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Simultaneous HPLC Analysis of Arachidonic Acid Metabolites in Biological Samples with Simple Solid Phase Extraction

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A reversed-phase high-performance liquid chromatogrphy (RP-HPLC) has been developed to analyze the metabolites of arachidonic acid based on the specificities of ultraviolet absorption of these various metabolites and is sensitive to the nanogram level. This procedure makes it possible to extract complex mixtures of eicosanoids efficiently with a single step and to analyze them simultaneously by RP-HPLC from biological samples using octadesylsilyl silica extraction column and PGB_2 as an internal standard. The cyclooxygenase products {prostaglandin (PG)D_2, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 (TXB_2)} and lipid peroxidation product, isoprostanes, of arachidonic acid were monitored by one isocratic HPLC system at 195 nm wavelength. The lipoxygenase products {leukotriene(LT)B_4, LTC_4, LTD_4, and 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, 15-HETE} were measured by another isocratic HPLC system at 280 nm for LTs and 235 nm for HETEs. This method provides a simple and reliable way to extract and assess quantitatively the final arachidonic acid metabolites.

Key Words: High-performance liquid chromatography, Arachidonic acid, Eicosanoid, Prostaglandin, Leukotriene, Hydroxyeicosatetraenoic acid, Isoprostane, Solid phase extraction

INTRODUCTION

Eicosanoids are a group of oxygenated products derived enzymatically from 20 carbon polyunsaturated fatty acids, including prostaglandins, thromboxanes, leukotrienes, hydroxyeicosatetraenoic acids, and isoprostanes. Their roles in the physiological process and cellular injury have been extensively studied. These compounds are now known as an important mediator of the physiological process and the cellular injury, resulting in human diseases such as cardiovascular disease, gastrointestinal disease, etc. (Raymond, 1993).

The major precursor of eicosanoids is an arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid) which has four nonconjugated double bonds, synthesized from linoleic acid of diet and stored in cell membranes esterified at glycerol C_2 of phospholipids. The activation of AA metabolism is initiated by the release of AA from the phospholipid pool of cellular membrames by the action of phospholipase A_2 (Sarafianos et al, 1990; Shimizu & Wolfe, 1990; Lewis et al, 1990). The released AA can be metabolized through two major metabolic pathways. The cyclooxygenase pathway consists of enzymes that make a bond in the AA molecule between carbons 8 and 12, forming a 5-carbon cyclic compound which is then metabolized into prostaglandins (PGs), thromboxane, prostacyclin (PGI_2), and their derivatives. Almost all mammalian cells except red blood cells produce prostaglandins and their related compounds. They have the various physiological effects at an extremely low concentration.

The lipoxygenase pathway produces leukotrienes (LTs) and mono-, or dihydroxy derivatives of arachidonic acids (Samuelsson et al, 1979). The lipoxygenase metabolites are named according to the position of the carbon of AA which was modified by the addition of a hydroperoxy group. The enzyme 5-

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lipoxygenase converts AA to 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE). The 5-hydroperoxy intermediate is then either converted to leukotriene A_4 (LTA_4) by 5-lipoxygenase (Rouzer et al, 1986), or reduced to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by a peroxidase (Borgeat et al, 1985). Leukotriene A_4 is metabolized by two pathways, one leading to the highly spasmogenic peptido-leukotrienes (LTC_4, LTD_4, and LTE_4) (Murphy et al, 1979) and the other to LTB_4 (Borgeat & Samuelsson, 1979), which is a potent chemotactic agent for PMNL (Ford-Hutchinson et al, 1980). In addition to these pathways, AA is also converted to 12- and 15-monohydroxy products (12-HETE and 15-HETE) by specific lipoxygenases (Needleman et al, 1986) and to epoxides by an epoxygenase related to cytochrome *P-450s* (Oliw et al, 1982). All of the above products, along with those derived from other eicosapolyenoic acids, are often referred to as eicosanoids (Corey et al, 1980).

Isoprostanes, a series of bioactive prostaglandin-like compounds which are produced in vivo by free radical-catalyzed peroxidation of AA independent of the cyclooxygenase enzyme were discovered by Morrow et al (1990). Isoprostanes have been shown to be a potent vasoconstrictor and a potential mediator of hepatorenal syndrome and artherosclerosis (Morrow & Roberts II, 1996). They may participate as a pathophysiological mediator in oxidant injury and are able to modify the fluidity and integrity of membranes, which is the known result of oxidative damage. One of isoprostanes, 8-iso-$PGF_{2\alpha}$ is used as an accurate marker of lipid peroxidation in animal models of oxidative stress and oxidative injury in association with several human diseases (Domenico et al, 1995; Ivana et al, 1996). The general metabolic pathways of AA and the final products we extracted and analyzed were schematically represented in Fig. 1.

Over the past years, a number of analytical techniques had been introduced for the measurement of PGs, LTs and HETEs levels. These included HPLC (Mathews et al, 1981: Terragno et al, 1981), radioimmunoassay (Levine et al, 1981; Salmon et al, 1982) and gas chromatography-mass spectroscopy (MacDermot et al, 1984). The advantages and disadvantages of each of these methods with respect to their routine applicability, sensitivity, and specificity have been reviewed (Palmblad et al, 1983). HPLC represented the best balance between these factors and applied for analyzing the complex mixtures of AA metabolites which are formed by many types of cells (Peter et al, 1983; Morgan & Laychock, 1988; Tordjman et al,

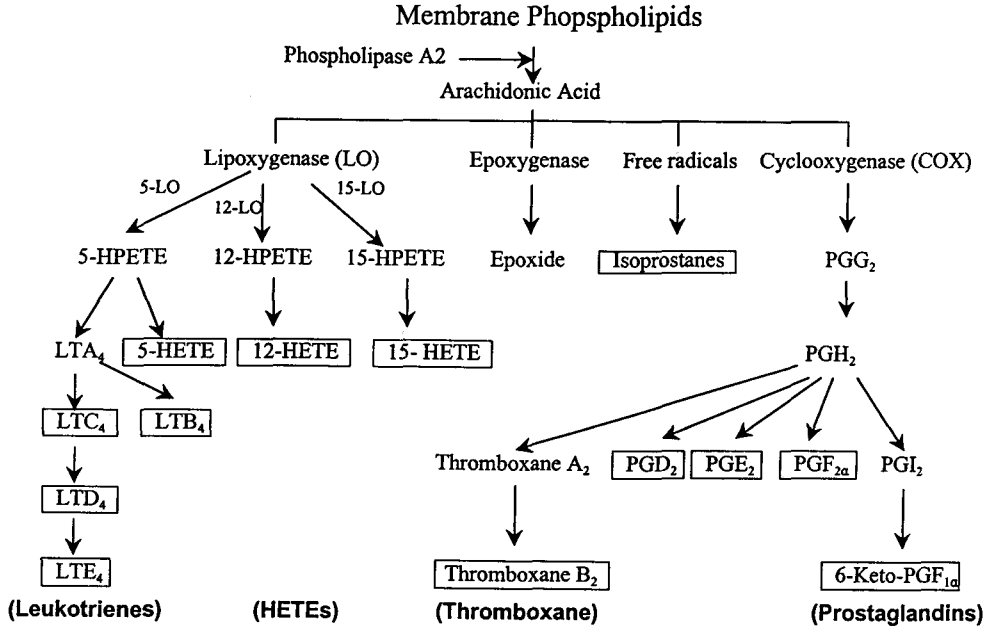

Fig. 1. The Process of arachidonic acid metabolism in mammalian cells. The metabolites we extracted and analyzed in this experiment are in □. HETE: Hydroxyeicosatetraenoic acid, PG: prostaglandin, HPETE: Hydroperoxyeicosatetraenoic acid, LT: Leukotriene.

1990). With respect to chromatographic behavior, there are two important classes of eicosanoids: (i) peptido-leukotrienes, which contain positively charged amino groups and (ii) all other eicosanoids, which contain carboxylic acid, hydroxyl, and, in some cases, oxo and epoxy group. These two classes of eicosanoids are affected quite differently by changes in both the stationary phase and the mobile phase. Reversed-phase (RP) HPLC is the only type of HPLC which gives good results with both classes of eicosanoids (Mathews et al, 1981). Normal-phase HPLC can be used for all types of eicosanoids except peptido-leukotrienes.

The use of a octadecylsilyl (ODS) silica cartridge (Powell, 1980) for a solid-phase extraction of the sample previous to the HPLC separation of the metabolites improved the results (Dawson & McGee, 1990; Graff et al, 1990). However, the separation achieved between closely related compounds was incomplete in published studies.

In this paper, we investigated various techniques for the extraction of eicosanoids from biological samples and their analysis by HPLC. Eicosanoids, including both cyclooxygenase, lipoxygenase products and isoprostanes could be rapidly extracted using Sep-Pak C_{18} cartridge and simultaneously quantitated with two isocratic HPLC systems.

METHODS

Reagents

PGs, LTs, HETEs, Thromboxane B_2 (TxB_2), and PGB_2 standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sep-Pak C-18 column was purchased from Waters Associates (Milford, MA, USA). Acetonitrile and methanol were HPLC grade and provided from E. Merck. Co. (Darmstadt, Germany).

8-iso-$PGF_{2\alpha}$ was purchased from R & D Systems (Minneapolis, MN, USA) as part of enzyme immunoassay kit.

Equipments

One HPLC system for PGs, TxB_2, and 8-iso-$PGF_{2\alpha}$ was consisted of Phamacia LKB · HPLC pump 2248, Rheodyne 7725 injector, Beckman Ultrasphere C18 (5 μm, 250×4.6 mm) column, Phamacia LKB 2141 UV detector, and Pharmacia LKB REC2.

The other HPLC system for LTs, HETEs, and PGB_2 was consisted of Phamacia LKB · HPLC pump 2248, Rheodyne 7125 injector, Rainin Microsorb RP-C18 (3 μm, 100×4.6 mm) column, Kontron HPLC UV detector 430, and Pharmacia LKB REC2.

Spin-Vac vacuum concentrator (Hanil industrial company, Korea) was used to evaporate solvents.

Sample preparation

The sample tissues or biologic fluids were frozen with liquid nitrogen immediately after sampling and stored. The frozen tissues were weighed and mixed with cold 0.5 ml of 100% methanol including 0.1% acetic acid and 100 ng PGB_2 as internal standard. After homogenation with Polytron tissue homogenizer (Ultra Turrax, IKA Lab., Germany), the homogenates were centrifugated at 15,000 rpm at 4°C for 20 min. The supernatants were brought to a 25% methanol by the addition of a 0.1% acetic acid.

Solid phase extraction

The resulting extracts were loaded onto a 2.0 ml Sep-Pak column prewashed with 5 ml of 0.1% acetic acid in methanol and 5 ml of 0.1% aectic acid. Then the Sep-Pak column was washed with 5 ml of 0.1% acetic acid and 5 ml of 0.1% acetic acid containing 25% methanol. AA metabolites were eluted with 2.4 ml of 0.1% acetic acid containing 90% methanol. The eluents were evaporated by vacuum concentrator (SpinVac) and reconstituted with a 200 μl of 50% methanol. Each 50 μl were injected into HPLC columns, and another 50 μl was used for enzyme immunoassay of 8-iso-$PGF_{2\alpha}$.

HPLC method for PGs and TxB_2

The composition of mobile phase was acetonitrile/water (32.8/67.2, v/v). The pH was adjusted to 3.3 with phosphoric acid. The peaks were monitored at 195 nm with 1.5 ml/min of flow rate.

HPLC method for PGB_2, LTs and HETEs

The composition of mobile phase was methanol/water (80/20, v/v) plus 0.1% trifluoroacetic acid (TFAA), 0.06% triethylamine (TEA). PGB_2 and LTs were monitored at 280 nm, and HETEs were detected

at 235 nm with 1.0 ml/min of flow rate.

RESULTS

HPLC method for PGs and TxB_2

Recoveries of PGs and TXB_2 were about 80 - 100% (Table 1). All peaks were separated clearly (Fig. 2) but the PGD_2 peak was shifted depending on the pH's of mobile phase. Therefore, the constant adjustment of pH with phosphoric acid in the range of 3.3~3.5 was important.

HPLC method for PGB_2, LTs and HETEs

Recoveries of peptido-LTs were about 50~80% and of HETEs were about 65~90% (Table 2). The relative low recoveries of peptido-LTs were probably by labile nature of peptido-LTs. The peaks of LTs were not completely separated. The peaks, especially of C4 and D4, were easily coeluted and showed

Table 1. Chromatographic behavior of cyclooxygenase metabolites on RP-HPLC

	Retention time (min)	Recovery (%)[a]
6-Keto $PGF_{1\alpha}$	3.8	92±4
6-Keto PGE_1	4.5	88±6
8-iso-$PGF_{2\alpha}$	6.8	98±3
TxB_2	7.5	87±4
$PGF_{2\alpha}$	8.9	96±5
PGE_2	12.3	93±3
PGE_1	13.8	84±4
PGD_2	15.2	85±5

[a]Standards (100 ng each) were used. Recoveries (mean± SD, n=6) were calculated by comparing peak height for each compound after extraction and injection into HPLC with a authentic standard on the same day.

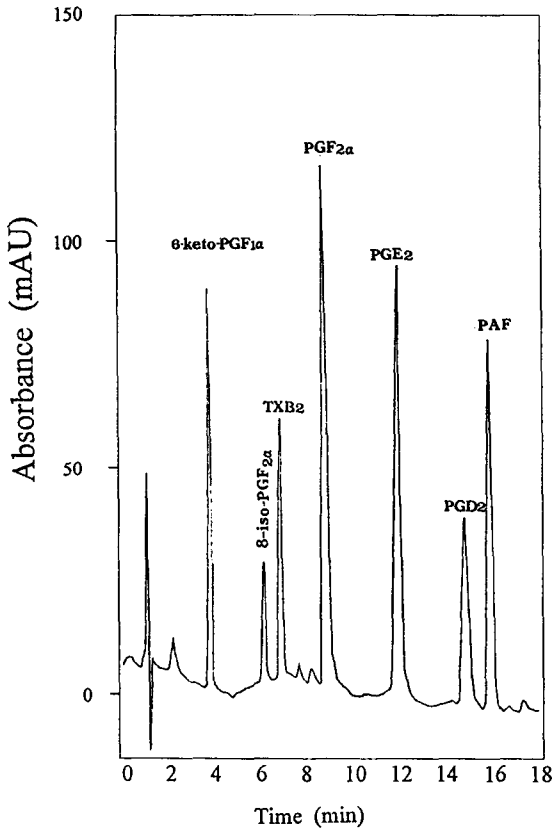

Fig. 2. RP-HPLC Chromatography of standards containing 100 ng each of 6-keto-PGF1a, thromboxane B_2 (TXB_2), PGF_2, PGE_2, PGD_2, 20 ng of 8-iso-PGF2a and 10 ng of platelet activating factor (PAF). Chromatogram was recorded at 195 nm.

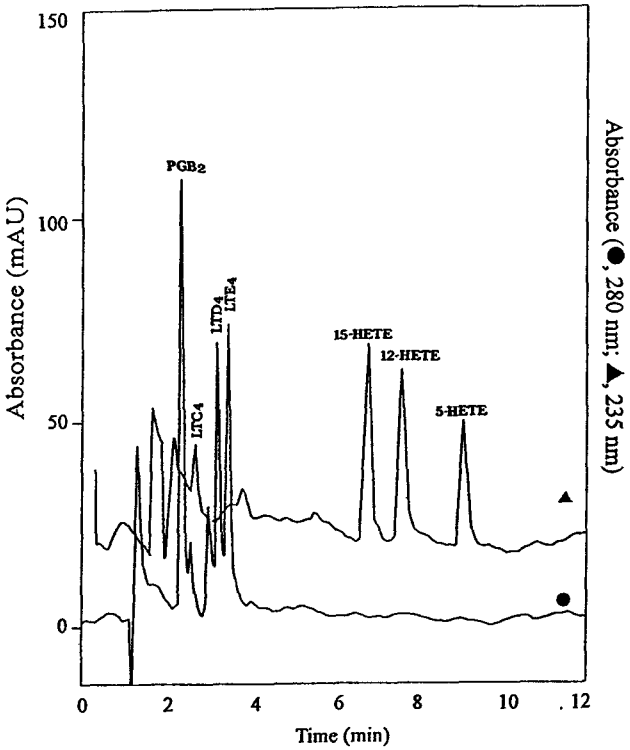

Fig. 3. RP-HPLC Chromatogram of standards containing 20 ng of PGB_2, 7.0 ng each of LTC_4, LTD_4, LTE_4, and 9.0 ng each of 15-HETE, 12-HETE, 5-HETE. Chromatogram was recorded at 280 nm for PGB_2 and leukotrienes and at 235 nm for HETEs.

broadened baseline. The composition of triethylamine is important to keep separate these two peaks. Triethylamine worked as ion pairing agent in this condition and made peaks sharper. The HETEs were well seperated, but the peak of 15-HETE was interfered by unknown peaks occasionally (Fig. 3).

Table 2. Chromatographic behavior of lipoxygenase metabolites on RP-HPLC

	Retention time (min)	Recovery (%)[a]
PGB_2	2.6	97±5
LTC_4	3.1	63±4
LTD_4	3.4	76±3
LTE_4	3.7	57±3
15-HETE	7.2	83±5
12-HETE	7.9	75±5
5-HETE	9.5	70±4

[a]Standards (50 ng each) were used. Recoveries (mean± SD, n=6) were calculated by comparing peak height for each compound after extraction and injection into HPLC with a authentic standard on the same day.

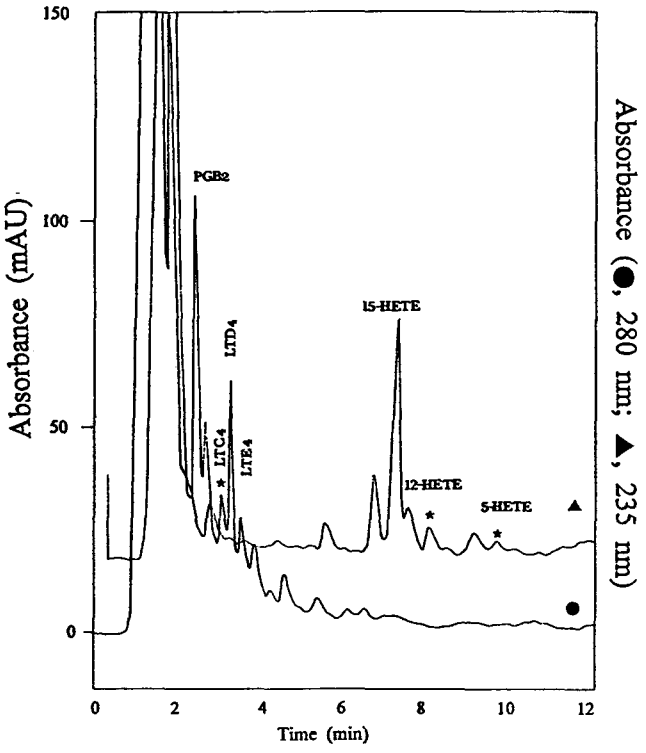

Fig. 4. RP-HPLC Chromatogram of leukotrienes and HETEs for 220 mg of tissue resected from a human nasal polyp.

HPLC analysis from biological samples

We measured the LTs and HETEs which extracted from resected tissue of human nasal polyp. LTs and HETEs were well seperated in Fig. 4. This means that the conditions we applied were very successful to extract and measure the even labile peptido-LTs in the biological samples.

From the comparison of chromatograms between otitis media exudate challenged by interleukin-α 0.1 U (Fig. 5) and otitis media exudate challenged by tumor necrosis factor 0.1 μg (Fig. 6), we could suggest that the inflammatory process or cellular injury by different insults were manifested differently and that the changes of AA metabolism by such stimuli would be detected by this system. This analytical method for whole AA metabolites was quite rapid and reproducible.

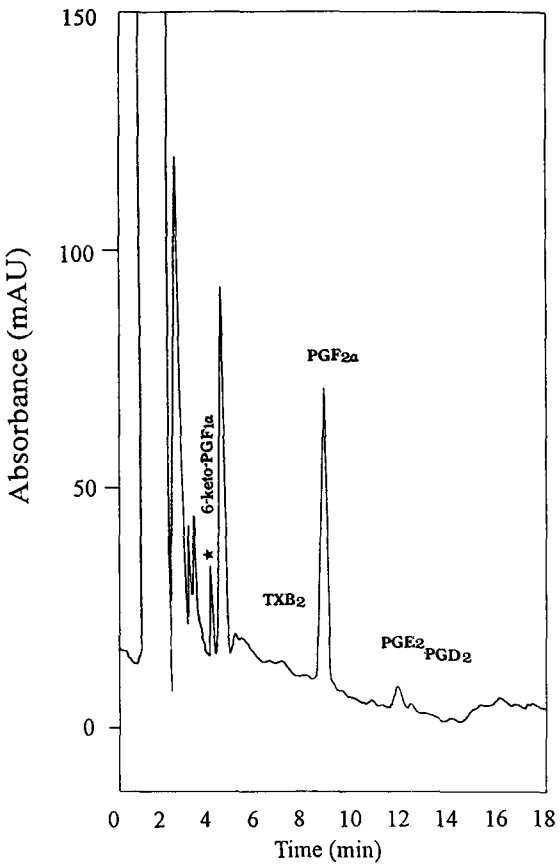

Fig. 5. RP-HPLC Chromatography of prostaglandins and thromboxane from the 38 μl of otitis media exudate of chinchilla challenged by IL-α 0.1 U.

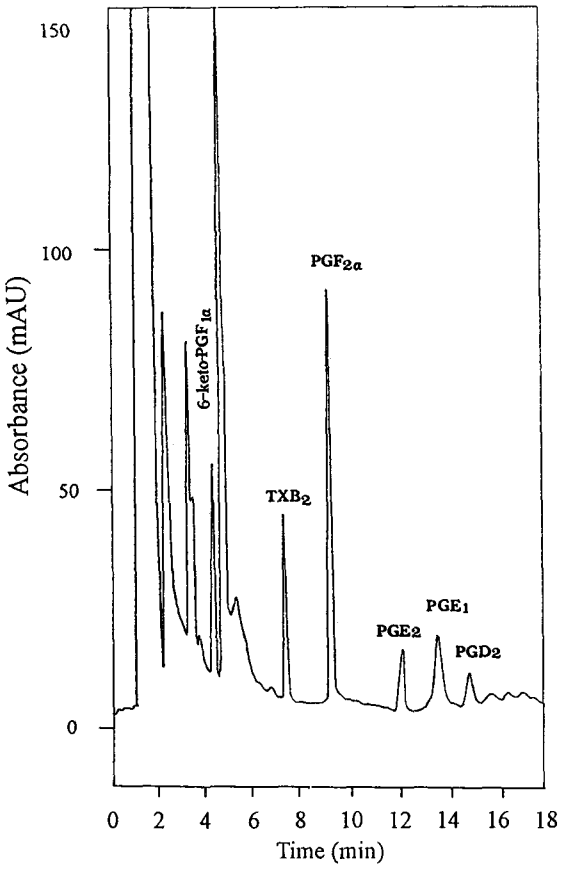

Fig. 6. RP-HPLC Chromatography of prostaglandins and thromboxane from the 41 μl of otitis media exudate of chinchilla challenged by TNF 0.1 μg.

DISCUSSIONS

Sep-Pak C_{18} extraction coupled to RP HPLC provides a convenient and efficient method for the analysis of complex mixtures of AA metabolites in a single step. The recoveries of AA metabolites when they are analyzed by Sep-Pak extraction/RP HPLC are all good and better than those obtained using other methods. The application of C_{18} (ODS) Sep-Pak cartridges on solid-phase extraction of arachidonic acid metabolites was introduced by Powell (1980). In his study, greater than 90% recovery of prostaglandin standards and approximately 50% recovery of 15-HETE from biological fluids were achieved, moreover, Eskra et al (1986) developed an extraction procedure with better recovery of HETEs and other lipoxygenase pathway products while minimizing the number of processing steps.

Lipoxygenase pathway metabolites are usually separated by reverse-phase HPLC using a mobile phase of aqueous methanol (60~70%) and acetic acid (0.1%). Mono- and di-HETEs can be resolved using this mobile phase and any good quality C_{18} packing. However, LTC_4, D_4, and E_4 are particularly difficult to analyze because of a variable and generally poor recovery, resolution, and coelution with other products of interests. This is probably due to the presence of multiple functional groups on the peptide portion of these molecules including hydroxyl, carboxyl, thioether, and amino moieties. Using an aqueous methanol mobile phase with trifluoroacetic acid and triethylamine at a flow rate of 1.0 ml/min, a chromatogram was obtained in which LTC_4, LTD_4, and LTE_4 were resolved in less than 5 min. The LTB_4 and LTD_4 were coeluted, and it was hard to separate these two peaks in spite of the extensive modification of trifluoroacetic acid and triethylamine. Considering the signal to noise ratio, the detection limit for PGs was about 1.5 ng while that for LTs and HETEs was about 0.8 ng.

Other than several minor extraneous peaks, the Sep-Pak C_{18} extract was remarkably free of UV-absorbing material from biological samples that could interfere with the analysis of PGs, leukotrienes, and HETEs.

Solid-phase extraction combined with the modified HPLC system described here provided a rapid, reproducible, economic, and sensitive method for the analysis of cyclooxygenase and lipoxygenase pathway metabolites. Besides above metabolites, this method has been used to extract isoprostanes simultaneouly. Therefore, this analytical method would be useful to reveal the complexities of the biochemical events of eicosanoids and their relationships to other mediators in the normal physiology and the pathophysiological processes of cellular injury.

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REFERENCES

Abe M, Kawazoe Y, Shigematsu N. Influences of salts on high-performance liquid chromatography of leukotrienes. *Anal Biochem* 144(2): 417−422, 1985

Bennett HP, Browne CA, Solomon S. Purification of the

two major forms of rat pituitary corticotropin using only reversed-phase liquid chromatography. *Biochemistry* 20(16): 4530−4538, 1981

Borgeat P, Nadeau M, Salari H, Poubelle P, Fruteau de Laclos B. Leukotrienes: biosynthesis, metabolism, and analysis. *Adv Lipid Res* 21: 47−77, 1985

Borgeat P, Samuelsson B. Arachidonic acid metabolism in polymorphonuclear leukocytes: unstable intermediate in formation of dihydroxy acids. *Proc Natl Acad Sci USA* 76(7): 3213−3217, 1979

Corey EJ, Niwa H, Falck JR, Mioskkowski C, Arai Y, Marfat A. Recent studies on the chemical synthesis of eicosanoids. *Adv Prostaglandin Thromoxane Res* 6: 19−25, 1980

Dawson M, McGee CM. Comparison between ethanol and methanol for the reversed-phase high-performance liquid chromatographic analysis of leukotriene B_4 and its metabolites in cell incubation supernatants. *J Chromatogr* 532(2): 379−386, 1990

Pratico D, Lawson JA, FitzGerald GA. Cyclooxygenase-dependent formation of the isoprostane, 8-epi-prostaglandin$F_{2\alpha}$. *J Biochemical Chemistry* 270(17): 9800−9808, 1995

Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJ. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 286(5770): 264−265, 1980

Graff G, Anderson LA, Jaques LW. Preparation and purification of soybean lipoxygenase-derived unsaturated hydroperoxy and hydroxy fatty acids and determination of molar absorptivities of hydroxy fatty acids. *Anal Biochem* 188(1): 38−47, 1990

Kawikova I, Barnes PJ, Takahashi T, Tadjkarimi S, Yacoub MH, Belvisi MG. 8-epi-$PGF_{2\alpha}$, a novel non-cyclooxygenase-derived prostaglandin, constricts airways in vitro. *Am J Respir Crit Care Med* 153: 590-596, 1996

Fletcher JR. Eicosanoids: Critical agents in the Physiological process and cellular injury. *Arch Surg* 128: 1192−1196, 1993

Eskra JD, Pereira MJ, Ernest MJ. Solid-phase extraction and high-performance liquid chromatography analysis of lipoxygenase pathway products. *Analytical Biochemistry* 154: 332−337, 1986

Levine L, Morgan RA, Lewis RA, Austen KF, Clark DA, Marfat A, Corey EJ. Radioimmunoassay of the leukotrienes of slow reacting substance of anaphylaxis. *Proc Natl Acad Sci USA* 78(12): 7692−7696, 1981

Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N Engl J Med* 323(10): 645−655, 1990

MacDermot J, Kelsey CR, Waddell KA, Richmond R, Knight RK, Cole PJ, Dollery CT, Landon DN, Blair IA. Synthsis of leukotriene B4, and prostanoids by human alveolar macrophages:analysis by gas chromatography/mass spectrometry. *Prostaglandins* 27(2): 163−179, 1984

Mathews WR, Rokach J, Murphy RC. Analysis of leukotrienes by high-pressure liquid chromatography. *Anal Biochem* 118(1): 96−101, 1981

Morgan RO, Laychock SG. Biosynthesis of peptidyl leukotrienes and other lipoxygenase products by rat pancreatic islets. Comparison with macrophages and neutrophils. *Prostaglandins* 35(4): 609−623, 1988

Murphy RC, Hammarstrom S, Samuelsson B. Leukotriene C: a slow-reacting substance from murine mastocytoma cells. *Proc Natl Acad Sci USA* 76(9): 4275−4279, 1979

Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. *Annu Rev Biochem* 55: 69−102, 1986

Oliw EH, Guengerich FP. Oxygenation of arachidonic acid by hepatic monooxygenases. Isolation and metabolism of four epoxide intermediates. *J Biol Chem* 257(7): 3771−3781, 1982

Palmblad J, Uden AM, Freberg I, Ringertz B, Radmark O, Lindgren JA, Hansson G, Malmsten CL. Effects of novel lipoxygenase products on neutrophil and eosinophil functions in vitro. *Adv Prostaglandin Thromboxane Leukot Res* 12: 25−31, 1983

Peters SP, Schulman ES, Liu MC, Hayes EC, Lichtenstein LM. Separation of major prostaglandins, leukotrienes, and monoHETEs by high performance liquid chromatography. *J Immunol Methods* 64(3): 335−343, 1983

Powell WS. Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. *Prostaglandins* 20(5): 947−957, 1980

Rouzer CA, Matsumoto T, Samuelsson B. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. *Proc Natl Acad Sci USA* 83(4): 857−861, 1986

Salmon JA, Simmons PM, Palmer RM. A radioimmunoassay for leukotriene B4. *Prostaglandins* 24(2): 225−235, 1982

Samuelsson B, Borgeat P, Hammarstrom S. Murphy RC. Introduction of a nomenclature: leukotrienes. *Prostaglandins* 17(6): 785−787, 1979

Sarafianos SG, Nair PP, Kumar S. AT32P-dependent estimation of nanomoles of fatty acids: its use in the assay of phospholipase A_2 activity. *Anal Biochem* 186(2): 374−379, 1990

Shimizu T, Wolfe LS. Arachidonic acid cascade and signal transduction. *J Neurochem* 55(1): 1−15, 1990

Terragno A, Rydzik R, Terragno NA. High performance liquid chromatography and UV detection for the

separation and quantitation of prostaglandins. *Prostaglandins* 21(1): 101−112, 1981

Tordjman C, Serkiz B, Rambaud F, Bonnet J, Volland JP. High-performance liquid chromatographic quantitation of cyclooxygenase and lipoxygenase metabolites of arachidonic acid from rat polymorphonuclear leukocytes. *J Chromatogr* 532(1): 135−143, 1990

William S. Powell. Precolumn extraction and reversed phase high-pressure liquid chromatography of prostag landins and leukotrienes. *Analytical Biochemistry* 164 117−131, 1987