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Selective separations of peptides with sequence deletions, single amino acid polymorphisms, and/or epimeric centers using macrocyclic glycopeptide liquid chromatography stationary phases

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Abstract

Separating closely related peptides (those differing by one or two amino acids or the chirality of a single amino acid) can be challenging using reversed-phase liquid chromatography (LC), ion-exchange LC, or using ion-pairing agents. Also, the mobile phases that give the best separations in these modes may not be electrospray ionization mass spectrometry (ESI-MS) compatible. Forty-two peptides from 11 peptide families were separated on three macrocyclic glycopeptide stationary phases in reverse-phase mode using ESI-MS-compatible mobile phases. The peptide classes studied were angiotensin, bradykinin, α-bag cell factor, β,γ-bag cell factor, β-casomorphin, dynorphin, enkephalin, leucokinin, lutinizing hormone releasing hormone, neurotinsin, substance P, and vasopressin. High selectivity was observed for single amino acid substitutions (achiral and chiral) regardless of the position of the substitution in the sequence. Mobile phase optimization, its effect on peptide elution behavior, and chromatographic efficiency is also discussed. Using LC–ESI-MS, a 2 ng limit of detection was obtained, two orders of magnitude lower than the UV detection limit.

Keywords: Gradient separation; Peptides; Enkephalins; Angiotensins; Bradykinins; Teicoplanin; Ristocetin A

1. Introduction

The separation and analysis of peptides continues to be of paramount importance in many areas of science and technology. Some of these areas include: (a) protein sequencing; (b) analysis, quantitation, and characterization of peptide hormones; (c) synthesis of new peptide drugs; (d) pharmacokinetic and pharmacodynamic studies of pharmacologically active peptides; and (e) other fields involving the environmental, biological, and geochemical sciences.

The separation of complicated peptide mixtures is one of the more important initial steps in protein sequencing. Also, there is increasing interest in detecting single amino acid polymorphisms in proteins [1–4] which would produce the resultant peptide polymorphs after digestion with proteolytic agents. These types of protein alterations emanate from certain single nucleotide polymorphisms [5,6] and have been linked to diseases by several researchers [7–10].

Low concentrations of peptide hormones are known to elicit a large spectrum of physiological effects [11,12]. Their identification and quantification in complex biological fluids can be problematic not only because of the complicated matrices, but also due to the large number and higher concentrations of interfering substances [12]. As a consequence of these peptides' profound activity, it is not surprising that pharmaceutical scientists are synthesizing an ever-increasing number of analogues. Frequently, this involves replacing specific amino acids with other natural or more frequently, non-natural amino acid analogues [13–17]. Non-natural amino acids can include: D-amino acids, β-amino acids, unusually substituted α-amino acids, cyclic and bicyclic-amino acids, as well as other useful permutations [14,15]. In all cases,

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active potential drug candidates must undergo pharmacokinetic and pharmacodynamic studies in which they and their metabolites must be distinguished from all other naturally occurring physiological components.

Liquid chromatography (LC) is the predominant separation method used for the analysis of peptides [18–34]. It is often coupled with other separation methods and/or mass spectrometry as part of a two-dimensional (2D) or multi-dimensional procedure [19,23,25,35]. Reversed-phase LC is the most prevalent method used because of its good resolving power, reproducibility, and ease of use [18,29,32,36]. It has become common practice to use mobile phases consisting of aqueous acetonitrile mixtures containing various ion-pairing agents [26]. Ion-exchange chromatography also has been used widely for the separation of peptides [24,27]. The composition of the mobile phase can be a problem if the separation is interfaced with electrospray ionization mass spectrometry (ESI-MS). Often, this is necessary to enhance both the sensitivity and selectivity of an analysis.

Given the wide variety of peptides, peptide mixtures and complex matrices in which they exist, there is a constant search for different selectivity separation approaches. For example, a porphyrin-based stationary phase was recently proposed for the separation of peptides [37]. When utilizing two-dimensional separations, it is usually desirable to have orthogonal separation methods. Orthogonality is more likely if the separation mechanisms are different from one another. However, the mobile phases have to be sufficiently compatible that the methods can be coupled (if using a continuous automated system).

Macrocyclic glycopeptide-based (i.e., containing teicoplanin, teicoplanin aglycone, or ristocetin A) chiral stationary phases are widely utilized for enantiomeric separations, including amino acids, dipeptides, and tripeptides [22,31]. They are known to selectively bind specific amino acids and sequences of amino acids via electrostatic, hydrogen bonding, and dipolar interactions [14,31]. It is highly likely that they also are selective for closely related peptides of any chain length. Their separation mechanism, and therefore selectivity, is significantly different from both C_{18} reversed-phase and ion-exchange LC. Furthermore, the mobile phases that are commonly used with teicoplanin-based stationary phases are ESI-MS compatible. The focus of this work is to evaluate the separation of a variety of closely related peptides on a teicoplanin stationary phase using isocratic elution with ESI-MS-compatible mobile phases.

1.1. Materials

Synthetic peptides used in this study were purchased from American Peptide Co. (APC; Sunnyvale, CA, USA) and Sigma (St. Louis, MO, USA). The peptides, their structure, and source are listed in Table 1.

Formic acid, 96%, ACS reagent grade (Sigma) was used as mobile phase additives. Acetonitrile (ACN; HPLC grade, Fisher Scientific, Pittsburgh, PA, USA) and deionized water (prepared in the laboratory) were used to make all mobile phases. All samples were dissolved in a water–methanol (50:50) solvent mixture at 1 mg/mL concentration unless mentioned otherwise. Triethylamine (TEA; HPLC grade, Sigma) and acetic acid (ACS grade, Fisher Scientific) were also used as mobile phase additives.

1.2. Instrument

The chromatographic methods were developed on a HP (Palo Alto, CA, USA) 1050 HPLC system, including one auto sampler, one quaternary pump, and one VWD detector operating under ChemStation software. All separations were carried out with analytical columns from Advanced Separation Technologies (ASTEC; Whippany, NJ, USA) at room temperature. The columns used were Chirobiotic T (250 mm × 4.6 mm), Chirobiotic R (250 mm × 4.6 mm), Chirobiotic TAG (250 mm × 4.6 mm). LC–MS analyses were carried out on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system with a photodiode array detection (DAD) system coupled with Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with ESI source. Xcalibur 3.1 was the operating software. Ultra-high purity helium gas (Linweld, Lincoln, NE, USA) was used as dampening gas. Praxair (Danbury, CT, USA) nitrogen was used as sheath gas and auxiliary gases.

2. Methods

All HPLC methods are listed in Table 2. Depending on mobile phase conditions, UV–vis detection was performed at wavelengths of 210, 232, or 254 nm. ESI conditions were set to the following: sheath gas = 50 arbitrary units, auxiliary gas = 40 arbitrary units, source voltage = 4.55 kV, capillary voltage = 30.6 V, tube lens offset = −15.0 V, and capillary temperature = 272 °C. LC–MS experiments were carried out using flow rates of 1.0 mL/min, unless noted otherwise.

3. Results and discussion

3.1. Peptide separations

Macrocyclic glycopeptide chiral stationary phases (CSPs) exhibited excellent selectivity in separating closely related peptides. Fig. 1 shows the baseline resolution of six enkephalin peptides on the Chirobiotic T (teicoplanin) column in single isocratic run. The enkephalin peptides are closely related structurally, differing from one another by only one amino acid or the chirality of a single amino acid. In addition, retention times can be reduced substantially, if desired, by utilizing gradient elution (see Section 3.3.1). Currie et al. had some success in resolving enkephalin peptides by using a phenyl-bonded column [38]. However, no baseline separation was achieved when four or more enkephalin peptides were present in the mixture. Underberg and co-workers

Table 1
Peptides, sequences and source

Name	Three-letter sequence	Single-letter sequence[a]	Source
Leu-Enkephalin	Tyr–Gly–Gly–Phe–Leu	YGGFL	Sigma
[D-Ala2,D-Leu5]-Enkephalin	Tyr–D-Ala–Gly–Phe–D-Leu	Y-dA-GF-dL	APC
[Ala2]-Leu-Enkephalin	Tyr–Ala–Gly–Phe–Leu	YAGFL	APC
Met-Enkephalin	Tyr–Gly–Gly–Phe–Met	YGGFM	Sigma
Met-Enkephalin [D-Ala2]	Tyr–D-Ala–Gly–Phe–Met	Y-dA-GFM	APC
[D-Ala2,Leu5]-Enkephalin	Tyr–D-Ala–Gly–Phe–Leu	Y-dA-GFL	APC
[D-Ala2,4,Tyr5]-β-Casomorphin (1–5), amide, bovine	Tyr–D-Ala–Phe–D-Ala−Tyr–NH_2	Y-dA-F-dA-Y-NH_2	APC
[D-Ala2,D-Pro4,Tyr5]-β-Casomorphin (1–5), amide	Tyr–D-Ala–Phe–D-Pro–Tyr–NH_2	Y-dA-F-dP-Y-NH_2	APC
[D-Ala2,Hyp4,Tyr5]-β-Casomorphin (1–5), amide	Tyr–D-Ala–Phe–Hyp–Tyr–NH_2	Y-dA-F-Hyp-Y-NH_2	APC
β-Bag cell factor	Arg–Leu–Arg–Phe–His	RLRFH	APC
γ-Bag cell factor	Arg–Leu–Arg–Phe–Asp	RLRFD	APC
α-Bag cell peptide (1–7)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr	APRLRFY	APC
α-Bag cell peptide (1–8)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr–Ser	APRLRFYS	APC
α-Bag cell peptide (1–9)	Ala–Pro–Arg–Leu–Arg–Phe–Tyr–Ser–Leu	APRLRFYSL	APC
Leucokinin I	Asp–Pro–Ala–Phe–Asn–Ser–Trp–Gly–NH_2	DPAFNSWG-NH_2	APC
Leucokinin II	Asp–Pro–Gly–Phe–Ser–Ser–Trp–Gly–NH_2	DPGFSSWG-NH_2	APC
Leucokinin VII	Asp–Pro–Ala–Phe–Ser–Ser–Trp–Gly–NH_2	DPAFSSWG-NH_2	APC
[Sar1,Thr8]-Angiotensin II	Sar–Arg–Val–Tyr–Ile–His–Pro–Thr	Sar-RVYIHPT	Sigma
Angiotensin II human	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe	DRVYIHPF	Sigma
[Val5]-Angiotensin II	Asp–Arg–Val–Tyr–Val–His–Pro–Phe	DRVYVHPF	Sigma
Angiotensin II antipeptide	Glu–Gly–Val–Tyr–Val–His–Pro–Val	EGVYVHPF	Sigma
[Sar1]-Angiotensin II	Sar–Arg–Val–Tyr–Ile–His–Pro–Phe	Sar-RVYIHPF	Sigma
Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	RPPGFSPFR	Sigma
des-Pro2-Bradykinin	Arg–Pro–Gly–Phe–Ser–Pro–Phe–Arg	RPGFSPFR	Sigma
Bradykinin fragment 2–7	Pro–Pro–Gly–Phe–Ser–Pro	PPGFSP	Sigma
Bradykinin fragment 2–9	Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	PPGFSPFR	Sigma
[Lys8]-Vasopressin	Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2	CYFQNCPKG-NH_2	APC
[Arg8]-Vasopressin/AVP	Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Arg–Gly–NH_2	CYFQACPRG-NH_2	APC
Dynorphin A (1–10), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro	YGGFLRRIRP	APC
Dynorphin A (1–10), amide, porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–NH_2	TGGFLRRIRP-NH_2	APC
[D-Ala6]-LH-RH	pGlu–His–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–Gly–NH_2	pEHWSY-dA-LRPG-NH_2	Sigma
[des-pGlu1]-LH-RH	His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2	HWSYGLRPG-NH_2	Sigma
[D-Lys6]-LH-RH	pGlu–His–Trp–Ser–Tyr–D-Lys–Leu–Arg–Pro–Gly–NH_2	pEHWSY-dK-LRPG-NH_2	Sigma
[D-Phe2,D-Ala6]-LH-RH	pGlu–D-Phe–Trp–Ser–Tyr–D-Ala–Leu–Arg–Pro–Gly–NH_2	Pe-dF-WSY-dA-LRPG-NH_2	Sigma
Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu	pELYENKPRRPYIL	Sigma
[Phe11]-Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Phe–Ile–Leu	pELYENKPRRPFIL	Sigma
[D-Trp11]-Neurotensin	Glp–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–D-Trp–Ile–Leu	pELYENKPRRP-dW-IL	APC
[D-Tyr11]-Neurotensin	Glp–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–D-Tyr–Ile–Leu	pELYENKPRRP-dY-IL	APC
[Gln4]-Neurotensin	Glp–Leu–Tyr–Gln–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu	pELYQNKPRRPYIL	APC
Neurotensin, guinea pig	Glp–Leu–Tyr–Glu–Asn–Lys–Ser–Arg–Arg–Pro–Tyr–Ile–Leu	pELYENKSRRPYIL	APC
[D-Pro10]-Dynorphin A (1–11), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–D-Pro–Lys	YGGFLRRIR-dP-K	APC
Dynorphin A (1–11), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys	YGGFLRRIRPK	APC
Dynorphin A (1–13), porcine	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys	YGGFLRRIRPKLK	APC
[D-Arg6]-Dynorphin A (1–13), porcine	Tyr–Gly–Gly–Phe–Leu–D-Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys	YGGFL-dR-RIRPKLK	APC
[Nor8]-Substance P	D-Pro–Gln–Gln–D-Trp–Phe–D-Trp–Leu–Nor–NH_2	dP-QQ-dW-F-dW-LN-NH_2	APC
Substance P	D-Pro–Gln–Gln–D-Trp–Phe–D-Trp–Leu–Met–NH_2	dP-QQ-dW-F-dW-LM-NH_2	APC

[a] The "d" denotes the chirality of D-amino acids. A lower case "d" had to be used to distinguish them from aspartic acid, which has the single letter abbreviation "D".

recently coupled size-exclusion chromatography (SEC) and CE to separate large proteins and enkephalin peptides [39]. Although separation was achieved, the system complexity and low chromatographic quality made the separation less desirable. In addition to the good selectivity observed in Fig. 1, the mobile phase is ESI-MS compatible (as will be shown and discussed for subsequent separations).

Excellent separations were commonly observed for most of the peptides listed in Table 2. Within each family (listed in Table 2), the individual peptides are listed in the order of

Table 2
Selectivity (α) and resolution (R_s) for peptides on Chirobiotic T, TAG and R stationary phases

Peptides	Columns											
	Chirobiotic T				Chirobiotic TAG				Chirobiotic R			
	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]
Enkephalins												
Y-**dA**-GF-**dL**	B	1			A	2			C	1		
Y-**dA**-GF**M**			1.09	1.75			1.14	2.31			1.24	2.71
Y**G**GF**M**			1.21	4.07			1.2	3.29			1.18	2.04
Y-**dA**-GF**L**			1.32	5.94			1.3	4.48			1.13	1.39
Y**G**GF**L**			1.26	5			1.24	3.56			1.06	0.06
Y**A**GF**L**			1.18	3.72			1	0			1.09	0.96
β,γ-Bag cell factors												
RLRF**H**	F	2							E	2		
RLRF**D**			2.41	11.58							2.96	2.58
β-Casomorphins												
Y-dA-F-**dA**-Y-NH_2	H	1							I	1		
Y-dA-F-**dP**-Y-NH_2			1.11	2.05							1.19	0.64
Y-dA-F-**Hyp**-Y-NH_2			1.32	5.44							1.95	2.56
α-Bag cell factors 7–9												
APRLRF**Y**					O	1.5			L	1		
APRLRFY**S**							1.32	2.29			1.15	1.3
APRLRFYS**L**							1.29	2.05			1.25	1.72
Leucokinins												
DP**A**F**S**SWG-NH_2	C	1.2							C	1		
DP**G**F**S**SWG-NH_2			1.07	1.54							1.11	0.82
DP**A**F**N**SWG-NH_2			1.12	2.81							1.28	1.74
Angiotensins												
EGVY**V**HP**F**	P	0.5							G	2		
DRVY**I**HP**F**			1.93	13.3							1.52	3.26
N-methyl-GRVY**I**HP**T**			1.06	1.25							1.45	2.93
N-methyl-GRVY**I**HP**F**			1.09	1.74							1.46	2.94
DRVY**V**HP**F**			1.07	1.39							1	
Substance P	Q	1							D	1		
dP-QQ-dW-F-dW-L**N**-NH_2											5.00	1.33
dP-QQ-dW-F-dW-L**M**-NH_2			5.93	21.2								
Bradykinins												
PPGFSFR	M	1							K	1.2		
PPGFSPFR			2.2	15.12							1.8	3.86
RPGFSPFR			2.37	15.39							2.16	2.16
RPPGFSPFR			1.1	1.82							1.31	1.31
Vasopressins												
CYFQNCP**K**G-NH_2	O	1			Q	1			J	1		
CYFQACP**R**G-NH_2							1.25	2.31			1.16	1.8
Dynorphins												
YGGFLRRIRP	Q	1.5										
TGGFLRRIRP-**NH**$_2$			1.26	2.3								
Lutenizing hormone releasing hormone												
pE-**dF**-WSY-**dA**-LRPG-NH_2	P	1							G	1		
pEHWSY-**dA**-LRPG-NH_2			1.38	5.16							2.34	7.68
pEHWSY-**dK**-LRPG-NH_2			1.71	8.56							2.14	3.48
HWSY**G**LRPG-NH_2			1.2	2.92							1.3	1.32
Neurotensins												
pELYENKPRRP-**dW**-IL	N	1										
pELYENKPRRP-**dY**-IL			1.26	1.63					G	1	1.15	1.24
pELYENKPRRP**Y**IL			1.08	0.83							1.28	2.04
pELYENKPRRP**F**IL			1.07	0.82							1.22	1.75

(*Continued*)

Peptides	Columns								
	Chirobiotic T				Chirobiotic TAG			Chirobiotic R		
	MP[a]	Flow rate (mL/min)	α[b]	R_s[b]	MP[a]	Flow rate (mL/min)	α[b] R_s[b]	MP[a]	Flow rate (mL/min)	α[b] R_s[b]
pELY**Q**NKPRRP**Y**IL			1.09	0.76						1.25 1.97
pELYENK**S**RRP**Y**IL			0	0						1.48 1.69
Dynophin 1–11										
YGGFLRRIR-**dP**-K	R	0.8								
YGGFLRRIR**P**K			1.12	1.53						
Dynophin 1–13										
YGGFL-**dR**-RIRPKLK	S	1								
YGGFL**R**RIRPK			1.19	1.95						

[a] Mobile phases: (A) ACN–water (65:35); (B) ACN–water (75:25); (C) ACN–water (85:15); (D) ACN–5 mM ammonium formate aqueous solution, pH 3 (55:45); (E) ACN–5 mM ammonium formate aqueous solution, pH 3 (60:40); (F) ACN–25 mM ammonium formate, pH 3.0 (30:70); (G) ACN–5 mM ammonium formate aqueous solution, pH 3 (65:35); (H) ACN–16 mM ammonium formate, pH as is (75:25); (I) ACN–16 mM ammonium formate, pH as is (90:10); (J) ACN–20 mM ammonium formate, pH as is (20:80); (K) ACN–32 mM ammonium formate, pH as is (50:50); (L) ACN–40 mM ammonium formate, pH as is (60:40); (M) ACN–0.06% formic acid aqueous solution (35:65); (N) ACN–0.1% formic acid aqueous solution (25:75); (O) ACN–0.1% formic acid aqueous solution (30:70); (P) ACN–0.1% formic acid aqueous solution (35:65); (Q) CAN–0.1% formic acid aqueous solution (40:60); (R) ACN–0.75% triethylamine, pH 2.8 (40:60); (S) ACN–1% triethylamine, pH 2.8 (20:80).

[b] $\alpha = k'_2/k'_1$, $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

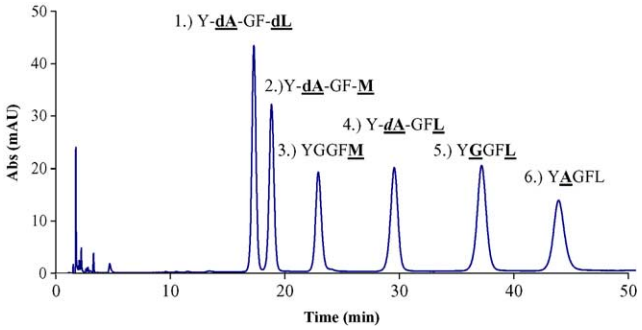

Fig. 1. Separation of six enkephalin peptides on Chirobiotic T column. Single amino acid polymorphisms (SAAP) occur in: (a) peaks 2 and 4; (b) peaks 3 and 5; (c) peaks 5 and 6. Examples of chiral amino acid polymorphisms are: (a) peaks 1 and 4; (b) peaks 4 and 6. Chromatographic conditions are given in Table 2.

their retention at the elution conditions specified. The selectivity (α) and resolution (R_s) values are reported for adjacent peptide peaks within each family. These values were calculated at optimized isocratic elution conditions for the separation of the entire peptide family. Other elution conditions can be found to further resolve any single pair of peptides within the family, if desired. Table 3 indicates the macrocyclic glycopeptide column that produced the most effective separations for each class (family) of peptides. The Chirobiotic T column produced the best separations for the largest numbers of families, but all three columns were needed to separate the all of the families.

3.2. Separation of peptides containing single amino acid polymorphism (SAAP)

As demonstrated in Fig. 1, the enkephalins were easily baseline separated from each other. Among these separations, enkephalin peaks 2 and 4, enkephalin peaks 3 and 5, enkephalin peaks 5 and 6 are different from each other only by a single amino acid. A particular separation of note is the SAAP represented in peaks 5 and 6. The glycine in position 2 of one peptide is replaced with an alanine. This difference in the side chains is one of the more subtle substitutions among native amino acids, yet it is easily separated. Enkephalin peaks 1 and 4, and enkephalin peaks 4 and 6 differ from each other only by the chirality of a single amino acid, making them epimers of one another. Interestingly, these single amino acid chirality polymorphism (SAACP) peptides were not eluted next to each other. At least one other peptide eluted between the epimers. The epimeric position in the peptide chain might play a critical role in determining if the separation is substantial enough to allow another peptide to elute between the epimers, as this behavior was not always observed.

Fig. 2 shows several separations of peptides with SAAP. Separation is achieved regardless of whether the amino acid substitution occurs at the N-terminus, middle, or C-terminus of the peptide chain. In each related sequence, the amino acid that is different is highlighted for easier comparison (Fig. 2 and Table 2). In general, the separation was easier to achieve

Table 3
Best separations for peptide classes by macrocyclic glycopeptide column

Chirobiotic T	Chirobiotic TAG	Chirobiotic R
Enkephalins	α-Bag cell factors	Neurotensins
β,γ-Bag cell factors	Vasopressins	
β-Casomorphins		
Bradykinins		
Angiotensins		
Dynorphins		
Leucokinins		
Substance P		

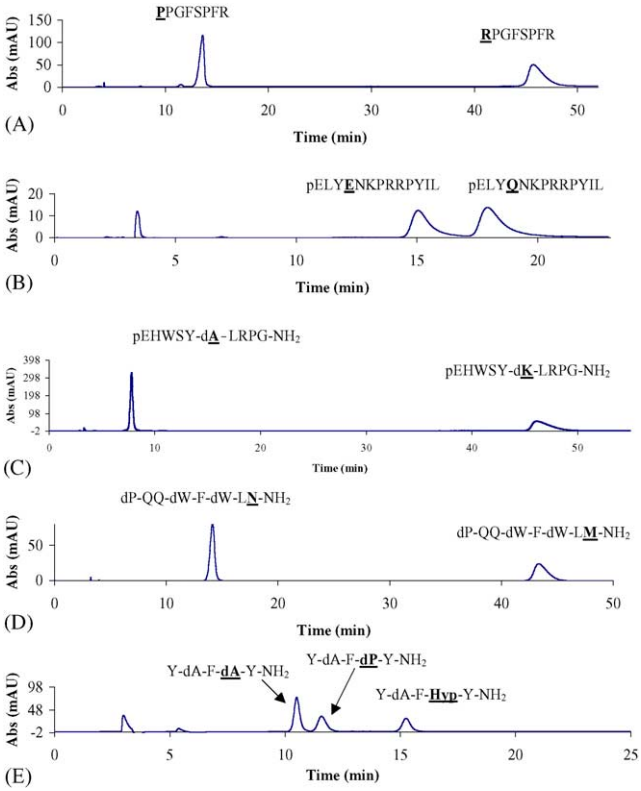

Fig. 2. Chromatograms showing the effect of the location of a SAAP within the peptide on the separation of the polymorphs. The polymorphism occurs at the: (A) N-terminus (bradykinin family); (B and E) position 4 (neurotensin, β-casomorphin families); (C) position 6 (lutenizing hormone releasing hormone family); or (D) the C-terminus (substance P family) of the peptide. Chromatograms A, C, D and E were generated on a Chirobiotic T column and chromatogram B was generated on a Chirobiotic R column. All chromatographic conditions same as in Table 1 using UV detection.

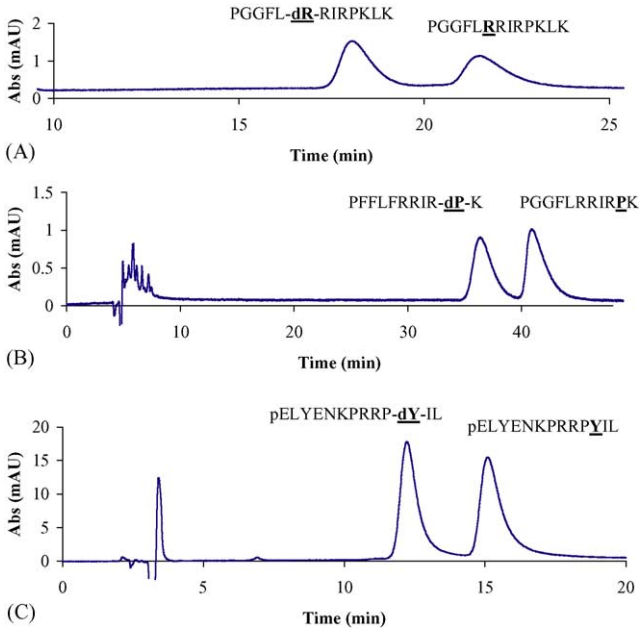

Fig. 3. Chromatograms showing the effect of the location of a chiral SAAP within the peptide on the separation of the polymorphs. The polymorphism occurs in: (A) position 6 (dynorphin 1–11 family); (B) position 10 (dynorphin 1–13 family); or (C) position 11 (neurotensin family). Chromatograms A and B were produced on a Chirobiotic T column and chromatogram C was produced on a Chirobiotic R column. All chromatographic conditions the same as in Table 2 using UV detection.

if the polymorphism occurred at or near the end of peptide chain. This is because functional groups on both ends provide stronger interaction with the stationary phase [31]. It is important to note that these separations were obtained under optimized elution conditions for the entire family of peptides. In the cases where a neutral amino acid is replaced with a positively charged amino acid (Fig. 2A and C), there is a tremendous difference in the retention of the peptides. This is largely due to the additional interaction of cationic side chains with the stationary phase. However, differences in electrostatic interactions are not solely responsible for the ultra-high selectivities. For example, the substitution of methionine for norleucine (Fig. 2D) also produces a tremendous change in the retention behavior of these peptides. Fig. 3 shows the separation of peptide epimers (i.e., where the single amino acid polymorphism is due to the opposite chirality of a single amino acid). In these cases, peptides with the chiral SAAP in the middle of the peptide chain were as easy to separate as those with more terminal groups. However, epimers in which the chiral SAAP is α or β to the C-terminal end (Fig. 1) appear to produce the most facile separations of this class of diastereomers. Interestingly, the epimer containing the D-amino acid always eluted before the other epimer, regardless of its position in the peptide chain (Figs. 1 and 3). It should be noted that this elution sequence is opposite to that observed for monomer native amino acids and dipeptides [31].

3.3. Optimization of peptide separations on Chirobiotic stationary phases

As with most separations of charged analytes in the reverse-phase mode, the percentage and type of organic modifier along with the pH of the mobile phase, must be optimized in order to produce the best separation. Since the macrocyclic glycopeptide stationary phases also have ionic sites, the ionic strength of the mobile phase must also be considered.

3.3.1. Organic modifier content and retention behavior

In separating small molecules in the reversed-phase mode, most macrocyclic glycopeptide stationary phases have shown the highest selectivity when methanol was used as the organic modifier [13–17,20–22,30–34]. While this also was true for the peptides examined here, methanol often produced broad peaks and inefficient separations. Efficiency was greatly improved when acetonitrile was used as the organic modifier. Acetonitrile was used in all the mobile phases reported here, as the increase in efficiency more than compensated for the loss in selectivity.

Regardless of organic modifier type, plots of mobile phase composition (i.e., percent organic modifier) versus retention

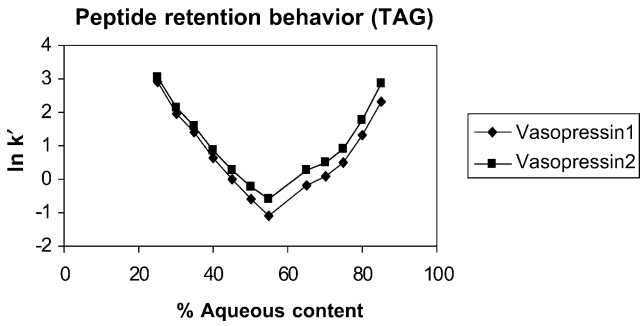

Fig. 4. Retention of vasopressin peptides on Chirobiotic TAG stationary phase. Increased retention at high organic modifier content is observed due to lower peptide solubility in the mobile phase. Chromatographic conditions: Chirobiotic TAG 250 mm × 4.6 mm column at a flow rate of 1 mL/min with UV detection at 210 nm. Aqueous solution included 0.1% formic acid.

produced U-shaped retention curve behavior on all macrocyclic glycopeptide stationary phases. Fig. 4 shows the elution behavior for two vasopressin peptides on a Chirobiotic TAG stationary phase. The peptides are more strongly retained under high organic content and high aqueous content mobile phases. The strongest eluting mobile phase was generally around half organic and half aqueous content, although the sequence of the peptide determines the exact location of the retention minimum for any retention versus composition curve. Similar U-shape retention behavior of peptides and proteins was commonly observed on alkyl bonded stationary phases [40–48], despite their differences in chemistry from macrocyclic glycopeptide stationary phases. Simpson and Moritz indicated that peptide retention, at high organic modifier concentration, was more like normal phase chromatography (polar stationary phase), which suggests that residual silanol groups also contribute greatly in retention [42]. Bij et al. proposed dual mechanisms, in which the combination of solvophobic and silanophilic interaction was thought to be the reason for retention inversion [48]. Early on, Armstrong and co-workers pointed out that the real reason for the inverse retention behavior at high organic modifier concentration (for many proteins, peptides, and even amino acids) was from the changes in their solubility as the organic concentration in the mobile phase is increased [40,41]. Under high aqueous mobile phase conditions, the classic reverse-phase mechanism (i.e., hydrophobic association) governs retention, where increased organic modifier amounts decrease retention. Under high organic mobile phase content, peptides become much less soluble in the mobile phase, which means longer retention times. The point of minimum retention (Fig. 2) can be approximated by coupling the reversed-phase retention curve and the solubility curve for any peptide of interest. In some cases, other specific interactions (electrostatic, etc.) can affect the exact location of the retention minimum. More choices in method development are available due to this U-shaped elution curve behavior. For example, the U-shaped elution curve behavior also indicates the possibility to carry out an inverse gradient on this class of stationary phases [40]. Thus, depending on the starting mobile phase composition, a traditional or inverse gradient can be used to decrease retention times, if desired. Most mobile phases in this work use higher organic modifier concentrations due to the increased efficiency observed with such mobile phases without gradient elution.

3.3.2. Mobile phase pH

The overall charge on a peptide is determined by the amino acids in the peptide and is a consideration in determining the optimized mobile phase. Under the operating pH range of the macrocyclic glycopeptide stationary phases (pH 2.8–7.5), peptides with basic side chain groups are generally protonated while peptides with acidic side chain groups mostly deprotonated. The additional positively charged side chains allow for increased interaction of the peptide with the stationary phase through its anionic sites. Thus, cationic peptides can be strongly retained [31]. Adding ammonium salts or acidifying the mobile phase appears to provide competing ions for the anionic sites or protonate them, respectively, thereby decreasing the retention of positively charged peptides. However, for neutral and anionic peptides, the ammonium salt or acid can overwhelm the interaction of the peptide with the stationary phase leading to insufficient retention. Some neutral peptides (e.g., enkephalins) elute near the void volume if salt or acid is added to the mobile phase. Additionally, the specific structure of the stationary phases must be considered as well. For the Chirobiotic T and TAG columns, the mobile

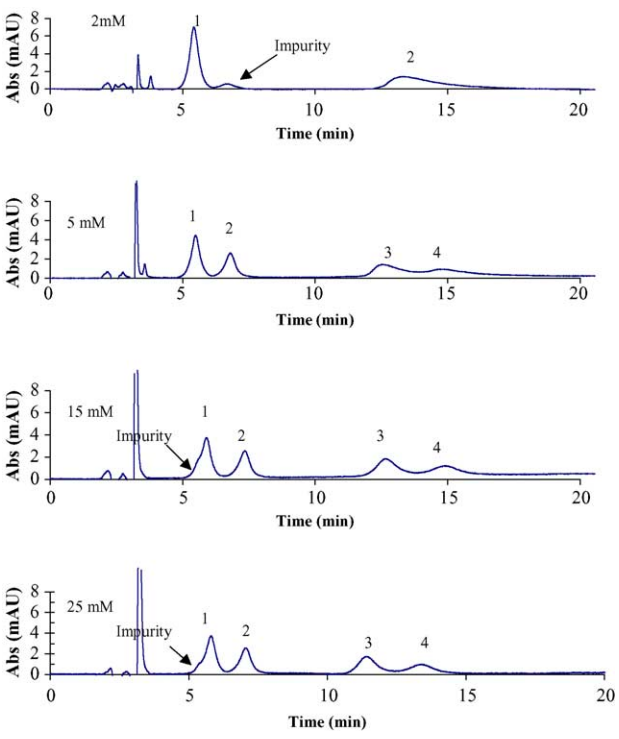

Fig. 5. The four chromatograms show the effect of ionic strength on the elution of charged peptides. Peptides are from the bradykinin family and the sequence is as follows: (1) PPGFSP; (2) PPGFSPFR; (3) RPGFSPFR; (4) RPPGFSPFR. Chromatographic conditions: Chirobiotic R column, acetonitrile–ammonium formate buffer, pH 3 (60:40), 1.0 mL/min, UV detection at 232 nm.

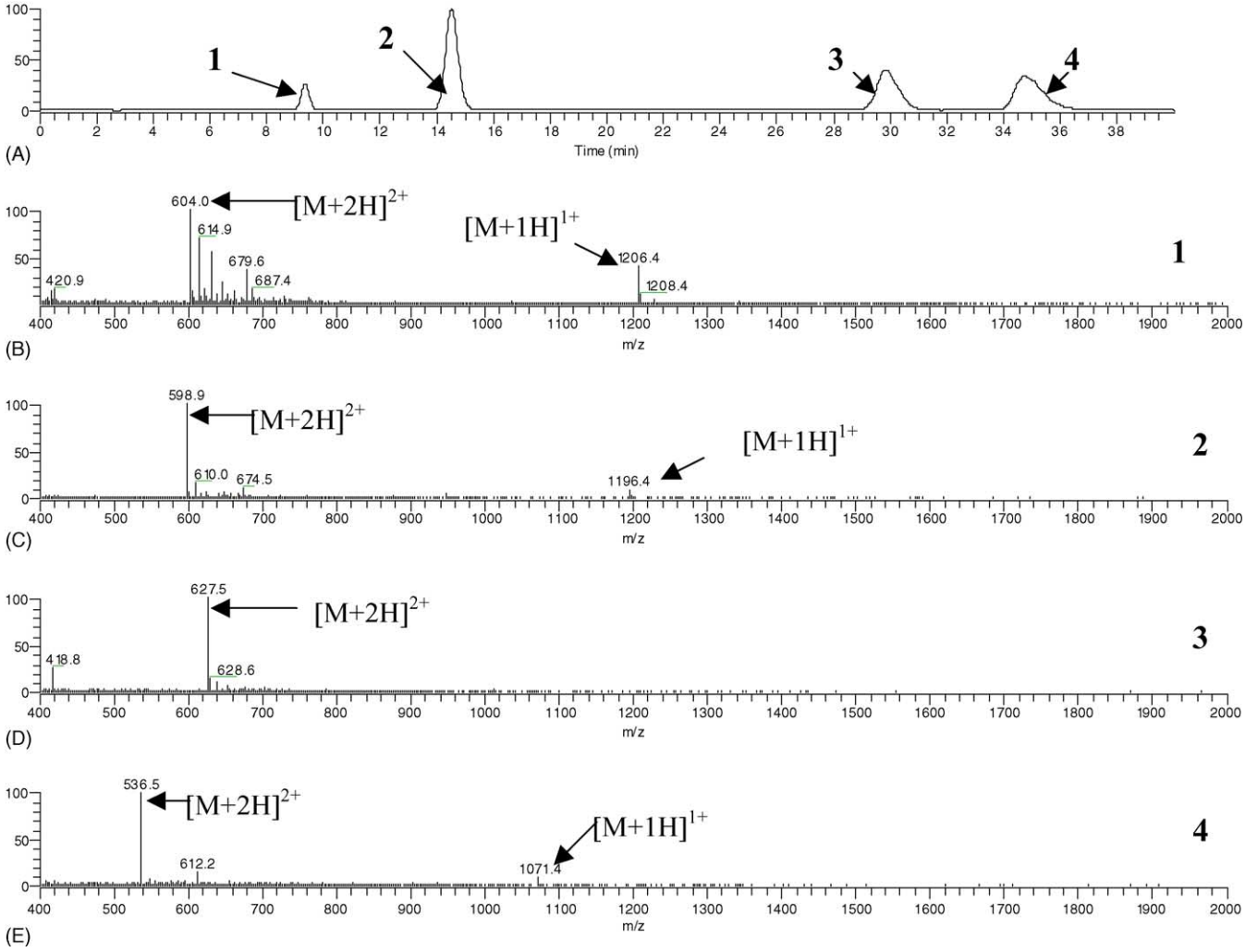

Fig. 6. LC–ESI-MS of the lutenizing hormone releasing hormone family. Panel A is the base peak chromatogram. Panels B–E are the mass spectra of each peak in panel A. HPLC conditions: Chirobiotic T 250 mm × 4.6 mm column; mobile phase composition: 60% formic acid (0.1%), 40% acetonitrile. Flow rate 1 mL/min.

phase additive formic acid was required to elute many of the peptides listed in Table 2. However, when the same mobile phase conditions used for separations on the Chirobiotic T or TAG columns are used on the Chirobiotic R column, the peptides elute near the void volume (data not shown). This change in behavior is due to the presence (or absence) of carboxylic acid sites on the stationary phase. Teicoplanin (Chirobiotic T) and the teicoplanin aglycone (Chirobiotic TAG) have a free carboxylic acid group while the corresponding acid site on the ristocetin (Chirobiotic R) has been esterified.

3.3.3. Mobile phase ionic strength

To illustrate the effect of mobile phase additives on retention, peak shape, and resolution, different amounts ammonium formate were added to the mobile phase while maintaining a constant pH and acetonitrile content. The bradykinin peptides were chosen because this family of peptides contains both neutral and cationic side chains. Peptides with cationic side chains contain up to two arginine residues. Fig. 5 shows chromatograms generated using mobile phases containing different ammonium formate concentrations of 2, 5, 15, and 25 mM. At 2 mM ammonium formate, RPGFSPFR and RPPGFSPFR did not elute after 100 min (data not shown). Only the first 20 min of the chromatogram is shown in order to compare the peak shape for the two peptides that did elute. The basic arginine group in PPGFSPFR produces much more pronounced tailing at this concentration relative to the PPGFSP peptide, which contains no amino acids with cationic side chains. Increasing the ammonium formate concentration to 5 mM drastically shortens the retention times of the peptides with two arginines residues. However, the more basic peptides still exhibit broad peaks with significant tailing. Raising the ammonium formate concentration to 15 and 25 mM continues to shorten the retention times of the basic peptides while leaving the retention of PPGFSP relatively constant. Greater efficiency is achieved at 15 and 25 mM compared to 5 mM, though changes tend to be less pronounced. It is expected that higher concentrations of ammonium formate

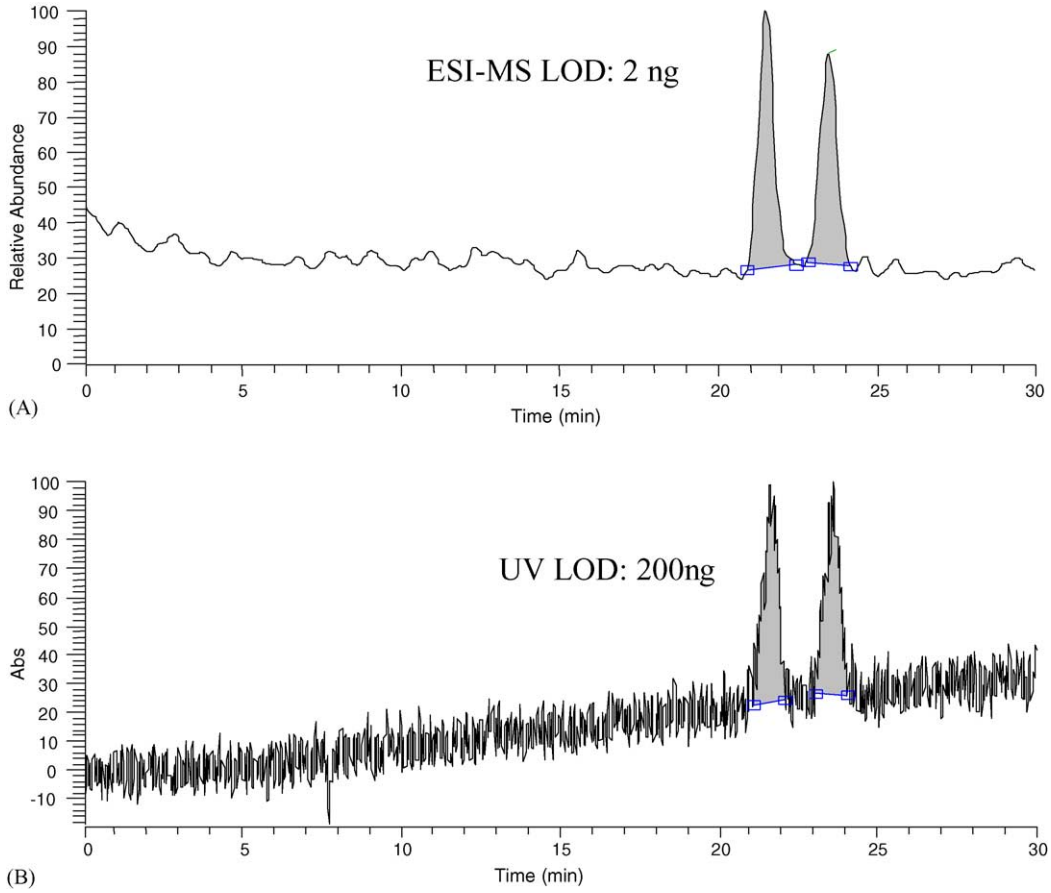

Fig. 7. The limits of detection for the vasopressin peptides were found for: (A) ESI-MS (selected-ion monitoring mode); (B) UV (210 nm) detection. S/N is the signal to noise ratio of the peaks. Injection volumes (2 μL) were identical for both panels. HPLC conditions: Antibiotic TAG 250 mm × 4.6 mm column, using a mobile phase composition of 60% formic acid aqueous solution (0.1%), 40% acetonitrile at 0.5 mL/min.

could further enhance peak shape of the more basic peptides. However, the most MS-compatible mobile phase would utilize the lowest salt concentration to give the desired detection sensitivity (as discussed in Section 3.4).

3.4. Electrospray mass spectrometry detection

Many previous LC methods, developed to separate peptides, used alkyl bonded phases with ion-pairing agents such as: trifluoroethylammonium phosphate [49], trifluoroacetic acid (TFA), trialkyl ammonium phosphate (TAAP) [50], or heptafluorobutyric acid (HFBA) [51,52]. By using these agents in mobile phases under appropriate pH conditions, charged analytes like peptides would form pairs of ions. Instead of eluting in the dead volume, peptides could be retained and separated due to their different hydrophobic interactions with the stationary phase. With the increasing popularity of electrospray ionization mass spectrometry coupled to HPLC, alternatives to the ion pair approach have been sought because of the adverse effects of ion pair reagents on ESI ionization efficiency [53,54]. In this study, all mobile phases developed but the two containing triethylamine were MS compatible. Triethylamine was added to the mobile phase in order to separate the epimers of the dynorphin family. The large number of basic amino acids present in this family of peptides caused them to interact very strongly with the stationary phase. Triethylamine at pH 2.8 provides stronger competition for the stationary phase than other mobile phase additives.

Fig. 6 shows an example separation of lutinizing hormone releasing hormone peptides using ESI-MS detection. The isocratic HPLC method is simple and ESI-MS compatible. From the mass spectra, the peaks can be easily identified according to their molecular weight. The most abundant ion was usually the $[M + 2H]^{2+}$ species, although sodium adduct products also were observed. This behavior is consistent with ESI spectra of peptides reported elsewhere [55].

In the recent literature, Desai and Armstrong reported the detection limits of amino acids at nanogram and sub nanogram levels by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [53]. In this study, APCI-MS gave the best sensitivity for small molecules under M_r 200 and similar sensitivity to ESI-MS for molecules between M_r 200 and 300. Above M_r 300, sensitivity increased for ESI-MS compared to APCI-MS. Fig. 7 compares the detection limit of ESI-MS and UV. A 2 ng peptide detection limit was easily achieved by ESI-MS in this study, consistent with the level

for single amino acids in Desai and Armstrong's report. This 2 ng detection limit is approximately two orders of magnitude lower than the detection limit obtained using UV detection at 210 nm under identical conditions.

The methods described here exhibit good detection linearity over wide range of peptide concentrations. For two vasopressin peptides, the calibration curve was linear over a concentration range of 0–1000 μg/mL with R^2 values of 0.991 for the first eluting peptide and 0.992 for the second eluting peptide. The methods developed in this study provide not only sensitive detection but also respectable detection linearity. This sensitive detection with a linear response is necessary for modern peptide assays.

4. Conclusions

Macrocyclic glycopeptide CSPs have great resolving power for closely related peptides separated by HPLC. In general, (1) terminal polymorphisms produced separations of greater resolution than those occurring in the middle of the peptide, (2) substituting a charged amino acid for an uncharged residue produced a separation of greater resolution than exchanging an uncharged amino acid for another uncharged amino acid or substituting like charged amino acids, and (3) all peptides containing a D-amino acid polymorphism eluted before the corresponding L-amino acid containing peptide. Most of the mobile phase conditions used are MS compatible and good limits of detection can be achieved by using ESI-MS. The peptides on macrocyclic glycopeptide CSPs exhibited U-shaped curves when retention is plotted against the concentration of organic modifier. Mobile phase composition, including the type and amount of organic modifier, mobile phase pH, and ionic strength, plays an important role in peptide elution and peak shape. The selectivity of the macrocyclic glycopeptide stationary phases for achiral and chiral polymorphisms using ESI-MS-compatible mobile phases should broaden their appeal for use in all areas where peptide separations are important.

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