Journal of Chromatography, 308 (1984) 316—321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2059

Note

Simple single-step high-performance liquid chromatographic method for the separation of cyclooxygenase and lipoxygenase enzyme metabolites of arachidonic acid

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(First received September 14th, 1983; revised manuscript received January 3rd, 1984)

Arachidonic acid (AA) is metabolized in numerous biological systems by the cyclooxygenase and lipoxygenase enzymes. The products of these pathways comprise several groups of metabolites including the primary prostaglandins (PGs) E_2, F_{2a} and D_2, thromboxane (Tx) A_2 and its stable hydrolysis product TxB_2, PGI_2 and its stable hydrolysis product 6-keto-PGF_{1a}, hydroxylated derivatives of AA (hydroxyeicosatetraenoic acids, HETEs), and leukotrienes (LTs), which have in addition to the AA-derived portion of the molecule all or part of the tripeptide glutathione [1].

Since both enzymatic pathways are functional in many cell types, individual samples are often analyzed for both cyclooxygenase and lipoxygenase products. The current approach to leukotriene separation is high-performance liquid chromatography (HPLC) with quantitation often dependent on biological assay or UV spectrometry [1, 2]. Thin-layer chromatography (TLC) and HPLC are the methods of choice for separating PGs and HETEs with additional quantification by radioimmunoassay or gas chromatography—mass spectrometry. HPLC of arachidonic acid metabolites has been recently reviewed by Hamilton and Karol [1].

To our knowledge there is no published simple single-step method for the simultaneous separation of the metabolites of both enzymatic pathways. Rouzer et al. [3] use HPLC to separate cyclooxygenase and lipoxygenase products of two types of pulmonary macrophages but must rechromatograph the fractions containing PGs to achieve separation of the various PGs. Several TLC systems allowing rapid separation and identification of metabolites of both pathways have been reported. One fails to resolve TxB_2 from PGE_2 satisfactorily and 15-HETE from 12- and 5-HETE [4] while others do not

0378-4347/84/$03.00

address the separation of LTs from other lipoxygenase [5] or cyclooxygenase products [6].

We report here a combination of solvent systems that separate 6-keto-PGF_{1a}, TxB_2, PGE_2, PGF_{2a}, PGD_2, HHT, LTB_4/LTC_4, LTD_4, 5-HETE, 12-HETE, 15-HETE and AA in a single 100-min HPLC run. The value of this method is two-fold. First, it reduces time in sample analysis by reducing the likelihood of needing additional chromatography, which in turn minimizes sample loss. Secondly, it can be performed without a separate solvent programming device, and, since a standard reversed-phase column is used, a radial compression device [7] is not required.

EXPERIMENTAL

Materials

Acetonitrile and methanol (distilled in glass) were purchased from Burdick & Jackson Labs. (Media, PA, U.S.A.). Water was deionized and purified with a Milli-R/Q system (Millipore, Bedford, MA, U.S.A.). Tritium-labeled standards were purchased from New England Nuclear (Boston, MA, U.S.A.) with the exception of [^{3}H]12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) which was synthesized by washed platelets from radiolabeled AA.

Chromatography

The HPLC apparatus consists of a single Waters 6000A pump (Milford, MA, U.S.A.) a Rheodyne Model 7125 injector (Cotati, CA, U.S.A.) and a 30 cm × 3.9 mm μBondapak C_{18} 10-μm particle size reversed-phase column and a phenyl Corasil packed guard column (Waters).

Elution was accomplished in less than 100 min with three different solvents. Flow-rate was 2 ml/min throughout and 1-min fractions were collected and counted in a scintillation counter. Samples were dissolved in 2 ml of solvent A, water—acetonitrile—benzene—acetic acid (767:230:2:1, v/v/v/v), prior to injection. Solvent A was pumped for 48 min before switching to solvent B, methanol—water—acetic acid (650:350:0.1, v/v/v, pH 4.2). Solvent B continued for 42 min before switching to solvent C, methanol—water—acetic acid (900:100:0.1, v/v/v), which was pumped for 10 min.

Blood platelets

Nine volumes of blood were drawn from the femoral vein of pentobarbital (30 mg/kg) anesthetized cats into one volume of 77 m*M* sodium EDTA and washed platelets prepared according to the method of Hamberg et al. [8]. Broken platelets were prepared by freezing and thawing the washed platelet suspension three times. Aliquots (475 μl) of the washed platelets were incubated in an aggregometer (Payton Assoc., Buffalo, NY, U.S.A.) at 37°C with constant stirring for 1 min before any additions were made. Platelets received 5 μl of a solution which contained 1 μCi [^{3}H]AA (62 Ci/mmol, New England Nuclear) and 0.5 μg of unlabeled AA (Nu Chek Prep., Elysian, MN, U.S.A.) as the sodium salt. The platelets and AA were incubated for 5 min. Aggregation is not induced by this concentration of AA. For some experiments indomethacin (Sigma, St. Louis, MO, U.S.A.) and sodium car-

bonate were weighed out (1:3, w/w), dissolved in distilled water and added to the platelets 1 min before AA to produce a final indomethacin concentration of 3 μg/ml of platelets. The pH of the indomethacin stock solution was measured to ensure it did not exceed 8, since alkaline solutions destroy indomethacin [9].

Following the 5-min incubation of the platelet suspensions the radiolabeled products were extracted and separated by HPLC. First the protein in each platelet incubate was precipitated with 300 μl of acetone and the pH adjusted to 3 with 0.4% formic acid. The incubate was then extracted twice with 3 ml of ethyl acetate. The ethyl acetate extract was dried under a stream of nitrogen, dissolved in 2 ml of chloroform and filtered with a 0.45-μm Millipore filter (Gelman, Ann Arbor, MI, U.S.A.). The chloroform was then removed with nitrogen and the residue dissolved in 2 ml of solvent A.

RESULTS AND DISCUSSION

The retention times of authentic standards separated by this HPLC method are seen in Fig. 1. Solvent A separates cyclooxygenase products, including PGD_2 and PGE_2. The same solvent run in like manner on a Waters Fatty Acid Analysis reversed-phase column does not resolve these two metabolites. Solvent B separates lipoxygenase products. However, note that LTB_4 and LTC_4 co-elute. Changes in solvent pH did not change this co-elution, nor did the use of a gradient from solvents A to B. It has been reported by others that LTB_4 and LTC_4 can be separated using a Nucleosil C_{18} column and a solvent system

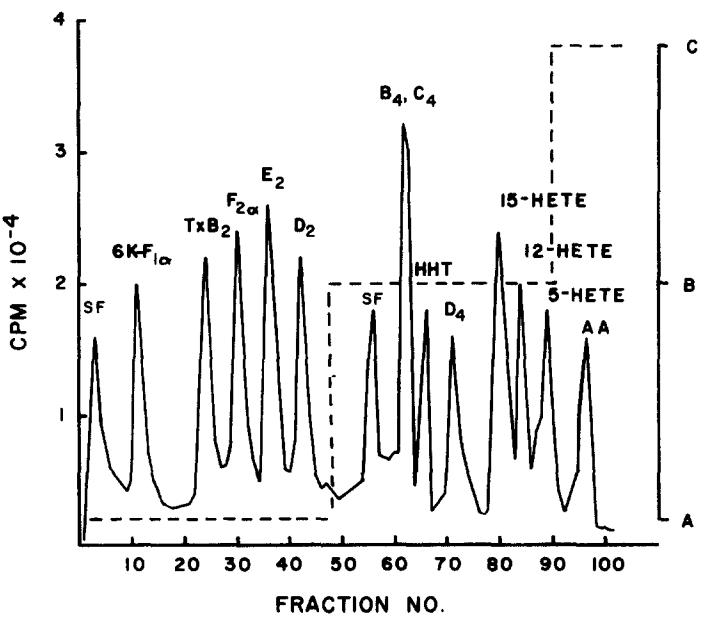

Fig. 1. Single-step HPLC separation of PGs, LTs, HETEs and AA. The labeled peaks are retention times of authentic standards. A μBondapak C_{18} column is used with a flow-rate of 2 ml/min (1-min fractions). The solvent composition is given in the text The dashed line indicates the time and manner in which the solvents are changed.

similar to solvent B but at a higher pH [6]. Elution of AA is readily accomplished with solvent C. Some investigators have reported poor or variable recoveries of leukotrienes using a C_{18} column [2]. Table I shows that the mean recovery of ^3H standards from a C_{18} column using our method was 82%. We feel that in light of the highly effective separation that this method accomplishes, these recoveries are acceptable. We might also note that this HPLC system can be more fully automated with the use of two pumps and a solvent switching device or a third pump used in conjunction with a Waters System Controller. Gradients can be run from one solvent to the next if desired, but retention times increase accordingly.

TABLE I

RECOVERY OF ^3H-LABELED STANDARDS

Compound	Recovery (%) (n = 3)
6-keto-PGF_{1a}	83
TxB_2	82
PGF_{2a}	57
PGE_2	79
PGD_2	90
LTB_4	80
LTC_4	85
LTD_4	72
15-HETE	79
12-HETE	74
5-HETE	78
AA	90
Mean	82

This HPLC solvent system has been applied successfully in our laboratory to study blood platelet metabolism of [^3H]AA. When washed, whole blood platelets are incubated for 5 min with [^3H]AA and the products extracted and run on this HPLC system a product profile is obtained, as shown in Fig. 2. We have used this HPLC system to compare the metabolism of AA in whole versus broken platelets. Table II shows that in whole cells 30% of the products were cyclooxygenase products, with approximately equal distribution between TxB_2 and HHT. The lipoxygenase product 12-HETE was twice as abundant as the cyclooxygenase products while only 9% of the [^3H]AA was not metabolized.

The percent distribution of TxB_2, HHT, 12-HETE and AA produced by broken cells was significantly different from the percent distribution of the same products in whole cells ($P < 0.01$, Student's t-test). With broken platelets, 12-HETE was half as abundant as in whole platelets while the percent cyclooxygenase products and unutilized AA both increased. The reason for this difference may be related to the fact that lipoxygenase is a soluble enzyme and in broken cells would be dispersed into the incubation buffer, thus decreasing its local concentration. This would allow more arachidonate to be

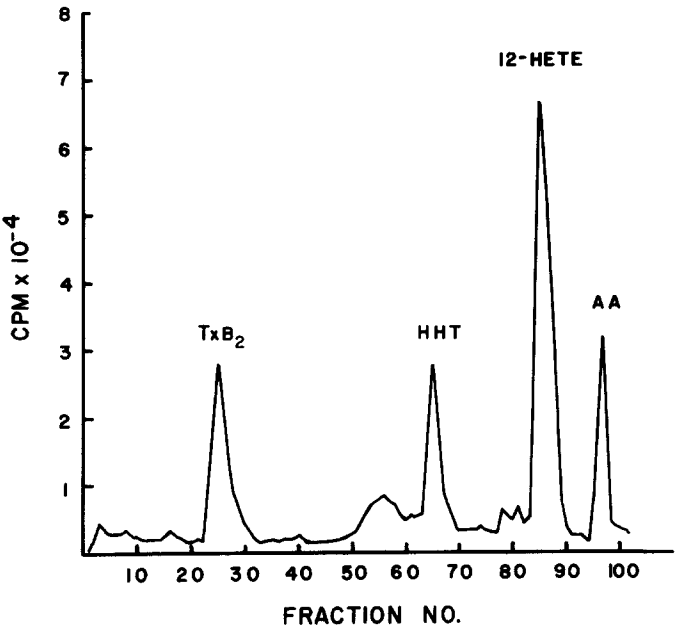

Fig. 2. Single-step HPLC separation of the cyclooxygenase and lipoxygenase products of whole washed cat platelets incubated with [^{3}H]AA.

TABLE II

METABOLISM OF [^{3}H]ARACHIDONIC ACID BY WHOLE OR BROKEN PLATELETS AND THE EFFECT OF INDOMETHACIN

	n	Product distribution of ^{3}H (mean ± S.E.M., %)			
		TxB_2	HHT	12-HETE	AA
Whole cells	4	13.5 ± 0.6	16.3 ± 1.4	61.3 ± 1.1	9.0 ± 1.0
Broken cells	3	14.0 ± 3.0	19.0 ± 4.8	32.6 ± 1.6	34.4 ± 7.9
Broken cells plus indomethacin (3 μg/ml)	6	0.4 ± 0.1	1.1 ± 0.1	48.2 ± 2.6	50.2 ± 2.6

utilized by the microsomal enzyme, cyclooxygenase, and also result in a decreased overall utilization of AA during the 5-min incubation period. In order to demonstrate that our experimental system is capable of detecting the effect of inhibitors, we pretreated broken platelets with the cyclooxygenase inhibitor, indomethacin. Indomethacin virtually abolished the cyclooxygenase products TxB_2 and HHT and increased the percent distribution in both 12-HETE and AA.

In summary, we have reported a relatively efficient HPLC method that uses only a single pump and column to achieve separation of twelve major AA metabolites. This capability will become increasingly important in light of the extensive work being done on the interrelationship of the cyclooxygenase and lipoxygenase pathway.

ACKNOWLEDGEMENTS

This work is supported by NS 12587 and a Grant-in-Aid from the American Heart Association. We thank Mrs. R. Harris for typing this manuscript.

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