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REVERSED-PHASE ION-INTERACTION CHROMATOGRAPHY OF LEUKOTRIENES, LIPOXINS AND RELATED COMPOUNDS

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SUMMARY

A novel mobile phase containing heptafluorobutyric acid has been used for ion-interaction high-performance liquid chromatography of leukotrienes, lipoxins and related compounds on octadecylsilane silica columns. The use of a hydrophobic perfluorinated carboxylic acid as an ion-interaction agent at optimized concentration and pH permitted rapid, isocratic separation of leukotrienes, lipoxins and monohydroxyeicosatetraenoic acids. A completely volatile mobile phase suitable for preparative chromatography was obtained by using triethylamine as the base for pH adjustment. With this novel mobile phase, leukotrienes and monohydroxyeicosatetraenoic acids were completely separated in less than 15 min.

INTRODUCTION

Since the discovery of leukotrienes, lipoxins and related hydroxy fatty acids, high-performance liquid chromatography (HPLC) has been a technique of major importance for separation and quantification of their formation by lipoxygenases in cells and tissues [1–4]. A great number of the developed techniques utilize reversed-phase chromatography on octadecylsilane (ODS) silica enabling direct analysis of aqueous samples after removal of proteins [5–13]. In a majority of these studies, a low concentration of acetic acid (0.1%) in the mobile phase has been used for suppression of ionization of the carboxyl groups. While ion suppression alone gives satisfactory chromatography with respect to peak symmetry and response for monohydroxyeicosatetraenoic acids (HETEs), leukotriene B_4 (LTB_4) including metabolites, the peptidoleukotrienes C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) still constitute a major separation problem. The retention times of peptidoleukotrienes, which contain free amino groups besides quite acidic carboxyl groups, are extremely dependent on the polarity and the pH of the mobile

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phase [5]. The pH obtained in the mobile phase by addition of a small amount of acetic acid is quite likely insufficient to bring about any significant suppression of ionization of the peptide carboxyl groups. Furthermore, there are also interactions between amino groups and the silica surface of the column packing material because there still remain about 50% unreacted surface hydroxyl groups despite maximized coverage and end-capping. These interactions can be of either dipole, hydrogen-bonding or ionic character, the last one attributed to the pH-dependent ion-exchange properties of silica [14]. The magnitude of interactions with the silica surface can be decreased by adding an amine to the mobile phase. It has been demonstrated earlier that ammonium formate and ammonium acetate in high concentrations can be effective for decreasing interaction with silanol groups [15,16]. Indeed, when the concentration of acetic acid in the mobile phase was raised to 1% with a concurrent increase in the amount of ammonium hydroxide, peak symmetry as well as response were increased for peptidoleukotrienes. A further improvement was obtained by substituting triethylamine for ammonium hydroxide [11]. The efficiency of tertiary amines for improving peak symmetry and recovery of amines and peptides by masking surface silanols has been reported in several investigations [17–19].

A more recent development of leukotriene chromatography has been the use of phosphoric acid or trifluoroacetic acid as ion-suppression agents and as ion-interaction agents for the amino groups [10,12,13]. These acids are presently widely used for reversed-phase chromatography of peptides. Phosphoric acid alone or in combination with triethylamine decreases the interaction of amino groups with surface silanols resulting in improved peak symmetry and recovery of peptides and proteins [20–22]. Perfluorinated carboxylic acids such as trifluoroacetic acid are excellent peptide solvents, volatile and UV-transparent down to 210 nm at 0.01 *M* level. Since their introduction, the use, particularly, of trifluoroacetic acid has been one of the more important methods for ion suppression of peptides and proteins [23–25].

In two recent studies, the use of trifluoroacetic acid for the separation of leukotrienes and related compounds is reported [12,13]. Although quite satisfactory separations were obtained, the pH of the mobile phase was very low which might lead to decomposition of the acid-labile leukotrienes or cause a hydrolytic cleavage of the stationary phase from the silica particles. These disadvantages and earlier reports about increased retention and separation of peptides using longer-chain perfluorinated carboxylic acids [24,25] led to the examination of possible alternatives. This report describes the use of heptafluorobutyric acid (HFBA) as a mobile phase additive for reversed-phase chromatography of leukotrienes and related compounds.

EXPERIMENTAL

Materials

Leukotrienes B_4, C_4, D_4, E_4 and lipoxins A and B were kindly donated by Dr. J. Rokach (Merck Frosst Labs., Montreal, Canada). 20-Carboxy-LTB_4 (20-COOH-LTB_4) and 20-hydroxy-LTB_4 (20-OH-LTB_4) were prepared from poly-

morphonuclear leukocytes (PMNLs) as described earlier [26]. HETEs were prepared as described elsewhere [1,27,28].

ODS C_{18} silica columns packed with 5-μm particles were either a 150 mm$\times$4 mm I.D. cartridge obtained from Perkin-Elmer (Norwalk, CT, U.S.A.) or an RP-18, 250 mm$\times$4.0 mm I.D. from E. Merck Labs. (Canadian Supplier: BDH).

Solvents were of HPLC grade and used as received from Fisher Scientific. HFBA, sequencher grade, was obtained from Pierce Chemicals (Rockford, IL, U.S.A.) and triethylamine of gold-label quality from Aldrich (Milwaukee, WI, U.S.A.).

Mobile phase preparation

The mobile phases used were either methanol–water, 8:2 or 7:3 (v/v) containing 0.1% (7.7 m*M*) HFBA adjusted to pH 3.0 with triethylamine. The mobile phases were prepared by adding HFBA to distilled water, titrating to desired pH with triethylamine and subsequent adjustment of the volume of water by adding distilled water. Finally, the appropriate amount of methanol was added and the solution sonicated for 20 min.

Equipment

HPLC was carried out using a Varian 5000 liquid chromatograph.

Preparation of human PMNLs

Preparation of PMNLs was performed as described elsewhere [26]. A concentration of 10^7 cells per ml of Tyrod's buffer was used. Incubations were allowed to proceed for 5 min in the presence of 1 μM ionophore A23187 and then terminated by centrifugation at 1000 *g*.

Incubation of human lung tissue

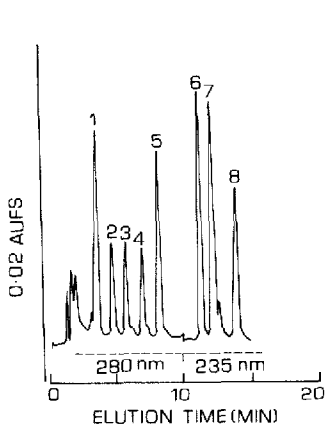
Fragments of human lung tissue (1 g wet weight) in 5 ml of Tyrod's buffer were incubated for 10 min with 10 μM arachidonic acid in the presence of 1 μM ionophore A23187. The incubation was terminated by centrifugation at 1000 *g*.

Extraction of leukotrienes

The supernatants from incubations with PMNLs or human lung tissue were extracted using an XAD-4 resin column essentially as described earlier [29]. The eluting solvent mixture was pyridine–water–tetrahydrofuran (50:45:5, v/v/v).

RESULTS AND DISCUSSION

The rapid separation that can be achieved by employing HFBA as a mobile phase additive is shown in Fig. 1. As can be seen, baseline separation is obtained between the three peptidoleukotrienes C_4, D_4 and E_4 on a rather short HPLC column at pH 3.0. Furthermore, optimal separation is obtained at considerably higher and for leukotrienes less detrimental pH than with the mobile phase systems using trifluoroacetic acid [12,13]. In one of these investigations, no base was used to adjust the pH of the mobile phase [12], while the other investigators added a small amount of triethylamine. The pH values in both of the mobile



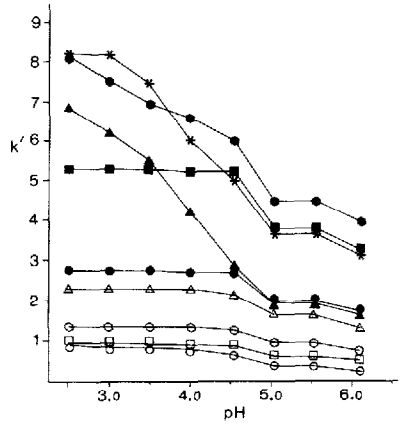


Fig. 1. Rapid separation of leukotrienes and HETEs with HFBA in the mobile phase. Mobile phase: methanol–water (8:2, v/v) containing 0.1% HFBA adjusted to pH 3.0 with triethylamine; column: Perkin-Elmer C_{18} cartridge, 150 mm × 4 mm I.D.; UV detection at 280 and 235 nm. Peaks: 1 = prostaglandin B_2 (internal standard); 2 = LTB_4; 3 = LTC_4; 4 = LTE_4; 5 = LTD_4; 6 = 15-HETE; 7 = 12-HETE; 8 = 5-HETE.

Fig. 2. Effect of pH on the separation of leukotrienes and lipoxins. Mobile phase: methanol–water (7:3, v/v) containing 0.1% HFBA adjusted to different pH values with triethylamine; column: LiChrosorb RP-18, 250 mm × 4.0 mm I.D.; UV detection at 280 and 310 nm. (*) LTD_4; (●) LTE_4; (▲) LTC_4; (■) LTB_4; (□) 20-OH-LTB_4; (○) 20-COOH-LTB_4; (△) lipoxin A; (○) lipoxin B; (●) PGB_2.

phases were well below 2. The choice of a low pH was very likely due to the increased retention and separation obtained. A similar pH effect is observed for HFBA as demonstrated in Fig. 2. It can be seen that by lowering the pH, the capacity factors (k') for the three peptidoleukotrienes C_4, D_4 and E_4 increase rapidly. However, an excellent separation is also obtained at pH 3.0. This pH likely represents less possibility for hydrolysis of the stationary phase than operating close to pH 2 as found in a recent investigation of peptide HPLC using trifluoroacetic acid [30]. In addition, the selectivity can be modulated by the pH as demonstrated by the reversed order of elution between LTE_4 and LTD_4 that occurs at a pH of about 3.8. The retention times of LTB_4 and lipoxins are virtually unaffected up to pH 4.0.

Moreover, it was observed that by increasing the concentration of HFBA to 0.17% (13 mM) at pH 3.0, which is equimolar to the concentration of trifluoroacetic acid (0.1%) used earlier [13], the retention times decreased with deteriorated separation as the result. On the contrary, upon decreasing the concentration of HFBA to 0.05% (4 mM), the retention times increased with a concomitant increased separation except between LTC_4 and LTE_4 due to severe tailing of the former. Thus, the chosen concentration of HFBA seems to be at optimum or very close to it.

The effects of different concentrations of HFBA indicate a dual effect on the chromatographic behavior of peptidoleukotrienes. The dramatic effect of a low

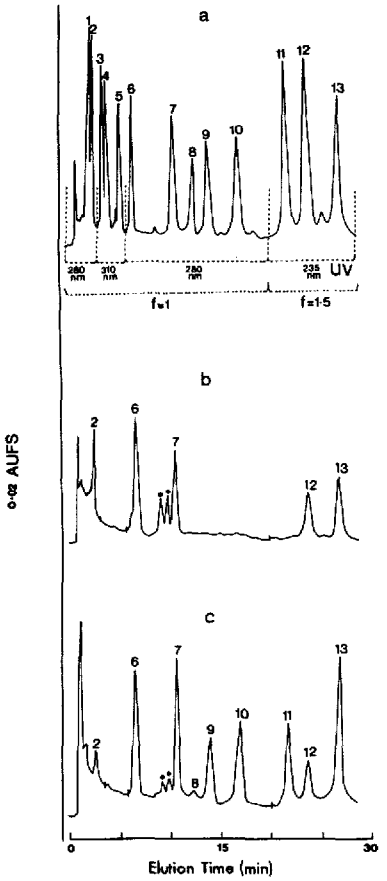


Fig. 3. Separation of leukotrienes, lipoxins and HETEs. Mobile phase: methanol–water (7:3, v/v) containing 0.1% HFBA adjusted to pH 3.0 with triethylamine; column: LiChrosorb RP-18, 250 mm × 4.0 mm I.D.; UV detection at 280, 310 and 235 nm. Peaks: 1 = 20-COOH-LTB_4; 2 = 20-OH-LTB_4; 3 = lipoxin B (14*S*); 4 = lipoxin B (14*R*); 5 = lipoxin A; 6 = prostaglandin B_2; 7 = LTB_4; 8 = LTC_4; 9 = LTE_4; 10 = LTD_4; 11 = 15-HETE; 12 = 12-HETE; 13 = 5-HETE; * = Δ^6-*trans*-isomers of LTB_4. (a) Standards; (b) leukotrienes and HETEs from human PMNLs stimulated with ionophore A23187; (c) leukotrienes and HETEs from human lung tissue stimulated with ionophore after addition of arachidonic acid.

concentration of HFBA on the peak shape of LTC_4 taken together with the generally increased retention times, might indicate the HFBA forms a primary layer of negatively charged ions on the stationary phase of the column packing material. Electrostatic forces from this layer would exert a greater repulsion on dicarboxylic acids such as peptidoleukotrienes and less on monocarboxylic acids such as LTB_4 [31]. The retention time of the latter was also found fairly constant for the concentration of HFBA used in this study. Furthermore, interactions between the amino groups of peptidoleukotrienes and HFBA were indicated by the improved separation as compared to the use of trifluoroacetic acid. This was also in accordance with earlier reports on the reversed-phase chromatography of peptides demonstrating that retention and in several cases selectivity increased upon

progressing from trifluoroacetic acid to undecacaproic acid [24,25]. However, the decreased retention times obtained upon increasing the concentration of HFBA was an effect contrary to observations made earlier with different perfluorinated carboxylic acids [25]. The finding in the present study indicates that interactions between HFBA and amino groups are less important than between HFBA and carboxyl groups. Moreover, a secondary positively charged layer of triethylamine molecules could also contribute to reduced attraction between amino groups and HFBA. The application of this novel ion-interaction chromatographic technique for arachidonic acid-derived compounds can be seen in Fig. 3. By utilizing a 25-cm column, the entire spectrum of lipoxygenase metabolites is separated isocratically in less than 30 min. In Fig. 3a it is demonstrated that the *R*- and *S*-isomers of lipoxin B (peaks 3 and 4) are well separated. Also, the two *trans*-isomers of LTB_4 are well separated from each other and from LTB_4 as can be seen in Fig. 3a and b. Thus the separation of lipoxins as free acids using HFBA for ion suppression is clearly superior to earlier results obtained with acetic acid in the mobile phase [4]. Unfortunately, LTB_4 coeluted with its 5*S*,12*S*-isomer using this solvent system but separation might be obtained by addition of acetonitrile as found earlier [10].

In conclusion, the presented ion-interaction method for isocratic separation of lipoxygenase metabolites will facilitate studies of the production of such compounds in various biological systems using a minimum of chromatographic hardware.

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REFERENCES

1 P. Borgeat and B. Samuelsson, J. Biol. Chem., 251 (1979) 7816.
2 P. Borgeat and B. Samuelsson, J. Biol. Chem., 254 (1979) 7865.
3 R.C. Murphy, S. Hammarstrom and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 4275.
4 C.N. Serhan, M. Hamberg and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 5335.
5 W.R. Mathews, J. Rokach and R.C. Murphy, Anal. Biochem., 118 (1981) 96.
6 H.J. Ziltener, P.-A. Chavaillaz and A. Jorg, Hoppe-Seyler's Z. Physiol. Chem., 364 (1983) 1029.
7 D.J. Osborne, B.J. Peters and C.H. Meade, Prostaglandins, 26 (1983) 817.
8 J. Verhagen, P. Walstra, G.A. Veldink and J.F.G. Vliegenthart, Prostaglandins Leukotrienes Med., 13 (1984) 15.
9 D.C. Henke, S. Kouzan and T.E. Eling, Anal. Biochem., 140 (1984) 87.
10 P. Borgeat, Prostaglandins, 27 (1984) 356.
11 M. Abe, Y. Kawazoe and N. Shigematsu, Anal. Biochem., 144 (1985) 417.
12 W.S. Powell, Anal. Biochem., 148 (1985) 59.
13 J.D. Eskra, M.J. Pereira and M.J. Ernest, Anal. Biochem., 154 (1986) 332.
14 S.G. Weber and W.G. Tramposch, Anal. Chem., 55 (1983) 1771.
15 K. Sugden, G.B. Cox and C.R. Loscombe, J. Chromatogr., 149 (1978) 377.
16 C.K. Lim and T.J. Peters, J. Chromatogr., 316 (1984) 397.

17 K.E. Bij, C. Horváth, W.R. Melander and A. Nahum, J. Chromatogr., 203 (1981) 65.
18 W.G. Tramposch and S.G. Weber, Anal. Chem., 56 (1984) 2567.
19 J.S. Kiel, S.L. Morgan and R.K. Abramson, J. Chromatogr., 320 (1985) 313.
20 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R. Harding and M.T.W. Hearn, J. Chromatogr., 153 (1978) 391.
21 J.E. Rivier, J. Liq. Chromatogr., 1 (1978) 343.
22 W.S. Hancock and J.T. Sparrow, J. Chromatogr., 206 (1981) 71.
23 H.P.J. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9.
24 H.P.J. Bennett, C.A. Browne and S. Solomon, J. Liq. Chromatogr., 3 (1980) 1353.
25 W.M.M. Schaaper, D. Voskamp and C. Olieman, J. Chromatogr., 195 (1980) 181.
26 G. Hansson, J.-A. Lindgren, S.-E. Dahlen, P. Hedquist and B. Samuelsson, FEBS Lett., 130 (1981) 107.
27 M. Hamberg and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 7 (1974) 3400.
28 E. Goetzl and F. Sun, J. Exp. Med., 150 (1979) 406.
29 H. Salari and S. Steffenrud, J. Chromatogr., 378 (1986) 35.
30 J.L. Glajch, J.J. Kirkland and J. Köhler, J. Chromatogr., 384 (1987) 81.
31 B.A. Bidlingmeyer, S.N. Deming, W.P. Price, Jr., B. Sachok and M. Petrusek, J. Chromatogr., 186 (1979) 419.