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ISOCRATIC DETERMINATION OF ARACHIDONIC ACID 5-LIPOXYGENASE PRODUCTS IN HUMAN NEUTROPHILS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method was developed to determine arachidonic 5-lipoxygenase products in calcium ionophore-stimulated neutrophils. This procedure allows the simultaneous measurement of leukotriene B_4 (LTB_4) and its ω-oxidation products without using a gradient elution system. 20-Carboxy-LTB_4, 20-hydroxy-LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, LTB_4 and 5s,12s-dihydroxyeicosatetraenoic acid can be separated and quantitated by reversed-phase chromatography using isocratic elution. The generation and degradation of 5-lipoxygenase products by human neutrophils following stimulation with calcium ionophore have been examined by this method.

INTRODUCTION

Leukotrienes (LTs), which are 5-lipoxygenase products of arachidonic acid, play an important role in disease processes such as inflammation and bronchial asthma. The peptide-LTs, LTC_4, LTD_4 and LTE_4, have been identified as slow-reacting substances of anaphylaxis, which are thought to be important mediators of immediate hypersensitivity. LTB_4 is a potent chemotactic and chemokinetic compound and stimulates the aggregation of polymorphonuclear leukocytes (PMNLs) [1]. For many years, human PMNLs have been used to study the production of LTC_4 and LTB_4. However, it has recently been established that eosinophils predominantly generate LTC_4 and LTB_4 is produced in neutrophils by incubation of the cells with calcium ionophore [2]. LTC_4 generated from eosinophils is converted into LTC_4 sulphoxides and 6-*trans*-isomers by eosinophil peroxidase [3]. As eosinophils comprise a small percentage of blood leukocyte preparation, the number of eosinophils obtained with high purity by a previously

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described method [4] was in general too small to generate sufficient amounts of LTC_4 and its metabolites for precise measurement. In contrast, we could collect a relatively large number of neutrophils with high purity. Human neutrophils generate LTB_4 as a predominant 5-lipoxygenase-derived product and subsequently convert it efficiently into 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 by ω-oxidation [5–7]. Therefore, a method capable of measuring these products is needed in order to assess 5-lipoxygenase-derived products in human neutrophils.

A high-performance liquid chromatographic (HPLC) method for LTB_4 and its ω-oxidation products reported previously required the use of two different solvent mixtures with gradient elution [5,6,8]. A method based on isocratic elution system has not been reported, despite the fact that such a system would be substantially simpler. The aim of this study was to develop an HPLC method using isocratic elution for the simultaneous determination of 5-lipoxygenase products in human neutrophils.

EXPERIMENTAL

Materials

The following reagents were purchased from commercial companies: calcium ionophore A23187 (Calbiochem, La Jolla, CA, U.S.A.), Lymphoprep (Nyegaard, Oslo, Norway), Percoll (Pharmacia, Uppsala, Sweden) and prostaglandin B_2 (PGB_2) (Funakoshi, Tokyo, Japan). 15-Hydroxyeicosatetraenoic acid (15-HETE) was synthesized by treatment of arachidonic acid with soybean lipoxygenase (type 1) (Sigma, St. Louis, MO, U.S.A.) and purified by HPLC.

Preparation of neutrophil suspension

The isolation of neutrophils was virtually identical with that described previously [4]. Briefly, mixed leukocytes were prepared from venous blood by dextran sedimentation and PMNLs were collected by centrifugation of the mixed leukocytes on Lymphoprep. The PMNLs were suspended in 1.5 ml of Percoll solution with a density of 1.070 g/ml. The leukocyte suspension was carefully layered on the top of five discontinuous Percoll gradients as follows: 1.080 g/ml, 2 ml; 1.085 g/ml, 3 ml; 1.090 g/ml, 3 ml; 1.095 g/ml, 3 ml; and 1.100 g/ml, 1.5 ml. After centrifugation at 400 *g* for 30 min, the cells collected from two interfaces between 1.080 and 1.085 g/ml and between 1.085 and 1.090 g/ml were mixed and used as neutrophils. After washing twice with Tyrode's buffer, the neutrophils were suspended in the same buffer. The cell preparations contained $>99.5\%$ neutrophils, $<0.2\%$ eosinophils, <5 erythrocytes per neutrophil and no platelets. The viability was greater than 98%, as determined by the trypan blue dye exclusion test.

Incubation procedure

A portion of $2 \cdot 10^6$ neutrophiles in 0.9 ml of Tyrode's buffer was placed in a siliconized glass tube. After preincubation at 37°C for a few minutes, calcium ionophore A23187 solution (1 μg per 0.1 ml) was added to the cells and the reaction was carried out in a final volume of 1 ml at 37°C for 10 min. A stock solution of calcium ionophore was prepared in dimethyl sulphoxide and the final

concentration of dimethyl sulphoxide was 0.05%. The reaction was stopped by cooling on ice and the supernatant was collected by centrifugation at 450 *g* for 5 min at 4°C. After washing with Tyrode's buffer, the cell pellet was saponified by incubation with 0.1 ml of 2 *M* sodium hydroxide solution and 0.4 ml of methanol as described by Laviolette et al. [9]. In some experiments, the cell pellet was extracted with 0.5 ml of methanol to determine the level of free HETE. All samples were stored at −70°C under argon until assay. All experiments were performed in duplicate.

Analysis of 5-lipoxygenase products

Prior to extraction, 25 ng of PGB_2 and 50 ng of 15-HETE were added to the sample as an internal standard. Lipoxygenase products were extracted with a disposable C_{18} column (NEN-Prep cartridge; DuPont, Wilmington, DE, U.S.A.) as described previously [10]. HPLC was performed using a Shimadzu 6A liquid chromatograph, equipped with an SPD-6A detector and a NovaPak C_{18} column (15 cm×3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.). LTs and HETEs were analysed separately.

For analysis of LTs, the column was eluted with acetonitrile–methanol–distilled water–trifluoroacetic acid (40:40:80:0.1, v/v), adjusted to pH 3 with triethylamine, at a flow-rate of 1 ml/min at 37°C. The UV absorption at 280 nm was monitored and peak-area ratios were calculated for each LT relative to PGB_2. HETEs were eluted with methanol–acetonitrile–distilled water (30:40:30) containing 0.01% phosphoric acid at a flow-rate of 1.0 ml/min. 5-HETE was measured relative to an internal standard (15-HETE) at 235 nm. The lipoxygenase products were determined from the UV absorbance, using molar absorption coefficients of 40 000 for LTs, 28 600 for PGB_2 at 280 nm and 29 500 for HETEs at 235 nm.

RESULTS

Reversed-phase HPLC analysis of the supernatant from ionophore-stimulated neutrophils revealed five major absorbance peaks at 280 nm, eluting at 3, 3.3, 24, 27 and 31 min. A typical chromatogram is shown in Fig. 1. Identification of peaks I and II in Fig. 1 was carried out as follows. First, the fraction with a retention time of 2–4 min was collected and converted into the corresponding methyl esters by treatment with ethereal diazomethane. The methyl esters of the products were then subjected to HPLC by using the same reversed-phase column eluted at a flow-rate of 1 ml/min with methanol–distilled water–acetic acid (60:40:1). These retention times were in agreement with those of methyl esters of authentic 20-carboxy-LTB_4 (10.5 min) and 20-hydroxy-LTB_4 (6 min). Next, UV spectra were obtained with a photodiode array detector (Shimadzu SPD-M1A) during reversed-phase HPLC. The UV spectra of the products, which exhibited maximal absorption at 270 nm and shoulders at 260 and 281 nm, were identical with those reported previously (Fig. 1). Based on co-elution with authentic standards and UV spectral analysis, peaks I and II in Fig. 1 were identified as 20-carboxy-LTB_4

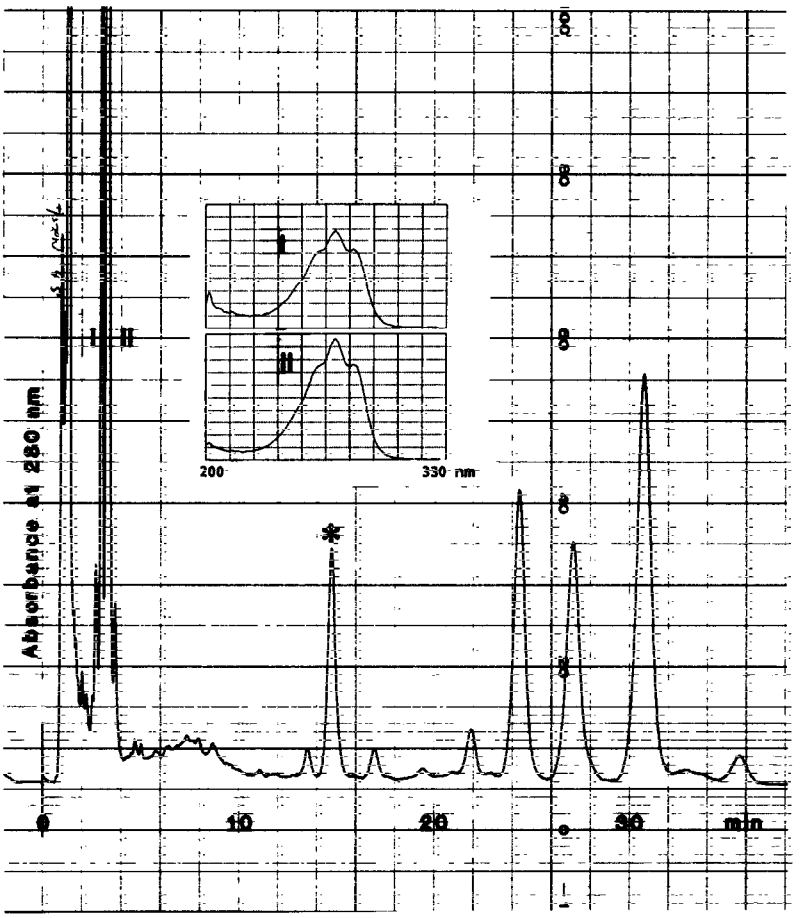

Fig. 1. HPLC trace of extracted supernatant from calcium ionophore-stimulated neutrophils and UV spectra of peaks I and II. The peak indicated with an asterisk is prostaglandin B_2, which was added as an internal standard.

and 20-hydroxy-LTB_4, respectively. The peaks at 24, 27 and 31 min were also identified as 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and LTB_4, respectively.

The peaks in Fig. 1 were reduced significantly by pretreating the cells with AA-861 (10^{-5}–10^{-8} *M*), which is a specific inhibitor of 5-lipoxygenase [11,12], and the peaks, except PGB_2, did not appear on the chromatogram in the presence of 10^{-5} *M* AA-861, suggesting that the peaks are derived from the 5-lipoxygenase pathway of arachidonic acid.

Fig. 2 shows the chromatographic profiles of 5-HETE analysis at 235 nm. the retention times for authentic 15-HETE, 12-HETE and 5-HETE were about 10, 12 and 13.5 min, respectively. We examined whether 10^{-5} *M* AA-861, which concentration completely inhibited the generation of LTB_4, prevented the generation of 5-HETE. The peak at 13.5 min disappeared from the chromatogram in the presence of AA-861 (Fig. 2C). The results in Fig. 2A–C suggest that there are no interfering peaks at retention times of 10 and 13.5 min and 5-HETE can be

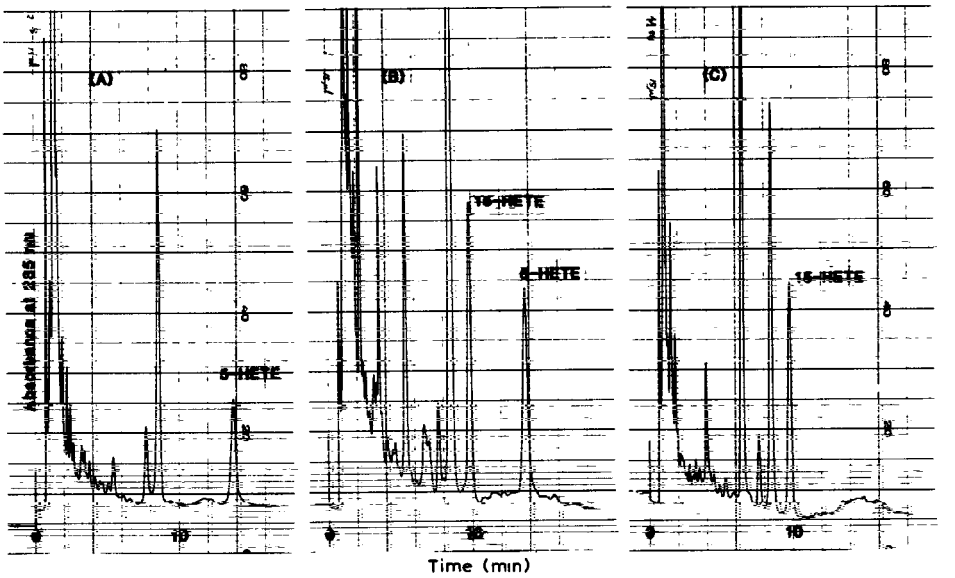

Fig. 2. HPLC profiles of 5-HETE generated from calcium ionophore-stimulated neutrophils. Prior to extraction, 15-HETE was added to the supernatants (B and C). The cells were stimulated with calcium ionophore in the presence of 10^{-5} *M* AA-861 (C).

measured at 235 nm. Similar chromatographic profiles were observed in the pellet of stimulated neutrophils.

In order to establish the specificity of the determination of ω-oxidation products, part of the sample was subjected to HPLC analysis with the solvent system described under Experimental and another part was analysed with the solvent system methanol–distilled water–acetic acid (40:50:0.1) at a flow-rate of 1.0 ml/min. Under these conditions, two ω-oxidation products were completely separated and the retention times of 20-carboxy-LTB_4 and 20-hydroxy-LTB_4 were 28 and 33 min, respectively. If compounds other than 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 were not present in the peaks, the ratios obtained by the two different solvent systems would be consistent with each other. When values obtained by HPLC with the two solvent systems were plotted, the correlation between the two methods was 0.89 ($p < 0.01$) (Fig. 3), indicating that compounds other than 20-carboxy-LTB_4 and 20-hydroxy-LTB_4 were not present in these peaks.

The calibration graph constructed from the results obtained with solutions containing various amounts of 20-hydroxy-LTB_4 together with a constant amount of PGB_2 (25 ng) was linear in the concentration range 4–33 ng. In addition, the observed ratio of 20-hydroxy-LTB_4 to PGB_2 was in agreement with the theoretical value, suggesting that the percentage of these compounds during extraction procedure is constant.

To confirm the specificity of determination of LTB_4, LTB_4 in the supernatant was simultaneously measured by HPLC and a radioimmunoassay method using a commercial kit (NEN, U.S.A.). A good correlation was found in the results obtained by these two techniques ($r = 0.981$, $p < 0.001$) (Fig. 4). The recovery of

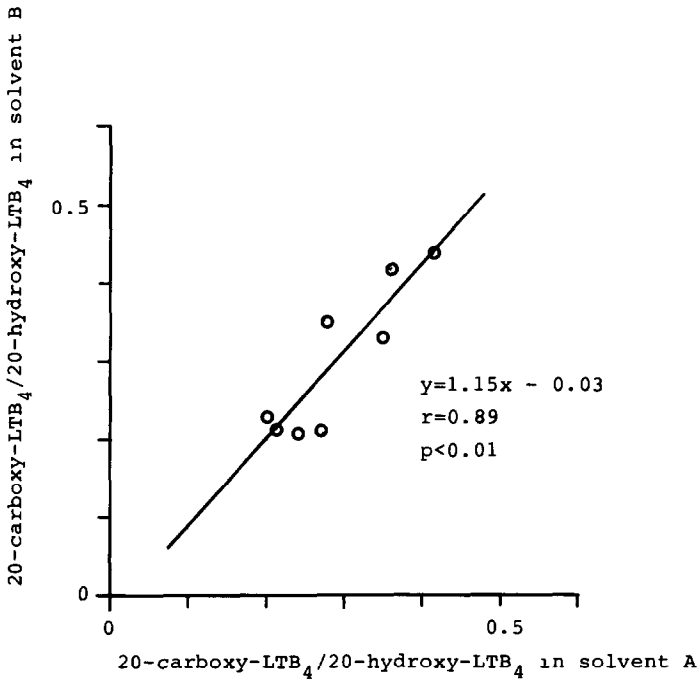

Fig. 3. Measurement of 20-carboxy-LTB_4 and 20-hydroxy-LTB_4 by HPLC in two different solvent systems. Solvent A, methanol–water–acetic acid (40:50:0.1, v/v); solvent B: acetonitrile–methanol–water–trifluoroacetic acid (40:40:80:0.1, v/v), pH 3.0, adjusted with triethylamine.

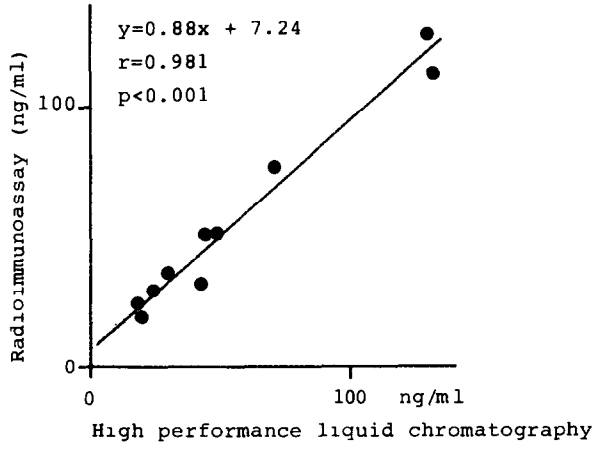

Fig. 4. Correlation between radioimmunoassay and HPLC results in measurement of LTB_4 released from calcium ionophore-stimulated neutrophils.

added [^{3}H]LTB_4 from the supernatant using the extraction procedure prior to HPLC was above 85%.

The precision of the method was determined from five consecutive analyses of a sample; the coefficient of variation was 3–10% (Table I). The intra-assay re-

TABLE I

REPRODUCIBILITY OF REPLICATE ANALYSES OF A SAMPLE FOR 5-LIPOXYGENASE PRODUCTS IN SUPERNATANT FROM IONOPHORE-STIMULATED HUMAN NEUTROPHILS

The data are expressed as ng per 10^6 neutrophils.

	20-Carboxy-LTB_4	20-Hydroxy-LTB_4	6-*trans*-LTB_4	12-epi-6-*trans*-LTB_4	LTB_4	5-HETE
	4.85	17.70	6.26	6.13	13.88	11.82
	4.82	18.47	6.41	7.48	14.16	10.83
	4.69	17.63	6.33	6.48	14.93	11.74
	4.90	18.96	6.94	7.94	14.37	11.60
	4.90	18.46	7.11	7.04	14.53	12.35
Mean ± S.D.	4.83 ± 0.09	18.24 ± 0.57	6.61 ± 0.39	7.01 ± 0.73	14.37 ± 0.39	11.67 ± 0.55
Coefficient of variation (%)	1.8	3.1	5.9	10.4	2.7	4.7

producibility of the method was determined by using five samples from the same neutrophil pool. The coefficient of variation for 5-lipoxygenase products ranged from 3 to 10% (data not shown).

Incubation of increasing numbers of cells from $1 \cdot 10^6$ to $6 \cdot 10^6$ cells/ml revealed that the generation of total LTB_4 was proportional to the number of cells. Therefore, the data were expressed as nanograms per 10^6 cells.

In order to investigate the time course for the generation and metabolism of LTB_4 and some of its isomers, neutrophils were incubated for various times with 1 μg/ml calcium ionophore and the products were measured by HPLC. The amount of LTB_4 was not significantly different with 1 or 2 μg/ml calcium ionophore after 10 min of incubation at 37°C. The time course for the generation of 5-lipoxygenase products by stimulated neutrophils is shown in Fig. 5. LTs are rapidly produced from endogenous arachidonic acid and LTB_4 generation reached a plateau at 5 min, remained at that level for 10 min and then decreased at 20 min, presumably owing to ω-oxidation. Two ω-oxidation products were observed after 2 min of incubation and they increased rapidly at longer incubation times. Spontaneous release of LTs was not observed in the absence of calcium ionophore after 10 min of incubation.

After 10 min of incubation, the supernatant contained LTB_4 16.4 ± 6.7, 6-*trans*-LTB_4 10.0 ± 3.2, 12-epi-6-*trans*-LTB_4 9.8 ± 3.1, 20-carboxy-LTB_4 3.5 ± 1.0, 20-hydroxy-LTB_4 10.7 ± 5.1 and 5-HETE 40.2 ± 15.2 ng per 10^6 neutrophils (mean ± S.D.; $n=6$) (Table II). Alkaline hydrolysis of the cell pellet released 37.4 ± 28.6 ng of 5-HETE and about 77% of the intracellular 5-HETE was found to be esterified to cellular lipid. We could not detect LTs, except 20-carboxy-LTB_4, in the pellet even after alkaline hydrolysis. 20-Carboxy-LTB_4 was detected in nearly equal amounts in both cells and the supernatant and the amount of 20-carboxy-LTB_4 retained in the cells was 4.5 ± 2.5 ng per 10^6 cells.

DISCUSSION

The activation of neutrophils with calcium ionophore results in the release of membrane-bound arachidonic acid, which is metabolized by 5-lipoxygenase to 5-

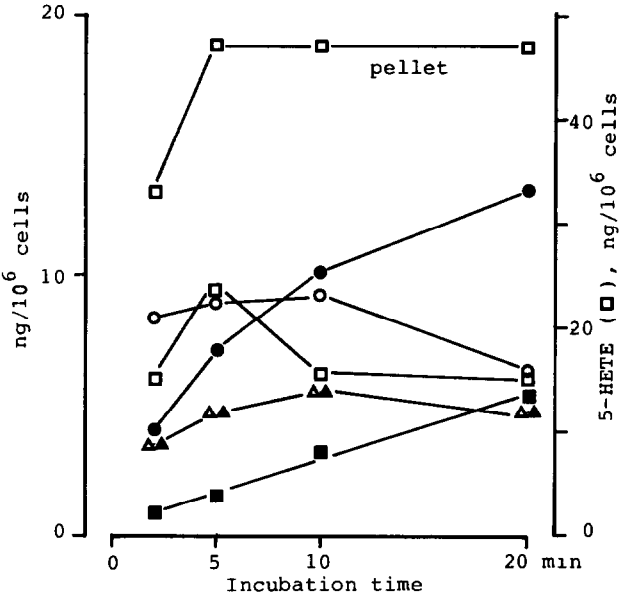

Fig. 5. Time course of generation of 5-lipoxygenase products by calcium ionophore-stimulated human neutrophils. (○) LTB_4; (▲, △) 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4; (■) 20-carboxy-LTB_4; (●) 20-hydroxy-LTB_4; (□) 5-HETE.

TABLE II

GENERATION OF 5-LIPOXYGENASE PRODUCTS IN IONOPHORE-STIMULATED HUMAN NEUTROPHILS

The data show the means ± S.D. (ng per 10^6 cells) for six different neutrophil preparations. Neutrophils were incubated with 1 μg/ml calcium ionophore A23187 for 10 min at 37°C. N.D. = not detected.

Sample	20-Carboxy-LTB_4	20-Hydroxy-LTB_4	6-*trans*-LTB_4	12-epi-6-*trans*-LTB_4	LTB_4	5-HETE
Supernatant	3.5±1.0	10.7±5.1	10.0±3.2	9.8±3.1	16.4±6.7	40.2±15.2
Pellet	4.5±2.5	N.D.	N.D.	N.D.	N.D.	37.4±28.6 (9.2±5.4)*

*Without hydrolysis of the pellet.

hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is converted either into 5-HETE or enzymatically into LTA_4. LTA_4 interacts with LTA_4 hydrolase to generate LTB_4. In addition, non-enzymatic hydrolysis of LTA_4 generates the biologically inactive 6-*trans*-isomers together with small amounts of 5,6-diHETE. In vitro, LTB_4 is metabolized by neutrophils by ω-oxidation to 20-hydroxy-LTB_4 and 20-carboxy-LTB_4, which are much less biologically active than LTB_4. 6-*trans*-LTB_4 is also converted into 20-hydroxy-6-*trans*-LTB_4 by LTB_4 20-hydroxylase in human PMNLs, but the rate of this reaction is lower than when LTB_4 is the substrate [13]. The observation that human neutrophils stimulated with calcium ionophore release 5-HETE, 6-*trans*-*LTB*$_4$, 12-epi-6-*trans*-LTB_4, LTB_4 and its ω-

oxidation products as 5-lipoxygenase-derived products needs a technique to assess these products.

An HPLC procedure for ω-oxidation products reported previously required the use of two different solvent mixtures with gradient elution [5,6,8]. The method based on isocratic elution used here is substantially simpler. The method can efficiently separate 20-carboxy-LTB_4, 20-hydroxy-LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, LTB_4 and 5s,12s-diHETE, but 5s,12s-diHETE and LTC_4 coeluted about 3 min later than LTB_4 in this system. Although 20-carboxy-LTB_4 and 20-hydroxy-LTB_4 are well resolved with the solvent system used, we examined whether certain UV-absorbing products coelute with these ω-oxidation products and influence the quantitation. The results in Fig. 3, together with the characteristic UV spectra, showed the specificity of quantitation of 20-carboxy-LTB_4 and 20-hydroxy-LTB_4, in spite of the relatively poor separation.

Kinetic analysis of LT generation shows that after ionophore stimulation, the LTB_4 levels peaked at 5 or 10 min and then declined with time. ω-Oxidation products of LTB_4 were detected even at 2 min and the levels increased linearly with time. Fig. 5 shows that about 60% of LTB_4 generated from neutrophils was converted into the ω-oxidation products at 10 min after incubation with calcium ionophore. When the percentage conversion of LTB_4 into its ω-oxidation products was calculated from the results, the apparent half-life of LTB_4 during the incubation was about 5–10 min. The rate of release and the metabolism were similar to those reported by Sun and McGuire [7]. The rapid metabolism of LTB_4 makes it necessary to determine both LTB_4 and its ω-oxidation products for evaluating 5-lipoxygenase products in human neutrophils.

Purified human neutrophils were challenged with 1 μg/ml calcium ionophore for 10 min and the 5-lipoxygenase products were measured. As summarized in Table II, the distribution of LTB_4 was predominantly extracellular in response to activation with calcium ionophore. Although Williams et al. [14] reported that a small percentage of generated LTB_4 remained intracellular, we could not observe LTB_4 retained in the cells, or the intracellular LTB_4 might be below the detection limit by this method.

The solvent system used here made it possible to determine both LTB_4 and the ω-oxidation products by HPLC with isocratic elution. The method should be useful for the study of the generation and metabolism of 5-lipoxygenase products in human neutrophils.

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