Hiroshima Journal of Medical Sciences
Vol.35, No.2, 99~105, June, 1986
HIJM 35-16

Quantitative Determination of Arachidonate 5-Lipoxygenase Metabolites in Human Polymorphonuclear Leukocytes by Reverse-Phase High-Performance Liquid Chromatography

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(Received February 20, 1986)

Key words: Leukotrienes, HETEs, High performance liquid chromatography

ABSTRACT

Quantitative analysis system of leukotriene B_4, C_4, D_4 (LTB_4, LTC_4, LTD_4), and 5-hydroxyeicosatetraenoic acid (5-HETE) by reverse-phase high-performance liquid chromatography was developed. The lower limit of detection was 1 ng of each compound with a very close correlation between the amount of each compound and its peak height. Normal peripheral blood polymorphonuclear leukocytes (PMN) stimulated with Ca ionophore A23187, produced detectable amounts of LTB_4, LTC_4, and 5-HETE, but no LTD_4 was produced in this condition. This analysis system was useful for measuring LTB_4, LTC_4, LTD_4, and HETEs from the biological samples.

Metabolites of arachidonic acid, prostaglandins, leukotrienes, and thromboxanes, and these analogues have been elucidated to be biologically active and have an important role in inflammation, immunity, and many pathophysiological states. In these metabolites, LTB_4, LTC_4, and 5-HETE are produced by human PMN, eosinophils, monocytes, and other cells[8]. LTB_4 is chemotactic for human PMN[3], increases the vascular permeability, enhances natural killing activity of human lymphocytes[9], and induces production of γ-interferon by human lymphocytes[6]. 5-HETE is also chemotactic for human PMN[4]. LTC_4 has been identified as one of the components of slow reacting substances of anaphylaxis (SRS-A), which constrict bronchial smooth muscle and guinea pig ileum, and reported to potentiate proliferation of epithelial cells of human kidney[2].

Much more findings about diverse potencies of these metabolites will be elucidated. It is valuable to establish the quantitative analysis system of these metabolites produced by biological samples including peripheral blood cells.

MATERIALS AND METHODS

Materials

Authentic LTC_4, LTD_4, two diastereoisomers of 6-*trans*-LTB_4, 5-, 8-, 9-, 11-, 12-, and 15-HETE, 1,4-dimethoxy-3-methyl-2-(3-methoxypropyl) naphthalene and specific 5-lipoxygenase inhibitor, AA861[12] were fine gifts from Dr. S. Terao (Central Research Division, Takeda Chemical Industry, Osaka, Japan). Synthetic LTB_4 and 5-HETE were generous gifts from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Ca ionophore A23187 was obtained from Calbiochem-Behring (La Jolla, CA), N-2-Hydroxyethylpiperadine-N'-2-ethanesulfonic acid (HEPES) from Nakarai Chemicals Ltd. (Kyoto, Japan). All solvents and distilled water were HPLC grade, obtained from Nakarai Chemicals Ltd. (Kyoto, Japan).

Cell preparation and Incubation

Peripheral blood PMN were isolated from heparinized venous blood, by dextran sedimentation followed by centrifugation on Ficoll-diatrizoate sodium gradients and hypotonic ly-

sis of erythrocytes[7]. After gentle wiping out of the inner wall of a centrifugal tube with Kimwipes®, PMN were washed three times with Hanks' balanced salt solution supplemented with 30 mM HEPES, pH 7.4 (Hanks-HEPES). During the washing the centrifugation was performed at 80 × g to avoid the platelet contamination. Platelet to leukocyte ratio was less than 1:100. PMN were suspended in Hanks-HEPES (1×10^7 PMN/ml). One ml PMN suspension were warmed at 37°C for 5 min prior to incubation with gentle shaking. Then, Ca ionophore dissolved in dimethylsufoxide (DMSO) at a concentration of 2 mM was added to a final concentration of 2μM, and the incubation was continued for an additional 5 min and stopped by adding 4 volumes of cold ethanol containing Prostaglandin B_2 and 1,4-dimethoxy-3-methyl-2-(3-methoxypropyl)naphthalene as an internal standard for LTB_4, LTC_4, and 5-HETE, respectively.

Extraction and Purification of Leukotrienes

Extraction and resolution of leukotrienes and HETEs by straight-phase (SP) and reverse-phase (RP) high-performance liquid chromatography (HPLC) were performed under the modified conditions described elsewhere[1,11]. The incubations were kept at 4°C for 60 min and the protein precipitates were removed by centrifugation at 800 × g for 10 min at 4°C. The supernatants were evaporated with a centrifugal evaporator (Model RD-42, Yamato Kagaku Co., Ltd., Japan) and 0.25 ml of methanol and 0.25 ml of distilled water were immediately added to each dried material, successively. An aliquot of the sample was applied to RP-HPLC. For characterization of leukotrienes, RP-HPLC was carried out on a Hiber Lichrosorb RP-18 column (4 × 250 mm, 5μm particle size) (Cica-MERCK, Kanto Chemical Co., Tokyo, Japan) at a flow rate of 1 ml/min in an isocratic solvent system of acetonitrile/methanol/water/acetic acid (30:10:40:0.2, v/v/v/v), pH 5.6 adjusted with triethylamine (solvent A). For characterization of HETEs, the same column was used at the same flow rate in a solvent system of acetonitrile/methanol/water/acetic acid (30:10:22:0.1, v/v/v/v), pH 4 (solvent B). A UV detector (Lambda-Max Model 410 LC Spectrophotometer, Waters Associates, Inc., Milford, MA), set at 236 nm for HETEs, and at 280 nm for LTB_4, LTC_4, recorded the absorption of the eluates. Each fraction co-chromatographed with an authentic compound on RP-HPLC was collected. For 5-HETE, further purification was performed by SP-HPLC column (3.9 × 300 mm, 10 μm particle size, μ-Porasil, Waters Associates, Inc., Milford, MA) eluted with hexane/isopropanol/acetic acid, (99:1:0.1, v/v/v) at 3 ml/min. The absorbance at 236 nm was recorded. 5-HETE fraction corresponding with an authentic compound on SP-HPLC was collected, concentrated, dissolved in methanol and its UV spectrum was monitored by a double wavelength-double beam spectrophotometer (Model 557, Hitachi, Japan). The peak corresponding with an authentic compound of LTB_4 on the RP-HPLC system was collected, concentrated, methylesterified with diazomethane, and applied to a SP-HPLC column (4.6 × 250 mm, 5 μm particle size, Nucleosil 50-5, Macherey Nagel Co., Düren, Germany) eluted with the solvent system of hexane/isopropanol/acetic acid (95:5:0.02, v/v/v) at flow rate 1 ml/min. The peak corresponding with authentic LTB_4 on the RP-HPLC showed a single peak on this SP-HPLC co-chromatographed with a standard of LTB_4 methylester, indicating no 5(S), 12(S)-diHETE, one of the diastereoisomers of LTB_4, was contained in LTB_4 peak on this RP-HPLC system[5]. Furthermore, UV spectrum of separated LTB_4 fraction on RP-HPLC was monitored with the spectrophotometer described above. Biological activity of LTB_4 and 5-HETE was assessed by their chemotactic activity for human PMN. LTC_4 identification was based on co-chromatography with standard material. The quantitation of LTB_4, LTC_4, and 5-HETE produced by PMN were determined by the absorption at 280 nm and 236 nm, respectively, compared with each authentic compound. In other experiments, cell suspension was preincubated with 10 μM specific 5-lipoxygenase inhibitor AA861 for 10 min at 37°C, followed by stimulation with A23187, extraction, and application to RP-HPLC. The HPLC pump (Model 510) and injector (U6K) were from Waters Associates, Inc., Milford, MA.

Assessment of PMN chemotaxis

PMN chemotaxis was assayed by modification of the Boyden chamber technique utilizing cham-

bers with a 0.2 ml blind-end stimulus compartment and filters with 3 μm pores (Millipore Corporation, Bedford, MA). Indicated concentrations of LTB_4 or 5-HETE were added in the lower stimulus compartment and their activities were compared with 10^{-8} M formyl-methionyl-leucyl-phenylalanine (fMLP) and 10% zymosan-activated serum (10% ZAS), which have been established as strong chemotaxins. 0.2 ml of PMN, suspended in Hanks-HEPES containing 0.1% bovine serum albumin at the concentration of 2×10^6/ml, was applied to the upper compartment, and the chambers were incubated for 45 min at 37°C.

The chemotactic response was expressed as the distance migrated by leading front cells.

RESULTS AND DISCUSSION

Chromatographic profiles of authentic compounds were shown in Fig. 1. LTB_4, LTC_4, LTD_4, and two diastereoisomers of 6-*trans*-LTB_4 were clearly separated on the RP-HPLC system with solvent A (Fig. 1a). 5-, 9-, 11-, and 15-HETEs were also completely separated with solvent B, but 8- and 12-HETE were overlapped with each other and incompletely separated (Fig. 1b).

But on SP-HPLC, all HETEs completely separated (Fig. 1c). The UV spectrum of LTB_4 showed three main bands at 281, 270, 260 nm (Fig. 2), suggesting that this compound contains three conjugated double bonds characteristic of

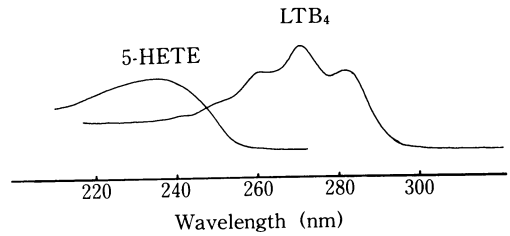

Fig. 2. The UV spectra of Leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE).

a triene. 5-HETE as well as other monoHETEs had a typical UV spectrum of a diene with max at 236 nm, as shown in Fig. 2. Each spectrum was identical to that reported previously[1,10]. In the experiments using specific 5-lipoxygenase inhibitor, AA861[12], production of all peaks corresponding with LTB_4, LTC_4, and 5-HETE was completely suppressed by AA861 at the reported concentration of 10 μM[12], indicating that these peaks were the products of 5-lipoxygenase. The purified LTB_4 was found to have a chemotactic potency for PMN comparable to fMLP and 10% ZAS (Fig. 3)[3,5]. Although less chemotactic than LTB_4, fMLP, and 10% ZAS, 5-HETE was also found to be a chemoattractant. All these findings described above indicate that purified LTB_4 and 5-HETE on this RP-HPLC satisfy the criteria for identifying these compounds as biologically active LTB_4 and 5-HETE, respectively. The lower limit of detection on this RP-HPLC system was 1 ng of each

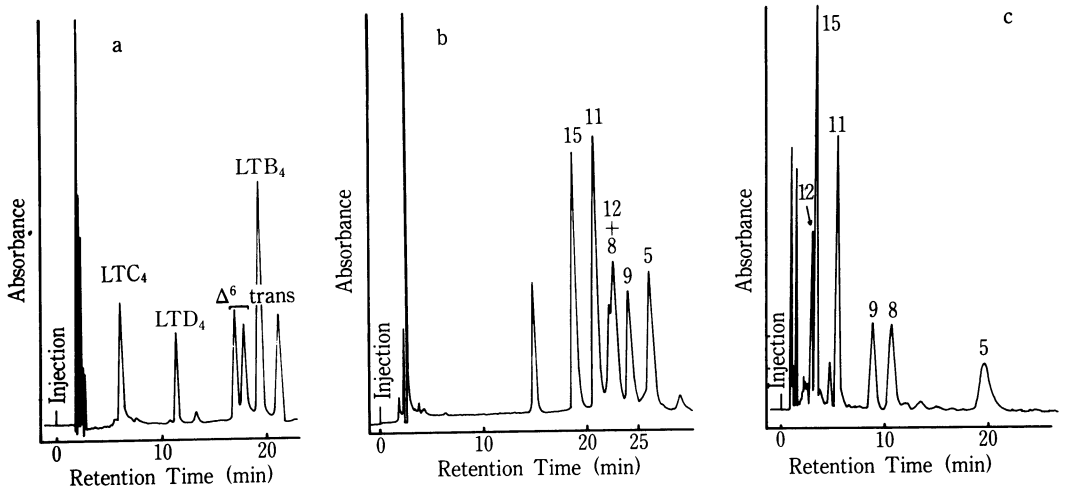

Fig. 1. Chromatographic profiles of authentic Leukotriene B_4, C_4, D_4 (LTB_4, LTC_4, LTD_4) on RP-HPLC with solvent A (a), and 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE) on RP-HPLC with solvent B (b) or SP-HPLC (c). Conditions of the assay in details were described in "Materials and Methods".

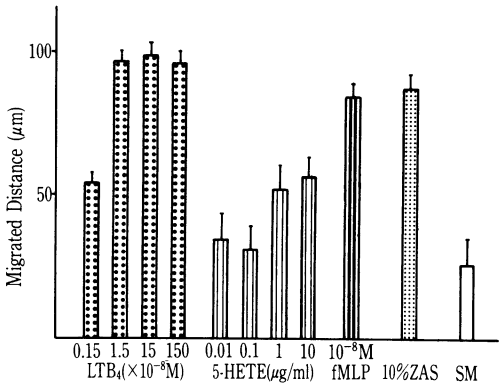

Fig. 3. Chemotactic activities of purified Leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) at the indicated concentrations compared with 10^{-8} M formyl-methionyl-leucyl-phenylalanine (fMLP), 10% zymosan-activated serum (10% ZAS), and spontaneous migration (SM) in the absence of a stimulus. Each bar and bracket depicts the mean ± S.D. of the results of two experiments performed in duplicate.

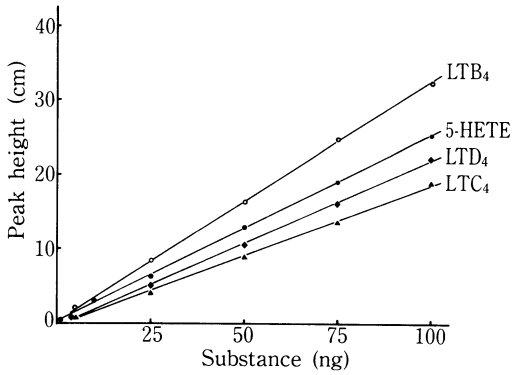

Fig. 4. Correlation between the concentration of each compound of LTB_4, LTC_4, LTD_4 and 5-HETE, and its peak height. Abbreviations used were the same as those in Fig. 1.

compound, and the amount of each compound was closely correlated with its peak height at the range from 1 ng to 100 ng as shown in Fig. 4. Representative chromatographic patterns of the products by A23187-stimulated normal PMN were shown in Fig. 5. PMN stimulated with A23187 produced detectable amounts of LTB_4, LTC_4, and 5-HETE (13.4 ± 2.4 ng/10^6 PMN,

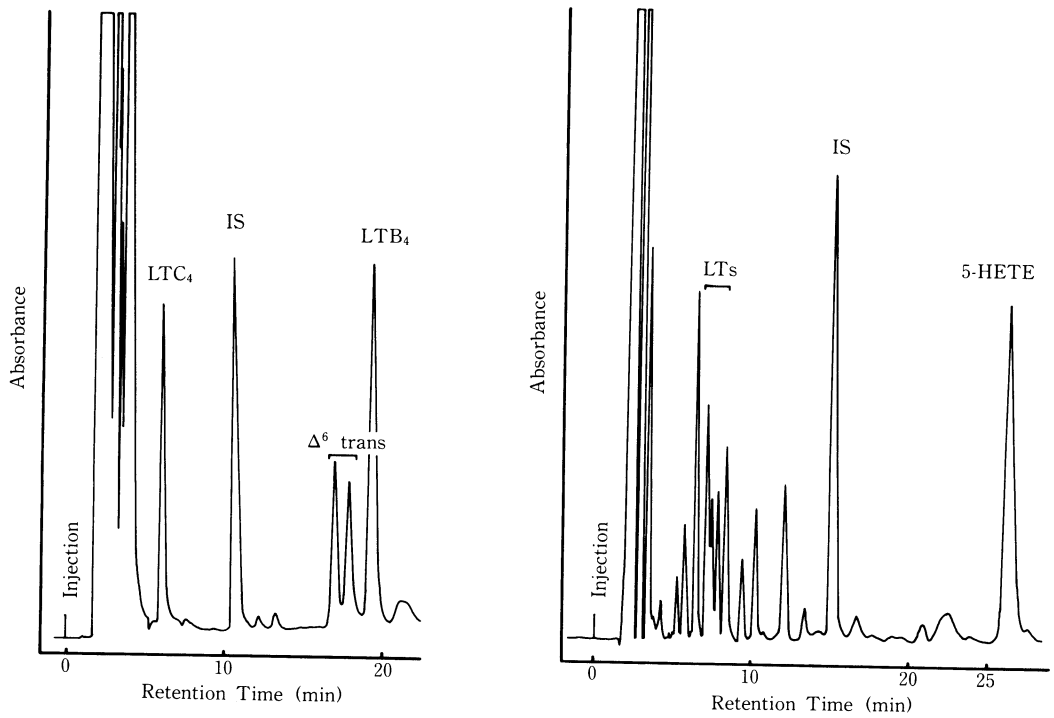

Fig. 5. Representative chromatographic profiles of the products of arachidonate 5-lipoxygenase by A23187-stimulated human polymorphonuclear leukocytes, separated on RP-HPLC with solvent A (*left*) and solvent B (*right*). Is denotes an internal standard and other abbreviations as the same as those used in Fig. 1. Assay conditions in details were presented in "Materials and Methods".

mean ± SD, n = 8, 13.2 ± 6.4 ng/10^6 PMN, 26.6 ± 7.1 ng/10^6 PMN, respectively). Furthermore, two diastereoisomers 6-*trans*-LTB_4 were also produced, but LTD_4 was not produced by normal PMN in this condition of stimulation.

In this report we described the quantitative analysis system for the metabolites of arachidonate 5-lipoxygenase using RP-HPLC. Arachidonate metabolites have been elucidated to have many biological activities. More detailed investigations will be carried out into the regulation of their *in vivo* and *in vitro* production and metabolism. This assay system is a useful and simple method to measure these metabolites from biological samples for the purpose of these investigations.

ACKNOWLEDGEMENT

We are greatly indebted to Dr. S. Terao, Central Research Division, Takeda Chemical Industry, Osaka, Japan, and Prof. S. Yamamoto, Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan, for their valuable advices for measuring leukotrienes and HETEs by RP-HPLC.

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