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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PEPTIDES ON A DIOL-GLY-PHE-PHE TRIPEPTIDE-BONDED PHASE

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SUMMARY

The retention characteristics of some selected peptides (mol. wt. <2000 a.m.u.) have been investigated on a diol-Gly-Phe-Phe partitioning phase, bound to 5-μm porous silica. The hydrophobic, positively charged peptides can be separated with mild mobile phases, containing only acetonitrile and phosphate buffer. The peptide selectivity of the diol-Gly-Phe-Phe-bonded phase is uniquely different from that of a C_8 column. The dependence of capacity factors on mobile phase pH, ionic strength, and organic solvent concentration demonstrated that the partitioning mechanisms of the diol-Gly-Phe-Phe phase involve multifunctional reversed-phase and cation-exchange processes.

INTRODUCTION

The need for increased high-performance liquid chromatographic (HPLC) selectivity is very important in the separation and characterization of peptides. The development of small, biologically active peptide derivatives for medicinal agents and the characterization of protein fragments by tryptic mapping has created an increasing need for better resolution of complex peptide mixtures. Current HPLC technology is based principally on the one-column/one-mechanism philosophy, and in many cases, chromatographers have optimized separations to the limits of the resolving power of their columns. Currently, the strategies of using multifunctional partitioning phases and more than one column of differing selectivity are being investigated.

Peptide separations by LC have been carried out by exploiting a wide variety of separation mechanisms including size-exclusion, ion-exchange, normal-phase, and reversed-phase techniques. Reversed-phase HPLC on C_8 and C_{18} bonded phase porous silica remains the method of choice for separating peptides with molecular weights of <2000 a.m.u.[1–4]. The rapid stationary phase mass transfer of solutes on alkyl-bonded phase with small particulates (3–5 μm), and the use of aqueous organic mobile phase gradients, produce the greatest number of resolvable peaks compared to other HPLC techniques. The reversed-phase separation of peptides typically involves the use of mobile phases containing aqueous trifluoroacetic acid (TFA) in combination with acetonitrile or methanol. The TFA primarily serves to maintain a pH below

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2 in order to protonate carboxylic acid groups. In addition, TFA acts as an ion-pairing agent by association with protonated amines, thus neutralizing the molecular charge and minimizing the interaction with surface silanols. Under optimized gradient conditions, such reversed-phase separations can achieve column peak capacities (*i.e.*, the theoretical maximum number of resolvable peaks) slightly over a hundred[5]. It might appear that such a resolving power is more than adequate for separating most complex mixtures. In reality, however, the most difficult separations demand discrimination between molecules with only slight differences; and given the use of only one separation mechanism, column peak capacities in adjoining regions of a chromatogram generally go unutilized.

It is well established that reversed-phase separations exploit the solvophobic properties of solutes to generate differences in chromatographic retention. These retention characteristics are proportional to the analyte hydrophobic surface area and the mobile phase interfacial surface tension[6]. It has been demonstrated that the retention difference between small peptides on reversed-phase columns can be predicted by the linear combination of hydrophobic fragment coefficients[1,2,7]. It becomes obvious that subtle differences in amino acid sequence or peptide configuration can lead to degenerate hydrophobic surface areas, which result in overlapping chromatographic peaks. With the use of ion-pairing agents and the adjustment of pH these degeneracies can often be eliminated; however, in complicated separations, peak overlap in other regions of the chromatogram usually result. One solution to this impasse is to vary selectivity by incorporating an additional separation mechanism and/or increasing the peak capacity by adding a second dimension. This is the common strategy employed in thin-layer chromatography and two-dimensional electrophoresis, which can provide peak capacities in the thousands. For qualitative purposes, two-dimensional thin-layer techniques remain unsurpassed in resolving power; however, for accurate quantification HPLC is superior.

In an attempt to achieve greater versatility in HPLC selectivity, researchers have investigated the use of multifunctional bonded phases[8]. Multifunctional bonded phases can be classified into three categories. The first involves the delineation of physical differences, as achieved with mixed-mode size-exclusion and partitioning mechanisms. The second involves the independent partitioning of solutes with separately bonded functional groups. In this category, stationary phases bound both with ion-exchange groups and alkyl groups have been evaluated[9]. The third involves the integrated partitioning of solutes with a single bonded phase moiety which functionally invokes different partition mechanisms (*i.e.*, van der Waals dispersive forces, hydrogen bonding, ion-exchange, etc.). Most chiral bonded phases fall into the latter category, where at least three points of interaction are necessary for a bonded phase to distinguish between enantiomers. Peptide-bonded phases can also be placed in this category, because of the multifunctional nature of peptides. The well known phenomenon of peptide–peptide intermolecular interaction has prompted researchers to evaluate peptide-bonded phases as potentially valuable in multifunctional separations.

The use of tripeptide-bonded phases in HPLC was introduced by Grushka and co-workers in the late 1970s[10–12], following the lead of Corbin *et al.*[13], who earlier used a similar strategy for the gas chromatographic separation of amino acid enantiomers. Grushka and co-workers produced three tripeptide-bonded phases, Val-Ala-

Ser[10], Val-Phe-Val[11], and Val-Ala-Pro[12]. Each was bound to silica via a methylphenylethane spacer and the tripeptide was bound to the spacer by a methyl ester linkage with the carboxyl terminus, thus leaving the terminal amine free. This effectively rendered the tripeptide bonded phases weak anion-exchangers. In 1985, Howard *et al.*[14] improved the bonding synthesis of the Val-Ala-Pro phase of Grushka and evaluated the selectivity over a broad pH range in the separation of dipeptides. In that same year, Hagestam and Pinkerton[15] introduced a diol-Gly-Phe-Phe-bonded phase, where the phenylalanine moieties on the external surface of the particulate had been removed by enzyme cleavage, in order to make the packing material non-adsorptive to proteins. The packing is referred to as internal surface reversed-phase (ISRP) material and is designed for the analysis of drugs in blood plasma or serum by direct injection[16]. The ISRP columns are now commercially available from Regis, Morton Grove, IL, U.S.A.

The ISRP diol-Gly-Phe-Phe-bonded phase is distinguished by three significant differences from previous tripeptide-bonded phases. First, the carboxyl terminus is a free functional group making the packing a weak cation exchanger[17]. This is advantageous, since many blocked peptides have net positive charges, and separation reproducibility is generally enhanced when ion-pairing agents are not used. Second, the peptide is attached to an underlying diol phase. The diol phase is present in *ca.* 300 μmol/g, whereas the tripeptide is present in only 60–80 μmol/g[18]. In HPLC, the attachment of partitioning moieties to a diol phase is well established[19–21]. The advantages of using an underlying diol phase are the non-absorptivity rended to large proteins in biological samples[22] and the inactivation of residual silanols[23], which are known to produce severe tailing of amines. Third, the diol-Gly-Phe-Phe phase is bound via a carbamate linkage, which is inherently more stable[24] than the ester linkages used in previous tripeptide bonded phases[10–13].

The following study has been undertaken in order to evaluate the selectivity of the diol-Gly-Phe-Phe-bonded phase in peptide separations. Assuming one can exploit the multifunctional nature (*i.e.*, reversed-phase and cation-exchange properties) of the diol-Gly-Phe-Phe phase to improve the resolution of selected peptides, relative to conventional alkyl-bonded phases, the need to increase column peak capacity becomes important because columns with greater selective discrimination are inherently less efficient. In order to gain higher peak capacities, isolated components can be further separated off line by another type of column or different columns can coupled via switching valves for two-dimensional LC. The combined use of two-dimensional LC with columns of differing selectivity provides the greatest promise for isolating heretofore unresolved peptides, as recently demonstrated by Tanaka *et al.*[25] in a peptide separation conducted with a two-dimensional LC strategy using an alumina column and a reversed-phase column. The inclusion of multifunctional bonded phases as one dimension further enhances the flexibility and potential resolving power of such two-dimensional LC systems. It is for this reason that the multifunctional nature of the diol-Gly-Phe-Phe-bonded phase has been investigated for use in peptide separations.

EXPERIMENTAL

Chromatographic system

Chromatograph. The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1090M liquid chromatograph with integrated ternary solvent delivery system, autosampler, column temperature controller, and photodiode-array detector. Chromatographic parameters, solvent delivery, and data acquisition were controlled by an HP9000 Model 310 computer (HPChemStation). The data was stored on either an HP 20BM hard disc or on a Bering Bernoulli 20MB removable cartridge. Retention times, peak-widths at half-height, and symmetries were determined with HPChemStation software. Additional chromatographic data including capacity factors, efficiency, selectivity, and resolution were calculated with custom macro programs, run on the HPChemStation. Hardcopy chromatograms were generated on an HP7440 plotter. Capacity factor *vs.* buffer concentration plots were created in Lotus software, run on an IBM PC.

Mobile phases. The ternary solvent system consisted of (A) deionized/photolyzed water, (B) 0.2 *M* ammonium phosphate (pH 6), and (C) acetonitrile–water (90:10). The ammonium phosphate buffer was prepared with Fisher A-685 HPLC-grade monobasic phosphate and Fisher A-686 ACS certified dibasic phosphate salts in an appropriate ratio to yield a pH of 6. The phosphate solution was filtered through a 0.2-μm Nylon filter prior to use. The water used for preparing the mobile phases was USP-grade water, which had been further purified with a MilliQ™ system (Millipore, Bedford, MA, U.S.A.). The water was then photolyzed in 2-l batches by a high-flux ultraviolet light source contained in a Barnstead (Boston, MA, U.S.A.) Organicpure™ unit. Photolysis of the water guaranteed the absence of organic impurity peaks in gradient elution with 214 nm detection. The acetonitrile used in eluent C was UV 015 grade 99.9% pure (Burdick & Jackson Labs., American Scientific Products, McGraw, IL, U.S.A.).

Columns. The diol-Gly-Phe-Phe tripeptide ISRP columns (GFF-S5-80) were purchased from Regis. The synthesis and characterization of the ISRP packing was described in detail elsewhere[18,26]. A 15 cm × 4.6 mm column (Ser. No. 22302) and a 25 cm × 4.6 mm (Ser. No. 21206) column were used throughout.

Detection. Using an HP photodiode-array detector, chromatograms were generated with optical responses at 214 nm and 254 nm. Detector settings for 214 nm included a 4-nm bandwidth and a reference wavelength region from 450 to 550 nm. Detector settings for 254 nm included a 8-nm bandwidth and a reference wavelength region from 400 to 450 nm. The photodiode-array was automatically balanced against the eluent prior to each injection to compensate for drift. The data sampling interval was typically 640 ms. When appropriate, the spectrum of each eluted component was recorded from 210 to 350 nm.

Procedures. Unless stated otherwise, general chromatographic parameters included injections of 20 μl, a flow-rate of 1.0 ml/min, and a column temperature of 40°C. For isocratic elutions, eluents A, B, and C were metered with the HP1090M solvent delivery system at constant rates.

The capacity factors of selected peptides were mapped over a range of buffer concentrations at constant pH and acetonitrile content. The peptides were chromatographed individually, in duplicate, with buffer concentrations ranging from 0.002

to 0.2 *M*. The buffer concentration was varied by metering selected percentages of eluents A and B with a constant percentage of C. When evaluating the change in capacity factor as a function of the acetonitrile concentration, eluents A and C were varied while B was held constant. In measuring the change in capacity factors as a function of pH, eluents A, B, and C were metered at constant rates, with the pH of B having been adjusted prior to the experiment with concentrated phosphoric acid.

The capacity factors (k) for each peptide were calculated in the standard fashion $k = (t_R - t_M)/t_M$, where t_R is the unadjusted retention time and t_M is the mobile phase penetration void volume. The t_M for each column was measured with a solution of sodium nitrate. The void marker was found to correspond to the first baseline perturbation observed in each chromatogram. It is presumed this perturbation was caused by conjugate salts in the peptide samples.

In order to optimize the resolution of a mixture of enzyme inhibitory peptide derivatives, a linear solvent strength gradient elution was employed with the ternary solvent system at a flow-rate of 0.7 ml/min. In all cases, an interval of at least 20 min was allowed for re-equilibration before each run. The ternary gradient program [%A–%B–%C (v/v/v) with linear elution] was as follows: start 89%–1%–10%; at 5 min, 77%–1%–22%; at 40 min, 72%–1%–27%; at 60 min, 35%–25%–40%; and at 70 min, return to initial conditions.

Peptide analytes

Fetirelin acetate (Conceral™), the proline ethylamide of luteinizing hormone releasing hormone (LHRH), was obtained from Takeda, Osaka, Japan. The remaining fetirelin derivatives were acquired from Sigma, St. Louis, MO, U.S.A.; these included LHRH (L-0507), LHRH [Gly-OH^{10}] (L-8008), LHRH des-Gly^{10} [D-Ala^6] (L-2383), LHRH des-Gly^{10} [D-Leu^6] (L-5009), LHRH des-Gly^{10} [D-Trp^6] (L-5386), and LHRH des-Gly^{10} [D-Phe^6] (L-8886). Selected amino acids, dipeptides, and tripeptides, including glycine, D-histidine, L-histidine, 1-methylhistidine, 3-methylhistidine, Gly-L-Phe, Gly-D-Phe, Gly-Gly-L-Phe, and L-Phe-Gly-Gly were obtained from Sigma. A peptide fragment mixture of dihydrofolate reductase (43–50) from *Escherichia coli*, D-Phe-Gly, D-Phe-D-Phe-Gly, D-Phe-L-Phe-Gly and derivatives of a proprietary enzyme-inhibitory peptide were obtained from The Upjohn Company. The Conceral derivatives were prepared at a concentration of 50 μg/ml in water. The enzyme-inhibitory peptide derivatives were dissolved in either absolute methanol or 50% aq. acetonitrile at concentrations ranging from 50 to 80 μg/ml.

RESULTS AND DISCUSSION

General selectivity of the diol-Gly-Phe-Phe ISRP columns

The retention characteristics for a wide variety of low-molecular-weight drugs have been determined on the ISRP columns[15–17]. The diol-Gly-Phe-Phe-bonded phase favors the retention of aromatic compounds[16] and separates species primarily by a reversed-phase mechanism. The reversed-phase properties have been established from linear plots of log k *vs.* fraction of organic solvent in the eluent[17]. The hydrophobic partitioning on an ODS column is not a good predictor of retention on the tripeptide-bonded phase, as shown by Sams and Evec[27] with the separation of 21 drugs. However, the diol-Gly-Phe-Phe-bonded phase exhibits a broader range of

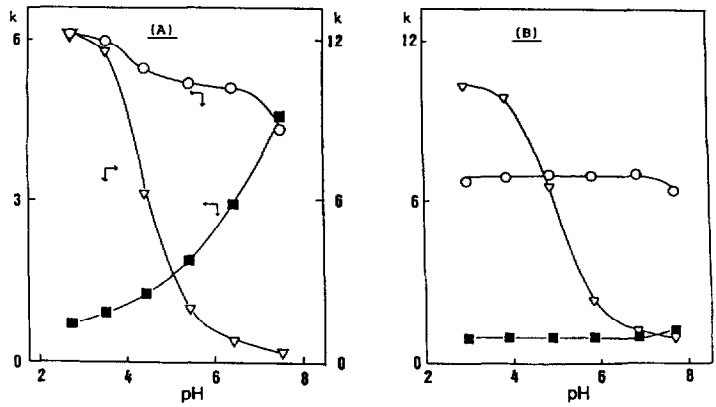


Fig. 1. Dependence of capacity factors of methylparaben (○), *p*-toluic acid (▽), and phenethylamine (■) on the pH of the mobile phase, observed with (A) diol-Gly-Phe-Phe column and (B) phenylsilica column; and mobile phases of (A) 0.1 *M* potassium phosphate buffer (ionic strength 0.2), (B) 0.1 *M* potassium phosphate buffer (ionic strength 0.20) – acetonitrile (80:20). Flow-rate, 1.0 ml/min; column size, 15 cm x 4.6 mm; detection, 220 nm. From ref. 17.

retentions than an alkyl-bonded phase and greater selectivity for some compounds. The carboxylic acid terminal of the diol-Gly-Phe-Phe acts as a weak cation exchanger and provides strong selective control for aromatic amines. Nakagawa *et al.*[17] demonstrated the secondary ion-exchange properties of the diol-Gly-Phe-Phe-bonded phase by contrasting it with a phenyl-bonded phase silica. Using *p*-toluic acid and phenylethylamine, Nakagawa *et al.*[17] plotted the change in capacity factors as a function of mobile phase pH on the two columns. As can be seen in Fig. 1, the capacity factor of *p*-toluic acid on both columns increases in a sigmoidal fashion as the pH is lowered. This is consistent with analyte protonation and reversed-phase partitioning of the neutral molecule. Phenethylamine, on the other hand, is positively charged in the pH range studied and is not well retained on the phenyl column over this range (Fig. 1). On the diol-Gly-Phe-Phe-bonded phase the capacity factor of the phenethylamine is seen to increase as the pH is raised. This implies that the carboxylic acid groups on the bonded phase become a weak cation exchanger as the groups are deprotonated. The retention of methylparaben, a neutral molecule, does not change appreciably with pH (Fig. 1). The ion-exchange properties of the diol-Gly-Phe-Phe-bonded phase are further exemplified by the change in capacity factors as a function of ionic strength, shown in Fig. 2. As the ionic strength increases, the capacity factors of the *p*-toluic acid on both columns increases slightly, as expected for reversed-phase partitioning. For the phenethylamine, as the ionic strength is decreased the capacity factor on the diol-Gly-Phe-Phe-bonded phase increases almost fifteen times over that of the phenyl column, indicating the distinct secondary cation-exchange properties of the peptide bonded phase. The retention of neutral compounds (methylparaben and phenol) remain constant with change in eluent ionic strength.

First peptide separations on ISRP columns

The separation of small biologically active peptides on diol-Gly-Phe-Phe ISRP columns was first demonstrated by Nakamura[28]. Using a 5 cm × 4.6 mm ISRP

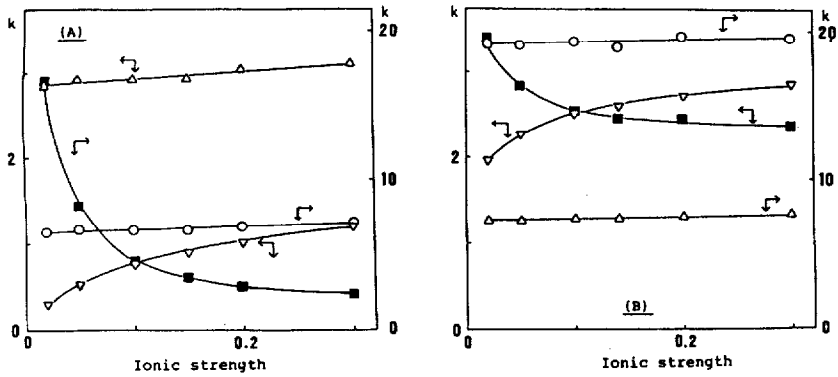


Fig. 2. Dependence of capacity factors on the ionic strength of the mobile phase at a constant pH of 6.7 with (A) diol-Gly-Phe-Phe column and (B) phenylsilica column; and mobile phases of (A) potassium phosphate buffer, (B) potassium phosphate buffer–acetonitrile (90:10). Symbol △ is for phenol. (For other information see Fig. 1.)

column, he measured the retention characteristics of a variety of proteins and peptides under both isocratic and gradient conditions (Table I). Small peptides exhibited retention, while the proteins with molecular weights > 14 000 a.m.u., were excluded and not adsorbed to the packing. Fig. 3 illustrates the resolution of four peptides by gradient elution. The amino acid sequences of peptides separated by Nakamura, and of fetirelin and DHFR investigated in this study, are shown on p. 136.

TABLE I

RETENTION CHARACTERISTICS OF SELECTED PROTEINS AND PEPTIDES ON A 5-cm DIOL-GLY-PHE-PHE COLUMN

Proteins and peptides separated by Nakamura[28] on a 5 cm x 4.6 mm ISRP column at a flow-rate of 1 ml/min with (A) an isocratic mobile phase of 0.1 *M* phosphate buffer (pH 6.7)–acetonitrile (80:20) and (B) a linear gradient of eluent A to B in 20 min, where A is 0.1 *M* phosphate buffer (pH 6.7) and B is 0.1 *M* phosphate buffer (pH 6.7)–acetonitrile (80:20). From ref. 28.

	mol. wt.	t_R *(min)*	
		A	*B*
Lysozyme	14 000	0.3	0.4
Bovine serum albumin	68 000	0.4	0.4
Catalase	240 000	0.4	0.4
Thyroglobulin	670 000	0.5	0.5
β-Lactoglobulin	18 400	0.6	0.5
Oxytocin	1007	1.1	3.2
Met-Enkephalin	574	1.3	1.5
Angiotensin II	1046	1.7	2.8
Neurotensin	1673	1.9	7.6
Angiotensin I	1297	2.0	11.2
Bradykinin	1060	5.3	14.6
Met-Lys-Bradykinin	1320	16.8	32.8

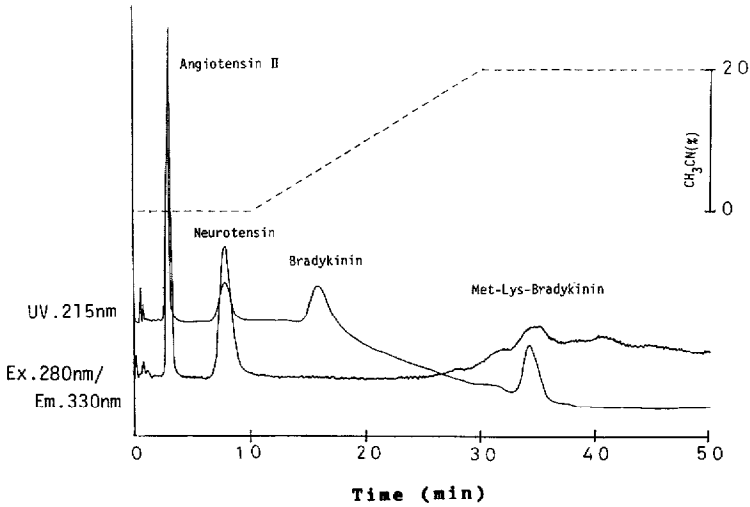
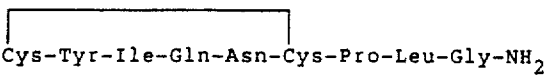


Fig. 3. Separation of biologically active peptides on a 5-cm diol-Gly-Phe-Phe column with linear gradient elution from eluent A to B, where A is 0.1 *M* phosphate buffer (pH 6.7) and B is 0.1 *M* phosphate buffer (pH 6.7)–acetonitrile (80:20). Flow-rate, 1 ml/min. (From ref. 28).

Angiotensin I
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin II
Asp-Arg-Val-Try-Ile-His-Pro-Phe
Bradykinin
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Oxytocin



Neurotensin
Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Methionine enkephalin
Tyr-Gly-Gly-Phe-Met
Fetirelin
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$NHCH_2CH_3$
Dihydrofolate reductase fragment (43–50)
Gly-Arg-His-Thr-Trp-Glu-Ser-Ile

All of these peptides have one or more of the aromatic amino acids (Phe, Tyr, or Trp), and some have one or more of the basic amino acids (Lys, Arg, or His).

Capacity factor mapping of fetirelin

Fetirelin is the proline ethylamide derivative of LHRH having a molecular weight of 1167 a.m.u. The peptide has nine amino acids, a pyroglutamyl group at the N terminus, and an ethylamide group blocking the carboxyl end. The hydrophobic

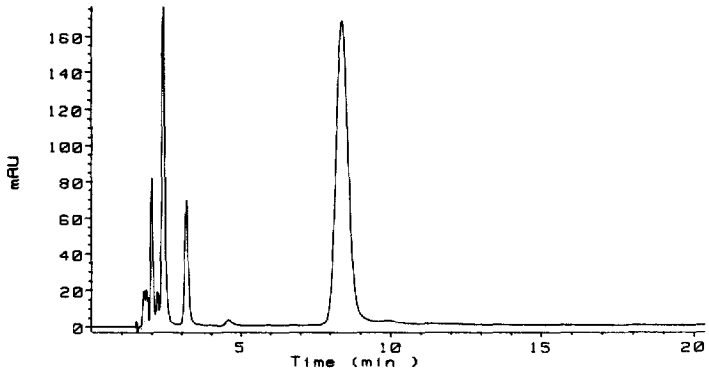


Fig. 4. Separation of fetirelin and degradation products on a 15-cm diol-Gly-Phe-Phe column with an isocratic mobile phase of 0.16 *M* ammonium phosphate (pH 6.1)–acetonitrile (82:18). Flow-rate, 1.0 ml/min; temperature 40°C; detection, 214 nm.

character of the peptide is primarily due to the Trp and Tyr, and its positive charge to His and Arg. The elution of fetirelin from a 15 cm x 4.6 mm diol-Gly-Phe-Phe column with a mobile phase of 0.16 *M* ammonium phosphate (pH 6.1)–acetonitrile (82:18) is shown in Fig. 4. Degradation products in the fetirelin sample are seen to be well separated.

By chromatographing derivatives of fetirelin one gains insights into its retention behavior relative to specific amino acid substitution. Modification of the sixth position with amino acids of increasing hydrophobicity, Ala < Leu < Phe < Trp, produces a proportional increase in the retention of the derivative on the diol-Gly-Phe-Phe-bonded phase. As shown in Table II, the increase in capacity factors parallel an increase in contribution to the log of the octanol–water partition coefficient (P)[2], and in the amino acid lipophilicity coefficient f, developed by Rekker[7]. Plots of f *vs.* log k or log P *vs.* log k for Leu, Phe, and Trp show linear relationships with correlation coeffficients of 0.95 and 0.99, respectively. This indicates that the diol-Gly-Phe-Phe reversed-phase partitioning responds systematically to a recognizable change in the molecular hydrophobic surface area. The capacity factors of fetirelin derivatives

TABLE II

RETENTION CHARACTERISTICS OF FETIRELIN DERIVATIVES

P is the amino acid octanol–water partition coefficient[2]. f is the lipophilicity side chain contribution according to Rekker[7]. k is the capacity factor of each fetirelin derivative determined on a 15-cm diol-Gly-Phe-Phe ISRP column under isocratic conditions by constant metering of the eluents A, B and C at 50%, 30% and 20%, respectively.

Amino acid substitution in sixth position	*log P*	*f*	*k*
D-Alanine	−2.94	0.53	6.56
D-Leucine	−1.71	1.99	7.21
D-Phenylalanine	−1.43	2.24	12.58
D-Tryptophan	−1.04	2.31	22.45

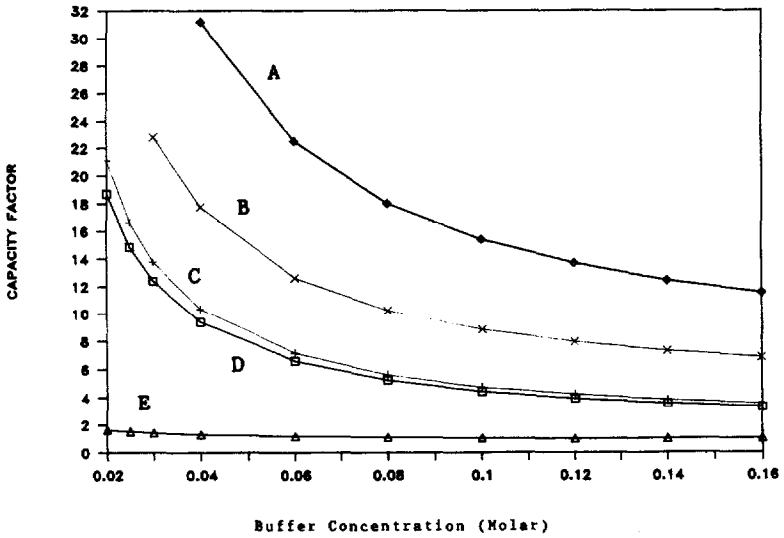


Fig. 5. Change in capacity factors of fetirelin derivatives as a function of mobile phase ionic strength at constant pH on a 15-cm diol-Gly-Phe-Phe column in isocratic elution with 0.16 *M* ammonium phosphate (pH 6.1)–acetonitrile (82:18). Other conditions same as in Fig. 4. (A) [D-Trp][6], (B) [D-Phe][6], (C) Conceral, (D) [D-Ala][6], and (E) [Gly-OH][10].

with Gly or Ala in the same position did not fall on this line, indicating these groups are too small to produce a significant change in hydrophobic surface area.

The effect of charge on the retention of the fetirelin derivatives is illustrated by the change in capacity factors as a function of mobile phase buffer concentration at constant pH (6.1) and acetonitrile concentration (Fig. 5). As expected, the capacity factors of the positively charged peptides increase with decreasing ionic strength in accordance with a cation-exchange partitioning mechanism. A decrease in pH produces a proportional increase in capacity factor of each peptide as a result of an increase in positive charge, brought on by further protonation. When the ethylamide group on the end is replaced by glycine with a free carboxylic acid terminal group [Gly-OH[10]], the molecular charge is neutralized, and the capacity factor does not increase with decreasing ionic strength (Fig 5).

Dihydrofolate reductase fragments

Another instance of the diol-Gly-Phe-Phe-bonded phase selectivity is illustrated with the separation of an *E. coli* dihydrofolate reductase (DHFR) fragment (43–50) and its N-formylated Trp derivative. Trp confers hydrophobic character on the fragment, while a positive charge is provided by Arg and His. The positive charge is offset by the negative charge produced by Glu and the C-terminal Ile. The separation, monitored with a photodiode-array detector, shows that the DHFR fragment is eluted at 8.2 min and the N-formylated Trp derivative is eluted at 9.6 min (Fig. 6). The N-formylated Trp derivative was clearly identified by its characteristic absorbances at 243 nm and 297 nm[29]. The peak at 2.9 min is an unidentified diastereomer of the DHFR fragment. This separation illustrates the selectivity difference produced on the

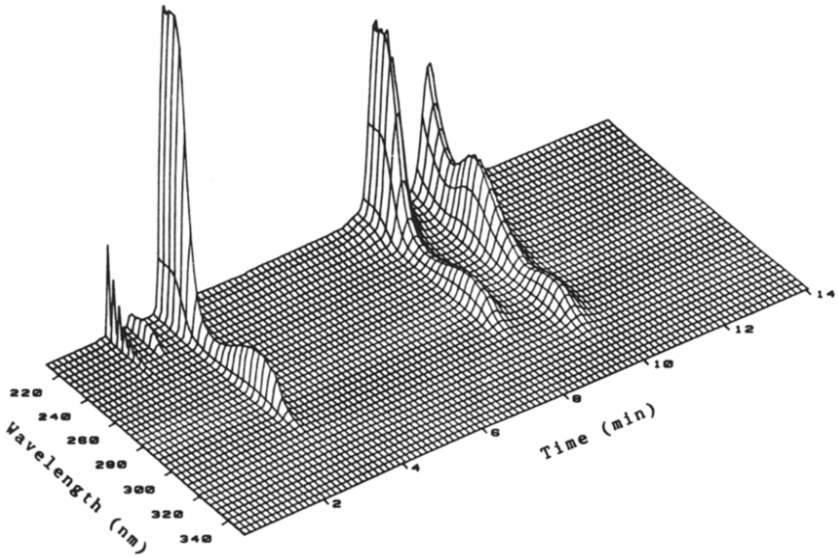


Fig. 6. Separation of DHFR (43–50) and the N-formyl-Trp derivative on a 15-cm diol-Gly-Phe-Phe column with an isocratic mobile phase of 95.5% 0.02 *M* ammonium phosphate buffer (pH 6.1)–acetonitrile (95.5:4.5). Flow-rate, 1.0 ml/min; temperature, 40°C; photodiode-array detection.

diol-Gly-Phe-Phe-bonded phase by a slight structural variation in a hydrophobic amino acid. By contrast, the components were difficult to resolve on an alkyl-bonded phase with conventional methods.

Enzyme-inhibitory peptide derivatives

Further selective discrimination can be illustrated with the separation of a synthetic mixture of peptide analogues and fragments, associated with a proprietary enzyme-inhibitory peptide. The peptide components range in molecular weight from about 450 to 940 a.m.u., and are protected on one end by a *tert.*-butyloxycarbonyl group and on the other end by methylpyridine. Hydrophobic interaction is provided by a phenylalanine residue, and positive charge by a histidine group. Fig. 7 shows a conventional isocratic separation of the mixture on a 25 cm x 4.6 mm Spherisorb C_8 column with a mobile phase of acetonitrile aqueous solution, 0.05 *M* in TFA and 0.04 *M* in triethylamine (40:60). Fig. 8 shows a ternary gradient separation of the same mixture on a 15 cm x 4.6 cm diol-Gly-Phe-Phe-bonded phase with an ammonium phosphate buffer (pH 6.1) and aq. acetonitrile. The gradient program maintains a constant buffer concentration of 0.002 *M* for the first 40 min of the chromatogram which is overlapped by a linear gradient elution with aq. acetonitrile. The program ends with a steep gradient increase in ionic strength, necessary to elute the last components. The details of the gradient program are given in the experimental section.

The numbering of the components is indicated by the elution order of the alkyl-bonded phase separation, thus illustrating discrimination by differences in the hydrophobic surface area of the analytes. The elution order on the diol-Gly-Phe-Phe phase is significantly different from that on C_8. The separation on the peptide-bonded phase is brought about by the multifunctional mechanisms of reversed-phase and

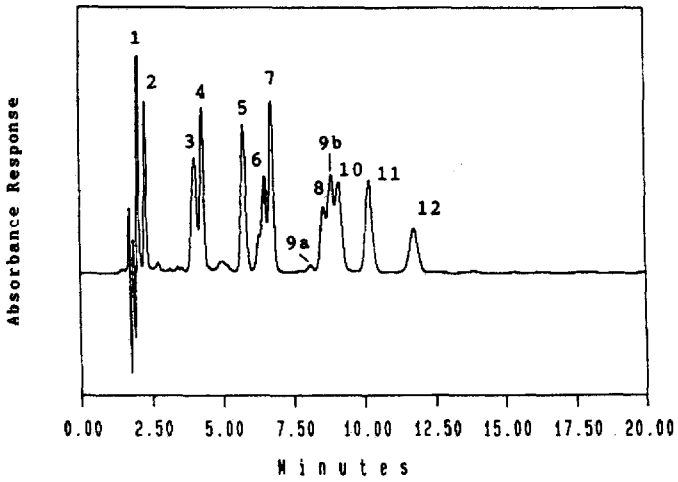


Fig. 7. Separation of an enzyme-inhibitory peptide mixture on a 25-cm Spherisorb C_8 column with an isocratic mobile phase of acetonitrile–aq. solution of 0.05 *M* in TFA and 0.04 *M* in triethylamine (40:60). Flow-rate, 1.5 ml/min; detection, at 254 nm.

cation-exchange chromatography. Components 1 and 2 are eluted early from the C_8 column and last from the diol-Gly-Phe-Phe column. These two components are unique in that the N-terminus is deblocked, thus providing an additional positive charge for the cation-exchange interaction on the diol-Gly-Phe-Phe phase. Component 13 is not eluted from the C_8. Components 8, 9a, 9b and 10 are eluted from the C_8 column in the same region, while the diol-Gly-Phe-Phe-bonded phase separation of these components differ greatly in their retention times, and the elution order is reversed. Components 9a and 9b are diastereomers, differing only in the configuration of the phenylalanine group; whereas 8 and 10 are isomers differing only in the position of a methyl group on the histidine residue (Fig. 9).

It may be postulated that the methyl group on the 3-methylhistidyl derivative (component 10) contributes more to the hydrophobic surface area of the molecule

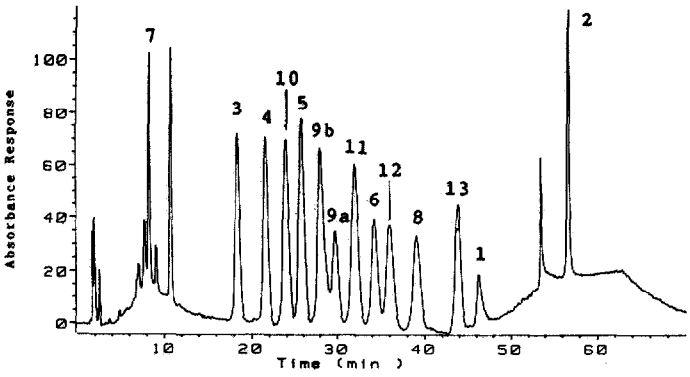


Fig. 8. Separation of the same peptide mixture as in Fig. 7 on a 15-cm diol-Gly-Phe-Phe column by gradient elution (see text for details). Components numbered according to elution order in Fig. 7.

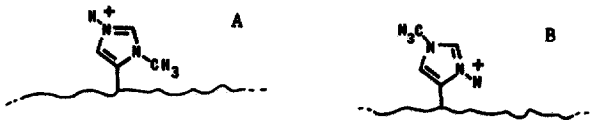


Fig. 9. Partial structures of (A) 1-methylhistidyl peptide derivative (component 8); (B) 3-methylhistidyl peptide derivative (component 10).

than does the 1-methylhistidyl derivative (component 8), where one might envision the methyl group being more imbedded in the molecule. This is consistent with the results of the C_8 separation, where one would expect the more hydrophobic molecule to have a greater retention. Conversely, on the diol-Gly-Phe-Phe-bonded phase the 1-methylhistidyl derivative (component 8) is much more strongly retained than the 3-methylhistidyl (component 10) derivative. This implies that the cation-exchange mechanism plays a critical role and that the more exposed the charged group is or the greater the differences in pK_a values, the greater is the partitioning interaction. This hypothesis is further supported by the change in capacity factors as a function of pH of the mobile phase, shown in Fig. 10. As the pH decreases from 7 to 5, the capacity factors increase as the methylhistidyl groups become more protonated. Below pH 5 the capacity factors drop off as the carboxylic acid terminal of the diol-Gly-Phe-Phe-bonded phase is protonated, thus eliminating its cation-exchange capacity. Also, the greater cation-exchange interaction of the 1-methylhistidyl derivative (component 8) is indicated by a greater increase in capacity factor compared to the 3-methylhistidyl derivative (component 10) with decreasing ionic strength of the mobile phase (Fig. 11). The degree of reversed-phase interaction is exhibited by the increase in capacity factors of the two components as a function of the acetonitrile content of the mobile

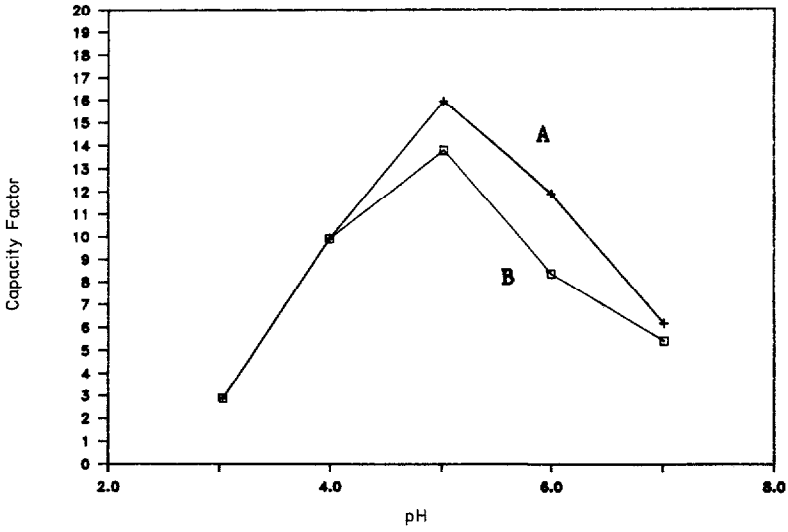


Fig. 10. Change in capacity factors of (A) 1-methylhistidyl and (B) 3-methylhistidyl derivatives as a function of pH with a 0.05 *M* ammonium phosphate–acetonitrile (82:18) isocratic mobile phase on a 25-cm diol-Gly-Phe-Phe column. Flow-rate 1.0 ml/min; temperature, 40°C.

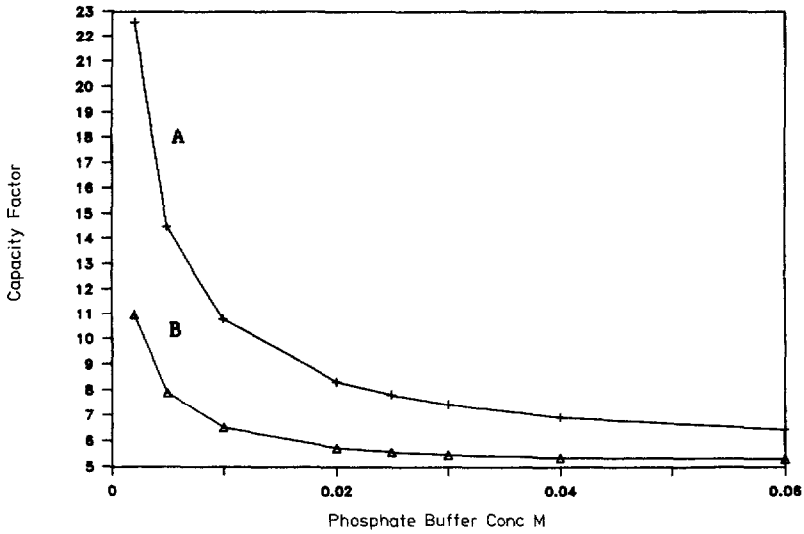


Fig. 11. Change in capacity factors of (A) 1-methylhistidyl and (B) 3-methylhistidyl derivatives as a function of mobile phase ammonium phosphate concentration at a constant pH of 6 and acetonitrile concentration of 18% with isocratic elution from a 15-cm diol-Gly-Phe-Phe column. Flow-rates 1.0 ml/min; temperature, 40°C.

phase (Fig. 12). Plots of log k *vs.* percent acetonitrile for the two components are linear and parallel with correlation coefficients of 0.99 and with indentical slopes of -0.05.

Amino acids, dipeptides, and tripeptides

Table III shows retention data for the elution of selected amino acids, dipeptides, and tripeptides for a 25 cm x 4.6 mm diol-Gly-Phe-Phe-bonded phase column under weak mobile phase conditions. As one can see, the amino acids glycine and phenylalanine and the dipeptides are not well retained. Although a retention time difference can be observed between 1-methylhistidine and 3-methylhistidine, they would not be resolved from one another. The glycylphenylalanine enantiomers are not distinguished from each other, but are slightly more retained than the D-phenylalanylglycine isomer; however, neither would be separable from one another. In like manner, the isomeric pair glycylglycyl-L-phenylalanine and L-phenylalanylglycylglycine exhibits a slight difference in retention, yet not enough to achieve resolution. On the other hand, the D-phenylalanyl-L-phenylalanylglycine and D-phenylalanyl-D-phenylalanylglycine diastereomers would be baseline resolved. This indicates that free amino acids, dipeptides, and some tripeptides could likely be resolved from larger peptides. However, minor modification in these small peptides will not enable separation of these isomers, except in the case of tripeptide diastereomers with sufficient hydrophobicity.

Column performance

Although the diol-Gly-Phe-Phe packings can provide unique selectivity for the separation of various peptides, the columns are inherently less efficient than alkyl-

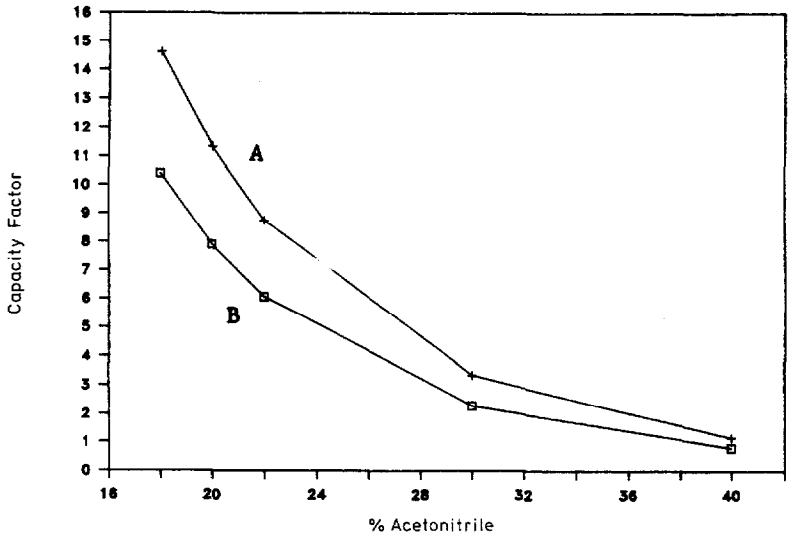


Fig. 12. Change in capacity factors of (A) 1-methylhistidyl and (B) 3-methylhistidyl derivatives as a function of mobile phase acetonitrile concentration combined with an aqueous 0.04 *M* ammonium phosphate buffer (pH 6) with isocratic elution from a 25-cm diol-Gly-Phe-Phe column.

TABLE III

SEPARATION OF SELECTED AMINO ACIDS, DIPEPTIDES, AND TRIPEPTIDES ON A DIOL-GLY-PHE-PHE-BONDED PHASE COLUMN

The separations were performed on a 25 cm x 4.6 mm diol-Gly-Phe-Phe ISRP column under isocratic conditions by constant metering of solvents A, B and C at 94%, 1% and 5%, respectively.

	t_R (min)	k
Amino acids		
L-Glycine	2.73	0.44
L-Phenylalanine	3.24	0.71
L-Histidine	11.59	5.10
3-Methylhistidine	13.34	6.03
1-Methylhistidine	13.88	6.31
Dipeptides		
D-Phe-Gly	3.05	0.61
Gly-L-Phe	3.20	0.68
Gly-D-Phe	3.20	0.68
Tripeptides		
Gly-Gly-L-Phe	2.94	0.55
L-Phe-Gly-Gly	3.10	0.67
D-Phe-L-Phe-Gly	5.59	1.94
D-Phe-D-Phe-Gly	7.31	2.85

bonded phases, because of slower stationary phase mass transfer. The 5-μm ISRP columns can typically achieve 30 000 plates/m, whereas conventional alkyl bonded phases can exhibit twice this amount. The column-to-column reproducibility of the packing is comparable to other silica-bonded phases. When comparing the 15-cm and 25-cm ISRP columns of this study, which were manufactured from different lots of packing, it was found that relative retentions of peptide components varied by an average of 5% between columns, but the slopes of log k *vs.* % acetonitrile for single components were identical between columns.

For the peptide-bonded phases produced by Grushka and co-workers[10–12] and Howard *et al.*[14], chemical stability was a limitation, because of the hydrolysis of the ester linkage connecting the peptides to the support spacers. The peptide-bonded phases produced in this manner were found to display a 50% drop in performance after about two months of use[14]. With the diol-Gly-Phe-Phe-bonded phases, the tripeptide is bound to a glycerylpropyl group by means of a carbonyldiimidazole reaction, producing a carbamate linkage between the peptide and the spacer, which is more stable[24]. A cursory evaluation of the diol-Gly-Phe-Phe-bonded phase stability can be obtained from the 15-cm ISRP column used in this study. After one year of periodic use for *ca.* 350 separations, retention times had decreased by an average of 40%. However, the relative retention between components varied by only 6%, and original retention times could be restored by decreasing the mobile phase strength. In some cases, resolution could not be restored between closely eluted components because of lost efficiency. It should be noted, that all separations were conducted without the use of guard columns. Previous studies have shown that efficiency loss can be prevented by the use of guard columns, even when serum is directly injected[30]. The diol-Gly-Phe-Phe-bonded phase with the carbamate linkage does appear to be more chemically stable than peptide-bonded phases produced with ester linkages, but definitive conclusions in this regard must await further investigation.

CONCLUSION

It was found that the diol-Gly-Phe-Phe silica bonded phase packings provide unique selectivity in the separation of positively charged hydrophobic peptides (<2000 a.m.u.) through multifunctional reversed-phase and cation-exchange partitioning mechanisms. The retention of peptide components can be controlled by alteration in either the mobile phase ionic strength or organic solvent composition. The use of ion-pairing agents, organic amine modifiers, or low pH is unnecessary, because selectivity differences are generated by the peptide-bonded phase itself. The disadvantages of the diol-Gly-Phe-Phe phases, compared to conventional alkyl-bonded phases, are lower efficiency, greater expense, and a lack of established theory for predicting multifunctional retention characteristics. These disadvantages are typical of specialty columns, and customarily, this is the price one pays for achieving a desirable end. In the case of the diol-Gly-Phe-Phe ISRP columns, it may be either the greater selectivity necessary to gain a desired separation between specific analytes with subtle structural differences or the reduction of sample clean-up procedures normally required for matrices containing unwanted proteins. In addition to blood serum or plasma, the latter category also includes plant samples, such as that recently analyzed by Meriluoto and Eriksson[31] with the ISRP separation of peptide toxins from cyanobacterial blooms.

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