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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ARACHIDONIC ACID METABOLITES BY PRE-COLUMN DERIVATIZATION USING 9-ANTHRYLDIAZOMETHANE

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SUMMARY

Arachidonic acid (AA) metabolites produced by washed human platelets and rat macrophages were analyzed by high-performance liquid chromatography (HPLC) using a pre-column derivatization method. The reagent, 9-anthryldiazomethane, used in this study and AA metabolites derivatized by the reagent were purified by gel permeation chromatography (PG-pak C column), prior to normal-phase HPLC analysis. A sample containing eleven derivatives (12-, 15- and 5-hydroxyeicosatetraenoic acid, 12-L-heptadecatrienoic acid, leukotriene B_4, prostaglandins B_2, D_2, E_2 and $F_{2\alpha}$, thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$) was separated on a normal-phase column (PG-pak B); the detection limit is better than 100 pg for all components.

INTRODUCTION

It is well known that arachidonic acid (AA) metabolites play an important role in various patho-physiological processes such as thrombus formation[1,2], inflammatory reactions[3,4] and allergic reactions[5,6]. These metabolites have been reported to be produced from AA through the cyclooxygenase pathway and several lipoxygenase pathways.

AA metabolites, including hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), prostaglandins (PGs) and thromboxane (TX) can be quantitated by radioimmunoassay, gas chromatography–mass spectrometry and high-performance liquid chromatography (HPLC).

Nanogram levels of HETE and LT can be quantitated using HPLC with an ultraviolet (UV) detector. However, similar concentrations of PG and TX cannot be detected by this method, because they show no specific UV adsorption. It has been reported that the derivatization of PGs with a fluorogenic reagent, 9-anthryldiazomethane (ADAM), offered highly sensitive detection[7]. In order to perform success-

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fully the HPLC–derivatization method, there are two constraints: (1) the reagent for pre-column derivatization must be extremely pure and (2) surplus reagent and impurities must be removed from the derivatized AA metabolites because they often cause many unexpected chromatographic peaks that overlap the target peaks.

We have succeeded in purifying and separating AA metabolites derivatized with ADAM by employing gel permeation chromatography (GPC) for the purfication of the reagent and the elimination of the surplus reagent from a derivatized sample. We have applied this method to the analysis of AA metabolites produced by stimulated washed human platelets and stimulated rat peritoneal macrophages.

EXPERIMENTAL

Reagents

PGI_2-Na, PGD_2, 6-keto-$PGF_{1\alpha}$, PGE_2, PGE_1, $PGF_{2\alpha}$, $PGF_{1\alpha}$ and TXB_2 were kindly provided by Ono Pharmaceutical (Osaka, Japan). 5-HETE, 12-HETE, 15-HETE, LTB_4 and ADAM were purchased from Funakoshi Chemical (Tokyo, Japan). [1-^{14}C]AA (50 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Thrombin was purchased from Mochida Pharmaceutical (Tokyo, Japan). Calcium ionophore A23187 was purchased from Calbiochem Böhling (San Francisco, CA, U.S.A.). [1-^{14}C]AA metabolites were prepared by incubating washed human platelets with [1-^{14}C]AA and then purified by reversed-phase HPLC on Fine-Pak C_{18}-5 (25 cm × 8 mm I.D.). All solvents were of HPLC grade (Wako, Osaka, Japan).

Preparation of washed human platelets

Washed human platelets were prepared as reported previously[8]. Citrated venous blood containing PGI_2-Na (0.1 μg/ml) was obtained from healthy volunteers. Blood samples were centrifuged at 150 *g* for 10 min at room temperature to obtain platelet-rich plasma (PRP). PRP was centrifuged at 900 *g* for 10 min and platelet pellets were gently resuspended in Tyrode solution (pH 7.4) containing PGI_2-Na (0.1 μg/ml). This platelet suspension was centrifuged at 800 *g* for 10 min at room temperature. Platelet pellets were gently resuspended in Tyrode solution without PGI_2-Na. The platelet count of the washed platelet suspension was adjusted to $5 \cdot 10^5/\mu l$ and this solution was stored at 4°C until use.

Preparation of rat peritoneal macrophages

Rat peritoneal macrophages were prepared as reported previously[9]. Male Wistar rats weighing around 300 g were decapitated and the whole blood was withdrawn. A 50-ml volume of sterile saline was injected into the peritoneal cavity. Following the collection of peritoneal fluid after abdominal massage, peritoneal macrophages ($3 \cdot 10^6$–$6 \cdot 10^6$) were resuspended in 1 ml of Eagles minimum essential medium (E-MEM; Nissui Pharmaceutical, Tokyo, Japan) containing 0.5% foetal bovine serum (FBS; Nissui Pharmaceutical). The cells were seeded on the culture dish (Nunc, Roskilde, Denmark; 35 mm) and were incubated for 3 h under carbon dioxide–air (5:95, v/v). The dish was washed vigorously with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline of pH 7.5 [PBS(−)] to remove non-adherent cells.

Extraction of AA metabolites produced by washed human platelets and rat peritoneal macrophages

A 1-ml volume of washed human platelets ($5 \cdot 10^8$/ml) were incubated with 0.5 μCi of [^{14}C]AA in the presence of thrombin (1 unit/ml) at 37°C for 10 min. A 1-ml volume of macrophage suspension ($3 \cdot 10^6$/ml FBS) were incubated with 1 μCi of [^{14}C]AA and 1 μM A23187 in the presence of 1 mM calcium chloride at 35°C for 10 min under carbon dioxide–oxygen (5:95, v/v). The incubation was terminated by the addition of 2 volumes of ethanol. AA metabolites in each incubation mixture were extracted using ODS silica mini-columns (Bond-elut C_{18}) as described previously[10]. The mini-columns were finally eluted with methanol and the methanol fractions were evaporated to dryness under nitrogen, suspended in ethyl acetate and stored at −20°C until the reaction with ADAM was performed.

HPLC system

The GPC system for ADAM and ADAM-derivatized AA metabolites consisted of a pump (BIP-1; Jasco, Tokyo, Japan), a 500-μl syringe-loading sample injector (Model 7125; Rheodyne, Berkeley, CA, U.S.A.) and a UV detector (UVIDEC 100-V; Jasco).

The HPLC system for analysing ADAM-derivatized AA metabolites consisted of a pump (TRI-V; Jasco), a solvent programmer (GPA-40; Jasco), a column bath (TU-300; Jasco), a 100-μl syringe-loading sample injector (Model 7125) and a fluorimetric detector (FP-210; Jasco).

Columns

PG-pak B (normal-phase column) was used in the separation of ADAM-derivatized AA metabolites. PG-pak C (GPC column) was also used in the preparation of ADAM-derivatized AA metabolites and the purification of ADAM. Fine-pak C_{18}-5 was packed in stainless-steel columns (25 cm × 4.6 and 8.0 mm I.D.) and used in the separation and preparation of [1-^{14}C]AA metabolites and ADAM-derivatized [1-^{14}C]AA metabolites.

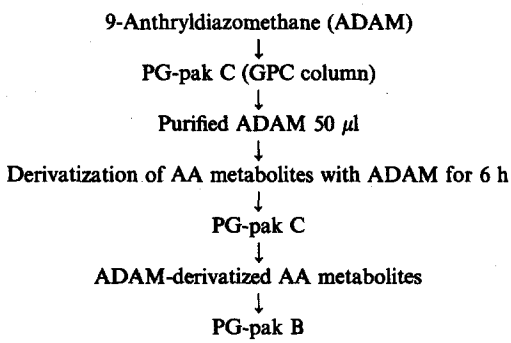
The packing materials for the PG-pak B and PG-pak C columns were kind of 5-μm silica gel and a 7-μm polystyrene gel, respectively.

Procedure

The analytical procedure is shown in Fig. 1.

Commercially available ADAM reagent contains various impurities and decomposed substances. These substances were resolved from ADAM itself by GPC using a PG-pak C column. Fig. 2 shows a chromatogram of ADAM and of ADAM-derivatized AA metabolites on PG-pak C using ethyl acetate as the eluent. The peak of ADAM appeared between 20 and 22 min and fractionated as purified ADAM. When 1 mg of ADAM was injected, the ADAM concentration of its fraction was *ca.* 500 μg/ml. AA metabolites were reacted with 50 μl of purified ADAM solution for 6 h (see *Conditions of ADAM derivatization*). The ADAM solution, once purified, is stable for only a few days at −20°C, so it has to be used almost immediately.

ADAM-derivatized AA metabolites were also purified using PG-pak C[7], and the peaks appeared between 11.5 and 15 min. Thus ADAM-derivatized AA metabolites were completely separated from surplus ADAM reagent (see Fig. 2). The frac-



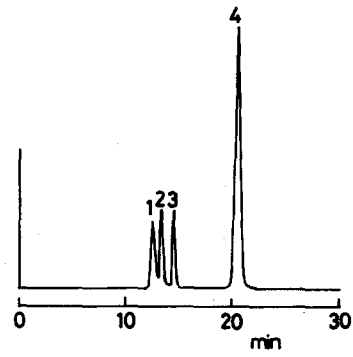


Fig. 1. Scheme of the analytical procedure.

Fig. 2. GPC of ADAM and ADAM-derivatized AA metabolites. Column, PG-pak C (50 cm × 0.72 cm I.D.); eluent, ethyl acetate; flow-rate, 1.0 ml/min; detection, UV (350 nm). Peaks: (1) ADAM-PGs and ADAM-TX; (2) ADAM-HETES; (3) ADAM-HHT; (4) ADAM.

tions between 11.5 and 15 min were collected and evaporated to dryness under a stream of nitrogen and dissolved in 100 μl of methylene chloride for injection on to the PG-pak B column. Fig. 3 shows the chromatogram of ADAM-derivatized standards of PGD_2, 6-keto-PGF_1, TXB_2, PGE_2 and PGF_2 (500 pg each as PGs and TXB_2) on PG-pak B using isooctane–ethyl acetate–ethanol–acetic acid (80:15:4:2) as the mobile phase. The detection limits are *ca.* 100 pg for all the components. The reproducibility will be reported elsewhere.

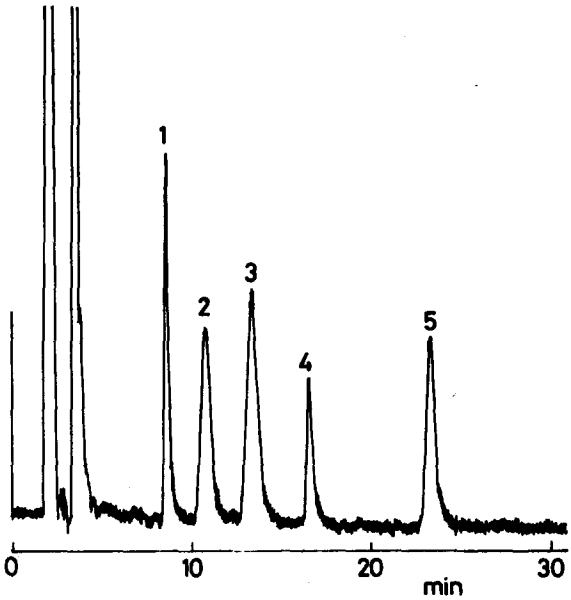


Fig. 3. High-sensitivity chromatogram of ADAM-derivatized standards of AA metabolites. Column, PG-pak B (25 cm × 0.46 cm I.D.); eluent, isooctane–ethyl acetate–ethanol–acetic acid (80:15:4:2, v/v); flow-rate, 1.2 ml/min; detection, fluorometric (exitation 365 nm, emission 412 nm); range × 10, × 1. Peaks: (1) ADAM-PGD_2; (2) ADAM-6-keto-$PGF_{1\alpha}$; (3) ADAM-TXB_2; (4) ADAM-PGE_2; (5) ADAM-$PGF_{2\alpha}$.

RESULTS AND DISCUSSION

Conditions of ADAM derivatization

It is well known that TXB_2, 12-HETE and 12-L-heptadecatrienoic acid (HHT) are produced by aggregated platelets. These three compounds were detected and measured by using the ADAM derivatization method.

When 10, 20 or 40 μl of Adam solutions purified by GPC were incubated with [1-^{14}C]-12-HETE (0.1 μCi/μl), the yields of ADAM derivatization yields were determined by measurement of the radioactivity appearing in ADAM-derivatized [1-^{14}C]-12-HETE after separation on a Fine-pak C_{18}-5 column (25 cm × 4.6 mm I.D.) using acetonitrile–water–acetic acid (90:10:0.1) as the mobile phase. The yield of ADAM-derivatized [1-^{14}C]-12-HETE using 40 μl of pure ADAM solution reached 90% after incubation for 4 h. Adding 50 μl of ADAM solutions to [1-^{14}C]TXB_2, [1-^{14}C]-12-HETE and [1-^{14}C]HHT, the reaction yields were also measured using the same method. The yields of ADAM-derivatized [1-^{14}C]TXB_2 and [1-^{14}C]-12-HETE reached over 90% after incubation for 4 h, whereas [1-^{14}C]HHT required incubation for 6 h to achieve the same yields as those of [1-^{14}C]TXB_2 and [1-^{14}C]-12-HETE. We therefore decided that the optimal incubation time and concentration of ADAM solution were 6 h and 50 μl, respectively.

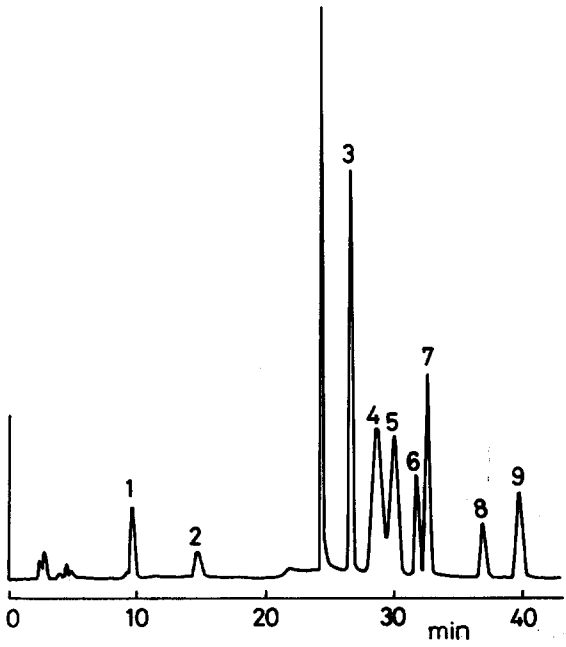


Fig. 4. Chromatogram of ADAM-derivatized standards of TXB_2, 12-HETE and HHT using stepwise elution. Eluents, A = isooctane–ethyl acetate–acetic acid (90:10:1, v/v), B = isooctane–ethyl acetate–ethanol–acetic acid (80:15:4:2, v/v). After pumping eluent A for 15 min, eluent B was pumped for 20 min by stepwise elution. Other conditions as in Fig. 3. Peaks: (1) ADAM-12-HETE; (2) ADAM-HHT; (3) ADAM-PGD_2; (4) ADAM-6-keto-$PGF_{1\alpha}$; (5) ADAM-TXB_2; (6) ADAM-PGE_2; (7) ADAM-PGE_1; (8) ADAM-$PGF_{2\alpha}$; (9) ADAM-$PGF_{1\alpha}$.

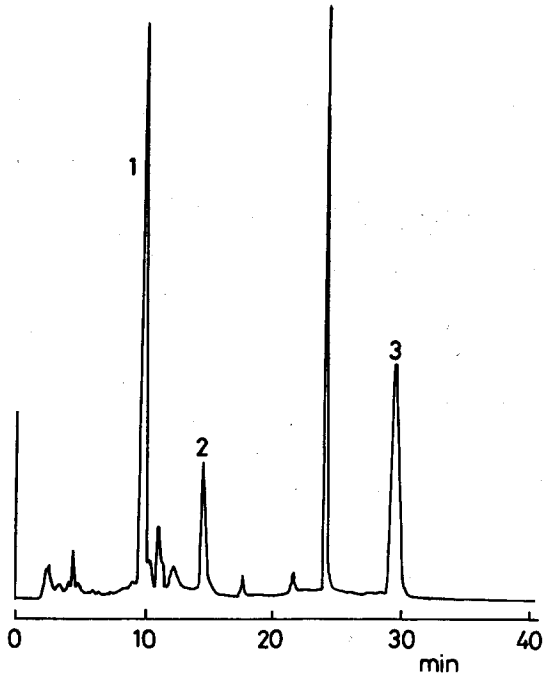


Fig. 5. Chromatogram of ADAM-derivatized AA metabolites produced by washed human platelets. Conditions as in Fig. 4. Peaks: (1) ADAM-12-HETE; (2) ADAM-HHT; (3) ADAM-TXB_2.

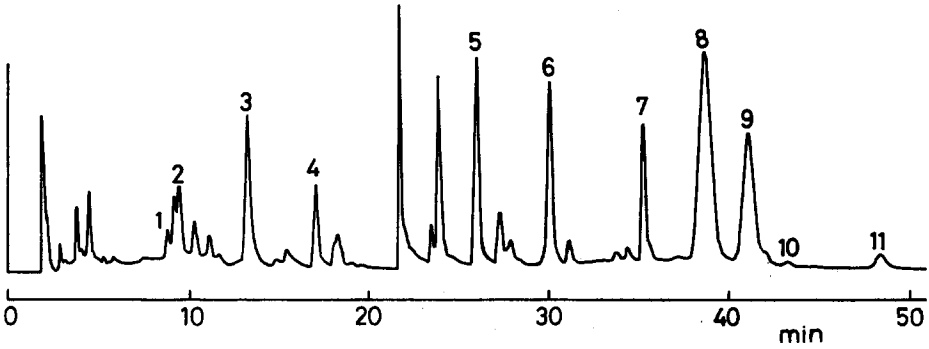


Fig. 6. Chromatogram of ADAM-derivatized AA metabolites produced in stimulated rat peritoneal macrophages. Eluents: A = isooctane–ethyl acetate–acetic acid (93:7:1, v/v), B = isooctane–ethyl acetate–ethanol–acetic acid (80:15:4:2, v/v). Three stepwise elutions as follows: eluent A for 12 min, then eluent A–B (1:1) for 15 min and eluent B for 20 min. Other conditions as in Fig. 4. Peaks: (1) ADAM-12-HETE; (2) ADAM-15-HETE; (3) ADAM-HHT; (4) ADAM-5-HETE; (5) ADAM-PGB_2 (IS); (6) ADAM-LTB_4; (7) ADAM-PGD_2; (8) ADAM-6-keto-$PGF_{1\alpha}$; (9) ADAM-TXB_2; (10) PGE_2; (11) ADAM-$PGF_{2\alpha}$.

Analysis of AA metabolites produced by washed human platelets and rat peritoneal macrophages

Fig. 4 shows the chromatogram of ADAM-derivatized standards of AA metabolites on PG-pak B using stepwise elution. Fig. 5* shows the chromatogram of the ADAM-derivatized AA metabolites produced by washed human platelets. ADAM-derivatized TXB_2, 12-HETE and HHT were well separated and clearly detected on the chromatogram.

Fig. 6* shows the chromatogram of ADAM-derivatized AA metabolites produced in rat peritoneal macrophages on PG-pak B using three stepwise elutions. In this analysis, we succeeded in the separation and detection of ADAM-derivatized 12-HETE, 15-HETE, 5-HETE, HHT, LTB_4, PGB_2, PGD_2, 6-keto-$PGF_{1\alpha}$, TXB_2, PGE_2 and $PGF_{2\alpha}$.

The identification of ADAM-derivatized AA metabolites produced by washed human platelets and rat peritoneal macrophages was based on a comparison with the retention times the standard compounds. As we did not have standard samples of the two *trans* isomers of LTB_4, and 5*S*,12*S*-di-HETE, we do not know where these peaks appear in the chromatogram.

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* We used a sample of 225 μl of $5 \cdot 10^8$/ml washed human platelet and 1 ml of $3 \cdot 10^6$/ml rat macrophages, respectively. A quarter of these amounts of derivatized samples were used in these analyses. Hence the minimum numbers of each kind of cell are about $5 \cdot 10^6$ platelets and $5 \cdot 10^5$ rat macrophages.