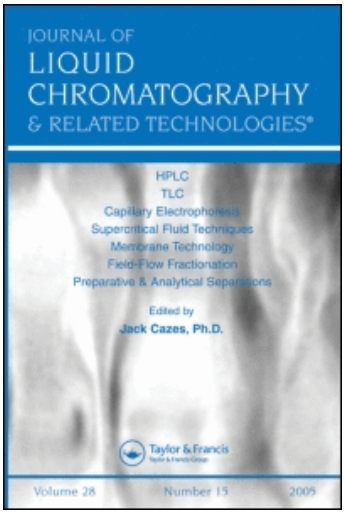

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QUANTIFICATION OF LEUKOTRIENE B_4 USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Leukotriene B_4 (LTB_4) was derivatized with p-(9-anthroyloxy)phenyl bromide forming the panacyl ester. The yield from this reaction was 95 percent. Subsequent separation of this ester using reversed phase high-performance liquid chromatography resulted in a greater than 10 fold increase in sensitivity as measured directly with ultraviolet light (absorption at 254 nm). Sensitivity was 16 ng and elution time was less than 15 minutes. We applied this technique to quantification of LTB_4 production by human polymorphonuclear leucocytes.

INTRODUCTION

Leukotrienes are metabolites of arachidonic acid produced by the lipoxygenase pathway. Leukotrienes induce a variety of biological effects including inflammation, contraction of smooth muscle and secretion of mucous, at very low concentrations. Leukotriene B_4 (LTB_4) is a non-peptidyl leukotriene with a number of biological actions. It affects adhesion of

neutrophils (1), is a potent chemotactic agent (1,2), and possesses ionophoric activity (3).

The activity of LTB_4 in a variety of cell systems suggests that its measurement in biological fluids may be important. Bioassay is non-specific in that a biological response may be generated by an interfering substance within the solution being tested (4). Radioimmunoassay is quite sensitive (4,5) and when combined with high-performance liquid chromatography (HPLC), is fairly specific. However, it is tedious and subject to the availability of antibodies. Furthermore, most of the polyclonal antibodies currently available cross react with other leukotrienes, dihydroxyeicosatetraenoic acids, and perhaps other unidentified molecules in a biological sample. Thus, radioimmunological assays frequently must be combined with HPLC. LTB_4 absorbs ultraviolet (uv) light maximally at 272 nm with a molar extinction coefficient of 50,000 cm^{-1} M^{-1}. Although there are claims of sensitivity of 1-2.5 ng/ml using HPLC and ultraviolet absorption (6,7), our experience with this technique has consistently provided a sensitivity of approximately 200 ng/ml. We have previously demonstrated the utility of derivatization in the measurement of monohydroxyeicosatetraenoic acids (mono-HETEs) (8). Therefore, to improve sensitivity, esterification of the dihydroxyeicosatetraenoic acid LTB_4 with p-(9-anthroyloxy) phenacyl bromide (PAB) forming the panacyl esters was carried out. The ester was separated using reversed phase HPLC and monitored by uv absorption directly at 254 nm. Sensitivity was increased to 16 ng/ml using this technique. This technique was applied to the quantification of LTB_4 by human peripheral blood polymorphonuclear leucocytes (PMN).

MATERIALS AND METHODS

Chemicals

Leukotriene B_4 was synthesized and generously provided by Dr. John Gleason, Smith Kline and French Laboratories and was greater than 98% pure as

determined by HPLC. ^{3}H-LTB_4 (30-60 Ci/mmol) was purchased from New England Nuclear, Boston, MA. p-(9-anthroyloxy)phenacyl bromide (PAB) was a gift from Dr. Walter Morozowich, The Upjohn Company, Kalamazoo, MI. Water, acetonitrile, ethanol, acetic acid, triethylamine (TEA) and tetrahydrofuran (THF) were all HPLC-grade (Fisher Scientific, King of Prussia, PA).

Instrumentation

HPLC (Waters Associates, Milford, MA) was performed with the following components: two Model 6000-A Solvent Delivery Systems, a Model 660 Solvent Programmer or a 720 Systems Controller, a Model 710B Waters Intelligent Sampling Processor, a Z Module Radial Compression Separation System, a Model 441 Fixed Wavelength Ultraviolet Absorbance Detector equipped with a mercury lamp and a 254 nm filter, and a Model 7000 Data Module. Separations were performed on an 8 mm internal-diameter (8mm x 20 cm) octadecylsilyl (ODS) (4 μ particle diameter) Radial Pak column (Waters Associates, Milford, MA).

The sample injection value was 200 ul. The mobile phase was acetonitrile and 0.1% aqueous acetic acid. The initial condition of the gradient was 56% acetonitrile. This was increased to 78% acetonitrile over 20 min using curve 2 of the solvent programmer. A flow rate of 5 ml per min was used throughout.

Derivatization

LTB_4 and ^{3}H-LTB_4 in methanol were dried under a stream of N_2. PAB was dissolved in a solution of acetonitrile:tetrahydrofuran (4:1, v:v). PAB solution (1 ml) and 3 ul triethylamine were added to each aliquot to be esterified. The stoichiometry was adjusted such that the molar ratio of PAB to LTB_4 was at least 4:1. Standards were placed in teflon-capped glass vials and incubated at 37^{o}C for 24 hr. Anyhydrous conditions are required to prevent hydrolysis of the ester back to the acid.

Cells

Heparinized peripheral venous blood was obtained from normal volunteers and PMN were prepared by isopycnic centrifugation using a Ficoll-Hypaque gradient, followed by sedimentation in 6% dextran (8). Contaminating erythrocytes were removed by hypotonic lysis and low-speed centrifugation. More than 98% of the recovered cells were PMN as determined by Wright stain, and greater than 95% of the cells were viable as determined by trypan blue exclusion. Cells were suspended in Hanks Balanced Salt Solution (GIBCO, Grand Island, NY).

RESULTS AND DISCUSSION

To show derivatization and to quantify the recovery from the derivatization, ^{3}H-LTB_4 was used (Fig. 1). The upper panel shows the absorbance of derivatized LTB_4. Aliquots of 2.5 ul fractions were collected and the radioactivity was counted using Beckman HpB fluor. The results were plotted as a function of time for nonderivatized ^{3}H-LTB_4 (middle panel of Fig. 1) and derivatized ^{3}H-LTB_4 (lower panel of Fig. 1). Derivatization of ^{3}H-LTB_4 resulted in a predictable increase in retention time since the panacyl ester is less polar than the free acid, and should be retained on the column longer in a reversed-phase system. In addition, it was determined in three separate experiments that greater than 95 percent of the total ^{3}H-LTB_4 was derivatized.

A convex gradient (curve 2) was utilized to elute panacyl-LTB_4 at 10.86 min in a sharp symmetrical peak, thus optimizing for sensitivity and separating derivatized LTB_4 from reaction impurities and byproducts. The maximum sensitivity achieved by this technique was 16 ng/ml. This technique was applied to quantification of LTB_4 production by human PMN: production of approximately 300 ng/5 x 10^6 cells after stimulation with 1 uM calcium ionophore A23187 for 5 min (Fig. 2) was noted. Quantification of LTB_4 production by this technique is at least ten-fold greater than that reported by other investigators using different systems (10,11).

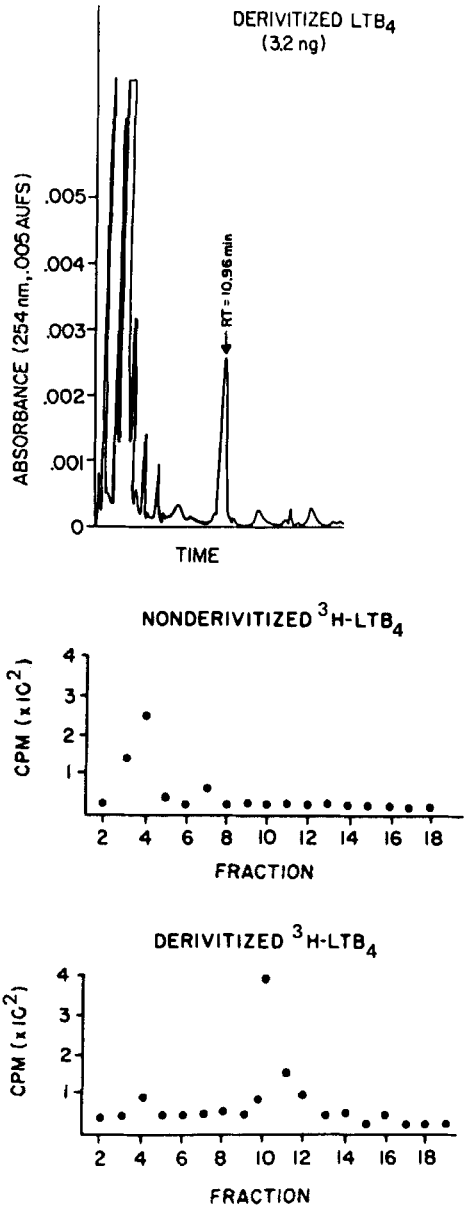

Figure 1. Derivatized LTB_4 (upper panel) and fraction collection of non-derivatized ^3H-LTB_4 (middle panel) and derivatized ^3H-LTB_4 (lower panel) injected into chromatograph: Flow rate 5.0 ml/min, acetonitrile:0.1% acetic acid, 56 to 78% of the former curve 2 over 20 min. Column is a 4 u particle-diameter ODS.

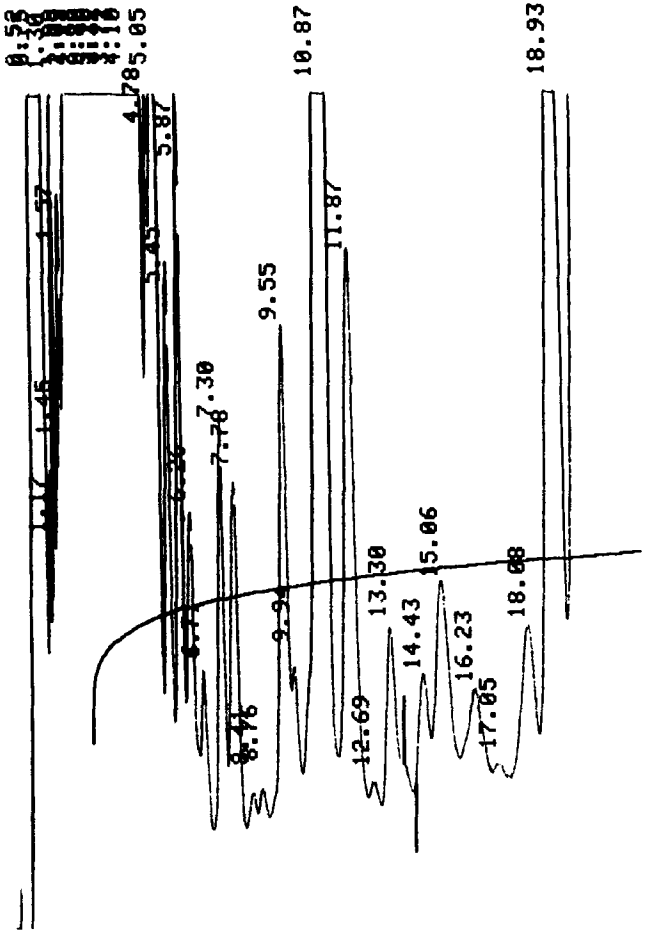

Figure 2. Derivatization and measurement of LTB_4 (RT=10.87 min) and 5-HETE (RT=18.93 min) from normal human peripheral blood polymorphonuclear leukocytes following stimulation with 1 uM calcium ionophore A23187 for 5 min.

Derivatization may be useful for improving detection by liquid chromatography (12). It has been reported that prostanoids and mono-HETEs can be derivatized using PAB and measured by HPLC (8,13). In this report we demonstrate that LTB_4, a di-HETE, can also be derivatized and measured by this procedure. This technique can be applied to measurement of these compounds in biological samples (Fig. 2). In addition to improved sensitivity, the procedure allows detection of all eicosanoids, except peptidyl leukotrienes, using a single wave length detector.

SUMMARY

LTB_4 was derivatized with p-(9-anthroyloxy)penacyl bromide forming the panacyl ester of LTB_4. Using HPLC and direct ultraviolet absorption at 254 nm, a sensitivity of 16 ng/ml was achieved. The technique is applicable to the analysis of biological specimens, including LTB_4 production by human PMN.

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