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CORRELATION OF CALCITONIN STRUCTURE WITH CHROMATOGRAPHIC RETENTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Chromatographic selectivity and position specificity were examined by using deletion and substitution analogues of calcitonin (CT), a 32-amino acid polypeptide. The biological activity of CT has been shown to be related to structural features, including a regular spacing of hydrophobic and hydrophilic residues in positions 8–22. The effect of structure on retention behavior in hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC) of 19 CT analogues was examined. No simple correlation was found between chromatographic retention and amino acid composition. Deletion of one leucine residue reduced retention in both chromatographic systems, but the magnitude of the change depended upon the site at which the deletion occurred. For example, deletion analogues des(Leu^{16})-, des-(Leu^{12})-, des(Leu^{19})-, des(Leu^{9})-, des(Leu^{4})-, and salmon calcitonin had retention times of 5.22, 8.44, 11.64, 13.53, 15.45, and 20.28 min, respectively, in HIC in contrast to RPC retentions of 15.19, 15.84, 28.53, 27.57, 25.92 and 34.79 min, respectively. Serine deletion was also shown to be position-specific. Non-amphiphilic analogues were eluted before amphiphilic ones. Circular dichroic spectral studies showed that the CT analogues possessed little secondary structure in the HIC solvents in contrast to α-helix formation in RPC solvents. The HIC data provided indirect evidence of secondary structure, induced in the amphiphilic CT analogues at the HIC solvent–chromatographic surface interface. Solute–solvent interactions contributed to differences observed between the selectivity of RPC and HIC.

INTRODUCTION

Changes in polypeptide primary and tertiary structure, such as racemization, oxidation, or incorrect disulfide coupling, can be quantitatively measured by high-performance liquid chromatography (HPLC)[1]. Work by Meek[2], Meek and Rossetti[3]

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and Parker *et al.*[4] showed that of the twenty common amino acids, some have a positive contribution to retention of peptides in reversed-phase chromatography (RPC), others have a negative contribution (earlier elution), while some amino acids, such as glycine, make little or no contribution to chromatographic retention of the peptide. Direct correlation of peptide retention in RPC fails when the peptide contains more than twenty residues[3].

The effect of structure on the RPC retention characteristics of larger molecules is illustrated by the study of 30 interleukin-2 muteins by Kunitani *et al.*[5]. Conformational differences resulting from changes in the residue positions of the disulfide bonds resulted in large changes in the chromatographically derived stoichiometry factor, Z, which is directly related to the polypeptide contact surface area with the sorbent surface. Proteins and peptides can undergo conformational changes during RPC resulting in loss of biological activity[6–9]. Both organic solvents and sorbents have been identified as causing protein denaturation with subsequent loss in biological activity in RPC.

Structural effects on retention in hydrophobic interaction chromatography (HIC) are not well understood. Fausnaugh and Regnier[10] studied avian lysozymes under HIC conditions to ascertain which surfaces of the protein are in contact with the hydrophobic sorbent surface. The proteins included in the study differed slightly in amino acid substitution but were nearly identical according to X-ray crystallography. It was shown that while residue substitution in some parts of lysozyme had an effect on retention behavior, substitution in other parts of the protein had no influence on HIC. It was concluded that one particular region of the lysozyme surface, that opposite the catalytic cleft, dominated chromatographic retention of lysozyme in HIC.

Surface-induced structural changes of polypeptides under HIC conditions have been reported. Wu and co-workers[11,12] studied temperature-induced conformational changes of α-lactalbumin, and the resulting stoichiometry factor, Z, retention time, and peak width with a photodiode array detector. Lau and co-workers[13,14] demonstrated that even relatively small two-stranded α-helical coiled coils underwent denaturation on the HIC sorbent. The importance of HIC for the separation of polypeptides warrants further understanding of solute–surface interactions in HIC and the relationship of structure to retention.

We have examined the application of HPLC to monitoring of the primary and secondary structure of polypeptides. The selectivity and positional specificity of both HIC and RPC were compared by using calcitonin (CT) analogues as models. CT is a single polypeptide chain of 32 amino acids containing a sequence from approximately residue 8 to residue 22 which can form an amphiphilic α-helix in which hydrophobic residues are on one face of the helix and hydrophilic residues are on the other[15,16]. The sequence shows considerable species variation but maintains regular spacing of hydrophobic amino acids in this region.

EXPERIMENTAL

Materials

Salmon calcitonin (sCT) and its related analogues were prepared by solid-phase peptide synthesis and purified by preparative HPLC[17]. Reduced sCT was pre-

pared by treating sCT with 2-mercaptoethanol and purified by HPLC[17]. Peptides of >90% purity were obtained. Synthetic human (hCT) was obtained from Ciba-Geigy (Summit, NJ, U.S.A.). Model CT (mCT-II) was obtained from Rockefeller University (New York, U.S.A.). Reagent chemicals were obtained from the following sources: ammonium sulfate (Bio-Rad, Richmond, CA, U.S.A.); disodium hydrogenphosphate, granular analytical-reagent grade (Mallinckrodt, St. Louis, MO, U.S.A.); HPLC-grade potassium dihydrogenphosphate (Fisher, Fair Lawn, NJ, U.S.A.); acetonitrile, UV grade (American Burdick & Jackson, Muskegon, MI, U.S.A.).

Apparatus

Separations were achieved on a Beckman (Beckman Instruments, Fullerton, CA, U.S.A.) Model 322 liquid chromatograph, equipped with 114M pumps, a Model 153 detector, and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator and a Waters (Milford, MA, U.S.A.) liquid chromatograph, equipped with Model 45 pumps, a Model 730 gradient programmer, a Model 490 detector, a Model 740 reporting integrator, and a Model 710 Waters Intelligent Sample Processor. Detection for HIC was at 280 nm; detection for RPC was at 220 nm. SynChropak propyl columns (100 × 4.6 mm I.D.) were obtained from SynChrom (Linden, ID, U.S.A.) and Partisil 5 ODS-3 RAC II columns (100 × 4.6 mm I.D.) from Whatman (Clifton, NJ, U.S.A.). For HIC the column was thermostatically controlled at 30°C, using a Haake (Saddle Brook, NJ, U.S.A.) Model FK constant temperature bath. Circular dichroic spectra were recorded on a JASCO (Easton, MD, U.S.A.) Model J500A or JASCO Model J41 recording spectropolarimeter at 25°C.

Methods

HIC. A flow-rate of 1 ml/min was used throughout. Separations were carried out as follows: eluent A, 1 *M* ammonium sulfate, 20 m*M* phosphate buffer (pH 7.0 or as indicated); eluent B, 20 m*M* phosphate (pH 7.0 or as indicated); gradient: 3 min at 100% A, then 30-min linear gradient from 100% A to 100% B. The system void volume was: column, 1.2 ml; mixer, 2.8 ml. Polypeptides were prepared at a concentration of 0.5 mg/ml in eluent B immediately before chromatography and injected, using a 50 μl loop.

RPC. A flow-rate of 2 ml/min was used. Separations were carried out as follows: eluent A, 20 m*M* phosphate buffer (pH 7) acetonitrile (9:1); eluent B, acetonitrile–20 m*M* phosphate buffer (pH 7) (3:2); 40-min linear gradient from 80% A (20% B) to 40% A (60% B). The system void volume was: column, 1.46 ml; mixer, 0.4 ml. Polypeptides were prepared at a concentration of 1 mg/ml in water immediately before chromatography and injected in a volume of 20 μl.

Circular dichroic spectra. The spectra of the peptides were measured in solvents having a composition similar to that of the chromatographic eluent at the time of peptide elution. Peptides were prepared at a concentration of 0.1 mg/ml in the solvents indicated below, and spectra were recorded as single scans, using in a 1-mm-path-length cell. Secondary structure was estimated with the aid of a computer program, based on the Chou and Fasman sets[18].

Hormone biological activity. The hypocalcemic activity was assayed *in vivo* by measuring the decrease in blood calcium levels following peptide administration to rats[19].

TABLE I

PRIMARY AMINO ACID SEQUENCE OF CALCITONIN ANALOGUES

Hydrophobic residues are indicated in italics.

Analogue	*Sequence*																													
	1							*5*									*10*													
Salmon CT	H_2N-	Cys-	Ser-	Asn-	*Leu-*	Ser-	Thr-	Cys-	*Val-*	*Leu-*	Gly-	Lys-	*Leu-*	Ser-																
Des(Leu[4])-sCT					–																									
Des(Leu[9])-sCT										–																				
Des(Leu[12])-sCT																														
Des(Leu[16])-sCT																														
Des(Leu[19])-sCT																														
Des(Ser[2])-sCT			–																											
Des(Ser[13])-sCT																														–
Des(Leu[19])Leu[15],Glu[16]-sCT																														
Des(Leu[16])Gly[8],D-Arg[24]-sCT																	Gly													
Des(Lys[11],Leu[12])-sCT																										–	–			
endo(Gly[18])-sCT	H_2N-	Cys-	Gly																											
Gly[8]-sCT																	Gly													
Des(Thr[21])-sCT																														
Des(Tyr[22])-sCT																														
Human CT	H_2N-	Cys-	Gly-	Asn-	*Leu-*	Ser-	Thr-	Cys-	Met-	*Leu-*	Gly-	Thr-	*Tyr-*	Thr-																
MCT-II*	H_2N-	Cys-	Ser-	Asn-	*Leu-*	Ser-	Thr-	Cys-	*Leu-*	*Leu-*	Gln-	Gln-	*Leu-*	Gln-																

* Ref. 20.

RESULTS AND DISCUSSION

HIC

The amino acid sequences of the polypeptides included in this study are shown in Table I. The hydrophobic residues of sCT and hCT calcitonin are indicated in italics, showing the regular spacing of hydrophobic and hydrophilic residues. hCT differs from sCT by 16 residues and in its isoelectric point; 8.7 for hCT *vs.* 10.4 sCT[21]. The retention behavior of five CT analogues on a weakly hydrophobic column, eluted with descending ammonium sulfate gradients at pH 5.0, 6.0, and 7.0 were examined. The results are depicted in Fig. 1. The calcitonin analogues were eluted as symmetrical peaks. Their retention decreased as the pH was lowered but no change in selectivity with change in pH was observed.

The HIC retention characteristics of the analogues listed in Table I were examined on the propyl column at pH 7 with linear descending ammonium sulfate gradients. A brief period (3 min), during which any impurities can be eluted by eluent A (1 *M* ammonium sulfate in buffer), was maintained before gradient elution. The results, summarized in Table II, show that HIC is very sensitive to changes in the polypeptide primary structure. Most analogues were well separated from the parent compound, sCT, the exception being des(Thr[21])-sCT, which had a retention time differing from sCT by only 0.4 min. Two analogues, reduced sCT and mCT-II, failed to elute under the described HIC conditions.

No direct relationship was found between amino acid composition and chro-

15	20	25	30
-Gln- Glu- *Leu*- His- Lys- *Leu*- Gln- Thr- *Tyr*- Pro- Arg- Thr- Asn- Thr- Gly- Ser- Gly- Thr- Pro- NH_2			
–			
	–		
Leu Glu	–		
–		D-Arg	
		–	
		–	
-Gln- Asp- *Phe*- Asn- Lys- *Phe*- His- Thr- *Phe*- Pro- Gln- Thr- Ala- *Ile*- Gly- *Val*- Gly- Ala- Pro- NH_2			
-Lys- *Leu*- *Leu*- Gln- Lys- *Leu*- Lys- Gln- *Tyr*- Pro- Arg- Thr- Asn- Thr- Gly- Ser- Gly- Thr- Pro- NH_2			

matographic retention. Deletion of one hydrophobic Leu residue reduced chromatographic retention, but the magnitude of the change depended upon the site at which the deletion occurred. For example, the analogues des(Leu[16])-, des(Leu[12])-, des(Leu[19])-, des(Leu[9])-, des(Leu[4])-, and sCT had retention times of 5.22, 8.44, 11.64, 13.53, 15.45, and 20.28 min, respectively. The degree of Leu contribution to retention was a function of its position in the molecule. Deletion of Ser changed retention, but the effect was also position-specific.

There is widespread interest in obtaining a correlation between biological activity and chromatographic properties for rapid toxicological screening purposes. No correlation was found between the serum calcium repression activity of the hormones and their retention in HIC (Table II). Total residue hydrophobicity for the CT analogues was calculated, using the residue hydrophobicity parameters derived by Rose *et al.*[22]. The residue parameters, derived from proteins of known structure, reflect the average area that each residue buries upon folding. Because HIC probes only the contact surface, little correlation between total molecular hydrophobicity and HIC retention was found.

The hydrophobic moments of seven CT analogues were reported by Epand *et al.*[23]. These values, included in Table II, showed a very good correlation with retention on HIC, with the exception of des(Ser[13])-sCT. The calculation of hydrophobic moments was according to the method of Eisenberg and co-workers[24–26] for residues 11–19, in which each amino acid is assigned a vector the magnitude of which is proportional to its hydrophobicity, and its direction is rotated 100° from the pre-

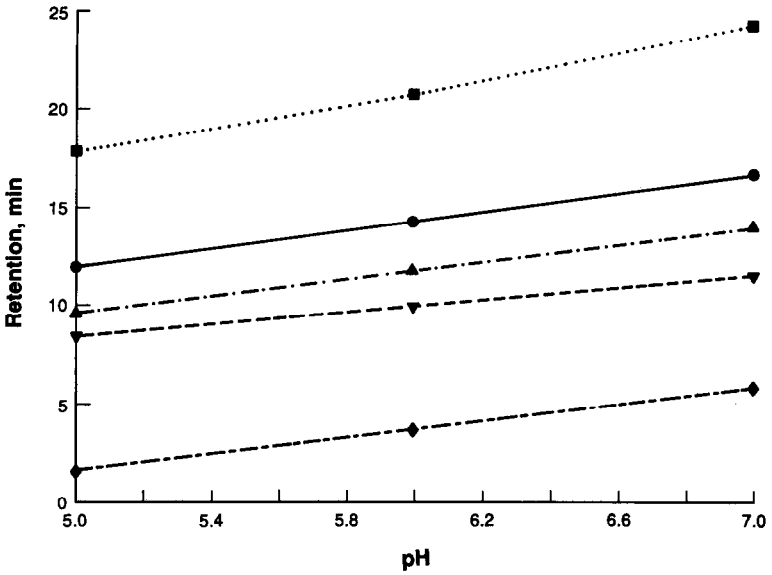


Fig. 1. Separation of CT analogues as a function of pH. Propyl column, 100 × 4.6 mm; flow-rate, 1 ml/min; eluent A, 1 *M* ammonium sulfate in 20 m*M* phosphate buffer (pH as indicated); eluent B, 20 m*M* phosphate buffer (pH as indicated); linear gradient from 100% A to 100% B over 30 min. (◆—·—◆) des(Leu^{12})-sCT; (▼— — —▼) des(Leu^{4})-sCT, (▲—·—▲) des(Ser^{2})-sCT, (●—●) sCT, (■···■) hCT.

ceeding residue for an α-helix. This concept may be more valuable than total hydrophobicity for the prediction of polypeptide retention, because the calculation includes consideration of both the hydrophobic and hydrophilic character of the residues and their relative positions to one another. Thus, hydrophobic moment is a measure of amphiphilicity within a segment of the polypeptide.

RPC

The retention characteristics of CT analogues were investigated by RPC on an octadecylsilane column, eluted with ascending acetonitrile gradients at pH 7 (Table III). The results were correlated with the total residue retention index using the retention coefficients described by Parker *et al.*[4], and the elution order was compared to that in HIC. No overall correlation was found between retention behavior and the total retention coefficient. Differences in elution order were noted between RPC and HIC. For example, sCT was eluted before hCT in HIC, whereas the opposite elution order was observed in the RPC. O'Hare and Nice[27] reported a similar elution order of hCT before sCT in RPC at pH 2 and postulated that secondary structure effects were responsible for the anomalous elution order. In HIC, des(Ser^{13})-sCT eluted after sCT, des(Leu^{4})-sCT was eluted after des(Leu^{19})-sCT, and Gly^{8}-sCT was eluted after des(Leu^{19})-sCT. In RPC, the elution order of each of these pairs was reversed. These results demonstrate the difference in selectivity between RPC and HIC. The complementary selectivity observed for the two chromatographic systems resulted from secondary (or higher) structure effects of interaction of the biopolymer with the sorbent surface.

TABLE II

COMPARISON OF ANALOGUE RETENTION IN HIC, TOTAL RESIDUE HYDROPHOBICITY, AND BIOLOGICAL ACTIVITY

N.D. = Not detected.

Analogue	*Retention time (min)*	*Total hydrophobicity**	*Hydrophobic moment***	*Biological activity (I.U./mg)*
Des(Leu^{16})-sCT	5.22	22.00	0.093	588
Des(Leu^{16})Gly^{8},D-Arg^{24}-sCT	5.77	21.86		2
Des(Leu^{12})-sCT	8.44	22.00	0.128	1014
Des(Leu^{19})Leu^{15},Glu^{16}-sCT	9.62	22.00	0.202	672
Des(Lys^{11},Leu^{12})-sCT	10.09	21.48	0.257	725
Des(Leu^{19})-sCT	11.64	22.00	0.372	7934
Des(Leu^{9})-sCT	13.53	22.00		350
Des(Ser^{2})Gly^{8}-sCT	13.99	22.05		3656
Gly^{8}-sCT	14.91	22.71		6991
Des(Leu^{4})-sCT	15.45	22.00		5397
Des(Ser^{2})-sCT	18.49	22.19		4318
endo(Gly^{1a})-sCT	18.49	23.57		4648
Des(Thr^{21})-sCT	19.88	22.15		5343
Salmon CT	20.28	22.85	0.419	5000
Des(Ser^{13})-sCT	21.68	22.19	0.185	4675
Human CT	31.05	23.73		150
mCT-II	N.D.	22.57		5000
Reduced	N.D.	22.85		4778

* Calculated according to ref. 22.
** Reported for residues 11–19 in ref. 23.

Circular dichroic spectra

The correlation of secondary structure with chromatographic retention was studied by measuring the circular dichroic (CD) spectra of six of the CT analogues in solvents having a composition similar to that of the chromatographic solvent at the time of elution. The spectra in ammonium sulfate solution were compared to those of the analogues in RPC eluents, (Fig. 2). The CT studied in ammonium sulfate had little, if any, helical content. The six spectra in aqueous 20 m*M* phosphate (pH 7) buffer were very similar even though the solvent varied in composition between 0.95 *M* and 0.05 *M* ammonium sulfate. Epand *et al.*[15] reported that little change in the CD spectrum of sCT was observed when the sodium chloride concentration was increased from 0.15 to 1.5 *M* in the aqueous buffer solution. We observed more ellipticity for sCT in ammonium sulfate solution than that reported by Epand *et al.*[15] in potassium chloride solution. The spectra of the six analogues in mixtures of acetonitrile and 20 m*M* phosphate buffer (pH 7) varied from one analogue to the other and differed from those in ammonium sulfate solution. Spectral differences in different solvents are not unexpected. Lau *et al.*[28] reported induction of α-helix and dimer formation in the CD spectra of peptides in 1.7 *M* ammonium sulfate solution (pH 7) in contrast to monomeric peptide having less helicity in 1.1 *M* potassium chloride (pH 7).

TABLE III

RETENTION IN RPC, RESIDUE RETENTION INDEX, AND NUMBER OF HYDROPHOBIC RESIDUES

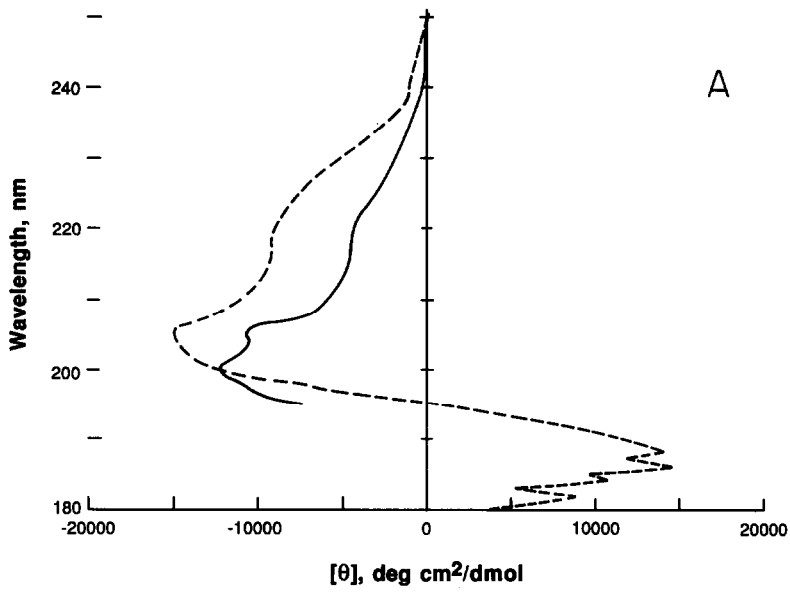
Analogue	*Retention (min)*	*Retention index**	*Total number of hydrophobic residues*					
			Ile	*Leu*	*Met*	*Phe*	*Tyr*	*Val*
Des(Leu^{16})Gly^{8},D-Arg^{24}-sCT	9.93	47.1		4			1	1
Des(Leu^{16})-sCT	15.19	52.8		4			1	1
Des(Leu^{12})-sCT	15.84	52.8		4			1	1
Des(Lys^{11},Leu^{12})-sCT	16.62	52.8		4			1	1
Gly^{8}-sCT	18.40	56.1		5			1	
Des(Ser^{2})Gly^{8}-sCT	18.53	56.6		5			1	
Des(Leu^{19})Leu^{15},Glu^{16}-sCT	20.12	52.8		4			1	1
Human CT	24.61	79.8	1	2	1	3	1	1
Des(Leu^{4})-sCT	25.92	52.8		4			1	1
Des(Leu^{9})-sCT	27.57	52.8		4			1	1
Reduced-sCT	28.28	61.8		5			1	1
Des(Leu^{19})-sCT	28.53	52.8		4			1	1
Des(Ser^{2})-sCT	30.49	62.3		5			1	1
Des(Tyr^{22})-sCT	32.46	57.2		5				1
endo(Gly^{10})-sCT	33.39	61.8		5			1	1
Des(Ser^{13})-sCT	33.49	62.3		5			1	1
Salmon CT	34.79	61.8		5			1	1
mCT-II	N.D.**	75.2		7			1	

* Calculated according to ref. 4.
** Not detected.

Secondary peptide structure was estimated from the CD data, using the Chou and Fasman model protein parameters[18]. The α-helical content of the analogues in ammonium sulfate solution was only 13%. According to the structures calculated for the peptides in solutions of acetonitrile and buffer (pH 7), the helical content was greater in the presence of acetonitrile (20–40% α-helix). It was concluded from the CD spectral data that the hormones possessed little α-helical structure in the HIC eluents, while acetonitrile contributed to more α-helix formation.

The CD spectra of the analogues were correlated with their chromatographic properties. In RPC, hCT was eluted before sCT, des(Ser^{13})-sCT was eluted before sCT, and des(Leu^{4})-sCT was eluted before des(Leu^{19})-sCT. The opposite elution order in HIC was observed for each of these pairs. Differences in the spectra of the various analogues were noted which were attributed to differences in the amount of α-helical structure among the analogues. Analysis of the CD data suggests that hCT possessed less helical structure than sCT in the acetonitrile–buffer solution (pH 7), des(Ser^{13})-sCT possessed less α-helicity than sCT, while des(Leu^{4})-sCT and des(Leu^{19})-sCT had the most helical structure of the six peptides. No correlation between helix formation in solution and chromatographic retention could be definitely established.

CT deletion and substitution analogues were used to analyze the effect of amino acid substitution on chromatographic retention in HIC. The chromatographic data were interpreted in terms of the model proposed for correlation of CT structure



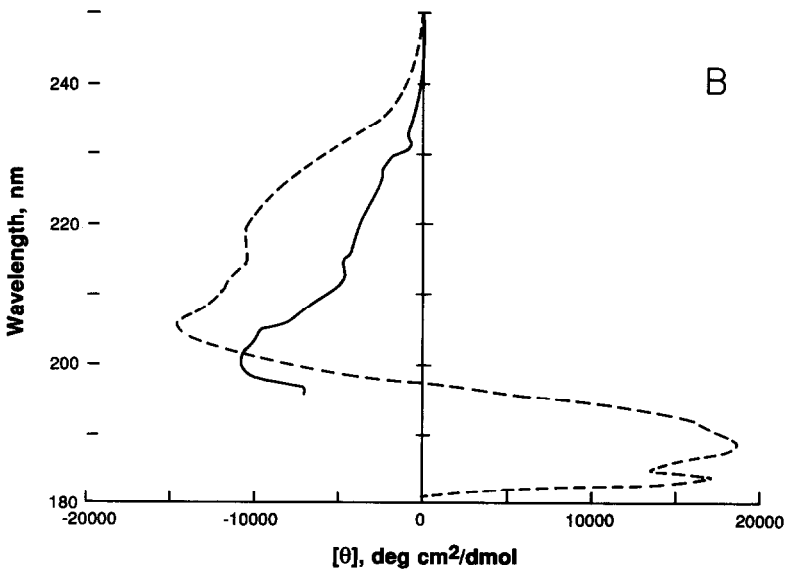


Fig. 2.

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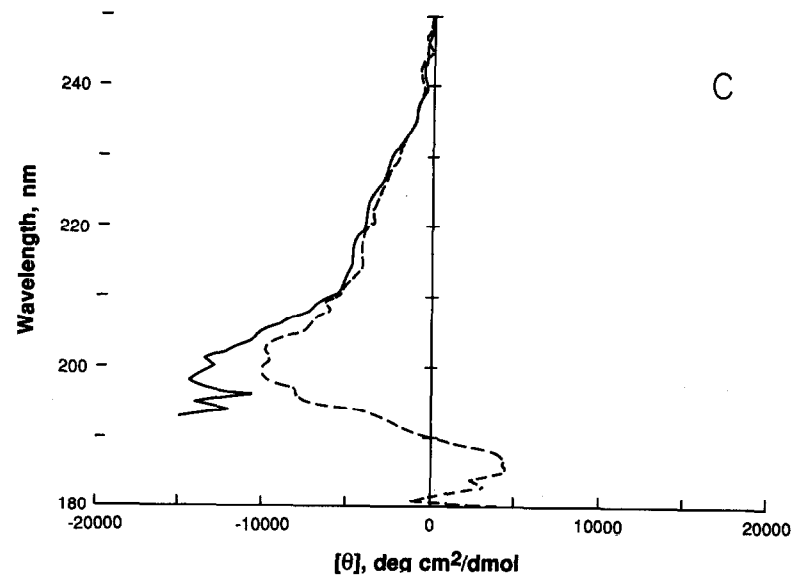
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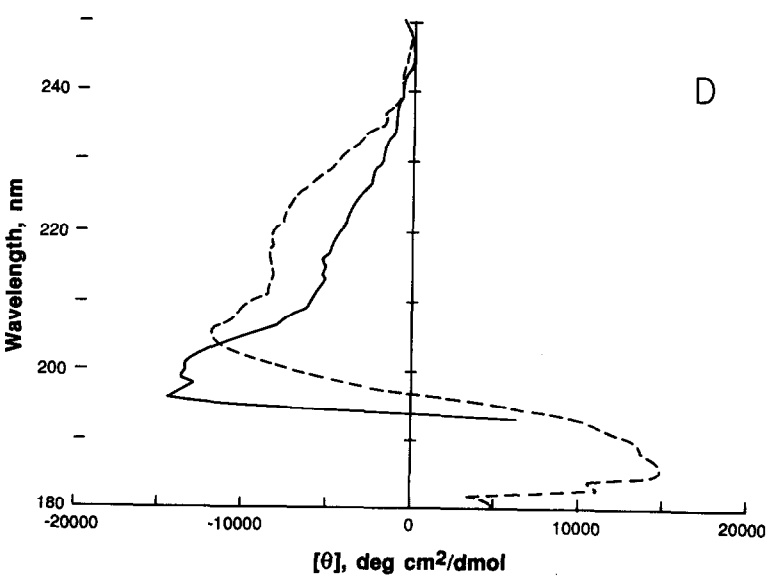
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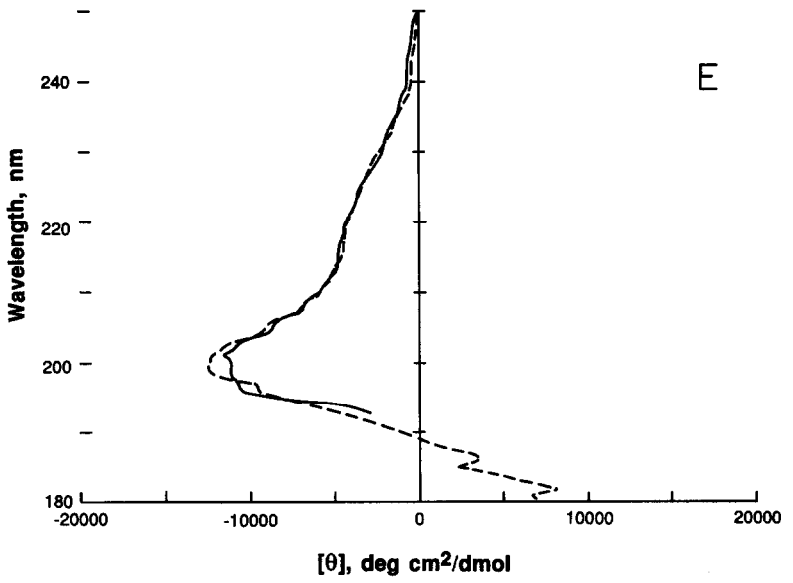


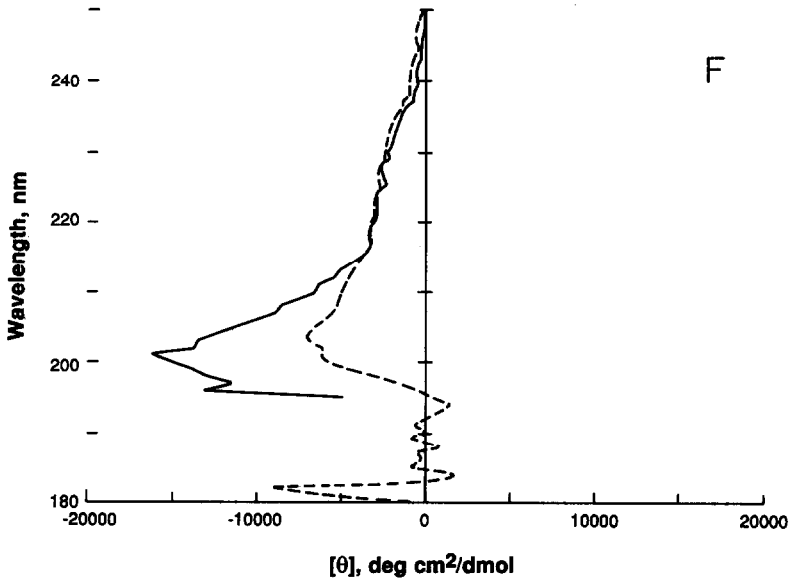


Fig. 2. Circular dichroic spectra of CT analogues, recorded in HIC and RPC eluents. (———) spectrum of peptide in ammonium sulfate and 20 m*M* phosphate buffer (pH 7); (· · ·) spectrum of peptide in acetonitrile and 20 m*M* phosphate buffer (pH 7). (A) sCT; 0.30 *M* ammonium sulfate; 52% acetonitrile. (B) Des(Leu^{4})-sCT; 0.70 *M* ammonium sulfate; 44% acetonitrile. (C) Des(Leu^{12})-sCT; 0.95 *M* ammonium sulfate; 35% acetonitrile. (D) Des(Leu^{19})-sCT; 0.85 *M* ammonium sulfate; 48% acetonitrile. (E) Des(Ser^{13})-sCT; 0.30 *M* ammonium sulfate; 52% acetonitrile. (F) hCT; 0.05 *M* ammonium sulfate; 44% acetonitrile.

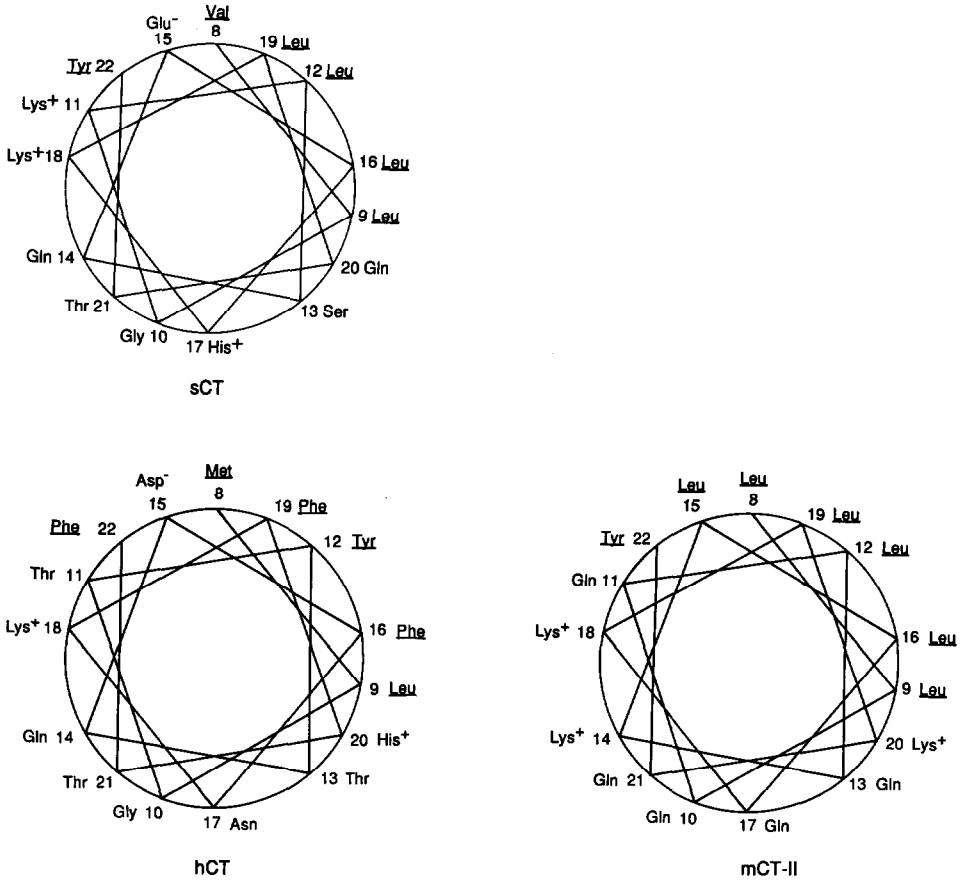


Fig. 3. Axial projections of α-helical region (residues 8–22) of salmon CT (sCT), human CT (hCT), and model CT (mCT-II).

to hypocalcemic activity[15,16]. Among the essential structural features related to function are the amphiphilic (*i.e.*, regular spacing of hydrophobic and hydrophilic residues such that formation of the α-helix results in segregation of hydrophobic and hydrophilic amino acid on opposite faces of the helix) α-helix from position 8 to 22.

The HIC retention characteristics of three different CT variants on the propyl column were compared. The CT from salmon (sCT), human (hCT), and a model peptide designed by Moe and Kaiser[20] (mCT-II) possessed widely different retention characteristics. The pair sCT and hCT was eluted at 20.28 and 31.05, respectively, while, mCT-II was never eluted. The results were interpreted in terms of the structure–function model[15,16]. The amino acid sequences of the CT analogues in the region 8–22 were viewed as axial projections of α-helices in a helical wheel, proposed by Schiffer and Edmundson[29] (Fig. 3). A segregation of five hydrophobic residues, one Val and four Leu residues, on one face of the helical cylinder of sCT can be seen in the figure. The hCT has five hydrophobic residues segregated on one face; one Met, two Phe, one Tyr, and one Leu. The analogue mCT-II, designed to have perfect

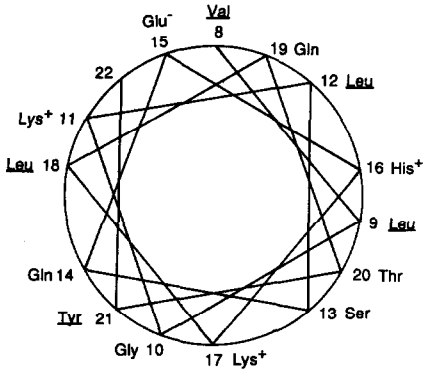
amphiphilicity, has seven hydrophobic residues on one face of the helical cylinder. This may explain its failure to be eluted in HIC under the above conditions. Although this analogue failed to be eluted in RPC under the reversed-phase conditions in this study (pH 7, acetonitrile gradient from 20 to 40%), Moe and Kaiser[20] chromatographed mCT-II on a C_{18} packing at pH 2.5 with acetonitrile gradient from 20 to 50%. The above analysis suggests that as hydrophobic groups accumulate on the face of the helix, chromatographic retention tends to increase.

The number of segregated hydrophobic residues is not the only factor contributing to retention. The longer retention of hCT may result from a combination of factors. The nature of the hydrophobic residues may also make an important contribution to retention. In positions 8–22 of hCT, three of the five amphiphilic hydrophobic residues are aromatic in contrast to the aliphatic Leu and Val in sCT. The number of charged amphiphilic residues on the hydrophilic faces of sCT and hCT also influence HIC retention. sCT has four charged amphiphilic residues, Glu^{15}, Lys^{11}, Lys^{18}, and His^{17}, in contrast to three charged residues in hCT, Asp^{15}, Lys^{11}, and His^{20}. Finally, the interaction of the terminal residues of the polypeptide with the sorbent surface must not be overlooked. The carboxyl terminus residues 23–32 of hCT differ markedly from those in sCT. The sCT analogue possesses a relatively hydrophilic C-terminus, including a charged Arg^{24} residue. On the other hand, hCT contains no charged residues in positions 23–32 but has hydrophobic amino acids, Ile^{27} and Val^{29}.

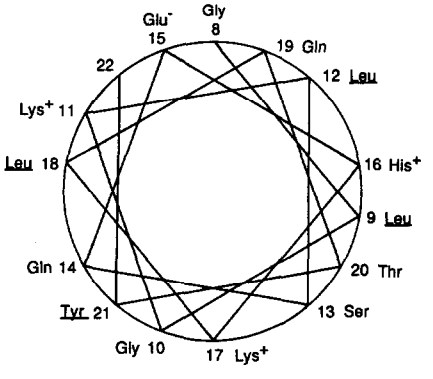
A similar analysis of the structures of the early-eluted analogues was conducted. Des(Leu^{16})-sCT was eluted under HIC conditions in 5.22 min, des(Leu^{16})Gly^{8}-D-Arg^{24}-sCT was eluted in 5.77 min, and des(Leu^{12})-sCT was eluted in 8.44 min. Examination of the axial projection models (Fig. 4) for the early eluted analogues shows hydrophobic residues in the region 8–22, isolated by clusters of hydrophilic residues, *i.e.* non-amphiphilic structures. The first two analogues differ only by the residue in position 8, des(Leu^{16})-sCT having Val^{8}, while the analogue eluted at 5.77 min has Gly^{8}. Des(Leu^{12})-sCT, on the other hand, possesses one hydrophobic pair of residues on one face, Leu^{15} and Val^{8}. All three analogues possess four charged amino acids. Thus, the small hydrophobic patch in des(Leu^{12})-sCT probably contributed to its being retained longer than the two earliest-eluted analogues.

The observation that des(Thr^{21})-sCT is eluted nearly simultaneously with sCT is also explained by this model (Fig. 5). Des(Thr^{21})-sCT has a hydrophobic face identical to that of sCT. Hence, the two analogues have very similar retention characteristics. The contribution of Val^{8} to chromatographic retention in an amphiphilic CT is apparent when the HIC retention of Gly^{8}-sCT (14.91 min) is contrasted with sCT (20.28 min). The projection model of the former (Fig. 5) shows four leucine residues on the hydrophobic face of Gly^{8}-sCT, compared to five hydrophobic residues (four Leu and one Val) on that of sCT.

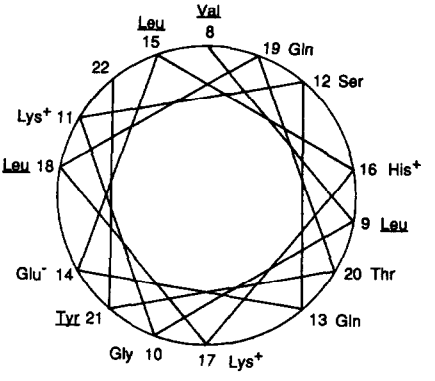
Many of the analogues displayed retention behavior in agreement with this model. For example, it can be shown that des(Leu^{19})Leu^{15}, Glu^{16}-sCT is predicted to be eluted before des(Leu^{19})-sCT. Their observed HIC retentions were 9.62 and 11.64 min, respectively. However, this analysis does not explain the observation that mCT-II failed to be eluted under HIC conditions. It is possible that retention cannot be predicted as a simple additive effect for each amphiphilic hydrophobic residue.



des (Leu^{16})-sCT

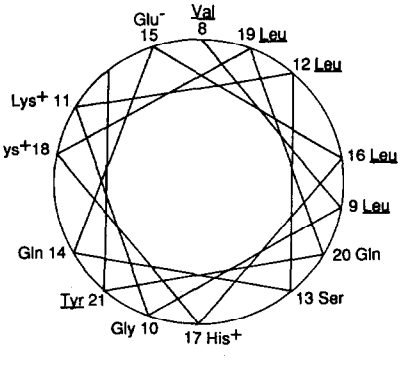


des(Leu^{16})Gly^{8}, D-Arg^{24}-sCT

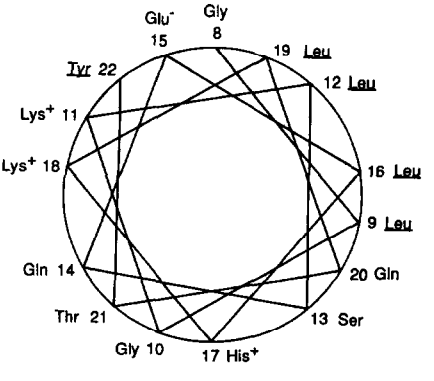


des(Leu^{12})-sCT

Fig. 4. Axial projections of α-helical region (residues 8–22) of the early-eluted analogues des(Leu^{16})-sCT, des(Leu^{16})Gly^{8}, D-Arg^{24}-sCT and des(Leu^{12})-sCT.



des(Thr^{21})-sCT



Gly^{8}-sCT

Fig. 5. Axial projections of α-helical region (region 8–22) of des(Thr^{21})-sCT and Gly^{8}-sCT.

Nor does this analysis explain the anomalous elution behavior of des(Ser^{13})-sCT and reduced sCT.

This discussion has centered on the effect of deletion or substitution in residues 8–22. The terminal portions of the peptide were also found to contribute to chromatographic retention. Leu in the 4-position was found to have a significant contribution to retention in HIC and RPC. Deletion of Leu^{4} reduced HIC retention from 20 to 15 min, while retention in RPC was reduced from 35 to 26 min. In contrast, Ser^{2} deletion reduced HIC retention only slightly, as did Gly addition between residues 1 and 2. The RPC retention of des(Ser^{2})-sCT on the other hand, was 30.49 min, (3 min earlier than sCT), while *endo*-(Gly^{1a})-sCT displayed a RPC retention time similar to that of sCT. This suggests that residues in positions 1–7 of the peptide structure interact with the sorbent surface in both RPC and in HIC. Secondary and tertiary structural differences contributed by the different eluents resulted in selectivity differences.

The differences in selectivity between RPC and HIC are compared in Fig. 6. In most instances, the analogues which were eluted closely together in one system were well resolved in the other system. The data of Parker *et al.*[4] predict that Ser deletion should have little effect on chromatographic retention. The present study showed that Ser deletion in the 2-position reduced both HIC and RPC retention. Ser^{13} deletion had a less pronounced but measurable effect on retention in both systems. The lack of correlation between a sum of the RPC retention coefficients according to Parker and the observed elution order may result from secondary (or higher) structural effects.

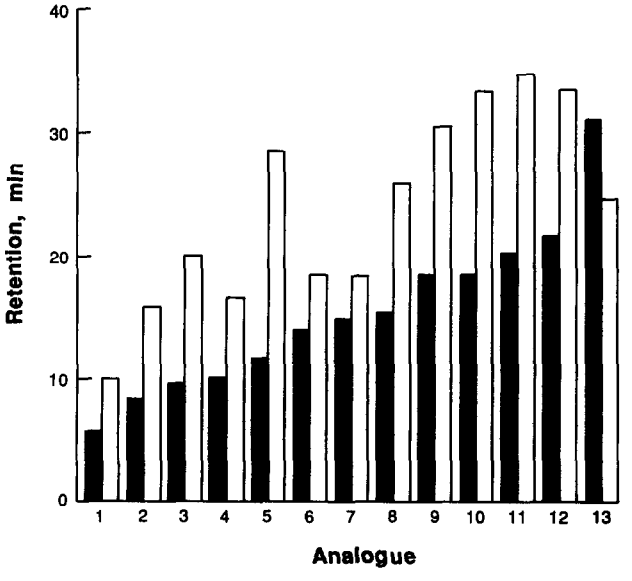


Fig. 6. Comparison of chromatographic resolution of CT analogues in HIC (black bars) and RPC (white bars). (1) Des(Leu^{16})Gly^{8}, D-Arg^{24}-sCT; (2) des(Leu^{12})-sCT; (3) des(Leu^{19})Leu^{15}, Glu^{16}-sCT; (4) des(Lys^{11}, Leu^{12})-sCT; (5) des(Leu^{19})-sCT; (6) des(Ser^{2})Gly^{8}-sCT; (7) Gly^{8}-sCT; (8) des(Leu^{4})-sCT; (9) des(Ser^{2})-sCT; (10) *endo*-(Gly^{1a})-sCT; (11) sCT; (12) des(Ser^{13})-sCT; (13) human CT.

Other authors have reported differences in polypeptide elution order between RPC and HIC. Guerini and Krebs[30] reported selectivity differences between HIC and RPC of tryptic fragments of calmodulin. Osthoff *et al.*[31] reported HIC resolution of snake venom proteins which are eluted closely together in RPC. Alpert[32] suggests that secondary and tertiary structural differences, imposed on the polypeptide in the chromatographic eluent influence the rate of migration. Houghton and Ostresh[33] reported that non-amphiphilic tridecapeptides were eluted before amphiphilic peptides in RPC, but no CD data were reported. Alpert contends that because polypeptides possess different structures in HIC eluents than those used for RPC, a complementary selectivity is observed when the two techniques are compared.

Correlation of chromatographic retention with Schiffer and Edmundson[29] projection models of residues 8–22 showed that a correlation existed between non-amphiphilicity and early elution in both HIC and RPC. There was also a correlation between amphiphilicity of CT and chromatographic retention. The correlation suggests that, although the polypeptides possessed little α-helical structure in the HIC eluents, the sorbent surface organizes the secondary structure. This induced secondary structure at the hydrophobic surface may be related to structural changes thought to occur in flexible polypeptide hormones at the membrane receptor surface[23,34], although the interactions contributing to biological potency are far more complex than in the chromatographic systems of the present study. All parts of the CT structure, including the N-terminus and C-terminus portions, appeared to affect chromatographic retention, just as all parts are important for biological function[15,16].

CONCLUSION

The effect of CT structure on chromatographic retention indicated that polypeptides interact with the sorbent surface in a manner unique to the chromatographic system. These interactions were dependent upon both the mobile and stationary phase composition and contributed to the chromatographic selectivity. CD spectra of CT analogues in HIC eluents indicated that the analogues possessed little α-helical structure in aq. ammonium sulfate solution while the HIC data indicated that chromatographic retention order of the analogues was proportional to the number of amphiphilic hydrophobic residues in positions 8–22 of CT. The chromatographic data indicated surface-induced structural changes in the polypeptide upon interaction with the HIC sorbent surface. Non-amphiphilic species were eluted before amphiphilic species in both HIC and RPC. Interactions at the RPC surface were different from those in HIC, as evidenced by the differences in selectivity observed between the two systems. These differences resulted, in part, from interactions imposed by the differences in the chromatographic eluents employed in HIC and RPC.

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