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RAPID EXTRACTION OF LEUKOTRIENES FROM BIOLOGIC FLUIDS AND QUANTITATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Previous methods for the recovery and quantitation of leukotrienes have involved tedious extraction procedures, and high-performance liquid chromatographic (HPLC) techniques with significant limitations. We have designed a method to extract leukotrienes from biologic fluids using commercially available silica mini-columns requiring minimal preparation. Sample clarification is followed by a sensitive and reproducible HPLC technique which separates and quantifies the leukotrienes LTC_4, LTD_4, LTB_4 (and at least three of their isomers). The entire procedure requires less than one hour per sample.

INTRODUCTION

Leukotrienes are a family of peptido-lipids derived from the metabolism of arachidonic acid and related long-chain polyunsaturated fatty acids via the lipoxygenase pathway. Since these compounds have potent effects upon white cell function, bronchial tone, and several other biological target systems, there has been considerable recent interest in quantifying these compounds in biological samples. Early assays employed one or more biologic endpoints for quantitation (for example, the contraction of the guinea pig ileum) [1]. Although such assays are sensitive to a few nanograms of leukotrienes, they are cumbersome and do not readily distinguish and quantify each of the various leukotrienes, such as LTB_4 and the components of slow-reacting substance of anaphylaxis, or "SRS-A" (leukotrienes C_4, D_4, and E_4). More recent techniques have employed high-performance liquid chromatography (HPLC). Early HPLC methods, using primarily preparative chromatography, also did not separate well the several components of SRS-A and/or did not simultaneously measure LTB_4 [2—6].

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Furthermore, most extraction methods employed the laborious combination of sequential XAD resin columns and silicic acid open-column chromatography [2, 3, 5—7]. A more recent method reported by us [8], using self-packed HPLC columns, was adequately sensitive and selective; however, use of commercially available HPLC columns was not assessed and the extraction procedure (XAD-7 resin) required extensive (1–2 days) preparation prior to use. We report herein a rapid and reliable extraction procedure for leukotrienes that employs commercially available extraction columns which retain leukotrienes (and several of their isomers), which can then be readily eluted and assayed. We also report several modifications of our original HPLC technique which permit improved reproducibility and sensitivity in the quantitation of leukotrienes when using commercial (rather than self-packed) HPLC columns.

MATERIALS AND METHODS

Silica and C_{18} (ODS) extraction columns (Sep-Paks) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Chromatography was carried out using a 5-μm ODS column (25 cm length) purchased from Altex Scientific (Berkeley, CA, U.S.A.). (Similar results to those reported were also obtained in preliminary studies using a 10-μm ODS column from Waters Assoc.) All solvents including water were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) or Waters Assoc. Samples were injected in 50—500 μl of water—methanol (30:70) through a Waters U6K injector. A Waters Assoc. guard column packed with Corasil (ODS) pellicular packing, a Waters 6000A pump, and a Model 441 UV detector with a 280-nm filter (generally set at 0.01 a.u.f.s.) were used for chromatography. Murine mastocytoma cells were stimulated to produce leukotrienes, as previously described [9]; the leukotrienes were then extracted and purified by HPLC [8]. Free fatty acid-free bovine serum albumin was purchased from Sigma (St. Louis, MO, U.S.A.) and A23187 (divalent ionophore) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Reagents for cell culture medium were purchased from GIBCO (Grand Island, NY, U.S.A.).

RESULTS

Chromatography technique

As we have previously observed, the chromatography of leukotrienes is exquisitely sensitive to changes in mobile phase pH (which has major effects on selectivity, α) and in methanol content (which has major effects on capacity factor, k', and lesser effects on α). Despite rigorous control of these variables, the HPLC system previously described by us proved not to be entirely adequate when commercially available columns (Waters C_{18}, 10 μm; Altex C_{18}, 5 μm) were used. Most dramatically, the recoveries (especially of leukotriene C_4) were variable and very poor; in addition, both α and k' varied unacceptably. After extensive empirical changes in mobile phase, optimum solvent characteristics were ascertained: 67% methanol, 33% water, 0.08% acetic acid, 0.04% ammonium hydroxide, brought to a final pH of 6.2. The addition of an excess of acetic acid and ammonium hydroxide (beyond that required to achieve an optimal pH) was empirically discovered to maximize peak sharpness and to

achieve reproducible and acceptable α and k' values. Although not pursued further, this effect was presumed to be due to the increase of ionic strength (ammonium acetate) of the mobile phase. Using this mobile phase at a flow-rate of 1 ml/min, a chromatogram could be obtained in which three leukotrienes (LTB_4, LTC_4, LTD_4), as well as the 11-*trans* isomer of LTC_4 and two non-enzymatically formed 5,12-dihydroxy isomers of LTB_4 could be separated in less than 30 min (Fig. 1). k' values using this mobile phase were: prostaglandin (PG) B_2, 3.8; LTC_4, 4.4; 11-*trans* LTC_4, 4.8; LTD_4, 7.6; LTB_4, 8.4. Although retention times proved reproducible from day to day, a standard curve using PGB_2 (as internal standard) and each of the leukotrienes was derived each day prior to injecting unknown samples.

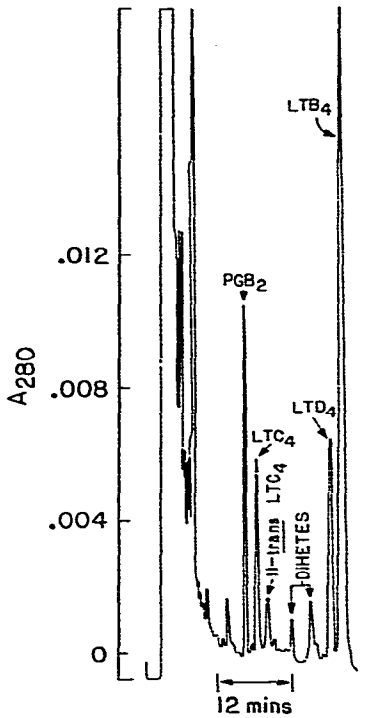

Fig. 1. Elution pattern of varying amounts of standards: PGB_2, LTC_4, 11-*trans* LTC_4, LTD_4, two non-enzymatically formed Δ^6-*trans*-LTB_4 [dihydroxyeicosatetraenoic acid (DiHETE)] isomers of LTB_4, and LTB_4. Column, 5 μm ODS (Altex); flow-rate 1 ml/min; mobile phase, methanol—water—acetic acid— ammonium hydroxide (67:33:0.08:0.04), pH 6.2.

Although it solved the problem of achieving a reproducible elution pattern, the above technique did not correct the variable and inadequate recovery of leukotrienes (especially LTC_4 and LTD_4) from the Altex column when nanogram to microgram quantities of standard (of known spectrophotometric absorbance) were injected onto the column. This problem was less severe for LTB_4 and minimal for the internal standard PGB_2, and therefore was presumptively ascribed to interactions between the peptide sequences of LTC_4 and LTD_4 and the column, leading to excessive retention. This problem was obviated by flushing the column overnight with several hundred ml of 3% diso-

dium EDTA (Fig. 2a) and maintaining the improvement by injecting 2–3 ml of EDTA through the U6K injector, guard column and column every morning prior to chromatography (Fig. 2b). This technique markedly increased recovery of LTC_4 (and the consequent sensitivity of the assay), such that reproducible and linear standard curves were achieved. Minimum detectable quantities were approximately 1 ng for LTB_4 and 2–3 ng for LTC_4 and LTD_4.

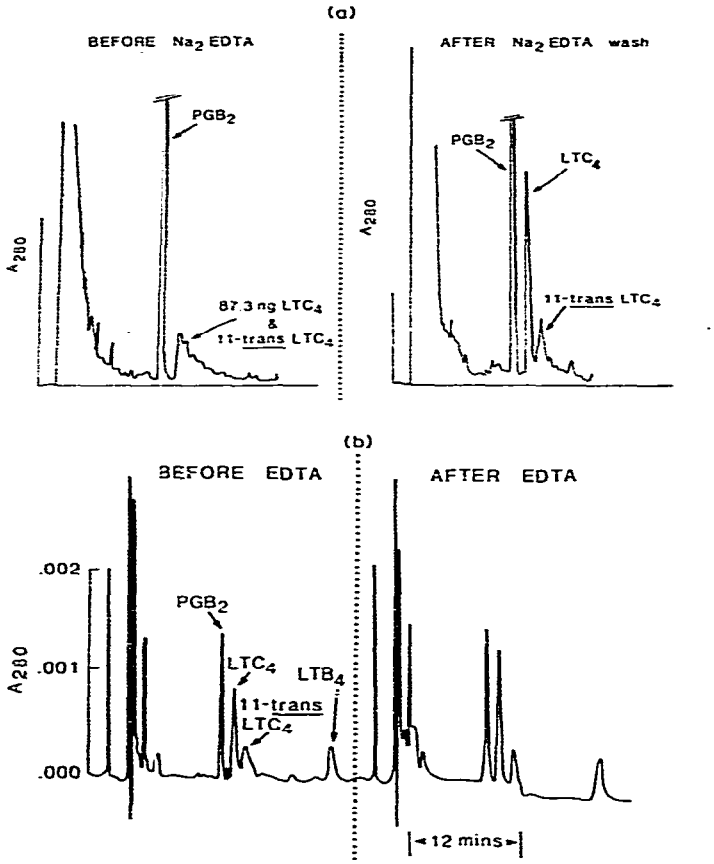

Fig. 2. (a) Effect on recovery of LTC_4 and 11-*trans* LTC_4 of an initial overnight rinse of the chromatographic column (Altex ODS) with disodium EDTA. (b) Effect of a brief (10 ml) morning rinse of EDTA to regenerate the HPLC column for the recoveries of leukotrienes after several hours' usage the preceding day. Note that the recoveries of LTC_4, 11-*trans* LTC_4 and LTB_4 are increased 44%, 25% and 22%, respectively, by EDTA, whereas that of PGB_2 is increased insignificantly (5%).

Extraction procedure

Initial attempts were made to develop a simple extraction technique using an ODS extraction column, following the work of Powell [10], who had previously observed greater than 90% recovery into methyl formate of prostaglandin standards (and about 50% recovery of 15-hydroxyeicosatetraenoic acid) added to biologic fluids, using this Sep-Pak. However, our initial studies demonstrated that recoveries of leukotrienes into methyl formate were poor. Although these

TABLE I

RECOVERY OF STANDARDS ADDED TO MASTOCYTOMA BUFFER (MCT, ref. 9) OR CULTURE MEDIUM (NCTC 135/MEDIUM 199) IN THE ABSENCE OR PRESENCE OF 1% FATTY ACID-FREE BOVINE SERUM ALBUMIN

Recoveries (mean ± S.D.) were assessed by measurement of peak heights of the samples and comparing them to values for a "standard curve" derived from external standard injections on the same day.

	PGB_2	LTB_4	LTC_4	LTD_4
Silica Sep-Pak				
MCT, no BSA*	82 ± 7 (n = 8)	76 ± 8 (n = 7)	93 ± 12 (n = 7)	86 ± 8 (n = 2)
MCT, with BSA	85 ± 8 (n = 5)	75 ± 6 (n = 5)	65 ± 10 (n = 5)	—
Medium, no BSA	91 ± 10 (n = 12)	76 ± 11 (n = 9)	81 ± 20 (n = 10)	—
Medium, with BSA	87 ± 10 (n = 3)	77 ± 6 (n = 3)	75 ± 5 (n = 3)	—
ODS (C_{18}) Sep-Pak				
Medium, no BSA	87 ± 15 (n = 7)	57 ± 20 (n = 6)	39 ± 18 (n = 6)	44 ± 8 (n = 2)

*BSA = bovine serum albumin.

recoveries were somewhat improved using methanol as the eluent (Table I), recoveries (particularly of LTC_4) were still unacceptable. Initial attempts to extract samples on silica columns instead of ODS were hindered by several artifactual peaks co-eluting near or with the leukotriene standards. These artifacts were traced to an unidentified contaminant washed off the silica in the Sep-Pak (and/or its container) after exposure to aqueous samples or water. It was empirically discovered that samples could be clarified of these peaks by a thorough pre-washing of the Sep-Pak using 120 ml of water followed by 20 ml of methanol and 20 ml of hexane. This procedure not only cleaned, but also pre-wetted, the Sep-Pak; contrary to the manufacturer's instructions, the latter was necessary for retention of compounds such as leukotrienes. (It was also noted that certain brands of plastic syringes used in the extraction procedure led to similar artifacts; therefore, only glass syringes were used thereafter.)

Following these washes, samples were placed on the extraction column and successively eluted with 10 ml of hexane, 5 ml of methylene chloride and 20 ml of pure methanol. The sample was then dried down using a rotary evaporator, brought up in methanol—water (30 : 70) and an aliquot injected on the column. For samples to which bovine serum albumin (1 g per 100 ml) had been added, the protein was first precipitated using 3 volumes of cold acetonitrile and 1 volume of cold methanol, followed by vigorous vortexing, 30 min of refrigeration and centrifugation. Following this the supernatant was decanted, evaporated and the residue brought up in 8 ml of water and placed on the Sep-Pak as described above.

Using these techniques, recoveries of leukotrienes and internal standard from

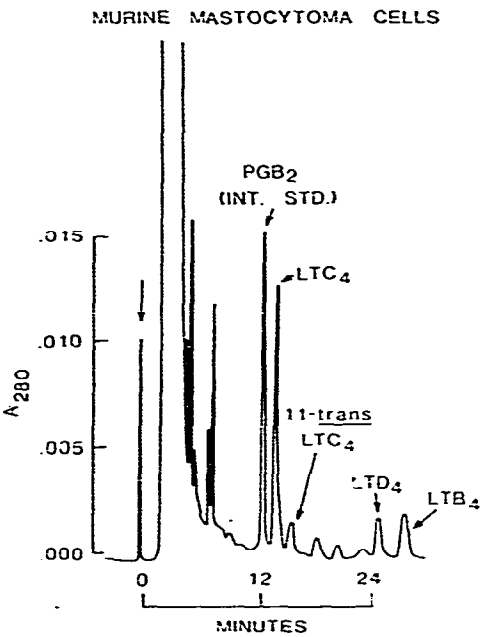

Fig. 3. Chromatogram of leukotrienes (and internal standard PGB_2) synthesized by murine mastocytoma cells, after protein precipitation, centrifugation, and sample extraction on silica Sep-Pak. Not labelled are several smaller peaks migrating between 11-*trans* LTC_4 and LTD_4 representing compounds which co-migrate with the non-enzymatically formed 5,12-dihydroxy isomers of LTB_4. Medium, minimum essential medium; extraction, silica (methanol); column, ODS (5 μm); mobile phase, methanol—water—acetic acid—ammonium hydroxide, pH 6.2; flow-rate 1 ml/min.

either buffer or culture medium were acceptably high and reproducible (in the presence or absence of exogenous bovine serum albumin) (Table I). However, it should be noted that in the case of LTC_4, the recovery appeared to be somewhat decreased by the presence of protein (Table I). Since the internal standard PGB_2 was not always recovered in an exact and equal proportion to each leukotriene under all circumstances, we generally correct the measured value of leukotrienes in biologic samples first for the recovery of the internal standard (PGB_2) and then for the ratio of the recovery of each leukotriene relative to PGB_2 for the sample type studied (i.e. medium or buffer) (Table I).

Using this system we measured the quantities of leukotrienes in one sample generated by murine mastocytoma cells [9] after extracting identical aliquots using three techniques: XAD-7 alone, XAD-7 followed by open silicic acid columns, and the simplified Sep-Pak extraction system described above (Fig. 3; Table II). Although recoveries after XAD-7 column alone were somewhat greater than with the silica Sep-Pak, the latter was comparable to the combination of XAD-7 and silicic acid open columns. Thus LTC_4 had a slightly greater recovery, LTB_4 isomers an identical recovery, and LTB_4 a slightly reduced recovery after Sep-Pak extraction, compared to XAD-7 and silicic acid columns (Table II).

TABLE II

COMPARISON OF THE RECOVERIES OF LEUKOTRIENES AFTER DIFFERENT EXTRACTION/PURIFICATION PROCEDURES

Aliquots of medium from one sample of murine mastocytoma cells, treated as previously described [9], were extracted on silica Sep-Pak columns (as described in text), or on XAD-7 columns (with or without open silicic acid columns) as previously described [8, 9]. Amounts of leukotrienes recovered after each method of extraction were quantified by HPLC on the same days using absolute absorbance units for quantitation. Values are expressed as percentage recovered (mean of two determinations each) relative to recovery from XAD-7 column alone (taken as 100%).

	Sep-Pak	XAD-7 and open silicic acid columns
LTC_4	83	69
11-*trans* LTC_4	63	63
Δ^6-*trans*-LTB_4 (isomer I)	66	68
Δ^6-*trans*-LTB_4 (isomer II)	63	63
LTB_4 (isomer III)	59	74

DISCUSSION

Most previous methods for the HPLC quantitation of leukotrienes either lack the required sensitivity, specificity, and selectivity for use in analytical separation of leukotrienes, or else they require tedious and time-consuming extraction processes (see, for example, refs. 2—7). For example, a new lot of XAD-7 can require more than a day in preparation alone before it can be used, and variable recoveries can result from impurities in some batches. We developed earlier an HPLC method which partly solved the first series of problems [8]. However, when adapted for use on commercially available columns, the reproducibility of the method and the recovery of leukotrienes proved to be inadequate. We discovered that a combination of increasing the ionic strength of the mobile phase and washing the column with 3% disodium EDTA, as well as exact preparation of solvent batches, resolved these problems. Although the mechanisms of the first two effects were not pursued further, they might be explicable in the first case, in part, by the increased buffering capacity of the system (thereby preventing even minimal changes in pH during the course of the HPLC runs), or, more likely, by a form of ion pairing with ammonium and acetate ions acting essentially to suppress both free carboxyl (COO^-) and amino (NH_3^+) functions on leukotrienes C_4 and D_4 which could not be simultaneously ion-suppressed by either acetic acid or ammonium hydroxide alone. It may be fruitful in the future to attempt to apply more conventional ion-pairing techniques (e.g. tetrabutylammonium ion in phosphate buffer) to the HPLC assay of leukotrienes. The EDTA effect suggests that negatively charged moieties on the leukotrienes were interacting with some cations retained on the column, leading to a destruction of the triene chromophore or to marked retention of all molecules except for a small proportion which were ion-suppressed at the pH used.

Although our initial attempts to use an ODS extraction column were not

encouraging, we found that we could reproducibly extract samples using a normal-phase silica extraction column providing that it was extensively washed beforehand to remove several spurious peaks co-eluting with leukotrienes and absorbing at 280 nm. Since extraction was carried out on a silica column using organic solvents, many unwanted contaminants with a lesser polarity than prostaglandins and leukotrienes (such as some neutral lipids) might be expected to be removed by the hexane or methylene chloride washes. This can be contrasted to XAD which primarily clarifies the sample of salts and polar compounds. Other investigators may wish to modify the choice of solvents used for particular purposes (e.g. substitute chloroform or ether for hexane or methylene chloride during the extraction), but ethyl acetate should be avoided since it will remove the internal standard PGB_2 and 5,12-dihydroxyeicosatetraenoic acids from retained LTC_4 and LTD_4 [9]. Some more polar compounds elute from the silica Sep-Pak with the leukotrienes but they migrate from the HPLC column in, or close to, the solvent front, with little or no baseline disturbance at 280 nm remaining by the elution time of the compounds of interest. However, proteins should first be removed by a denaturation/centrifugation step and cells, cell fragments and other particulates should likewise be removed by filtration or centrifugation. Thus, the current technique combines an extraction technique based on the retention of hydrophilic (polar) compounds on silica with an HPLC column which retains progressively more hydrophobic (non-polar) compounds with increasing avidity. The leukotrienes (being long-chain fatty acids with polar constituents) are selectively recovered after the combination of these two steps.

A sample can be extracted and quantitated on HPLC using the system described above in less than 1 h compared to 1–1.5 days required for the initial clean-up of the XAD, the extraction, then open silicic acid column chromatography, and finally HPLC. Furthermore, data obtained measuring aliquots of a sample of leukotrienes generated by mastocytoma cells suggest that the current technique yields results similar to those derived using the more time-consuming extraction techniques. Although recoveries are lower than those after XAD alone, the loss is modest and more than compensated for by the ease and speed of processing samples. Furthermore, this system can be used analytically or semi-preparatively (i.e., microgram quantities of leukotrienes). Thus this system should facilitate the measurement of leukotrienes in large numbers of biologic samples.

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