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A REEVALUATION OF THE CHEMOTACTIC POTENCY OF LEUKOTRIENE B_4 (LTB_4)*

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SUMMARY: The chemotactic potency of leukotriene B_4 (LTB_4) was reevaluated based on an improved purification procedure which combines reversed phase and straight phase high pressure liquid chromatography (RP-HPLC and SP-HPLC). So-called LTB_4 isomer III prepared by RP-HPLC contains two double oxygenated 5,12-dihydroxy acids in addition to LTB_4. On a molar basis, the chemotactic activity of LTB_4 repurified by SP-HPLC was far greater than that of the other two 5,12- dihydroxy acids and comparable to that of formyl-methionyl-leucyl-phenylalanine (fMLP). The chemotactic activity of LTB_4 isomer III is dependent upon the relative concentrations of the double oxygenated 5,12-dihydroxy acids and LTB_4. Further purification of peak III by SP-HPLC is required before assessing the biologic activity of LTB_4.

Leukotriene B_4 (5S, 12R-dihydroxy-6,8,10,14 (cis, trans, trans, cis)-icosatetraenoic acid) is formed in polymorphonuclear leukocytes (PMNL) from arachidonic acid by a 5-lipoxygenase. It exhibits a variety of biologic effects such as binding to its cell membrane receptors (1); and, stimulation of calcium entry (2), enzyme release (3) and chemotaxis (4,5). In most studies, LTB_4 has been obtained from incubations of PMNL with the calcium ionophore, A23187, with or without arachidonic acid, and purified by the reversed phase high pressure liquid chromatography (RP-HPLC) procedure reported by Borgeat et al (6). Furthermore, an extinction coefficient of 40,000 corresponding to an ultraviolet absorption band at 280 nm has been routinely utilized to calculate its concentration (4-8). I wish to report that the chemotactic potency of LTB_4 has been underestimated, based on two observations: 1) the so-called isomer III on RP-HPLC contains, in addition to LTB_4, two other leukotrienes, both double oxygenated; 2) the maximum UV absorption of LTB_4 occurs at 270 nm, which corresponds to an extinction coefficient of 51,000 and not 40,000.

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MATERIALS AND METHODS

Human PMNL obtained by leukopheresis from healthy individual donors were isolated by dextran sedimentation and hypotonic hemolysis as described previously (9,10). Cells (50 ml of 50-100 x 10^6 cells/ml) were suspended in Hank's buffer pH 7.4 containing Hepes (6 g/L) and were incubated at 37°C for 10 minutes in the presence of 15 µM arachidonic acid (Nu Chek Prep, Inc., Elysian, MN 56028) and 5 µM ionophore A-23187 (Calbiochem-Behring Corp., La Jolla, CA 92037) each in 50 µl of ethanol. Reaction was stopped with 75 ml of methanol and the leukotrienes were extracted with 300 ml of methyl t-butyl ether* at pH 3.0. The extract was washed twice with 200 ml and 100 ml of distilled water and evaporated in vacuo. The residue was dissolved in 1 ml of hexane/methyl t-butyl ether 85/15 V/V and applied into a 30 x 1 cm glass column packed with 2 g of silicar (CC-4, Mallinckrodt, Inc., Paris, KY 40361). Substances in the column were sequentially eluted with 60 ml of hexane/methyl t-butyl ether 85/15 V/V which removes arachidonic acid and other unpolar substances and 60 ml of methyl t-butyl ether/methanol 95/5 V/V which removes mono-, di- and trihydroxy acids including the leukotrienes. The methyl t-butyl ether/methanol extract was evaporated to dryness, dissolved in 250 µl of methanol/water/ acetic acid 60/40/0.01 and injected into a Waters HPLC equipped with a model U6K injector, a model M-45 solvent delivery system, a model 450 variable wavelength detector and an Omni Scribe B-500 recorder (Houston Instruments, Austin, Texas 78753). Leukotrienes were eluted from a reversed phase Radial-PAK cartridge (C_{18}, 8 mm ID x 10 cm, 5 µ) in a Z-module system at 4 ml/min. Peaks detected at 270 nm were collected, reinjected into a straight phase Radial-PAK cartridge (SI, 8 mm ID x 10 cm, 5 µ) in a Z-module system at 4 ml/min and eluted with methyl t-butyl ether/ hexane/acetic acid 40/60/1 V/V/V. Peaks at 270 nm were collected, dried down, dissolved in 1 ml of methanol, quantitated by UV spectroscopy and assayed for chemotaxis. For the purpose of comparison, a portion of the methyl t-butyl ether/methanol extract was injected into a 30 cm x 3.9 mm, µ Bondapak C_{18} reversed phase column and eluted with methanol/water/acetic acid 75/25/0.01 V/V/V (6). Peaks at 270 nm were then reinjected into the straight phase HPLC system described above.

Additional incubations (50 ml of 100 x 10^6 cells/ml) were carried out under vacuum, in the presence of O_{18} and 5 µM ionophore A-23187 at 37°C for 10 minutes. Extraction, column chromatography and HPLC were as described above.

CHEMOTACTIC ASSAY

A modification of the chromium-51 method of Gallin et al (11) was used. Twenty-five milliliters of blood from the antecubital vein of normal subjects was drawn into a 60 ml heparinized plastic syringe, mixed with 25 ml of 2% dextran in saline and left in the vertical position at room temperature for 30 minutes. The supernatant was transferred to a 50 ml plastic tube and centrifuged at 4° C, 200 g for 8 minutes. The leukocyte pellet was suspended in 20 ml of ammonium chloride-Tris buffer pH 7.4 (9 parts of 0.83% ammonium chloride and 1 part of Tris buffer) and incubated at 37° C for 10 minutes. The cell suspension was centrifuged again at 4° C, 200 g for 8 minutes and the leukocyte pellet was suspended in 5 ml of Hank's buffer pH 7.4 containing 6 g/L Hepes. Five milliliters of Ficoll-Hypaque (Pharmacia Fine Chemicals Division of Pharmacia, Inc., Piscataway, NJ 08854) was added and the mixture was centrifuged at 4° C, 1000 g for 30 minutes. Sedimented PMNL were suspended in 5 ml of Hank's buffer, counted and incubated with chromium-51 as sodium chromate in saline, (200-500 µCi/µg, New England Nuclear, Boston, MA 02118) 1 µCi per 10^6 cells at 37° C for 1 hour with continuous shaking. The PMNL suspension was centrifuged at 4° C, 200 g for 8 minutes and the pellet was washed twice with 20 ml of Hank's buffer. The final pellet containing more

* methyl t-butyl ether, methanol and hexane were HPLC quality, Burdick-Jackson Laboratories, Inc., Muskegon, Michigan 49442.

than 99% PMNL was suspended in Hank's buffer containing 2% bovine serum albumin, 2% potassium penicillin and 2% streptomycin sulfate. Volume was adjusted to obtain a PMNL concentration of 5 x 10^6 cells/ml. Chemotaxis was investigated in Boyden chambers (5/16" diameter wells, Neuro probe, Cabin Road, MD 20818). Chromium-51-labeled PMNL (2.5 x 10^6 cells in 0.5 ml) were placed in the upper compartment, the chemoattractant was dissolved in 1.5 ml of Hank's buffer was placed in the lower compartment and two-3μ cellulose nitrate filters (Neuro probe) were placed in between. The leukotrienes were dissolved in ethanol and Hank's buffer was added to a final ethanol concentration of 0.002-0.2%. The chambers were incubated at 37° C, 100% humidity, for 3 hours, the filters were removed, immersed briefly in normal saline and counted in a Packard Gamma Scintillation Spectrometer. Chemotaxis was expressed as percent of the counts in 10^6 PMNL that were present in the lower filter.

$$\frac{\text{c.p.m in lower filter x 100}}{\text{c.p.m. in } 10^6 \text{ PMNL}}$$

ULTRAVIOLET SPECTROMETRY

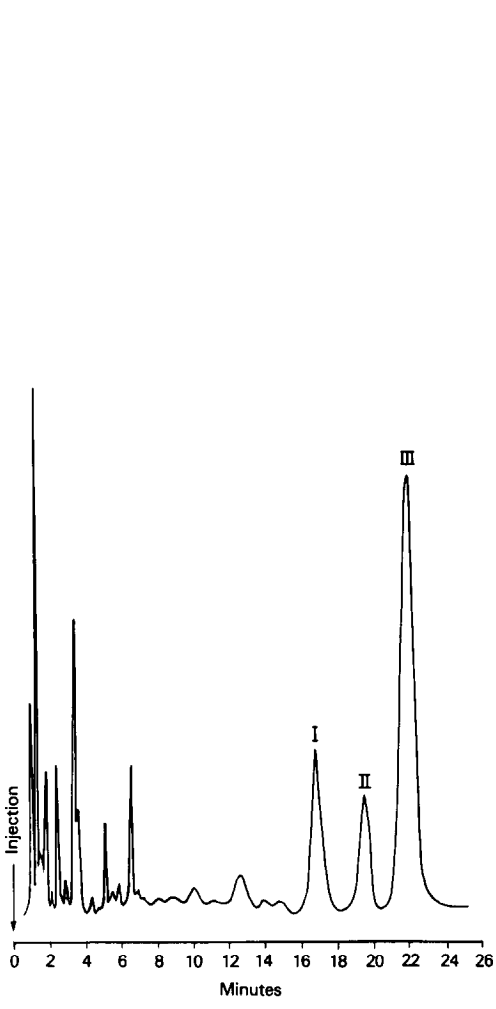
Scanning ultraviolet spectrometry was performed in a Cary-219 spectrophotometer. Samples were dissolved in methanol.

GAS CHROMATOGRAPHY - MASS SPECTROMETRY

Mass spectrometry was performed in a Dupont DP-102 GC/MS/DS, single focusing magnetic sector. Temperature of the injector was 225° C and the oven temperature was programmed from 150° C to 270° C at 10° per minute. Temperature of the jet was 200° C and the energy of ionization was 70eV. The column was 3 foot-long, 2 mm ID and packed with 100-120 mesh 3% SP-2250. Prior to injection into the GC/MS/DS instrument, samples were methylated with diazomethane and derivatized with bis (trimethylsilyl) trifluoroacetamide (Merck) as described previously (12).

RESULTS

The RP-HPLC chromatogram of an extract from incubations of PMNL with arachidonic acid and ionophore is shown in Figure 1. Three peaks eluting between 16 and 23 minutes were resolved. Separation of the three peaks under these conditions was much better than that obtained with the RP-HPLC procedure of Borgeat et al (6). Peaks I and II correspond to LTB_4 isomers I (5S, 12R-dihydroxy-6,8,10,14 (trans, trans, trans, cis)-icosatetraenoic acid) and II (5S, 12S-dihydroxy-6,8,10,14 (trans, trans, trans, cis)-icosatetraenoic acid). The third peak, commonly referred to as "LTB_4 isomer III" in the literature, contained at least three major compounds. Peak III on SP-HPLC with methyl t-butyl ether/hexane/acetic acid 40/60/1 V/V/V mobile phase was resolved into two distinct peaks (III-1 and III-2, Figure 2). Peak III-1 contained two double-oxygenated 5,12-dihydroxy acids having three UV absorption bands (258 nm, 268 nm and 278 nm). They could not be separated by HPLC



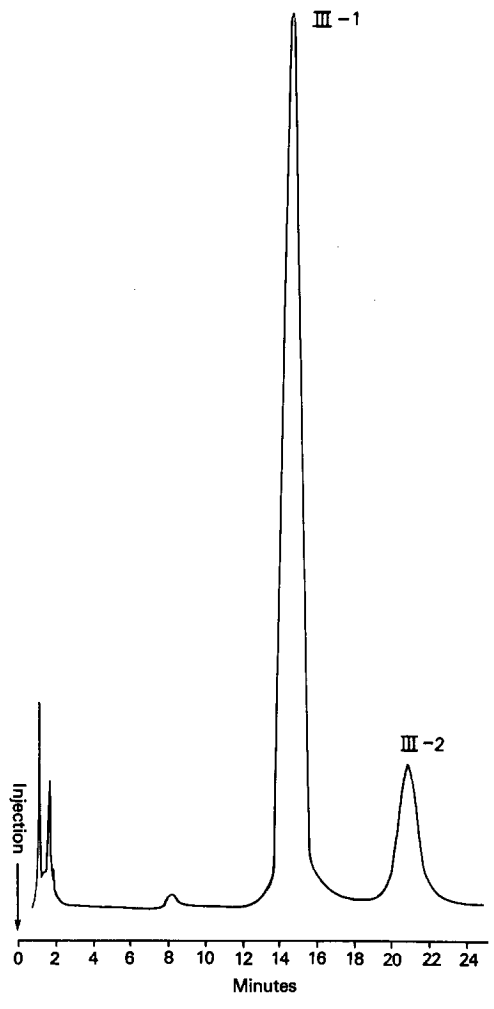
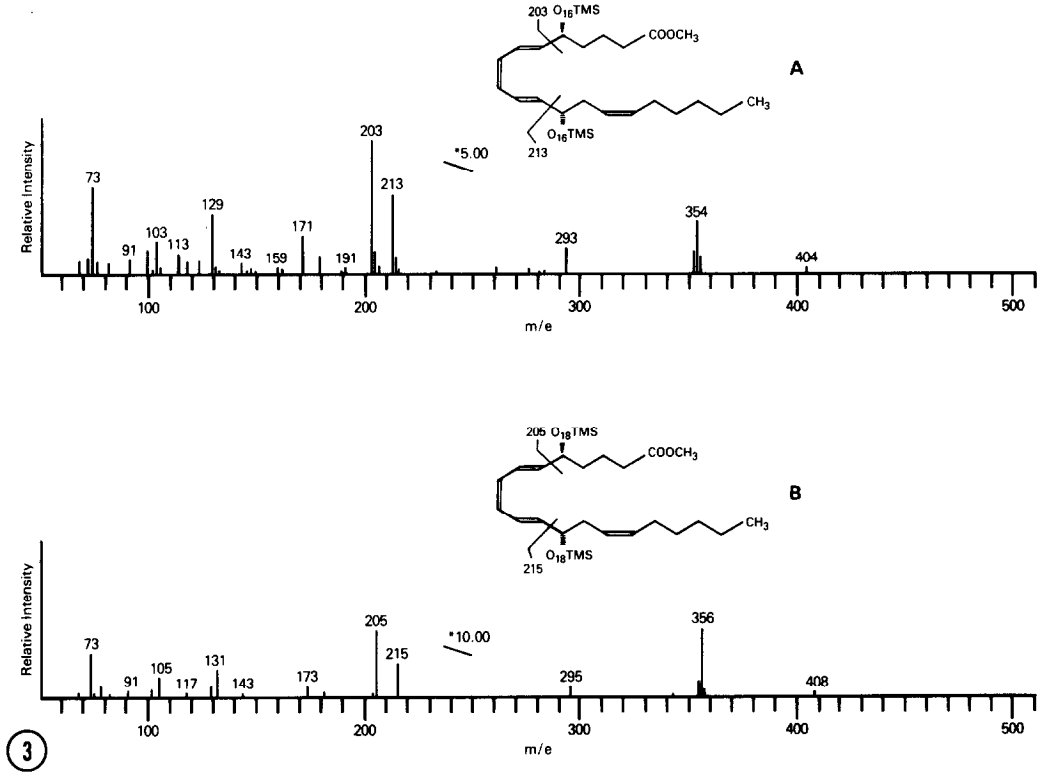


Figure 1: RP-HPLC chromatogram of the methyl t-butyl ether/methanol extract from an incubation of human PMNL (10 ml of 100 x 10^6 cells/ml) with 15 μM arachidonic acid and 5 μM ionophore for 10 minutes, Z-module system, Radial-PAK cartridge C_{18}, 8 mm ID x 10 cm, 5 μ. System: methanol/water/acetic acid 60/40/0.01 V/V/V. Pump rate: 4 ml/min. Ultraviolet absorption was monitored at 270 nm.

Figure 2: SP-HPLC of peak III from RP-HPLC, Z-module system, Radial-PAK cartridge SI, 8 mm ID x 10 cm, 5 μ. System: methyl t-butyl ether/hexane/ acetic acid 40/60/1 V/V/V. Pump rate: 4 ml/min. Ultraviolet absorption was monitored at 270 nm.

using a multiplicity of systems and procedures, but gas chromatography allowed the identification of two compounds with different retention times and mass spectra. Peak III-2 gave the characteristic UV pattern (absorption bands at 260 nm, 270 nm and 280 nm) and mass spectrum of 5S,12R-dihydroxy-6, 8,10,14 (cis, trans, trans, cis)-icosatetraenoic acid (LTB_4). Mass-spectra of the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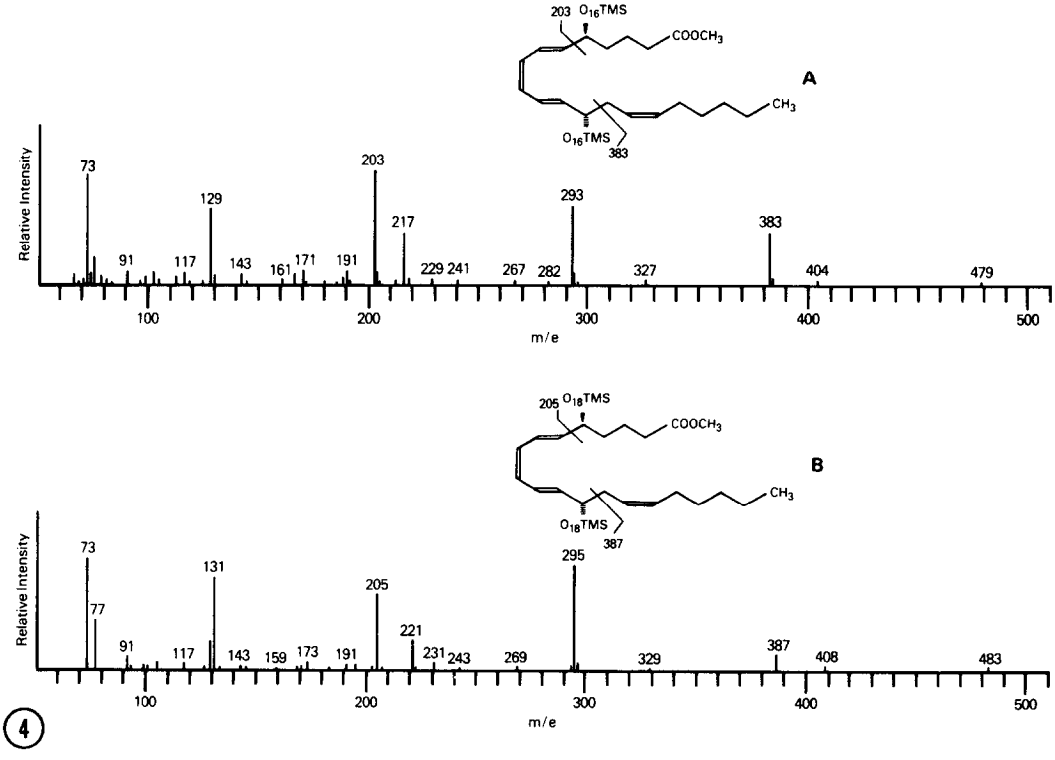
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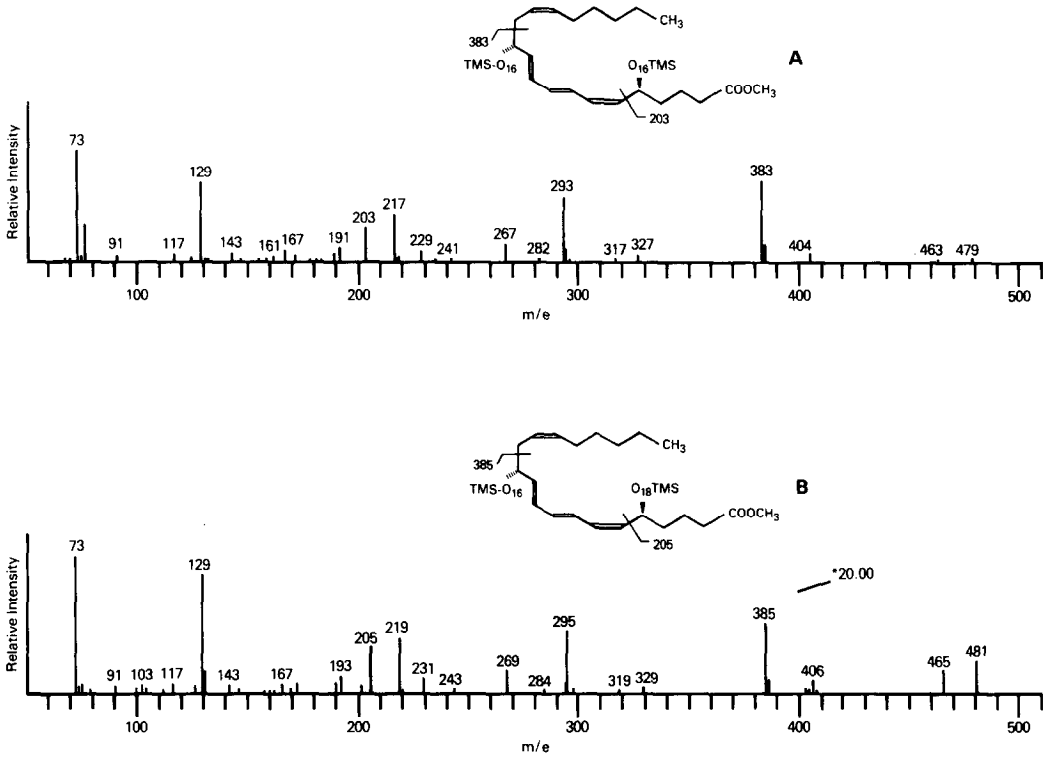

Figure 5: Mass spectra of compound III-2, C value 23.6. A = O_{16}, B = O_{18}. Shift of ion 203 to 205 and of ion 383 to 385 indicate incorporation of O_{18} only at C_5. Shift of ions 404 (M-90), 463 (M-31) and 479 (M-15) by two mass units indicate incorporation of one O_{18} molecule. The structure corresponds to 5S, 12R-dihydroxy-6,8,10,14 (cis, trans, trans, cis)-icosatetraenoic acid (17,18).

three 5,12-dihydroxy acids obtained after incubations of PMNL with ionophore in the presence or absence of O_{18} are depicted in Figures 3, 4 and 5. Equivalent chain lengths (C value) for the two compounds in peak III-1 were 22.4 and 23.6. Both compounds incorporated O_{18} at C_5 and C_{12} indicating double lipoxygenation at those positions (Figures 3 and 4). The C value of 23.6 and the mass spectrum of the compound in peak III-2 were those of LTB_4

Figure 3: Mass spectra of compound III-1, C value 22.4 A = O_{16}, B = O_{18}. Shift of ion 203 to 205 and of ion 213 to 215 indicate incorporation of O_{18} at C_5 and C_{12}. Shift of ion 404 (M-90) to 408 indicates an increase of 4 mass units in the molecular ion resulting from incorporations of two molecules of O_{18}. The structure corresponds to 5S, 12S-dihydroxy-6,8,10,14 (trans, cis, trans, cis)-icosatetraenoic acid (14).

Figure 4: Mass spectra of compound III-1, C value 23.6. A = O_{16}, B = O_{18}. Shift of ion 203 to 205 and of ion 383 to 387 indicate incorporation of O_{18} at C_5 and C_{12}. Shift of ion 404 (M-90) to 408 indicate an increase of 4 mass units in the molecular ion resulting from incorporation of two molecules of O_{18}. The structure corresponds to 5S, 12S-dihydroxy-6,8, 10,14 (trans, cis, trans, cis)-icosatetraenoic acid (15,16).

TABLE 1: Comparison of fMLP and compounds III, III-1 and III-2 as chemoattractants at a concentration of 10^{-7} M. All compounds were dissolved in Hank's buffer pH 7.4 containing 0.6% Hepes. A final alcohol concentration of 0.2%, present in all chemoattractant preparations and the control (Hank's buffer) did not inhibit chemotaxis.

Values represent $\frac{\text{c.p.m in lower filter x 100}}{\text{c.p.m in } 10^6 \text{ PMNL}}$

An extinction coefficient of 56,000 was used to calculate concentration of 5,12-dihydroxy acids in peak III for Experiment No. 1 (predominantly double oxygenated compounds with peak UV absorption at 268 nm). An extinction coefficient of 51,000 was used to calculate concentration of 5,12-dihydroxy acids in peak III for Experiment No. 2 (predominantly LTB_4 with peak UV absorption at 270 nm).

10^{-7}M is the optimal chemotactic concentration for both fMLP and compound III-2 (LTB_4). The chemotactic potency of compounds in peak III-1 increases at higher concentrations (10^{-5}M, 10^{-6}M).

	fMLP	III	III-1	III-2	Control
Experiment No. 1					
	24.7	6.0	4.7	31.7	4.0
	22.1	9.5	4.1	22.5	4.0
	21.2	4.2	1.0	31.0	1.0
mean (SD)	22.7 (1.8)	6.6 (2.7)	3.3 (2.0)	28.4 (5.1)	3.0 (1.7)
p		<0.009*	<0.001	NS	<0.001
Experiment No. 2					
	20.2	26.0	2.0	25.9	1.8
	21.3	23.7	2.9	21.4	2.9
	22.4	24.3	3.9	21.1	3.8
	19.1	21.2	3.9	23.2	3.8
mean (SD)	20.8 (1.4)	23.8 (2.0)	3.2 (0.9)	22.9 (2.2)	3.1 (1.0)
p		NS	<0.001	NS	<0.001

* All p values in reference to fMLP.

and incorporation of O_{18} occurred only at C_5 (Figure 5). Data on chemotaxis are presented in Table 1. The chemotactic activities of peak III before separation and of its component peaks, III-1 and III-2 were compared with that of the synthetic peptide formyl-methionyl-leucyl-phenylalanine (fMLP).

Material from peaks III, III-1 and III-2 were obtained in two separate experiments. In the first experiment, peak III-1 predominated whereas in the second experiment, peak III was composed almost exclusively of peak III-2. It can be observed (Table 1) that the chemotactic activity of peak III is variable and that this variability is dependent upon the concentration of peak III-2 (LTB_4). These observations were confirmed with material obtained from several other experiments.

DISCUSSION

The biologic activity of LTB_4 as a chemotactic agent has been confirmed by a number of investigators (4,5,13). However, from the data presented here accuracy of those reports is questioned. Two observations lend support to this conclusion: First, an extinction coefficient of 40,000 has been used to calculate LTB_4 concentrations from the ultraviolet spectrum. The peak absorption band for LTB_4 occurs at 270 nm giving a correct extinction coefficient of 51,000. Second, it has been assumed that so-called LTB_4 isomer III is a homogeneous peak which contains only LTB_4. We have shown that in addition to LTB_4, peak III contains two double oxygenated 5,12-dihydroxy acids which are much less potent than LTB_4 as chemoattractants. Because the peak ultraviolet absorption band for these compounds occurs at 268 nm, an extinction coefficient of 56,000 should be used to calculate their concentrations. In addition, the concentration of LTB_4 in peak III varies considerably from incubation to incubation. All of these factors have contributed to an underestimation of the chemotactic potency of LTB_4. We have found that on a molar basis LTB_4 is at least as potent as fMLP.

The two double oxygenated 5,12-dihydroxy acids have been identified by two other groups of investigators (14,15). However, it is noteworthy that only one of the two compounds was found by either group. Several factors may have contributed to this phenomenon. In the first place, the two dihydroxy acids have not been separated by HPLC. Secondly, the GC peak of the compound with a C value of 22.4 is not sharp and often is contaminated with other substances. Lastly, the C value of 23.6 of the other double oxygenated dihydroxy

acid is identical to that of LTB_4 and the mass spectra of these two compounds contain identical ions. In fact, it is possible that some of the mass spectra of LTB_4 which appeared in early publications may have contained a mixture of ions of the double oxygenated and the single oxygenated (LTB_4) 5,12-dhydroxy acids.

In summary, caution should be exercised in the isolation and purification of leukotrienes and other lipoxygenase products of arachidonic acid. Neither RP-HPLC nor SP-HPLC alone is sufficient for preparation of highly purified compounds. Purification by a combination of RP-HPLC and SP-HPLC is advisable.

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