High-Performance Liquid Chromatography of Arachidonic Acid Metabolites and Its Application to the Determination of Leukotriene B_4 in Stimulated Leukocytes

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A highly sensitive high-performance liquid chromatography with fluorescence detection for the determination of arachidonic acid metabolites is described. The metabolites are converted into corresponding fluorescent derivatives by reaction with 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone in the presence of potassium hydrogen carbonate and 18-crown-6 in acetonitrile. The derivatives are separated on a reversed-phase column (Inertsil ODS) with aqueous acetonitrile and detected fluorimetrically. The detection limits are 5—15 fmol at a signal-to-noise ratio of 3 in a 10-μl injection volume. The method is applied to the determination of leukotriene B_4 produced in stimulated leukocytes.

Keywords 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone; arachidonic acid metabolite; high-performance liquid chromatography; leukocyte; fluorescence detection

Many kinds of arachidonic acid metabolites, including hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), thromboxanes (TXs) and prostaglandins (PGs), are present in biological materials, and play physiologically important roles at trace levels in the living body. Thus, a highly sensitive and simple method for the determination of the metabolites is required for biological and biomedical investigations.

Some high-performance liquid chromatographic (HPLC) methods with fluorescence detection using anthryldiazomethane,[1,2] *p*-(9-anthroyloxy)phenacyl bromide,[3,4] 4-bromomethyl-7-methoxycoumarin,[5] 4-bromomethyl-7-acetoxycoumarin,[6] 1-pyrenyldiazomethane[7] and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone,[8] have been developed for the determination of PGs, LTs, TXs and/or HETEs.

Recently, we reported that 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone (Br-MMEQ),[9] a fluorescence derivatization reagent for carboxylic acids, is more sensitive than conventional reagents. This paper presents a sensitive HPLC method utilizing Br-MMEQ for the microanalysis of six arachidonic acid metabolites (see Fig. 1). In order to ascertain the practical applicability of the method to biological samples, the quantification of LTB_4 produced in stimulated leukocytes was investigated using PGB_2 as an internal standard.

Experimental

Reagents and Solutions All chemicals and solvents were of analytical reagent grade, unless otherwise stated. Deionized and distilled water was used. (5*S*,6*Z*,8*Z*,10*E*,12*S*,14*Z*)-5,12-Dihydroxytetraenoic acid (5*S*,12*S*-diHETE), TXB_2, $PGF_{2\alpha}$, PGE_2, PGB_2, LTB_4 and calcium ionophore A23187 were purchased from Sigma (St. Louis, Mo., U.S.A.). Ionophore (9.5 μM) solution was prepared in dimethylsulfoxide. Br-MMEQ was prepared as described previously.[9] Br-MMEQ (6.7 mM) and 18-crown-6 (1.9 mM) solutions were prepared in acetonitrile. The Br-MMEQ solution could be used for more than one week when stored in a refrigerator at 4 °C. HEPES-buffered saline contains 135 mM NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgSO_4$, 2 mM glucose and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4).

HPLC Apparatus and Conditions A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow-cell operating at 370 nm excitation and 455 nm emission was used. The column was an Inertsil ODS (250 × 4.6 mm i.d.; particle size, 5 μm; Gasukuro Kogyo). The column temperature was ambient (20—27 °C). Aqueous 42% and 55% (v/v) acetonitrile were used for the separation of MMEQ derivatives of TXB_2, $PGF_{2\alpha}$ and PGE_2, and PGB_2, LTB_4 and 5*S*,12*S*-diHETE, respectively.

Corrected fluorescence excitation and emission spectra of the euates were measured with a Hitachi 650—60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Preparation of Leukocytes and Incubation Procedure Human blood cells were isolated from venous blood of healthy volunteers. Polymorphonuclear leukocytes (PMNL) were prepared by dextran sedimentation, hypotonic lysis and the Conray–Ficoll method as described by Böyum.[10] The leukocytes were suspended in the HEPES-buffered saline in the concentration range of 0.3—5.0 × 10^6 cells/ml. The suspensions of PMNL consisted of 95—98% neutrophils, 1—3% lymphocytes and less than 1% eosinophils. Portions (1.0 ml) of the suspension were preincubated at 37 °C for 2 min. Incubations were started by the addition of the ionophore solution and were performed at 37 °C. Incubations were terminated by the addition of 3.0 ml of ice-cold CH_3OH containing 30 pmol of PGB_2. The resulting solution was centrifuged at 1000 *g* for 20 min at −4 °C. The supernatant (3.6 ml) was evaporated under a stream of nitrogen. The aqueous residue (*ca.* 1 ml) was poured onto a Centricon 30 ultrafilter (Amicon, Danvers, Mass., U.S.A.) and centrifuged at 1500 *g*. The eluate was diluted with 2.0 ml of water and the resulting solution was adjusted to pH 3.0—3.5 with 0.1 M HCl, and extracted twice with 3 ml of ethyl acetate. The combined extracts were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 μl of acetonitrile. The final solution was used as the sample solution.

Derivatization Procedure A 100-μl portion of the sample solution was placed in a screw-capped 10-ml vial, to which were added *ca.* 10 mg of potassium hydrogen carbonate and 100 μl each of the Br-MMEQ and 18-crown-6 solutions. The vial was tightly closed and warmed at 50 °C for 15 min in the dark. After cooling, 10 μl of the resulting mixture was injected into the chromatograph.

The calibration graph was prepared according to the same procedure, except that ice-cold methanol (3 ml) containing 2.0—750 pmol of LTB_4 was used and the order of addition of the methanol and ionophore solution was reversed. The net peak-height ratios of LTB_4 and PGB_2 were plotted against the concentrations of the spiked LTB_4.

Results and Discussion

Derivatization Conditions Figures 1A and 1B show typical chromatograms obtained with a standard mixture of six arachidonic acid metabolites. The individual metabolites gave single peaks in the chromatogram.

Br-MMEQ gave the most intense peaks at concentrations greater than *ca.* 5 mM in the solution; 6.7 mM was used as a sufficient concentration. Maximum and constant peak heights were attained at 18-crown-6 concentrations in the solution in the range of 1—4 mM; 1.9 mM was selected for the procedure. The peak heights due to the metabolites were maximal and constant at amounts of potassium hydrogen carbonate larger than 5 mg; *ca.* 10 mg was employed in the procedure.

The derivatization reaction of the six metabolites with

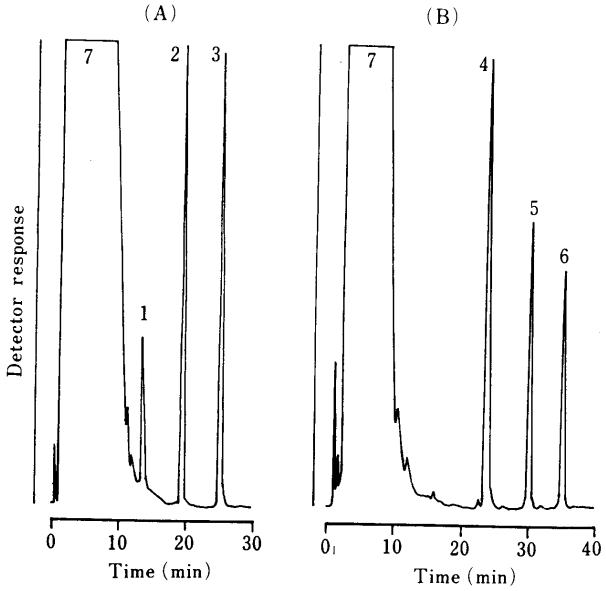

Fig. 1. Chromatograms of MMEQ Derivatives of Arachidonic Acid Metabolites

A portion (100 μl) of a mixture of the six metabolites (2.0 nmol/ml each) was treated according to the standard procedure. Peaks: 1, TXB_2; 2, $PGF_{2\alpha}$; 3, PGE_2; 4, PGB_2; 5, LTB_4; 6, 5*S*,12*S*-diHETE; 7, reagent blank. HPLC conditions: column, Inertsil ODC (260 × 4.6 mm i.d.; particle size, 5 μm); eluent, (A) CH_3CH–H_2O (42:58, v/v), (B) CH_3CN–H_2O (55:45, v/v).

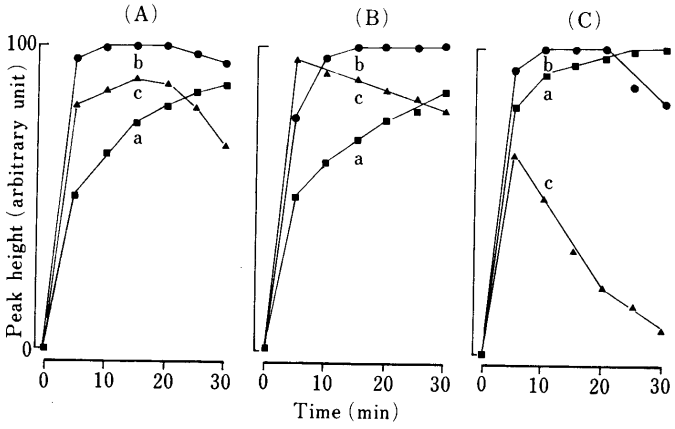

Fig. 2. Effect of Reaction Temperature and Time on the Peak Heights of (A) LTB_4, (B) PGE_2 and (C) TXB_2

Portions (100 μl) of a standard mixture of the six metabolites were treated according to the standard procedure, except for temperature. Temperature: a, 37 °C; b, 50 °C; c, 80 °C.

Br-MMEQ, which apparently occurred even at 37 °C, was accelerated by higher temperatures (Fig. 2). However, at 80 °C, the peak heights decreased after prolonged heating (5—30 min). The decrease was remarkable for TXB_2. At 50 °C, the peak heights for all the metabolites reached almost maxima after warming for 15 min; 15-min warming at 50 °C was recommended in the procedure. The MMEQ derivatives were stable for at least 24 h in the dark at 4 °C.

The within-day precision was examined by performing ten separate analyses using standard mixtures of the six metabolites (20 and 0.5 pmol/100 μl each); the relative standard deviation did not exceed 3.7% for any of the metabolites. The detection limits (fmol) were 15 (TXB_2), 5 ($PGF_{2\alpha}$, PGE_2 and PGB_2), 8 (LTB_4) and 10 (HETE) in a 10-μl injection volume at a signal-to-noise ratio of 3. The sensitivity was higher than those of the methods with

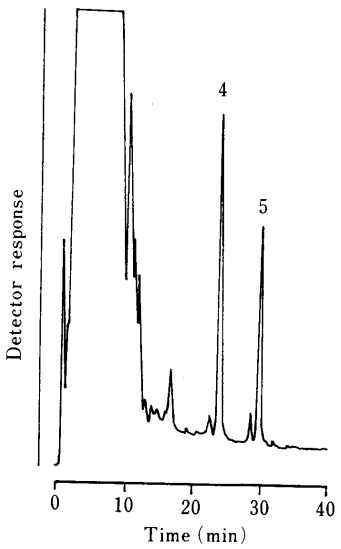

Fig. 3. Chromatogram Obtained with Stimulated Leukocytes

A portion (1.0 ml) of the PMNL suspension (1.0 × 10^6 cells/ml) was stimulated by the ionophore for 5 min. Peaks, see Fig. 1.

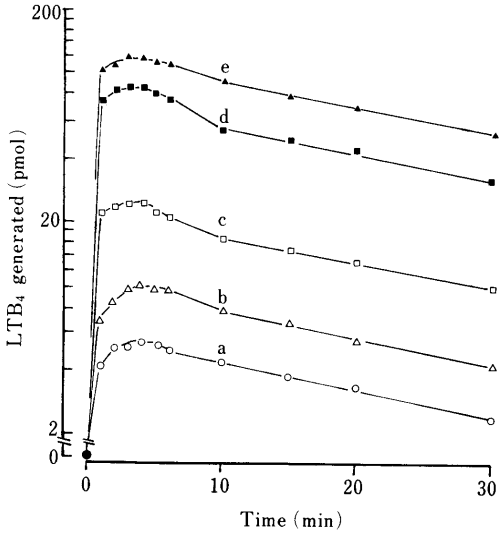

Fig. 4. Time-Course of the LTB_4 Generation by Stimulated Leukocytes

Portions (1.0 ml) of the PMNL suspensions were incubated with the ionophore for various times. PMNL suspensions (× 10^5 cells/ml): a, 3; b, 5; c, 10; d, 30; e, 50.

conventional fluorescence derivatization reagents.[1-8] The relationships between the peak heights and the amounts of the six individual metabolites were linear up to at least 10 nmol per injection volume (10 μl).

Determination of LTB_4 in Stimulated Human Leukocytes Figure 3 shows a typical chromatogram obtained with the PMNL suspension (1.0 × 10^6 cells/ml), which was stimulated by calcium ionophore A23187 for 5 min. Peak 5 was identified as LTB_4 on the basis of the retention time and fluorescence excitation and emission spectra of the eluate by comparison with the standard compound, and also by co-chromatography of the standard and the leukocyte sample with aqueous 50—100% acetonitrile or methanol as the mobile phase.

A linear relationship was observed between the peak height ratios of LTB_4 to PGB_2 and the amounts of LTB_4 added in the range of 2.0—750 pmol to 1 ml of PMNL suspension.

The within-day precision was established by repeated determinations ($n=10$) using a PMNL suspension (1.0×10^6 cells/ml) stimulated by the ionophore for 5 min. The relative standard deviation was 4.8%. The recovery (%, mean $\pm$ S.D., $n=8$) of LTB_4 added to a PMNL suspension was 89.2 ± 3.8%, when the recovery of PGB_2 was taken as 100.

Figure 4 shows the time-course of LTB_4 generated by human PMNL. The pattern of the curves was almost identical with those obtained by the previously reported methods.[11,12]

The present HPLC method using fluorogenic Br-MMEQ is highly sensitive; the sensitivity permits the assay of LTB_4 generated in a small number of leukocytes. The method should be useful for biological and biomedical investigations of arachidonic acid metabolites.

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