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SEPARATION AND QUANTITATION OF LEUKOTRIENES BY REVERSED-PHASE HIGH--PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid and sensitive reversed-phase HPLC procedure is reported which allows the simultaneous separation and quantitation of LTC_4, 11*t*-LTC_4, LTD_4, LTB_4, 12*epi*,6*t*,8*c*-LTB_4, 6*t*-LTB_4 + 12*epi*,6*t*-LTB_4, two trihydroxy-eicosatetraenoic acids tentatively identified as 20-OH--LTB_4 and 20-OH,12*epi*,6*t*,8*c*-LTB_4 and several not yet identified 15--series leukotrienes produced by the cytosol of porcine polymorphonuclear leukocytes.

INTRODUCTION

Unsaturated fatty acids containing three or more methylene-interrupted *cis* double bonds can be dioxygenated by lipoxygenases to yield mono- and dihydroperoxy fatty acids. Bild et al. (1) have reported on the double dioxygenation of all-*cis*-5,8,11,14-eicosatetraenoic acid (ETE, arachidonic acid) by soybean lipoxygenase-1. This reaction has subsequently been studied by Van Os et al. (2) who showed that two major products were formed, both containing two hydroperoxide groups, which were identified as 8,15-DHPETE and 5,15-DHPETE. The 8,15-DHPETE has three conjugated double bonds and hence has a characteristic triene UV-spectrum with absorption maxima at 259, 268.5 and 279 nm. It was shown that all chiral centers have the S-configuration (2).

In animal systems, lipoxygenases also produce oxygenated unsaturated fatty acids, which can be enzymically converted into leukotrienes. The latter compounds have pronounced biological activities.

In the presence of glutathione or L-cysteine the sulfur-containing leukotrienes C_4 and D_4 can be formed which were shown to be potent bronchoconstrictors (3). Therefore these leukotrienes may play an important role as mediators in immediate hypersensitivity reactions (4).

LTB_4, which is formed from LTA_4 by enzymic hydrolysis, and to a lesser degree 20-OH-LTB_4, formed from LTB_4 through ω-hydroxylation, show a strong chemotactic behaviour towards human neutrophils (5) and induce degranulation of human neutrophils (6). These physiological activities suggest an important role for these leukotrienes in inflammation reactions.

Upon incubation of arachidonic acid with leukocyte preparations, usually a complex mixture of leukotrienes with different structural features is formed. The composition of the mixture depends on the species used as well as on the incubation conditions i.e., the concentration of Ca^{2+}-ions and the presence of Ca-ionophore and glutathione. The simultaneous presence of sulfidopeptide-leukotrienes, various DHETE's and THETE's then constitutes a serious analytical problem. Previously published RP-HPLC methods did not resolve LTB_4 and 12*epi*,6*t*, 8*c*-LTB_4 (7,8). Recently, Masters and McMillan (Annual Symposium on Leukotrienes, London, 1982) presented a RP-HPLC procedure which allows the separation of LTB_4 and 12*epi*,6*t*,8*c*-LTB_4 but these authors did not simultaneously measure sulfidopeptide-leukotrienes and 15-series leukotrienes.

In this paper we present a rapid, quantitative and sensitive method for a complete analysis of sulfidopeptide-leukotrienes, 5- and 15--series DHETE's, and THETE's in a single isocratic RP-HPLC run.

MATERIALS AND METHODS

MATERIALS: Synthetic LTB_4, LTC_4 and LTD_4 were a generous gift of Dr J. Rokach, Merck-Frosst Laboratories, Pointe-Claire/Dorval, Quebec, Canada. Arachidonic acid (purity >99%) was obtained from Fluka AG, Switzerland. Reduced glutathione, Ca-ionophore A23187 and PGB_2 were purchased from Sigma Chemical Company, St. Louis, Mo, U.S.A.. Human blood was obtained from healthy volunteers. Porcine blood came from the local slaughterhouse. All solvents (including water) were of HPLC quality and purchased from Baker, Deventer, The Netherlands. Dextran grade A (MW 200,000 - 275,000) was from BDH, Poole, U.K.. Ficoll-Paque was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

METHODS: Human and porcine polymorphonuclear leukocytes (PMNL's) were isolated as follows: platelet-rich plasma was removed by centrifugation at 275 g for 15 min. After collection of the buffy coat, leukocytes were separated from erythrocytes by dextran sedimentation. PMNL's were separated from lymphocytes and monocytes by centrifugation on Ficoll-Paque at 650 g for 45 min. The remaining erythrocytes were removed by ammoniumchloride lysis at 0°C and subsequent centrifugation. The purity of the cell preparations was better than 97%. The porcine PMNL cytosol was prepared by ultrasonication of a PMNL suspension (in Dulbecco's salt solution, pH 7.4) at 0°C for 2 x 1.5 min at 20 kHz and centrifugation of the homogenate at 27,000 g for 10 min. Suspensions of human PMNL's in Dulbecco's salt solution (pH 7.4, ~ $3x10^7$ cells/ml) or porcine PMNL cytosol (from ~ $4x10^7$ cells/ml) were then incubated during 10 min at 37°C and pH 7.4 with 80 μM arachidonic acid, 20 μM A23187, 1 mM (extra) $CaCl_2$ and, in case of human PMNL's, 5 mM glutathione. The reaction was stopped by the addition of ice to the solution, followed by centrifugation at 46,000 g for 10 min at 4°C. The supernatant was then applied to a C18 reversed-phase extraction column

(Baker, Deventer, The Netherlands). The adsorbed leukotrienes were eluted with ~ 3 ml methanol.

RP-HPLC was carried out on Nucleosil 5C18 column (250 x 4.6 mm, Chrompack, Middleburg, The Netherlands) attached to a Perkin Elmer Series 1 pump fitted with a Rheodyne injector (20 µl sample loop) and a Perkin-Elmer LC85 detector. The solvent system was tetrahydrofuran--methanol-water-acetic acid (25/30/45/0.1, by volume) which was brought to pH 5.5 with ammoniumhydroxide. The aqueous phase contained 0.1% EDTA to prevent binding to the column of cations, which could lead to excessive retention of sulfidopeptide leukotrienes (cf. 8). A flow rate of 0.9 ml/min was maintained and the effluent was monitored at 280 nm. Peak areas were determined with a Hewlett-Packard 3390A integrator and related to the internal standard PGB_2 using molar absorption coefficients at 280 nm of 28,650 for PGB_2 and 40,000 for the leukotrienes.

RESULTS AND DISCUSSION

Incubation of human PMNL's with arachidonic acid in the presence of Ca^{2+}, Ca-ionophore A23187 and glutathione leads to the formation of LTC_4, 11*t*-LTC_4, LTB_4, 12*epi*,6*t*,8*c*-LTB_4, a racemic mixture of non--enzymically formed 6*t*-LTB_4 and 12*epi*,6*t*-LTB_4 and two trihydroxy-eicosatetraenoic acids, identified as 20-OH-LTB_4 and 20-OH,12*epi*,6*t*,8*c*-LTB_4 (9). The relative amounts of the leukotrienes were found to be dependent on the incubation conditions as well as on the PMNL preparation. The sulfidopeptide leukotrienes LTC_4 and 11*t*-LTC_4 are only formed if glutathione or L-cysteine is also present. The relative amounts of 20-OH-LTB_4 and 20-OH,12*epi*,6*t*,8*c*-LTB_4 were found to be strongly dependent on the length of the incubation period (Masters and McMillan, Annual Symposium on Leukotrienes, London, 1982). In the absence of Ca^{2+} and Ca-ionophore, considerable amounts of 15-series leukotrienes are synthesized (10). The production of the double dioxygenation product 12*epi*,6*t*,8*c*-LTB_4 and its ω-hydroxylated metabolite 20-OH,12*epi*,6*t*,8*c*--LTB_4 strongly increases when the PMNL preparation is contaminated with thrombocytes (11). To get a proper insight into the absolute quantities of the leukotrienes formed under well-defined conditions, we developed a rapid and sensitive RP-HPLC assay that allows the simultaneous separation and quantitation of all the leukotrienes mentioned above.
A major disadvantage of the solvent systems generally employed sofar in the RP-HPLC analysis of leukotrienes is that LTB_4 and the double dioxygenation product 12*epi*,6*t*,8*c*-LTB_4 are not resolved. Unfortunately, the solvent system proposed by Masters and McMillan (Annual Symposium on Leukotrienes, London, 1982) though considerably improving the resolution of LTB_4 and 12*epi*,6*t*,8*c*-LTB_4, does not satisfactorily separate the sulfidopeptide leukotrienes and the 15-series leukotrienes from the 5-series DHETE's. Suitable retention times for LTC_4 and LTD_4 can be obtained by changing the pH of the mobile phase because the retention of these leukotrienes depends strongly on the pH of the solvent system, due to the presence of 3 and 2 carboxyl groups in these compounds, respectively. Furthermore, increasing the tetrahydrofuran content improves the resolution of LTB_4 and 12*epi*,6*t*,8*c*-LTB_4 but deteriorates the separation of LTB_4 and the non-enzymically formed DHETE's. Careful adjustment of the composition of the mobile phase resulted in a solvent system that allows the separation and quantitation of all leukotrienes mentioned above within a reasonable period of time (~ 40 min).

Fig. 1 shows the RP-HPLC chromatogram of an artificial mixture of leukotrienes, prepared by mixing appropriate amounts of synthetic LTB_4, LTC_4 and LTD_4 with various leukotriene samples produced by human PMNL's.

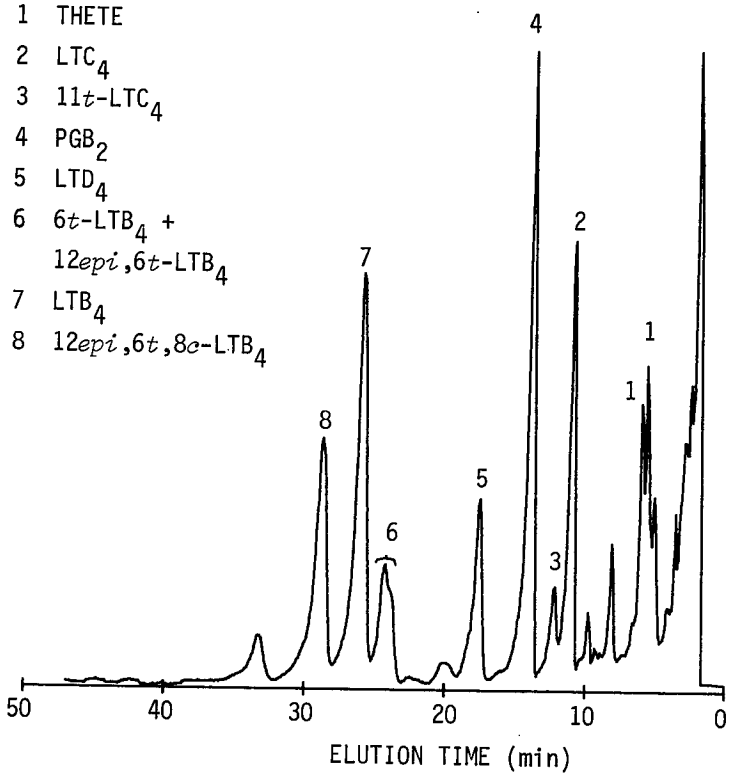

Fig. 1 RP-HPLC chromatogram of synthetic LTB_4, LTC_4, LTD_4 and the leukotrienes produced by human PMNL's. PGB_2 was added as an internal standard. Absorbance ranges: 0 - 7 min 0.08 AUFS; 7 - 50 min 0.04 AUFS

The 12*epi*,6*t*,8*c*-LTB_4, the THETE's and the non-enzymically formed DHETE's were identified by their characteristic UV-spectra and by comparing their retention times on RP-HPLC and SP-HPLC with values obtained from the literature with various solvent systems. Although 6*t*-LTB_4 and 12*epi*,6*t*-LTB_4 are not completely resolved with the mobile phase used, no information is lost since these leukotrienes are formed from LTA_4 by non-enzymic hydrolysis in 1 : 1 ratio. Porcine PMNL cytosol produces from arachidonic acid, under the conditions described in Materials and Methods, 15-series leukotrienes and 5-series leukotrienes, which are also formed by human PMNL's. A RP-HPLC chromatogram of a sample of leukotrienes prepared by mixing synthetic LTB_4, LTC_4 and LTD_4 with the leukotrienes produced by porcine PMNL cytosol and human PMNL's is shown in Fig. 2.

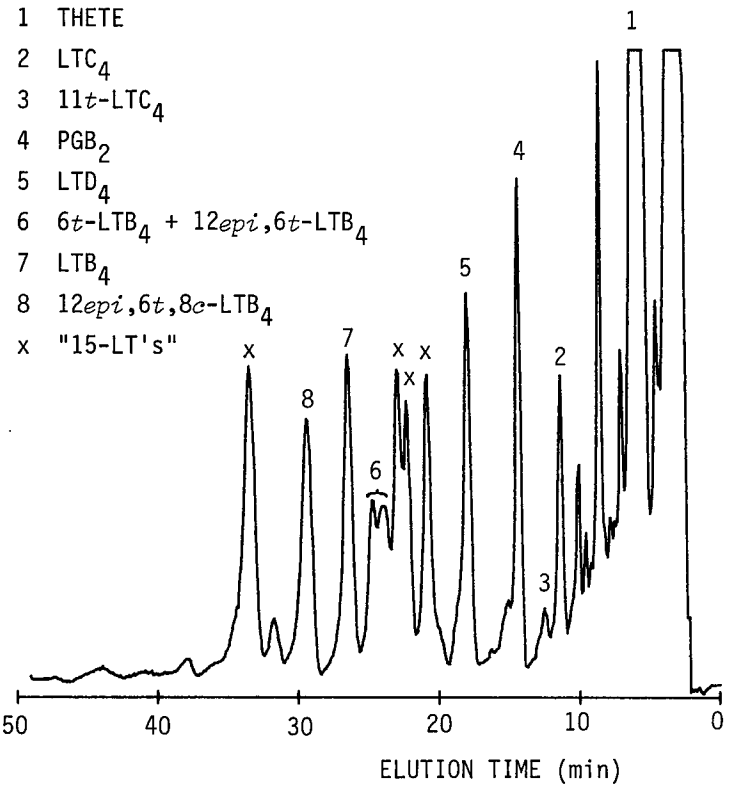

Fig. 2. RP-HPLC chromatogram of synthetic LTB_4, LTC_4, LTD_4, the leukotrienes produced by human PMNL's and the leukotrienes produced by porcine PMNL cytosol. PGB_2 was added as an internal standard. Absorbance range: 0.02 AUFS

Although a full structural analysis of the x-marked leukotrienes (Fig. 2) has not yet been completed, these leukotrienes belong to the 15-series since the RP-HPLC chromatogram of the leukotrienes produced by porcine lymphocytes from 15(S)-HPETE shows peaks with identical retention times and UV-spectra (unpublished results).

This RP-HPLC assay is now routinely used by us to measure leukotriene production of PMNL's purified from human blood. Since the detection limit for leukotrienes was found to be below 10 ng in this analysis and only one HPLC-run is needed to quantitate all leukotrienes, relatively small blood samples (<20 ml) are sufficient to study the leukotriene synthesis by human blood PMNL's.

Abbreviations used:
DHPETE: dihydroperoxy-eicosatetraenoic acid, DHETE: dihydroxy-eicosatetraenoic acid, THETE: trihydroxy-eicosatetraenoic acid.
12*epi*,6*t*,8*c*-LTB_4 is sometimes referred to elsewhere as 5S,12S-DHETE.

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