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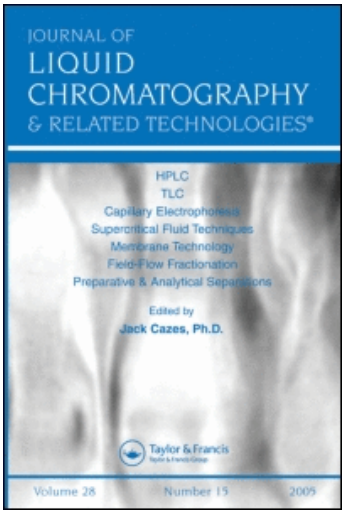

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An Improved Technique for Extraction, Identification, and Quantification of Leukotrienes

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AN IMPROVED TECHNIQUE FOR EXTRACTION, IDENTIFICATION, AND QUANTIFICATION OF LEUKOTRIENES

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ABSTRACT

An improved method for extraction, separation and quantification of leukotrienes is described. Critical steps include extraction in thoroughly cleaned Sep-Pak C_{18} mini columns, elution with 90 percent methanol in water, addition of 0.25 percent Na_4EDTA to the solvent system of methanol/water 60/40, pH 7.4, injection of the samples in 1 ml of the same system after lowering the pH to 3.0 with glacial acetic acid and utilization of a Z-module containing a 5 μm C_{18} reverse phase cartridge. Recovery of the leukotrienes was 94 ± 8% mean ± SD for LTC_4, 89 ± 5% for LTD_4, 89 ± 3% for LTB_4 and 84 ± 6% for LTE_4. We had no problems with precipitation of Na_4EDTA and occlusion of the HPLC fittings. The method is simple, reproducible and easily adaptable to any research laboratory.

INTRODUCTION

Following the discovery of the leukotrienes, a large number of investigators have attempted to elucidate the role of these interesting

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compounds in physiologic and pathologic processes. An important aspect of this area of research is the availability of reliable techniques to isolate and quantify the several leukotrienes. High performance liquid chromatography (HPLC) has been the traditional technique for purification and quantification of the leukotrienes (1,2). Initially, Amberlite XAD-7 was introduced to extract peptidoleukotrienes but the method is cumbersome (2,3). Later, silica mini-columns were recommended to simplify the extraction procedure (4) and washings of the HPLC columns with disodium ethylenediaminetetraacetic acid (Na_2EDTA) were intended to improve resolution of the leukotriene peaks (5). These modifications have contributed in a favorable manner to the methodology of leukotriene research. However, some problems still exist, particularly reproducibility of the techniques and recovery of peptidoleukotrienes. We report here a technique for extraction, separation and quantification of leukotrienes based on utilization of Sep-Pak C_{18} columns and addition of Na_4EDTA into the mobile phase of the HPLC.

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EXPERIMENTAL

Extraction Procedures

Before the first sample was applied, the C_{18} Sep-Pak columns were washed four times with 30 ml of high purity water (Burdick and Jackson Labs. Inc., Muskegon, MI 49421) twice with 20 ml of methanol (Pierce Chemical Company, Rockford, IL 61105) twice with 30 ml of water and once with 20 ml of methanol. Samples were dissolved in normal saline (1 to 25 ml), applied to the Sep-Pak columns and eluted with 15 ml of 90 percent methanol in water. The eluent was evaporated under vacuum and dissolved in 1 ml of the solvent system prior to injection into the HPLC. Between samples, the washing procedure of the Sep-Pak columns consisted of the successive application of 30 ml water, 30 ml methanol, 30 ml water and 30 ml methanol.

HPLC

Apparatus

Samples were injected into a Waters HPLC equipped with a model U6K injector, a model M-45 solvent delivery system, a model 450 vari-

able wavelength detector and an Omni Scribe B-500 recorder (Houston Instruments, Austin, TX).

Procedures

Three HPLC procedures were compared. In the first two, a reverse phase stainless steel column (μ BONDAPAK, 30 cm x 3.9 mm) was utilized. A Z-module containing a 5 μm radial-PAK C_{18} reverse phase cartridge (10 cm x 0.8 cm) was employed in the third procedure. The first chromatography system was identical to that developed by Anderson et al (5). In short, the column was washed overnight with 0.5% Na_2EDTA in water/methanol 10/90 and the mobile phase consisted of methanol/water/acetic acid 67/33/0.02 adjusted to pH 5.7 with ammonium hydroxide. Pump rate was 1 ml/min. In the second procedure, the column was not washed with Na_2EDTA but the solvent system, which consisted of methanol/water 60/40, adjusted to pH 7.4 with glacial acetic acid contained 0.25% ethylenedinitrilotetraacetic acid tetrasodium salt (Na_4EDTA), Matheson Coleman and Bell, Norwood, Ohio 45212. Pump rate was 1 ml/min. An identical solvent system was utilized in the third procedure but the stainless steel column was replaced by the Z-module and the pump rate was 2 ml/min. In all of the procedures, samples were dissolved in 1 ml of the solvent system and injected into the HPLC without and with lowering of the pH to 3 with glacial acetic acid. Peaks were detected at 280 nm. The HPLC fittings and the cartridge were washed with 100 ml of water before and immediately after using the solvent system containing Na_4EDTA.

Recovery Experiments

Multiple recovery experiments were carried out. LTC_4, LTD_4, and LTE_4 (400 ng each) and 100 ng of LTB_4 (all leukotrienes were kindly supplied by Dr. Joshua Rokach, Merck-Frosst Lab, Pointe Clair, Quebec, Canada) were added to 25 ml of normal saline and extracted as described above. Same amounts of the leukotrienes were injected directly into the HPLC. In some of these experiments, the leukotriene peaks were collected, evaporated under vacuum at room temperature, dissolved in 1 ml of the solvent system and reinjected into the HPLC. Concentration of the leukotrienes could be calculated by comparing the area under the peak with that of known amounts of leukotrienes.

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TABLE 1

Recovery of Leukotrienes. Data from 10 Separate Experiments.

Leukotriene	Percent Recovery (Mean ± SD)
LTC_4	94 ± 8
LTD_4	89 ± 5
LTB_4	89 ± 3
LTE_4	84 ± 6

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RESULTS

Representative chromatography patterns of the three procedures are shown in Figures 1-3. Separation of the leukotrienes was poor with overnight washing of the column with Na_2EDTA, and acidification of the sample prior to injection improved recovery but it did not improve the resolution of the peaks (Figure 1). Addition of Na_4EDTA to the buffer improved resolution of the peaks and acidification of the samples led to better recovery but LTD_4 could not be separated from LTB_4 (Figure 2). The third procedure utilizing the Z-module was the best in that excellent resolution of the peaks was obtained and recovery was increased by lowering the pH of the sample prior to injection (Figure 3).

Recovery of all leukotrienes was excellent (Table 1) but a loss of 0-30% occurred during collection, evaporation and reinjection of the peaks.

DISCUSSION

We have developed a procedure for extraction, separation and quantification of leukotrienes which is simple, reproducible and easily adaptable to any laboratory. The cardinal features of the procedure include extraction in Sep-Pak C_{18} mini columns, elution

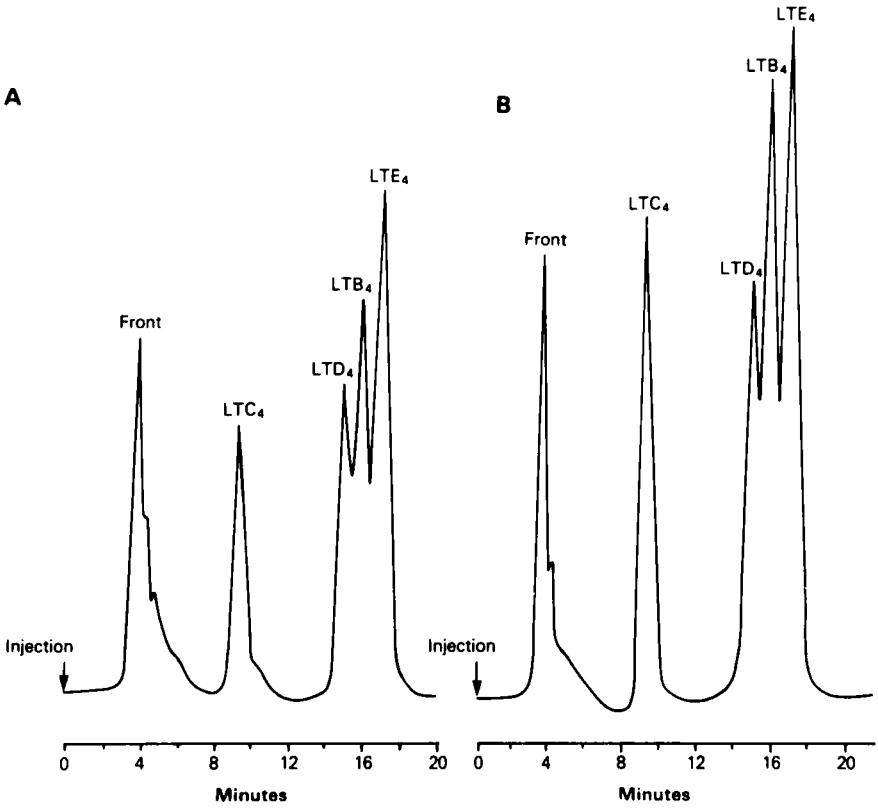

Figure 1: Reverse phase HPLC chromatogram of leukotrienes separated in an stainless steel column (μ BONDAPAK, 30 cm x 3.9 mm). Solvent system: methanol/water/acetic acid 67/33/0.02, pH 5.7. Pump rate 1 ml/min. Peaks were detected at 280 nm. Samples were dissolved in 1 ml of the solvent system and injected without (A) or with (B) acidification to pH 3.0 with acetic acid.

with 90 percent methanol in water, separation in a 5 μm C_{18} reverse phase cartridge, addition of Na_4EDTA to the solvent system, dilution of the sample in the same system and acidification to pH 3.0 prior to injection. To avoid artefacts the mini-columns need to be cleaned thoroughly prior to each extraction. We had no problem with precipitation of Na_4EDTA and obstruction of the HPLC fittings. Precipitation of EDTA is pH and time dependent, occurring faster at lower

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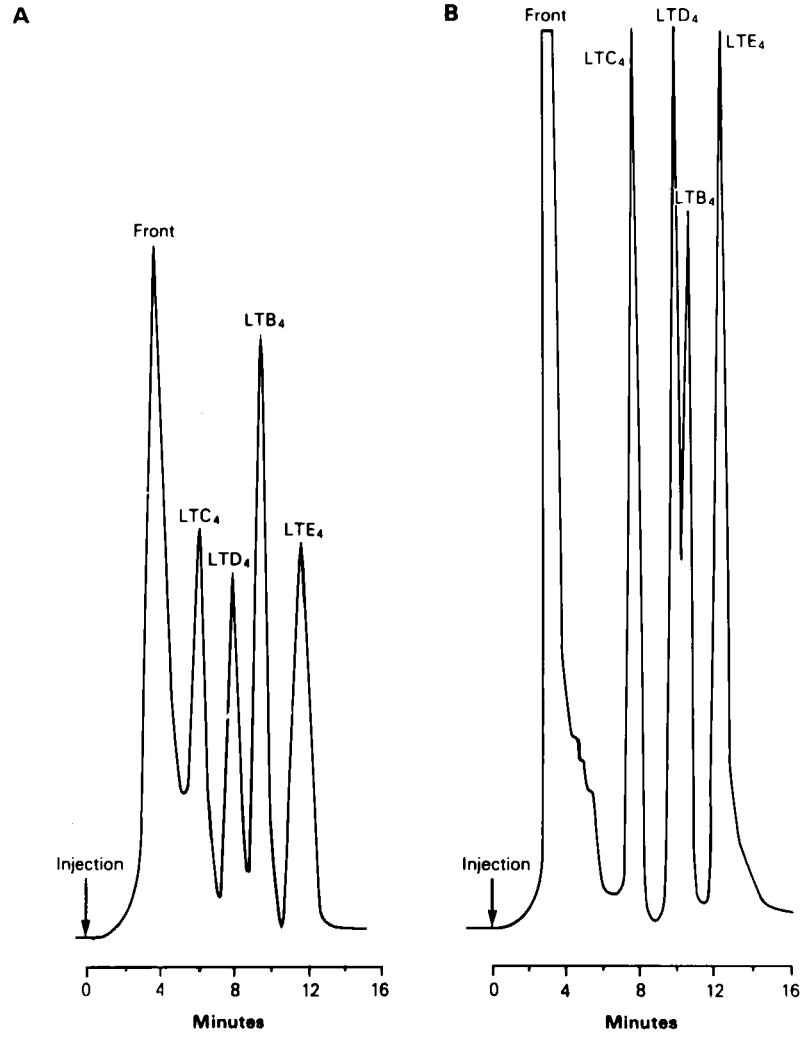

Figure 2: Reverse phase HPLC chromatogram of leukotrienes separated in a stainless steel column (μ BONDAPAK, 30 cm x 3.9 mm). Solvent system: methanol/water 60/40, pH 7.4 containing 0.25% Na_4EDTA. Pump rate = 1 ml/min. Peaks were detected at 280 nm. Samples were dissolved in 1 ml of the solvent system and injected without (A) or with (B) acidification to pH 3.0 with acetic acid.

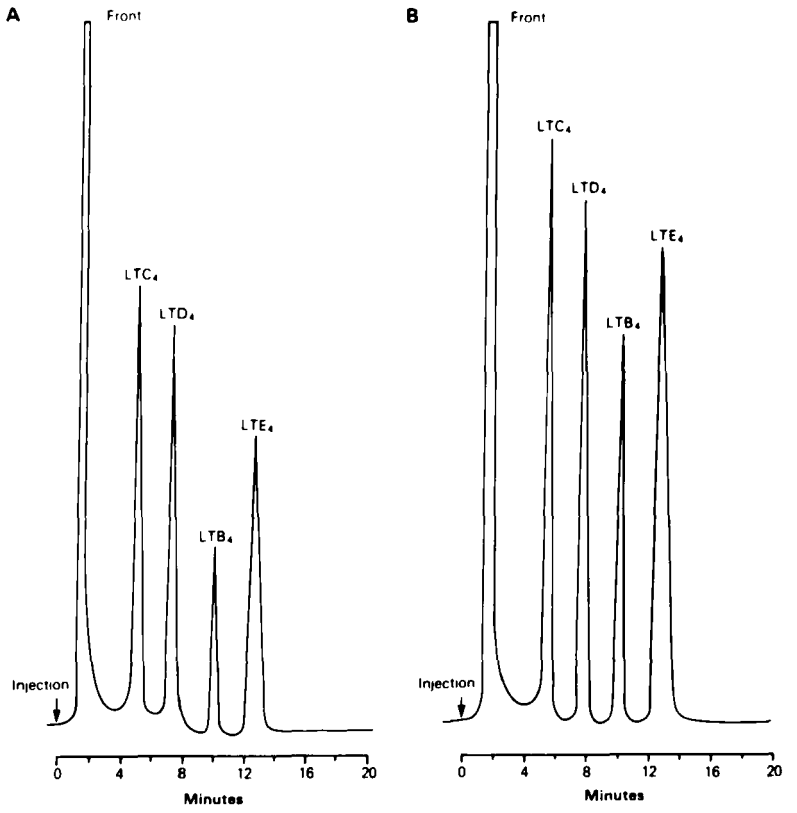

Figure 3: Reverse phase HPLC chromatogram of leukotrienes separated in a Z-module containing a 5 μm radial-PAK C_{18} cartridge (10 cm x 0.8 cm). Solvent system: methanol/water 60/40 pH 7.4 containing 0.25% Na_4EDTA. Pump rate = 2 ml/min. Peaks were detected at 280 nm. Samples were dissolved in 1 ml of the solvent system and injected without (A) or with (B) acidification to pH 3.0 with acetic acid.

pH. Na_4EDTA remains in solution at pH 7.4. It is critical to wash the entire HPLC apparatus with water before and after using the solvent system containing Na_4EDTA. Methanol mixtures will precipitate Na_4EDTA if added immediately before or after running solvent systems containing the chelating agent. Acidification of the samples prior to injection into the HPLC improves peak resolution and increases recovery.

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