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Note

Purification of leukotriene B_4 by semi-preparative high-performance liquid chromatography

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Leukotriene B_4 [5(*S*), 12(*R*)-dihydroxy-6-*Z*-8,10-*E*-14-*Z*-eicosatetraenoic acid] (LTB_4) is a major product of arachidonic acid metabolism via the 5-lipoxygenase pathway in a variety of human tissues[1]. It induces aggregation, chemotaxis and chemokinesis of human polymorphonuclear leukocytes (PMN) *in vitro*[2] and produces increased vascular permeability and PMN infiltration in the presence of cyclooxygenase products *in vivo*[3]. Its major role as a mediator of inflammation[1] has generated the need to examine its functional properties in a wide variety of inflammatory models and to elucidate the mechanism of its pharmacological action.

Studies of the metabolism of arachidonic acid in PMN leukocytes led to the discovery of LTB_4 by Borgeat and Samuelsson[4] in 1979. More recently, several chemical syntheses have been developed[5–10]. It has been shown that minor structural modifications of LTB_4 have led to molecules which are either biologically inactive or antagonize the actions of LTB_4[11]. Because of the possibility of contamination of both natural and synthetic LTB_4 with geometric and stereoisomers, high-performance liquid chromatography (HPLC) has been an important technique for assuring the use of high purity LTB_4 in pharmacological studies. Although analytical HPLC conditions for LTB_4 have been reported[12], there have been no details of the preparative HPLC purification of LTB_4 described in the literature.

LTB_4 has been prepared in our laboratory by the convergent synthesis* depicted in Fig. 1. Final hydrolysis of the protected LTB_4 ester yields a mixture of isomers of which the naturally occurring 5(*S*),12(*R*) isomer is the major component (III) (Fig. 1). The identity and approximate amount of each minor LTB_4 isomer is shown in Table I. We have developed a semi-preparative HPLC method capable of producing 100-mg quantities of highly purified LTB_4.

EXPERIMENTAL

The analytical HPLC system consisted of an Altex Model 110A solvent pump,

* LTB_4 benzoate methyl ester was synthesized by Wittig reaction of I and II (ref. 5). Improved synthetic routes to precursors I and II will be published separately.

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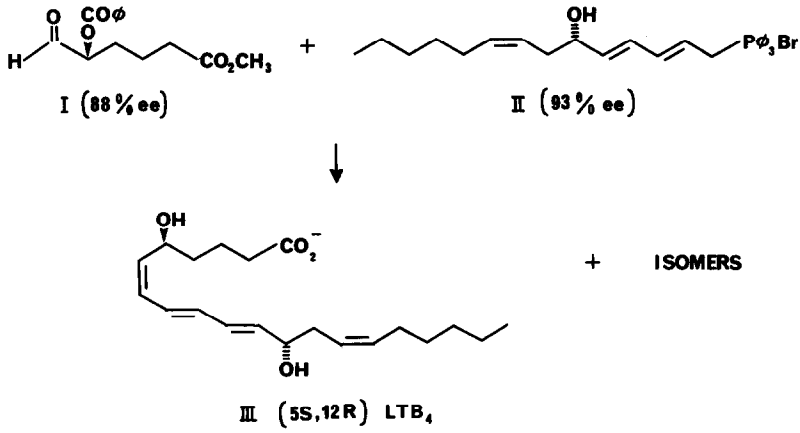

Fig. 1. Synthesis of Leukotriene B_4. ee = Enantiomeric excess.

Rheodyne Model 7125 injector with 20-μl loop, LDC Spectromonitor III variable-wavelength UV detector, and Shimadzu Model C-RIA integrator.

The semi-preparative HPLC system consisted of a Perkin Elmer Series 3B pump, Rheodyne Model 7125 injector with 2-ml loop, LC 75 variable-wavelength UV detector, and Sigma 15 data system.

The following new analytical HPLC columns (25 cm × 4.6 mm; 5 μm) were used in this study: Lichrosorb RP-18 (Merck), Partisil ODS-3 (Whatman), PE C_{18} (Perkin Elmer) and Zorbax ODS (Dupont). Each column was first tested for compliance with the manufacturer's specifications of efficiency and asymmetry, using test standards (toluene, naphthalene and benzyl alcohol). A Zorbax ODS column (25 cm × 21.2 mm I.D.; 7 μm) was used for semi-preparative HPLC work.

The HPLC mobile phase used throughout this study was made by mixing 670 ml methanol, 330 ml distilled water, and 1.0 ml glacial acetic acid. The mobile phase pH was adjusted to 6.2 by the addition of concentrated ammonium hydroxide.

TABLE I

HPLC ASSAY OF CRUDE LTB_4

Identity	*% Concentration (UV area integration)*
5(*S*),12(*R*)-LTB_4*	56
6*E*-LTB_4	17
EPI-LTB_4**	13
6*E*-EPI-LTB_4	7
Unknown	7

* The LTB_4 purified by HPLC is estimated to contain less than 1% of the 5(*R*),12(*S*) enantiomer, based on the chiral purity of the synthetic precursors, (I and II in Fig. 1).

** EPI-LTB_4 refers to a mixture of the 5(*S*),12(*S*) and 5(*R*),12(*R*) enantiomers.

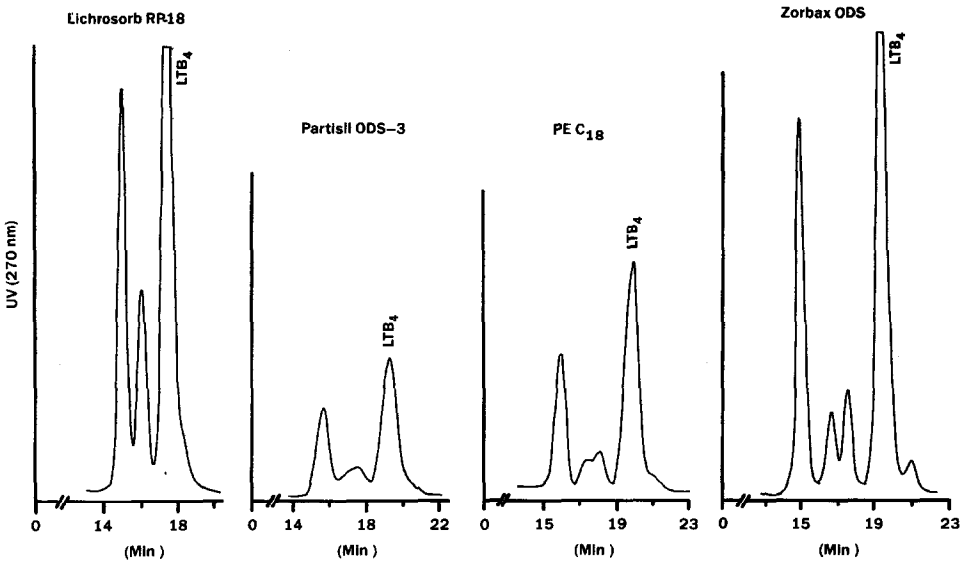

Fig. 2. Analytical HPLC of LTB_4 mixture. Columns, 25 cm × 4.6 mm I.D.; mobile phase, methanol–water–ammonium acetate (67:33:0.1) (pH 6.2); flow-rate, 1.0 ml/min.

RESULTS

Our strategy for the purification of synthetic LTB_4 involved three steps: (1) selection of an appropriate analytical C_{18} column; (2) use of a volatile buffer for ease of isolation of pure LTB_4; and (3) scale-up to semi-preparative HPLC using a column with the same selectivity as the corresponding analytical column.

For the initial column study, we chose a mobile phase consisting of methanol–water–ammonium acetate (67:33:0.1) (pH 6.2), similar to that reported by Metz *et al.*[12] for the analysis of peptidoleukotrienes. Several C_{18} columns were evaluated for selectivity in the separation of the LTB_4 isomer mixture. The results, shown in Fig. 2, indicate significant selectivity differences among the analytical columns. The Zorbax ODS column gave the best resolution of the isomer mixture, and was chosen for scale-up to semi-preparative HPLC.

Using the Zorbax analytical column, the following mobile phases were examined and found to give decreased resolution of the isomer mixture: (1) methanol–10 m*M* potassium dihydrogen phosphate (67:33) (pH 6.2); (2) acetonitrile–10 m*M* potassium dihydrogen phosphate (50:50) (pH 6.2); (3) methanol–10 m*M* ammonium acetate (67:33) (pH 4.0).

In order to test the efficiency and selectivity of the Zorbax semi-preparative column, an analytical size sample (50 μg) of LTB_4 isomer mixture was chromatographed. As shown in Fig. 3, the efficiency and selectivity are identical to that of the corresponding Zorbax analytical column (Fig. 2).

The first semi-preparative HPLC separation of crude LTB_4 is shown in Fig. 4. Because of the high extinction coefficient (ε = 50 000 l mol^{-1} cm^{-1}) of LTB_4 at 270 nm, it was necessary to detune the UV detector to 300 nm in order to obtain a

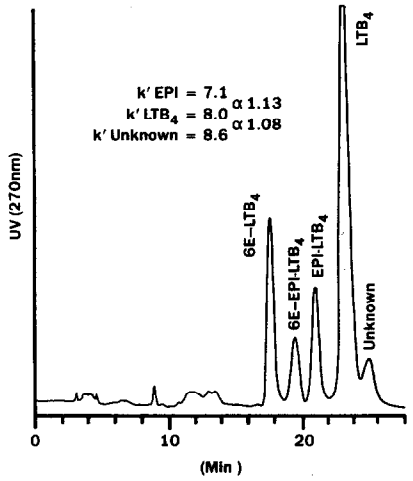

Fig. 3. Analytical HPLC of LTB_4 mixture on Zorbax Semi-Prep column (25 cm × 21.2 mm I.D.). Mobile phase, methanol–water–ammonium acetate (67:33:0.1) (pH 6.2); flow-rate, 20 ml/min.

satisfactory semi-preparative HPLC chromatogram. Using an injected mass of 9 mg mixture (0.2 mg/g packing), LTB_4 was isolated with an HPLC purity >99% and a recovery of *ca.* 90% (based on 56% purity of crude LTB_4). The purified LTB_4 was identical by UV and HPLC to material prepared by a different synthetic route[10].

In order to maximize preparative throughput, the injected mass was increased to 45 mg (0.9 mg/g packing). As shown in Fig. 5, there is a drop in resolution, but the major fraction of LTB_4 (*ca.* 75% recovery) could still be isolated with an HPLC purity of 99%.

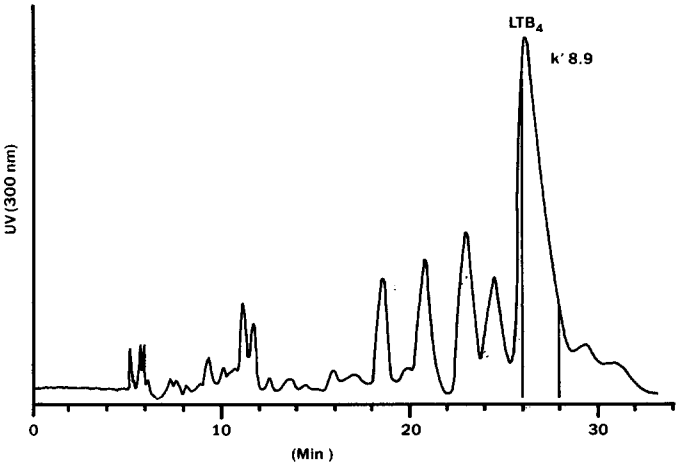

Fig. 4. Semi-preparative HPLC purification of LTB_4 (9 mg mixture, 0.2 mg/g). Column, Zorbax ODS (25 cm × 21.2 mm I.D.); mobile phase, methanol–water–ammonium acetate (67:33:0.1) (pH 6.2); flow-rate, 20 ml/min.

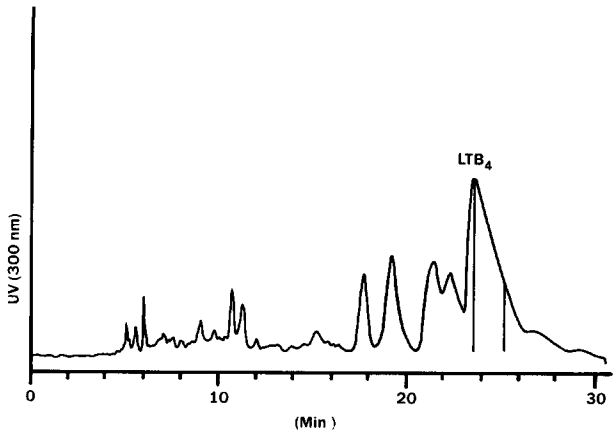

Fig. 5. Semi-preparative HPLC purification of LTB_4 (45 mg mixture, 0.9 mg/g). Column, Zorbax ODS (25 cm × 21.2 mm I.D.); mobile phase, methanol–water–ammonium acetate (67:33:0.1) (pH 6.2); flow-rate, 20 ml/min.

DISCUSSION

In liquid chromatography, the selectivity factor (α) indicates the degree of difficulty of a separation, according to the equation:

$$R = \tfrac{1}{4}(\alpha - 1)[k'/(k' + 1)]\sqrt{N}$$

where $\alpha = k'_2/k'_1$; capacity factor (k') = $(V_R - V_0)/V_0$ (V_R = retention volume, V_0 = retention volume of unretained compound); plate number (N) = $5.54\,(V_R/V_0)^2$. In general, separations with α values between 1.1 and 1.2 are very difficult, and require the use of a high efficiency semi-preparative HPLC column, operated in the non-overload condition to maintain acceptable resolution[13]. In the case of the LTB_4 isomer mixture, the EPI-LTB_4 isomer and the unknown LTB_4 isomer are very difficult to resolve from LTB_4, with α values of 1.13 and 1.08, respectively.

Optimization of the α value is required in order to maximize the throughput of a mixture. This is usually accomplished by varying the components of the mobile phase. In the case of the LTB_4 isomer mixture, the α value was maximized by varying the C_{18} bonded phase. This observation is consistent with literature data describing subtle differences in the chromatography characteristics of C_{18} bonded phases[14].

Using the Zorbax semi-preparative HPLC column, the purification of crude LTB_4 was scaled-up to 45 mg mixture per run. This represents the maximum throughput per injection, and is consistent with operation of the column in the non-overload condition (1 mg/g packing)[13]. By using repetitive injections, 100 mg of highly purified LTB_4 can be obtained in about 2.5 h.

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