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Fast enantioseparation of arylglycine amides by capillary electrophoresis with highly sulfated-β-cyclodextrin as a chiral selector

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Abstract

Nine racemic arylglycine amides were synthesized and successfully enantioseparated by capillary electrophoresis (CE) using highly sulfated β-cyclodextrin (HS-β-CD) as a chiral selector. Baseline enantioseparation of the analytes was obtained around neutral pH but not in the acidic conditions that are commonly used. HS-β-CD content, buffer pH, type and concentration, and organic modifier concentration were studied and optimized for fast and efficient separation. A chiral CE separation system composed of 1.5% (w/v) HS-β-CD, 0 to 10% (v/v) methanol and 20 m*M* 3-(*N*-morpholino)propanesulfonic acid at pH 6.5 was shown suitable for baseline enantioseparation of the mentioned amides within 6 min, including simultaneous enantioseparation of three positional isomer series (methyl-, methoxyl or chloro-substituted). By using this system, D-enantiomers migrated ahead of the L-enantiomers and the enantiomeric resolution order of arylglycine amides was more or less parallel to the pK_a order of the analytes.

Keywords: Enantiomer separation; Arylglycine amides; Amides; Cyclodextrins

1. Introduction

Arylglycine amides are important precursors for the synthesis of amino acids and have special importance in the pharmaceutical industry [1]. They are broadly employed as chiral auxiliaries and ligands in asymmetric reactions [2]. Enantioseparation of them is hence important but only a few methods are available at present. In most cases, HPLC is the only tool for choice and special chiral stationary phases (CSPs) are required. Crown-ether-based chemicals [3–5] have been shown to be effective as CSPs but need to operate under strong acidic solutions (in sulfuric acid [3] or perchloric acid [4,5]). Temperature modulation was sometimes required to improve the separation [4]. In such conditions, the enantioseparation normally took a long time, about 30 min [4,5], and the simultaneous enantioseparation of positional isomers was not mentioned in the literature [3–5]. Due to the difficulty of producing a stable chiral column with a long life, the operation involved high cost. Such a HPLC method also

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suffered from its large volume consumption of samples. A microscaled technique with low cost and high efficiency was thus considered, and chiral capillary electrophoresis (CE) was tried.

CE has been shown to be powerful in chiral analysis, allowing a rapid development of separating conditions in treating different racemic compounds. It consumes only a small amount of chiral selectors and samples. Furthermore, different types of CE chiral selectors are now available [6–8], and such versatility offers a great chance to achieve high resolution when new chiral analytes are encountered. In the present work, great attention has been paid to the anionic chiral selector of highly sulfated β-cyclodextrin (HS-β-CD), so that electrostatic interaction between the anionic selector and the investigated cationic analytes can be imposed in the separation to improve the enantioseparation. One of the most important benefits of charged chiral selectors is their strong electrostatic interaction with the charged solute, and fundamental contributions to the state of the art should be attributed to the work dealing with charged CD reported by Chankvetadze and co-workers [8–10], Fanali [11], Nishi and Terabe [12], Williams and Vigh [13], and Gahm and Stalcup [14]. The electrostatic interaction is deduced from experimental phenomena such as the resolution power of charged CD is so high that its concentration is usually much lower than neutral CD [9–14], or from related techniques such as nuclear magnetic resonance (NMR) [9,15–18] and computational studies [19].

Highly sulfated CDs (with average substitution degree of 11, 12, 13 for α-, β- and γ-CD, respectively) were firstly synthesized and employed in chiral CE by Chen and Evangelista [20] of Beckman Coulter. More than 140 chiral compounds have been separated with high resolution, including neutral, acidic and basic drugs of different structures [20–25]. Nevertheless, highly sulfated CDs remain rarely used in the enantioseparation of amino amides except for tryptophanamide and phenylalanineamide (PAA) [22]. It is interesting whether or not HS-β-CD is generally applicable to amides. Experiments were hence conducted and the data demonstrated that fast and high enantioresolution could be achieved without too much difficulty when some key factors were optimized, such as buffer pH and HS-β-CD content. The concentration of organic modifier was also shown to be important for fine and fast separation of positional isomers simultaneously. Finally, the present work demonstrated that the enantioseparation order of the arylglycine amides was almost parallel to their pK_a in their partially dissociated status.

2. Experimental

2.1. Chemicals and solutions

HS-β-CD (sodium as the counter ion) was obtained as 20% (w/v) (ca. 84 m*M*) aqueous solution from Beckman Coulter (Fullerton, CA, USA). 3-(*N*-Morpholino)propanesulfonic acid (MOPS) was purchased from Wako (Osaka, Japan) while *N*-(2-acetamino)-2-aminoethansulfonsaure (ACES) and tris-(hydroxymethyl)aminomethane (Tris) were from Fluka (Buchs, Switzerland). β-Cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD, molecule substitution=0.6), heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD), heptakis (2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD) and 18C6 (18-crown-6) were all provided by Aldrich (Milwaukee, WI, USA). Other chemicals were all of analytical reagent grade from Beijing Chemical Work (Beijing, China).

CE running buffers were prepared by addition of appropriate volumes of HS-β-CD and methanol (when required) in 20 m*M* MOPS aqueous solution, adjusted to a required pH with 1 *M* ammonium acetate or acetic acid. Before use, the running buffer solutions were filtered through a membrane with 0.45 μm pores and degassed by sonication for 4 min. All the solutions were prepared with double-distilled water produced using a distillation apparatus model SZ-93 (Ya Rong Biochemical Instrument, Shanghai, China).

2.2. Sample preparation

Arylglycine amides were synthesized in the laboratory. The detailed synthesis and characterization have been reported elsewhere [26]. In brief, an arylglycine nitrile [27] was prepared by mixing the corresponding aldehyde, ammonium chloride, potassium cyanide and alumina in acetonitrile. The mixed solution was constantly stirred at 50 °C for 12–24 h.

After filtration to remove the alumina, the solution was bubbled with hydrogen chloride gas and arylglycine nitrile hydrochloride was precipitated. The filtered solid product was added with cold concentrated sulfuric acid (98%) dropwise and the resulting mixture was stirred first at 0 °C for 30 min and then at ambient temperature for 12 h. The solution was poured onto ice, adjusted to pH 9–10 with aqueous ammonia (28%) and further stirred at below 10 °C for 1 h. The final aryglycine amide was collected by filtration [28] and identified by IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analysis (data not shown). For CE, the identified product was just dissolved in distilled water to form a solution of 0.1 mg/ml.

The nine arylglycine amide enantiomers synthesized could be classified into four series as listed in Table 1. The first series features different amino groups. The second is actually a class of isomers, having the methyl group located at a different position. The third and fourth series bear methoxy and chloro groups on the benzyl ring at the *p*- or *m*-position, respectively; they are positional isomers.

2.3. Electrophoresis

All capillary electrophoresis experiments were performed using a P/ACE model MDO system (Beckman Coulter). A bare fused-silica capillary (J&W Scientific, Folsom, CA, USA) of 40.2 cm (30 cm to the detector)×50 μm I.D. was mounted in the machine. Prior to every injection, the capillary was sequentially washed with 0.1 *M* sodium hydroxide solution, distilled water and running buffer for 2 min each. Samples were introduced by electrokinetic injection at 4 kV for 2 s, and separated at 16 kV and 20 °C. The separated bands were detected by UV absorption at 214 nm through a slit aperture of 100×200 μm. The data were recorded at 2 Hz and processed with 32Karat software. The target peaks were identified by spiking one or more standard enantiomers in the sample solution. The running buffer was renewed after every 10 runs.

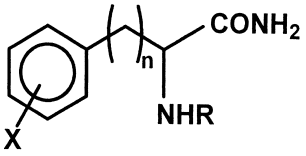
Resolution is calculated from the equation $R_s = 2(t_2 - t_1)/(w_2 + w_1)$, where t_1 and t_2 are the migration times of the two enantiomers, respectively, and w_1 and w_2 their peak widths. The resolution value is an average of three injections.

3. Results and discussion

3.1. Chiral selector

In our preliminary experiments, different neutral cyclodextrin chiral selectors were tried to achieve

Table 1
Structures of racemic arylglycine amides synthesized



Series	Name	Abbreviation	*n*	R	X
I	Phenylglycine amide	PGA	0	H	H
	N-Methylphenylglycine amide	NME-PGA	0	CH_3	H
II	4-Methylphenylglycine amide	*p*ME-PGA	0	H	CH_3 (*p*)
	2-Methylphenylglycine amide	*o*ME-PGA	0	H	CH_3 (*o*)
	Phenylalanine amide	PAA	1	H	H
III	4-Methoxylphenylglycine amide	*p*OME-PGA	0	H	OCH_3 (*p*)
	3-Methoxylphenylglycine amide	*m*OME-PGA	0	H	OCH_3 (*m*)
IV	4-Chlorophenylglycine amide	*p*Cl-PGA	0	H	Cl (*p*)
	3-Chlorophenylglycine amide	*m*Cl-PGA	0	H	Cl (*m*)

resolution of the racemic arylglycine amides. Most of the commonly used neutral CDs such as β-CD, HP-β-CD, DM-β-CD and TM-β-CD under acidic (15 m*M* each of the above CDs in 20 m*M* citrate buffer, pH 3.0) or neutral (15 m*M* DM-β-CD in 20 m*M* Tris buffer, pH 7.2) conditions were investigated but yielded no enantioresolution. Considering that crown ether can form a complex with a primary amine that assists cyclodextrins to improve their resolving ability [29,30], 18C6 was used as an additive to the above tested systems. Not much improvement has ever been obtained except for the DM-β-CD/18C6 (20 m*M* citrate buffer, pH 3.0) system which could indeed generate some enantioseparation for *p*OME-PGA and *m*OME-PGA but further improving the resolution became impossible.

Electrostatic interaction [9–14] was then taken into consideration since the analytes are positively charged below about pH 7 [31]. The anionic chiral selector HS-β-CD was tried and promising results were obtained. HS-β-CD can be negatively charged over a wide pH range and will migrate toward the anode (injection) end [24], against the cationic analytes. The electrophoretic mobility of HS-β-CD which is directed opposite to the migration of the solute is one of the primary factors for the enantioselectivity [10]. Such a countercurrent interaction has been proved to improve the enantioseparation or make the enantioseparation easier; this point was also demonstrated to be true by performing the separation at a low concentration of HS-β-CD. Fine enantioseparation could still be observed at 0.5% (w/v, ca. 2 m*M*) HS-β-CD for some arylglycine amides.

3.2. Buffer pH, type and concentration

Buffer pH is a critical factor controlling both the enantioresolution and migrating time. Low pH at around 2.5 was tried under reversed voltage polarity [20–25] with 0.5% HS-β-CD added but was shown to be not suitable for the separation of these weak bases. However, the best pH was found at pH around 7.0 and fast (6 min) enantioseparation of all the investigated analytes can easily be achieved with normal voltage polarity in a buffer containing 0.5% HS-β-CD. At a pH below 6.0, serious peak tailing was normally observed, and the running speed was decreased. While at a pH above 7.5, most of the enantioseparation was also destroyed, especially when the concentration of HS-β-CD was below 1.0% (see Fig. 1). Lower pH yielded higher resolution but higher pH speeded up the migration. pH 6.5 was finally adopted in this study.

In addition to pH, buffer type and its concentration were studied. At pH 6.5, two inorganic buffers (ammonium acetate and phosphate) and three organic buffers (ACES, MOPS, Tris) have been tested at a

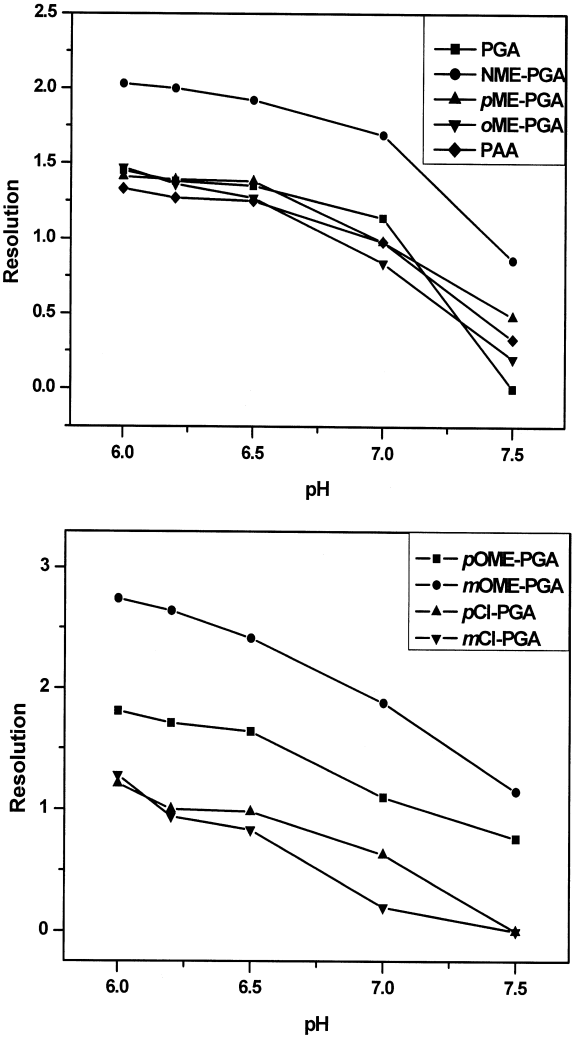


Fig. 1. Influence of buffer pH on enantioselectivity. Buffer, 0.5% (w/v) HS-β-CD in 20 m*M* MOPS; injection, 4 kV, 2 s; separation voltage, 16 kV; capillary, 40.2 cm (30 cm effective length)× 50 μm I.D.; capillary temperature, 20 °C.

concentration of 20 m*M*, but no significant difference was observed in respect of the enantioresolution. MOPS buffer was finally selected due to its low electric conductivity and high buffer capacity. The data revealed that MOPS increased the usage time. Its running current could be kept constant for more than 10 runs. More importantly, the resolution of each arylglycine amide depended slightly on the concentration of MOPS in the range of 10–50 m*M*. This was very convenient in practice. However, it should be noted that the concentration of MOPS could have an influence on the migration time. The shortest migration happened at 30 m*M* for most analytes but comigration of some analytes would happen. The concentrations ranging between 10 and 20 m*M* were actually useable. We adopted the value of 20 m*M* for the purpose of high reproducibility.

3.3. Concentration of HS-β-CD

Fig. 2 shows that the enantioresolution increases with the concentration of HS-β-CD, where three typical cases can be found: (i) near linear increase, (ii) one-stepped increase (the constant step located in between 0.5% and 1.0% HS-β-CD), and (iii) multiple-stepped increase. Analytes of series I, III and IV fall into the first case while *p*ME-PGA and *o*ME-PGA of series II are in the second. Only PAA falls into the third case. Importantly, no matter which case is concerned, HS-β-CD is better added at a concentration above 1.0% to obtain baseline resolution. But too high a concentration is not preferred for fast separation because the migration time will be largely prolonged. For instance, the migration time was doubled as HS-β-CD increased from 0.2% to 2.0%. A value of 1.5% HS-β-CD is suggested as a compromise of separation speed and resolution.

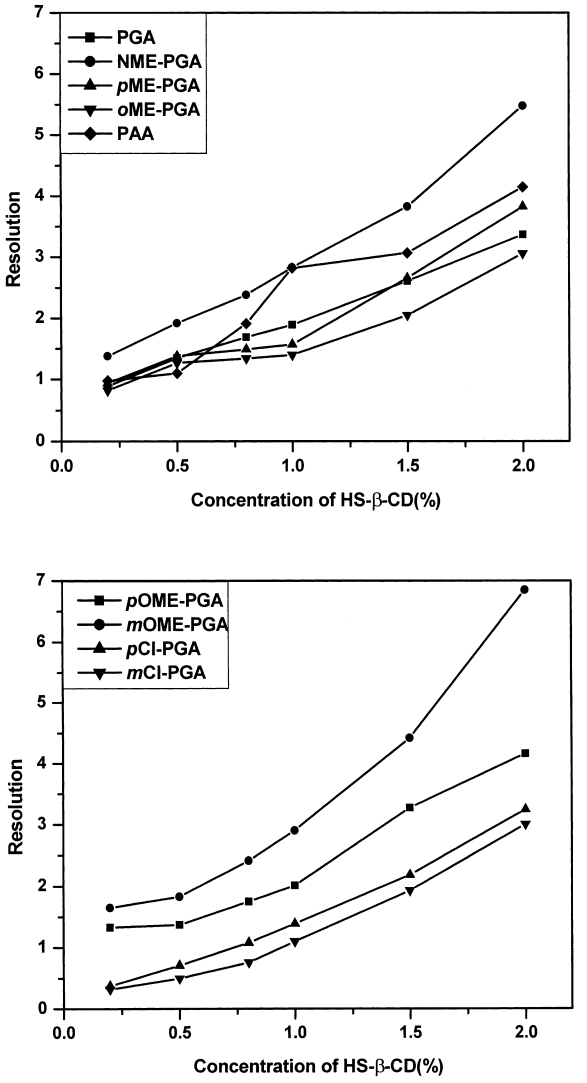


Fig. 2. Influence of HS-β-CD concentration on enantioselectivity. Buffer, *x*% (w/v) HS-β-CD in 20 m*M* MOPS at pH 6.5; other conditions as in Fig. 1.

3.4. Organic modifier

One problem was still left after the optimization of the above discussed parameters: L-*p*OME-PGA and D-*m*OME-PGA partially comigrated (see Fig. 3 curve 3). The organic modifier, methanol, was thus added at a concentration below 20% (v/v) to adjust the affinity of the hydrophobic moiety of the analytes by increasing their solubility. It is recognized that organic modifiers compete with analyte for the relatively hydrophobic cavity of negatively charged β-CD [32]. When methanol concentration reached 10%, the resolution between *p*OME-PGA and *m*OME-PGA increased from 0 to 0.69 (no HS-β-CD was added), and simultaneous enantioseparation of them became possible when 1.5% HS-β-CD was present (see Fig. 3 curve 2). However, further raising the methanol concentration degraded the enantio-

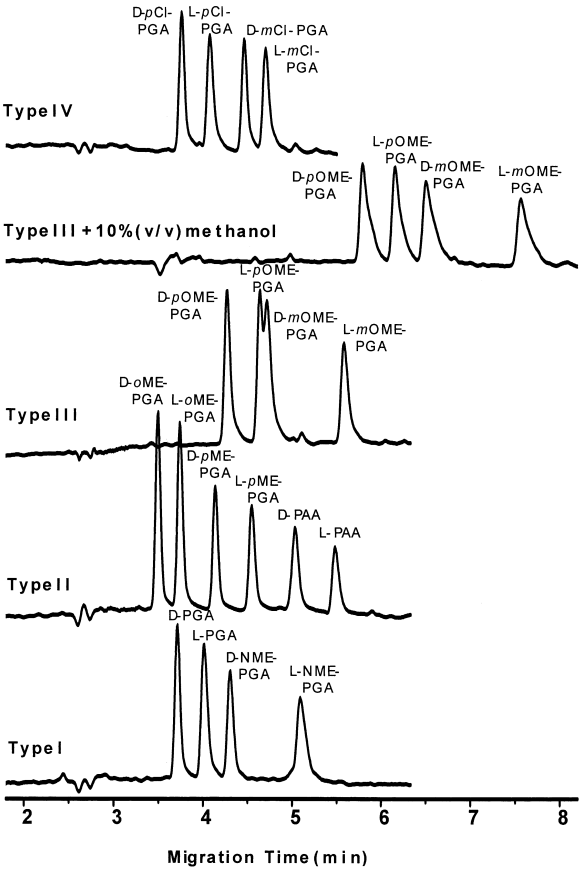


Fig. 3. CE enantioseparation of arylglycine amides under optimized conditions. Buffer, 1.5% (w/v) HS-β-CD in 20 m*M* MOPS at pH 6.5 (or apparent pH 6.5 when methanol added); other conditions as in Fig. 1.

separation of *p*OME-PGA; 10% methanol was thus adopted as the working condition.

3.5. Optimized running conditions

After systematic method optimizations, a running system was obtained, which was composed of 20 m*M* MOPS buffer at pH 6.5, 1.5% (w/v) HS-β-CD and 0 or 10% methanol, running in a bare fused-silica capillary at +16 kV and 20 °C (typical current of ~41 μA with no methanol or 33 μA with 10% methanol added). Reproducibilities of the migration time and peak area percentage of each enantiomer of racemic arylglycine amides (see Table 2) proved that the precision of this established method was reliable.

3.6. Enantioresolution order

The enantioresolution order of four *para*-substituted arylglycine amides was *p*Cl-PGA < PGA < *p*ME-PGA < *p*OME-PGA, which is almost parallel to their pK_a or electron donor order (denoted by σ values, the more negative the σ value is, the higher electron-donating ability the group will have [33]) as shown in Table 3. The same order was found for the two *meta*-substituted compounds: *m*Cl-PGA < *m*OME-PGA.

At the optimized experimental pH of 6.5, most of the investigated arylglycine amides were partially dissociated, the slight difference in pK_a values might change the charging density of the analytes. The higher the pK_a value is, the more cationized will be the analyte, and it seemingly leads to more electrostatic interaction with the anionic chiral selector. Although the electrostatic interaction is not stereoselective, it might contribute to the stereoselective interactions [10]. From the point of view of σ values, it is well-known that a higher electron-donating ability of the groups attached to the benzyl ring would increase the positive charging density of the analyte. Higher positive charge status maybe leads to more electrostatic interaction with oppositely charged chiral selectors.

However, it should be noted that NME-PGA in the four methyl (or methylene) substituted positional isomers (*o*ME-PGA, *p*ME-PGA, PAA and NME-PGA) which has the smallest pK_a had a different behavior, the largest resolution of NME-PGA indicating that the hindrance introduced by an N-substituted methyl group should contribute to the enantioseparation. The hindrance effect is further supported by the larger methoxy group of arylglycine amides *p*OME-PGA and *m*OME-PGA which also improves the enantioseparation more than the charge status.

4. Conclusion

HS-β-CD was shown to be suitable for chiral CE of arylglycine amides. The resolution was shown to

Table 2
Reproducibility of the migration times and the peak area percentage of racemic arylglycine amides in CE ($n=3$)

Analyte	Enantiomer form	Average migration time (min) (RSD, %)	Average peak area percentage (%) (RSD, %)
PGA	D-	3.71 (2.30)	50.67 (1.41)
	L-	4.01 (2.61)	49.33 (1.44)
NME-PGA	D-	4.30 (2.27)	50.88 (1.33)
	L-	5.08 (2.16)	49.12 (1.38)
*p*ME-PGA	D-	4.13 (1.86)	50.45 (0.93)
	L-	4.54 (1.31)	49.55 (0.95)
*o*ME-PGA	D-	3.49 (1.65)	50.41 (0.97)
	L-	3.73 (0.96)	49.59 (0.99)
PAA	D-	5.03 (1.10)	50.80 (0.78)
	L-	5.48 (1.53)	49.20 (0.81)
*p*OME-PGA	D-	4.27 (1.11)	50.56 (0.76)
	L-	4.63 (1.27)	49.44 (0.78)
*m*OME-PGA	D-	4.71 (1.21)	50.70 (0.63)
	L-	5.58 (1.61)	49.30 (0.65)
*p*Cl-PGA	D-	3.75 (0.92)	49.35 (0.19)
	L-	4.07 (0.91)	50.65 (0.19)
*m*Cl-PGA	D-	4.45 (1.55)	49.59 (0.68)
	L-	4.70 (0.44)	50.41 (0.67)

Optimized conditions: buffer, 1.5% (w/v) HS-β-CD, 20 m*M* MOPS at pH 6.5; injection, 4 kV×2 s; separation voltage, 16 kV; capillary, 40.2 cm (30 cm effective length)×50 μm I.D.; capillary temperature, 20 °C.

Table 3
Relationship of enantioresolution with the pK_a values and Hammett substituent constants. Conditions as in Table 2

Symbol	*p*Cl-PGA	PGA	*p*ME-PGA	*p*OME-PGA	*m*Cl-PGA	*m*OME-PGA	*o*ME-PGA	PAA	NME-PGA
σ[a]	0.23	0	−0.17	−0.27	0.37	0.12			
pK_a[b]	6.74±0.31	7.00±0.29	7.12±0.31	7.11±0.31	6.72±0.50	6.94±0.50	7.06±0.50	7.50±0.33	7.03±0.20
R_s	2.18	2.61	2.66	3.27	1.93	4.42	2.05	3.07	3.83

[a] σ denotes the Hammett substituent constant calculated from thermodynamic equilibrium constants of benzoic acids in water at 25 °C [33].
[b] From Ref. [31].

be dependent on CD concentration, buffer pH, organic modifier and the structure of analytes. An effective chiral CE separation system is composed of 1.5% HS-β-CD, 0 to 10% methanol and 20 m*M* MOPS at pH 6.5. Using this system, baseline enantioseparation of all nine arylglycine amides can be achieved within 6 min. Simultaneous enantioseparation of positional isomers can also be achieved. The D-enantiomer migrated ahead of the L-enantiomer for all nine pairs of arylglycine amide enantiomers, showing that the D-form has a weaker interaction with the chiral selector than the L-form. Electrostatic interaction was suggested to contribute to the stereoselective interactions and the hindrance effect has an influence on this enantioseparation.

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