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Fast chiral separation of amino acid derivatives and acidic drugs by co-electroosmotic flow capillary electrophoresis with vancomycin as chiral selector

Jing-Wu Kang*, Yong-Tan Yang, Jin-Mao You, Qing-Yu Ou

Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China

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Abstract

A fast chiral separation method for acidic enantiomers, including 9-fluorenylmethyl chloroformate (FMOC) amino acid derivatives and ketoprofen, with vancomycin as chiral selector is presented. In this method, hexadimethrine bromide (HDB), a polycationic polymer, was added to the run buffer as electroosmotic flow (EOF) modifier. The reversed EOF migrated in the same direction as the anionic analytes. Consequently, the separation time was shortened. Another advantage of using HDB as buffer additive is that the adsorption of vancomycin onto the capillary wall was minimized, hence, the separation efficiency was improved. The effects of buffer pH and vancomycin concentration on separation were investigated. Base line chiral separation of 12 FMOC-amino acid derivatives, ketoprofen and drug intermediate 4,4′-dimethoxy-5,6,5′6′-bis-methylenedioxybiphenyl-2,2′-dicarboxylic acid were obtained. The separation time for each enantiomers was not more than 4.5 min, and the average efficiency of $3.2 \cdot 10^5$ plates/m was obtained.

Keywords: Enantiomer separation; Buffer composition; Amino acids; Vancomycin; Ketoprofen

1. Introduction

The analysis of chiral compounds is of great importance in pharmacy, medicine and biological sciences. During the past several years, capillary electrophoresis (CE) has been demonstrated to be a powerful tool for chiral separation [1]. A number of chiral compounds have been separated with various chiral selectors by CE. The chiral selectors used in capillary electrophoresis include: cyclodextrins and their derivatives [2–6], crown ether [7], proteins [8,9], chiral surfactants [10] and optical metal chelate complexes [11]. Recently, Armstrong and coworkers [12–14] have demonstrated that vancomycin is a useful chiral selector for enantiomer separation in capillary electrophoresis.

Vancomycin consists of three fused macrocyclic rings and two side chains, a carbohydrate dimer and *N*-methylleucine [14]. Its three fused macrocyclic rings are formed by ether and peptide linkages. The chiral selectivity of vancomycin should be related to the following structural features: (i) the semirigid basket shaped aglycan [14] facilitates the formation of host–guest inclusion complexes with chiral analytes; (ii) the chiral environment formed by 18 asymmetrical centers and various functional groups

*Corresponding author.

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are known to provide the essential interactions for chiral recognition. Dramatically high chiral selectivity could be obtained for the chiral compounds containing a free carboxylic acid functional group. However, the separation efficiency was relatively poor due to the adsorption of vancomycin onto the capillary wall [12]. Further, the analysis time was relatively long. Rundlett and Armstrong [13] improved the separation efficiency by adding sodium dodecyl sulfate (SDS) to the run buffer, however, this led to a decrease of chiral resolution, and the separation time was still somewhat long. Vespalec et al. [15] used a coated capillary to improve the separation efficiency and achieved fast chiral separation of AQC amino acid derivatives. The average efficiency was 250 000 plates/m. Wan and Blomberg [16] chirally separated the 9-fluorenylmethyl chloroformate (FMOC) derivatives of amino acids, di- and tripeptides. They recommended that the separation efficiency could be improved by using the buffer with pH above the pI value of vancomycin and adding 2-propanol in the buffer. Another disadvantage of using vancomycin as chiral selector in CE is the detection problem, because it absorbs strongly at wavelengths below 254 nm. A commonly used method [12–16] to overcome this problem is to perform the detection at 254 nm. Recently, the partial filling-counter current method has been introduced by two groups [17,18] to improve the detection sensitivity. In this method, the capillary was partially filled with the buffer containing vancomycin from the injection end. Since the analyte and vancomycin migrated in the opposite direction, the vancomycin zone had left the detection window before the analyte reached, thus the window was clear and the detection sensitivity was improved.

In the separation of anionic analytes with normal CE, the analysis time is relatively long due to the migration of anions in the opposite direction to the inherent electroosmotic flow. The analysis time of anionic analytes can be reduced by adding cationic surfactants (cetyltrimethylammonium bromide, CTAB) as well as a polycationic polymer (hexadimethrine bromide, HDB) in the buffer to reverse the electroosmotic flow (EOF) direction. CE with reversed EOF, referred to as co-electroosmotic flow electrophoresis [19], has been used for the separation of anions [20–25], phenolic compounds [19,26] and proteins [27].

In the present paper, a fast and highly efficient chiral separation method for FMOC amino acid derivatives and other acidic chiral compounds is presented. The capillary wall was dynamically modified with HDB added to the run buffer. HDB is a polycationic polymer which tends to adsorb on the capillary inner wall via coulombic interaction. Consequently, the capillary inner surface carried a positive charge, and the direction of EOF was altered. Thus, the separation time was reduced. Meanwhile the adsorption of vancomycin onto the capillary wall was minimized, and significant improvement in separation efficiency was obtained.

2. Experimental

2.1. Instrumentation

Separations were performed on a BioFocus 3000 capillary electrophoresis system (Bio-Rad Labs., Hercules, CA, USA). An untreated fused capillary (Yongnian, Hebei Province, China) with 50 μm I.D., 350 μm O.D. and 35 cm total length (30 cm to detector window) was used throughout the experiments.

2.2. Reagents and materials

The amino acids, ketoprofen, 9-flurenylmethyl chloroformate (FMOC), vancomycin and hexadimethrine bromide (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) (HDB) were purchased from Sigma (St. Louis, MO, USA). Tris-(hydroxymethylaminomethane) (Tris) and phosphoric acid were purchased in China. 4,4′-Dimethoxy - 5,6,5′6′ - bismethylenedioxybiphenyl - 2,3′ - dicarboxylic acid (DBDA) was a gift from Beijing Institute of Pharmacy. Other reagents used as organic additives were of analytical grade. Stock solutions of 200 m*M* borate buffer (pH 9.0), 1% (w/v) hexadimethrine and 400 m*M* Tris were prepared. The running buffer was composed of 50 m*M* Tris and a certain amount of HDB (0.0002%–0.005%). The buffer pH was adjusted with phosphoric acid solution to a suitable value. If necessary, a certain amount of organic solution was added. A certain amount of vancomycin was dissolved in the buffer.

2.3. Derivatization procedure

Derivatization of amino acids with FMOC was performed according to the literature [28]. 200 μl of 10 m*M* FMOC solution in acetonitrile was added to 200 μl of 3 m*M* amino acid in 200 m*M* borate buffer (pH 9.0). This mixture was kept for 2 min, then extracted with 0.5 ml pentane twice to remove excess reagent. The samples were diluted ten-fold with water prior to injection.

2.4. Electrophoresis

Before use, the capillary was rinsed with 0.1 *M* NaOH for 10 min, then with water for 4 min. The capillary was conditioned with buffer containing 0.002% HDB in the absent of vancomycin for 10 min and then with run buffer for 4 min. After ten runs, the capillary needed to be rinsed with 0.1 *M* H_3PO_4 for 10 min, then with water, buffer without vancomycin and finally with run buffer for 4 min respectively. When changing the run buffer, the same steps were performed. The capillary was thermostatted at 20°C. Applied voltage of −18 kV (anode at detection end) was used throughout the experiments. Determination was performed at UV 254 nm. Samples were pressure injected by 4 p.s.i. s (1 p.s.i.= 6894.76 Pa).

3. Results and discussion

3.1. Effect of HDB

In this experiment, using HDB as buffer additive is based on the following consideration: (i) the adsorption of vancomycin on the capillary wall could be diminished by dynamically modifying the capillary wall with polycationic polymer, hence the separation efficiency was improved; (ii) the reversed EOF shortened the separation times of anionic analytes via co-electroosmotic flow electrophoresis; (iii) the migration orders of enantiomers were reversed due to the reversed EOF. This is very useful for the determination of trace enantiomeric impurity, since it allows the small peak to be eluted before the main peak. Although cationic surfactants, such as CTAB, can be used as EOF modifier, a complex mechanism [13] had to be involved in the chiral separation and may reduce the chiral selectivity of vancomycin due to the interaction of vancomycin with micelle. On the other hand, the HDB molecule contains shorter aliphatic chains and relatively lower concentration is required in the buffer compared with CTAB. In addition, the adsorption of HDB onto the capillary wall was strong enough to provide a stable electroosmotic flow [29,30]. Therefore, it is suitable for use as an EOF modifier in our experiments for chiral separation.

The influence of HDB concentration on EOF and the electrophoresis mobility of FMOC-amino acids was investigated at pH 6.0. The HDB concentration varied from 0.0002% to 0.005% (w/v), and each buffer contained 0.5 m*M* vancomycin. The EOF increased with raising HDB concentration up to 0.002% HDB, then remained almost constant implying the saturate adsorption of HDB onto the capillary wall. For FMOC-amino acids, the situation is somewhat complex due to the interaction between FMOC-amino acids and HDB [30]. The apparent mobility of FMOC-amino acids increased with the rise in HDB concentration, then decreased slowly with rising HDB concentration when HDB concentration was above 0.002%. However, it was found that the separation efficiency decreased with rising HDB concentration. Therefore, 0.002% of HDB was selected for the following experiments.

It should be noted that, in principle, the adsorption of HDB and vancomycin onto the capillary wall is a competition process, therefore, the pretreatment of the capillary wall with buffer in the absence of vancomycin for 10 min is necessary. In fact, after using the capillary for about 5 h, an obvious drop in separation efficiency and a prolonged EOF were observed. This behavior should be ascribed to the gradual accumulation of vancomycin on the capillary wall. The vacomycin adsorbed onto the capillary wall could be cleaned by rinsing the capillary wall with 0.1 *M* H_3PO_4 for 10 min. It was found that washing the capillary with H_3PO_4 solution was more effective than NaOH or HCl solutions with the same concentration.

3.2. Effect of buffer pH

The effect of the buffer pH on chiral separation of five FMOC-amino acids is shown in Fig. 1. There is a maximum value at pH 6.0 for FMOC-Ala, Phe and

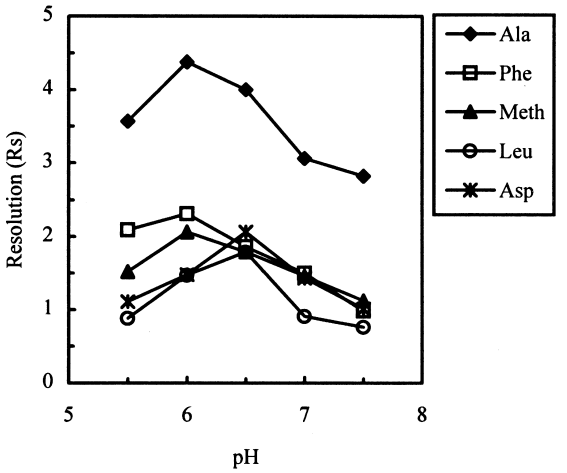

Fig. 1. Effect of buffer pH on chiral resolution. Conditions: separation capillary, 35 cm (31 cm effective length)×50 μm I.D.; 50 m*M* Tris–H_3PO_4 buffer containing 0.5 m*M* vancomycin and 0.002% HDB; applied voltage, −18 kV, current, 18 μA; Column temperature, 20°C. Dimethylformamide was used as neutral marker.

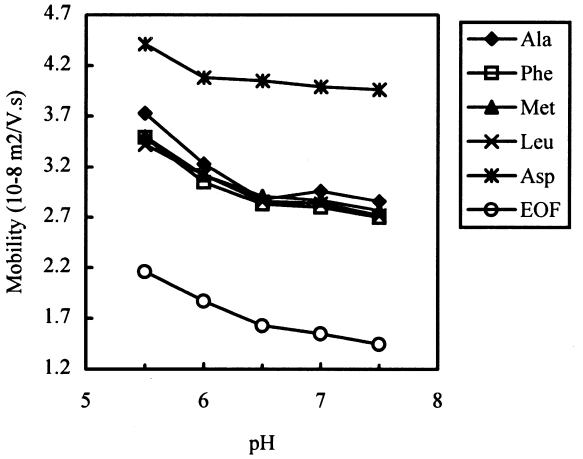

Fig. 2. Effect of buffer pH on mobility. Conditions are the same as in Fig. 1.

Met. For FMOC-Asp and Leu, the maximum pH is about at 6.5. When the buffer pH was above 7.0, obvious peak tailing was observed, possibly due to another kind of adsorption of vancomycin onto the modified capillary wall which carried positive charge. Since the vancomycin molecule has one carboxyl group and two amino groups, it has an isoelectrophoretic point (pI) which has been reported as 7.2 [12] and 7.5 [31] measured under different conditions. With raising the buffer pH value, the ratio of the negative to positive charges increased, consequently, the adsorption of vancomycin onto the positively charged capillary wall increased resulting in a decrease in separation efficiency.

The mobility of FMOC-amino acids and EOF decreased with raising buffer pH (Fig. 2). This behavior could be explained by the fact that the higher ionic strength of alkaline electrolytes reduced the EOF [25]. When the buffer pH was lower than pH 6.0, the chiral resolution became poor. The determined optimum pH value is pH 6.0.

3.3. Effect of vancomycin concentration

The effect of vancomycin concentration on chiral separation is shown in Fig. 3. The chiral resolution increased with increasing vancomycin concentration in the range from 0.5 to 2 m*M*. But for FMOC-Phe, it had a maximum value at 1.5 m*M*. Above this concentration, efficiency decreased with increasing vancomycin concentration (see Fig. 4). In Fig. 4, each point represents the average value of the efficiency of two enantiomers. At pH 6.0, 12 FMOC-amino acid derivatives could be baseline separated with 2 m*M* vancomycin. The separation results are summarized in Table 1. Unfortunately, for those FMOC-amino acids, such as His, Trp, Tyr, Lys and

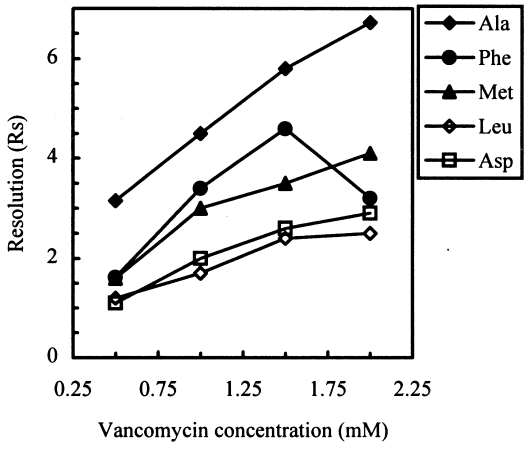

Fig. 3. Effect of vancomycin concentration on chiral resolution. Conditions: buffer, 50 m*M* Tris–H_3PO_4 buffer (pH=6.0) containing 0.002% HDB; applied voltage, −18 kV, current, 18 μA; other conditions as in Fig. 1.

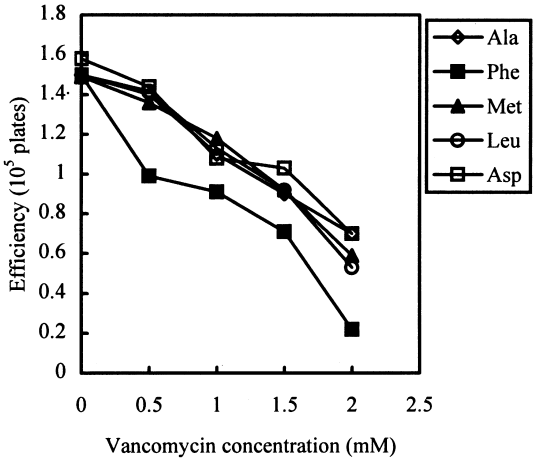

Fig. 4. Effect of vancomycin concentration on separation efficiency. Conditions are the same as in Fig. 3.

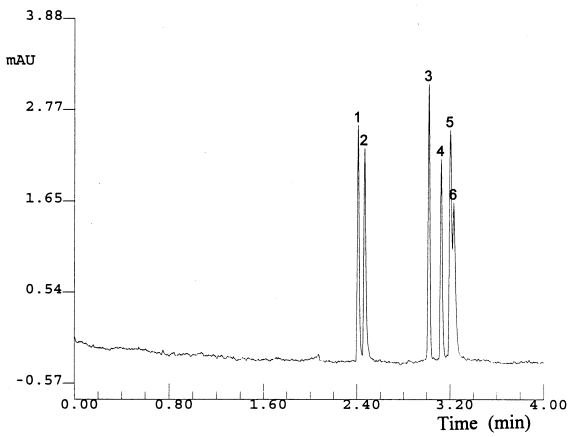

Fig. 5. A typical electropherogram of the chiral separation of FMOC-amino acids enantiomers. Conditions: buffer, 50 m*M* Tris–H_3PO_4 buffer (pH=6.0) containing 0.002% HDB and 1 m*M* vancomycin; applied voltage, −18 kV, current, 18 μA; other conditions as in Fig. 1; peaks: 1=L-Asp, 2=D-Asp, 3=L-Ala, 4=L-Met, 5=D-Ala, 6=D-Met.

Arg, their peaks coeluted with EOF and the EOF reversed peak disturbed the separation. A probable explanation of this phenomenon is that they have relatively strong hydrophobicity which makes the ion-pair interaction with HDB so strong that their mobilities are reduced. This problem could not be resolved by adding 2-propanol (15%, v/v) or acetonitrile (15%, v/v) to the run buffer. A typical electropherogram for chiral separation of FMOC-amino acids is shown in Fig. 5.

Beside FMOC-amino acid derivatives, two acidic chiral compounds, ketoprofen and DBDA were also chirally separated with excellent resolution by this method (Table 1). DBDA is an intermediate of a new

Table 1
Enantiomer resolution, migration time and separation efficiency[a]

Chiral compounds	Time (1) (min)	Time (2) (min)	R_s	Efficiency (10^5 plates)
FMOC-Asp	2.87	3.03	2.9	1.0
Norval	3.69	3.99	5.1	1.3
Val	4.17	4.38	2.6	0.9
Pro	3.85	3.92	1.4	0.9
Asn	3.45	3.54	2.3	1.5
Isoleu	3.95	4.25	4.6	1.0
Norleu	3.87	4.13	4.3	1.1
Ser	3.88	4.05	3.0	1.2
Glu	3.21	3.34	2.2	0.7
Ala	3.56	4.02	6.7	1.1
Phe	3.96	4.45	3.2	0.6
Met	3.80	4.13	4.1	0.9
Leu	3.84	4.05	2.5	0.8
DBDA	2.79	2.88	1.6	0.8
Ketoprofen	3.32	3.61	5.6	1.1

[a] Conditions: separation capillary, 35 cm (31 cm effective length)×50 μm I.D.; buffer, 50 m*M* Tris–H_3PO_4 buffer (pH=6.0) containing 0.002% (w/v) HDB and 2 m*M* vancomycin; applied voltage, −18 kV; current, 18 μA; column temperature, 20°C. Efficiencies listed are the average values of two enantiomers; Time (1) and time (2) represented the first and second eluted peaks of enantiomers. For FMOC-amino acids, L-amino acid was eluted before the D-amino acid. For ketoprofen, the *S* enantiomer was eluted first.

Table 2
Reproducibility of analyte migration time and efficiency[a]

Chiral compounds	R.S.D. (%)			
	Migration time		Efficiency	
	Run to run[b]	Day to day[c]	Run to run[b]	Day to day[c]
FMOC-Ala	0.39	4.0	0.50	6.3
Phe	0.41	5.4	0.62	6.5
Met	0.29	3.8	0.41	3.9
Leu	0.35	4.0	0.48	5.1
Asp	0.33	3.2	0.32	4.4
Ketoprofen	0.27	2.1	0.53	3.2
EOF	0.84	5.9		

[a] Conditions as in Table 1.
[b] $n=5$.
[c] $n=3$.

antihepatic drug named biphenyl diester. It could not be chirally separated with cyclodextrins (CDs) including β-CD and γ-CD.

3.4. Reproducibility

Five FMOC-amino acid derivatives, ketoprophen and EOF were selected for reproducibility test of migration time and efficiency. Reproducibility was expressed in terms of percent relative standard deviation (R.S.D.). The results are summarized in Table 2. It can be seen that run-to-run reproducibility of both migration time and efficiency is good, but that of day-to-day is somewhat worse than the former, though the capillary was pretreated with the same washing procedure every day before use.

4. Conclusions

Fast chiral separation of acidic chiral compounds could be obtained by co-electroosmotic flow electrophoresis with vancomycin as chiral selector. Since the capillary wall was dynamically coated with a polycationic polymer HDB, the adsorption of vancomycin on capillary wall was minimized resulting in improvement of separation efficiency. The average efficiency of 15 acidic enantiomers was $3.2 \cdot 10^5$ plates/m. Twelve FMOC-amino acids, ketoprofen and an acidic chiral compound DBDA could be baseline separated with 2 m*M* vancomycin. The defect of this method is that the separation of some FMOC-amino acids, such as His, Tys, Lys, Tyr and Arg were interfered with by the EOF peak.

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