ELSEVIER

Journal of Chromatography B, 762 (2001) 175–180

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Improved high-performance liquid chromatographic method for the combined analysis of phospholipase, lipoxygenase and cyclooxygenase activities

Denis Reynaud[a], Andrea Sun[a], Peter Demin[a], Cecil R. Pace-Asciak[a,b,*]

[a]*Programme in Integrative Biology, Research Institute, The Hospital for Sick Children, Toronto M5G 1X8, Canada*
[b]*Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto M5A 1A8, Canada*

Received 26 January 2001; received in revised form 8 June 2001; accepted 31 July 2001

Abstract

We report herein an improved method for the high-performance liquid chromatographic separation and analysis of eicosanoids formed during the stimulation of human platelets in vitro with collagen. Since the products of interest, excepting arachidonic acid, contain hydroxyl groups (one to several), our method involves the conversion of the hydroxyl groups into acetates (pyridine/acetic anhydride) after derivatization with anthryl diazomethane (ADAM) rendering the compounds with much decreased polarity for separation on a reversed-phase column. This procedure is superior to that involving ADAM esters only, i.e. with free hydroxyl groups, as it leads to the excellent separation of the desired compounds from each other and from extraneous peaks observed due to the ADAM reagent and sharpens the peak of thromboxane. We have successfully applied the method to investigate the formation of thromboxane B_2 and 12-hydroxyheptadecatrienoic acid (HHT) (products of cyclooxygenase and thromboxane A_2 synthase), 12-hydroxyeicosatetraenoic acid (12-HETE, a 12-lipoxygenase product) and arachidonic acid (AA, product of phospholipase A_2) formed during the in vitro aggregation of human platelets induced by collagen. A correlation between the inhibition of aggregation by aspirin and thromboxane/HHT formation was observed. All four compounds can be chromatographed in a single run. We employed prostaglandin B_1 (PGB_1) as internal reference standard to quantify the products. The method is useful to investigate selectivity of drugs which may affect either or all of these enzyme pathways at the same time.

Keywords: Enzymes; Phospholipase; Lipoxygenase; Cycloxygenase

1. Introduction

High-performance liquid chromatography (HPLC) coupled with simple quantitative derivatization procedures for sensitive detection (fluorescence), affords a rapid procedure for high sample throughput analyses. The ADAM esterification of carboxylic acids ranging from fatty acids [1] through to the diverse products in the eicosanoid pathway has been reported [2–4]. We previously demonstrated that ADAM esterification is even useful in the rapid extractive derivatization and stabilization of the unstable hepoxilins for subsequent analysis by HPLC [5,6]. In our experience, this method proved often superior to

*Corresponding author. Programme in Integrative Biology, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. Tel.: +1-416-813-5755; fax: +1-416-813-5086.

E-mail address: pace@sickkids.on.ca (C.R. Pace-Asciak).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00363-2

GC–MS in ease and simplicity of operation especially when thermally unstable compounds as the hepoxilins are involved [7]. One of the troublesome technical problems that we often observed with the ADAM derivatization was that many spurious peaks appeared which interfered with sample analysis, peaks that were derived from the reagent itself. To overcome this and to avoid tedious purification of samples, we attempted to 'move' the desired compounds to regions in the chromatogram that were essentially devoid of these reagent interferences. One approach was to make the desired compounds less polar. Since the compounds of interest possess one to several hydroxyl groups, a simple and convenient way was to convert the compounds into acetates. In this report we describe results of such a two-step procedure to resolve eicosanoids as diverse as thromboxane B_2, HHT, 12-HETE and AA from a given sample. This method is useful for the analysis of 'crude' extracts. We also describe the usefulness of this method to the analysis of eicosanoids released from platelets during aggregation with collagen and inhibition of only thromboxane/HHT by aspirin.

2. Experimental

2.1. Chemicals

Authentic standards of arachidonic acid, 12-HETE, HHT, prostaglandin B_1 and thromboxane B_2 were purchased from Cayman (Ann Arbor, MI, USA). ADAM reagent was from Research Organics (Cleveland, OH, USA). All solvents were reagent grade and distilled in glass (Caledon Laboratories, Georgetown, Canada). Human platelets were prepared essentially as previously described [8].

2.2. Sample extraction

Platelet suspensions in which aggregation was monitored for 6 min at 37°C in a platelet aggregometer (PAP-4C) were transferred to test tubes, PGB_1 internal standard (100 ng) was added. The samples were extracted with ethyl acetate (3×2 ml) after sample acidification to pH 3. The ethyl acetate extracts were combined, washed twice with 0.5 ml water to eliminate traces of acid, and the samples were taken to complete dryness with a gentle stream of N_2 gas in a well-ventilated fume hood. Half of the sample was taken for analysis.

2.3. Derivatization and detection

Authentic compounds (1 μg) or biological samples (half of the extracts derived from incubations of $350 \cdot 10^6$ platelets) were dissolved in 0.2 ml ethyl acetate containing ADAM reagent (20 μg/sample), and the samples were left at 23°C for 2 h in the dark [5,6]. The solvent was removed with N_2 gas and the residue was dissolved in pyridine–acetic anhydride (100:40, v/v) for 16 h at 23°C. The reagents were evaporated with N_2 gas and the residue was dissolved in 100 μl acetonitrile–water (80:20, v/v) and injected onto the HPLC column.

2.4. HPLC

A Hewlett-Packard HPLC 1100 series was used with solvent program capabilities and with an on-line Shimadzu fluorescent detector (RF-10AXL). The excitation wavelength was set at 364 nm, emission at 411 nm. A reversed-phase column (NovaPak C_{18}, 300×3.9 mm, Waters, Toronto, Canada) was used. The running solvent (1.5 ml/min) at injection was acetonitrile–water (80:20, v/v) maintained for 10 min, followed by a linear gradient to 100% acetonitrile during 20 min, where it was maintained for another 10 min until the end of the run. At the end of each run, the column was washed at 2.5 ml/min for 10 min with 100% acetonitrile, then the running solvent was recycled back to starting solvent for the analysis of the next sample.

2.5. Confirmation of derivatization by mass spectrometry

Authentic compounds (5 μg) were converted into the ADAM/acetate derivatives and purified by HPLC. Each compound was then subjected to structural confirmation by positive ion electrospray MS (Sciex API III Plus triple quadrupole MS) with direct injection of the samples (1 μg each) in 1 μl acetonitrile–1 m*M* NH_4OAc (4:1, v/v) into the ion source.

2.6. Statistical analysis

Values derived from aspirin treatment were compared to corresponding values in the absence of aspirin. Analysis was carried out with a Macintosh STATVIEW statistical software and are reported as mean±SD from three separate experiments.

3. Results and discussion

ADAM esterification of eicosanoids has worked well both in sensitivity as well as in ease of operation as the level of compounds requiring analysis was in the high picogram–low nanogram range. In fact we and others used it to monitor the generation of 12-HETE and the hepoxilins formed in skin of rats after addition intradermally of bradykinin [9] or in human psoriatic and normal skin [10,11]. In the present study we needed to investigate the eicosanoid profile formed during the collagen-induced aggregation of platelets. Preliminary studies indicated that the compounds of interest especially thromboxane B_2 had to be slowed down as they overlapped significantly with reagent peaks. Hence we investigated the use of acetylation to 'move' the compounds to a relatively clean portion of the chromatogram. In so doing, we established a good and workable separation between all the compounds of interest, i.e. thromboxane B_2, PGB_1, HHT (a product of the intermediate endoperoxide PGH_2), 12-HETE, and arachidonic acid. All compounds showed an $[M+NH_4]^+$ consistent with structure, i.e. m/z 704 for thromboxane B_2 (three acetates), m/z 586 for PGE_1 (one acetate), m/z 530 for HHT (one acetate), m/z 570 for 12-HETE (one acetate) and m/z 512 (no acetate) for AA (see Fig. 1). Samples were extracted and analyzed as described in Table 1. Fig. 2 illustrates an HPLC of the separation of authentic standards as ADAM esters (Fig. 2B) and ADAM esters and acetates (Fig. 2C). The reagent background showing reagent contaminants is shown in Fig. 2A. It is clear that the eicosanoids of interest are well separated from each other as acetates but not as the unacetylated compounds. The thromboxane peak is merged with the solvent front in Fig. 2B where all the reagent elutes. Additionally, the thromboxane peak is much sharper in the acetate form (Fig. 2C)

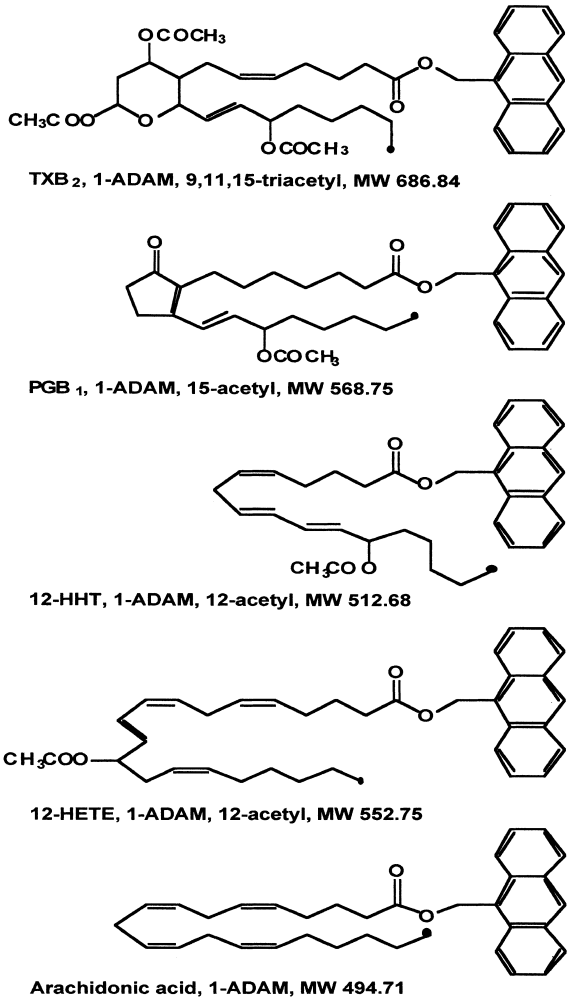

Fig. 1. Structures of products and derivatives detected in this paper. M_w was confirmed by direct injection positive ion electrospray MS as the $[M+NH_4]^+$ ion occurring as the base peak in the spectrum.

than previously described as the free hydroxyl form [12] making quantitation more feasible. Fig. 3A shows HPLC comparisons between samples derived from (a) control platelet suspension in the absence of any additions and in which no aggregation took place (see Fig. 3B, line a). Hence it will be seen that relative to the internal standard, PGB_1, no thromboxane B_2 or HHT or 12-HETE or arachidonic acid are observed. In contrast, considerable amounts of these eicosanoids are generated when platelet aggregation is evoked by the addition of 2 μg of collagen (Fig.

Table 1
Preparation of samples for analysis

1. Sample extraction
Sample derived from platelet incubation (0.5 ml)
Add 100 ng PGB_1 as internal standard
Extract with 4 vols of ethyl acetate after acidification to pH 3
Separate ethyl acetate phase and wash to neutrality with water (2×0.5 ml)
Separate ethyl acetate phase and take to dryness
Resuspend sample in 0.4 ml ethyl acetate and take 1/2 sample
2. Derivatization
ADAM esterification — add 20 μg ADAM reagent to sample in ethyl acetate — mix and leave 2 h in dark
Acetylation — take sample containing ADAM reagent to dryness and add 140 μl pyridine–acetic anhydride (100:40) — leave overnight at 23°C
Take sample to dryness and redissolve in acetonitrile–water (80:20, v/v)
3. Analysis
Inject into HPLC 1/10th of derivatized sample (see Experimental section for HPLC conditions)

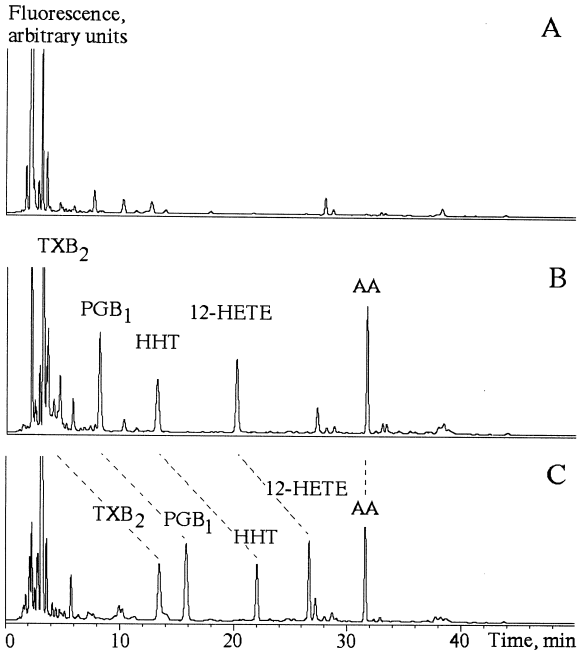

Fig. 2. HPLC profile of authentic standards of thromboxane B_2, PGB_1 internal standard, HHT, 12-HETE and arachidonic acid as the ADAM esters (B) before and (C) after acetylation; (A) reagent alone. A 1 μg amount of each compound as a mixture was derivatized and 1/10th of the sample (100 ng) was analyzed as described in Experimental. Note the absence of change in retention time for arachidonic acid as it does not acetylate, while all the other compounds elute later after acetylation. Note the migration of thromboxane B_2 within the early minutes of the run mixed with reagent peaks when unacetylated (B), but is 'moved backwards' after acetylation.

3A, panel b, and the corresponding line b in Fig. 3B). In panel c (Fig. 3A) and line c (Fig. 3B) is shown the effect of aspirin (1 μg) added to the platelet suspension 2 min prior to the addition of collagen on the formation of the eicosanoids (section A) and the corresponding aggregation response (section B). Clearly, thromboxane B_2 and HHT are partially blocked, while 12-HETE and arachidonic acid are largely unaffected demonstrating the well known selectivity of aspirin at this low dose to inhibit prostaglandin H synthase through acetylation. Panel d in Fig. 3A shows the greater blockade of thromboxane and HHT by 5 μg of aspirin. A positive correlation between the inhibition of formation of thromboxane B_2 and HHT with the inhibition of platelet aggregation by aspirin (line d, Fig. 3B) can be seen. Quantitation of the thromboxane B_2 and HHT peaks using PGB_1 as internal standard under the various conditions afforded the results in ng/$350 \cdot 10^6$ platelets shown in Table 2. The corresponding results for inhibition of platelet aggregation by aspirin are shown in Table 3.

These experiments illustrate the usefulness of the method described employing two simple and quantitative derivatization procedures, i.e. ADAM esterification and acetylation, to analyze a series of eicosanoids by HPLC and to correlate both ex vivo platelet aggregation with eicosanoid production. Thromboxane A_2 is a well known powerful prothrombotic mediator [13–15] and vasoconstrictor of smooth muscle [16], implicated in a variety of

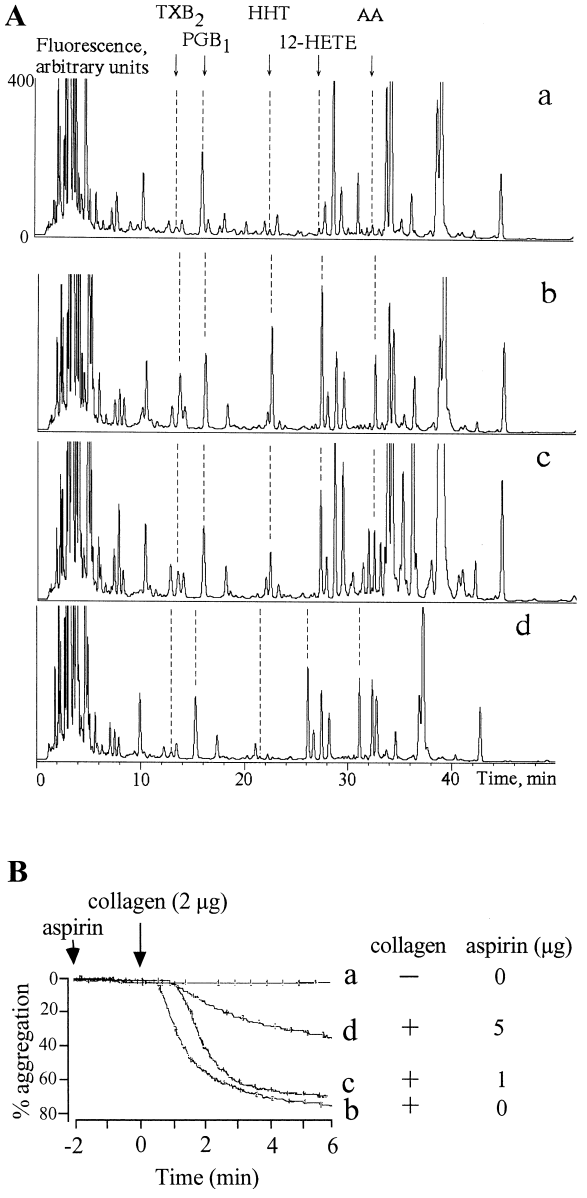

Fig. 3. Comparison of the HPLC elution profiles (A) of crude extracts of platelets ($350 \cdot 10^6$ cells) derivatized as the ADAM ester acetates with the corresponding platelet aggregation charts (B). In both sections (a) represents control platelets (no additions), (b) represents platelets treated with collagen (2 μg) for 6 min, and (c) and (d) platelets pretreated for 2 min with aspirin (1 or 5 μg, respectively), then with collagen for 6 min. Note the correlation between thromboxane and HHT formation (A) with aggregation (B) for panel b (collagen treated) or its inhibition panels c and d (collagen + aspirin). PGB_1 (100 ng) was added as internal standard prior to sample extraction.

Table 2
Quantitation of various eicosanoids in platelet suspensions under various conditions of aggregation

Compound measured	Collagen (2 μg) Aspirin (μg)		
	0	1	5
TxB_2	105.0±10.4	38.0±0.75[a]	13.5±4.0[a]
HHT	96.7±11.8	42.3±6.7[a]	5.1±2.2[a]
12-HETE	106.7±22.6	79.4±17.5[b1]	99.0±9.7[b2]
AA	53.7±3.4	56.3±22.6[c1]	85.8±10.7[c2]

P values compared to 0 aspirin; $n=3$, mean±SD.
[a] $P=0.0005$.
[b1] $0.05<P<0.1$
[b2] $0.1<P<0.375$
[c1] $P>0.4$.
[c2] $0.01<P<0.025$.

Table 3
Inhibition of collagen-evoked (2 μg collagen) aggregation by aspirin

	Aspirin (μg)		
	0	1	5
Aggregation (%)	74.0±2.6	71.3±3.6[d1]	44.7±7.4[d2]

P values compared to 0 aspirin; $n=3$, mean±SD.
[d1] $0.1<P<0.375$.
[d2] $0.0005<P<0.005$.

syndromes including septic shock [17–19]. The method described herein can be highly effective in the screening of a variety of drugs as inhibitors of thromboxane synthesis with potential application to thromboxane-mediated syndromes. The method also provides an insight into the mechanism involved in the blockade of aggregation by monitoring the phospholipase A_2 step (i.e. arachidonic acid), PGHS step (i.e. thromboxane and HHT) or the thromboxane synthase step (i.e. thromboxane B_2). The method is simple and suitable for expansion to the detection of other prostaglandins and related substances. Because of the relatively inexpensive equipment needed, this method can be easily set up in most laboratories.

Acknowledgements

This study was supported by a grant (MT-4181) to CRP-A from the CIHR.

References

[1] S.A. Barker, J.A. Monti, S.T. Christian, F. Benington, R.D. Morin, Anal. Biochem. 107 (1980) 116.
[2] K. Yamaki, S. Oh-Ishi, Chem. Pharm. Bull. (Tokyo) 34 (1986) 3526.
[3] M. Kurosawa, Y. Igarashi, H. Kobayashi, S. Konayashi, S. Abe, Ann. Alerg. 64 (1990) 464.
[4] M. Kurosawa, H. Kobayashi, S. Kobayashi, J. Asthma 27 (1990) 349.
[5] P. Demin, D. Reynaud, C.R. Pace-Asciak, Anal. Biochem. 226 (1995) 252.
[6] P. Demin, D. Reynaud, C.R. Pace-Asciak, J. Chromatogr. B 672 (1995) 282.
[7] C.R. Pace-Asciak, S.P. Lee, J.M. Martin, Biochem. Biophys. Res. Commun. 147 (1987) 881.
[8] C.R. Pace-Asciak, S. Hahn, E.P. Diamandis, G. Soleas, D.M. Goldberg, Clin. Chim. Acta 235 (1995) 207.
[9] M.M. Wang, D. Reynaud, C.R. Pace-Asciak, Biochim. Biophys. Acta Lipids Lipid Metab. 1436 (1999) 354.
[10] R. Antón, L. Vila, J. Invest. Dermatol. 114 (2000) 554.
[11] R. Antón, L. Puig, T. Esgleyes, T.M. De Moragas, L. Vila, J. Invest. Dermatol. 110 (1998) 303.
[12] W.R. Schafer, H.P. Zahradnik, E. Arbogast, B. Wetzka, K. Werner, M. Breckwoldt, Placenta 17 (1996) 231.
[13] M. Hamberg, B. Samuelsson, Proc. Natl. Acad. Sci. USA 71 (1974) 3400.
[14] M. Hamberg, J. Svensson, B. Samuelsson, Proc. Natl. Acad. Sci. USA 72 (1975) 2994.
[15] S. Hammarstrom, U. Diczfalusy, Adv. Prostaglandin Thromboxane Res. 6 (1980) 267.
[16] M. Hamberg, P. Hedqvist, K. Strandberg, J. Svensson, B. Samuelsson, Life Sci. 16 (1975) 451.
[17] M. Zaitsu, Y. Hamasaki, M. Matsuo, M. Miyazaki, R. Hayasaki, E. Muro, S. Yamamoto, I. Kobayashi, T. Ichimaru, S. Miyazaki, Eur. J. Haematol. 63 (1999) 94.
[18] S.M. Heidemann, A.P. Sarnaik, Prostaglandins, Leukotrienes and Essential Fatty Acids 56 (1997) 473.
[19] U. Sibelius, U. Grandel, M. Buerke, D. Mueller, L. Kiss, H.J. Kraemer, R. Braun-Dullaeus, W. Haberbosch, W. Seeger, F. Grimminger, Circulation 101 (2000) 78.