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Short communication

Analysis of benzylpenicillin by capillary electrophoresis

Zhu Yongxin, J. Dalle, A. Van Schepdael, E. Roets, J. Hoogmartens*

Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, K.U. Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium

Abstract

The applicability of capillary electrophoresis for the separation of benzylpenicillin (penicillin G, PG) and its related substances was investigated. The CE method proposed uses sodium phosphate 40 m*M*–sodium dodecyl sulfate 150 m*M*, pH 6.5, as a buffer. This method is as selective as that of existing liquid chromatography (LC) methods. The limit of detection was 10 pg for PG and the limit of quantitation was 20 pg. PG samples were analysed by CE as well as LC. The analysis of variance showed no significant difference between the results obtained by the two techniques. © 1997 Elsevier Science B.V.

Keywords: Pharmaceutical analysis; Benzylpenicillin; Antibiotics

1. Introduction

Benzylpenicillin (PG) was the first natural penicillin. Assay methods of PG drug substance include microbiological assay [1], mercurimetric titration [2] and chromatographic assays [3–5]. Liquid chromatography (LC) has so far been the most popular technique for the separation of impurities and degradation products of PG [6].

Recently, capillary electrophoresis (CE) has proven to be a significant and versatile technique for the analysis of β-lactam antibiotics [7–10]. Most of these CE methods involving micellar electrokinetic capillary chromatography (MECC), utilize micellar buffer solutions which exhibit a differential partitioning effect especially with neutral and weakly ionic molecules, such as penicillin antibiotics. Previously capillary zone electrophoresis methods were developed for determination of PG in pharmaceuticals and monitoring of PG decomposition in gastric contents [11,12]. However, the separation of PG and its potential impurities has not been discussed.

In this paper, we report the separation of PG and its related substances by MECC. A micellar system using sodium dodecyl sulfate (SDS) in a phosphate buffer of pH 6.5 can satisfactorily separate PG from eight of its related substances. Commercial samples of PG were analysed by using both CE and LC methods.

2. Experimental

2.1. Instrumentation

CE experiments were carried out on a Spectra Phoresis 1000 (Thermo Separation Products, Fremont, CA, USA), which was driven by CE software (version 3.01) operating under IBM OS/2TM (version 1.2). The vacuum system of the instrument applies a constant negative pressure of 5.17 kPa for the injection. Hydrodynamic injection was performed

*Corresponding author.

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for 5 s during the selectivity study and 10 s during quantitative work. Fused-silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA): 44 cm×50 μm I.D. and 36 cm effective length. UV detection was set at 225 nm. The capillary was washed at the beginning of the day with 0.1 *M* NaOH for 5 min, followed by a water wash for 5 min. Before every analysis the capillary was washed 5 min with running buffer.

LC experiments were performed with an L-6200 pump (Merck–Hitachi, Darmstadt, Germany), an autosampler (Marathon, Spark Holland, The Netherlands) with a 20-μl loop, a Hypersil C_{18} 5-μm (25×0.46 cm I.D.) column, a Model L-4000 UV detector (Merck–Hitachi) set at 225 nm and an integrator Model 3396 Series II (Hewlett-Packard, Avondale, PA).

2.2. Reagents and samples

Milli-Q water (Millipore, Milford, MA, USA) was used throughout. Reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Chimica, Geel, Belgium). The running buffer for MECC was prepared by dissolving SDS in sodium dihydrogenphosphate solution, the pH of the buffer was adjusted using NaOH.

The mobile phase for LC was composed of 0.5 *M* phosphate buffer (pH 3.5)–methanol–water (10:36:54, v/v/v).

PG is commercially available (Gist-Brocades, Delft, The Netherlands). Related substances originate from the biosynthesis or from degradation. The structures of the available related substances are shown in Fig. 1. Phenylacetic acid (**2**) (Acros Chimica) and 6-aminopenicillanic acid (6-APA) (**3**) (Gist-Brocades) are the basic constituents of PG. 4-Hydroxybenzylpenicillin (**4**) can arise from the biosynthesis. The other related substances are decomposition products. Related substances **4**, **5**, **6**, **7** and **8** were prepared in the laboratory [13,14]. Electrophoretic parameters were determined using mixtures containing approximately equal amounts of PG and its related substances in a concentration of 0.5 mg/ml water. For the analysis of PG, the concentration of commercial samples was 2 mg/ml water and 1 mg/ml water for CE and LC, respectively.

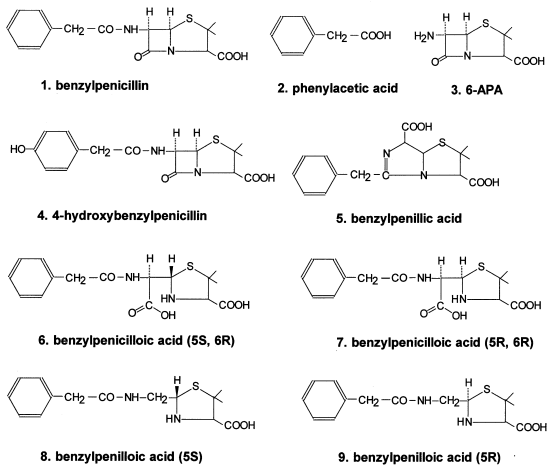

Fig. 1. Structures of benzylpenicillin and its related substances.

3. Results and discussion

MECC is especially powerful for the separation of complex mixtures such as natural products and weakly ionic molecules [15]. In order to develop a method for the separation of PG and its related substances, SDS was applied in the running buffer for the separation of PG and its related substances, i.e., 40 m*M* sodium phosphate buffer (pH 6.5) containing 150 m*M* SDS. Under these conditions, PG can be completely separated from all eight related substances.

The influence of different parameters on the separation was investigated. Since small differences in pK_a can cause the separation of closely related substances, the pH is critical for method development. Experiments were done using sodium phosphate (40 m*M*)–SDS (150 m*M*) buffer. The applied voltage was 12 kV and the temperature 25°C. The pH was varied between 6 and 8 with steps of half a pH unit. The influence of pH of the electrolyte on migration time (t_m) is shown in Fig. 2. The apparent mobility of related substance **5** decreased much more than that of other compounds from pH 6.5 to 8.0. A similar result was obtained for its electrophoretic mobility. This is due to the pK_{a2} value of **5**, which is about 7.7, while the pK_a value of other compounds is about 3 [16]. Good selectivity can be obtained at different pH values. A pH 6.5 phosphate buffer was

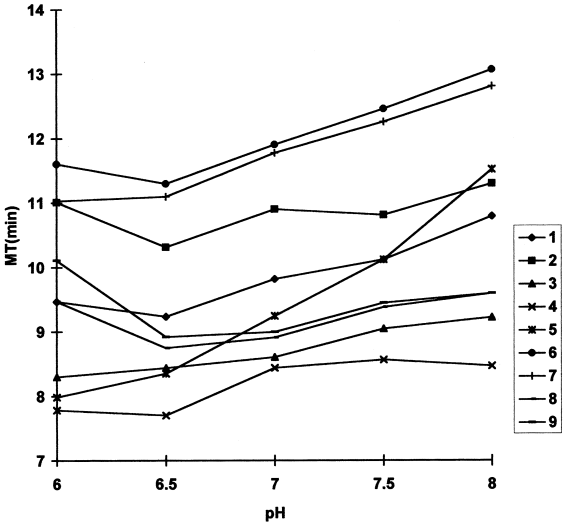

Fig. 2. Influence of pH on migration time (t_m) of PG and its related substances. Capillary: fused silica, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium dihydrogenphosphate (40 mM)–SDS (150 mM) buffer pH x; voltage, 12 kV; temperature, 25°C. See Fig. 1 for identification of compounds.

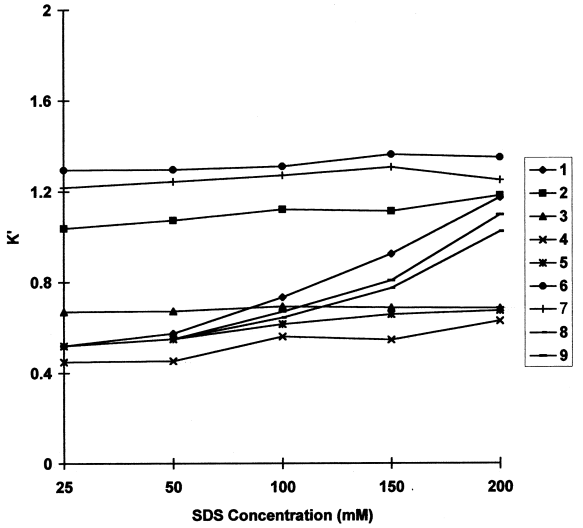

Fig. 3. Influence of SDS concentration on capacity factors (k') of PG and its related substances. Capillary: fused silica, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium dihydrogenphosphate (40 mM)–SDS (x mM) buffer, pH 6.5; voltage, 12 kV; temperature, 25°C.

retained for further study because PG has a good stability at this pH [17].

The concentration of SDS in the buffer was also investigated. It was varied between 25 and 200 mM, keeping the phosphate concentration at 40 mM and the pH at 6.5. The results are shown in Fig. 3. It can be seen that as the number of micelles is increased, the concentration of solute in the micelles is increased which is resulting in a lower mobility. SDS has more influence on the mobility of PG and related substances **8** and **9** and less influence on the mobility of other related substances. This was also reflected in the electrophoretic mobility of PG, **8** and **9**, which decreased much more than other compounds as SDS concentration increased from 25 to 200 mM. The observed effect is related to a hydrophobic effect. PG and compounds **8** and **9** are more hydrophobic than the other compounds and therefore they interact more strongly with the micelles. This resulted in fast change of their k' values as a function of SDS concentration. 150 mM SDS was selected for further experiments as it gave the best selectivity.

The next parameter to be investigated was the concentration of phosphate buffer. It was varied between 20 and 50 mM. An increase in the buffer concentration resulted in a decrease in the EOF due to compression of the double layer [18] and an increase in t_m values of the solutes. Buffer concentration slightly influenced the selectivity; 40 mM was selected as it gives the best compromise in terms of running time, current generated and efficiency of separation.

The effect of temperature on the selectivity was investigated between 20 and 35°C. A decrease in temperature resulted in decreased EOF due to higher electrolyte viscosity and therefore in increase of t_m values and better separation. However, the selectivity was only slightly affected; 25°C was selected as it gives the best compromise between the selectivity and running time.

The effect of varying the voltage on the selectivity was examined from 8 to 14 kV. An increase in voltage resulted in increased EOF due to higher electric field strength and therefore in decrease of t_m values, which led also to poor separation of PG and related substances; 12 kV was selected as a good compromise for resolution and running time. Fig. 4 shows an electropherogram of a mixture of PG and its related substances. Fig. 5 shows an LC chromatogram of PG and its related substances obtained on a

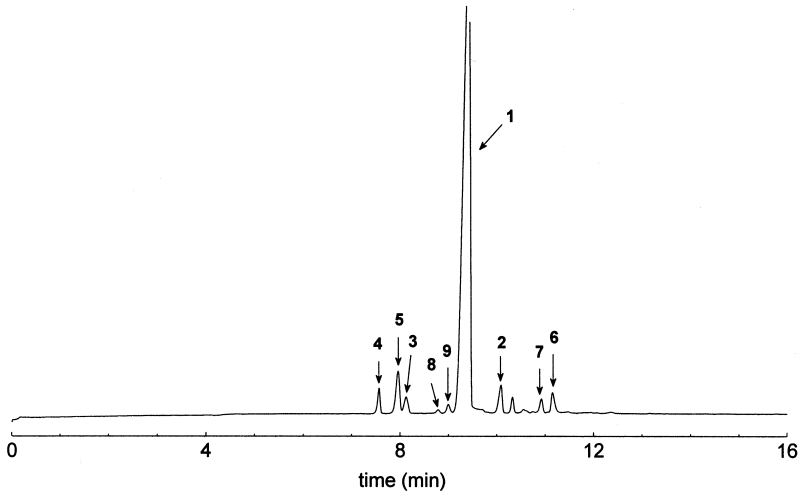

Fig. 4. Electropherogram of a mixture of PG and its related substances. Capillary: fused silica, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium dihydrogenphosphate (40 mM)–SDS (150 mM), pH 6.5; voltage, 12 kV; temperature, 25°C.

Hypersil C_{18} column [6]. It can be seen that with both techniques PG and its related substances are well separated from each other.

The quantitative aspects of this method were examined and the data are shown in Table 1. Five commercial samples of PG were analyzed by both CE and LC. The LC method has been proposed for use in the European Pharmacopoeia [19]. Sample 1 is sodium salt, samples 2–5 are potassium salts. Sample 2 was used as the reference substance to which a content of 99% (w/w) was assigned. The content of PG in each sample was calculated by comparison of the peak area and the concentration with those of the reference sample. The impurities were quantified by comparison of the peaks with that obtained with a 1% dilution of the reference solution and are therefore expressed as PG. The mean values and the corresponding R.S.D. values for PG and sum of impurities of each sample, obtained by the two methods, are shown in Table 2. The ratios of the

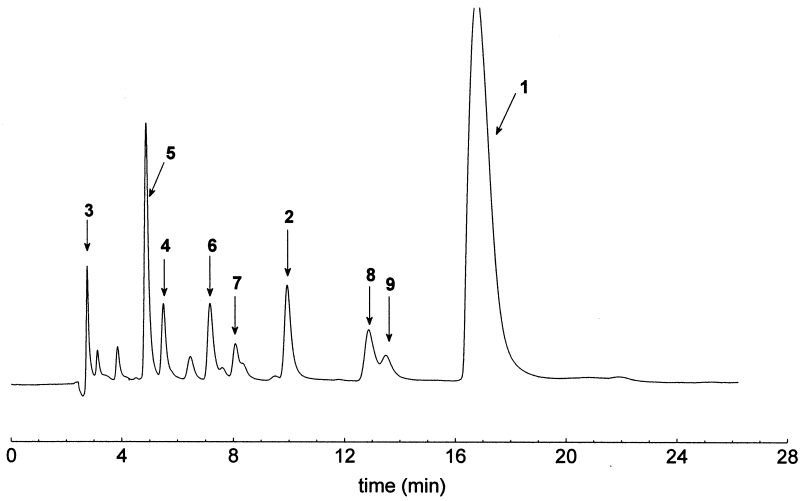

Fig. 5. Chromatogram of a mixture of PG and its related substances using liquid chromatography. Column: Hypersil C_{18} 5 μm; mobile phase, 0.5 M phosphate buffer (pH 3.5)–methanol–water (10:36:54, v/v/v); flow-rate: 1 ml/min; column temperature, 30°C.

Table 1
Quantitative performance test for CE

Parameter	PG
Within-day repeatability (n=4)	
Migration time	R.S.D.=0.9%
Corrected area	R.S.D.=0.7%
Day-to-day repeatability (n=3)	
Migration time	R.S.D.=4.0%
Corrected area	R.S.D.=1.2%
Linearity	
y=corrected area	y=32576x+3290
x=PG concentration in mg/ml	r=0.9993
range=1.22–2.81 mg/ml	$S_{y,x}$=706
number of concentrations=5	
total number of analyses=15	
LOD (S/N=3)	10 pg
R.S.D.=31% (n=6)	0.03%
LOQ (S/N=6)	20 pg
R.S.D=11% (n=6)	0.06%

Fused-silica capillary, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium dihydrogenphosphate (40 mM)–SDS (150 mM), pH 6.5; temperature, 25°C; voltage, 12 kV; hydrodynamic injection, 10 s (corresponding to an injection volume of about 17 nl). The solution of 2 mg/ml was diluted for the LOD and LOQ tests.

mean values for the main component obtained by CE and LC are also shown in Table 2. From these data, the mean ratio and the standard deviation of ratios (S.D.) were calculated and an analysis of variance was performed [20]. Since t(calculated)< t(tabulated), the mean ratio is not significantly different from 1.0, and therefore there is no significant difference (at 99% confidence limits) between the results obtained with the two methods. It has been reported previously that the repeatability of LC is better than that of CE [21]. To verify this the means of the R.S.D. values obtained by both methods were calculated, as well as the standard deviations. The analysis of variance shows significant difference between CE and LC. However, the R.S.D. values for CE are still quite low. It should be mentioned that two different integration systems were used. A HP 3396 Series II integrator was used in LC, while TSP PC 1000 software was used for the peak processing in CE.

4. Conclusion

The CE method is suitable not only for the assay but also for the determination of impurities. The results obtained by CE and LC were not significantly different. The CE method may be a valuable alternative technique to LC in the analysis of benzylpenicillin.

Acknowledgements

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Table 2
Comparison of CE and LC results for the analysis of PG samples

Sample	CE		LC		Ratio (CE/LC)
	PG	Sum of impurities	PG	Sum of impurities	PG
1	94.96(0.6)	1.27	95.53(0.4)	0.51	0.9940
2	99[a](0.8)	0.18	99[a](0.2)	0.11	
3	99.21(0.9)	0.52	97.55(0.3)	0.32	1.0170
4	100.5(0.7)	0.05	99.29(0.4)	0.05	1.0171
5	97.98(0.6)	0.44	97.55(0.4)	0.24	1.0044
Mean	(0.72)		(0.34)		1.0081
S.D.	0.13		0.09		0.0111
t (calc.)	4.81				1.46
$t_{0.99}$	3.36				3.75

The sample concentration is 1 mg/ml for LC and 2 mg/ml for CE, about 17 nl was injected in CE and 20 μl was injected in LC. R.S.D. values are given in parentheses.
[a]99% (w/w) is assigned for the content of PG in sample 2.

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