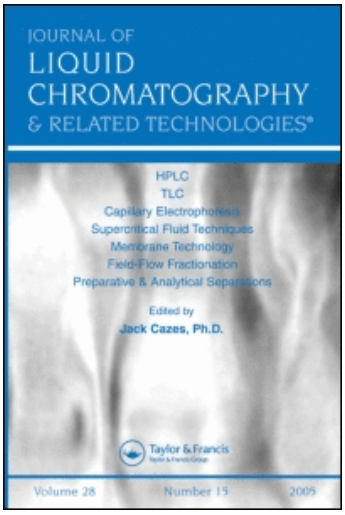

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bennett, H. P. J. , Browne, C. A. and Solomon, S.(1980) 'The Use of Perfluorinated Carboxylic Acids in the Reversed-Phase HPLC of Peptides', Journal of Liquid Chromatography & Related Technologies, 3: 9, 1353 — 1365
To link to this Article: DOI: 10.1080/01483918008062781
URL: http://dx.doi.org/10.1080/01483918008062781

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(9), 1353-1365 (1980)

THE USE OF PERFLUORINATED CARBOXYLIC ACIDS IN THE REVERSED-PHASE HPLC OF PEPTIDES

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ABSTRACT

The relative effectiveness of trifluoroacetic acid (TFA), pentafluoropropanoic acid (PFPA), heptafluorobutyric acid (HFBA) and undecafluorocaproic acid (UFCA) as hydrophobic counter-ions in the reversed-phase high performance liquid chromatography (RP-HPLC) of peptides was assessed. Four solvent systems were compared each containing 0.01M of a perfluorocarboxylic acid throughout. Twelve standard peptides and proteins were loaded onto the RP-HPLC column which was eluted with a linear gradient of 20-58.4% aqueous acetonitrile over 90 minutes. The retention times of the peptide standards were different in each solvent system. In progressing from TFA to PFPA, HFBA and UFCA all the peptides showed greater retention times. However, the effect was most marked with peptides having the greatest number of basic groups. By exploiting this behaviour a different type of chromatography can be introduced into the RP-HPLC purification of peptides. For instance, column fractions obtained from the use of the TFA solvent system can be re-chromatographed in a solvent system containing HFBA. It is possible by this procedure to purify naturally occurring peptides on the basis of their overall positive charges. At 0.01M each acid solution is sufficiently U.V. transparent to permit monitoring of column effluents at 210 nm. TFA, PFPA, HFBA and UFCA solvent systems are also completely volatile and this property facilitates the bioassay, radioimmunoassay and amino acid analysis of column fractions.

INTRODUCTION

The potential of reversed-phase high performance liquid chromatography (RP-HPLC) (1) in the purification of naturally occurring peptides has been demonstrated by several investigators (2,3,4). RP-HPLC has also proved extremely useful in the final purification of synthetic peptides (5). In most cases the use of RP-HPLC is confined to final purification

following more conventional gel-filtration and ion-exchange chromatography. Work in this laboratory has demonstrated that the resolving power of reversed-phase liquid chromatography is sufficiently high that this technique alone can be used to isolate pure peptides from pituitary tissue (6,7). Peptides are adsorbed from tissue homogenates onto octadecylsilyl-silica (ODS-silica) (8,9) and extracts are subsequently subjected to gradient elution RP-HPLC. In general pure peptides may be obtained following a further isocratic elution step. In these studies trifluoroacetic acid (TFA) was used as a hydrophobic acidic counter-ion in the HPLC solvent system. Since only one type of chromatography was used in these peptide isolations, one cannot be completely certain about the purity obtained. The purpose of this paper is to compare the the properties of the perfluorinated carboxylic acids, trifluoroacetic acid, pentafluoropropenoic acid (PFPA), heptafluorobutyric acid (HFBA) and undecafluorocaproic acid (UFCA) as counter-ions. The effects of these different acids on the retention times of various peptide standards and proteins is illustrated. Their potential as a tool in the preparation of synthetic and naturally occurring peptides with a high degree of purity will be discussed

MATERIALS AND METHODS

Trifluoroacetic acid (TFA laboratory reagent grade) was obtained from BDH Chemicals Ltd., Montreal, Canada. Pentafluoropropanoic acid (purum grade) and heptafluorobutyric acid (purissimum grade) were purchased from Tridom Chemical Inc., Hauppauge, N.Y., U.S.A. Undecafluorocaproic acid (experimental grade) was kindly donated by 3M Co., St. Paul, Mn, U.S.A. Acetonitrile (HPLC grade) was purchased from Fisher Scientific Co., Montreal, Canada.

Many of the peptides used as chromatographic standards were generous gifts: Dr. W. Rittel, Ciba-Geigy Ltd., Basel, Switzerland provided the synthetic α-MSH and C-41795-Ba (1-18 ACTH analogue), Synacthen, human ACTH and human calcitonin, synthetic Met- and Leu-enkephalin and somatostatin were from Dr. R. Degenghi, Ayerst Laboratories, Montreal, Canada, Dr. E. L. Grinnan, Eli Lilly Co., Indianapolis Ind., U.S.A. provided the natural bovine insulin, synthetic human β-endorphin was from Dr. N. Ling and Dr. R. Guillemin of the Salk Institute, La Jolla, Ca., U.S.A.; Synthetic LH-RH was purchased from Bachem Fine Chemicals, Marina Del Rey, Ca., U.S.A.

A Waters HPLC system was used which consisted of two 6000A pumps, a 660 solvent programmer, U6K injector and a C18 μBondapak reversed-phase column (Waters Scientific Ltd., Mississauga, Canada). Column eluates were continously monitored for absorbance at 210 nm using an LC75 flow-through continous wavelength spectrophotometer from Perkin-Elmer Ltd., Montreal, Canada.

HPLC grade water was prepared as described previously (7): glass distilled deionized water was passed slowly under gravity over a small bed of ODS-silica (the contents of a C18 Sep-Pak (Waters Associates) packed within a glass syringe) and stored in glass at 4° until used. Prior to the preparation of HPLC solvents, water was filtered using a Millipore all-glass filter apparatus and 0.5 μm Fluoropore Filters (Millipore Corporation, Bedford, Ma, U.S.A.). These Teflon filters function efficiently provided that they are wetted with acetonitrile before use. Stock solution (0.1M) of TFA, PFPA, HFBA and UFCA were prepared using filtered water and each was passed through a Sep-Pak cartridge. Immediately before solvent preparation water was degassed for 20 minutes and acetonitrile for 1 to 2 minutes. The 0.1M stock acid solu-

tions were diluted such that HPLC solvents consisted of 0.01M aqueous acid (solvent A) and 0.01M aqueous acid in 80% aqueous acetonitrile (solvent B). Linear gradients of increasing acetonitrile were formed from mixtures of solvents A and B controlled by the solvent programmer.

A standard mixture of peptides and proteins was prepared in 0.01M TFA. For each chromatographic run 50 μl containing 5 μg of each peptide and protein was injected onto the column via the U6K injector. This was followed by 50 μl of 0.01M TFA to ensure that the standards were not trapped within the injector system. The HPLC column was eluted in each case with a linear gradient of 20 to 58.4% acetonitrile containing either 0.01M TFA, PFPA, HFBA or UFCA over 90 minutes at a flow rate of 1.5 ml per minute. The column was eluted at room temperature (21°C). Individual peaks were identified by injecting each peptide and protein alone and observing the retention time.

RESULTS AND DISCUSSION

A characteristic of peptides is that they have hydrophobic (e.g. Trp, Phe, Tyr) as well as hydrophilic (e.g. Asp, Glu, Arg, Lys) amino acids. However, with few exceptions they are generally considered to be highly polar molecules, and are extremely water soluble. This property would suggest that they are poor candidates for efficient RP-HPLC. This is in contrast to steroids for which successful RP-HPLC systems have been established using simple aqueous solvent mixtures for column elution. A solvent system such as aqueous methanol or acetonitrile gives rise to very poor chromatography of peptides. Reversed-phase columns are composed of uniform silica particles to which a hydrophobic surface (e.g.

C18) has been covalently linked via the silanol groups in a process similar to the siliconization of glassware (10). Despite secondary derivitization or "end-capping" procedures used by manufacturers to ensure maximal coverage, sufficient silanol groups remain to provide a surface to which peptides may adsorb. In a highly polar state peptides interact inefficiently with the reversed-phase column support and adsorb strongly to free silanol groups. This results in very long retention times and broad peaks (5). This situation can be largely rectified by adding an ionic component, frequently a strong acid, to the solvent system. They have the dual function of "salting-out" the silanol groups and, by lowering the pH, tend to protonate aspartate and glutamate residues. The polarity of peptides at low pH is therefore markedly reduced and because their adsorption to bare silica is inhibited they interact more efficiently with the reversed-phase support. This gives rise to acceptable chromatography with unique selectively based on the relative hydrophobic character of peptides. It is noteworthy that column efficiency for peptides in terms of theoretical plates is very low compared with small non-polar molecules. It is the novel selectivity which makes this chromatographic method so useful. A strongly basic ionic modifier would produce interesting chromatographic results but is not feasible because alkaline solutions tend to dissolve silica and destroy HPLC columns.

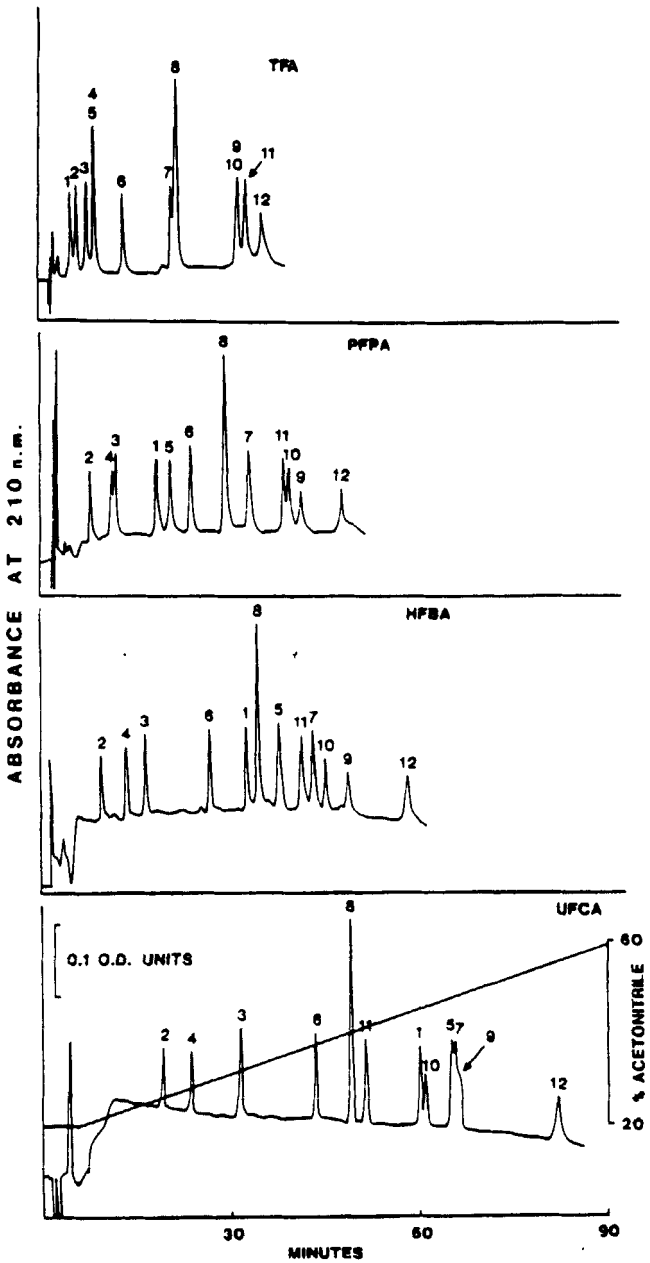
The concept of "ion-pairing" in RP-HPLC is very useful in both developing and understanding the mechanism of action of solvent systems modified with acids and salts (11). Many peptide RP-HPLC solvent systems have a pH of three or below. Under these conditions not only do aspartate and glutamate residues in peptides tend to be protonated but

also lysine, arginine and histidine residues are fully charged. It has been suggested that anions associate or "ion-pair" with these basic amino acids. The resulting effects on chromatographic behaviour are dependent on the nature of the anion. Numerous ionic solvent modifiers have been suggested including trifluoroacetic acid (6,7,8,12), phosphoric acid (5), perchloric acid (3), sodium dihydrogen phosphate acidified with phosphoric acid (4), pyridinium acetate or formate (3), triethylammonium phosphate or formate (2,15), tartrate buffer containing sodium sulphate and sodium butanesulphonate (16), sodium chloride acidified with hydrochloric acid (14) and sodium perchlorate with phosphate buffer or phosphoric acid (17). The pH of an aqueous solvent mixture is only an apparent value since organic solvents tend to suppress ionization. The apparent pH of the buffered RP-HPLC solvent systems may not be a very critical parameter provided that it is well below the pK of the peptide carboxyl groups (3.0-4.7 for Asp, approx. 4.4 for Glu and 3.0-3.2 for the C-terminal carboxyl group) (18). Indeed simple unbuffered solutions of trifluoroacetic (8), phosphoric (5) perchloric (13) and hydrochloric (14) acids give rise to reproducible RP-HPLC of peptides. For maximum inhibition of adsorption to underivatized silanol groups, a molarity of at least 0.1M is desirable (8,14) but this may not be feasible in some instances (6,7).

The hydrophobic ion-pairing reagents trifluoroacetate, acetate, formate and alkylsulphonates tend to increase the affinity of peptides for the reversed-phase support and increase retention times and resolution. The hydrophilic ions phosphate and chloride probably have little effect on retention times other than inhibiting adsorption to underivatized silica. While the hydrochloric acid/sodium chloride system is

simple and efficient it should never be used with HPLC pumps with vital stainless steel components (i.e. check valve seats) since acid halides corrode even inactivated stainless steel. The RP-HPLC solvent system used routinely in this laboratory consists of aqueous acetonitrile containing 0.1% TFA. This has the advantage of being non-corrosive, sufficiently U.V. transparent to permit monitoring of column eluates at 210 nm, and completely volatile. The property of volatility is particularly useful in the preparation of natural peptides since column fractions may simply be dried in vacuo prior to radioimmunoassay, bioassay or amino acid analysis. However, this system is a compromise since some column efficiency is lost due to the relatively weak ionic strength of 0.1% TFA solutions. Solvent systems with higher ionic strength (e.g. 1% TFA (8), 0.1M sodium dihydrogen phosphate (14), 0.1M sodium chloride (14), or 0.1M sodium perchlorate (17)) inhibit peptide adsorption to silica more efficiently. The ionic strength of 0.1% TFA solutions cannot be altered without affecting acidity, volatility or the capacity for U.V. monitoring at 210 nm.

In previous reports from this laboratory, it was demonstrated that bovine ACTH and rat α-MSH can be extracted from pituitary tissue and purified to apparent homogeneity by reversed-phase liquid chromatography (6,7). Peptides were adsorbed onto ODS-silica from tissue homogenates. These preliminary extracts were then loaded onto a RP-HPLC column and subjected to gradient elution using the 0.1% TFA aqueous acetonitrile solvent system. Peaks of immunoreactive hormone were finally purified by rechromatographing under isocratic conditions. A criticism of this approach is that only one type of chromatography is used in this isolation procedure. Also because the final step is an isocratic elution



procedure, the compound of interest is contained in a relatively large volume of solvent. This is a distinct disadvantage when isolating peptides in microgram quantities. The properties of other perfluorinated carboxylic acids have been evaluated in order to investigate the possibility that they might introduce a different chromatographic principle into the peptide hormone isolation procedure.

Perfluorinated carboxylic acids are powerful partitioning reagents and have high surface active properties (19). The three, four and six carbon acids, like TFA, are all liquids at room temperature, are strong acids and volatile. These properties make them suitable as RP-HPLC counter-ions. In order to compare the acids directly the RP-HPLC column, the acetonitrile gradient, the acid concentration and the and flow rate were kept constant. Thus changes in elution time for each of the test peptides could be attributed solely to efficacy of TFA, PFPA, HFBA and UFCA as hydrophobic counter-ions. The relative elution positions of the twelve test peptides and proteins in the four systems is shown in Fig.1. As one progresses up the carboxylic acid series profound differences in retention times are observed. These changes are most apparent with the most basic peptides (at pH 2). The effect can best be appreciated by

FIGURE 1

A comparison of the efficacy of TFA, PFPA, HFBA and UFCA as hydrophobic counter-ions. (See Methods section for chromatographic conditions.) The peptides and proteins tested were as follows (the number of basic amino acid residues at pH 2 for each peptide is given in brackets - see discussion: 1. C-41795-Ba, 1-18 ACTH analogue (8), 2. Met-enkephalin (1), 3. LH-RH (1), 4. Leu-enkephalin (1), 5. Synacthen, 1-24 ACTH (9), 6. α-MSH (3), 7. Human ACTH 1-39 (9), 8. Somatostatin (3), 9. Bovine insulin (6), 10. Human β-endorphin (7), 11. Human calcitonin (3), 12. Bovine cytochrome C (24).

The UFCA chromatogram shows the linear aqueous acetonitrile gradient employed and a bar indicating 0.1 absorbance units at 210 nm. Both these parameters are the same in the TFA, PFPA and HFBA chromatograms.

studying the behaviour of the 1-18 ACTH analogue (C-41795-Ba) which has 8 positive charges. From having the shortest retention time with TFA, (C-41795-Ba) elutes progressively later with PFPA (fourth) and HFBA (fifth) until with UFCA it elutes as seventh in the series. More importantly the absolute elution position of all the peptides is changed. The degree of change is related to the number of positive charges available for ion-pairing (the number of basic amino acids at pH 2 (including histidine residues) of each peptide and protein is given in the legend to Fig. 1).

These results show that PFPA, HFBA and UFCA are highly effective hydrophobic "ion-pairing" reagents and can be used to turn a RP-HPLC column into what has been termed a "dynamic ion-exchanger" (11). It was immediately apparent that these systems could be applied to the peptide purification procedure outlined earlier. UFCA is, unfortunately, not of high enough purity for this purpose. The initial rise and subsequent gradual fall in the baseline observed in Fig. 1 is a reflection of hign U.V. absorbing UFCA contaminants eluting from the column. The same effect is true of HFBA but to a much lesser extent. The final purification of peptide hormones in this laboratory now includes a reversed-phase chromatographic step using HFBA as counter-ion. Thus by running a tissue extract first in the TFA solvent system described earlier and then re-running appropriate fractions on the same reversed-phase column using HFBA, peptides can be obtained in a concentrated and highly pure form. It is now possible to isolate and purify peptides with the minimum of manipulation using only reversed-phase liquid chromatography. This technique has been used in our laboratory to isolate α-MSH N, O-diacetyl-Ser^1 α-MSH (20), corticotropin-like intermediary lobe peptide (21) and ACTH from rat pituitaries (22) and human parathyroid hormone from para-

thyroid gland adenomas (23). The technique is so successful for ACTH that an analytical RP-HPLC column has been used to isolate 6 mg of this hormone from an ODS-silica extract of 32 grams of bovine anterior pituitaries (24).

ACKNOWLEDGEMENTS

The authors would like to thank Dr. H. Niall for helpful discussions. They are also indebted to Dr. W. Rittel, Dr. R. Degenghi, Dr. E.L. Grinnam and Dr. R. Guillemin and Dr. N. Ling for generously donating the peptides and proteins used as HPLC standards and to Dr. R. A. Guenthner of the 3M Co. for kindly providing the undecafluorocaproic acid. This work was supported by Medical Research Council of Canada grant no. MT-1658, United States Public Health Service grant no. HD04365 and National Foundation March of Dimes grant no. 1-694.

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1. Abreviations: RP-HPLC, reversed-phase high performance liquid chromatography, ODS-silica, octadecylsilyl-silica, TFA, trifluoroacetic acid, PFPA, pentafluoropropenoic acid, HFBA, heptafluorobutyric acid, UFCA, undecafluorocaproic acid, Met-endkephalin, methionine enkephalin, Leu-enkephalin, leucine-enkephalin, LH-RH, luteinising hormone-releasing hormone, α-MSH, α-melanocyte stimulating hormone, C-41795-Ba, [Dser1, Lys17,18]-corticotropin-(1-18)-octadecapeptide amide, Synacthen, corticotropin-(1-24)-tetracosapeptide, h-ACTH, human adrenocorticotropin.

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