9993, a standard error of estimate

Method 3

▼ PG — 2 ✳ 3 ■ 4 ✕ 5 ♦ 6 ▲ 7 ⊠ 8 ● 9

Column

A

C

D

```
0        2        4        6        8  k'
```

Method 4

▼ PG — 2 ✳ 3 ■ 4 ✕ 5 ♦ 6 ▲ 7 ⊠ 8 ● 9

Column

A

B*

C

D

```
0        2        4        6        8  k'
```

Fig. 3. Capacity factors of benzylpenicillin and its related substances according to methods 3 and 4.

$(S_{y.x})$ of 61.5 for the intercept and a standard error of 3.0 for the slope. The repeatability was checked by analysing the same solution (20 μg injected) six times. The relative standard deviation (R.S.D.) on the PG peak was 0.29%. In a related substances test, 80 μg of PG may be injected in order to better detect

Methods 5 and 6

▼PG ─ 2 ✳3 ▪ 4 ✕5 ◆ 6 ▲7 𝕏 8 ●9

Column

Fig. 4. Capacity factors of benzylpenicillin and its related substances according to methods 5 and 6. Columns E, F and G were used in method 5, column H was used in method 6.

Method 7

▼PG ─ 2 ✳3 ▪ 4 ✕5 ◆ 6 ▲7 𝕏 8 ●9

Column

Fig. 5. Capacity factors of benzylpenicillin and its related substances according to method 7.

the impurities. For this quantity, the LOD of PG, with $S/N=3.5$, was 0.025% (0.02 μg). The LOQ was 0.05% (0.04 μg) ($n=6$, R.S.D.=11%).

3.3. Robustness of method 7

The robustness of method 7 was examined by

Table 4
General information on method performance using method 7

Column	k' Penicillin G	S Penicillin G	N Penicillin G	R_s Penicillin G phenylacetic acid
A	5.90	1.33	5770	8.4
B [a]	5.66	2.72	15 170	10.3
C	4.34	1.65	2320	7.1
D	5.34	1.42	1330	6.1
E	5.24	2.07	1430	7.0
F	4.89	2.08	1850	7.3
G	4.36	1.83	2150	6.3

k'=capacity factor; S=symmetry factor; N=number of theoretical plates; R_s=resolution.
[a]Column used in laboratory B.

applying a full factorial experimental design [16,17]. Robustness is an important aspect of method validation [18]. One evaluates the influence of small changes in the operating or environmental conditions (variables) of the analytical procedure on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is transferred between different laboratories, different experimenters, or using different equipment [19].

In this study, four important chromatographic parameters (variables), governing the separation process, were examined (low and high values are mentioned in parentheses): The concentration of methanol (34%, 38%) as organic modifier in the mobile phase; the pH of the buffer (3.0, 4.0); the concentration of phosphate buffer (6%, 14%) and the column temperature (25°C, 35°C). The measured responses were the retention times of PG (1), phenylacetic acid (2), benzylpenicilloic acids (6, 7)
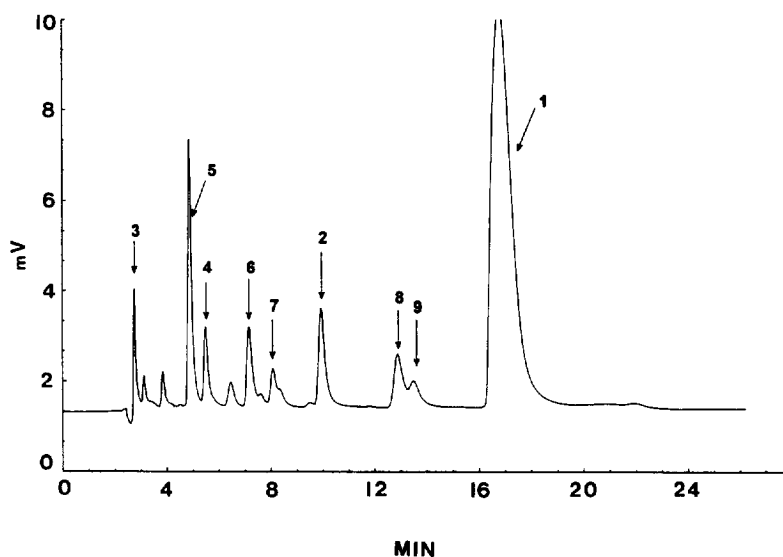

Fig. 6. Typical chromatogram of benzylpenicillin and its related substances according to method 7. Mobile phase, 0.5 M phosphate buffer (pH 3.5)–CH$_3$OH–H$_2$O (10:36:54, v/v/v); stationary phase, Hypersil C$_{18}$, 5 μm; column temperature, 30°C; flow-rate, 1 ml/min; detection, 254 nm.

and benzylpenilloic acids (**8**, **9**). The selectivity ($\alpha =$ k_1'/k_2') between **6** and **7**, **8** and **9**, PG and **9** and PG and **2** were calculated. This was because the pairs **6** and **7**, **8** and **9**, and PG and **9** are always eluted closely and PG and **2** are used for the resolution test. The application of this factorial design, analysis of the measured response variables and multivariate regression calculation were supported by the statistical graphic software system 'STATGRAPHIC', version 6.0 (STSC, Rockville, MD, USA). It enabled us

to obtain estimated parameters for main effects, analysis of variance (ANOVA) tables, standardised pareto charts for each compound and response surface plots. Standardised pareto charts for the selectivity of **6** and **7** (α_{6-7}), **8** and **9** (α_{8-9}), PG and **9** (α_{1-9}) and PG and **2** (α_{1-2}) are shown in Fig. 7. The codes A, B, C and D mean the effect of methanol, the pH of the mobile phase buffer, the concentration of buffer and the column temperature on the α values for **6–7**, **8–9**, **1–9** and **1–2**,
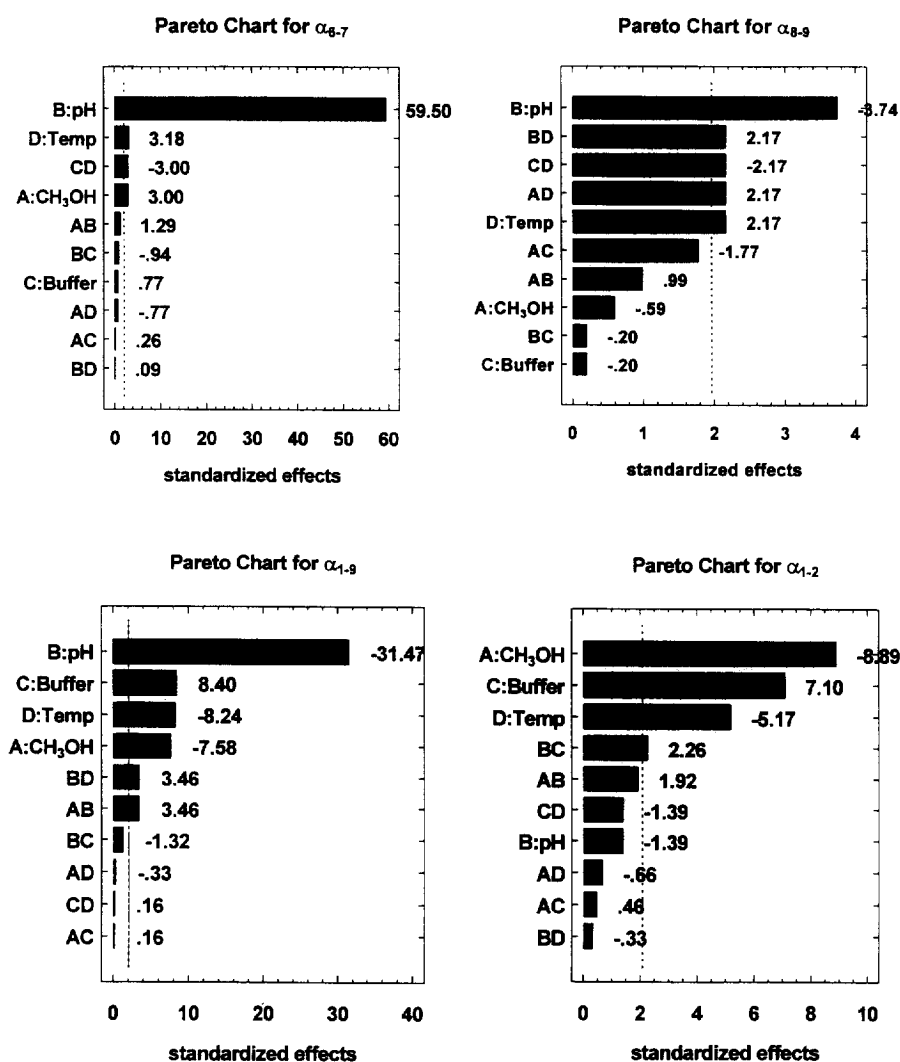

Fig. 7. Standardised pareto charts for the selectivity of related substances **6** and **7** (α_{6-7}), Benzylpenicillin and **2** (α_{1-2}), **8** and **9** (α_{8-9}) and benzylpenicillin and **9** (α_{1-9}).

respectively. The combination of two codes means the interaction between the two parameters. The standardised pareto chart consists of bars that are displayed in size order of the effects, and of a vertical line at a critical t-value ($\alpha=0.05$). Parameter effects for which the bars are smaller than the critical t-value are considered as not being significant and not affecting the response variables. It can be seen that the separation of the diastereoisomers **6, 7** and **8, 9** is influenced in a different way. For the former, the pH is a much more significant parameter than for the latter. Both the resolution test and the separation **1–2** are sensitive to methanol content, buffer concentration and column temperature, but the former is highly sensitive to pH variations, while the latter is not. This means that the separation **1–2**, which is used in the system performance test or resolution test, is more sensitive to variations. Interaction effects never have an important influence on the separations examined.

The response surface plots were constructed with the retention times as a function of the most important chromatographic parameters: i.e. the pH of the buffer and the concentration of methanol. Under all of the conditions examined, it was found that changes of the parameters did not hamper the separation of PG from **9**. The results of this study show that the method is robust.

4. Conclusion

It can be concluded that method 7 reproducibly shows good selectivity. It also shows good repeatability, linearity and sensitivity. The method is robust and can be used for the assay and purity control of benzylpenicillin. Phenylacetic acid may be used in a resolution test. The performance of this method will be further examined in a collaborative study.

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