Journal of Chromatography, 491 (1989) 193–199
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4722

Note

Rapid and simple assay method to study the content and the biosynthesis of peptide leukotrienes in whole blood

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(First received October 12th, 1988; revised manuscript received February 3rd, 1989)

Peptide leukotrienes (LTs) LTC_4, LTD_4 and LTE_4 as well as dihydroxylated LTB_4 are biologically active metabolites of arachidonic acid from human polymorphonuclear leukocytes [1]. Several analytical methods have been employed to measure LTs [2]. Among these, high-performance liquid chromatography (HPLC) with UV detection has been used to analyse LTs formed in stimulated isolated leukocytes [3]. However, this method has some disadvantages, such as the problems that arise when chemically unrelated substances that adsorb at ca. 270–280 nm are coeluted with LTs [2]. This problem is particularly serious when LTs are analysed in biological samples containing many unknown substances. Unknown UV-absorbing peaks could be mistaken for LTs when only retention times are available for identification. These pitfalls in the HPLC method necessitate another positive identification method for UV-absorbing substances, in addition to retention times. Indeed, HPLC–UV has been useful in the study of the production and metabolism of LTs in the pure isolated leukocytes. However, recent in vitro studies have pointed out that other blood cells, as well as plasma protein and free arachidonic acid in the blood, can affect the production and metabolism of LTs in the blood [4,5].

0378-4347/89/$03.50

The method used in studying the production of LTs in stimulated whole blood is applicable only to LTB_4 [7], not to LTC_4, LTD_4 and LTE_4.

Taking these considerations into account, we have developed a rapid and simple method to assay the production of LTs (LTB_4, LTC_4, LTD_4, LTE_4) in stimulated whole blood, using a computerized photodiode array detector for peak identification.

This method makes use of the metabolism of LTC_4 to LTE_4 via LTD_4 by γ-glutamyltranspeptidase activities and dipeptidase activities present in plasma protein and leukocytes [7]. The biosynthesis of the peptide LTs was followed by measuring the amount of LTE_4 formed by the conversion of LTC_4 and LTD_4 into LTE_4.

EXPERIMENTAL

Blood samples

Blood was collected from patients with bronchial asthma (9–15 years old) during the quiescent state. The percentage of eosinophils in the total leukocyte count was less than 6%. None of the donors had taken either steroidal or non-steroidal anti-inflammatory drugs for at least two weeks prior to blood sampling.

Chemicals

The following drugs were used: synthetic LTB_4, LTC_4, LTD_4, LTE_4 (Cayman, Ann Arbor, MI, U.S.A.) and prostaglandin B_2 (PGB_2) and calcium ionophore A23187 (Sigma, St. Louis, MO, U.S.A.). Azelastine was kindly provided by Eisai (Tokyo, Japan) [8]. Octadecylsilyl silica extraction columns (Sep-Pak C_{18} cartridges) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Solvents were HPLC grade (Nacalai Tesque, Kyoto, Japan). All other chemicals used were of analytical grade.

Apparatus

The HPLC equipment consisted of a Model M6000 pump, a U6K injector, a 990J computerized photodiode array spectrophotometer (all from Waters Assoc.) and a Shimadzu Chromatopac R-3A integrator. The mobile phase for HPLC was acetonitrile–methanol–distilled water–acetic acid–EDTA (300:100:420:0.8:0.5, v/v) and the pH was adjusted to 5.1 with ammonium hydroxide. The separations were carried out using a Cosmosil $5C_{18}$ packed column (Nacalai Tesque, 150 mm $\times$ 4.6 mm I.D., particle size 5 μm) at a flow-rate of 1.0 ml/min. The UV absorbance was continuously monitored in the range 240–320 nm, and the identification of compounds was based on the comparison of elution times as well as the UV spectra with those of synthetic LTB_4, LTC_4, LTD_4 and LTE_4. In some cases, the HPLC–UV data were compared with those obtained by radioimmunoassay (RIA). The RIA kits for LTB_4 were obtained

from Amersham (Amersham, U.K.) and that for LTC, LTD and LTE from NEN (Boston, MA, U.S.A.)

Method for biosynthesis and purification of LTs

A 2-ml volume of heparinized human whole blood (10 U heparin per ml of whole blood) was preincubated for 30 min at 37°C in the absence or presence of lipoxygenase inhibitor [30 μM azelastine or 3 μM nordihydroguaiaretic acid (NDGA)], and thereafter incubated in the presence of 20 μM ionophore A23187 for another 80 min at 37°C. Reactions were terminated by cooling the reaction tubes in ice-cold water. Extraction was performed essentially as described by Verhagen et al. [9], without a prior protein precipitation step. In brief, following centrifugation at 400 *g* for 10 min at 4°C, the supernatant plasma was diluted with four volumes of ice-cold phosphate-buffered saline (PBS) and applied directly to a Sep-Pak C_{18} cartridge, which was initially conditioned with 10 ml of methanol, 5 ml of water and then 5 ml of 0.1% EDTA solution. The cartridge was washed twice with 5 ml of water, and the adsorbed lipid was eluted with 3 ml of methanol. To achieve optimal extraction from plasma without protein precipitation, the volume of diluted plasma was limited to 10 ml per Sep-Pak C_{18} cartridge used. The speed of application was also a critical factor, hence the diluted plasma was usually applied to the cartridge at a speed of 2.5 ml/min. A high application speed or the use of vacuum brought about poor recoveries of LTs. The methanol fraction was carefully evaporated to near dryness under a stream of nitrogen gas. Complete drying also resulted in poor recoveries, and the extract was reconstituted in 100 μl of methanol. The recoveries of LTs from Sep-Pak C_{18} cartridges were obtained by adding known amounts of synthetic LTs prior to and after extraction. PGB_2 was added to the diluted plasma as an internal standard during the extraction and analysis. The effluent from the HPLC column was monitored at 280 nm, and standard curves were obtained for each experiment.

RESULTS

Chromatography

Fig. 1 shows the typical reversed-phase HPLC separations of the synthetic mixtures (LTB_4, LTC_4, LTD_4 and LTE_4) or the extracts from whole blood stimulated by ionophore A23187 under various experimental conditions, as monitored by UV absorbance at 280 nm. The pH of the eluting solvent was carefully studied to obtain a satisfactory separation of LTs, as well as to avoid any interference by UV-absorbing impurities, and the value of 5.1 was chosen. The good separation of the mixture containing 5 ng each of synthetic LTC_4, LTD_4 and LTE_4 and 15 ng of LTB_4 is shown in Fig. 1A. UV analysis of peaks corresponding to synthetic LTC_4, LTD_4 and LTE_4 by the photodiode array spectrophotometer revealed virtually identical UV absorbance maximum at

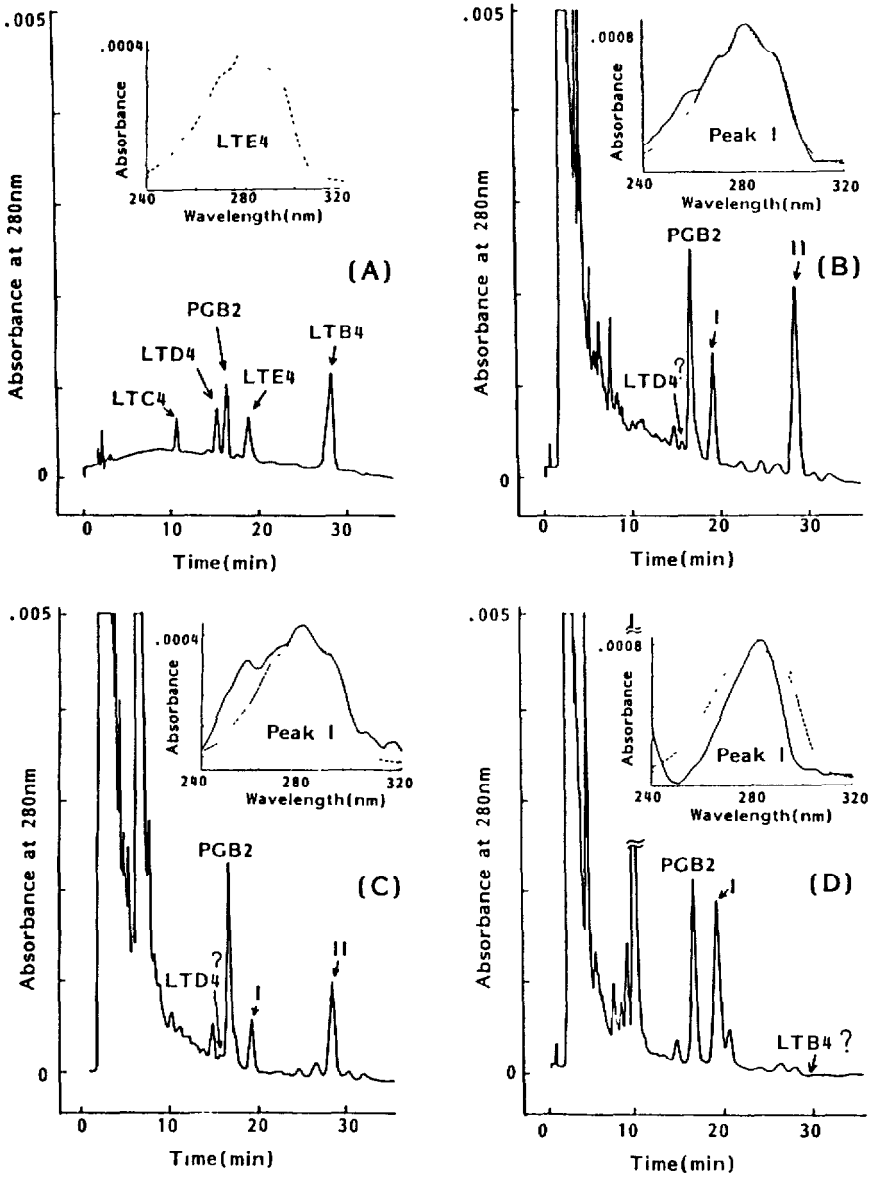

Fig. 1. Reversed-phase HPLC profiles and UV spectra of 5 ng each of synthetic LTC_4, LTD_4 and LTE_4 and 15 ng of LTB_4 (A) or those of biologically formed leukotrienes in 2 ml of whole blood stimulated by 20 μM A23187 in the absence of any inhibitor (B) or in the presence of 30 μM azelastine (C) or 3 μM NDGA (D). The insets in each panel show the UV spectrum corresponding to synthetic LTE_4 or those of peak I, which coelutes with synthetic LTE_4. In the insets of (B), (C) and (D), dotted lines represent the UV spectrum of synthetic LTE_4 scaled for comparison with that of peak I. No measurable peak was seen corresponding to LTC_4 or LTD_4 in the chromatogram from extracts of stimulated whole blood.

280 nm with shoulders at 270 and 290 nm, as reported for LTC_4, LTD_4 and LTE_4 (only the UV spectrum of LTE_4 is shown in the inset of Fig. 1A) [10], and the peak corresponding to synthetic LTB_4 also showed a UV absorbance maximum at 270 nm with shoulders at 260 and 280 nm, as reported for LTB_4 (data not given) [10]. However, no measurable peaks corresponding to those of synthetic LTC_4 and LTD_4 were observed in the chromatogram of the extracts from stimulated whole blood, whereas the peaks (I and II) corresponding to those of synthetic LTE_4 and LTB_4 were detected. (Fig. 1B, C and D). Peak I as LTE_4 (Fig. 1B and C) was confirmed by the characteristic UV spectrum shown in the insets of Fig. 1B and C, and the RIA analysis, in addition to the coincidence of the retention time with that of synthetic LTE_4. Except for the UV absorbance at ca. 258 nm probably due to an impurity, UV spectra of peak I in Fig. 1B and C closely coincided with that of the synthetic LTE_4 (insets of Fig. 1B and C). The amounts of LTE_4 formed in stimulated whole blood were found to be 16.6 ± 6.1 ng/ml of whole blood for 80 min ($n=5$; mean $\pm$ S.D.) in the absence of any inhibitor and 4.3 ± 0.7 ng/ml in the presence of 30 μM azelastine.

Peak II was identified as LTB_4 by the same methods, including RIA and gas chromatography–mass spectrometry (GC–MS) [11]. Similary, the amounts of LTB_4 formed were also calculated to be 35.2 ± 10.3 ng/ml of whole blood in the absence any inhibitor and 16.3 ± 7.5 ng/ml of whole blood in the presence of 30 μM of azelastine. When the stimulation was carried out following preincubation of whole blood with 3 μM NDGA for 30 min, the peak-area ratio of peak I to PGB_2 (internal standard) was greater than that when stimulation was done in the absence of any inhibitor. However, peak II, which corresponds to synthetic LTB_4, was not evident in the extract from whole blood stimulated after preincubation with NDGA (Fig. 1D). Nevertheless, RIA analysis has demonstrated the disappearance of both immunoreactive substances for anti-LTE_4 antibody and anti-LTB_4 antibody from peaks I and II, respectively (data not given). Furthermore, detailed analysis of the UV spectrum of peak I in Fig. 1D showed that the UV spectrum of peak I from the stimulated whole blood preincubated with NDGA was apparently different from that of synthetic LTE_4, exhibiting a non-characteristic UV absorbance maximum at 293 nm with no shoulder (inset of Fig. 1D). Finally, peak I from the stimulated whole blood preincubated with NDGA was confirmed to be contaminated with impurities from the NDGA added to inhibit the production of LTs.

Conversion of LTC_4 into LTD_4 and LTD_4 into LTE_4

Preliminary experiments showed that the peak-area ratio of the peak corresponding to LTC_4 to that of the internal standard increased up to ca. 10 min of incubation time, then decreased below the detection limit at ca. 80 min of incubation time, whereas the peak-area ratio of the peak corresponding to LTE_4 to that of the internal standard gradually increased up to ca. 80 min. In order

to confirm that the 80 min incubation time is long enough to effect a complete conversion of LTC_4 formed in stimulated whole blood, 20 ng each of synthetic LTC_4 and LTD_4 were separately added to the whole blood after 20 min of incubation time with ionophore A23187 and incubated for another 60 min, after which LTs were analysed as described. Similar experiments were performed in the absence of synthetic LTs. In none of these experiments was a substantial peak corresponding to LTC_4 or LTD_4 detected. The recoveries from Sep-Pak C_{18} cartridges of synthetic LTs added to whole blood and incubated were estimated to be between 75 and 85%, which is comparable with recovery from Sep-Pak C_{18} cartridges of synthetic LTE_4 added to diluted plasma.

Recovery and sensitivity

Recoveries of synthetic LTs from Sep-Pak C_{18} cartridges were found to be 78% for LTB_4, 38% for LTC_4, 50% for LTD_4 and 73% for LTE_4 when synthetic LTs were added to the diluted plasma. The recovery of PGB_2 was consistently ca. 85%. Since both LTC_4 and LTD_4 were shown to be almost quantitatively converted into LTE_4 in stimulated whole blood, only the sensitivity of LTE_4 was evaluated in this investigation: it was less than 5 ng for the analysis of the UV spectrum, as judged from the insets of Fig. 1A and C, and less than 1 ng when the UV absorbance was monitored at 280 nm.

DISCUSSION

We have devised a rapid and simple method to study the content and biosynthesis of LTs in whole blood for purposes of minimizing the loss of peptide leukotrienes (LTC_4, LTD_4 and LTE_4) during the process and increasing the specificity of UV detection. Fig. 1B shows a result consistent with the conversion of most of the LTC_4 formed in whole blood into LTE_4 via LTD_4, supposedly by enzymic activity in plasma or leukocytes [7]. Likewise, the experiment in which synthetic LTs were added to the stimulated whole blood similarly confirmed the almost complete conversion of endogenously formed LTC_4 into LTE_4. LTC_4 production in whole blood could be measured as the amount of LTE_4, which is reportedly much more stable than LTC_4 and LTD_4 [12].

We utilized Sep-Pak C_{18} cartridges to extract LTs without protein precipitation step. Verhagen et al. [9] also successfully extracted LTs in the presence of 100 mg of albumin, using a Baker octadecyl extraction column. In our study using Sep-Pak C_{18} cartridges, we also showed that LTE_4 could be recovered moderately efficiently from up to 10 ml of five-fold diluted plasma per cartridge. Inasmuch as LTC_4 and LTD_4 were recovered relatively poorly in the presence of plasma protein, the conversion of LTC_4 and LTD_4 proved to be useful in obtaining higher recoveries. Moreover, the simplicity and speed of this method render it useful for assaying, especially in a large number of biological samples.

The use of a computerized photodiode array spectrophotometer enabled us to identify the peak corresponding to LTE_4 and to check the purity of LTE_4 even after 75% inhibition of the production by 30 μM azelastine. Likewise, this prevented an overestimation of the amount of LTs due to the presence of NDGA. Many investigators have employed RIA, bioassay or mass spectrometry (MS) to identify the peaks in the HPLC chromatogram, since extracts of biological samples contain many impurities that adsorb at ca 280 nm [2]. Although LTB_4 can be identified with quite a good sensitivity by GC–MS [2, 11], LTC_4, LTD_4 and LTE_4 must be identified by thermospray LC–MS or fast atom bombardment MS with relatively poor sensitivity (microgram level for identification). In the present study, we easily obtained a UV spectrum with a high signal-to-noise ratio from ca. 5 ng of LTE_4 present in stimulated whole blood (Fig. 1D).

Finally, biosynthetic activities of LTs in whole blood could be measured in several millilitres without the step of leukocyte preparation.

Our method may be useful in screening the effects of various drugs on LT formation as well as in monitoring biosynthetic activities of LTs in patients with allergic or inflammatory diseases.

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