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Analysis of Leukotrienes, Lipoxins, and Monooxygenated Metabolites of Arachidonic Acid by Reversed-Phase High-Pressure Liquid Chromatography

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Arachidonic acid can be converted to a large number of metabolites by various lipoxygenases, cyclooxygenase, and other enzymes. Because of the complex profiles of products formed by many types of cells, high-pressure liquid chromatography (HPLC) has proved to be an invaluable technique for their purification and analysis. In the present study we have developed improved methods for the analysis of complex mixtures of eicosanoids by HPLC using binary gradients containing trifluoroacetic acid (TFA), which allows considerable manipulation of the retention times of the cysteine-containing leukotrienes (LTs) LTC₄, LTD₄, and LTE₄ relative to those of other eicosanoids. With a gradient between 0.003 and 0.005% TFA and a 4.6-mm-i.d. column of Spherisorb ODS-2, cysteinyl-LTs are very well resolved from one another and are separated as a group with retention times longer than those of all other major eicosanoids. These conditions can be used for the analysis of prostaglandin B₂ (PGB₂), LTB₄, monohydroxyeicosatetraenoic acids (HETEs), and cysteinyl-LTs in only 30 min. Slightly longer analysis times must be used for the separation of more polar eicosanoids such as hydroxy metabolites of LTB₄ and lipoxins. We have also developed methods for the analysis of eicosanoids using a midbore (3.2 mm i.d.) column containing Spherisorb ODS-2, which improves sensitivity and reduces solvent consumption. In this case higher concentrations (0.04 to 0.05%) of TFA have been used, resulting in retention times for cysteinyl-LTs between those of the cyclooxygenase product 12-hydroxy-5,8,10-heptadecatrienoic acid and the HETEs. This approach permits analysis of PGB₂, LTB₄, HETEs, and cysteinyl-LTs in only 20 min. Samples which also contain hydroxy-LTB₄ and lipoxins can be analyzed in 40 min. The above techniques are

highly reproducible and give baselines which are free of interfering peaks. © 1995 Academic Press, Inc.

Arachidonic acid is oxygenated in a variety of tissues to a large array of biologically active compounds including monohydroxyeicosanoids (HETEs),² leukotrienes (LTs), lipoxins (LXs), prostanoids, epoxy metabolites, and hepxilins (1). High-pressure liquid chromatography (HPLC) has been instrumental in the identification and analysis of many of these products. The most important pathways for the metabolism of arachidonic acid are generally considered to be the cyclooxygenase pathway, which is responsible for the formation of prostaglandins and thromboxanes, and the 5-lipoxygenase pathway, which results in the formation of leukotrienes and a variety of other products. With the exception of 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHTrE), none of the major products of the cyclooxygenase pathway possess conjugated double bonds and can thus not be easily detected at low levels on the basis of uv absorbance. However, products formed by the 5-lipoxygenase pathway possess at least two conjugated double bonds and have characteristic uv spectra.

The initial product of the 5-lipoxygenase pathway, 5-hy-

² Abbreviations used: LT, leukotriene; 5S,12S-diHETE, 5S,12S, dihydroxy-6,8,10,14-eicosatetraenoic acid; LX, lipoxin; 13-HODE, 13-hydroxy-9,11-octadecadienoic acid; 5,6-diHETEs, 5S,6R- and 5S,6S-dihydroxy-7,9,11,14-eicosatetraenoic acids; 12-HHTrE, 12-hydroxy-5,8,10-heptadecatrienoic acid; 5-oxo-EETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; PGB₂, prostaglandin B₂; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; t_R, retention time; ODS, octadecylsilyl; PMNL, polymorphonuclear leukocytes.

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droperoxy-6,8,11,14-eicosatetraenoic acid, is rapidly converted to either 5-HETE (2) or LTA₄ (3). The latter compound can be converted enzymatically to LTB₄ (4) or LTC₄ (4) by the addition of either water or glutathione, respectively. LTB₄ is a potent proinflammatory agent (5), whereas LTC₄ and its metabolites LTD₄ (6,7) and LTE₄ (8) are potent constrictors of airway smooth muscle (9). LTB₄ is metabolized to ω - (10,11) and β - oxidation products (12) as well as to 12-oxo (13) and 10,11-dihydro metabolites (14). 5-HETE, although not itself very potent biologically, is converted by a dehydrogenase to 5-oxo-ETE (15), which is a potent agonist of both neutrophils (16,17) and eosinophils (18). In addition to products formed directly by 5-lipoxygenase and their metabolites, other groups of eicosanoids can be formed by the combined actions of 5-lipoxygenase with either 12-lipoxygenase or 15-lipoxygenase, resulting in the formation of 5S,12S-diHETE (19), 5S,15S-diHETE (20), and the lipoxins (1,21).

The retention times (t_R 's) of most eicosanoids with reversed-phase (RP)-HPLC can be reasonably well predicted from the numbers of hydroxyl groups, oxo groups, and double bonds they possess. There are important selectivity differences depending, for example, on the relative amounts of methanol and acetonitrile in the mobile phase (22). However, these differences do not usually result in large changes in retention time from one mobile phase to another. In contrast, the cysteinyl-leukotrienes LTC₄, LTD₄, and LTE₄ behave quite differently from other eicosanoids because they contain a positively charged amino group in addition to the negatively charged carboxyl group which is common to all eicosanoids. This results in dramatic changes in selectivity with different acidic modifiers (e.g., acetic acid, trifluoroacetic acid, or phosphoric acid) of the mobile phase as well as with different stationary phases.

Advantage has been taken of the above selectivity changes with certain stationary phases to cause the cysteinyl-LTs to be eluted as a group after all of the other eicosanoids. We reported one such approach using a binary gradient with a Beckman Ultrasphere octadecylsilyl (ODS)-silica column (22). However, the nature of this stationary phase, although sold under the same name, has since been changed, and the reported separation cannot be obtained with currently available columns. We (23) and others (24) have since reported the use of ternary gradients utilizing either increasing concentrations of trifluoroacetic acid (23) or increasing pH (24) to elute cysteinyl-LTs at the end of the gradient. We now report two systems, both based on binary gradients, which give excellent separation of a large number of lipoxygenase products. These systems are simpler and have the advantage of improved baselines compared to the previously reported ternary gradients.

MATERIALS AND METHODS

Materials. Prostaglandin B₂ (PGB₂) and arachidonic acid were purchased from Sigma and Nuchek Prep

Inc., respectively. A23187 was obtained from Calbiochem-Behring. LTB₄, LTC₄, LTD₄, LTE₄, and *N*-acetyl LTE₄ were kindly provided by Dr. A. W. Ford-Hutchinson of Merck-Frosst (Pointe Claire, Quebec, Canada). The cysteinyl-LTs were kept in water at -80°C in plastic vials. Lipoxins A₄ and B₄ were obtained from Cayman Chemical Co. 5-HETE was prepared by incubating arachidonic acid and A23187 with porcine leukocytes in the presence of 5,8,11,14-eicosatetraenoic acid (25). 15-HETE was prepared using soybean lipoxygenase (Sigma) (26). 12-HETE and 12-HHTe were prepared by incubation of arachidonic acid with human blood platelets (27). 5-Oxo-ETE was synthesized as previously described (15). All of the products prepared in our laboratory were extracted using cartridges containing ODS silica (C₁₈ Sep-Paks, Waters Associates) as previously described (28) and were purified either by RP- or normal-phase HPLC.

Preparation of rat lung cells. Crude lung cells were prepared from male Sprague-Dawley rats by enzymatic digestion (29). Rats (200–300 g) were anesthetized with sodium pentobarbital (60 mg/kg, ip). Heparin (1000 units/kg) was injected via the sublingual vein before midthoracotomy. The inferior vena cava was then cut and the heart and lungs were removed. The lungs were perfused via the pulmonary artery with 50 ml Krebs buffer supplemented with 2% albumin to remove blood. After the lungs were cleared of blood, they were perfused with 0.06% protease (type XXIV, Sigma) via the trachea at a flow rate of 10 ml/min for 15 min at 37°C. The trachea and external bronchi were then removed and the lungs were dissociated with forceps to remove large bronchi and vessels. The gelatinous lung tissue was subsequently dispersed by six in-and-out flushings, using a 60-ml syringe, with Krebs's buffer (50 ml) containing 50–70 Kunitz units of DNase (DN25, Sigma). The dissociated cells were filtered and washed twice. Cell viability was determined using trypan blue.

Preparation of rat pleural polymorphonuclear leukocytes (PMNL). Rat PMNL were prepared from pleural exudates obtained 4 h after the injection of λ -carrageenan (1 mg in 0.1 ml of sterile saline) into the pleural cavities of male rats. The cells were then purified by centrifugation over Ficoll-Paque.

Preparation of human leukocytes and platelets. Peripheral blood obtained from healthy volunteers was mixed with 6% Dextran T-500 in saline (5:1) to remove red blood cells. The upper layer was removed 45 min later and centrifuged at 250g for 10 min to obtain leukocytes. The resulting supernatant was then centrifuged at 2200g for 10 min to obtain platelets. Contaminating erythrocytes in both leukocyte and platelet fractions were removed by hypotonic lysis with distilled water. The leukocyte and platelet fractions were then washed twice before being resuspended in incubation medium.

TABLE 1

Composition of Solvents Used to Prepare Mobile Phases

Solvent	Volume (%)			
	Water	Acetonitrile	Methanol	TFA
A	70	—	30	—
B	20	80	—	0.005
C	20	55	25	0.009
D	70	—	30	0.04
E	20	60	20	0.05
F	25	55	20	0.04

Incubation conditions. Cells were suspended in Hanks' buffer containing calcium (1.66 mM) and magnesium (1.2 mM) and incubated with A23187 (1 μ M) for either 15 or 45 min. The reactions were terminated by addition of cold methanol to give a final methanol concentration of 30%. The samples were stored at -80°C before HPLC analysis. Prior to analysis, samples were centrifuged at 2200g for 10 min.

Analysis of eicosanoids by precolumn extraction/RP-HPLC. Precolumn extraction/RP-HPLC analysis of eicosanoids was performed as previously described (23,25) using a six-port switching valve coupled to a precolumn, an analytical column, a solvent delivery system for the analytical column, and a pump to load the sample onto the precolumn via an automatic injector. The sample was first pumped from a WISP automatic injector (Waters-Millipore) via a WAVS automated switching valve (Waters-Millipore) in the "load" position onto a precolumn cartridge (μ Bondapak C_{18} Guard-Pak cartridge, Waters-Millipore), which had been preequilibrated with precolumn solvent (15 ml of either 15% or 30% methanol in water containing 2.5 mM phosphoric acid). The precolumn was then washed with 18 ml of precolumn solvent. The six-port WAVS automated switching valve was then switched to the "inject" position, placing the precolumn in line with a HPLC pump (Model 600, Waters-Millipore) and an analytical octadecylsilyl silica HPLC column (Spherisorb ODS-2, either 4.6×250 mm or 3.2×250 mm; Phenomenex), which was also attached to the WAVS six-port valve. Leukotrienes and other eicosanoids retained on the precolumn cartridge were then separated by the mobile phase used for the analytical column. The uv spectrum of the column eluate was continuously recorded between 220 and 330 nm using a Waters 991 photodiode array detector. Eicosanoids in biological samples were identified by comparison of their retention times and uv spectra with those of authentic standards.

Preparation of mobile phases. Water was obtained from a Milli-Q water purification system (Waters-Millipore) and further purified by passing it through a Millipore Norganic cartridge (Millipore Corp., Bedford, MA).

Stock solutions of aqueous 2% TFA were purified by passing them through C_{18} Sep-Paks prior to use. Organic solvents and phosphoric acid (HPLC grade) were from BDH and Fisher Scientific. The compositions of the solvents used to prepare the mobile phases used in this study are shown in Table 1.

RESULTS

Conditions for the Precolumn Extraction of Eicosanoids

Precolumn extraction coupled to RP-HPLC is a rapid and sensitive method for the analysis of eicosanoids. We previously showed that excellent recoveries of a variety of lipoxygenase products, including 20h-LTB₄, LTB₄, LTC₄, and 15-HETE, could be obtained when samples were loaded onto a precolumn at neutral pH in medium containing between 15 and 20% methanol (23). However, we observed that repeated analysis of biological samples extracted in this manner sometimes led to deteriorations in peak shape and selectivity, possibly due to the presence of proteins in the samples. We found that this problem could be alleviated by raising the concentration of methanol in the sample to 30% and freezing at -80°C , followed by removal of precipitated material by centrifugation. Since it was possible that increasing the concentration of methanol in the loading medium could result in reduced retention of the more polar eicosanoids by the precolumn, we evaluated the recoveries of 20-hydroxy-LTB₄, LXA₄, LTB₄, and LTC₄ using these conditions (Table 2). A mixture of the above compounds was injected onto a HPLC column using either a conventional injector with a sample loop (sample volume, 50 μ l) or via a precolumn connected to an automatic injector (sample in 4 ml of 30% methanol at neutral pH). In the latter case, the precolumn was first equilibrated with 30% methanol containing 2.5 mM H_3PO_4 . The components were separated by RP-HPLC on a 3.2×250 -mm

TABLE 2
Recoveries of Eicosanoids after Precolumn Extraction
with 30% Methanol

Eicosanoid	Recovery (%)
20h-LTB ₄	94 $\pm$ 2
LXA ₄	95 $\pm$ 12
LTB ₄	91 $\pm$ 2
LTC ₄	101 $\pm$ 13

Note. The precolumn was first washed with 30% methanol in water containing 2.5 mM H_3PO_4 (15 ml). Standards were then loaded onto the precolumn in 30% methanol at neutral pH in a volume of 4 ml and the precolumn was then washed with a further 18 ml of 2.5 mM H_3PO_4 in 30% methanol. Recoveries were calculated by comparing the peak areas of standards with those obtained from an identical sample which had been injected via a conventional loop injector. All values are means $\pm$ SD of triplicate determinations.

Spherisorb ODS-2 column as described below. Recoveries for the precolumn extraction procedure were calculated by comparison of the peak areas of standards obtained after precolumn extraction with those obtained by direct injection using a conventional injector with a sample loop. As shown in Table 2, the recoveries of all of the standards investigated were in excess of 90% when the sample was loaded onto the precolumn in 30% methanol.

Analysis of Eicosanoids on a 4.6-mm-i.d. Spherisorb ODS-2 Column

We developed two systems which are useful for the analysis of lipoxygenase products using a 4.6×250 -mm Spherisorb ODS-2 column. The first was designed primarily for the analysis of cysteinyl-LTs, and some of the less polar eicosanoids, whereas the second could be used for a wide range of lipoxygenase products.

The first system referred to above utilizes a mobile phase consisting of a linear gradient between solvents A (methanol/water (30:70)) and B (acetonitrile/water/TFA (80:20:0.005)) as follows: 0 min, 60% B; 30 min, 100% B with a flow rate of 1.5 ml/min. The separation of a mixture of standards (15–25 ng of each), first loaded onto a precolumn as described above, is shown in Fig. 1A. Using the above conditions, there was baseline separation of all of the standards injected (LTB₄, LTC₄, LTD₄, and LTE₄, PGB₂, 5-HETE, 12-HETE, 15-HETE, and 12-HHTrE). Raising the concentration of TFA reduced the retention times of cysteinyl-LTs but did not affect those of other eicosanoids (cf. Ref. 23). For example, with 0.01% TFA in solvent B, all of the above standards can be separated within 20 min, but there was some overlap between cysteinyl-LTs and HETEs (data not shown). The separation of uv-absorbing eicosanoids produced when a mixture of rat lung cells was incubated with A23187 (1 μ M) for 45 min is shown in Fig. 1B. The major products detected were leukotrienes B₄, C₄, D₄, and E₄, 12-HHTrE, 5-HETE, and 12-HETE, all of which were very well resolved from one another.

Although the conditions described above give excellent resolution of cysteinyl-LTs and HETEs, the separation of a number of polar eicosanoids is not optimal because of their short retention times. For example, the 6-*trans* isomers of LTB₄ are not well resolved from LTB₄, and lipoxins and ω -oxidation products of LTB₄ are not well separated. To analyze these compounds, methanol can be added to solvent B and the concentration of acetonitrile can be reduced to 55% to give a mobile phase consisting of a linear gradient between solvents A and C (water/methanol/acetonitrile/TFA (20:25:55:0.009)) as follows: 0 min, 50% C; 40 min, 100% C. These conditions can be used to analyze mixtures of eicosanoids containing LTB₄ and its isomers and metabolites, lipoxins, cysteinyl-LTs, 5-oxo-EETE, 12-HHTrE,

and HETEs (Fig. 2A). The separation of eicosanoids formed by incubation of rat lung cells with A23187 (1 μ M) for 15 min is shown in Fig. 2B. The profile of eicosanoids formed under these conditions is similar to that shown in Fig. 1B, except that the retention times are longer and LTC₄ has a retention time similar to that of 5-HETE. Since LTE₄ is formed from LTD₄, only very small amounts are present after incubation of lung cells with A23187 for 15 min (Fig. 2B) compared to 45 min (Fig. 1B).

Figure 2C shows a chromatogram of the products obtained after incubation of carrageenan-elicited rat neutrophils with A23187 (5 μ M) and arachidonic acid (10 μ M) for 30 min. These cells do not synthesize detectable amounts of cysteinyl-LTs, but extensively metabolize LTB₄ by reduction of the 10,11-double bond to give 10,11-dihydro-LTB₄, (dh-LTB₄) and by ω -oxidation (30). Unlike human neutrophils, which metabolize LTB₄ via a 20-hydroxylase, rat neutrophils convert LTB₄ and 10,11-dihydro-LTB₄ to 18- and 19-hydroxy metabolites (30).

Analysis of Eicosanoids by HPLC on a 3.2-mm-i.d. Spherisorb ODS-2 Column

When the diameter of a HPLC column is reduced, the flow rate can be lowered, resulting in a reduction in the volume in which solutes are eluted from the column. This both increases the sensitivity and has the economic benefit of reduced solvent consumption. By slightly modifying solvents A and C and raising the concentration of TFA in the mobile phase, excellent separation of all of the eicosanoids described above was obtained using a 3.2×250 -mm column. As shown in Fig. 3, complete separation of LTB₄, cysteinyl-LTs, HETEs, and 5-oxo-EETE was obtained using a linear gradient between solvents D (water/methanol/TFA (70:30:0.04)) and E (water/methanol/acetonitrile/TFA (20:20:60:0.05)) as follows: 0 min, 75% E; 20 min, 95% E. The flow rate was 0.75 ml/min. Under these conditions, the cysteinyl-LTs have shorter t_R 's relative to those of other eicosanoids obtained using the 4.6-mm-i.d. column shown in Figs. 1 and 2, and they all eluted between 12-HHTrE and 15-HETE. It should also be noted that LTE₄ has a shorter t_R than LTD₄ in contrast to the chromatograms shown in Figs. 1 and 2 in which case the reverse is true.

The advantage of the conditions described above is that the analysis is very rapid, the time required being only 20 min. If it is necessary to separate a more complex mixture of lipoxygenase products, a weaker mobile phase can be used, consisting of a linear gradient between solvents D and F (water/methanol/acetonitrile/TFA (25:20:55:0.04)) as follows: 0 min, 50% F; 40 min, 100% F. With a flow rate of 0.75 ml/min mixtures containing almost all of the major lipoxygenase products can be resolved in 40 min (Fig. 4A). Thus, excellent sep-

FIG. 1. Reversed-phase high-pressure liquid chromatograms of standards (A) and products formed by isolated rat lung cells (B). The lung cells (4 ml; 5×10^6 cells/ml) were incubated with A23187 (1 μ M) for 45 min at 37°C. The incubation was terminated by addition of 1.7 ml methanol (final concentration: 30% methanol). PGB_2 (25 ng) was added as an internal standard. The standards and samples were directly injected for analysis by precolumn extraction/RP-HPLC as described under Materials and Methods. The stationary phase was a column of Spherisorb ODS-2 (4.6×250 mm), whereas the mobile phase consisted of a linear gradient between solvents A (water/methanol (70:30)) and B (water/acetonitrile/TFA (20:80:0.005)) as follows: 0 min, 60% B; 30 min, 100% B. The flow rate was 1.5 ml/min. The uv spectrum of the column eluate was continuously recorded between 220 and 330 nm using a Waters photodiode array detector. The eicosanoids were identified by their retention times and uv spectra.

aration of LTB_4 , its 6-*trans* isomers, 20-hydroxy- LTB_4 , cysteinyl-LTs, HETEs, 5-oxo-E Δ^5 ETE, and 13-HODE was obtained. Analysis of eicosanoids released from lung cells incubated with A23187 (1 μ M) for 15 min using the above conditions is shown in Fig. 4B. This chromato-

gram closely resembles that obtained for a similar sample analyzed using the 4.6-mm-diameter column (Fig. 2B) except that the retention times of the cysteinyl-LTs are considerably shorter. The HPLC profile of eicosanoids synthesized by mixed human leukocytes (1 ml; 30

FIG. 2. RP-HPLC of standards (A) and eicosanoids formed by isolated rat lung cells (B) or rat pleural PMNL (C). The isolated lung cells (4 ml ; $5 \times 10^6 \text{ cells/ml}$) were incubated with calcium ionophore A23187 ($1 \mu\text{M}$) for 15 min, whereas the pleural PMNL (1 ml ; $30 \times 10^6 \text{ cells/ml}$) were incubated with arachidonic acid ($10 \mu\text{M}$) and A23187 ($5 \mu\text{M}$) for 30 min at 37°C . The eicosanoids were analyzed by precolumn extraction/RP-HPLC on a Spherisorb ODS-2 column ($4.6 \times 250 \text{ mm}$) with a linear gradient between solvents A (water/methanol (70:30)) and C (water/methanol/acetonitrile/TFA (20:25:55:0.009)) as follows: 0 min, 50% B; 40 min, 100% B. The flow rate was 1.5 ml/min. 18h-LTB₄, 18-hydroxy-LTB₄; 19h-LTB₄, 19-hydroxy-LTB₄; 20h-LTB₄, 20-hydroxy-LTB₄; 6t-LTB₄, 6-*trans*-LTB₄; 12e-6t-LTB₄, 12-*epi*-6-*trans*-LTB₄; 5o-EETE, 5-oxo-EETE; 11h, 11-HETE; 12h, 12-HETE; 15h, 15-HETE.

$\times 10^6 \text{ cells/ml}$) containing platelets ($60 \times 10^6 \text{ cells/ml}$) stimulated with A23187 ($5 \mu\text{M}$) and arachidonic acid ($10 \mu\text{M}$) is shown in Fig. 4C. This mixture of cells converts arachidonic acid to a large variety of products due in part to transcellular metabolism. For example, platelets can convert neutrophil-derived LTA₄ and 5-HETE to LTC₄

(31,32) and 5S,12S-diHETE (19), respectively, since they contain LTC₄ synthase and 12-lipoxygenase.

Regeneration of Column after Sample Analysis

A problem which may be encountered in the analysis of cysteinyl-LTs by HPLC is that the reproducibility de-

FIG. 2—Continued

teriorates with column use, making accurate and sensitive measurements of these compounds difficult. We observed that with mobile phases containing low concentrations of TFA such as those used with the 4.6-mm-i.d. column (solvents B and C), the retention times of cysteinyl-LTs gradually decreased with increasing col-

umn use, up to the point where LTD₄ and LTE₄ were no longer resolved. One possible explanation for this is that materials such as peptides and proteins could accumulate on the column because they may require a higher concentration of TFA to be completely eluted. Concentrations (0.1%) of TFA higher than those used in the

FIG. 3. RP-HPLC analysis of a mixture of standards on a midbore Spherisorb ODS-2 column (3.2 × 250 mm). The mobile phase consisted of a linear gradient between solvents D (water/methanol/TFA (70:30:0.04)) and E (water/acetonitrile/methanol/TFA (20:60:20:0.05)) as follows: 0 min, 75% E; 20 min, 95% E. The flow rate was 0.75 ml/min. dh-LTB₄, 10,11-dihydro-LTB₄; 5o-ETE, 5-oxo-ETE.

FIG. 4. RP-HPLC analysis of standards (A) and eicosanoids formed by isolated rat lung cells (B) or a mixture of human leukocytes and platelets (C). Isolated lung cells (2 ml, 5×10^6 cells/ml) were incubated with $1 \mu\text{M}$ A23187 for 15 min. The mixture of human leukocytes and platelets (30×10^6 leukocytes and 60×10^6 platelets in a volume of 1 ml) was incubated with arachidonic acid ($10 \mu\text{M}$) and A23187 ($5 \mu\text{M}$) for 30 min at 37°C . The products were analyzed on a midbore Spherisorb column (3.2×250 mm). The mobile phase consisted of a linear gradient between solvents D (water/methanol/TFA (70:30:0.04)) and F (water/methanol/acetonitrile/TFA (25:20:55:0.04)) as follows: 0 min, 50% F; 40 min, 100% F. The flow rate was 0.75 ml/min. 5S,12S-dh, 5S,12S-diHETE; dh-LTB₄, 10,11-dihydro-LTB₄; other abbreviations are as described in the legend to Fig. 2.

present study are widely used for the separation of peptides by RP-HPLC (33). Thus, washing the HPLC column with a mobile phase containing a higher concentration of TFA seemed like a potentially useful approach to regenerate columns used for the analysis of cysteinyl-

LTs. We found that purging the column with acetonitrile/water/TFA (80/20/0.1) for 5 min after each chromatography dramatically improved reproducibility in the analysis of cysteinyl-LTs. Even if this treatment is not used routinely, it can be used to regenerate columns

FIG. 4—Continued

which have lost their capacity to resolve these compounds. In contrast, deteriorating resolution of cysteinyl-LTs was not a serious problem for analyses using the 3.2-mm-i.d. column, presumably because the concentration of TFA in the mobile phases employed was higher (0.04% in solvents D and F and 0.05% in solvent E; see Table 1). However, the resolution of some of the other eicosanoids became worse with increasing column use. We found that this problem could be alleviated by treating the column with methanol/water (50:50) at a flow rate of 0.75 ml/min for 5 min after each chromatography.

DISCUSSION

Analysis of mixtures of eicosanoids not containing cysteinyl-LTs by reversed-phase HPLC on ODS-silica is relatively straightforward, since the t_R 's and peak shapes are relatively independent of the particular brand of ODS-silica used as well as the nature of the acidic modifier (TFA, acetic acid, phosphoric acid, etc.). In this case, a major consideration is the relative amounts of methanol and acetonitrile in the mobile phase, since there are substantial selectivity differences between these two solvents (22).

The HPLC systems described here were designed for the analysis of mixtures of eicosanoids which include cysteinyl-LTs. For the analysis of cysteinyl-LTs, both the precise nature of the ODS-silica and the acidic modifier present in the mobile phase are critical because of the positively charged amino groups of these compounds. It should be pointed out that the LTE_4 me-

tabolite *N*-acetyl- LTE_4 (34) exhibits chromatographic behavior similar to the nonamino acid-containing eicosanoids since its amino group is acetylated.

In addition to the dramatic changes in selectivity between cysteinyl-LTs and other eicosanoids induced by altering the concentration of TFA, there are also changes in selectivity between LTD_4 and LTE_4 . As previously reported (22), when the mobile phase contains very low concentrations of TFA, the t_R of LTD_4 is greater than that of LTE_4 (Figs. 1 and 2). However, with mobile phases containing higher concentrations of TFA (Figs. 3 and 4), LTE_4 has a slightly longer t_R than that of LTD_4 (cf. Ref. 22).

We have analyzed mixtures of cysteinyl-containing leukotrienes on many different stationary phases and have found that, in general, TFA affects their t_R 's in a manner similar to that reported previously (22) (data not shown). However, there were considerable differences between stationary phases in the degree of retention of cysteinyl-LTs and the sensitivity to changes in the concentration of TFA. In addition, we found that certain stationary phases, such as Ultrasphere ODS, give rather poor peak shapes with these compounds. Although we previously described mobile phases that gave good resolution of eicosanoids with this stationary phase (22), the manufacturing process has since been changed and it is no longer suitable for the analysis of cysteinyl-LTs. Compared to other stationary phases, Spherisorb ODS exhibits a high degree of retention of cysteinyl-LTs at low concentrations of TFA. The t_R 's of these compounds are quite sensitive to changes in the concentra-

tion of TFA, allowing them to be readily manipulated, and excellent peak shapes are obtained. We previously took advantage of these properties to develop ternary gradients for the separation of eicosanoids. It should be noted that the separations obtained using the mobile phases reported here with types of ODS-silica other than Spherisorb ODS-2 may be quite different, depending on the degrees of end capping and carbon loading.

The chromatograms shown in Figs. 1 to 4 show that complex mixtures of lipoxygenase products can be analyzed on Spherisorb ODS-2 columns using mobile phases consisting of binary gradients containing low concentrations of TFA. The addition of acidic modifiers such as TFA (22,23), heptafluorobutyric acid (35,36), or phosphoric acid (22,24) to mobile phases has been shown to be very useful for the chromatography of cysteinyl-LTs, since sharp peaks are obtained without the use of EDTA (37) to complex divalent cations. TFA is particularly useful, since it is volatile and can therefore be readily removed from column fractions. However, it is important to note that since TFA is a relatively strong acid, the acidity of the fraction will increase as the solvent is evaporated, resulting in a very low pH just prior to complete removal of the solvent. This can cause degradation of cysteinyl-LTs and low recoveries. To circumvent this problem, ammonium hydroxide can be added to the column fractions prior to evaporation, resulting in excellent recoveries of cysteinyl-LTs after evaporation of the column solvent.

Although we (23) and others (24) have shown that ternary gradients give excellent resolution of a large number of eicosanoids, with cysteinyl-LTs appearing at the end of the gradient, the baselines obtained using these conditions may contain spurious peaks which can interfere with the analysis. This is presumably due to the accumulation of various contaminants on the column during the first phase of the chromatography which utilizes either low concentrations of TFA (23) or phosphoric acid (24). These contaminants are rapidly eluted from the column early in the second phase of the gradient when the concentration of TFA or the pH is increased. These problems are largely circumvented by the use of the binary gradients reported in the present study.

In conclusion, several approaches to the analysis of mixtures of eicosanoids have been developed in the present study. The optimal conditions for a particular application will vary, depending on the metabolites to be measured. For example, the conditions shown in Fig. 1 are very useful for the analysis of cysteinyl-LTs since they are well separated and have t_R 's longer than those of nonamino acid-containing eicosanoids. On the other hand, if it is necessary to analyze a larger number of eicosanoids, the conditions illustrated in either Figs. 2 or 4 can be used. The narrower bore column shown in Figs. 3 and 4 gives very good separation of a large number of eicosanoids. Since the cysteinyl-LTs have t_R 's between

12-HHTrE and 15-HETE, analyses can be completed in as little as 20 min (Fig. 3) or somewhat longer if it is necessary to analyze more polar eicosanoids such as hydroxylated metabolites of LTB₄ or lipoxins (Fig. 4). This column also has the advantage of conserving solvents, since the flow rates can be reduced by 50% compared to the wider bore column used in this study.

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