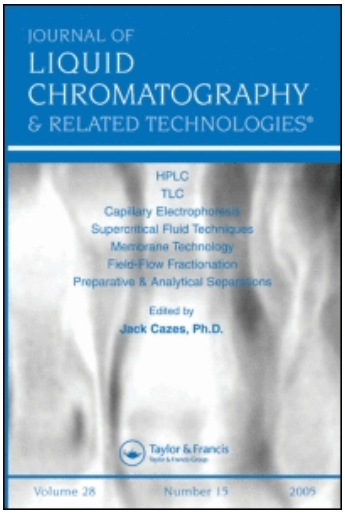

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Formation of Isoindole Derivatives of Sulfidopeptide Leukotrienes by Reaction with O-Phthalaldehyde and Separation by Reverse Phase High Performance Liquid Chromatography

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FORMATION OF ISOINDOLE DERIVATIVES OF SULFIDOPEPTIDE LEUKOTRIENES BY REACTION WITH o-PHTHALALDEHYDE AND SEPARATION BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method is described for the formation of fluorescent conjugates of the sulfidopeptide leukotrienes (LTC_4, LTD_4, and LTE_4) by reaction of the primary amine moiety of these metabolites with o-phthalaldehyde. Separation of the fluorescent derivatives was achieved by reverse-phase high performance liquid chromatography in less than 30 minutes using a convex gradient of methanol-50 mM Na Acetate-5% Tetrahydrofuran pH 5.5. Detection limits realized under the conditions described were 0.35 ng, 3.8 ng and 3.7 ng for LTC_4, LTD_4, and LTE_4, respectively. This represents an increased sensitivity over detection of these metabolites by ultra-violet spectroscopy. The leukotriene-OPA derivatives are fully stable for 50 minutes at 23°C and for at least 4 hours at 0°C. The method is applied to the detection of LTC_4 generated by zymosan stimulated murine peritoneal macrophages.

INTRODUCTION

Leukotrienes C_4, D_4. and E_4, recognized as the slow-reacting substance of anaphylaxis, exert potent effects on a variety of tissues. These and other products of the lipoxygenase-pathway have been implicated in the pathology of asthma and hypersensitivity reactions (1,2). The recent interest in the biochemistry of the leukotrienes has necessitated the development of sensitive and reliable methods for the detection of these metabolites. To date the primary detection methods available for the analysis of leukotrienes include reverse-phase high performance liquid chromatography (RP-HPLC) with detection by ultra-violet (UV) absorbance, bioassay with the guinea pig ileum, mass spectrometry, and radioimmunoassay (RIA) (3,4). Though RIA offers an exquisite degree of sensitivity its specificity is questionable, due to cross-reactivity with other arachidonate metabolites. The use of UV absorbance subsequent to RP-HPLC affords selectivity, but lacks a high degree of sensitivity. Fluorescence detection offers selectivity at specific excitation and emission wavelengths, while increasing the sensitivity over UV limits (13).

The limits on the spectroscopic detection of sulfidopeptide leukotrienes may be increased by reaction of the primary amine of LTC_4, LTD_4, and LTE_4 in the formation of a fluorescent conjugate. The fluorogenic reaction of primary amines with o-phthalaldehyde (OPA) in the presence of a thiol yields strongly fluorescent 1,2 disubstituted isoindoles (5-9). Pre-column derivatization of primary amines with OPA in the presence of 2-mercaptoethanol followed

by RP-HPLC has become a popular method for the detection of amino acids (10-12) and catecholamines (13). Though other reagents can react similarly with primary amines to form fluorescent conjugates the use of OPA has been found to be superior to either fluorescamine (13,14) or ninhydrin (11).

The present report demonstrates the relative ease and sensitivity of using OPA as a pre-column derivatization reagent for detection of peptidoleukotrienes. The method is applied to the detection of LTC_4 released from zymosan stimulated mouse peritoneal macrophages (15).

MATERIALS

Synthetic LTC_4, LTD_4, and LTE_4 were a gift of Dr. J. Rokach, Merck-Frosst, Quebec, Canada. [^{3}H] LTC_4, [^{3}H] LTD_4, [^{3}H] LTE_4, [^{3}H] LTB_4, [^{3}H] 15-HETE, [^{3}H] 12-HETE, [^{3}H] 5-HETE and [^{3}H] arachidonic acid were products of New England Nuclear, Boston, MA. o-Phthalaldehyde, γ-glutamyltranspeptidase and zymosan were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were of HPLC grade quality.

The HPLC system consisted of two Waters model 510 pumps operated through a Waters Automated Gradient Controller and Autochrom solvent selector. Injections were made with a Waters U6K injector and detection monitored in series with a Waters model 481 variable wavelength detector set at 280 nm and a McPherson model 749 flow-through spectrofluorometer set at an excitation and emission wavelength of 340 nm and 455 nm, respectively. The cell volume was 0.02 ml. Separations were achieved on a 4.6 x 250 mm Altex 5 µm Utrasphere-ODS column.

Aqueous mobile phase was prepared by dissolving 6.804 g of Na Acetate in 700 ml of double distilled water. Fifty ml of Tetrahydrofuran (THF) was added and the solution mixed. The solvent was adjusted to pH 5.5 with 12N HCl and the volume adjusted to 1 liter. Organic mobile phase consisted of 100% methanol. Mobile phases were filtered and degassed daily under vacuum.

o-Phthalaldehyde was prepared essentially as described (12). Briefly, 270 mg OPA, in absolute ethanol, was added to 40 ml of 0.4M boric acid and the pH adjusted to 9.5 with 5N NaOH. To this was added 0.2 ml of 2-mercaptoethanol and the volume brought to 50 ml with buffer. The solution was shielded from light and reagent strength maintained by the addition of 0.02 ml of 2-mercapto-ethanol every 4 days. The reagent was used for approximately one month.

The concentrations of stock solutions of leukotriene standards were determined immediately prior to use by absorbance at 280 nm and assuming a molar extinction coefficient of 40,000 M^{-1} (3). All dilutions were made with double distilled water and kept at 4°C for one week. The 11-trans isomer of LTC_4 was prepared as described (16) and the N-acetyl derivative of [^{3}H] LTE_4 was synthesized by reaction with acetic anhydride in Na_2CO_3 (17).

METHODS

Mixed solutions of leukotriene standards were reacted with at least a 400-fold mole excess of OPA for 2 minutes at room temperature. An aliquot of the reaction mixture, 2-10 µl, was then

injected and separated by gradient elution at ambient temperature at a flow rate of 1 ml/min. Initial elution was at 50% methanol-50% 50 mM Na Acetate-5% THF pH 5.5 for 4 minutes after which a convex gradient (curve 3) was initiated to 75% methanol-25% 50 mM Na Acetate-5% THF pH 5.5 over 10 minutes and held constant for an additional 9 minutes. The column was then washed with 100% methanol for 10 minutes and brought to initial conditions by increasing the aqueous percentage of mobile phase at the rate of 10% per minute. Total run time including reequilibration was 45 minutes.

LTC_4 was treated with γ-glutamyl transpeptidase (γ-GTPase) prior to derivatization with OPA. LTC_4 (32 μM) in 0.1M KH_2PO_4 pH 8.0, was reacted with 0.1 mg of γ-GTPase for 30 minutes at 37°C with constant shaking. The reaction was terminated by the addition of 0.5 volume of 0.5% acetic acid in methanol. Following centrifugation, an aliquot of the supernatant was reacted with 0.5 volume of OPA for 2 minutes. Control reactions received protein after quenching and were derivatized as above.

Mouse macrophages were obtained by peritoneal lavage as described (15). Cells were plated on 35 mm plastic dishes in RPMI medium containing 10% fetal calf serum at a density of 2×10^6 cells per well. After 2-3 hours at 37°C in 5% CO_2 in air the nonadherent cells were removed by PBS washings. Adherent cells were incubated overnight in RPMI medium containing 10% fetal calf serum with 1 μCi of [^{3}H] arachidonic acid. Following incubation, the medium was removed and the cells incubated in RPMI medium

containing 0.40 mg of zymosan for 2 hours. The supernatant was centrifuged and extracted into ethanol (16).

RESULTS AND DISCUSSION

Optimization of Reaction and Chromatography Conditions

Previous reports have determined that the response of OPA derivatives of amino acids is independent of reagent composition as long as at least a 200-fold excess of reagent is maintained (12). For a fixed reaction time of 2 minutes the relative fluorescence of the leukotriene-OPA (LT-OPA) derivatives was maximal at a 300 to 400-fold mole excess of reagent to analyte.

The effect of mobile phase pH on the fluorescence and separation of the LT-OPA derivatives was investigated from pH 3.0 to pH 6.5. Maximal fluorescence and resolution was observed at a mobile phase pH of 5.5. The percentage of methanol and THF present in the mobile phase yielded optimal resolution of all LT-OPA conjugates at a final concentration of 75% and 1.25%, respectively. Higher concentrations of either methanol (i.e. 76-100%) or THF (i.e. 10-35%) interfered with the resolution of LTD_4-OPA from LTE_4-OPA.

The fluorescence excitation and emission wavelengths were varied from 310 nm to 350 nm and 435 nm to 465 nm, respectively. Maximal response was obtained with an excitation wavelength between 330 to 350 nm and an emission wavelength of 455 nm.

The reaction was judged to be essentially complete after 2 minutes at room temperature when the appropriate ratio of

reagent to analyte was used. This was determined by the absence of an UV response corresponding to an underivatized standard. As expected, formation of the LT-OPA conjugate increased the hydrophobicity of the molecule and its retention on the column matrix. Underivatized LTD_4 and LTE_4 were not separated in this system.

Figure 1 shows the separation obtained for the OPA conjugates of the peptidoleukotrienes. Separation was achieved in about 25 minutes with complete resolution of the three conjugates. Peaks labeled 1 and 2 were identified as glutamate and glycine, respectively. The identity of fluorescent peaks eluting between 11-16 minutes was not ascertained. Use of radiolabeled leukotriene standards found less than 10% of the radioactivity eluting in this region. Peaks labeled 3, 4, 5, and 6 represent the OPA conjugates of LTC_4, 11-trans LTC_4, LTD_4, and LTE_4 respectively, as identified by the comigration of a fluorescent response with the major fraction of radiolabel. Recoveries of the tritium label coeluting with fluorescent peaks for LTC_4, LTD_4, and LTE_4 were found to be 86%, 56%, and 83%, respectively. The [^{3}H] LTD_4 preparation also showed about 12% of the label migrating as LTE_4-OPA. Separation of 11-trans LTC_4-OPA was not achieved in this system and appears as a small shoulder on the LTC_4-OPA peak. Identification of the derivatives was further characterized by UV spectroscopy, enzymatic, and chemical treatment of leukotriene standards prior to derivatization.

The UV spectra of the isolated LT-OPA derivatives are shown in Figure 2. Both LTC_4-OPA and LTD_4-OPA exhibit the charac-

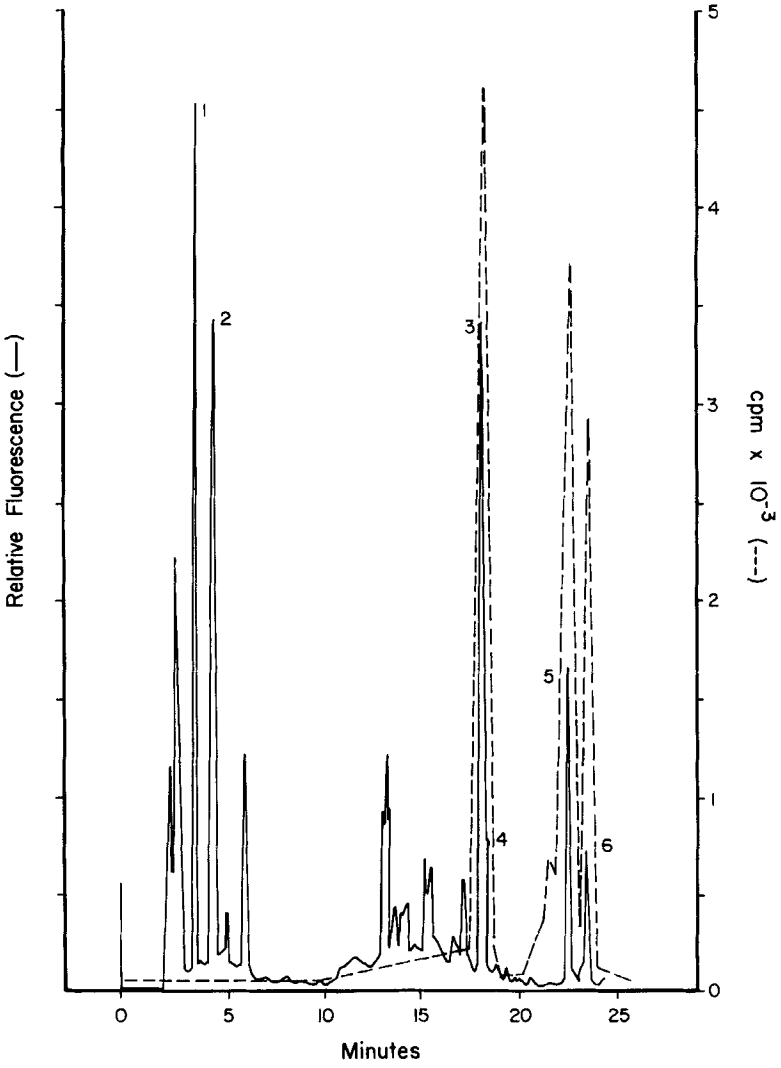

FIGURE 1. Elution Profile of [^{3}H] Leukotriene-OPA Derivatives. [^{3}H] LTC_4 (0.86 nmol), 11-trans LTC_4 (0.40 nmol), [^{3}H] LTD_4 (1.30 nmol) and [^{3}H] LTE_4 (0.91 nmol) were reacted with 1.6 µmols of OPA for 2 minutes at 23°C. An aliquot of the reaction mix was chromatographed as described in METHODS and fractions of 1 ml collected and quantitated by scintillation spectrometry. Fluorescent (——) and radioactive (----) peaks were identified as (1) glutamate, (2) glycine, (3) LTC_4-OPA, (4) 11-trans LTC_4-OPA, (5) LTD_4-OPA and (6) LTE_4-OPA.

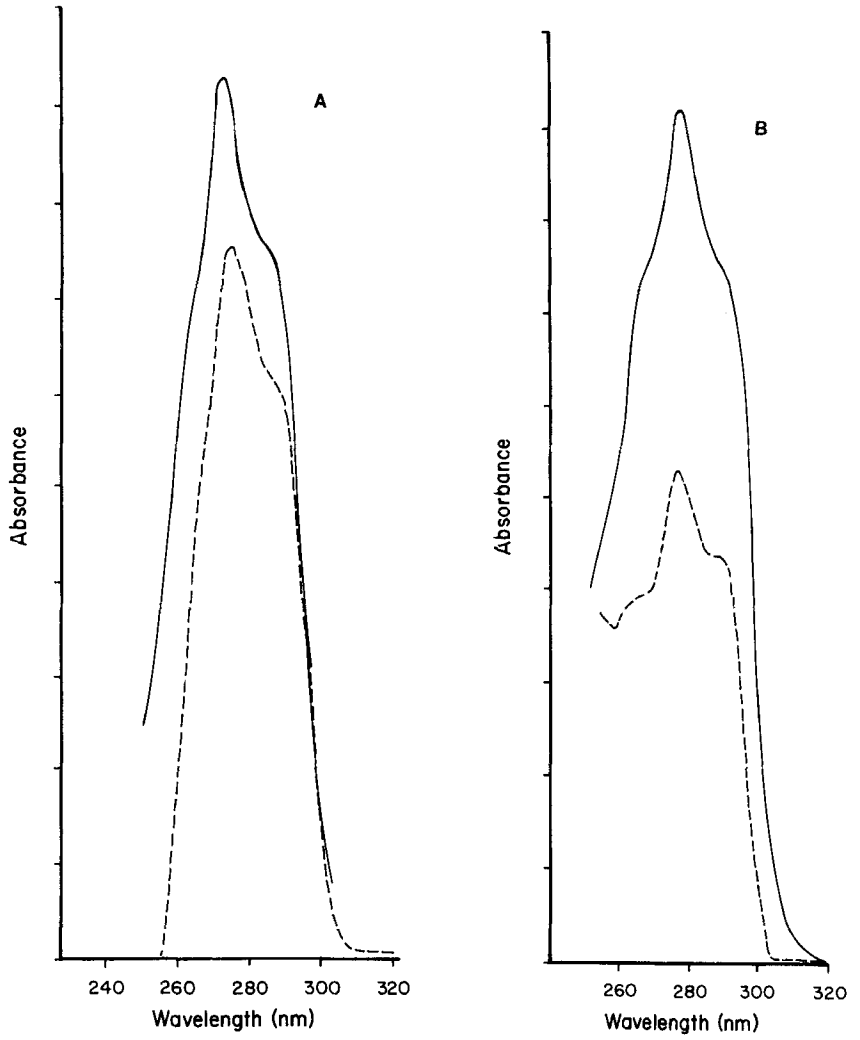

FIGURE 2. Ultra-Violet Spectra of Leukotriene-OPA Derivatives. (A) Spectra of 0.30 nmols of LTC_4 before (——) and after (----) derivatization with 80 nmols of OPA. (B) Spectra of 0.40 nmols of LTD_4 before (——) and of 0.20 nmols of LTD_4 after (----) derivatization with 80 nmols of OPA. (C) Spectra of 0.46 nmols of LTE_4 before (——) and after (----) derivatization with 90 nmols of OPA. All spectra were taken in 0.25M borate pH 9.5 with NaOH before and after a 2 minute reaction at 23°C in a volume of 0.5 ml using a Beckman 2000 Spectrophotometer.

(continued)

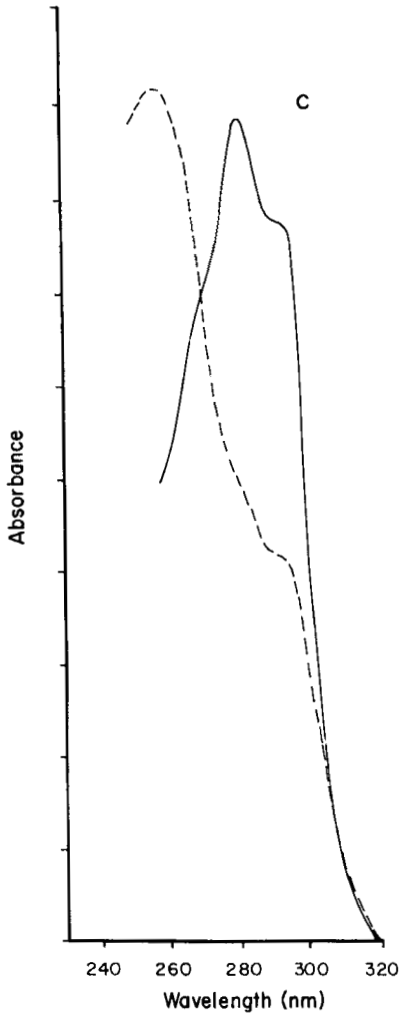

FIGURE 2C

teristic absorption maximum at 280 nm with a shoulder at 292 nm. However, the absence of a shoulder at 271 nm was observed with the LTC_4-OPA conjugate, while a broad shoulder was apparent at 272 nm with increasing absorbance at shorter wavelengths with the LTD_4-OPA derivative. The absorption spectrum of LTE_4-OPA displays the characteristic shoulder at 292 nm, but exhibits a strong hypsochromic shift of 22 nm. The coelution of a fluorescent peak with the tritium label of authentic standards of LTC_4, LTD_4, and LTE_4 and the above UV spectral data strongly supports the structure of the products as being a 1-alkylthio-2-glutathionyl, cysteinylglcyl, or cysteinyl-5-hydroxy eicosatetranoate substituted isoindole (5,9) (Figure 3). The characterization of these products by UV spectroscopy indicates that isoindole formation has no effect on the absorption maximum elicited by the α-substituted conjugated triene of LTC_4 and LTD_4. However, isoindole formation does interfere with the absorption at higher frequencies. This may be due to an increasing loss in coplanarity of the bonds of the parent lipid as the peptide substituents are removed and steric effects increase. This phenomenon would account for the 22 nm hypsochromic shift resulting from formation of the LTE_4 isoindole, which should be the most sterically hindered of the derivatives (Figure 3) (18).

Enzymatic reaction of LTC_4 by γ-GTPase prior to derivatization hydrolyzed 90% of the substrate. This was estimated by comparing the unhydrolyzed LTC_4 (Fig. 4a) with the enzymatically treated LTC_4, and the appearance of a peak with a retention time

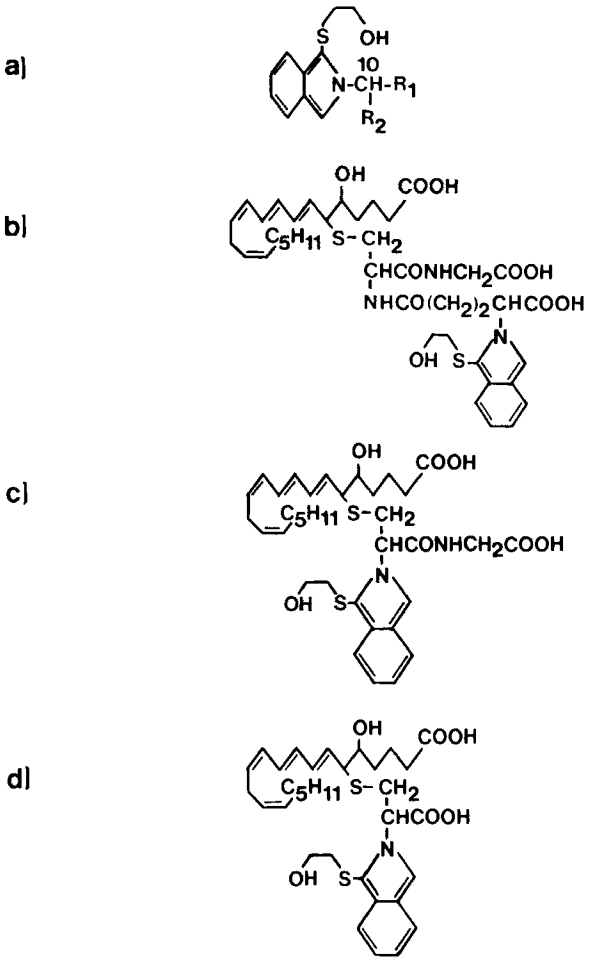

FIGURE 3. Postulated Structures of Leukotriene-OPA Derivatives. Substituted isoindole (a), LTC_4-OPA (b), LTD_4-OPA (c), and LTE_4-OPA (d).

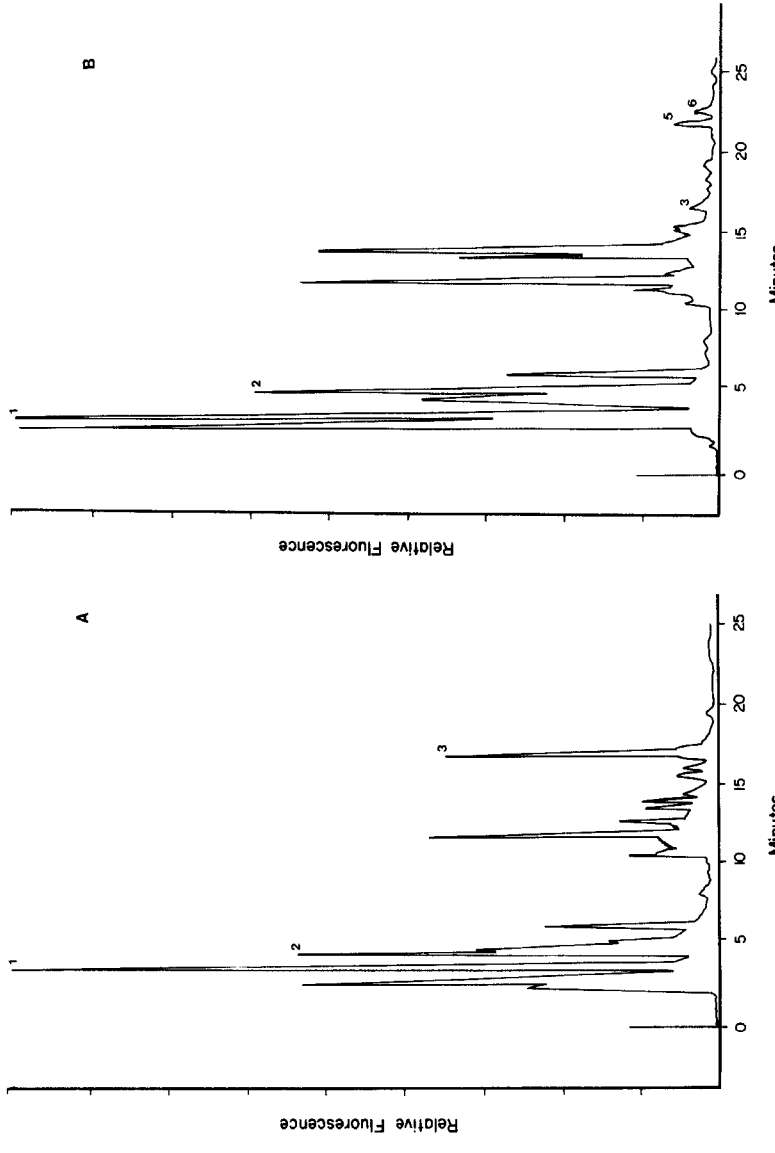

FIGURE 4. Treatment of LTC_4 with γ-GTPase Prior to OPA Derivatization and Response of [^{3}H] LTE_4 to OPA After Acetylation. Chromatography of 0.55 nmols of LTC_4 (A) enzyme control, (B) after reaction with 0.10 mg of γ-GTPase as described in METHODS. (C) Response of 1.8 nmols of LTE_4 after reaction with a 400-fold excess of OPA. (D) Response of 0.28 nmols of N-acetyl-[^{3}H] LTE_4 following reaction with a 400-fold excess of OPA. Chromatography was performed as described in METHODS. Peaks are as in Figure 1.

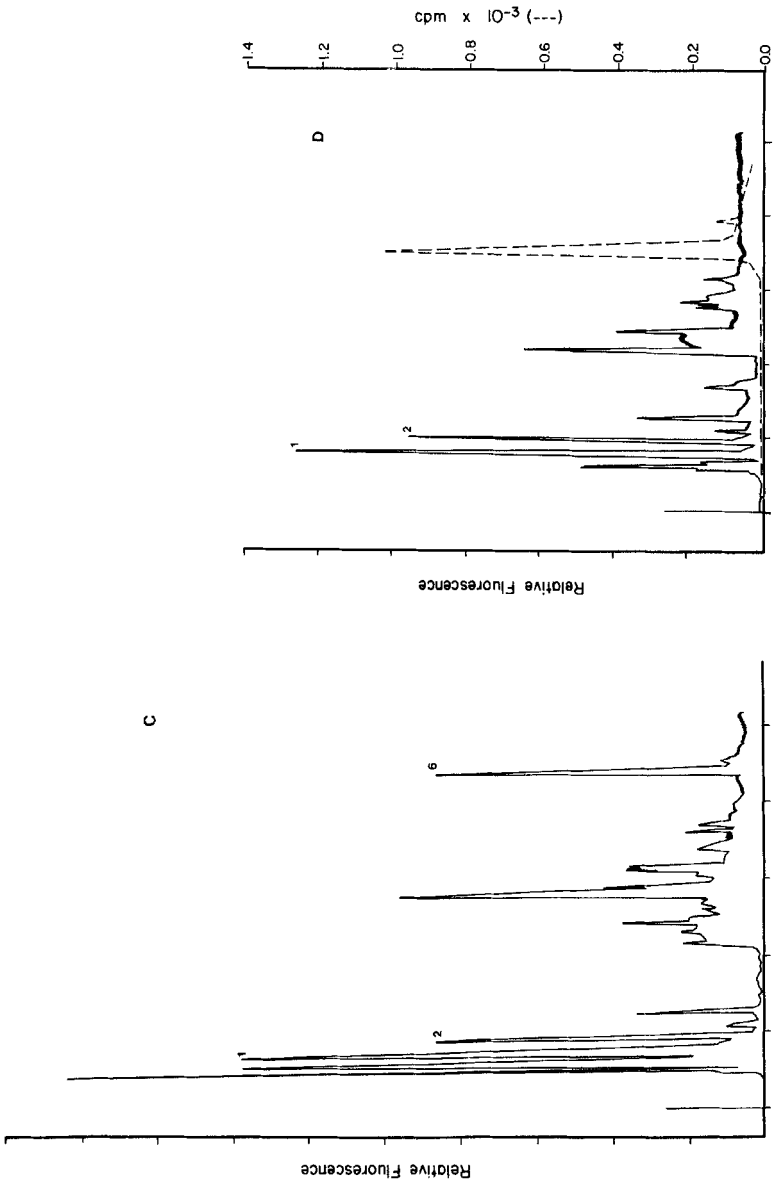

FIGURE 4D

FIGURE 4C

similar to LTD_4-OPA (Figure 4b). Identification of LTD_4-OPA was based on its UV spectrum and migration as a radiolabeled fluorescent peak with a retention time between that of LTC_4-OPA and LTE_4-OPA. The identification of the LTE_4-OPA conjugate was further verified by reacting [^{3}H] LTE_4 with acetic anhydride (17). This renders the molecule resistant to reaction with OPA. Elution of N-acetyl[^{3}H] LTE_4 was at 18 minutes and lacked a fluorescent response (Figure 4).

Linearity and Conjugate Stability

Figure 5 depicts the response of increasing concentrations of the leukotrienes following derivatization. Linearity is seen from 0.6 ng to 22 ng for LTC_4-OPA, from 3.8 ng to 26 ng for LTD_4-OPA and from 3.7 ng to 28 ng for LTE_4-OPA. The detection limits of the leukotrienes under the conditions described were 0.57 pmols (0.35 ng), 7.6 pmols (3.8 ng), and 9.0 pmols (3.7 ng) for LTC_4, LTD_4 and LTE_4, respectively. Reaction of equimolar amounts of leukotriene did not result in a fluorescent response of equal intensity, even though the reaction of each was judged to be complete.

The stability of the LT-OPA conjugates was monitored for 4-5 hours at 23°C and 0°C (Figure 6 and Table 1). At 23°C the conjugates appear relatively stable for about 1 hour, after which a steady rate of degradation is apparent. Approximately 66% of the initial fluorescence was lost after 5 hours. After derivatization, while maintaining the conjugates at 0°C, the fluorescence of the LTC_4 and LTD_4 derivatives appeared fully stable for at least

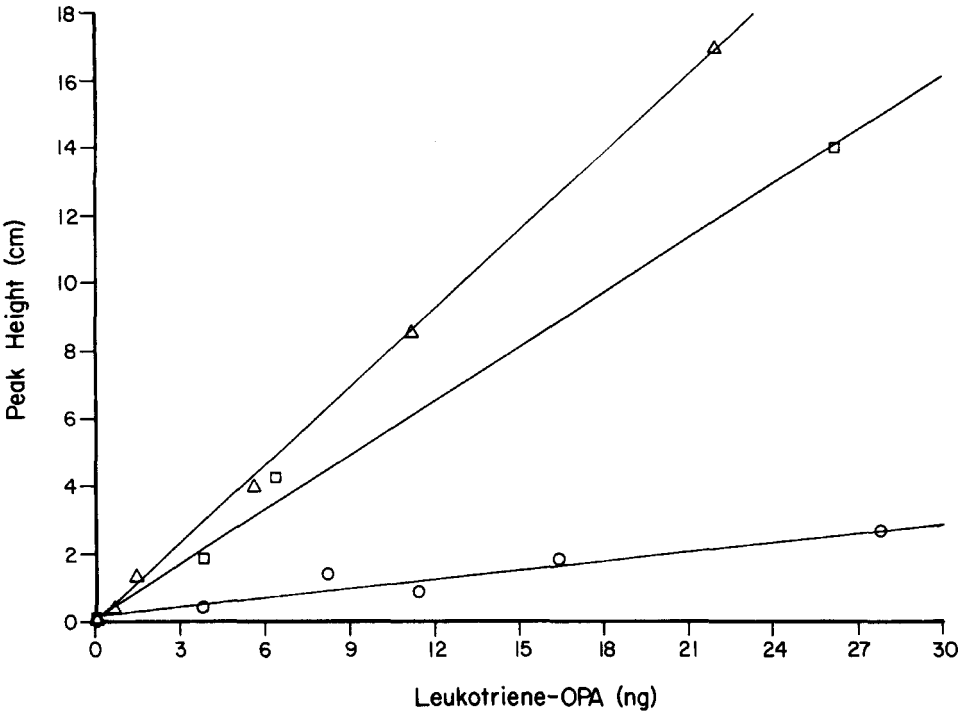

FIGURE 5. Linearity of Response of LTC_4, LTD_4 and LTE_4-OPA Derivatives. LTC_4 (32 ng, △), LTD_4 (40 ng, □), and LTE_4 (46.1 ng, ○) were reacted with 80 nmols of OPA, the reaction mix diluted with water, and 0.002 ml injected to give the the indicated amounts. Chromatography was performed as described in METHODS.

TABLE 1

Stability of LT-OPA Conjugates. LTC_4 (1.6 ng) and LTD_4 (2.7 ng) were reacted with 0.070 ml of OPA for 2 minutes at 23°C in a final volume of 0.1 ml and kept at 0°C. Injections of 0.01 ml were made at the indicated times and chromatographed as described in METHODS.

	Time after LT-OPA conjugate formation (minutes)				
	0	48	94	140	228
Conjugate	% Remaining				
LTC_4 -OPA	100	104	102	101	102
LTD_4 -OPA	100	110	108	110	110

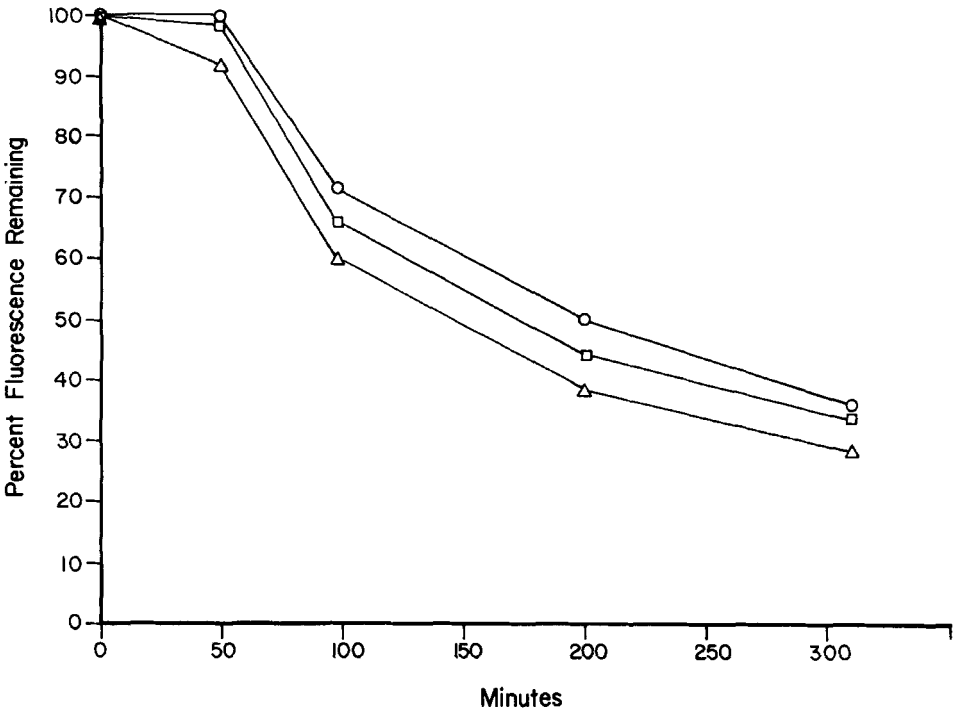

FIGURE 6. Stability of LTC_4, LTD_4 and LTE_4-OPA Derivatives at 23°C. LTC_4 (321 ng,△), LTD_4 (408 ng,□) and LTE_4 (237 ng,○) were reacted with 2.8 µmols of OPA in a final volume of 0.10 ml. An aliquot, 0.01 ml, was injected after 2 minutes, the remaining mix kept at 23°C, and 0.01 ml injections made at the indicated times. Data is presented as percent response remaining after initial 2 minute injection (time 0).

228 minutes. The rate of degradation of the LT-OPA conjugates revealed an unusual degree of stability for substituted isoindoles (5,9). Previous reports have noted that slight increases in stability occur as the N-substituents of the isoindole become larger (Figure 3) as well as when the N-substituent is additionally substituted at carbon-10 (5,12). Also, increased stability of isoindoles has been observed if the substituent at C-10 was a car-

boxylate anion (5,12,19). The LT-OPA conjugates of LTC_4 and LTE_4 fullfil both of these requirements, while the LTD_4 conjugate lacks a carboxyl group at the C-10 position (Figure 3). However, the stability of LTC_4-OPA < LTD_4-OPA < LTE_4-OPA (Figure 6). Thus, it is postulated that the bulky lipid moiety of these conjugates serves to stabilize the molecule more than the presence of a carboxylate anion at C-10 of the N-substituent. When the peptide moieties are removed, stability is enhanced due to increased steric effects (5). It is possible that the stability of these molecules can be further enhanced by the use of a bulkier thiol compound (5,9).

Biological Application

The utility of this method in detecting biologically derived LTC_4 was demonstrated using zymosan stimulated mouse peritoneal macrophages (15). Figure 7 shows the fluorescent response of an aliquot of the macrophage extract before and after treatment with γ-GTPase. The samples were diluted 120-fold after enzyme treatment to minimize background fluorescence due to OPA reacting with endogenous primary amines. Enzyme treatment abolished 72% of the response, compared to the nontreated control. Chromatography of the extract in a step gradient of methanol:water:acetic acid (60:40:0.08 v/v), pH 6.2 with NH_4OH, to 100% methanol over 60 minutes (16) separated the leukotrienes and dihydroxy eicosatetranoates produced by the stimulated cells (15) and showed 25% of the total radioactivity migrating with a retention time similar to

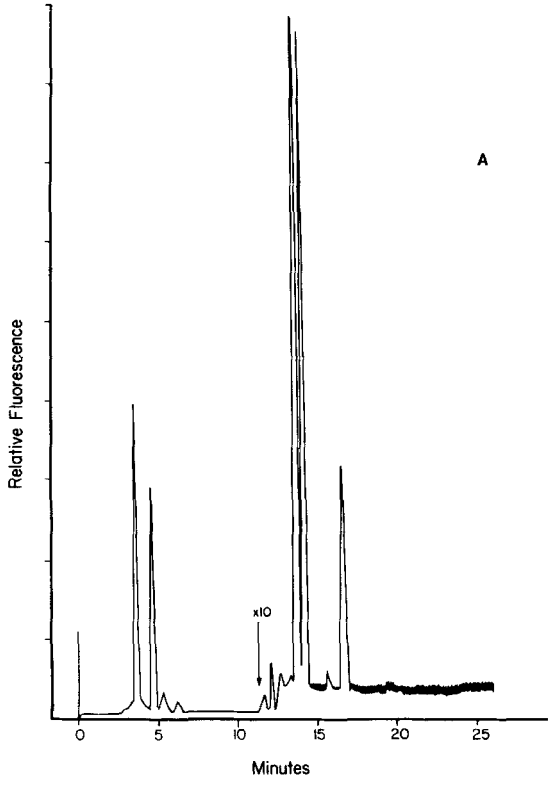

FIGURE 7. Derivatization of Mouse Macrophage Extract Before and After Treatment with γ-GTPase. An 0.01 ml aliquot of the extracted macrophage supernatant was reacted with 0.10 mg of γ-GTPase in 0.05 ml of 0.1M KH_2 PO_4 pH 8.0 for 30 minutes at 37°C. The reaction was terminated by adding 1 volume of 0.5% acetic acid in methanol and the mix centrifuged. The control reaction received enzyme after quenching. Both mixes were diluted 20-fold with water, 0.01 ml reacted with 80 nmols of OPA for 2 minutes at 23°C, and 2 µl of the reaction mix chromatographed as described in METHODS. The response of LTC_4 in the enzyme control (A) and enzyme treatment (B) co-chromatographed with authentic LTC_4-OPA.

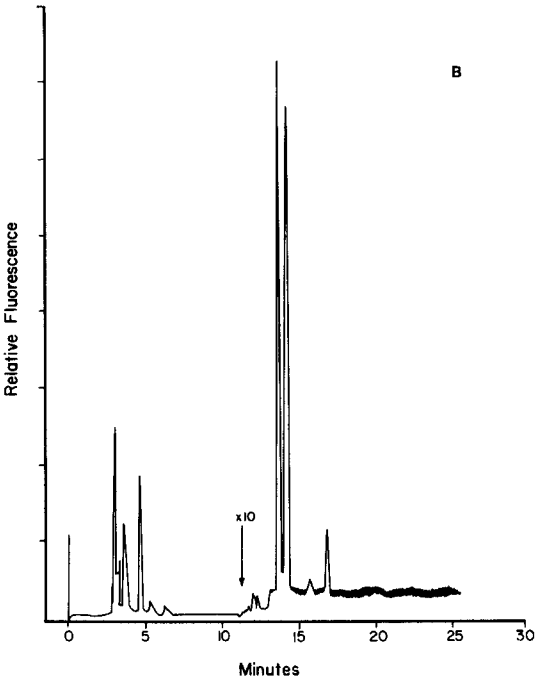

FIGURE 7B

authentic [^{3}H] LTC_4 (Figure 8). The absence of LTD_4 and LTE_4 was confirmed in both systems. The ability of the extract to induce a sustained contraction on an isolated segment of guinea pig ileum, inhibitable by the leukotriene receptor antagonist FPL 55712, further confirmed the presence of biologically active slow-reacting substance of anaphylaxis (data not shown) (3,4).

In summary, OPA may be used to form highly fluorescent derivatives of peptidoleukotrienes, which can be separated by RP-HPLC in less than 30 minutes. The derivatives are formed in a 2 minute reaction in aqueous solution and appear to be fully

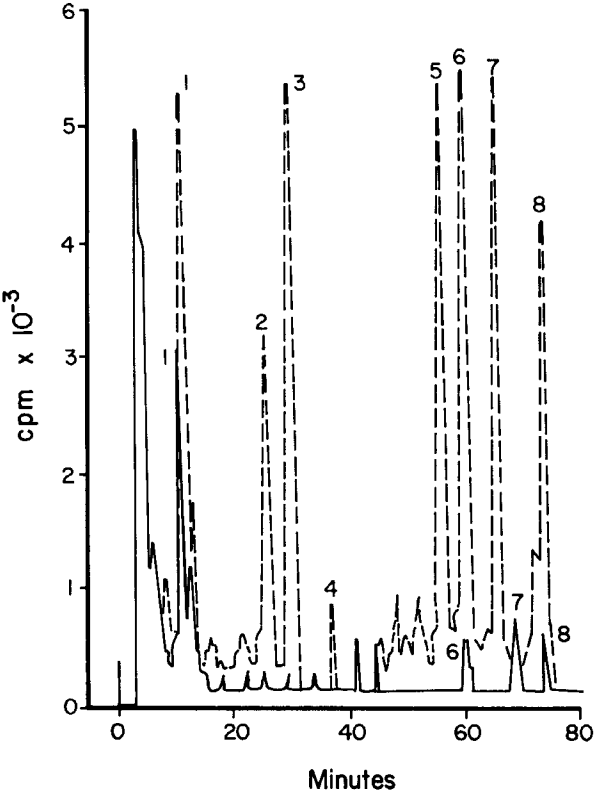

FIGURE 8. Radioactive Elution Profile of Products of Zymosan Stimulated Mouse Macrophages. A 0.75 ml aliquot of the final methanol extract was concentrated under argon, brought to 0.1 ml with 30% methanol, and chromatographed in a step gradient of methanol:water:acetic acid (60:40:0.08 v/v/v) pH 6.2 with NH_4OH for 30 minutes to methanol:water:acetic acid (70:30:0.08 v/v/v) pH 6.2 for 30 minutes to 100% methanol. Products present in the macrophage extract (——) were identified by comigration with tritiated standards (----). Peak 1, LTC_4; Peak 2, LTD_4; Peak 3, LTB_4; Peak 4, LTE_4; Peak 5, 15-HETE; Peak 6, 12-HETE; Peak 7, 5-HETE; and Peak 8,arachidonic acid.

stable for 1 hour at room temperature. The procedure is inexpensive, affords an increased sensitivity over UV detection, especially for LTC_4, and offers another means for the physical characterization of biogenically derived peptidoleukotrienes.

ACKNOWLEDGEMENTS

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