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Micellar electrokinetic capillary chromatography for the separation of phenoxymethylpenicillin and related substances

Yongxin Zhu, 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

A micellar electrokinetic capillary chromatography (MECC) method has been developed for the separation of phenoxymethylpenicillin and its related substances. This method permits phenoxymethylpenicillin to be completely separated from eight of its related substances. The separation buffer contained 40 m*M* sodium dihydrogen phosphate and 100 m*M* sodium dodecyl sulfate (SDS) adjusted to pH 7.0. The influence of various parameters on the separation such as pH of the buffer, SDS concentration, buffer concentration, temperature, voltage and different length and internal diameter of capillary were investigated. The method shows good repeatability, linearity and sensitivity. It is suitable not only for the related substances test but also for the assay. The results obtained by MECC were also compared with those obtained by liquid chromatography. The analysis of variance showed no significant difference between the results obtained by the two methods.

Keywords: Buffer composition; Pharmaceutical analysis; Phenoxymethylpenicillin; Penicillins; Antibiotics

1. Introduction

Micellar electrokinetic capillary chromatography (MECC) has become a popular mode among several capillary electrophoresis (CE) techniques. Recently several papers described the application of capillary electrophoresis for the analysis of β-lactam antibiotics, most of them using MECC because of its good selectivity and wide applicability [1–7]. The perspectives of MECC in drug analysis were reviewed by Nishi et al. [8].

Phenoxymethylpenicillin (penicillin V, pen V) is a natural penicillin. Liquid chromatography (LC) has so far been the most popular technique for the determination of impurities and degradation products and for the assay [9–13].

The present paper explores the applicability of CE for the analysis of pen V. A micellar system using SDS in a phosphate buffer of pH 7.0 is described for the separation of pen V from its related substances. Commercial samples of pen V were analyzed by using both MECC and LC methods and the results were compared.

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

*Corresponding author.

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applies a constant negative pressure of 5170 Pa for the injection. Hydrodynamic injection was performed for 5 s during qualitative work and 20 s during quantitative work. Fused-silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA): 70 cm (62 cm effective length)×75 μm I.D.; 44 cm (36 cm effective length)×50 μm I.D. UV detection was set at 225 nm. The capillary was rinsed 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 for 5 min with running buffer.

LC experiments were performed with a L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa-N60 Valco injector (Houston, TX, USA) with a 20 μl loop, a Hypersil C_{18} 5 μm (25×0.46 cm I.D.) column, a Model D 254 nm fixed-wavelength UV monitor (LDC/Milton Roy, Riviera Beach, FL, USA) and an integrator Model 3396 SERIES II (Hewlett-Packard, Avondale, PA, USA).

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 the SDS in sodium dihydrogen phosphate 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)–water–methanol (10:50:40, v/v/v).

Pen V was obtained from 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. 6-Aminopenicillanic acid (6-APA) (**2**) (Gist-Brocades) and phenoxyacetic acid (**3**) (Acros Chimica) are the basic constituents of pen V. 4-Hydroxyphenoxymethylpenicillin (**4**) (Gist-Brocades) and benzylpenicillin (**5**) (Gist-Brocades) can arise from the biosynthesis. The other related substances are decomposition products. Phenoxymethylpenicilloic acid (5*R*,6*R*) (**6**), phenoxymethylpenicilloic acid (5*S*,6*R*) (**7**), phenoxymethylpenilloic acid (5*R*) (**8**) and phenoxymethylpenilloic acid (5*S*) (**9**) were prepared as described by Munro et al. [14]. Electrophoretic parameters were determined using mixtures containing approximately equal amounts of pen V and its related substances in a concentration of 0.2 mg ml^{-1}. For the assay of pen V, the concentration of commercial samples was 1 mg ml^{-1}.

3. Results and discussion

In order to develop a method for the separation of pen V and its related substances, five buffers were tested by using free solution capillary electrophoresis (FSCE), each in a concentration of 40 m*M* and at pH 8.0: sodium phosphate, Tris, sodium tetraborate, sodium carbonate and EDTA. It was observed that none of them gave satisfactory separation of pen V and its related substances in the FSCE operating mode. It was also found that phosphate buffer gave the best selectivity. MECC then was applied for the separation of pen V and its related substances, i.e., 40 m*M* sodium phosphate buffer (pH 7.0) containing 100 m*M* sodium dodecyl sulfate (SDS). Under these conditions, pen V 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 (100 m*M*) buffer on a fused-silica capillary of 70 cm length and 75 μm I.D. The applied voltage was 15 kV and the temperature was 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 times (t_m values) is shown in Fig. 2. The mobility of related substances **8** and **9** was influenced much more than that of other compounds from pH 6.0 to 6.5. This is due to their pK_a value, which is about 4.8 while the pK_a value of other compounds is about 2.7 [15]. Good selectivity can be obtained from pH 6.5 to 8.0. A pH 7.0 phosphate buffer was retained for further study because it has a high buffer capacity ($pK_{a2}=7.2$) and gives a consistent electroosmotic flow (EOF), causing migration of compounds towards the cathode. At this pH, pen V also has a good stability [16].

The concentration of SDS in the buffer was also

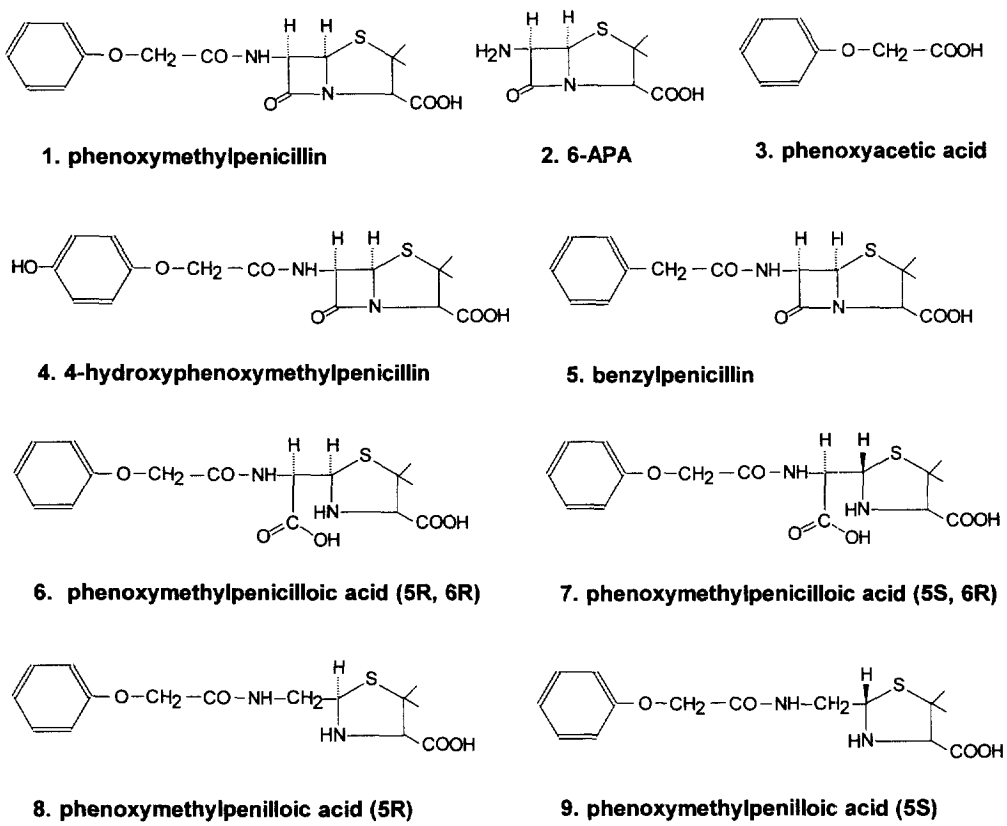

Fig. 1. Structures of phenoxymethylpenicillin and its related substances.

investigated. It was varied between 25 and 150 m*M* keeping the phosphate concentration at 40 m*M* and the pH at 7.0. 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 results in its low mobility. SDS has more influence on the mobility of pen V and related substances **4**, **5**, **8** and **9** and less influence on the mobility of related substances **2**, **3**, **6** and **7**. The observed effect is related to a hydrophobic effect. Pen V and compounds **4**, **5**, **8** and **9** are more hydrophobic than other compounds. Therefore, they interact more strongly with the micelle. This resulted in fast change of their t_m values as a function of SDS concentration. 100 m*M* 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 60 m*M*. The influence of phosphate buffer concentration on migration time is shown in Fig. 4. An increase in the buffer concentration resulted in a decrease in the EOF due to compression of the double layer [17] and an increase in t_m values of the solutes. It can be seen that pen V and its related substances can be well separated from each other in the buffer concentration from 35 m*M* to 60 m*M*. 40 m*M* was selected as it gives the best compromise in terms of run time, current generated and efficiency of separation.

The effect of temperature on the selectivity was investigated between 20°C and 30°C. A decrease in temperature resulted in decreased EOF due to higher electrolyte viscosity, and, therefore, in an increase in t_m values. This also improves the resolution. A temperature of 25°C was selected as it gives the best compromise between resolution and run time.

At this stage it was found opportune to try to shorten the analysis time, which amounted to up to

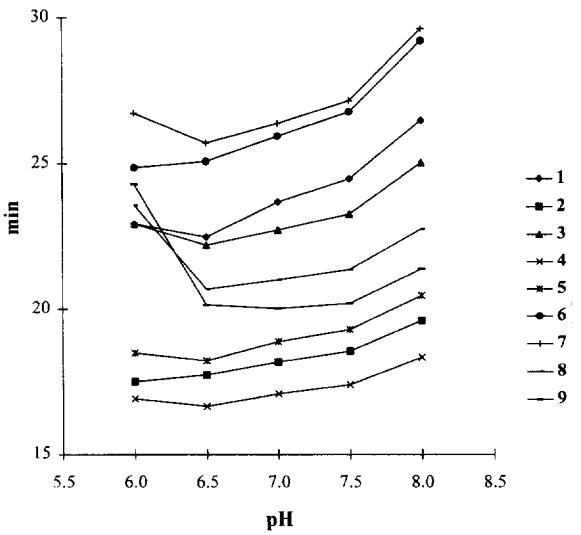

Fig. 2. Influence of pH on migration time (min) of pen V and its related substances. Capillary: fused-silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium phosphate (40 mM)–SDS (100 mM) buffer; voltage; 15 kV; temperature, 25°C. See Fig. 1 for numbering of compounds.

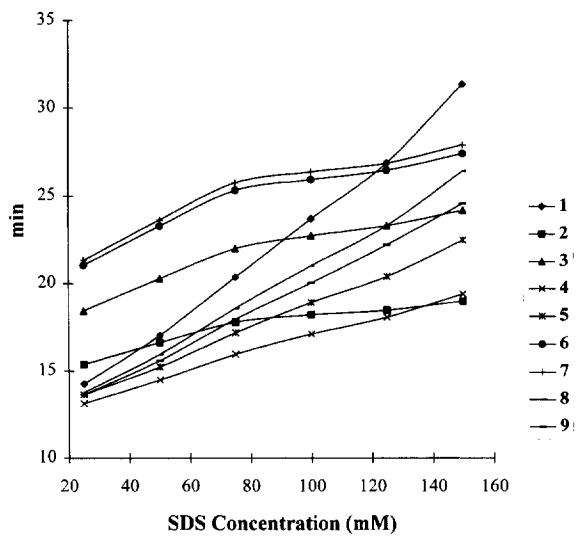

Fig. 3. Influence of SDS concentration on migration time of pen V and its related substances. Capillary: fused-silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium phosphate (40 mM)–SDS (x mM) buffer, pH 7.0; voltage; 15 kV; temperature, 25°C.

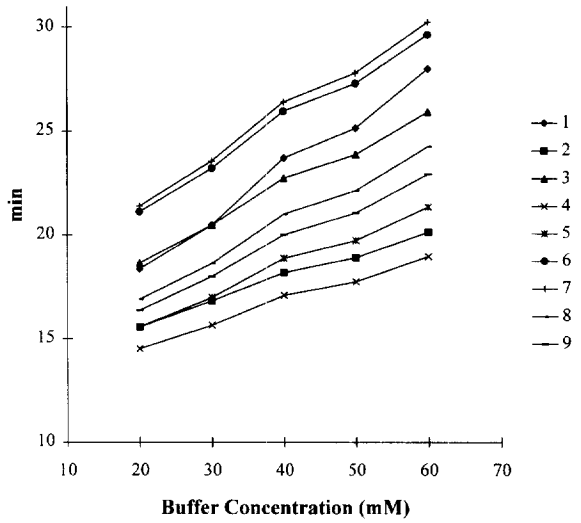

Fig. 4. Influence of buffer concentration on migration time of pen V and its related substances. Capillary: fused-silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium phosphate (x mM)–SDS (100 mM) buffer, pH 7.0; voltage; 15 kV; temperature, 25°C.

25 min. Therefore, studies were undertaken with a shorter capillary (44 cm) of which the internal diameter was 50 μm. Since similar selectivity for the separation was obtained with diminishing length of the capillary and since a considerable time gain was achieved, it was attempted to slightly adjust the applied voltage for the short capillary. The effect of varying the voltage from 10 to 15 kV on the short capillary was investigated using the same experimental conditions as those for the long one. The selectivity of separation was slightly affected. Fig. 5 shows electropherograms of a mixture of pen V and its related substances analyzed on the long and short capillary. In both cases pen V and 4-OH-pen V, which is a biologically active derivative, present in many commercial samples, are well separated from other related substances.

The quantitative aspects of this method were examined by using the short capillary and the data are shown in Table 1. In the limit of detection (LOD) and limit of quantification (LOQ) tests, a solution of pen V (25.6 mg/25 ml) was diluted gradually. The solutions corresponding to 0.03% and 0.06% were found to correspond to the LOD and LOQ, respectively. The injection volume was about 37 nl. Five commercial samples of pen V were

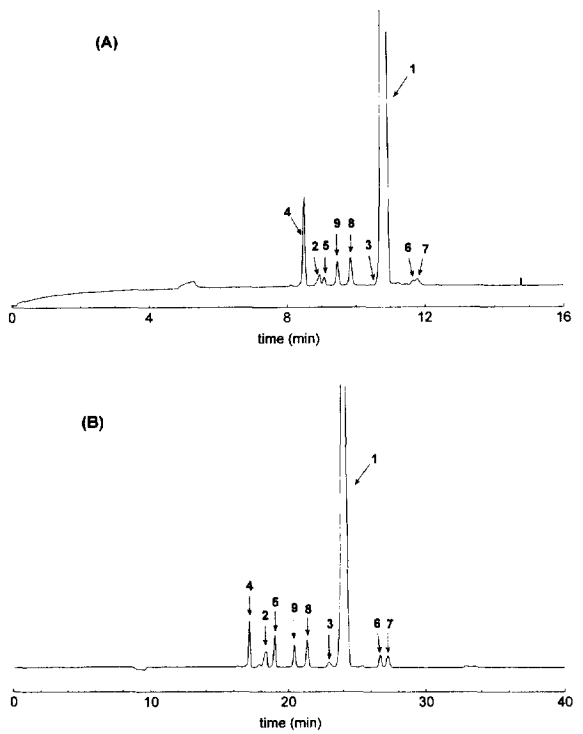

Fig. 5. Electrophoregram of a mixture of pen V and its related substances. (A) Capillary: fused-silica, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium phosphate (40 mM)–SDS (100 mM) buffer, pH 7.0; voltage, 10 kV; temperature, 25°C. (B) capillary: fused-silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium phosphate (40 mM)–SDS (100 mM) buffer, pH 7.0; voltage, 15 kV; temperature, 25°C.

analyzed by both MECC and LC. For the LC method, UV detection was set at 254 nm. This method has been proposed for use in the European Pharmacopoeia [13]. Samples 1–3 are sodium salts, samples 4 and 5 are potassium salts. The mean values and the corresponding R.S.D. values for **1** and **4** of each sample, obtained by the two methods, are shown in Table 2. In sample 4 compound **4** was measured only once by LC. The ratios of the 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 [18]. Since t(calculated)<t(tabulated), the mean ratio is not significantly different from 1.0, and, therefore, there is no significant difference (at 95% confidence limits) between the results obtained

Table 1
Quantitative performance test for CE

Parameter	Pen V
Within-day repeatability (n=6)	
Migration time	R.S.D.=1.1%
Corrected area	R.S.D.=0.63%
Day-to-day repeatability (n=3)	
Migration time	R.S.D.=2.7%
Corrected area	R.S.D.=0.64%
Linearity y=corrected area	y=1113x+38
x=pen V concentration (mg ml^{-1})	r=0.9995
Range=0.66–1.54 (mg ml^{-1})	
Number of concentrations=5	
Total number of analyses=18	
LOD (S/N=3)	11.8 pg
R.S.D. 23% (n=6)	0.03%
LOQ (S/N=6)	23.6 pg
R.S.D. 10.3% (n=6)	0.06%

Fused silica capillary, L=44 cm, l=36 cm, I.D.=50 μm; background electrolyte, sodium dihydrogen phosphate (40 mM)–SDS (100 mM), pH 7.0; temperature, 25°C; voltage, 15 kV; hydrodynamic injection, 20 s (corresponding to an injection volume of about 37 nl).

with the two methods. It has been reported previously that the repeatability of LC is better than that of CE [19]. 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 no significant difference between CE and LC. However, this may be partly due to the fact 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 MECC method is suitable not only for the assay but also for the related substances test. The results obtained by MECC and LC were not significantly different. The MECC method may be a valuable alternative technique to LC in the analysis of phenoxymethylpenicillin.

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

Sample	CE		LC		R=CE/LC (pen V)
	pen V	4-OH-pen V	pen V	4-OH-pen V	
1	98.0[a]	1.55	98.0[a]	1.48	
	(0.77)	(0.48)	(0.69)	(6.9)	
2	98.47	1.94	97.48	1.85	1.0101
	(0.18)	(1.3)	(0.29)	(3.9)	
3	96.76	3.84	95.64	3.48	1.0117
	(0.33)	(0.36)	(0.41)	(2.4)	
4	98.06	0.22	98.37	0.18	0.9968
	(0.47)	(2.3)	(0.47)		
5	98.59	1.25	98.39	1.12	1.0020
	(0.95)	(3.9)	(0.40)	(2.2)	
Mean	(0.54)		(0.45)		1.0051
S.D.	0.32		0.15		0.0070
t (calc.)	0.51				1.46
$t_{0.95}$	2.57				2.78

[a] 98.0% is the assigned content of pen V in Sample 1.
The sample concentration is 1 mg ml^{-1} for both CE and LC, about 37 nl was injected in CE and 20 μl was injected in LC.
The number of analyses for Sample 1 is 6. For the other samples, the number of analyses for CE is 3, the number of analyses for LC is 4; the R.S.D. values are given in parentheses.

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