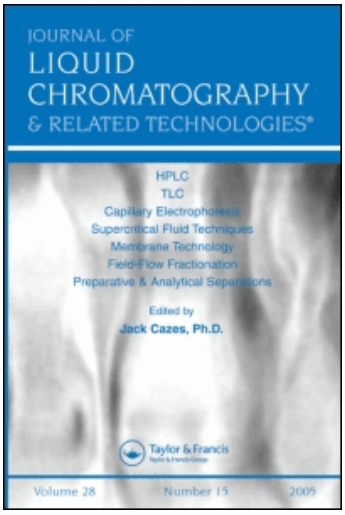

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Use of Trialkyl Ammonium Phosphate (TAAP) Buffers in Reverse Phase HPLC for High Resolution and High Recovery of Peptides and Proteins

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USE OF TRIALKYL AMMONIUM PHOSPHATE (TAAP) BUFFERS IN REVERSE PHASE HPLC FOR HIGH RESOLUTION AND HIGH RECOVERY OF PEPTIDES AND PROTEINS

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ABSTRACT

A new buffer system compatible with reverse phase high pressure liquid chromatography (RP-HPLC) column packings and giving high recoveries and high resolution for peptides and proteins is described. The TEAP buffer (triethylammonium phosphate), one of several TAAP buffers (trialkylammonium phosphate) compatible with RP-HPLC, gives high resolution and high recovery for closely related peptides or proteins as well as day to day reproducibility. It is UV transparent to less than 200 nm, extremely versatile, and could probably be used for several other classes of compounds (phosphatidyl cholines and sphingomyelin, for example). Furthermore, this system is compatible with in vitro and in vivo biological systems after removal of the organic solvent. We have applied our system to the purification of radioactive peptides, i.e. the separation and total recovery of tritiated luteinizing hormone releasing factor (LRF) from closely related radioactive impurities. Peptides and proteins as different as LRF, somatostatin, insulin and cytochrome C are easily separated and recovered. Application to peptide mapping is also described. The influence of flow rate, temperature and column packings on resolution is discussed and illustrated. Linearity between load and integrated response is demonstrated over four order of magnitude while retention time for a particular component remains constant. A flow rate of 1-1.5 ml/min and ambient or lower temperatures gives optimal resolution. Furthermore, it was found that the higher the hydrophobicities of the support was, the higher the resolution, but also possible the higher the chances for peptide or protein loss.

INTRODUCTION

The emphasis of our original report (1) on high pressure (or performance) liquid chromatography (HPLC) was to demonstrate its power in the resolution of unprotected peptides. Systems compatible with reverse phase (RP) chromatography* were developed, and peptides ranging from three (TRF) to twenty-nine (glucagon) amino acids were shown to be easily separated, quantitated and recoverable after lyophilization of the volatile buffers. This was amply illustrated by showing that numerous closely related analogs of the decapeptide luteinizing hormone releasing factor (LRF) and of the tetradecapeptide somatostatin (SS), as well as some stereoisomers, could be separated from each other or from the parent compound. Isolation of peptides of biological interest from synthetic mixtures, as well as from natural sources, became possible. Rivier et al. (5) and Rivier and Brown (6) applied such systems (ammonium acetate buffer/CH_3CN) to characterize neurotensin and bombesin analogs respectively. Ling et al. (7) isolated α- and γ-endorphins from natural sources. Rivier et al. (8) demonstrated the homogeneity of synthetic somatostatin and glucagon selective somatostatin analogs made in gram quantities by solid phase procedures.

Although at this time reports on separation of underivatized peptides are still very limited, Schecter (9) has described the separation of proteins on porous silica deactivated by carbowax. Pickart and Thaler (10) have reported on the use of HPLC on silica gel to partition growth-promoting peptides and proteins as well as histones, and Tsuji and Robertson (11) have used RP-HPLC to separate peptide antibiotics. Since then, our results using RP-HPLC with hydrocarbonaceous bonded phases were indepen-

* See Horwath and Melander (2) among others for the theoretical treatment of RP-HPLC, Cox (3) for the practical aspects of bonded phase chromatography, and Knox (4) for the practical aspects of LC theory.

dently confirmed by Hancock et al. (12) and shown very versatile by Hansen et al. (13), Krummen and Frise (14) and Molnar and Horvath (15). More recently, Hancock et al. (16) reported on the use of dilute phosphoric acid in combination with CH_3CN for improved resolution when dealing with small peptides, whereas we used those systems for the development of a racemisation test (17, 18).

During the course of these studies we became aware of poor recoveries, especially when dealing with polypeptides (β-endorphins) and proteins. Conditions in Fig. 1 for example, are those that were used to separate human β-endorphin (the sequence of which is H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Gly-Glu-OH), from ovine β-endorphin (identical to [His^{87}, Gln^{91}]-human β-LPH (61-91))and from porcine β-endorphin (identical to [Val^{83}, His^{87}, Gln^{91}]-human β-LPH (61-91)). It should be noted that the only difference between β_o-endorphin and β_p-endorphin is the presence of one extra methyl group at position 83 of the original β-LPH sequence or the substitution of one Ile residue for Val. The conditions used for that separation were, however, found to be detrimental to the column, which finally collapsed because of the postulated slow dissolution of the support.

We were thus faced with having to condemn columns after about ten runs or finding new systems that would be more compatible with the column supports. The loss of the peptides seemed to be essentially due to irreversible binding to the column through high non-specific hydrophobic interactions and the effect of high temperatures; and basic conditions leading to the slow dissolution of the column pointed the way to the obvious use of detergents (or ion pairs), as originally suggested by G. Hawk (from Waters Associates) for full recovery. We had shown that the presence of sodium dodecyl sulfate (SDS) in our buffer dramatically increased recoveries, but were reluctant to systematically use it even for analytical purposes since recovery of samples of

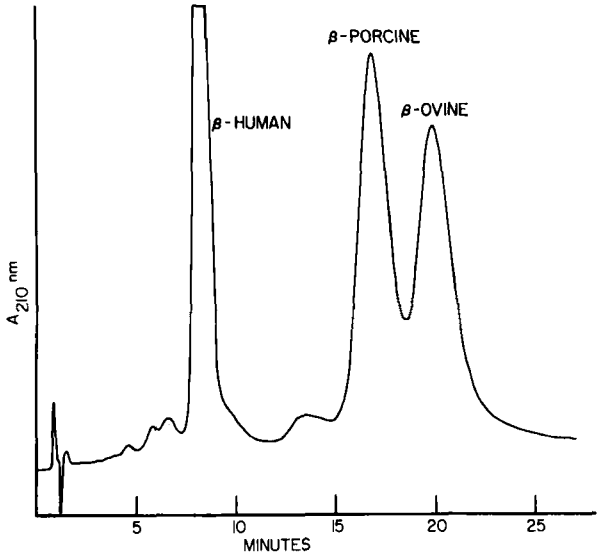

Figure 1: One .4 X 30 cm μCyanopropyl column. Back pressure = 1800 psi. Flow rate - 2 ml/min. Isocratic conditions were 32% CH_3CN, 68% buffer. Buffer was 0.05 M in H_3PO_4, pH was adjusted to 7.50 with NaOH. Load was ca. 45 μg each. 0.1 AUFS

biological interest required further purification steps which made it impractical.

By using sodium and ammonium phosphate buffers (17, 18), which were non-absorbing in the UV range where the absorption of a peptide bond is maximal (i.e. 190nm) (19,20), we found that when running gradients we could keep flat baselines by avoiding the absorption of the carbonyl group of the acetate or formate counter ions originally used. We were thus determined to use 200-210nm as the wavelength of observation so that we could keep high sensitivity and reliable response for any peptide bond containing components of our test mixtures.

We thought the ideal system should give high resolution and high recovery for closely related peptides or proteins; the ideal system would also be sensitive (non-abosrbant in the UV); would give reproducible results from day to day; be versatile, so that it could possibly be used for the separation of other classes of compounds; be compatible with the support and the pumps (thus be non-corrosive, or not too viscous) and, last but not least, be compatible after the elimination of the organic solvent with *in vitro* and *in vivo* biological systems, so that simple procedures such as lyophylization, gel filtration or dialysis could be avoided in the screening of columns for biological activities.

Once we had narrowed down the properties an ideal system should have, we realized that only a few parameters could be studied systematically. These were the choice of the column, the choice of the buffer composition and pH, the choice of an appropriate temperature and the choice of an appropriate flow rate.

While searching for a better system, we had observed under conditions of reverse phase chromatography with a combination of CH_3CN and an acetate buffer that: a) retention times for a particular molecule (the decapeptide LRF, for example) on a μCN column were smaller than those on a μC_{18} column under the same conditions, or, in other words, elution of a particular peptide at a fixed retention time required a higher concentration of the organic component of the buffer system in the case of the μC_{18} versus the μCN column; b) higher resolution was obtained if the ionic strength of the buffer was increased or if the pH was lowered from 7 to 2; c) higher temperatures were giving smaller retention time and lower resolution, which is the opposite of what is generally observed for ion exchange or adsorption chromatographies; and d)flow rates of around 1.5 ml/min were found adequate when an analytical column (4mm diameter) was used.

This paper describes a trialkylammonium phosphate (TAAP) buffer which (as shown below) is fully compatible with either pumps, RP columns or standard *in vivo* and *in vitro* tests, allows

for high recovery of peptides and proteins as large as Myelin basic protein (MBP), is highly resolutive and is UV transparent down to 200nm or below, thus allowing for high sensitivity. Influence of physical parameters such as the choice of the reverse phase support, temperature, flow rate, pH and composition of the buffer, as well as the uniqueness and some of the limitations of the system, are described.

MATERIAL AND METHODS

Apparatus

The apparatus consisted of Waters Associates Models: 204 Liquid Chromatograph, UK6 Injector, two 6000A Pumps, 660 Programmer, Infotronics Model 110 Integrator, Schoeffel Model 770 Multiwave Length Detector and Linear Instruments Corp. Model 445 Chart Recorder. The following analytical columns of the Bondapak series were used in these studies: μCN #51928, μalkyl phenyl # 54413 and μC_{18} #26332. Full scale UV absorbance expressed as AUFS.

Composition of the TEAP Buffer

The TEAP buffer was obtained by bringing the pH of .25N phosphoric acid to 3-3.5 with triethylamine. Both H_3PO_4 and Et_3N should be free of UV absorbing materials, and once made should be: a) Millipore filtered to eliminate any solid particles that might plug the columns, b) degassed to avoid the possibility of bubble formation during decompression of the solvent before the detection system, and c) refrigerated to decrease the possibility of bacterial contamination.

When running gradients of CH_3CN, the A buffer was pure TEAP buffer, pH 3.0, while the B buffer generally had a composition of 40% TEAP buffer and 60% CH_3CN. We arrived at this ratio because most peptides can be eluted from the μCN columns by using gradients from 12-48% CH_3CN. This corresponds to 20-80% B which is at a flow rate of 1.5ml/min, the range in which both pumps will deliver at one time or another a minimum of .3ml/min. This particular

composition of B was found to be much less detrimental to the B pump than 100% CH_3CN, which has a tendency to dry the O ring around the sapphire piston, leading to squeaking of the pump and leakage.

Conditions

All experiments were run at room temperature unless indicated otherwise. The columns and conditions used in each experiment are described in the legends of each table or figure. Solvents were glass distilled (Burdick and Jackson), had low absorbance in the range of dilution; aqueous buffers were filtered through 0.5 x μ Millipore filter. Distilled water came from our institution's distilled deionized water supply. All solvents were thoroughly degassed prior to use, a procedure that had to be repeated every hour or two when high temperature (60°C) was used. High temperature was controlled within 1°C by immersing the column (s) in a thermoregulated water bath. Chromatography with programmed concentration gradients was generally used to determine proper solvent systems for individual compounds but isocratic solvent mixtures gave larger separation factors with closely related compounds.

Peptides used in these studies were synthesized by solid phase and purified by conventional methods (5,6,8).

[^{3}H]-LRF was purchased from NEN; cytochrome C Type III was bought from Sigma, No. C-2506. Bovine endorphin was synthesized in collaboration with Dr. N. Ling; crystalline porcine insulin was obtained from the Lilly Laboratories (Lot No. 615-D63-10). Ovine myelin basic protein was isolated in our laboratory by Dr. J. Villarreal, who performed the tryptic digest. L-α-Lysophosphatidyl choline Type I (lot 27C-8075) was bought from Sigma.

RESULTS AND DISCUSSION

Studies on Recovery

It is evident than any chromatographic system which does not allow for complete recovery of the different components of a given mixture cannot be used as a quantitative analytical tool. We were especially concerned by that particular aspect of HPLC when analy-

zing small synthetic peptides for homogeneity (5,6,8). Indeed, one could imagine that a non-representative percentage of a polypeptide mixture applied onto the column might irreversibly adsorb to the column, or simply disappear in the system (more specifically, in the injector), thus invalidating quantitation.

Since recovery of weighable material was difficult to accurately achieve and biological activities were not giving us sufficiently accurate measures of recovery for this purpose, we arrived at the conclusion that the only way to assess recovery was to follow counts per minute (cpm) emitted by a radioactive peptide with the proviso that, loaded cpm and eluted cpm, would be measured under the same strict conditions to eliminate such problems as quenching of the solvent in the case of a β emitter Technically (and it is not trivial), we also had to make sure that no material was lost in the injector. The only way to reliably do so, was to push the solution of the peptide contained in a syringe in front of a plug of pure buffer which had been introduced in the syringe prior to the peptide solution.

Figure 2 shows the elution profile (cpm x elution time) obtained when a preparation of [^{3}H]-LRF (10,000 $\pm$ 1000 cpm) was run under the condition described in the legend. A total of 11,000 $\pm$ 1000 cpm were collected in the different fractions, indicating that all the counts had been recovered. This experiment could be repeated and was reproducible not only in terms of high recovery, but also in terms of resolution. A second experiment not shown here showed 50,140 cpm recovered and a similar profile, when 50,000 cpm were injected.

It is noteworthy that this impure preparation of [^{3}H]-LRF, when run on TLC, was thought be to homogenous and co-eluted with cold LRF. Perrin, Rivier and Vale (in preparation) have shown that only the fraction which exhibited the same retention time as cold LRF had the expected ability to specifically bind to pituitary cell membranes. Thus $\sim$ 1ng [^{3}H]-LRF, corresponding to less than 10,000 cpm of a preparation presumed to have a specific radioactivity of 35 Cu/mmole is being eluted with the same retention time as 10^4 times as much cold LRF (10μg) under the same conditions. Similarily, we

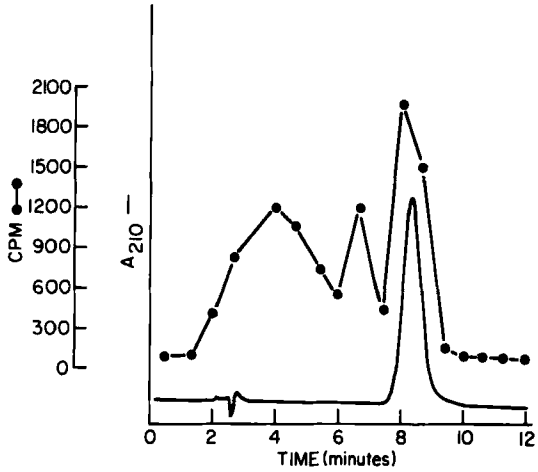

Figure 2: One .4 X 30 cm μCyanopropyl column. Back pressure = 1000 psi. Flow rate = 1.5ml/min. Isocaratic conditions were: 12% CH_3CN (40% A, 60% B: B = 20% CH_3CN), 88% TEAP buffer. Load was 4 μg LRF for ——— and 10.000 cpm [^{3}H-LRF]- for •——•——•. 0.1 AUFS

had already shown than less than 1/1000 of a dipeptide such as L-Phe-L-Leu could easily be isolated from its stereoisomer L-Phe-D-Leu.

To check whether we could get equally good recovery with larger peptides or small proteins, we loaded a mixture of LRF (MW: 1181, 5μg), somatostatin (MW: 1636, 5μg), insulin (MW: ∿5630, 5μg) and cytochrome C (MW: 12,750, 5μg) under the conditions shown in the legend of Fig. 3. Positive identification of each peak was done by amino acid analysis. Next to the names identifying the different peaks of the chromatogram is the integrated value obtained for each component. If we assume that for all peptides or proteins there is, as an average, the same number of peptide bonds per unit of weight, then by observing the UV absorption at 210nm (end absorption of the amide bond), one should have the same molar absorptivity (ε) for each component. That is, the

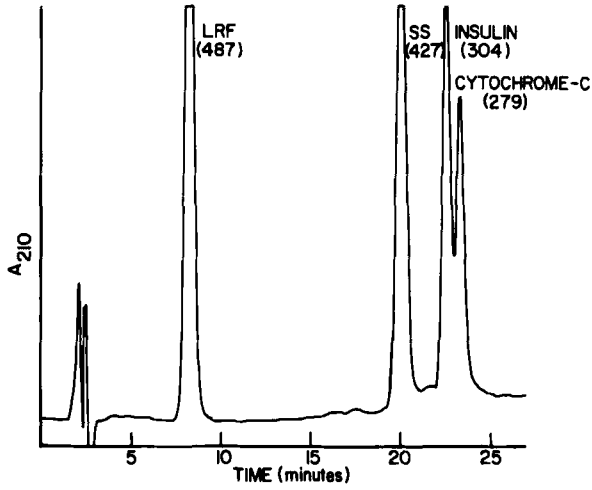

Figure 3: One .4 X 30 cm μCyanopropyl column. Back pressure = 1000 psi. Flow rate = 1.5ml/min. Gradient 12% CH_3CN to 33% CH_3CN (20% B to 55% B: B = 60% CH_3CN) in 30 min was started at time 0. Load was 5 μg each LRF, somatostatin, insulin and cytochrome C. 0.1 AUFS

same integrated value will be obtained per unit of weight of peptide or proteins whatever their molecular weight.

In this particular case we were surprised to find that the integrated peaks for LRF and SS were not only identical but also 30% larger than those of insulin and cytochrome C. Before jumping to conclusions, let us first comment on the postulated total recovery of SS. The fact that Spiess and Rivier (21) have shown good recovery of immunoactivity for somatostatin during their program for isolation of pancreatic small somatostatin from the pigeon and, that SS (5μg) gives the same integrated value as the same amount of LRF are two strong pieces of evidence in the demonstration of high (if not total) recovery of SS and probably of similar (in size and charge) peptides, when using the appropriate columns and the TEAP buffer system.

The data presented in Fig. 4 are consistent with those reported in Fig. 3 in that there is an apparent lower recovery of cytochrome C. It is noteworthy that both with SS and cytochrome C (Fig. 4), a linear relationship between load and response is observed. The most likely explanation for the apparent lower recovery for cytochrome C and insulin (see Fig.3)

is a smaller ε value at 210nm for both proteins. It could also be explained on the basis of a lower peptide content/unit of weight, i.e. the samples would be contaminated with a certain amount of UV transparent material. Both explanations are now being investigated but, in the end, the only convincing evidence for full recovery of larger peptides will come from studies similar to those we described earlier with radioactively labelled materials.

Studies on Resolution

Resolution, or the ability of a particular system to separate closely related molecules is dependent upon several factors, only a few of which we will comment upon from the practitioner's point of view. The theoretical treatment of resolution has been recently

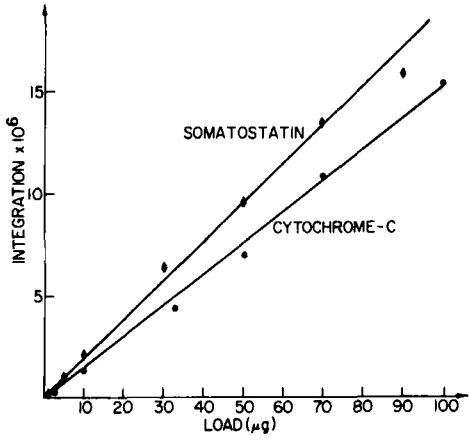

Figure 4: Load-response curves for somatostatin and cytochrome C.

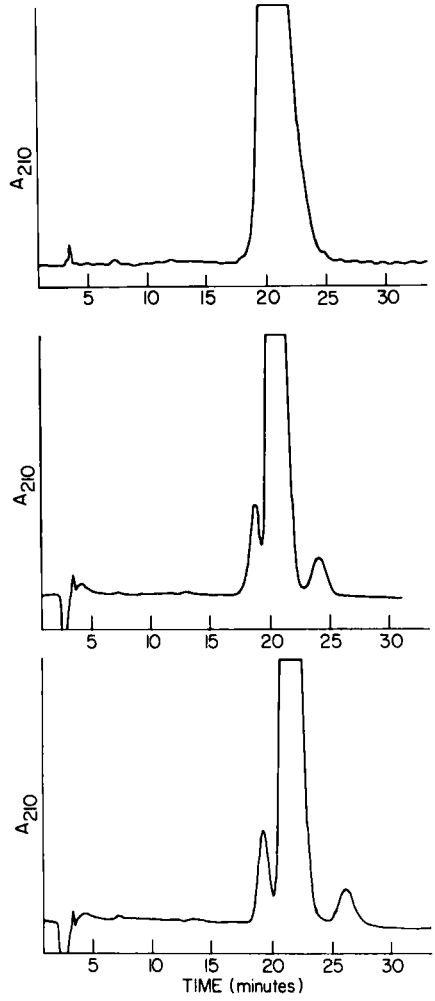

Figure 5: Top trace: One .4 X 30 cm μCyanopropyl column. Back pressure = 1000 psi. Flow rate = 1.5ml/min. Isocratic conditions were: 7.2% CH_3CN (88% A, 12% B: B = 60% CH_3CN) 92.8% TEAP buffer. Load was 50 μg LRF, #14-136-06.

summarized by J. Knox (4) who tackled the practical aspects of LC theory and commented upon such things as : column preparation and testing, optimization of column length and particle size; size of the column bore; sample dilution and sample volume, and dimensions of connectors.

Since we are using a standard HPLC system (see experimental section) and commercially available columns, we will address ourselves to other experimental parameters such as the influence of the reverse phase packing material of the columns, flow rate, temperature and pH of the buffer using our TEAP buffer and a standard synthetic peptide mixture.

Role of Column Support

Fig. 5 demonstrates the critical role of the packing material for resolution of different components of a standard peptide mixture. All parameters such as flow rate, temperature, amount loaded and detection system, were kept constant. Different concentrations of the organic buffer had, however, to be used so that identical retention times would be obtained for the major component of the mixture.

Figure 5 (continued)

Middle trace: One .4 X 30 cm μalkylphenyl column, Back Pressure = 1300 psi. Flow rate = 1.5ml/min. Isocratic conditions were: 15% CH_3CN (75% A, 25% B, B: 60% CH_3CN) 85% TEAP buffer. Load was 50 μg LRF #14-136-06. Bottom trace: One .4 X 30 cm Bondapak C_{18} column. Back pressure = 1400 psi. Flow rate = 1.5ml/min. Isocratic conditions were: 15.6% CH_3CN (74% A, 26% B, B = 60% CH_3CN) 84.4 TEAP buffer. Load was 50 μg LRF #14-136-06. 0.1 AUFS

It is noteworthy that the best resolution was obtained on the most hydrophobic columns as judged by the higher concentration of organic solvent needed to elute the peptide. Even though we have not studied recoveries in that system (i.e. alkylphenyl or μC_{18} columns), it does not seem to be a problem for small peptides.

Role of Flow Rate

Fig. 6 illustrates the role of different flow rates on resolution while all other parameters are kept constant. Table 1 shows that interestingly enough, elution.volume is very much constant. It is apparent that the slower the flow rate, the more easily recoverable would be the different components of a given mixture.

Role of Temperature

Fig. 7 illustrates the influence of temperature upon resolution and retention time under otherwise constant parameters. Note that

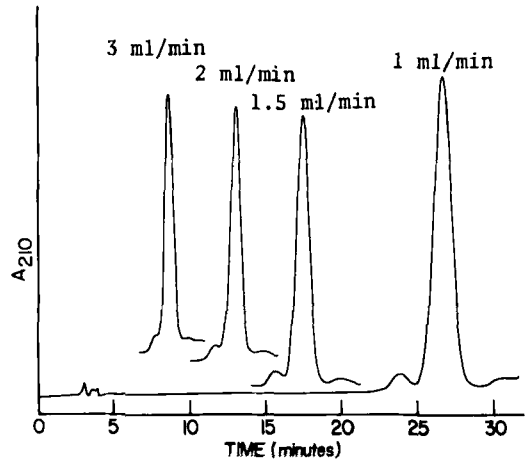

Figure 6: One .4 X 30 cm μalkylphenyl column. Back pressure = 300,700, 900 and 1400 psi going from flow rates 1 to 1.5, 2.0 and 3.0ml/min respectively. Isocratic conditions were: 15% CH_3CN (75% A, 25% B: B = 60% CH_3CN) 85% TEAP buffer. Load was 5 μg LRF #16-136-06. 0.1 AUFS

TABLE I. Influence of flow rate on retention times of LRF

FLOW RATE (mls/min^{-1})	RETENTION TIME (min)	ELUTION VOLUME (mls)
1.0	26.6	26.6
1.5	17.3	25.95
2.0	12.9	25.80
3.0	8.5	25.50

whereas we used the μalkylphenyl column for the study on flow rate, studies on the influence of temperature were run using the μC_{18} column.

It is obvious that the higher the temperature, the lower the resolution. This is quite the opposite of what is generally observed for ion exchange chromatography (amino acid analyses for example) or adsorption chromatography (the resolution of PTH amino acids on a Zorbax column is, for example, essentially due to the presence of residual unsilanized groups on that column (22)). In both of those cases, temperatures of approximately 60°C greatly improve resolution.

It is interesting that even if we keep the retention volume the same at two different temperatures, resolution at the higher temperature is definitely poorer than at the lower one. We have not tried to lower the temperature below room temperature, but we would expect better resolution before encountering problems due to high viscosity of the eluent.

Role of the Buffer's Composition and pH

We have not extensively studied this parameter but have observed that for neutral or basic peptides, a better resolution was obtained at lower pH (2.5 - 3-5). This is the range in which

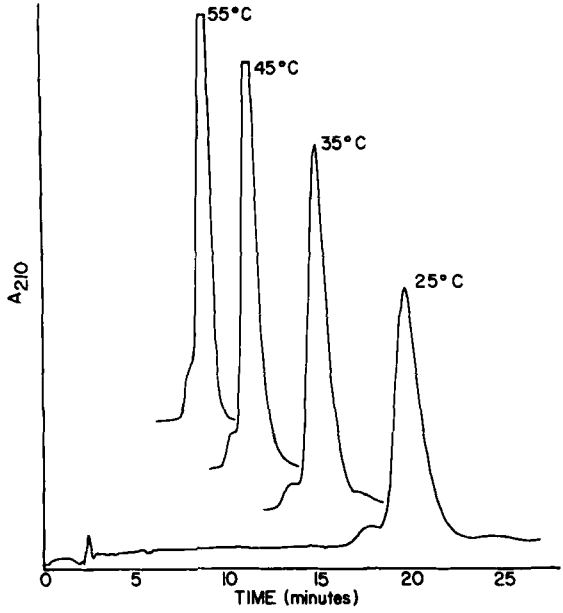

Figure 7: One .4 X 30 cm μBondapak C_{18} column. Back pressure = 2000, 1800, 1600, 1400 psi, going from temperature of 25°C to 35,45 and 55°C respectively. Flow rate was 1.5ml/min. Isocratic conditions were: 15% CH_3CN (75% A, 25% B: B = 60% CH_3CN) 85% TEAP buffer. Load was 10 μg LRF #16-146-06. 0.1 AUFS

most acidic functional groups are not dissociated and all basic groups protonated. This pH range has the advantage of being compatible with the column packing materials as exemplified by the excellent performance of columns used for more than a thousand different runs. The uniqueness of this buffer was demonstrated by using β porcine endorphin as a model peptide and, except for the conditions described in the legend of Fig. 1, no other buffer (ammonium acetate, carbonate and formate; sodium acetate or phosphate) at any pH (3-7) or concentration (.01 - .5N) were found to give good recoveries based on OD coupled with good resolution. Triethylammonium formate

(.25N, pH3) was used and shown to give good recoveries and resolution for somatostatin and β human endorphin, but was not used with β_p-endorphin; this latter system has the advantage of being lyophilizable, but can only be used when large enough quantities of material are available so that the problems due to absorption of the buffer, such as shifting of base line when running gradients, and lower sensitivity in the 200-230 nM region can be avoided.

Other bases such as methylmorphine have also been used with success, but no general studies on the influence of the hydrophobicity of the phosphoric acid counter ions were performed. From our past experience, we would expect subtle but significant differences when going from trimethylammonium phosphate to the tributyl ammonium phosphate, for example, and a particular problem might well be solved by one particular combination but not by another. Obviously, the need for a better system arising from a particular problem will be the only driving force for finding the right polypeptide or protein model, as well as the right buffer composition and pH.

Unusual Separations Made Possible With TEAP Buffer

Fig. 8 shows the elution profile generated after injection of a commercially available L-α-phosphatidyl choline preparation under the conditions described in the legend. It is noteworthy that the different components of the mixture could be clearly separated and quantitated.

Fig. 9 shows the elution profile generated after injection of the three β-endorphins shown in Fig. 1 under acidic conditions (TEAP buffer). In terms of resolution the acidic system, when run at room temperature, seems slightly more effective. It is interesting to note that β_h-endorphin, which is more acidic than the other two β-endorphins (see above for sequences), has now become more hydrophobic. This was expected since the different available side chain and C-terminal carboxyl groups are now in their unionized form. It should be pointed out that β_p- and β_o-endorphins are still eluted in the same order, indicating that

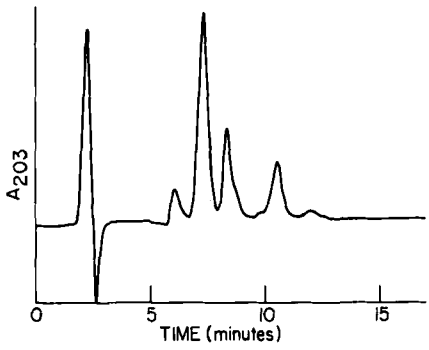

Figure 8: One .4 X 30 cm μCyanopropyl column. Back pressure = 1000 psi. Flow rate = 1.5ml/min. Isocratic conditions were: 40% CH_3CN 60% TEAP buffer. Load was 250 μg L-α-lysophosphatidyl-choline. 0.1 AUFS

whatever the pH, the ratio of the partition coefficients of those two peptides remained the same. This is also to say that if any conformational change took place as a result of lowering the pH, residue 23 of β_o- and β_p-endorphin must still remain exposed for interaction with the support. (Residue 23 of β_o-endorphin is the same as residue 83 of β_o-LPH).

Studies on Reproducibility

Day to day reproducibility of a particular separation has to be discussed in terms of observed retention times and resolution. It has been our experience that retention times will vary from day to day, whereas the general features of a particular elution profile (i.e., resolution, ratios of peaks, shape of loading "artifact") will remain constant.

Variations in retention times are essentially dependent upon two parameters: the flow rate and the composition of the buffer.

The first parameter is generally easily controllable and has given us little concern. Different composition of the buffer mixture in terms of the organic portion, however, will dramatically alter elution time or volume of a compound to the point where

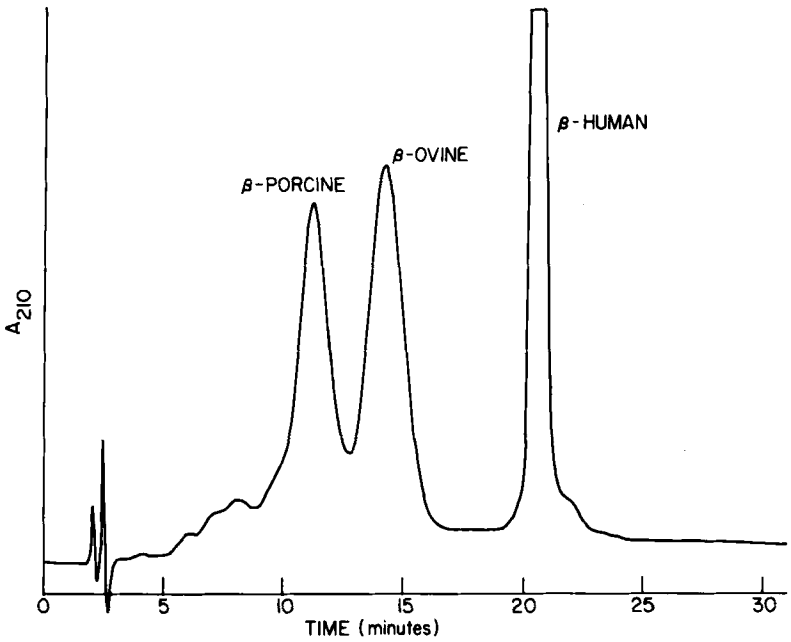

Figure 9: One .4 X 30 cm μCyanopropyl column. Back pressure = 1000 psi. Flow rate = 1.5ml/min. Isocratic conditions for 14 min were 17.4% CH_3CN (71% A, 39% B: B = 60% CH_3CN) 82.6% TEAP buffer. Isocratic conditions were followed by a 3 min gradient to 24% CH_3CN (40% B) followed by isocratic conditons at 24% CH_3CN. Load was 15 μg each of β-porcine endorphin, β-ovine endorphin and β-human endorphin. 0.1 AUFS

different degassing time from one batch to the other of the B buffer (CH_3CN-containing buffer) will alter its composition enough to allow variations in elution time as large as 1 to 2 minutes. Salt concentration of buffer A or B does not seem as critical and small variations from one batch to the next will pass unnoticed. This also holds true for slight variation of pH (± .05 to .1 pH unit) around pH 3.00. If all the above mentioned parameters are kept constant (and this is possible when one

analyzes consecutive runs), the variations in terms of retention time which are observed are fairly small and in the range of ± 0.3 min, for a material eluting at 15 to 20 min. Under these conditions, most of the peptides that we have studied (5,6,8), including closely related analogs of a natural sequence, will separate by more than one minute. It is thus tempting and in our opinion, legitimate, to use retention time under controlled conditions as one criterion of identity of two substances, whether of synthetic, or of synthetic and natural sources.

Studies on Sensitivity

The amount of material detectable using UV absorption (aside from the quality of the detector, the configuration of the flow cell, etc.) is dependent on the molar absorptivity (ε). In peptides where one predominantly observes the absorption of the amide bond, the lower the wavelength (down to 190nm), the higher the extinction coefficient (ε) (19-20). In our opinion, the most valuable property of the TEAP buffer is its UV transparence down to < 200nm. This has allowed us to routinely follow at 200nm the absorbance of 50ng of a peptide or protein. We have already shown (see Fig. 2) that even smaller amounts of peptide (∿ 1ng of H^3-LRF) will behave, in terms of elution patterns, in a manner similar to 1μg or even 100μg of the same peptide. Relating these numbers to the distribution of somatostatin-like activity (SLA) as demonstrated by radioimmunoassay and bioassay, 50ng of immunoreactive SLA was shown to be present in the hypothalamus of one pigeon or one rat, while a whole rat brain would contain 12 times as much and a pigeon pancreas almost a 1,000 times more SLA (23).

Possible Applications and Limitations

There are limitations to one's ability to vary parameters in a controlled manner unless a particular problem is to be solved. We have not, for example, studied the role of methanol versus that of acetonitrile, but are aware that it might give slightly different and possibly better results. It is obvious that a new system compatible with RP-HPLC offering good recoveries and high

resolution will find many more applications than we can presently dream of. We have used it as an analytical tool to demonstrate the optical purity (racemization test (17,18)) and chemical purity of synthetic oligopeptides and polypeptides (5,6,8). We are using this system to isolate new natural substances (21) and in one particular instance, for peptide mapping as illustrated in Fig. 10. This elution profile shows the separation of several peptide fragments generated by tryptic digestion of ovine myelin basic protein isolated in our laboratory by Villarreal et al. (24). This early experiment is certainly encouraging enough to justify further work in that area. Other

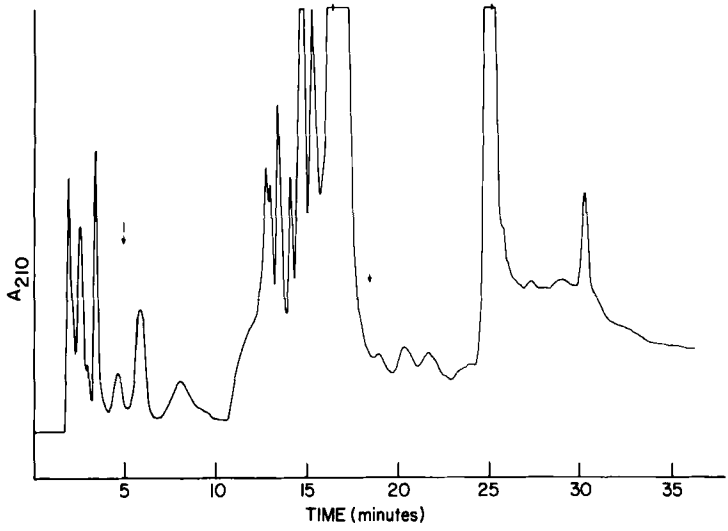

Figure 10: One .4 X 30 μBondapak C_{18} column. Back pressure = 900 psi. Flow rate = 1.5ml/min. Isocratic conditions for 5 min were 0% CH_3CN 100% TEAP buffer. It was followed by 5 min gradient to 18% CH_3CN then isocratic for 8 min at that concentration followed by 5 min gradient to 48% CH_3CN. Load was 50 μg of a tryptic digest of myelin basic protein. 0.1 AUFS

experiments in which we tried to separate all PTH or dansyl derivatives of a 20-amino acid mixture certainly do not look as promising since several overlaps could not be resolved. The use of an adaptation of our racemization test for amino acid analysis looks much more promising, however, especially since it was shown by Mitchell et al. (25) that the O-succinimide active ester of Leu (it would be Phe in our case) could be quantitatively coupled to a mixture of amino acids.

CONCLUSION

The studies here are by no means exhaustive in determining the ideal system for individual peptides, but show that a readily available buffer system which is UV transparent down to below 200nm, will allow for high resolution and quantitative recovery of proteins as large as cytochrome C. The evaluation of several parameters affecting RP-HPLC leads us to conclude that low pH, low temperature, relatively low flow rates and the use of the μCN column for larger proteins and of the μalkyl phenyl or μC_{18} columns for oligopeptides should be used for greater chances of success. The lack of availability of large proteins free of microheterogeneity which we might resolve was the real limiting parameter to the assessment of the TEAP or a TAAP buffer as a general panacea in the field of liquid chromatography of peptides and proteins.

We certainly look forward to a larger array of column supports, the availability of a preparative set-up which would allow for separation and isolation of gram quantities of pure polypeptides if not proteins, and possibly new solvent systems which could be used to chromatograph large biological substances such as glycoproteins and enzymes.

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