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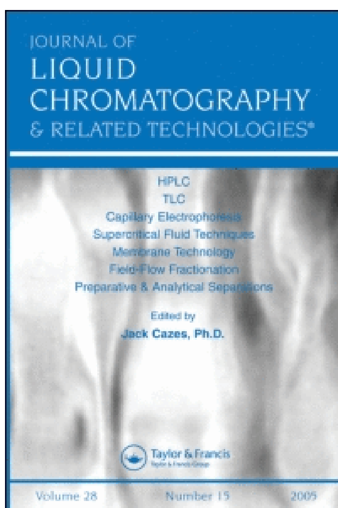

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ION EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF LEUKOTRIENES

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ABSTRACT

A novel anion exchange liquid chromatographic system has been developed for isocratic separation of leukotrienes. Hydrophobic as well as ionic forces were found to influence the separation. By optimization of solvent strenght, ionic strenght and pH, amphoteric peptidoleukotrienes could be separated simultaneously with hydroxy fatty acids such as leukotriene B_4 and its ω-oxidized metabolites. To obtain a good buffering capacity of the mobile phase at optimum pH, a multicomponent buffer was developed.

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INTRODUCTION

Separation of lipoxygenase metabolites of arachidonic acid has commonly been performed by normal phase HPLC (1-3) or reversed phase HPLC (4-12).

The rapid and efficient separations that can be achieved by these HPLC techniques were of major importance for the isolation of such lipoxygenase metabolites as monohydroxyeicosatetraenoic acids (HETEs), leukotrienes and lipoxins prior to characterization by UV spectroscopy and gas chromatography-mass spectrometry (13-17). Particularly, reversed phase has been very versatile for preparative separations as well as quantitations by UV detection. Since reversed phase chromatography is performed with water containing mobile phases, a direct application of sample onto the column is possible after a simple protein removal (9).

Already in one of the earliest studies of the chromatographic behaviour of leukotrienes on reversed supports, a strong pH dependence was found for peptidoleukotrienes (4). Later investigations demonstrated the favourable influence of phosphoric acid (9) and high salt concentrations (10) on peak shape and resolution of leukotrienes upon reversed phase chromatography. All these chromatographic data taken together indicate that despite the use of a support

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maximally covered with a hydrophobic stationary phase and further endcapped, the free silanol groups still available play an important role in influencing the chromatographic behaviour of leukotrienes. The influence of pH, salt concentration and the kind of acid employed, all indicate that ion-exchange mechanisms originating from free silanols are as important as solvophobic interactions in governing reversed phase separations of leukotrienes.

Accordingly, by using a chromatographic support containing a high number of free silanol groups (i.e. a non-endcapped support), lipoxygenase products of a wide range of polarities could be separated by a combination of solvent and pH gradients (9). This latter HPLC system was realized by taking advantage of the cation exchange characteristics of silica based reversed phase supports (18).

Thus, the purpose of the present study was to investigate ion-exchange mechanisms governing the chromatographic behaviour of leukotrienes on a dedicated ion-exchange support. An intermediate base anion exchanger of moderate hydrophobicity was chosen for the present study. Results are presented for the chromatography of leukotrienes using either a phosphate buffer or a mixed phosphate-acetate buffer.

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EXPERIMENTAL

Materials

Leukotrienes B_4 (LTB_4), C_4 (LTC_4), D_4 (LTD_4), E_4 (LTE_4) and N-acetyl-E_4 were kindly donated by Dr. J. Rokach, Merck Frosst Laboratories, Montreal, Que.. 20-hydroxy-LTB_4 (20-OH-LTB_4) and 20-carboxy-LTB_4 (20-COOH-LTB_4) were prepared as described (19).

The column packing material, 5 um dimethylamino-propyl silica (Macherey & Nagel, North American supplier: Alltech Associates) was slurry packed into a stainless steel column, 125 x 4.6 mm (i.d.)

HPLC grade acetonitrile, acetic acid and phosphoric acid (85%) were used as received from Fisher Scientific.

Triethylamine (Gold Label, Aldrich) was distilled before use.

Amberlite XAD-4, 50-100 um was obtained from Serva Feinbiochemica (Canadian supplier: Terochem Laboratories Ltd., Edmonton)

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Preparation of mobile phase

Buffers were prepared by addition of phosphoric acid to distilled water, titration to desired pH with triethylamine and final adjustment of the volume by addition of water. Then acetonitrile was added and the solution degassed by sonication for twenty minutes.

Preparation of biological material

Samples of 1 g (wet weight) of human lung tissue was prepared by extraction on Amberlite XAD-4 as described earlier (20).

Instrumentation

The temperature of the HPLC column was maintained at 25+0.5°C by a water jacket connected to a Polytemp (Polyscience) circulating water bath.

The mobile phase was delivered at 1.0 ml min^{-1} by a Waters M45 pump attached to a Rheodyne injector. Eluting peaks were detected by a Beckman 163 variable UV detector connected to an Omniscribe chart recorder (Houston Instruments).

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RESULTS AND DISCUSSION

Fig. 1 demonstrates the separation of various leukotrienes as a function of pH with 40% acetonitrile and 20 mM triethylamine phosphate in the mobile phase. As can be seen, the maximum retention for all compounds is achieved at pH 5.0, which is partly dependent on the dissociation constants of the carboxyl groups of the fatty acids. The rapidly decreasing capacity factors upon a further increase of the pH are presumably due to the second ionization (pKa_2 7.21) of phosphoric acid resulting in a divalent anion. The increased negative

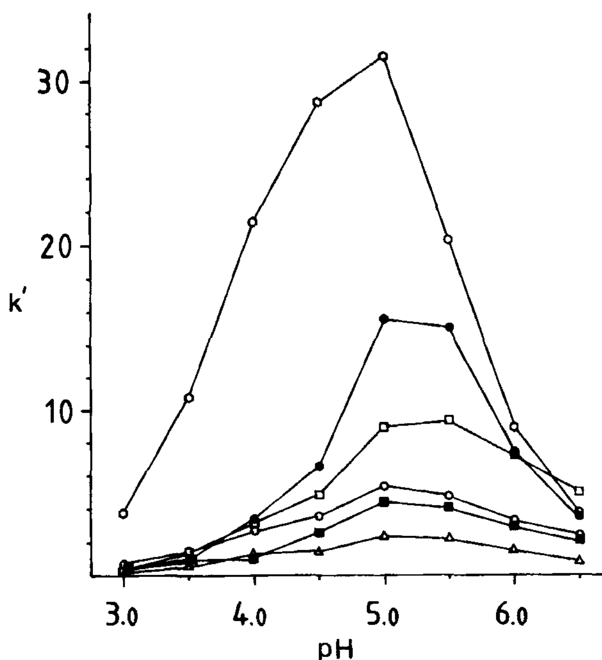

Fig. 1. Effect of pH on the separation of leukotrienes. Mobile phase: acetonitrile/water, 4:6 containing 20 mM triethylamine phosphate; UV detection at 270 nm; compounds: LTC_4 (O-O), LTD_4 (□-□), LTE_4 (O-O), 20-COOH-LTB_4 (●-●), 20-OH-LTB_4 (Δ-Δ), LTB_4 (■-■).

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charge in the mobile phase compete effciently with the solutes for ion exchange sites with decreased capacity factors as the result (21).

In this chromatographic system, however, N-acetyl-LTE_4 was strongly retained on the column and was eluted at pH <3 or pH >6 only (data not shown in Fig. 1). A likely reason for the much higher capcity factors observed with N-acetyl-LTE_4 as compared to LTE_4, is the

difference in electrostatic Donnan forces (21,22). The fixed positive charges on the support repel the positive charge on the nitrogen of the leukotrienes C_4, D_4 and E_4 but N-acetylated compounds lack this positive charge.

Increasing the amount of acetonitrile to 60%, decreased the capacity factors for all solutes, including N-acetyl-LTE_4 which demonstrated a chromatographic behaviour similar to that of LTC_4 (Fig. 2). This decrease of the capacity factors indicates the presence of rather strong hydrophobic (solvophobic) interaction for the leukotrienes. In fact, the hydrophobic interaction likely contributes to the large difference in the capacity factors for LTE_4 and N-acetyl-LTE_4 since the latter clearly has a stronger hydrophobic character. A further increase of the content of organic modifier in the mobile phase from 60% to 80% resulted in capacity factors lower than shown in Fig. 2 (data not shown). Although an increased retention was observed earlier using an identical chromatographic support as in the present study (23), it was found that those earlier data were likely obtained under non-equilibrium conditions.

On the other hand, an increased ionic strength of a mobile phase containing 40% acetonitrile, accelerated the elution of all solutes, clearly demonstrating the importance of ionic forces (Fig. 3). The excellent

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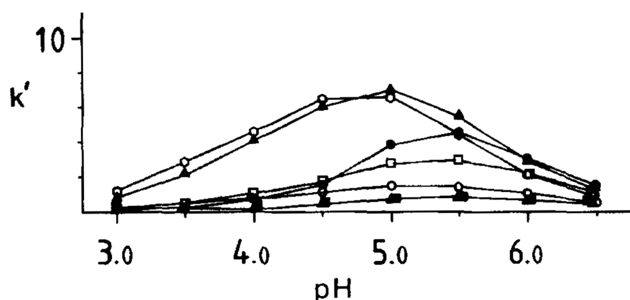

Fig. 2. Effect of pH on the separation of leukotrienes. Mobile phase: acetonitrile/water, 6:4 containing 20 mM triethylamine phosphate; UV detection at 270 nm; compounds: LTC_4 (⬡-⬡), LTD_4 (□-□), LTE_4 (○-○), N-acetyl-LTE_4 (▲-▲), 20-COOH-LTB_4 (●-●), 20-OH-LTB_4 (△-△), LTB_4 (■-■).

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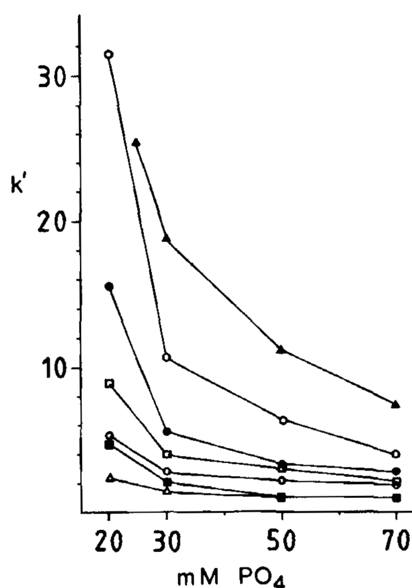

Fig. 3. Effect of ionic strenght on the elution of leukotrienes. Mobile phase: acetonitrile/water, 4:6 adjusted to pH 5.0 with triethylamine; UV detection at 270 nm; compounds: LTC_4 (⬡-⬡), LTD_4 (□-□), LTE_4 (○-○), N-acetyl-LTE_4 (▲-▲), 20-COOH-LTB_4 (●-●), 20-OH-LTB_4 (△-△), LTB_4 (■-■).

separation obtained between LTB_4 and 20-OH-LTB_4, however, is probably not possible with pure ion exchange interactions (Fig. 4 and 5) and also supports involvment of hydrophobic interactions. Thus, the retention of leukotrienes appears to be governed by mixed mode forces on this particular ion exchange support.

The simultaneous isocratic separation of seven different leukotrienes of widely different polarities are examplefied in Fig. 4. In order to elute N-acetyl-LTE_4 without compromising the separation of the other solutes, the phosphate concentration was increased to 25 mM. As can be seen, all solutes exhibit a satisfactory chromatographic behaviour.

A disadvantage with the use of phosphate buffer, however, is the poor buffering capacity obtained at pH 5.0, between pKa_1 and pKa_2 of phosphoric acid (24). With a larger sample applied to the column, buffering components in the mobile phase may be completely consumed and the resulting poor control of the pH will lead to assymetrical peaks and altered retention times.

The maximum buffering capacity is obtained with an equimolar solution of a conjugate acid-base pair, at a pH close to the pKa values of the buffering components. Since the pKa value of acetic acid (4.75) is close to the pH (5.0) of the mobile phase, it was considered as a

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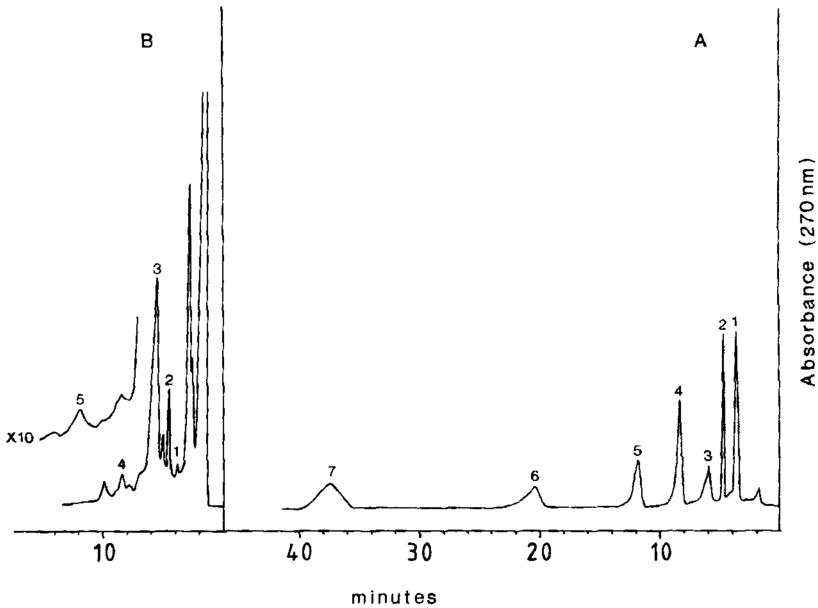

Fig. 4. Isocratic separation of leukotrienes with UV detection at 270 nm. Mobile phase: acetonitrile/water, 4:6 with 25 mM triethylamine phosphate at pH 5.0; compounds: 1. 20-OH-LTB_4, 2. LTB_4, 3. LTE_4, 4. LTD_4, 5. 20-COOH-LTB_4, 6. LTC_4, 7. N-acetyl-LTE_4.
Right panel: Standards
Left panel: Extract of human lung tissue, 1/5 injected.

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good candidate for enhancement of the buffering capacity of the mobile phase. Unfortunately, a mobile phase containing triethylamine acetate was found too weak for elution of LTC_4 except at low pH (3.0) and a concentration of 0.1 M. However, by decreasing the amount of phosphate to 10 mM and adding 20 mM acetate, a mixed buffer was obtained which gave the same elution pattern as with phosphate only in the mobile phase (Fig.

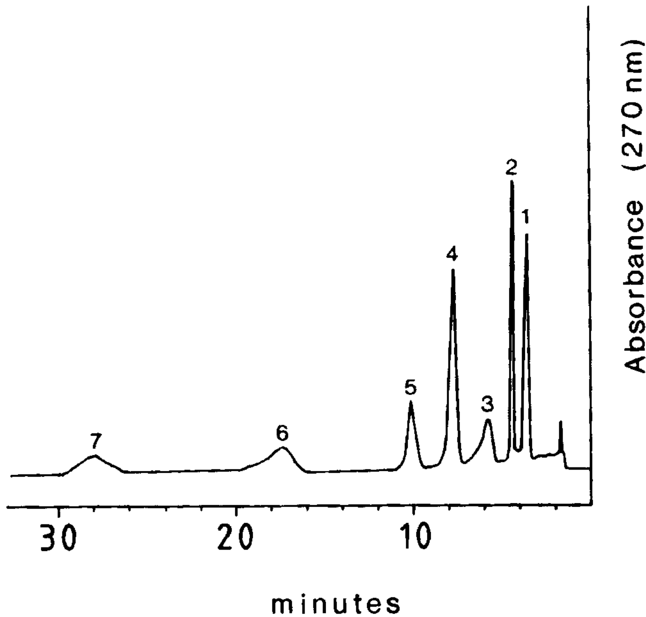

Fig. 5. Isocratic separation of leukotrienes with UV detection at 270 nm. Mobile phase: acetonitrile/water, 4:6 with 10 mM triethylamine phosphate and 20 mM triethylamine acetate at pH 5.0; compounds: 1. 20-OH-LTB_4, 2. LTB_4, 3. LTE_4, 4. LTD_4, 5. 20-COOH-LTB_4, 6. LTC_4, 7. N-acetyl-LTE_4.

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5). Calculated by the use of the formula $\beta_{max} = 0.58C$, where β is the buffering capacity and C is the molar concentration of a buffer component, the buffering capacity of the mixed buffer is at least 100 times higher at pH 5.0 than with phosphate only in the mobile phase. This mixed buffer caused, however, more tailing of LTE_4.

Another approach for enhanced buffering capacity that was investigated, is the substitution of an acidic

diamine for triethylamine as reported earlier (24). However, N-methylpiperazine with a pKa value of 4.94, when substituted for triethylamine, gave deteriorated peak shape and separation for several solutes.

In conclusion, the anion exchange chromatographic system presented in this study provides a complementary second dimension in the separation of lipoxygenase products. The different selectivity may be used to assess the identity of compounds separated by reversed phase chromatography. Moreover, it is compatible with subsequent immunological or biological assays after removal of the organic modifier (25). The intermediate pH of the mobile phase and the use of liquid buffer components, permit complete removal of the mobile phase under reduced pressure without salt precipitation or decomposition of acid labile leukotrienes prior to gas chromatography-mass spectrometry or various spectroscopic techniques.

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