Communications to the Editor

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9-ANTHRYLDIAZOMETHANE-HPLC METHOD FOR DETECTION OF PROSTAGLANDINS AND THROMBOXANE: AN APPLICATION TO THE MEASUREMENT OF THE PRODUCTS OF STIMULATED RABBIT PLATELETS

Kohji Yamaki and Sachiko Oh-ishi*

Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minatoku, Tokyo 108, Japan

The cyclooxygenase products of rabbit platelets stimulated with arachidonic acid were measured by HPLC. Prostaglandins and thromboxane were extracted, derivatized with 9-anthryldiazomethane, and then loaded on an ODS-column. The fluorescence of the eluate was monitored. Thromboxane B_2 was a major product, which returned to the control level in the presence of indomethacin or the thromboxane synthetase inhibitor OKY-046. Pretreatment with OKY-046 shifted the cyclooxygenase products to increased levels of PGE_2, PGD_2 and $PGF_{2\alpha}$.

KEYWORDS——TXB_2; PGE_2; PGD_2; 9-anthryldiazomethane; OKY-046; platelet aggregation; HPLC

Prostaglandins (PGs) and thromboxane (TX) in biological fluids have been assayed by radioimmunoassay with good sensitivity and selectivity. They also have been separated by high-performance liquid chromatography (HPLC), but the sensitivity of UV-monitoring is not sufficient to detect a trace amount of the metabolites in biological samples.[1] The fluorescent labeling of prostaglandins with 9-anthryldiazomethane (ADAM) was reported by Hatsumi et al,[2] but frequent noise levels interfered with the procedure. We previously reported an improved ADAM-method with a clean-up procedure and applied it to the products of isolated rat pleural cells stimulated with phorbol myristate acetate.[3] With this clean-up procedure, better resolution of the chromatogram was obtained. This method can be applied to various other biological samples. Here we report the application of this method to the measurement of the products of rabbit platelet-rich plasma (PRP) stimulated with arachidonic acid (AA).

Arachidonic acid (>99%, Nu Chek Prep, Inc., Elysian, Minn.) and indomethacin (Sigma) were purchased. OKY-046 ((E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride) was a gift from Kissei Pharmaceutical Co. PGE_2, 6-keto-$PGF_{1\alpha}$, PGD_2, $PGF_{2\alpha}$, TXB_2 and anti-TXB_2 antiserum were gifts from Ono Pharm. Co. Osaka. ADAM was prepared by Funakoshi Pharm Co. Tokyo. [5,6,8,11,12,14,15(n)-^{3}H]-PGE_2 (160 Ci/mmol, Amersham Int. Plc.) and [5,6,8,9,11,12,14,15-^{3}H(N)]-TXB_2 (100 Ci/mmol, New England Nuc., Boston), and anti-PGE_2 antiserum (Institut Pasteur

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Production, Paris) were purchased. Blood was collected from the carotid artery of male albino rabbits (Japanese white, 3-5 kg, Doken, Ibaragi), under light ether anesthesia, into a polypropylene tube containing 1/10 volume of 3.8% trisodium citrate solution. PRP was obtained by centrifugation at 280 x g for 20 min. The supernatant plasma was further centrifuged at 2,200 x g to yield platelet-poor plasma (PPP). The platelets were counted with a Sysmex platelet counter (PL-110, Toa Electric Co.). The number of PRP platelets was finally adjusted with PPP to 5×10^5 cells/μl.

Generation of cyclooxygenase metabolites: One hundred μl of Hanks' blanced salt solution containg 0.1% bovine serum albumin and 800 μl of PRP were taken into polypropyrene tube (Falcon #2306), and preincubated at 37°C for 2 min with gentle shaking in the presence or absence of 1 mM OKY-046 or indomethacin. AA was then added to the platelet suspensions to a final concentration of 0.3 mM, and further incubated for 10 min. The reaction was terminated by the addition of a chilled solution of indomethacin (0.1 mM) and the sample was processed for extraction.

Procedure for ADAM-HPLC method: PGs and TX were extracted by the previously reported[3] modified method of Powell.[4] ADAM-derivatization and the clean-up method were also reported previously.[3] Briefly, samples were extracted through Sep-pak C_{18} (Waters), evaporated, then dissolved in methanol. The solution was mixed with 0.2% ADAM solution in ethylacetate (1:1), and left overnight at room temperature. The reaction mixture was evaporated and cleaned up through Sep-pak silica cartridge (Waters). Finally ADAM-PGs were eluted with acetonitrile/methanol (4:1). The recoveries of TXB_2, $PGF_{2\alpha}$ and PGE_2 were 84.7±2.0% (n=4), 89.5±3.8% (n=4) and 92.7±1.3% (n=4) respectively. The HPLC instruments used were a TriRoter V (Jasco, Tokyo); Uvidec-V (Jasco) and RF-530 (Shimazu) detectors, Zorbax-ODS (250x4.6 mm, Dupont), ERC-ODS (30x6 mm, Erma) and Develosil-C_{18} (Nomura Chem., Seto) columns with particle size of 5 μm. The solvent, acetonitrile/water/phosphoric acid (60:40:0.1), flowed at 0.7 ml/min at 30°C. The fluorescence of the ADAM-derivatives were monitored at 412 nm with excitation at 365 nm. Radioimmunoassay (RIA) was performed as previously described.[1]

An HPLC chromatogram HPLC of ADAM derivatives of 5 ng of authentic 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2 and TXB_2 is shown in Fig. 1. TXB_2 and $PGF_{2\alpha}$ migrated at a similar retention time when ODS (Zorbax) was eluted with acetonitrile/phosphoric acid system. TXB_2 formed a rather broad peak, but other PGs were clearly separated. Figure 3 shows typical chromatograms of ADAM derivatives of the AA metabolites of rabbit PRP stimulated with 0.3 mM AA for 10 min. The major peaks

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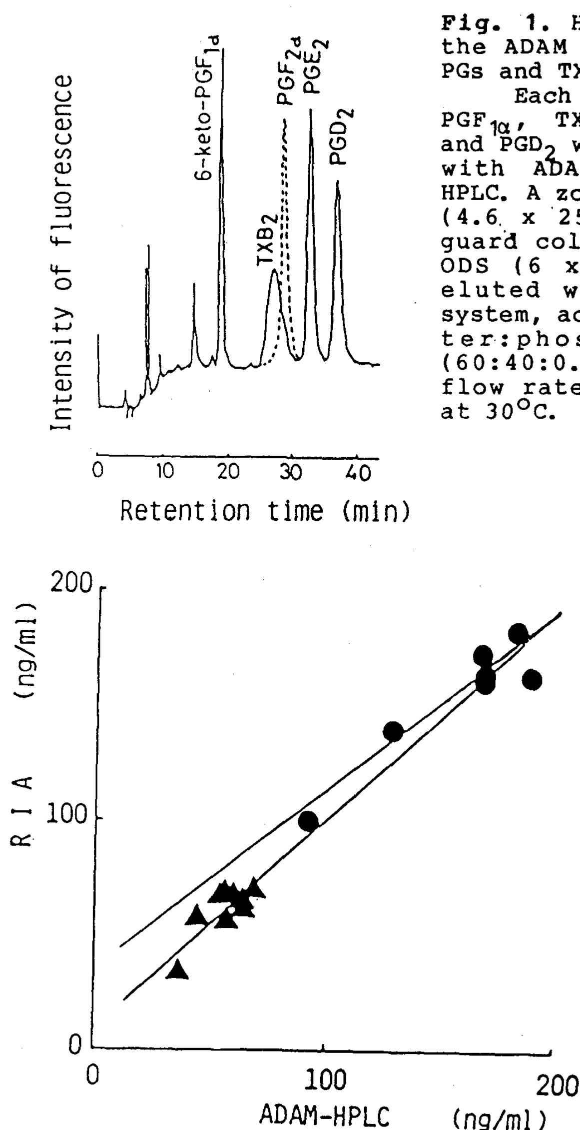

Fig. 1. HPLC Profile of the ADAM Derivatives of PGs and TXB_2

Each 5 ng of 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 were derivatized with ADAM and loaded on HPLC. A zorbax ODS column (4.6 x 250 mm) with a guard column, Deverosil ODS (6 x 30 mm), was eluted with a solvent system, acetonitrile:water:phosphoric acid (60:40:0.1,v/v/v) at a flow rate of 0.7 ml/min at 30°C.

Fig. 2. Relationship between the Estimates Obtained by ADAM-HPLC Method and Those by RIA

Values are expressed as ng/ml incubation mixture of rabbit PRP and AA (0.3 mM) with or without OKY-046 (1 mM). (●) TXB_2 and (▲) PGE_2. Correlation coefficients are 0.94 ($p<0.01$) and 0.80 ($p<0.01$) for TXB_2 and PGE_2, respectively.

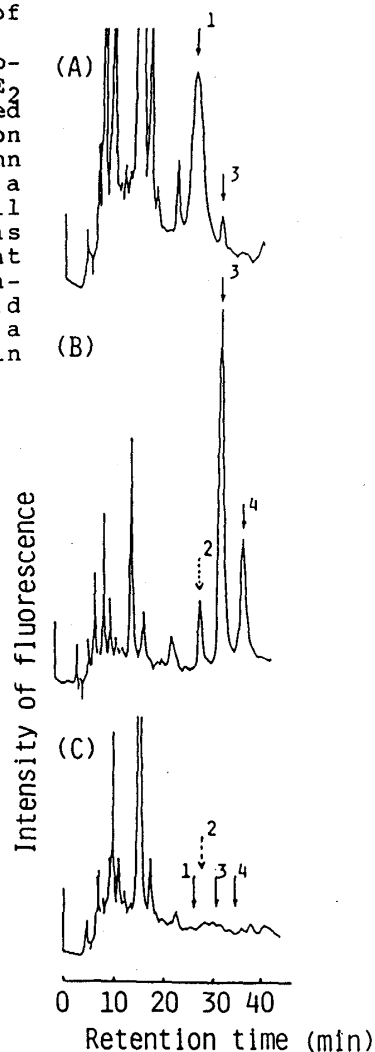

Fig. 3. Typical Chromatograms of ADAM-Derivatives of Arachidonic Acid Metabolites Produced by the Stimulated Rabbit Platelets

(A) Profile of the products of rabbit platelets stimulated with 0.3 mM arachidonic acid (control).
(B) Profile of the above incubation mixture in the presence of 1 mM OKY-046.
(C) Profile of the above incubation mixture in the presence of 1 mM indomethacin.
1;TXB_2, 2;$PGF_{2\alpha}$, 3;PGE_2, 4;PGD_2. Details are described in the text.

TABLE I. The Effect of Pretreatment with OKY-046 (1mM) on the Arachidonic Acid Metabolites of Rabbit Platelets (ng/ml)

	TXB_2	PGE_2	PGD_2	$PGF_{2\alpha}$
Control	161.8 ± 9.4	12.3 ± 0.6	<3	<3
OKY-046	<5	192.5 ± 14.4	76.6 ± 5.3	35.8 ± 2.4

Values are means and standard errors of the determinations by the ADAM-HPLC method (n=5).

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were TXB_2 and PGE_2. In the presence of 1 mM OKY-046, a TXA_2 synthetase inhibitor,[5] the TXB_2 peak disappeared (Fig. 3B). Instead the PGE_2 peak height increased to 192.5 ± 14.4 ng/ml (n=5) as shown in Table I. Simultaneously the PGD_2 peak (76.6 ± 5.3 ng/ml, n=5) appeared, but there was no detectable 6-keto-$PGF_{1\alpha}$ peak. Platelet aggregation was monitored with a light-transmission detector (Rikadenki, Tokyo) simultaneously with the experiment of Fig. 3. OKY-046 and indomethacin completely inhibited the AA-induced aggregation of rabbit PRP. The values of PGE_2 and TXB_2 in the incubation mixtures of rabbit PRP with AA (0.3 mM) were also measured by RIA and plotted against those obtained by ADAM-HPLC as shown in Fig. 2. The actual values of the samples used in Fig. 2 were: for TXB_2, 155.8±12.9 ng/ml (n=7, ADAM method) and 155.2±10.5 ng/ml (n=7, RIA), and for PGE_2, 56.2±3.0 ng/ml (n=10, ADAM method) and 61.1±3.4 ng/ml (n=10, RIA). For the PGE_2 assay the sample was incubated in the presence of OKY-046 (1 mM). By this HPLC the separation of the PGs and TX was demonstrated within 30 min. The detection limit is about 100 pg for standard PGs and TXB_2. Although the $PGF_{2\alpha}$ peak coeluted with the broad TXB_2 peak in this HPLC condition, fortunately significant amount of $PGF_{2\alpha}$ was not generated in the rabbit platelet aggregation, even when assayed with the solvent of the methanol/acetonitrile system, which was previously reported for the separation of the two metabolites.[3] The results coincid nicely with the reports of Smith et al[6] and Rajtar et al.[7] The former reference describes how in rabbit platelets, OKY-1581, a selective inhibitor of TX synthetase, inhibits the generation of TX and increases PGE_2 and $PGF_{2\alpha}$. The second paper reports that dazoxiben also inhibited TX synthesis and caused reorientation of cyclooxygenase products towards PGE_2, PGD_2 and $PGF_{2\alpha}$. As shown in Fig. 3C, the sample incubated with indomethacin (1 mM) did not yield a detectable amount of PGs and TXB_2 even when stimulated with 0.3 mM AA, further proving the validity of ADAM-HPLC method.

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