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Note

Determination of 5-lipoxygenase activity in human polymorphonuclear leukocytes using high-performance liquid chromatography

S.R. McCOLL*, W.H. BETTS, G.A. MURPHY and L.G. CLELAND

Rheumatology Unit, The Queen Elizabeth Hospital, Woodville Road, Woodville, South Australia 5011 (Australia)

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Products of arachidonate metabolism (prostaglandins, leukotrienes and thromboxanes) are of central importance in vascular/platelet homeostasis [1], reproduction [2], allergy [3—5] and inflammation [4—6]. A variety of analytical procedures have been used to quantify and/or isolate these products. The procedures used range from bioassay [7,8], radioimmunoassay (RIA) [9,10], thin-layer chromatography (TLC) [11,12], high-performance liquid chromatography (HPLC) [13—16] and gas chromatography—mass spectrometry (GC—MS) [13,15,17]. All methods suffer from disadvantages. For example, quantification in bioassay is difficult and may lack specificity. RIA, which affords high sensitivity, necessitates the use of radioisotopes and suffers occasionally from insufficient antibody specificity to discriminate between closely related compounds. TLC allows a broad range of products to be detected but is not suitable for quantification of biological samples without use of radiolabelled precursor molecules. GC—MS provides not only specific and very sensitive results but also structural information. However, constraints of cost, time involved in sample preparation and assay time limit this method to special studies. HPLC provides both specific and reproducible results combined with great sensitivity and is an ideal method for simultaneous analysis of compounds which are chemically closely related. Use of radioisotopes is not necessary in the case of compounds with favorable UV absorption or fluorescent characteristics.

EXPERIMENTAL

Chemicals

Calcium ionophore A23187, 15-lipoxygenase (soy-bean), sodium tetraborate

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and arachidonic acid (AA) were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium borohydride was purchased from Laboratory Chemicals (Ajax Chemicals, Auburn, Australia) and leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) standards were a generous gift from Merck, Sharp and Frosst (Canada) and the Department of Organic Chemistry at the University of Adelaide. All solvents (HPLC grade) were purchased from Waters Millipore (Milford, MA, U.S.A.). 15-HETE was biosynthesised as described below.

Apparatus

All chromatographic equipment was from Waters Assoc. The system included a Model 510 pump, 730 data module, 490 variable-wavelength UV detector and 710B WISP autoinjector. The mobile phase for HPLC was methanol–water–acetic acid (72:28:0.08) and the pH was 6.2 (adjusted with ammonium hydroxide). The separations were carried out using a Waters Nova-Pak C_{18} (15 cm × 3.9 mm) column (5 μm average particle size) at a flow-rate of 1.0 ml/min. A C_{18} guard column (10 μm) was used at all times. Chromatography was carried out at room temperature and the mobile phase was constantly bubbled with helium to remove dissolved oxygen. Detection of HETEs was carried out at 234 nm UV at 0.02 a.u.f.s. and LTB_4 at 280 nm UV at 0.005 a.u.f.s. Identification of compounds was verified using pure standards.

Method for purification of blood cells

Human polymorphonuclear leucocytes (PMNLs) were isolated, stimulated and their 5-lipoxygenase products extracted as follows: whole blood was taken from healthy volunteers by venepuncture and mixed with 4.5% EDTA (5:1). Red blood cells were sedimented at 37°C by addition of dextran (5:1 of Dextraven 150). The white blood cell rich supernatant was carefully layered onto a double Percoll (Pharmacia, Uppsala, Sweden) gradient (specific gravities of 1.070 and 1.092) and spun at 450 *g* for 20 min. The PMNLs were collected from the interface between the two Percoll layers, washed twice in Dulbecco's phosphate-buffered saline before a final resuspension in Hank's balanced salt solution at the desired cell concentration (approximately $2 \cdot 10^6$ cells per ml). Using this procedure cell purity was greater than 97% as determined by gentian violet staining. Cells were stimulated by addition of 10 μl of 1 m*M* AA followed rapidly by 5 μl of 1 m*M* A23187 to 1 ml of cells at 37°C and the reaction was terminated after 2 min by addition of 5 ml of ethyl acetate. Internal standard (170 ng of 15-HETE) was added and the samples were vortexed for 15 s and centrifuged at 1000 *g* for about 5 min. The ethyl acetate layer was withdrawn and evaporated to dryness under vacuum using a centrifugal evaporator (Savant, Hicksville, NY, U.S.A.) and the samples were reconstituted in 100 μl of methanol. Aliquots of 25 μl were injected onto the column (Fig. 1). Standard curves were performed for each experiment.

Biosynthesis of 15-HETE

15-Lipoxygenase (10 μl, 7350 U) was added to 1 ml of sodium tetraborate buffer (200 m*M* at pH 9.0) at 25°C followed by 10 μl of 1 *M* AA. The reaction mixture was stirred for 20 min prior to addition of 10 μl of 100 mg/ml sodium

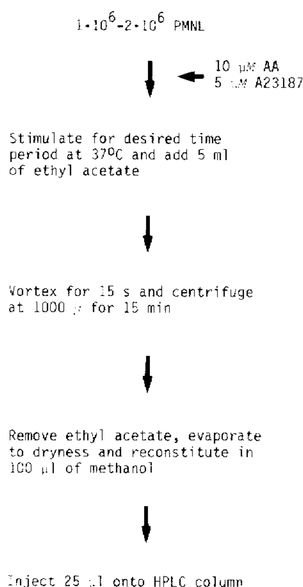

Fig. 1. Procedure for stimulation of cells and extraction of AA metabolites prior to HPLC.

borohydride [added to reduce 15-hydroperoxyeicosatetraenoic acid (15-HPETE) to 15-HETE]. The reaction mixture was then stirred continuously overnight. 15-HETE was extracted into ethyl acetate, evaporated to dryness and reconstituted in benzene (for storage) at a concentration of 0.19 m*M*. Aliquots were dried and reconstituted in methanol for use as internal standard. Quantification was performed in methanol using a spectrophotometer (E = 30 500 at 234 nm [14]). 15-HETE (170 ng) was added as internal standard.

Synthesis and determination of radiolabelled AA metabolites

Isolated neutrophils were stimulated for 2 min at 37°C with 0.1 μCi [^{14}C]AA (40–60 mCi/mmol) and 5 μM A23187. The AA metabolites were extracted, evaporated to dryness and reconstituted prior to HPLC (as described above). The samples were monitored by UV spectrophotometry and 1-min aliquots were collected for liquid scintillation counting.

RESULTS

Chromatography

Figs. 2 and 3 are typical chromatograms of LTB_4, 5-HETE and 15-HETE. Fig. 2 shows a chromatogram of a mixture of pure standards and Fig. 3 shows a typical chromatogram of a sample from cells stimulated with A23187. The k' values for LTB_4, 15-HETE and 5-HETE were 3.2, 11.8 and 16.2 min, respectively.

Recovery

The procedure for stimulation, extraction and chromatographic preparation was found to be rapid and free from interferences. Recoveries were found to be

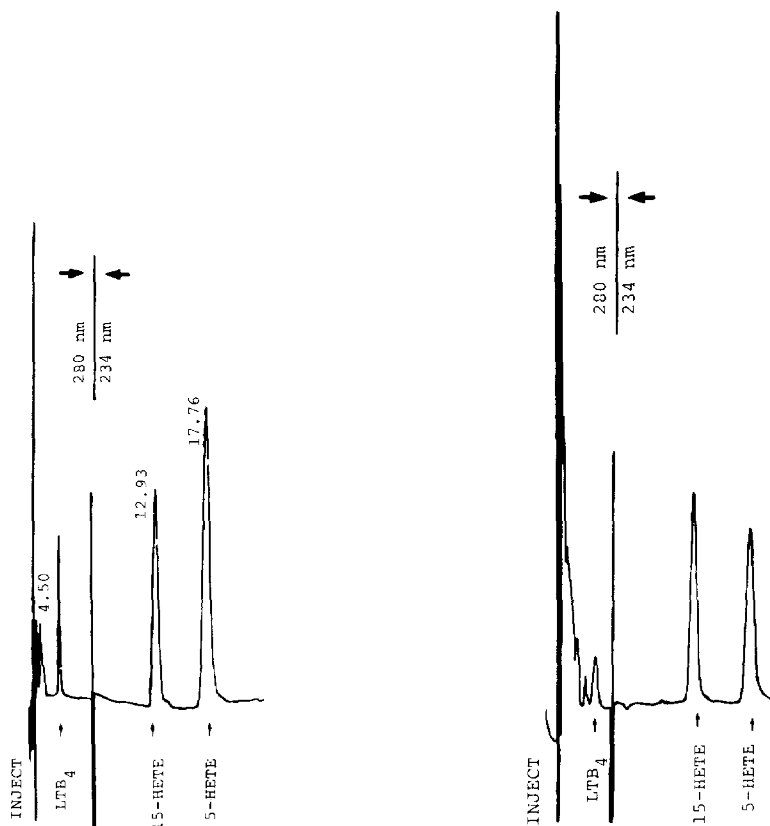

Fig. 2. Chromatogram of pure standards.

Fig. 3. Chromatogram of cell samples stimulated for 2 min with 5 μM A23187 and 10 μM AA (final concentrations).

58% for LTB_4 (range 0—50 ng) and 92% for the mono-HETEs (range 0- 250 ng).

Standard curves and sensitivity

Standard curves were obtained for each experiment in the range 0—250 ng (5-HETE) and 0—50 ng (LTB_4). Using the linear regression analysis, the coefficients of correlation for 5-HETE and LTB_4 were 0.999 and 0.996, respectively. Sensitivity was less than 1 ng (as determined at a signal-to-noise ratio of 2:1) when extracted from $1 \cdot 10^6$—$5 \cdot 10^6$ cells.

Precision

Coefficients of variation for both intra- and inter-assay variability were calculated for both 5-HETE and LTB_4. Intra-assay variability was determined by measuring quadruplicate samples at high and low levels of the respective metabolites following production by cells. The low level (point A) was obtained by measuring products in cell samples stimulated for 30 s and the high level (point B) by measuring products after 5 min of stimulation (see Figs. 4 and 5). The coefficients of variation of the assay for 5-HETE at these two points were 6.5 and 5.9% and those for LTB_4 were 9.8 and 12.6% (points A and B, respective-

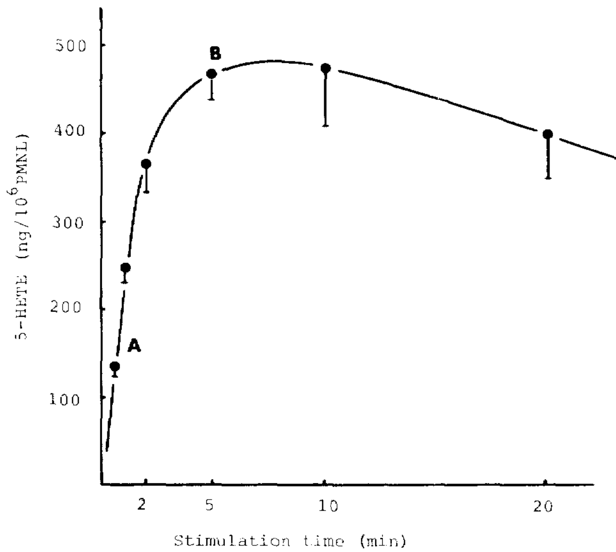

Fig. 4. Time course of production of 5-HETE. $1 \cdot 10^6$ PMNLs were stimulated with 5 μM A23187 and 10 μM AA for designated time periods.

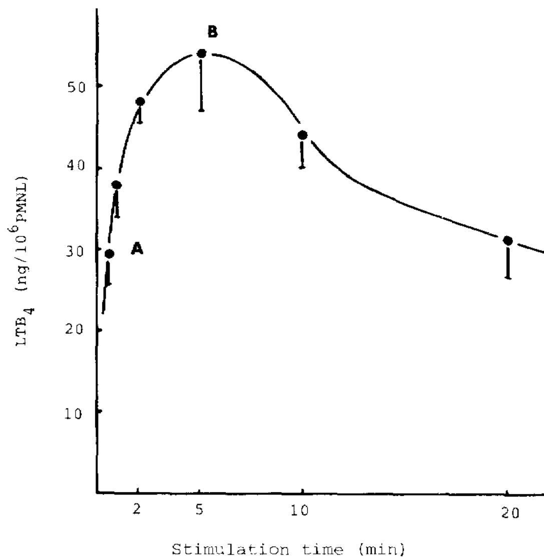

Fig. 5. Time course of production of LTB_4. $1 \cdot 10^6$ PMNLs were stimulated with 5 μM A23187 and 10 μM AA for designated time periods.

ly). The value for inter-assay variability was obtained by quadruplicate measurement of a known standard in separate assays over a period of twenty experimental days by two analysts. The coefficients of inter-assay variation were 5.6% for LTB_4 and 4.2% for 5-HETE.

Choice of internal standard

Cells stimulated with 5 μM A23187 and radiolabelled AA produced large quantities of ^{14}C-labelled LTB_4 and 5-HETE. No ^{14}C-labelled 15-HETE was produced under these conditions.

DISCUSSION

Several HPLC methods for analysis of leukotrienes and hydroxy fatty acids have been described [13—16]. The present assay was specifically designed to measure the two major eicosanoid products PMNLs, 5-HETE and LTB_4. A 10-ml volume of venous blood is sufficient to produce quantifiable amounts of these products using this method. The extraction and guard column clean-up procedures are simple and rapid compared to other methods, and the analysis time for assaying both LTB_4 and 5-HETE is ca. 20 min. Using this technique we experienced no difficulty in resolving LTB_4 from its isomers and ω-oxidation products. Sensitivity and precision are high.

Under the experimental conditions described, no 15-HETE was detected. There have been several previous reports indicating that PMNLs synthesize 15-HETE [18, 19] but these studies were not performed using intact PMNLs. Therefore, in an intact-PMNLs experimental system, 15-HETE is a suitable internal standard, providing the correct controls are performed.

The assay cannot be used to measure the isomers or the ω-oxidation products of LTB_4 or the peptidoleukotrienes without significantly increasing analysis time. Therefore, in its current form the assay is limited to measuring the two major biologically active 5-lipoxygenase products in inflammatory cells such as PMNLs.

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