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Note

Separation of lipoxins and leukotrienes from human granulocytes by high-performance liquid chromatography with a Radial-Pak cartridge after extraction with an octadecyl reversed-phase column

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Leukotrienes (LT) and lipoxins (LX) have potent biological actions. The cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 (for all abbreviations, see Experimental) contract smooth muscle cells and increase vascular permeability. LTB_4 activates polymorphonuclear leukocytes (PMNLs) and causes the release of superoxide radicals, chemotaxis, aggregation and degranulation of PMNLs [1]. Interaction between the 5- and the 15-lipoxygenase pathways leads to the formation of LXA_4 and LXB_4 and some isomers of both LXA_4 and LXB_4 [2,3]. If added to neutrophils, LXA_4 stimulates superoxide anion generation without provoking aggregation [4]. In addition, LXA_4 and LXB_4 alter the human killer cell activity [5] and activate protein kinase C in vitro [6].

As the isomers of LXA_4 and LXB_4 do not share these biological activities there is a need for the separation of all the lipoxin isomers. Moreover, the separation is desirable in order to elucidate the biosynthetic pathways for lipoxin formation in various tissues.

In the last few years there has been considerable progress in the analysis of lipoxygenase products. Rapid, simple and efficient extraction of arachidonic acid metabolites from biological samples was achieved using octadecylsilica-based extraction columns [7–13]. The high-performance liquid chromatographic (HPLC) resolution of the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 was improved using trifluoroacetic acid (TFA) instead of acetic acid as the

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acidic component of the mobile phase. Furthermore, higher sensitivity was achieved with the electrochemical detection of LTB_4 [14], lipoxins [15] and other arachidonic acid metabolites [16,17]. However, the separation of LTB_4 from 5*S*,12*S*-diHETE on reversed-phase columns eluted with water–methanol is still unsatisfactory. Unlike methanol–water–TFA, acetonitrile–water–TFA separates LTB_4 well from 5*S*,12*S*-diHETE, but the 6-*trans* isomers are not separated from LTB_4 [13]. In addition, the resolution of the lipoxin isomers is poor with both mobile phases. Here, we describe a rapid method for the extraction of lipoxins from biological samples and the application of a Radial-Pak column, which allows a sufficient separation of the lipoxin isomers and the baseline separation of LTB_4 and its isomers, including 5*S*,12*S*-diHETE, within 8 min.

EXPERIMENTAL

Chemicals

The lipoxin standards were generous gifts from Dr. J. Rokach (Merck Frosst, Quebec, Canada) and the abbreviations used are as follows: LXA_4, (5*S*,6*R*,15*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 6*S*-LXA_4, (5*S*,6*S*,15*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 11-*trans*-LXA_4, (5*S*,6*R*,15*S*)-trihydroxy-7,9,11,13-*trans*-eicosatetraenoic acid; 6*S*-11-*trans*-LXA_4, (5*S*,6*S*,15*S*)-trihydroxy-7,9,11,13-*trans*-eicosateraenoic acid; LXB_4, (5*S*,14*R*,15*S*)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 14*S*-LXB_4, (5*S*,14*S*,15*S*)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid, 8-*trans*-LXB_4, (5*S*,14*R*,15*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid; 14*S*-8-*trans*-LXB_4, (5*S*,14*S*,15*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid.

LTB_4, (5*S*,12*S*)-dihydroxy-6,10-*trans*-8,13-*cis*-eicosatetraenoic acid (5*S*,12*S*-diHETE) and the other lipoxygenase metabolites were obtained from Paesel (Frankfurt, F.R.G.) and TFA and Ca^{2+} ionophore A23187 (free acid) from Sigma (St. Louis, MO, U.S.A.). All solvents used were of analytical-reagent grade. PM16 supplemented with human albumin, glucose and Ca^{2+} served as an incubation buffer.

Apparatus

The HPLC equipment consisted of a Model 481 variable-wavelength detector, a Model 590 pump and a U6K injector, all from Waters Assoc. (Milford, MA, U.S.A.). The chromatograms were recorded on a Waters M740 Data Module.

Ultraviolet spectra were recorded with a Beckman DU-50 spectrophotometer connected on-line with the HPLC equipment.

Column and mobile phase

The separations were carried out on a Waters Radial-Pak cartridge (100 mm×5 mm I.D.) packed with 4-μm Novapak C_{18} material obtained from

Waters (Eschborn, F.R.G.) or on a 5-μm Nucleosil C_{18} column (250 mm $\times$ 4.6 mm I.D.). The solvent was either methanol–water–TFA or acetonitrile–water–TFA. A flow-rate of 1.2 ml/min was used.

Peaks were identified by co-chromatography with the standards and by UV spectrophotometry. A molar absorptivity of 50 000 (at 301 nm) was used for calculations.

Incubation of the PMNLs and extraction procedure

Suspensions of human PMNLs were prepared according to the method of Hjorth et al. [18] and incubated as described previously [19], briefly as follows. Human PMNLs ($2.0 \cdot 10^7$ cells per ml) suspended in Serva PM16 buffer salt solution supplemented with 1 m*M* Ca^{2+}, 0.1% glucose and 0.1% human albumin were preincubated with 15-HETE (10 μM) for 3 min. The reaction was started with Ca^{2+} ionophore (5 μM). After 15 min, the reaction was stopped with methanol and the tubes were chilled on ice, diluted with buffer to a methanol content lower than 30% and centrifuged (800 *g*, 10 min). Extraction of the eicosanoids was done with a modified Baker 10 extraction system using Baker C_{18} disposable columns (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns were conditioned with 2 ml of methanol and 2 ml of water. The samples were loaded on the columns, which were washed with 2 ml of water and 2 ml of 25% methanol. Finally, the lipoxins were eluted with 100% methanol and the extract was evaporated to dryness under a stream of nitrogen and resuspended in a small volume of methanol. A 10-μl aliquot of this solution was injected into the HPLC apparatus.

RESULTS AND DISCUSSION

The extraction of lipoxins has generally been carried out with ethyl acetate or diethyl ether after acidification of the samples. Using this extraction procedure, the acid-catalysed isomerization of lipoxins cannot be ruled out when the samples are evaporated to dryness. However, the acidification step can be omitted if the extraction is done with reversed-phase material. Powell [7] used Sep-Pak cartridges and Luderer et al. [8] used Baker disposable columns for the extraction of other eicosanoids. Verhagen et al. [9] reported the extraction of leukotrienes without acidification of the samples, thus avoiding the isomerization. Comparison of the Sep-Pak cartridges with the Baker material showed that the Baker disposable columns give better recoveries of the leukotrienes, particularly in the presence of proteins [9].

The present paper describes an extraction method for the lipoxins that takes these previous results into account. In order to determine the recoveries of LXA_4 and LXB_4, 13 ng of LXA_4 and 26 ng of LXB_4 were extracted (Table I) and the recoveries were 86 ± 4 and 85 ± 2%, respectively. Approximately 5% of

TABLE I

RECOVERIES OF LXA_4 AND LXB_4 ($n=3$)

Compound	Amount applied in 1-ml volume (ng)	Recovery (mean ± S.D.) (%)	Conversion to all-*trans* isomers (%)	Total recovery (mean ± S.D.) (%)
LXA_4	13	86 ± 6	5	91 ± 8
LXB_4	26	85 ± 3	5	90 ± 5

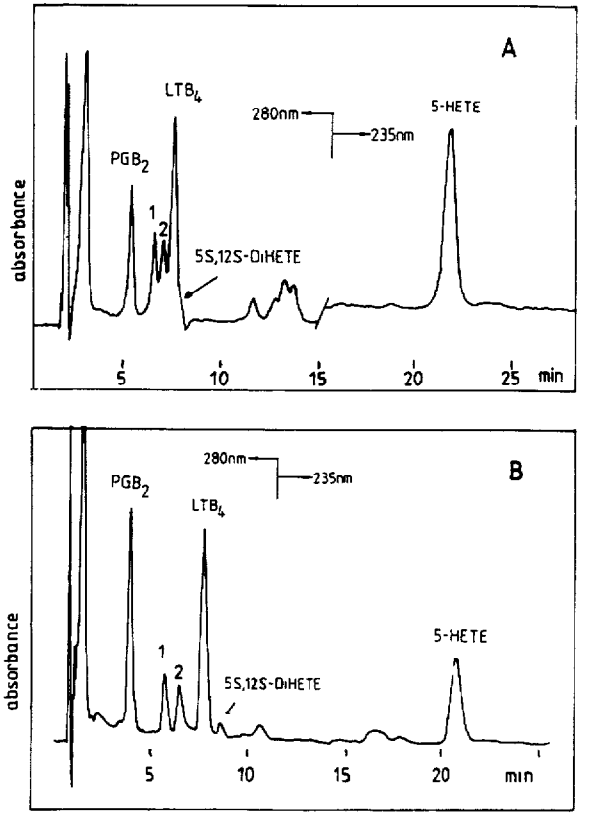

Fig. 1. HPLC of the products formed after incubation of human leukocytes with A23187 (5 μM). Mobile phase: methanol–water–acetic acid (70:30·0.01, v/v). Columns: (A) conventional; (B) Radial-Pak (Novapak). Peaks: 1 = 6-*trans*-LTB_4; 2 = 12*S*-6-*trans*-LTB_4.

LXA_4 and LXB_4 were converted to the corresponding *trans* isomers during the extraction procedure so that the total recovery was ca. 90%.

The most commonly used mobile phase for the separation of leukotrienes and lipoxins has been methanol–water–acetic acid. An extract of human PMNLs, stimulated with the Ca^{2+} ionophore, was used to investigate the re-

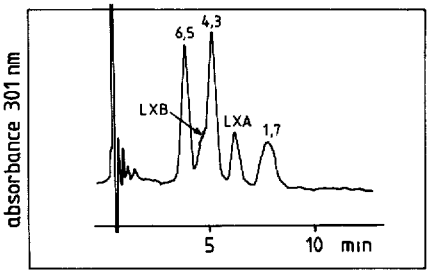

Fig. 2. HPLC of lipoxin standards. Mobile phase: methanol–water–acetic acid (60:40·0.01, v/v). Column: Radial-Pak. Peaks: 1 = 6*S*-LXA_4; 3 = 6*S*-11-*trans*-LXA_4; 4 = 11-*trans*-LXA_4; 5 = 14*S*-8-*trans*-LXB_4; 6 = 8-*trans*-LXB_4; 7 = 14*S*-LXB_4.

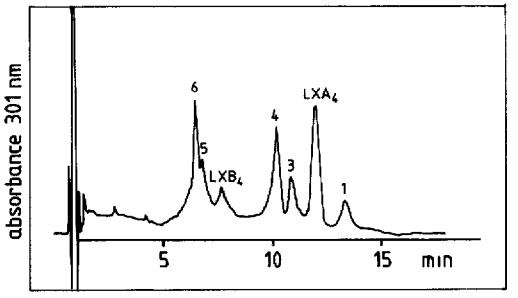

Fig. 3. HPLC of lipoxins extracted from human PMNLs. Mobile phase: acetonitrile–water–TFA (32:68:0.004, v/v). Column: Radial-Pak. Peaks: 1 = 6*S*-LXA_4; 3 = 6*S*-11-*trans*-LXA_4; 4 = 11-*trans*-LXA_4; 5 = 14*S*-8-*trans*-LXB_4; 6 = 8-*trans*-LXB_4.

solving power of the Radial-Pak cartridge. Fig. 1 shows the separation of LTB_4 from its 6-*trans* isomers and 5*S*,12*S*-diHETE, achieved with the conventional (Fig. 1A) and Radial-Pak columns (Fig. 1B). Only the Radial-Pak column completely separated LTB_4 and its three major dihydroxy isomers within 8 min; the conventional column did not separate LTB_4 from 5*S*,12*S*-diHETE. This may be due to the higher resolving power of the cartridge employing the radial compression technology. As the separation of all the lipoxin isomers produced by human granulocytes is still unsatisfactory [2–4], we tried to improve the analysis of the lipoxins using the Radial-Pak cartridge. Human PMNLs were incubated and extracted as described under Experimental.

When the Radial-Pak cartridge was eluted with methanol–water–acetic acid, the lipoxins were separated as illustrated in Fig. 2. LXA_4 was the only peak that consisted of a single compound. LXA_4 and LXB_4 were both separated from their corresponding *trans* isomers, but LXB_4 was co-eluted with 11-*trans*-LXA_4. When TFA was used instead of acetic acid the separation was relatively unaffected (data not shown). The methanol–water system led to a poor resolution of the lipoxins. The use of the acetonitrile–water system gives a better

separation of the lipoxins. When the column was eluted with acetonitrile–water–TFA (32:68:0.004, v/v), LXA_4 and LXB_4 were separated from the corresponding *trans* isomers ($\alpha \geq 1.12$ and 1.14, respectively). Additionally, there was a good resolution of the *trans* isomers of LXA_4 ($\alpha = 1.08$) (Fig. 3). The separation of the two *trans* isomers of LXB_4 was more critical ($\alpha = 1.06$) and 14*S*-LXB_4 was not separated from 11-*trans*-LXA_4.

The chromatogram (Fig. 3) shows a typical product profile obtained from a 1-ml suspension of human leukocytes. LXA_4 (25 ng) and its 11-*trans* isomer (20.5 ng) are the predominant metabolites, while LXB_4 (2.5 ng) and 6*S*-LXA_4 (2.5 ng) are produced only in minor amounts. No interfering material was detected in any of the extracts analysed. The detection limit for the lipoxins, based on a signal-to-noise ratio of 5:1, was 0.6 ng. Under these chromatographic conditions, all seven lipoxin isomers produced in human PMNLs could be separated. When acetic acid was used instead of TFA there was no significant difference in the chromatographic properties of the lipoxins.

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