Journal of Chromatography, 232 (1982) 176—179
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1348

Note

Simple method for the separation of monohydroxy fatty acid metabolites of arachidonate metabolism

GUNDU H.R. RAO*

Department of Laboratory Medicine and Pathology, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

K. RATNAMMAL REDDY

Department of Pediatrics, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

and

JAMES G. WHITE

Departments of Laboratory Medicine and Pathology and Pediatrics, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

(First received February 22nd, 1982; revised manuscript received April 13th, 1982)

Prostaglandins play a major role in cardiovascular physiology [1]. Arachidonic acid is the most abundant of the polyunsaturated fatty acids in platelet membranes and is transformed to biologically active intermediates by two different pathways [2,3]. Active compounds of the cyclooxygenase pathway are prostaglandin endoperoxides (PGG_2, PGH_2) and thromboxane A_2 [4]. Hydroperoxy acids (HPETE), hydroxy acids (HETE) and leukotrienes are generated by the lipoxygenase pathway [5,6]. Methods have been developed to monitor several of the stable metabolites of the labile compounds as an index of the enzyme activity in various tissue. Methods of choice have been thin-layer chromatography (TLC), high-performance liquid chromatography, radioimmunoassay, gas chromatography (GC) and massspectrometry (MS) [7]. Recently, Vincent et al. [8] have suggested using ratios of thromboxane B_2 (TxB_2); 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT); and 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), as an index of arachidonic acid metabolism by platelets in health and disease. By-and-large, the method used to separate HETE from HHT involve methylation followed by TLC [7,9,10]. In addition, separation achieved by this method may not be complete. In the

0378-4347/82/0000—0000/$02.75

present paper we describe a simple procedure in which total separation of the two hydroxy acids could be achieved without derivatization.

MATERIALS AND METHODS

Blood for these studies was obtained from normal volunteer donors. The procedures used to obtain blood, mix the samples with trisodium citrate—citric acid—dextrose (CCD) buffer, (0.1 *M* citrate, 7 m*M* citric acid, 0.14 *M* dextrose, pH 6.5), in a ratio of nine parts of blood to one part anticoagulant and isolate platelet rich plasma (PRP) by centrifugation at room temperature have been described in several publications [11—13]. To obtain platelets free of plasma, PPR was centrifuged, 0.34 *M*—PRP EDTA (9:1), with EDTA as anticoagulant in a cold centrifuge for 20 min (860 *g*). Plasma was discarded and platelets resuspended in Hanks' balanced salt solution (HBSS) free of calcium and magnesium ions. Each sample was mixed with equal volumes of CCD and the procedure was repeated twice to obtain washed platelets. Platelets thus prepared were suspended in normal HBSS and cell counts were made with a Coulter counter.

[^{14}C] Arachidonic acid metabolism by intact platelets

For measurement of arachidonic acid metabolism each reaction mixture containing $1.5 \cdot 10^9$ cells suspended in 1 ml of regular HBSS was stirred on an aggregometer for 5 min at 37°C with 1 μg of labelled arachidonic acid ([1-^{14}C]-AA; New England Nuclear, Boston, MA, U.S.A.). At the end of the experiment 1 ml of ethyl acetate was added to each reaction mixture and acidified with 10 μl of 0.5 *M* citric acid. After thorough mixing the ethyl acetate layer was separated and the reaction mixture was once more extracted with an equal volume of ethyl acetate [14]. Fractions of the organic phase were pooled, concentrated over nitrogen and saved for further analysis.

Separation of thromboxane B_2 and hydroxy acids

Pooled concentrates were plated on a silica gel G plate. The solvent system used for the separation of TxB_2 was diethyl ether—methanol—acetic acid (135:3:3). Hydroxy acids (HETE,HHT) were separated by using a different solvent system consisting of petroleum ether (60—70°C)—diethyl ether—acetic acid (60:39:1). Radioactivity was monitored with a Berthold radiolabel scanner and quantitation was achieved by separation of the spots and counting for radioactivity in a Beckman LS3133T scintillation counter. For characterization of the compounds identified as peaks I and II, the extracts from the plate containing the hydroxy acids were concentrated, derivatized to methyl esters by using diazomethane. Methyl esters were silated by reacting with 25 μl of TRI-SIL/TBT (Pierce, Rockford, IL, U.S.A.) at 50°C for 10 min. Derivatives of the hydroxy acids were subjected to electron impact MS using an LKB 9000 mass spectrometer with a digital PDP-8e data processor. The GC separation of the hydroxy acids were achieved on a 3% OV-1 column with an initial temperature of 180°C programmed for linear increase of 6°C/min.

RESULTS AND DISCUSSION

[1-^{14}C] AA was transformed to TXB_2 and hydroxy acids using intact washed

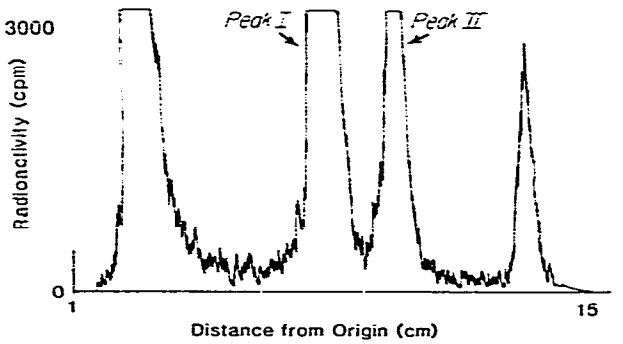

Fig. 1. Radiochromatogram showing the separation of the two hydroxy fatty acids generated by intact human platelets.

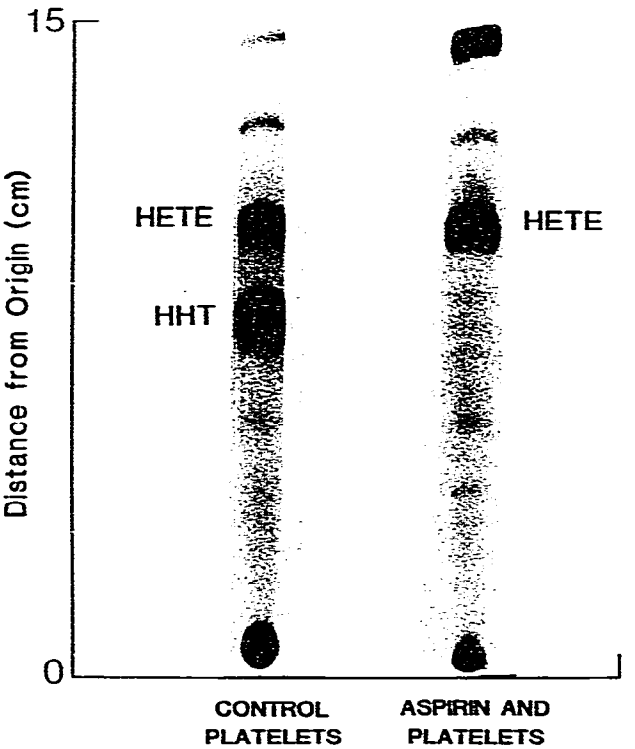

Fig. 2. Photograph of an X-ray film developed on a thin-layer plate. The two hydroxy fatty acids generated by intact blood pletelets are separated completely from one another. Aspirin treated placelets generated only 12L-hydroxy 5,8,10,14-eicosatetraenoic acid (HETE).

platelets as the source of cyclo-oxygenase and lipoxygenase enzymes. The reaction mixtures were extracted, concentrated, plated on a TLC plate without any derivatization step and the metabolites separated. The solvent system selected for the separation of metabolites of arachidonic acid, separated the hydroxy acids completely from one another (Figs. 1 and 2). Retention times for the peaks I and II were 5.8 and 8.4 min, respectively. The mass spectrum of peak I showed ions of high tensity at *m/e* 366, 351, 335, 295, 276, and 225

whereas the major fragments for peak II were at *m/e* 406, 391, 375, 295, 229, 205, and 173. The fragmentation pattern matched the expected pattern for HHT and HETE, respectively. There was less than 1% cross contamination of these products in the individual peaks obtained by this technique. The method is very simple, needs no derivatization of the metabolites and gives complete separation of the two hydroxy acids.

ACKNOWLEDGEMENTS

This work was supported by USPHS grants HL-11880, HL-16833, HL-20695, CA-21737, GM-22167, AM17697, GM-24558. The authors wish to thank Thomas P. Krick of the Department of Chemistry, University of Minnesota, and The Universtiy of Minnesota Agricultural Experimental Station for providing help in the characterization of compounds by GC—MS.

REFERENCES

1 T.M.A. Elatter, J. Oral Pathol., 7 (1978) 175.
2 A.J. Marcus, J. Lipid, Res., 19 (1978) 793
3 W.E.M. Lands, Ann. Rev. Physiol., 41 (1979) 633.
4 M. Hamberg, J. Svensson and B. Samuelsson, Proc, Nat. Acad. Sci. U.S.A., 72 (1975) 2994.
5 D.H. Nugteren, Biochim. Biophys. Acta, 380 (1975) 299.
6 P. Borgeat and B. Samuelsson, J. Biol. Chem., 254 (1979) 7865.
7 K. Green, M. Hamberg, B. Samuelsson and J.C. Frolich, in J.C. Rolich (Editor), Advances in Prostaglandin and Thromboxane Research 5, Raven Press, New York, 1978, pp. 15—95.
8 J.E. Vincent, F.J. Zijlstra and H. van Vliet, Prostaglandins Med., 5 (1980) 79.
9 M. Hamberg and B. Samuelsson, Proc. Nat. Acad. Sci. U.S.A., 71 (1976) 3400.
10 T.K. Bills, J.B. Smith and M.J. Silver, Biochim. Biophys. Acta, 424 (1976) 303.
11 J.G. White, Blood, 31 (1968) 604.
12 J.G. White, Amer. J. Pathol., 69 (1972) 439.
13 G.H.R. Rao and J.G. White, Amer. J. Hematol., 11 (1981) 355.
14 G.H.R. Rao, A.C. Cox, J.M. Gerrard and J.G. White, Biochim. Biophys. Acta, 628 (1980) 468.