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Multiplex quantitative analysis of eicosanoid mediators in human plasma and serum: Possible introduction into clinical testing

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ABSTRACT

Eicosanoid mediators play important roles in maintaining the physiological and pathophysiological homeostasis in the body. Their measurements, however, are rarely performed in clinical practice. In the present study, we analyzed 30 varieties of eicosanoid mediators that were detectable in human plasma and serum collected from healthy donors, using liquid chromatography-tandem mass spectrometry from the viewpoint of the clinical application of the multiplex quantitation of eicosanoid mediators. Wider variety of eicosanoid mediators were detected in serum (27 out of 30) than in plasma (14 out of 30), since the serum was thought to contain lipid mediators released from activated platelets. Larger inter-individual variations were observed in the plasma and serum eicosanoid levels. On the other hand, the concentrations of eicosanoids were not affected by the platelet count but were affected by the concentration of arachidonic acid (AA) within the reference interval (17.4–40.5 × 10^{10}/L). When serum samples from patients with hematological disorders were analyzed, the concentrations of AA were positively correlated with the platelet count. When the patients underwent ASA therapy, a marked decrease in the concentrations of thromboxane B_2 (TXB_2) and 12-hydroxyl-heptadecatrienoic acid (12-HHT) was observed. Considering the availability of serum samples in clinical settings, the serum analysis of eicosanoids may be clinically useful.

1. Introduction

Eicosanoid mediators are bioactive lipids derived from 20-carbon arachidonic acid (AA) and are classified as thromboxane (TX), prostaglandins (PGs), leukotrienes (LTs), and hydroxy-eicosatetraenoic acid (HETE); they also include 17-carbon 12-hydroxy-heptadecatrienoic acid (12-HHT) [1]. AA, which is liberated from membrane phospholipids by PLA_2, is metabolized by the downstream enzymes (cyclooxygenase [COX], cytochrome P450 [CYP], and lipoxygenase [LOX]); TX and PGs are produced by COX, and LTs are produced by LOX. These mediators play central roles in maintaining the physiological and pathophysiological homeostasis of inflammation and work in each tissue as an autacoid [2]. As recognized well, eicosanoid mediators have been targets for the discovery of new medications to control the biological activities of these mediators [3–5].

Since the excessively elevated or defective production of eicosanoid mediators is reported to be associated with the onset or the progression of diseases, the importance of measuring the concentrations of eicosanoid mediators for differential diagnoses and as markers of treatment effects has been understood [6–12]. Unfortunately, however, the measurement of eicosanoid mediators is rarely performed in clinical practice. Blood samples, especially plasma samples, are typically used for such measurements since eicosanoids are released from tissues or blood cells into the blood; however, the concentrations of eicosanoids in plasma do not reflect them in tissues accurately, since eicosanoid mediators work as an autacoid at considerably low concentrations and are rapidly metabolized [13]. Also, almost all PGs are known to be rapidly metabolized through the pulmonary circulation, since a large amount of PG dehydrogenase inactivates PGs in the lung [14]. Eicosanoid mediators such as TX and 12-HETE are also produced in large

Abbreviations: ASA, aspirin; AA, arachidonic acid; CID, collision induced dissociation; COX, cyclooxygenase; CYP, cytochrome P450; DiHDoHE, dihydroxy-docosahexaenoic acid; DiHOME, dihydroxy-octadecenoic acid; DHA, docosahexaenoic acid; DHET, dihydroxyeicosatrienoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; EpETE, epoxy eicosatetraenoic acid; EpOME, epoxy-octadecenoic acid; ET, essential thrombocytosis; HpODE, hydroperoxy-octadecadienoic acid; 12-HHT, 12-hydroxyl-heptadecatrienoic acid; HETE, hydroxy-eicosatetraenoic acid; ITP, immune thrombocytopenia; LT, leukotriene; LXB_4, lipoxin B_4; LOX, lipoxygenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MPNs, myeloproliferative neoplasms; NSAIDs, non-steroid anti-inflammatory drugs; OEA, oleoylethanolamide; PAF, platelet-activating factor; PG, prostaglandin; PGEM, prostaglandin E metabolite; PLA_2, phospholipase A_2; SRM, selected reaction monitoring; TX, thromboxane

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amounts once blood has clotted [15], so it can be difficult to prove whether the detected eicosanoids were derived *in vivo* or *in vitro*, even if the plasma or serum samples are analyzed quantitatively. Therefore, great care is needed when evaluating such results, since the resulting concentrations can vary considerably depending on how the samples were obtained, handled, and measured.

Plasma is the supernatant of blood containing an anticoagulant, like a calcium chelator (such as ethylenediaminetetraacetic acid [EDTA] or sodium citrate), and can be analyzed in the absence of blood clotting [16]. Plasma is suitable for analyzing the dynamics of eicosanoid mediators *in vivo*. Indeed, numerous clinically significant results have been reported using plasma [17,18], even though the accurate sampling of plasma samples is extremely difficult in clinical practice. The concentration of eicosanoid mediators in serum differs from that *in vivo*, however, yielding useful information. Therefore, considering the availability of serum samples, the analysis of eicosanoid mediators in serum may be promising for clinical testing [19–21].

The function of each eicosanoid mediator has typically been discussed individually, and the concentrations of such mediators are usually analyzed quantitatively using an enzyme-linked immunosorbent assay (ELISA) [22]. Although ELISA is characterized by a simple pretreatment, high sensitivity, high repeatability and simultaneous measurement of multiple samples for a kind of eicosanoid mediators, it is not suitable for the multiplexed analysis of eicosanoid mediators [23]. Various physiological functions are based on the balance of multiple eicosanoid mediators *in vivo*. Recently, liquid chromatography-tandem mass spectrometry (LC–MS/MS) has been reported to be useful as a highly sensitive quantitation method for multiple eicosanoid mediators [24–27] and is expected to be employed in clinical practice.

To consider the application of the multiplex quantitation of eicosanoid mediators in serum using LC–MS/MS, we conducted a basic study by measuring and analyzing the concentrations of eicosanoids in plasma and serum samples from healthy adult donors using LC–MS/MS.

2. Materials and methods

2.1. Chemicals

All lipid reference compounds including 16 deuterium-labeled internal standards were obtained from Cayman Chemical (Ann Arbor, MI). LC/MS-grade acetonitrile, high-performance liquid chromatography (HPLC)-grade formic acid, HPLC-grade methanol, ethanol, petroleum ether, NaCl, KCl, $MgCl_2{\cdot}6H_2O$, and glucose were obtained from WAKO (Osaka, Japan). Fibrinogen, HPLC-grade prostaglandin I_2 (PGI_2) sodium salt, and fatty acid free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). $CaCl_2$ was obtained from Ishidu (Osaka, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Nacalai Tesque (Kyoto, Japan). $NaH_2PO_4{\cdot}H_2O$ was obtained from Merck (Darmstadt, Germany). Thrombin receptor activator peptide-6 (TRAP-6) amide trifluoroacetate salt (referred to as "TRAP" in this report) was obtained from BACHEM (Bubendorf, Switzerland). Acid-citrate-dextrose (ACD) solution was obtained from TERUMO (Tokyo, Japan). Ultrapure water was prepared using the milli-Q system (Millipore, Billerica, MA).

2.2. Washed platelets preparation

Washed platelets (WPs) were purified using a modified method as previously described [28]. Briefly, written informed consent was obtained from the subjects, and blood was then collected from healthy donors via a 21-guage needle into 4.5-mL vacuum plasma separator tubes with 0.5 mL of 3.2% sodium citrate, Venoject II (TERUMO, Tokyo, Japan), and centrifuged (90*g*, 15 min, room temperature [RT]). The platelets were washed twice in a modified Tyrode's buffer (138 mM NaCl, 2.9 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 0.1% glucose, 0.1% BSA, pH7.4) containing 15% ACD and 100 nM PGI_2. WPs were resuspended at 1–100 $\times$ 10^{10}/L in Tyrode's buffer with 1 mM $CaCl_2$. Two microliters of fibrinogen (final concentration [f.c.], 500 μg/mL) was added just prior to stimulation. Twenty microliters of TRAP solution (f.c., 1.0 μM) was added to 180 μL of WPs solution as described above, and the mixture was incubated with stirring at 37° C for 1 h. Finally, 1 mL of methanol was added, and the samples were stored at −80 °C before use.

2.3. Healthy adult subjects

Twenty-seven subjects (12 men and 15 women) participated in this study after providing written informed consent, as described in detail in Supplementary Table 1. The subjects were healthy and never-smoker adult donors who were not taking any medicines or supplements and did not have any diseases. We did not obtain further personal information, such as body weight, height, or individual dietary information other than their age and sex. This study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, the University of Tokyo.

2.4. Sample preparation

After the subjects had fasted, blood was collected into 8-mL vacuum serum separator tubes containing a certain amount of silica and thrombin (Insepack II-D SQ3; SEKISUI, Tokyo, Japan), 4.5-mL plasma tubes containing 0.5 mL of 3.2% sodium citrate, and 2.5-mL vacuum plasma separator tubes containing 2.5 mg of EDTA-2K (NIPRO, Osaka, Japan). The blood cell counts were measured using EDTA-2K samples. Serum samples were immediately inverted 10 times, left standing for 5 min at RT, and then centrifuged (2300 $\times$ *g*, 5 min, RT). The citrated plasma samples were immediately inverted 10 times and centrifuged (1700 $\times$ *g*, 15 min, 4 °C), and both the plasma and serum fractions were stored at −80 °C before use.

2.5. Clinical samples

The concentrations of eicosanoids in serum were also measured using residual blood samples remaining after the completion of routine laboratory analyses. Patients with non-hematological disorders, those who had taken non-steroid anti-inflammatory drugs (NSAIDs) or aspirin (ASA), and those who were diagnosed as having immune thrombocytopenia (ITP), myeloproliferative neoplasms (MPNs), or essential thrombocytosis (ET) were enrolled after obtaining informed consent. Only eicosanoids that are strongly associated with platelets (TXB_2, 12-HHT, 12-HETE, and AA) were analyzed.

2.6. Eicosanoid extraction procedure

An internal standard method was used for quantification. A mixture of 16 internal standards containing 0.2 μg/mL each of tetranor-prostaglandin E metabolite-d6 (tetranor-PGEM-d6), TXB_2-d4, PGE_2-d4, PGD_2-d4, LTC_4-d5, LTB_4-d4, 15(S)-HETE-d8, $PGF_{2\alpha}$-d4, platelet-activating factor C16-d4 (PAF C16-d4), 6-keto-$PGF_{1\alpha}$-d4, 5(S)-HETE-d8, and 12(S)-HETE-d8, 0.1 μg/mL of oleoylethanolamide-d4 (OEA-d4), and 4 μg/mL each of eicosapentaenoic acid-d5 (EPA-d5), docosahexaenoic acid-d5 (DHA-d5) and arachidonic acid-d8 (AA-d8) was prepared. Firstly, solid-phase extraction was performed as described previously [24,27,29,30]. Two hundred microliters of each sample type (serum, plasma, and WPs) was diluted with 1 mL of methanol, and 10 μL of the internal standard mixture was added to each sample. The samples were then mixed and centrifuged (20,000 $\times$ *g*, 10 min, 4° C), and the supernatants were diluted with 3 mL of 0.03% formic acid in water.

The solid phase extraction of eicosanoids was performed using Oasis HLB 1-mL (10 mg) extraction cartridges (Waters) set on a vacuum