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Short communication

Perfluorinated acid alternatives to trifluoroacetic acid for reversed-phase high-performance liquid chromatography

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Abstract

Over the past decade trifluoroacetic acid (TFA) has become the ion-pairing agent (IPA) of choice for reversed-phase high-performance liquid chromatography (RP-HPLC) of peptides and proteins. Reagent grade TFA is highly pure, water soluble, transparent at 220 nm and readily volatile. A drawback of this universal appeal is that several alternative perfluorinated carboxylic acids tend to be overlooked when TFA does not work in a particular separation. Examples are given comparing TFA selectivity with those of pentafluoropropionic acid, heptafluorobutyric acid, perfluoropentanoic acid, perfluorohexanoic acid and perfluoroheptanoic acid. We have found that increasing the IPA *n*-alkyl chain length can resolve sample components that otherwise co-elute in the void volume of TFA-based RP-HPLC. Examples are given for the resolution of an oligoglycine series and enhanced selectivity for a bovine hypothalamic extract.

Keywords: Ion-pairing reagents; Mobile phase composition; Perfluorinated acids; Peptides; Oligoglycines

1. Introduction

Trifluoroacetic acid (TFA) is the most commonly chosen ion-pairing agent (IPA) used in reversed-phase high-performance liquid chromatography (RP-HPLC) separations of peptides and proteins. This is partially due to the fact that TFA is volatile and available in a highly pure form. We have been interested in resolving small hydrophilic components from biological samples that elute in the void volume (V_0) of TFA-mediated RP-HPLC separations. We have examined the use of longer *n*-alkyl chained perfluorinated carboxylic acids as an alternative to TFA. Bennett et al. [1] demonstrated that longer perfluorinated carboxylic acid homologs increase peptide retention times compared to TFA. We have extended this observation to show that many solutes that elute in the V_0 fraction of TFA ion-pairing systems can be retained and resolved by replacing TFA with longer alkyl chained perfluorinated carboxylic acid homologs. Sometimes these non-retained solutes can be separated by ion-exchange or size-exclusion methods, but these methods introduce exogenous salts which can be difficult to remove from small biomolecules and effect bioassay data. In this report we demonstrate that the use of longer alkyl chained perfluorinated carboxylic acids may be an option to solve such separation problems. These IPAs, like TFA, are volatile. The main disadvantage of these longer perfluorocarboxylic acids is their purity as obtained from commercial sources. One exception to this is heptafluorobutyric acid (HFBA), which is available in HPLC/sequenal grade and used most often as a TFA alternative [2–7]. Due to this

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purity issue, alternative perfluorinated carboxylic acids (other than HFBA) are best employed in initial reversed-phase fractionations of solutes that would otherwise co-elute with salts and other non-retained molecules in TFA ion-pairing systems. We will illustrate the selectivity advantage of long *n*-alkyl chained perfluorinated carboxylic acids for the separation of small hydrophilic oligoglycine peptides and a complex bovine hypothalamic extract.

2. Materials and methods

2.1. High-performance liquid chromatography

HPLC was performed on a Varian Model 5500 HPLC equipped with a UV-200 UV detector and a 601 Data System (Varian Instruments, Walnut Creek, CA, USA). All separations were done using a 250× 4.6 mm Alltima C_{18} RP-HPLC column (Alltech, Deerfield, IL, USA) containing 5 μm 100 Å pore diameter silica. The chromatographic conditions were 10 m*M* of the indicated IPA as buffer A and 80% acetonitrile in 10 m*M* of the indicated IPA as buffer B. The column flow-rate was 1 ml/min. The elution conditions are described in the individual experiments. The HPLC used for obtaining hypothalamic extract was a Waters Delta-Prep equipped with a Model 481 UV detector (Waters Instruments, Milford, MA, USA) and a Waters Prep-Pak C_4 Preparative HPLC column (10 μm silica, 300 Å pores).

Ion-pairing agents: TFA and HFBA, both HPLC/sequenal grade, were obtained from Pierce (Rockford, IL, USA). Perfluoropentanoic acid (PFPN) and perfluoroheptanoic acid (PFHP) were purchased from PCR (Gainesville, FL, USA). Pentafluoropropionic acid (PFPA) was from Aldrich (Milwaukee, WI, USA). Perfluorohexanoic acid (PFHX) was obtained from Marshallton Labs. (Winston-Salem, NC, USA). The PFHX used for data in [1] (and referred to as "UFCA") was from the 3M Co. (St. Paul, MN, USA) and is no longer available (personal communication, H.P.J. Bennett). Oligoglycine standards were purchased from Sigma (St. Louis, MO, USA). HPLC grade water and acetonitrile were from EM Science (Gibbstown, NJ, USA).

2.2. Glycine oligomer standards

Glycine oligomers ($n=1$ to 6) were purchased from Sigma. Each standard was solubilized in water stock solution and diluted with either 10 m*M* TFA or 10 m*M* PFHX for HPLC injection. It should be noted that the smaller peaks for the longer glycine oligomers in Fig. 1 were due to poor solubility in water during sample preparation.

2.3. Preparation of bovine hypothalamic extracts

Bovine hypothalami were homogenized in 1% (v/v) glacial acetic acid containing 10 m*M* dithiothreitol and heated at 100°C for 30 min. The material was then lyophilized and resuspended in 1% TFA–

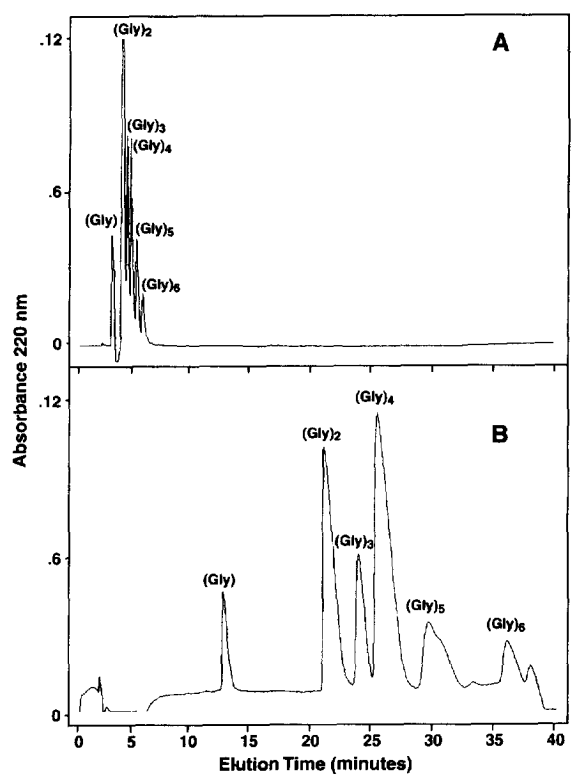

Fig. 1. Comparison of TFA and PFHX for resolution of oligoglycine peptides. 100 μl of oligoglycine peptides ($n=1\rightarrow6$) were injected onto an Alltima C_{18} RP-HPLC column at a flow-rate of 1 ml/minute. Conditions: isocratic elution at 1 ml/min using indicated ion-pairing agent on an Alltima C_{18} (250×4.6 mm, 5 μm, 100 Å) column at ambient temperature. (A) 10 m*M* TFA; (B) 10 m*M* PFHX. Detection at 220 nm.

40% methanol and centrifuged at 10 000 *g* for 30 min. The resulting supernatant was lyophilized and then resuspended in 0.1% TFA and injected onto a preparative C_4 HPLC column with a flow-rate of 50 ml/min. The V_0 peak (as monitored at 220 nm) was collected as a pool, divided into 1% aliquots, lyophilized and stored in a freezer. Aliquots were re-suspended in the various IPAs shown in Fig. 2 for RP-HPLC separations. The first IPA tested was TFA, followed in order of increasing *n*-alkyl chain lengths all on the same HPLC column. Several blank gradients with new IPA were run in order to wash out old IPA before a new extract aliquot was chromatographed. The chromatograms shown in Fig. 2A–F are representative of at least two reproducible profiles for each IPA examined.

3. Results and discussion

Bennett has previously reported, for a collection of twelve peptide and protein standards, that increasing the alkyl chain length of the perfluorocarboxylic acid IPA increases retention times during gradient elution [1]. We wished to determine whether longer *n*-alkyl chained perfluorinated carboxylic acids could also be used to resolve solutes that co-elute in the V_0 of gradient TFA gradient systems. Glycyl residues have minimal contribution to RP-HPLC retention according to empirically derived reversed-phase retention tables developed by Meek and co-workers [8,9]. Therefore, a collection of glycine oligomers was prepared as probes for V_0 selectivity. As shown in Fig. 1A, these small hydrophilic glycine oligomers elute as a V_0 cluster when applied onto a C_{18} column and eluted with 10 m*M* TFA. Baseline resolution of all glycine oligomers was achieved when the IPA was changed to 10 m*M* PFHX (Fig. 1B). This illustrates the utility of long chain perfluorinated acid IPAs for resolving solutes that elute in the V_0 of TFA systems.

Fig. 2 illustrates the selectivity differences of various alkyl chain perfluorinated carboxylic acids when used in the fractionation of bovine hypothalamic extract. When TFA was used as the IPA, peaks elute as a cluster in and near the V_0 of the column and again as a cluster between 25–35 min into the gradient (Fig. 2A). Increasing the alkyl chain length of the perfluorinated IPA can readily change selectivity as shown in Fig. 2B–F. The overall peak arrays show less V_0 elution and higher column capacity factors as a function of increasing IPA alkyl chain length. Although individual peaks were not identified in this complex hypothalamic extract, it was evident that increasing IPA alkyl chain length resulted in non-uniform increases in component elution. This differential selectivity was also noted in Bennett's work [1] using peptide hormone standards.

TFA, HFBA and, to a lesser extent PFPA, are clear liquids which are commercially available in high purity. PFPN, PFHX and PFHP are not available in ultrapure grade and the latter two are especially prone to developing a yellowish color over time. This may explain the gradient baseline drift observed in Fig. 2D–F. These drifts, including the UV spike at 49 min for PFHP (Fig. 2F) were reproducible in the blank gradients (data not shown). This is most likely due to the purity grade of the longer alkyl chained IPAs and remains a disadvantage for their use as compared to TFA or HFBA. PFHP was the only IPA tested that was not a liquid at room temperature. It was semi-solid, but warming the reagent bottle with cupped hands and transferring with a warm pipet allowed accurate delivery of liquified PFHP. The next IPA in the series, perfluorooctanoic acid, was a white solid at room temperature and not tested in this study.

All of the 10 m*M* perfluorocarboxylic acid solutions had pH values of 2.0 to 2.1, except for the longest homolog, PFHP, which had a pH of 2.35. This suggests that selectivity differences were not mediated by ionic interactions, but rather by differential IPA hydrophobic complexing. According to the literature, the homologous C_4–C_{12} alkanesulfonate IPA series are interchangeable in their surface concentrations [10]. However, a mechanistic study by Patthy [11] has shown that TFA and HFBA differ significantly in their interaction with column stationary phase. HFBA selectivity may be explained by coating the stationary C_{18} alkyl chain in a concentration dependent manner, whereas TFA forms a layer that does not vary significantly with mobile phase concentration. Thus it was proposed [11] that HFBA selectivity is concentration dependent (a dynamic ion-exchange mechanism) while TFA gives much less selectivity as a function of concentration

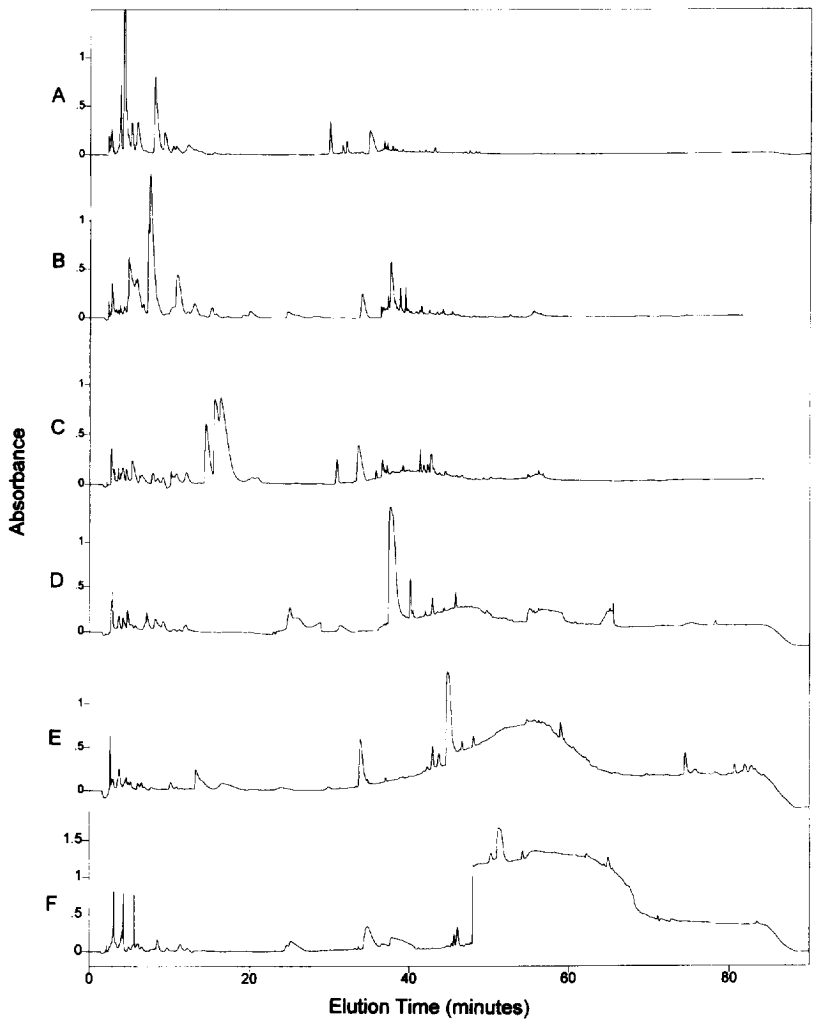

Fig. 2. Comparison of various ion-pairing agents for RP-HPLC of bovine hypothalamic extract. Hypothalamic extract aliquots were resuspended in 1 ml of indicated IPA and injected onto the Alltima C_{18} RP-HPLC column at 1 ml/min. Column was eluted isocratically for 10 min followed by gradient elution from 0–60% acetonitrile in 60 min. Detection was at 220 nm and chromatography was performed at ambient temperature. IPAs for injection and mobile phases were the following: (A) TFA (trifluoroacetic acid); (B) PFPA (pentfluoropropionic acid); (C) HFBA (heptafluorobutyric acid); (D) PFPN (perfluoropentanoic acid); (E) PFHX (perfluorohexanoic acid) and (F) PFHP (perfluoroheptanoic acid).

(a surface absorption mechanism). This would suggest that like HFBA, varying the concentration of PFPN, PFHX and PFHP may enhance selectivity for complex biological samples.

In conclusion, long-chain perfluorinated carboxylic acids serve as useful IPAs to separate small hydrophilic molecules that typically elute in the V_0 of TFA-based RP-HPLC systems. Examples using oligoglycine peptides and a bovine hypothalamic extract demonstrate their utility.

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