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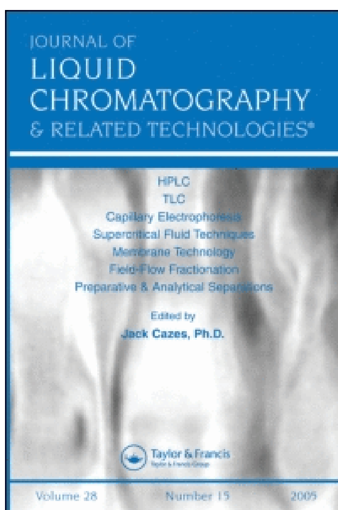

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMPHIBIAN PEPTIDES. SELECTIVITY CHANGES INDUCED BY pH.

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ABSTRACT

The effect of pH on the retention behavior under reversed- phase liquid chromatography conditions of a series of peptides was examined. Isocratic conditions were used with either methanol or acetonitrile as organic modifiers. The intrinsic hydrophobicity of the peptides was altered by changes in the pH of the eluent mixture. Increased retention at pH 7 relative to pH 4 was correlated with the presence of a histidine residue in a hydrophobic environment. An experimental parameter, α_{pH}, was defined as the positive quotient of capacity factors at pH 4 and pH 7 for a given eluent. These α_{pH} values are interpreted as reflecting changes in peptide hydrophobicity introduced by variations in solvent and pH. Identical α_{pH} values were obtained for homologous peptides, particularly histidine containing peptides. This approach to selectivity effects yielded diagnostic conditions for the analysis of bombesin, a peptide touted as a potential marker for human small-cell lung carcinoma.

INTRODUCTION

The combined use of reversed-phase HPLC and radioimmunoassay (RIA) has evolved as a powerful tool for the identification and quantitation

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of peptides in tissue extracts [1-3]. Problems inherent to radioimmunoassays such as low specificity or cross-reactivity with other structurally related compounds present in samples [4], are minimized by the prior separation of the peptides by reversed-phase HPLC [3]. The resolving power of this chromatographic method allows discrimination among peptide analogs based on minimal structural differences [4]. However, despite the impressive results obtained by the joint application of these techniques to the analysis of peptides from tissue samples the peptides are commonly defined as having "immuno-like reactivity" only because structural homology is defined by antigenic sites. Therefore, we investigated ways of enhancing and exploiting specific chemical characteristics of peptides and their interaction with the bonded phase during HPLC.

Our approach was based on the notion that a predominant factor in the separation of peptides on reversed-phase HPLC is the extent and magnitude of hydrophobic interactions between the bonded-phase material and the peptide molecule [5]. Quantitative expressions have been developed to establish this correlation [6-11]. Based on these assumptions, if one could modify the intrinsic hydrophobicity of a peptide in a predictable fashion, this altered hydrophobicity might be anticipated on theoretical and experimental bases to be reflected in the reversed-phase HPLC behavior of a peptide. This modified behavior could then be compared with the "immuno-like reactivity" found in tissue samples, providing an experimental parameter directly correlated to the parent peptide. The variables chosen for this study were pH, with emphasis on ionic changes induced in the imidazole ring of

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histidine residues, and the organic solvent components of the mobile phase. The peptides selected for the initial studies belong to the bombesin family and selected tachykinins (physalaemin-related peptides) [12]. This choice was based on the increased attention given to the possible application of bombesin and physalaemin as a potential markers for human lung small-cell carcinoma [13-15], and the presence of immunoreactivities to these peptides in mammalian tissues [16].

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MATERIALS AND METHODS

The peptides physalaemin, physalaemin, kassinin, eledoisin, litorin, and ranatensin were obtained from Penisula Laboratories, San Carlos, CA; bombesin was purchased from Bachem Inc., Torrance, CA. Peptide solutions for HPLC were prepared in 30% methanol/water at a concentration of 1 mg/ml. From these stock solutions, the peptide was diluted in the isocratic solvent and injections of 6-10 µg of peptide material were made. The peptide solutions were made fresh at weekly intervals.

The buffer solutions used for HPLC elution were: a) 15 mM ammonium acetate (ca. 1g/liter) brought to pH 4 with glacial acetic acid; and b) 10 mM tris-(hydroxymethyl)aminomethane (Tris-base) buffered to pH 7 with concentrated phosphoric acid. Methanol (50% v/v) and acetonitrile (30% v/v) were used as organic modifiers. The HPLC eluent consisted of a premixed solution of buffer and organic solvent; i.e., a single pump isocratic elution.

The instrumentation used consisted of a M6000A pump, 440 UV absorbance detector (280 nm), U6K injector, 720 system controller, and a

730 data module, all from Waters Associates. The column used was a Whatman Partisil-5 ODS (4.6 mm ID x 25 cm) equipped with a Brownlee 5 micron Spherisorb RP-18 precolumn. The flow rate was 1 ml/min. The eluted peaks of bombesin and physalaemin were further identified by RIA analyses described elsewhere [14,17] to ensure that the UV absorbance trace indeed correlated with the peptide in question.

RESULTS AND DISCUSSION

The rationale behind this study was the premise that hydrophobic binding interactions play a major role in the separation of peptides by reversed-phase HPLC [5]. Supporting evidence for this argument is found in work coming from different laboratories [6-11]. This hydrophobic effect has been quantitatively expressed and determined to be an additive property reflecting the cumulative hydrophobicity of the amino acid residues present in the peptide. A correlation was found between the sum of hydrophobic constant values and the elution order of peptides which was qualified as having a predictive value for peptides ranging from 5-20 residues [6-11].

Another important aspect, not as fully explored, is the accesibility and extent of the peptide surface available for hydrophobic binding [20]. It was this particular feature, namely the modification of the accessible or effective surface on the peptide, that we felt could be specifically exploited in the case of bombesin. By operating at acid (pH 4) and neutral (pH 7) conditions the ionic character of carboxyl and imidazole groups would be effected: lysine and arginine residues would remain unchanged at both pH valves; the carboxyl group

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would be ionized at pH 7 and the negatively charged residues would show little affinity for the HPLC column, whereas the imidazole group (histidine) would be neutralized at pH 7 (pKa 5.5-7) with an anticipated higher retention (Fig. 1).

In the case of bombesin, the histidine residue at position 12 may be manipulated to produce a disrupted peptide surface by protonation (pH 4) or an extended hydrophobic area by neutralization (pH 7). In order to establish that changes in elution were correlated to the presence of histidine residues, a series of histidine (H) and non-histidine (NH) containing peptides was examined (Table 1). The solvent strength was maintained constant so as to isolate the effect of pH. Isocratic conditions also prevented fluctuations in pH due to varying amounts of organic solvent in the mobile phase [21].

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The first set of experiments using methanol as organic modifier is illustrated in Fig. 2. Litorin and bombesin (H-peptides) and physalaemin (NH), were analyzed isocratically (50% methanol) at pH 4 and pH 7. It is evident from Fig. 2 that, in accordance with the expectations, bombesin experienced a dramatic shift in retention. Results for all peptides examined are shown in Table 2. The Δk' is the difference in k' values at pH 7 and pH 4. For the NH-peptides, physalaemin and eledosin showed no k' variation while for kassinin there is a small, but significant decrease in k' at pH 7. For the H-peptides a more consistent pattern developed: litorin and ranatensin experienced substantial increases in k' at pH 7, and for bombesin this effect was magnified with a dramatic shift in k' (Δk' = +30.7).

In order to establish the role of organic solvent on this pH induced selectivity effect, we also examined acetonitrile as a

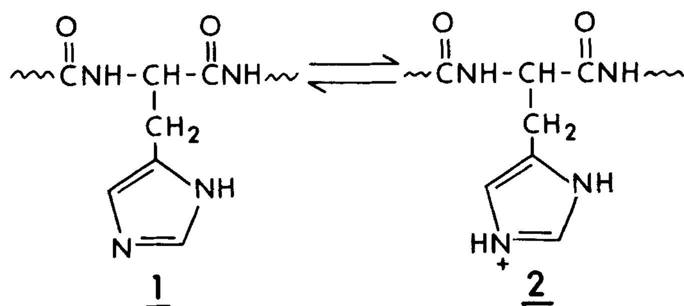

Figure 1. Ionic equilibrium for a histidine residue. The unionized imidazole (1) is anticipated to bind more effectively to a RP-HPLC column.

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TABLE 1. Peptides Used In This Study

Peptide	Sequence
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Glu-Phe-Val-Gly-Leu-Met-NH_2
Litorin	pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Ranatensin	pGlu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

modifier. The results obtained with acetonitrile (30% v/v) are illustrated in Fig. 3. From the peptides examined only bombesin showed a significant increase in k' as shown in Table 3. The NH-peptides showed a modest decline in k' at pH 7. The H-peptides, litorin and ranatensin were unaffected by pH whereas bombesin showed a substantial increase in k' at pH 7.

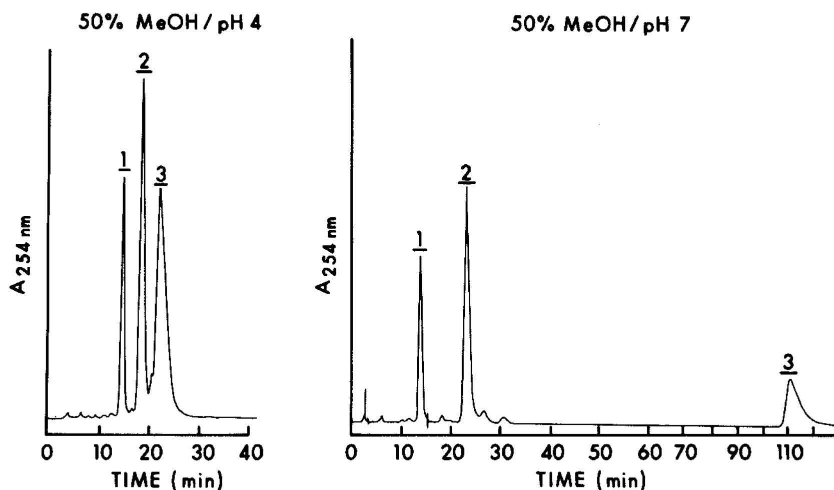

Figure 2. RP-HPLC of Physalaemin (1), Litorin (2) and Bombesin (3) using 50% methanol in 0.015 M ammonium acetate, pH 4 (left panel) and in 0.01 M Tris-phosphate, pH 7 (right panel); 2 and 3 are histidine-containing peptides.

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Table 2. Reversed-phase HPLC capacity factors (k') for bombesin and related peptides. Isocratic elution with 50% (v/v) methanol.

Peptides	pH 4	pH 7	Δk' at pH 7 relative to pH 4
Non-histidine (NH)			
Physalaemin	3.6 (0.22)[a]	3.4 (0.23)	-0.2
Eledoisin	0.2 (0.21)	2.4 (0.22)	+0.5
Kassinin	3.6 (0.21)	2.4 (0.22)	-1.2
Histidine (H)			
Litorin	4.6 (0.23)	6.6 (0.22)	+2.0
Ranatensin	15.1 (0.40)	21.1 (0.34)	+6.0
Bombesin	5.1 (0.15)	35.8 (0.26)	+30.7

[a]Mean ± S.D. in parenthesis; n=6 except for bombesin where n=3.

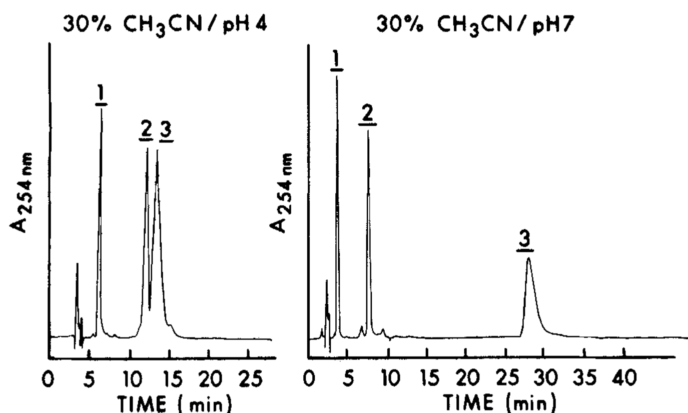

Figure 3. RP-HPLC of Physalaemin (1), Litorin (2) and Bombesin (3) using 30% acetonitrile in 0.015 M ammonium acetate, pH 4 (left panel) and in 0.01 M Tris-phosphate, pH 7 (right panel); 2 and 3 are histidine-containing peptides.

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Table 3. Reversed-phase HPLC capacity factors (k') for bombesin and related peptides. Isocratic elution with 30% (v/v) acetonitrile.

Peptides	pH 4	pH 7	Δk' at pH 7 relative to pH 4
Non-histidine (NH)			
Physalaemin	0.9 (0.11)[a]	0.7 (0.06)	-0.2
Eledoisin	1.8 (0.04)	1.1 (0.06)	-0.7
Kassinin	0.6 (0.00)	0.4 (0.04)	-0.2
Histidine (H)			
Litorin	2.4 (0.08)	2.4 (0.06)	0
Ranatensin	5.1 (0.06)	4.9 (0.06)	-0.2
Bombesin	3.1 (0.04)	11.0 (0.00)	+7.9

[a]Mean ± S.D. in parenthesis; n=3.

The results obtained are in agreement with the basic postulate that protonation of histidine residues results in disruption of the hydrophobic surface of the peptide with a concomitant effect on k' values. Litorin, ranatensin, and bombesin illustrate this effect (Tables 2 and 3). A complementary solvent effect was uncovered with litorin and ranatensin in which no k' variation occurred in the presence of acetonitrile (Table 3). The magnitude of the shift observed for bombesin is larger than that anticipated from only the hydrophobic residues involved. The amino acid residues adjacent to the histidine group in litorin and bombesin are illustrated in Table 4. The extended surface generated at pH 7 would have a Phe in litorin versus a Leu in bombesin. Although Phe is more hydrophobic than Leu, this is apparently not reflected in a proportional increase in k' values. This observation, plus the lower magnitude of the effect of acetonitrile on bombesin relative to methanol, is suggestive that other factors are operating in bombesin. A conformational effect is an attractive possibility, hydrogen bonding between Arg^{3} and His^{12} may occur at pH 7 and the resulting folded conformer exhibits retention characteristics different from those predicted by the hydrophobic theory. An example of stable conformers of a cyclic peptide separable by HPLC has been reported [5].

The effect of pH on the retention of these peptides, particularly H-peptides, may be more easily visualized by defining a new experimental parameter, α_{pH}, as the positive quotient of k' values at pH 7 and pH 4 for a given mobile phase. These values are tabulated in Table 5. The α_{pH} parameter is interpreted as reflecting changes in hydrophobi-

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Table 4. Relative hydrophobicity of selected amino acid residues in the vicinity of histidine residue in litorin and bombesin.

Peptide	Carboxyl terminal sequence
Litorin	Gly-His-Phe-Met NH_2
Bombesin	Gly-His-Leu-Met NH_2

Relative hydrophobicity[a]: Trp > Phe > Ile ≃ Leu > Tyr

[a]References [6-11]

Table 5. Selectivity expressed as a function of pH (α_{pH}).

	α_{pH}[a]	
Peptides	50% CH_3OH	30% CH_3CN
Non-histidine (NH)		
Physalaemin	1.06	1.29
Eledoisin	1.05	1.64
Kassinin	1.50	1.50
Histidine (H)		
Litorin	1.43	1.00
Ranatensin	1.40	1.04
Bombesin	7.02	3.55

[a]Ratio of k' values at the pH 4 and pH 7.

city introduced by variations in solvent and pH. For physalaemin and eledoisin, α_{pH} values with methanol indicated low sensitivity toward changes and these small selectivity differences were of equal magnitude in both cases; this parallelism was lost with acetonitrile where

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eledoisin showed a more pronounced response, i.e., a larger α_{pH}, than physalaemin. Kassinin showed that selectivity was affected by pH (Table 2), but not by organic solvent as evidenced by the identical α_{pH} values. Litorin and ranatensin showed identical behavior under both solvent conditions; i.e. selectivity effects were greater with methanol (α_{pH} = 1.40), and negligible with acetonitrile (α_{pH} = 1.00). It should be emphasized that the magnitude of the shift is the same in both cases, a situation resembling that of the NH-peptides physalaemin and eledoisin (Table 5). Bombesin is particularly sensitive to pH variations, (large α_{pH} values) and also experiences a solvent effect evidenced by a lower α_{pH} with acetonitrile (Table 5).

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The two major factors contributing to this selectivity effect are: a) pH, which changes the ionic nature of the peptide; and b) organic solvent, which modifies both the column bonded-phase and the eluate. Considering these factors, a possible scheme emerges which may help explain the differences observed: for physalaemin and eledoisin the ionizable groups are identical (Asp, Lys) but whose position in the peptide chain differ relative to each other (Table 1), whereas litorin and ranatensin only contain a single histidine residue at the same position in the carboxyl terminal region. In each of these pairs, the ionic changes introduced are identical: when the residue undergoing this change is in a homologous sequence (litorin and ranatensin) an identical α_{pH} value is observed. On the other hand, if these ionizable groups differ in their relative sequence (physalaemin and eledosin), this lack of structural continuity is detected by a solvent effect.

For the peptides examined, increased retention in reversed-phase HPLC at pH 7 relative to pH 4 correlates well with the presence of a

histidine residue in a hydrophobic environment. By using a solvent effect, this predictive shift becomes diagnostic for bombesin which satisfies the original objective of this study. As to why other H-peptides do not exhibit this quality when acetonitrile is used might be answered by examining the comformational behavior of bombesin as a function of pH.

In summary, the experimental parameter α_{pH} represents a potentially valuable observation for the identification and structural correlation of peptides by HPLC. Peptides may be distinguished by reversed-phase HPLC on the basis of their intrinsic chemical properties due to hydrophobic and ionizable residues (k' values), their hydrophobicity altered by changing pH (acid vs neutral) and affecting retention with organic modifiers (methanol and acetonitrile) at both pH limits to give α_{pH} values. By following this protocol substantial information regarding the degree of structural homology among a group of peptides may be obtained in a non-destructive manner using only minute amounts of sample as required for RIA. Further work along these lines is in progress.

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