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Note

Rapid and simple methods for the investigation of lipoxygenase pathways in human granulocytes

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Metabolites of arachidonic acid (or analogues), produced by the lipoxygenase pathway, play an important role in many physiological and pathophysiological processes. However, many questions related to their metabolism and exact function remain unanswered [1–6].

Current methods for the measurement of arachidonic acid metabolites require large blood samples and time-consuming extraction and concentration procedures. Moreover, high-performance liquid chromatographic (HPLC) methods applied to the separation of lipoxygenase products use complex gradients and give long retention times [7–10]. In view of future research in this area, an urgent need exists for rapid and convenient micromethods. This paper describes a rapid HPLC method for the determination of leukotrienes and hydroxy fatty acids originating from in vitro stimulated peripheral blood cells.

The advantages of the system are that (i) the use of microtitre plates enables a large number of experiments to be performed very efficiently, (ii) no time-consuming extraction or concentration procedures are required before HPLC analysis and (iii) the sample capacity is 144 per day.

EXPERIMENTAL

Materials

Arachidonic acid (AA) and prostaglandin B_2 (PGB_2) were obtained from Sigma (St. Louis, MO, U.S.A.) and calcium ionophore A 23187 from Calbiochem (La Jolla, CA, U.S.A.). Standard leukotrienes [leukotriene B_4 (LTB_4), C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4)] were a gift from Dr. J. Rokach of Merck Frosst (Pointe Claire, Quebec, Canada). Microtitre plates (flat-bottomed 96-well ELISA plates) were purchased from Costar (Cambridge, MA, U.S.A.). Tetrahydrofuran

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was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and methanol (HPLC grade) from Rathburn (Walkerburn, U.K.). Methanol for standard solutions was of Uvasol quality (Merck, Darmstadt, F.R.G.)

Blood samples were obtained from human volunteers.

High-performance liquid chromatography

For HPLC analysis of leukotrienes a modification of the method of Verhagen et al. [11] was used. HPLC columns (120 mm×4.6 mm I.D.) were packed in our laboratory with Nucleosil 120-5 C_{18} material (Macherey-Nagel, Düren, F.R.G.). For analysis of hydroxy fatty acids, a Nucleosil 120-3 C_{18} column (60 mm×4.6 mm I.D.) was used.

The HPLC system consisted of a Knauer pump (Model FR 30), a Gilson sample injector (Model 231), a Techmation column oven (Tracor 925), an LDC/Milton Roy variable-wavelength UV–visible detector (Spectromonitor D) and an LDC/Milton Roy integrator system (Model CI-10). The mobile phase used for analysis of leukotrienes was tetrahydrofuran–methanol–0.1% EDTA/0.01 *M* sodium acetate in water, adjusted to pH 5.5 with acetic acid (25:30:45, v/v). With a flow-rate of 1.2 ml/min, the analysis time was 10 min. The mobile phase used for analyses of hydroxy fatty acids was methanol–water–acetic acid (77.5:22.45:0.05, v/v). The flow-rate was 1.2 ml/min and the analysis time was 10 min. The detection wavelength for leukotrienes was 278 nm and for hydroxy fatty acids 235 nm. Supernatants from stimulation experiments with washed leukocytes could be injected without further treatment.

Granulocyte experiments

Leukocytes were isolated from blood by dextran precipitation [12] and resuspended in a phosphate buffer to a final concentration of 10^7 cells per ml. The composition of the buffer was 137 m*M* sodium chloride–2.7 m*M* potassium chloride–8.1 m*M* Na_2HPO_4–1.5 m*M* KH_2PO_4 (pH 7.4). From 30 ml of human blood the yield of leukocytes was mostly between $5 \cdot 10^7$ and $1 \cdot 10^8$. The cells (240 μl) were incubated at 37 °C in microtitre plates before the experiments. After at least 5 min, 30 μl of a solution of calcium and magnesium with or without AA (800 μM AA, 20 m*M* calcium chloride, 5 m*M* magnesium chloride in buffer, pH 7.4) were added, mixed and incubated at 37 °C for 5 or 15 min. The reaction was started by adding 30 μl of a solution containing the ionophore A 23187 and glutathione (0.1 mg/ml ionophore A 23187, 15.4 μg/ml glutathione in buffer, pH 7.4). The reaction was performed at 37 °C with constant mixing. After exactly 15 min, the reaction was stopped by cooling the cell suspensions as fast as possible on ice. For constant mixing during the reaction a plate shaker was used.

After centrifugation at 2200 *g* for 10 min at 4 °C, supernatants could be collected for HPLC analysis. A centrifuge with buckets for microtitre plates was used. The microtitre plate containing the supernatants was placed directly on a Gilson sample injector, which has standard programmes for handling microtitre plates.

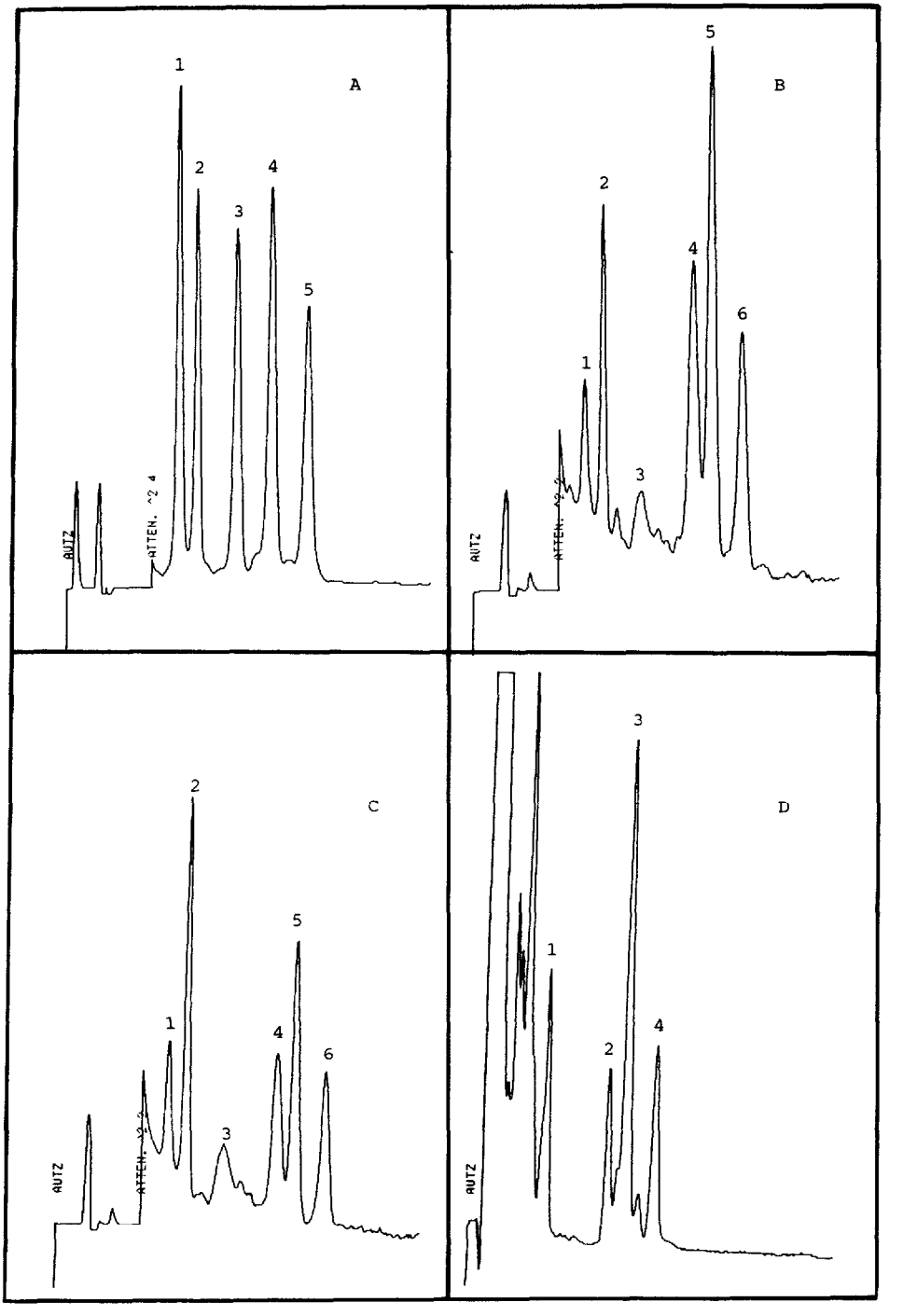

Fig. 1. (A) Standard leukotrienes: (1) LTC_4; (2) PGB_2 (internal standard); (3) LTD_4; (4) LTE_4; (5) LTB_4. (B) Leukotrienes produced by leucocytes with 80 μM exogenous arachidonic acid: (1) LTC_4; (2) PGB_2; (3) LTD_4; (4) 6*t*-LTB_4+12*epi*,6*t*-LTB_4; (5) LTB_4; (6) 12*epi*,6*t*,8*c*-LTB_4. (C) Leukotrienes produced by leukocytes without exogenous AA: (1) LTC_4; (2) PGB_2; (3) LTD_4; (4) 6*t*-LTB_4+12*epi*,6*t*-LTB_4; (5) LTB_4; (6) 12*epi*,6*t*,8*c*-LTB_4. (D) Hydroxy fatty acids produced by leukocytes with 80 μM exogenous AA: (1) HHT; (2) 15-HETE; (3) 12-HETE; (4) 5-HETE.

RESULTS AND DISCUSSION

Separation of the leukotriene standards could be performed within 10 min (Fig. 1A) by using the 120 mm × 4.6 mm I.D. C_{18} column and the solvent system described by Verhagen et al. [11].

The detection limit was 500 pg for each leukotriene in the standard solution. The use of a column oven improved the reproducibility of retention times but is not essential when standard solutions are injected before and after every ten samples.

Fig. 1B and C show chromatograms of leukotrienes obtained from supernatants of leukocytes that were stimulated either in the presence (Fig. 1B) or absence (Fig. 1C) of AA. In the latter experiment only metabolites from endogenous AA could be produced. LTE_4 was never seen in our samples, but the chromatogram shows a baseline at the retention time of LTE_4, so it is possible to detect LTE_4 in other types of samples.

Granulocyte experiments were mostly carried out with a suspension of total blood leukocytes. A microscope slide of the cell suspension was always made for a differential cell count. When necessary, mononuclear cells were removed by using Ficoll–Paque. In a stimulation experiment with 10^7 mononuclear cells per ml, however, no leukotrienes could be detected in the supernatant.

Fig. 1D shows the pattern of hydroxy fatty acids produced in the same experiment as shown in Fig. 1B. 12-L-Hydroxyheptadecatrienoic acid (HHT) and 12-hydroxyeicosatetraenoic acid (12-HETE) are mainly produced by platelets in the cell suspension. The detection limits for the hydroxy fatty acids were approximately 0.5–1 ng. As washed cell suspensions were used, no interfering UV-absorbing peaks were found when supernatants from unstimulated cells were injected.

There were no changes in the chromatograms of leukotrienes or hydroxy fatty acids within 20 h when the supernatants were kept at 4 °C under nitrogen. The overall coefficients of variation ranged from 2.1% for LTC_4 to 5.3% for LTD_4 when 10-ng amounts of standards were injected in 75 μl ($n = 20$ over four days).

CONCLUSION

Rapid and simple methods for the investigation of lipoxygenase pathways in human granulocytes have been described. With the use of an automatic injector with cooling facilities the HPLC method for lipoxygenase products has the sample capacity of a radioimmunoassay method (144 samples per day). This, together with the use of microtitre plates and multi-channel pipettes for incubations, makes it possible to perform large numbers of experiments. This work may encourage more laboratories to employ these techniques in the investigation of arachidonic acid metabolism in humans, animals or in vitro systems and for diagnostic procedures.

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