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Chiral separation of nipecotic acid amides

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ABSTRACT

1-Decyl-3-(N,N-diethylcarbamoyl)piperidine (1) and α,α′-bis[3-(N-benzyl-N-methylcarbamoyl)piperidinol]-*p*-xylene (2) represent mono-N-substituted and bis-N-substituted carbamoylpiperidines, or nipecotic acid amides, respectively. Initially, several attempts were made to resolve these compounds using β-cyclodextrin, cellulose carbamate and Pirkle-type columns. However, the interactions of the stereoisomers of the two compounds with these stationary phases did not differ enough to permit satisfactory separations. Baseline resolution was achieved using an α_1-acid glycoprotein (AGP) chiral column. The mobile phase was phosphate buffer (pH 7.0). Tetrabutylammonium (TBA) was used as the cationic modifier and ethanol as the uncharged modifier. Circular dichroism was used to identify the enantiomers. Compound 1 was resolved into positive and negative enantiomers and 2 into positive and negative enantiomers and a *meso* diastereomer. The influence of pH, buffer ionic strength, cationic and uncharged modifier concentrations on retention, chiral selectivity and resolution were evaluated. Based on the results, it is suggested that both ionic and hydrophobic interactions may be responsible for retention and resolution.

INTRODUCTION

Many drugs are marketed as racemates although one enantioner may contribute to virtually all of the therapeutic activity [1]. In a few instances, the less active isomer happens to be the more toxic one [2]. Therefore, chiral resolutions of biologically active compounds assume considerable therapeutic importance.

Our laboratory has been engaged in the synthesis of nipecotic acid amides (Fig. 1) of types I and II as potential antithrombotic agents. Although the precise mechanism of the antiplatelet action of these compounds has not yet been elucidated, it has been hypothesized that they interact with anionic phospholipids, *e.g.*, phosphatidylserine and phosphatidylinositol, of the platelet membrane [3,4] and of certain intracellular organelles [5,6]. As these phospholipid molecules contain many chiral centers, it is conceivable that stereoselective interactions occur with xenobiotics. Types I and II compounds have chiral centers at the 3-position of the piperidine ring and therefore could exhibit stereoselective interactions with anionic phospholipids. With a view to investigating such properties, we have attempted the resolution of these compounds. 1-Decyl-3-(N,N-diethylcarbamoyl)piperidine hydrobromide (**1**; type I, n = 9, R, R′ = Et) and α,α′-bis[3-(N-benzyl-N-methylcarbamoyl)piperidino]-*p*-xylene dihydrobromide (**2**; type II, R = Bz, R′ = Me) were chosen for resolution as representatives of the two structural types. Compound **1** has been reported to inhibit ADP-induced human platelet aggregation *in vitro* (Ic_{50} 174.3 μ*M*) [7]. Compound **2** was potent in inhibiting platelet aggregation *in vitro* (Ic_{50} 27.3 μ*M*) [8] and in protecting mice from thromboembo-

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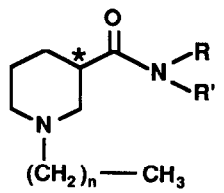
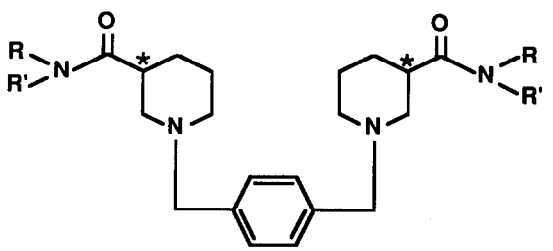


Type I. 1 - [3 - (N, N - dialkylcarbamoyl) piperidino]-alkanes



Type II. α,α′-Bis [3 - (N, N - dialkylcarbamoyl) piperidino]-*p*-xylenes

Fig. 1. Structures of nipecotic acid amides. The chiral center is indicated with an asterisk.

lic death upon challenge with collagen and epinephrine [9]. Compound **1** can exist as *R* and *S* enantiomorphs and **2**, which has two chiral centers, can exist as *R,R* and *S,S* enantiomers and *R,S* and *S,R* diastereomers. The *R,S* and *S,R* forms are superimposable and, therefore, achiral. Consequently, they are represented as the *meso* configuration. This paper describes the resolution of **1** and **2** on a chiral α_1-acid glycoprotein (AGP) column.

EXPERIMENTAL

Materials

Tetrabutylammonium (TBA), hydrogensulfate, bromide, chloride and hydroxide were purchased from Sigma (St. Louis, MO, USA). Anhydrous Na_2HPO_4 (analytical-reagent grade) was obtained from Mallinckrodt (St. Louis, MO, USA) and NaH_2PO_2 (reagent grade) from MCB Manufacturing Chemists (Cincinnati, OH, USA). Platinum-(IV) oxide monohydrate was purchased from Aldrich (Milwaukee, WI, USA). High-performance liquid chromatographic (HPLC) solvents including water were supplied by Burdick and Jackson (Muskegon, MI, USA). All solvents and buffers were filtered using nylon-66 (0.45-μm) filters (Rainin Instruments, Woburn, MA, USA). The solvents were continuously degassed by purging with helium (ultrahigh purity; Liquid Air, Walnut Creek, CA, USA).

Instrumentation

The chromatographic system consisted of a Waters U6K injector, a Model 600E Powerline multi-solvent delivery system, a Model 484 tunable UV–VIS detector, a NEC PowerMate SX plus computer with an NEC P5200 printer/plotter and Baseline 810 Chromatography Workstation software. A chiral AGP (5-μm) analytical column (100 × 4.0 mm I.D.) was purchased from Regis Chemical (Morton Grove, IL, USA). The following chiral HPLC columns (250 × 4.6 mm I.D.) (5 μm) were used in the exploratory part of this investigation: (*R*)-naphthylalanine (covalent), (*S*)-naphthylleucine (covalent) (both from Regis Chemical), (*R*)-naphthylurea and (*R*)-(3,5-dinitrobenzoyl)phenylglycine (covalent) (both from J. T. Baker, Phillipsburg, NJ, USA) and (*S*)-naphthylethylcarbamate-derivatized β-cyclodextrin (Cyclobond I SN) (from Astec, Whippany, NJ, USA). Chiralcel AD and Chiralpak OD were both obtained from Chiral Technologies (Exton, PA, USA).

Synthesis

Compound **1** was synthesized as described [10]. Compound **2** was obtained by the catalytic reduction (PtO_2–H_2) of α,α′-bis[N-benzyl-N-methylcarbamoyl)pyridinium]-*p*-xylene dibromide, which was prepared by the condensation of α,α′-dibromo-*p*-xylene and N-benzyl-N-methylnicotinamide; the latter was obtained by reaction of nicotinoyl chloride with N-benzylmethylamine [8].

HPLC

In the exploratory phase of the chiral separations, the mobile phases consisted of various ratios of solvents at flow-rates ranging from 0.4 to 0.9 ml/min. The following are typical examples, where either very poor or no resolution occurred and **1** and **2** eluted as fused, or single peaks: with (*S*)-naphthylleucine column, *n*-hexane (with and without 0.0025

M triethylamine)–methanol, 2-propanol or 2-butanol, *n*-hexane (0.0025 *M* triethylamine)–2-butanol and chloroform, k' = 1.5–14 when triethylamine was added to *n*-hexane and >60 when it was not added; with (*R*)-naphthylalanine column, *n*-hexane (0.0025 *M* triethylamine)–2-propanol–chloroform, k' = 4–19; with Cyclobond I SN column, *n*-hexane–2-propanol, acetonitrile–triethylammonium acetate (pH 4.0 and 7.0), k' = 4–14; and with Chiralcel AD column, 2-propanol–n-hexane, ethanol–*n*-hexane and methanol–*n*-hexane, k' = 4–14.

Resolution of compounds **1** *and* **2** *on a chiral* α_1*-AGP column*

A systematic study was carried out with a view to (i) establishing how separation is affected by altering experimental parameters such as pH, ionic strength and flow-rate, and (ii) obtaining an optimum separation of the isomers. The HPLC conditions and parameters were gradually changed and the resultant effects on the capacity factor (k'), selectivity (α) and resolution (R_s) were calculated using the following equations:

$$k' = (V_1 - V_0)/V_0$$

$$\alpha = (V_2 - V_0)/(V_1 - V_0)$$

$$R_s = (V_2 - V_1)/[1/2(W_1 + W_2)]$$

where V_1 and V_2 = retention volumes of the first- and second-eluted components, respectively, V_0 = void volume and W_1 and W_2 = widths of peaks A and B, respectively. Optimum conditions consisted of a mobile phase of 10% ethanol in 0.025 *M* phosphate buffer (PB) (pH 7.0) containing 0.025 *M* TBA · HSO_4. The flow-rate was 0.9 ml/min and the detector was set at 225 nm. Compounds **1** and **2** were dissolved in the mobile phase for injection into the column.

Circular dichroic spectra

A Jasco (Tokyo, Japan) J-600 spectropolarimeter was used to obtain circular dichroic (CD) spectra. The collected chromatographic fractions were extracted with diethyl ether (pH 9.0), the solvent was evaporated and the residues were dissolved in 2.2 ml of cyclohexane (HPLC grade, Aldrich). Dissolution was completed with the help of a vortex stirring device. A 1.0-cm path-length cylindrical quartz cell (Jasco) was used to hold the sample solution. The conditions for CD data acquisition were as follows: band width = 2 nm; slit width = auto; time constant = 2.0 s; step resolution = 1 nm; scan speed = 50 nm/min; number of scans = 2; wavelength set from 180 to 500 nm. The raw data was further smoothed using a set data interval of 1.

RESULTS AND DISCUSSION

Compound **1** was resolved into two components, **1A** and **1B** (Fig. 2), and **2** was separated into three components, **2A**, **2B** and **2C** (Fig. 3), achieving a baseline separation on the α_1-AGP column with a mobile phase consisting of 10% ethanol in 0.025 *M* PB containing 0.025 *M* TBA · HSO_4 (pH 7.0) at a flow-rate of 0.9 ml/min. It is apparent from the CD spectra that **1A** has a positive Cotton effect at 230 nm and that **1B** is its optical antipode with a negative Cotton effect at the same wavelength (Fig. 4). Components **2A** and **2C** appear to be enantiomers, giving negative and positive Cotton effects respectively, at 235 nm (Fig. 5). Fraction **2B**, which shows no Cotton effect, should be the *meso* isomer.

Several attempts were made to resolve these compounds on β-cyclodextrin, cellulose carbamate and Pirkle-type columns. The interactions of the two enantiomers of **1** and of the three isomers of **2** with the stationary phases of these columns did not differ sufficiently to afford satisfactory resolution. The α_1-AGP column, derived from human plasma, has

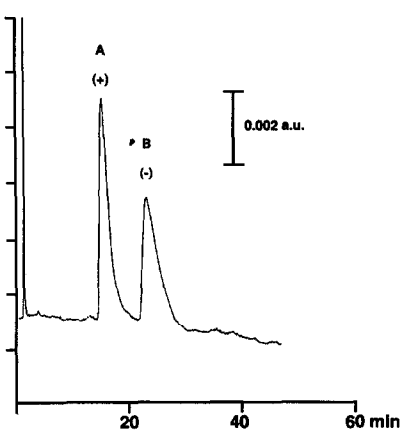


Fig. 2. Chromatogram showing resolution of **1** (2 μg injected). A chiral AGP (5-μm) analytical column (100 mm × 4 mm I.D.) was used with a mobile phase consisting of 10% ethanol in 0.025 *M* PB (pH 7.0) containing of 0.025 *M* TBA · HSO_4. Flow-rate, 0.9 ml/min; temperature, ambient.

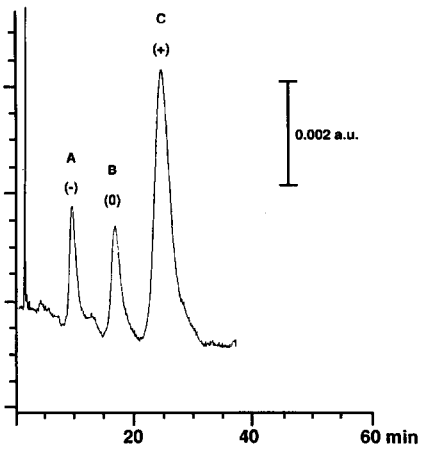


Fig. 3. Chromatographic resolution of **2**. Conditions as in Fig. 2.

been found to be useful for the separation of a number of basic pharmaceutical compounds, presumably because this acidic protein can interact with protonated bases by ion exchange [11]. It therefore appeared suitable for the resolution of nipecotic acid amides (3-carbamoylpiperidines). α_1-AGP, with an isoelectric point at pH $\approx$ 2, has a net negative charge at pH 7.0 [11]. The piperidine nitrogen of **1** and **2** is believed to be protonated at pH 7.0 and can be expected to be tightly bound to the stationary phase. Also, because of the presence of a C_{10} alkyl chain in **1** and the xylylene moiety in **2**, both ionic and hydrophobic interactions are possible. The cationic modifier TBA could compete with **1** and **2** for ionic bonding with anionic groups such as sialic acid residues present on the glycoprotein [11].

α_1-AGP contains several chiral centers with different binding characteristics. It is therefore possible

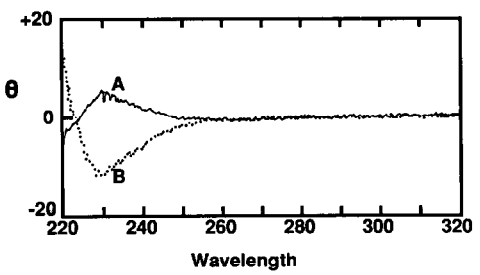


Fig. 4. CD spectrum of fractions A and B from **1**. Approximate concentrations: **1A** = 0.1 and **1B** = 0.5 mg/ml in cyclohexane. Wavelength in nm; θ in millidegrees.

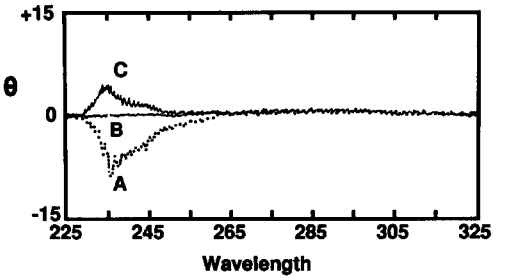


Fig. 5. CD spectrum of fractions A, B and C separated from **2**. Approximate concentrations: **2A** = 0.4, **2B** = 0.2 and **2C** = 0.5 mg/ml in cyclohexane. Wavelength in nm; θ in millidegrees.

to alter the retention time, selectivity and resolution by varying the mobile phase pH, molarity and organic and ionic modifiers, in order to arrive at an optimum separation.

Effect of pH

The influence of pH was more pronounced than the effects of organic and ionic modifiers. Retention and resolution increased with increasing pH, suggesting that an increase in the negative charge of the AGP molecule results in a greater ion-exchange binding of the positively charged sample molecules (Table I). Thus, for **1**, changing the pH from 6.0 to 7.0 increased α from 1.14 to 1.58 and increased R_s from 0.43 to 1.28, providing baseline separation of the two components. At pH 7.0, the three components of **2** showed baseline separation (α_1 = 1.87, α_2 = 1.51) but lower pH values resulted in incomplete resolution or no resolution at all. Because of the low efficiency of AGP columns. selectivity factors >1.5 are believed to be required to achieve baseline separations [12].

It may be noted that with **1** and **2**, a cationic N^+ moiety (at pH 7.0 of the mobile phase) is separated from the chiral center by one carbon atom and also the hydrogen-bonding amide group is coupled directly to the chiral center, features deemed favorable for chiral resolution on an α_1-AGP column [13].

Effect of uncharged modifier

Organic solvents influence resolution and selectivity by causing conformational changes in the protein molecules. The retention, selectivity and resolution of **1** decreased when the organic modifier was changed from ethanol to 2-propanol and from

TABLE I

EFFECT OF pH ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase: 0.025 *M* phosphate buffer containing 10% ethanol and 0.025 *M* TBA · HSO_4. Flow-rate, 0.9 ml/min.

pH	1			2				
	k'^a	α	R_s	k'^b	α_1	α_2	$R_{s1}{}^c$	$R_{s2}{}^d$
6.0	10.82	1.14	0.43	One peak				
6.5	17.15	1.21	0.76	Two peaks				
7.0	19.91	1.58	1.28	21.65	1.87	1.51	1.29	0.88

[a] Capacity factor of the second-eluted peak.
[b] Capacity factor of the third-eluted peak.
[c] Resolution of peaks A and B.
[d] Resolution of peaks B and C.

TABLE II

EFFECT OF ORGANIC MODIFIER ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase: 0.025 *M* phosphate buffer (pH 7.0) containing 10% of organic modifier and 0.025 *M* TBA · HSO_4. Flow-rate, 0.9 ml/min.

Organic modifier	1			2				
	k'^a	α	R_s	k'^b	α_1	α_2	$R_{s1}{}^c$	$R_{s2}{}^d$
Ethanol	19.91	1.58	1.28	21.65	1.87	1.51	1.29	0.88
2-Propanol	10.94	1.40	0.76	12.49	1.46	1.32	0.93	0.62
Acetonitrile	9.31	1.18	0.69	10.49	1.35	1.62	0.84	1.33

[a] Capacity factor of the second-eluted peak.
[b] Capacity factor of the third-eluted peak.
[c] Resolution of peaks A and B.
[d] Resolution of peaks B and C.

TABLE III

EFFECT OF MOBILE PHASE SOLVENT RATIO WHEN USING ETHANOL AS ORGANIC MODIFIER ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase: 0.025 *M* phosphate buffer (pH 7.0) containing ethanol and 0.025 *M* TBA · HSO_4. Flow-rate, 0.9 ml/min. The values represent averages of three independent determinations.

Ethanol–buffer (v/v)	1			2				
	k'^a	α	R_s	k'^b	α_1	α_2	$R_{s1}{}^c$	$R_{s2}{}^d$
20:80	10.98	1.52	1.11	10.08	1.33	1.32	0.61	0.76
15:85	17.25	1.59	1.34	18.48	1.36	1.45	0.75	0.92
10:90	22.32	1.60	1.36	26.35	1.57	1.53	0.96	1.10
7:93	41.84	1.70	1.61	55.19	1.77	1.45	1.85	1.34
3:97	107.86	1.73	1.97	153.99	3.11	1.44	3.54	1.48

[a] Capacity factor of the second-eluted peak.
[b] Capacity factor of the third-eluted peak.
[c] Resolution of peaks A and B.
[d] Resolution of peaks B and C.

TABLE IV

EFFECT OF BUFFER CONCENTRATION ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase: Phosphate buffer (pH 7.0) containing 10% ethanol and 0.025 *M* TBA · HSO_4. Flow-rate, 0.9 ml/min. The values represent averages of three independent determinations.

Buffer concentration (*M*)	1			2				
	k'^a	α	R_s	k'^b	α_1	α_2	$R_{s1}{}^c$	$R_{s2}{}^d$
0.01	24.52	1.40	1.21	29.04	1.46	1.61	0.84	1.28
0.015	24.19	1.49	1.28	28.56	1.57	1.56	0.88	1.14
0.025	22.32	1.60	1.36	26.35	1.57	1.53	0.96	1.10
0.035	18.00	1.60	1.37	25.32	1.69	1.43	1.00	0.81

[a] Capacity factor of the second-eluted peak.
[b] Capacity factor of the third-eluted peak.
[c] Resolution of peaks A and B.
[d] Resolution of peaks B and C.

2-propanol to acetonitrile (Table II). Similarly, the k', α_1 and R_{s1} values of the first and second components of **2** decreased when the organic modifier was changed from ethanol to 2-propanol and then to acetonitrile. Consequently, ethanol was chosen as the organic modifier, although a better resolution of the second and third peaks of **2** was observed with acetonitrile than with ethanol as the modifier.

As would be expected, the retention times increased with decreasing ethanol concentration (Table III). The selectivity and resolution of both compounds increased when the ethanol concentration was decreased from 20% to 3%. The separation factor (α_2) for peaks B and C of **2** increased with decreasing ethanol concentration from 20% to 10%. Further reduction to 3% resulted in a decrease in α_2.

Effect of buffer and cationic modifier concentrations

The concentration of PB was increased stepwise from 0.01 to 0.035 *M* while maintaining the TBA · HSO_4 concentration at 0.025 *M* (Table IV). Increasing the buffer concentration resulted in a decrease in k', suggesting an ion-exchange retention

TABLE V

EFFECT OF CATIONIC MODIFIER (TBA · HSO_4) ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase: 0.025 M phosphate buffer (pH 7.0) containing 10% ethanol and various concentrations of TBA · HSO_4. Flow-rate, 0.9 ml/min. The values represent averages of three independent determinations.

TBA · HSO_4 concentration (*M*)	1			2				
	k'^a	α	R_s	k'^b	α_1	α_2	$R_{s1}{}^c$	$R_{s2}{}^d$
0.01	40.10	1.42	1.18	52.46	1.39	1.61	0.71	1.33
0.015	29.01	1.45	1.35	40.93	1.47	1.58	0.89	1.15
0.025	22.32	1.60	1.36	26.35	1.57	1.53	0.96	1.10
0.035	19.29	1.60	1.72	24.49	1.67	1.44	1.18	0.89

[a] Capacity factor of the second-eluted peak.
[b] Capacity factor of the third-eluted peak.
[c] Resolution of peaks A and B.
[d] Resolution of peaks B and C.

process. The selectivity and resolution increased with increasing buffer concentration. However, α_2 and R_{s2} decreased with increasing buffer concentration.

The relationship between selectivity and concentration of the charged modifier was also studied while the molarity of PB was maintained constant. Increasing the concentration of TBA from 0.01 to 0.035 *M* resulted in a decrease in the retention times and an increase in selectivity and resolution. However, the α_2 and R_{s2} values of **2** decreased with increasing TBA concentration (Table V). A change in the modifier concentration resulted in a change in selectivity, and as retention appears to be based on an ion-pairing mechanism, 3-carbamoylpiperidines can be presumed to bind to multiple chiral and achiral sites on the glycoprotein. If, on the other hand, the enantiomers were to be retained by a single type of binding site, then the stereoselectivity would not change with changes in the cationic modifier concentration [14].

The separation (α) and resolution (R_s) of the two enantiomers of **1**, and those of peaks A and B of **2**, followed the expected behavior. However, the α_2 and R_{s2} values of peaks B and C of **2** showed unexpected variability (Tables III–V). It is possible that stereochemical interactions between the latter two peaks and the chiral stationary phase are controlled by factors other than ion-exchange and hydrophobic interactions.

A flow-rate of 0.9 ml/min was chosen because of inefficient resolution at lower flow-rates. Ambient temperature was used for analysis. Higher or lower temperatures did not improve resolution.

The conditions that were chosen for further resolution of **1** and **2** consisted of a mobile phase of 10% ethanol in 0.025 *M* PB containing 0.025 *M* TBA · HSO_4 (pH 7.0) with a flow-rate of 0.9 ml/min. These conditions afforded baseline chiral resolution of **1** and **2**.

Column stability

Initially (1 week), the selectivity factor α for **1** was 1.60. After 4 weeks of use (200 injections), α was 1.47. During this period, the column was cleaned daily with water followed by 25% aqueous 2-propanol. It was also occasionally flushed with 0.01 *M* PB (pH 3.5) followed by 50% aqueous ethanol. The routine and diverse use of the column for 4 months and approximately 400 injections resulted in a deterioration of its performance, and **2** was resolved into only two peaks (α = 1.46) instead of three.

CONCLUSIONS

The resolution of mono- and bis-substituted nipecotic acid amides was not possible on many conventional chiral HPLC columns. Adequate separation was achieved on the α_1-AGP column. Complete baseline chiral separation of **1** and **2** was achieved on this column with a mobile phase consisting of 10% ethanol in 0.025 *M* PB containing 0.025 *M* TBA · HSO_4 at pH 7.0. Compound **1** was separated into two enantiomers and **2** into two enantiomers and a diastereomer, as corroborated by their CD spectra. The effects of pH, ionic strength and ionic and uncharged modifiers were evaluated. Based on the results, an ion-pairing mechanism and hydrophobic interactions appear to be responsible for retention and resolution.

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