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Short communication

Simultaneous quantification of PGI_2 and TXA_2 metabolites in plasma and urine in NO-deficient mice by a novel UHPLC/MS/MS method

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ABSTRACT

The balance between vascular prostacyclin (PGI_2) generated mainly *via* cyclooxygenase-2 (COX-2) and its physiological antagonist platelet-derived thromboxane A_2 (TXA_2) formed by cyclooxygenase-1 (COX-1) determines cardiovascular homeostasis. In the present work, a novel bioanalytical method for simultaneous quantification of stable plasma and urinary metabolites of PGI_2 (6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$) and TXA_2 (TXB_2, 2,3-dinor-TXB_2) using ultra high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC/MS/MS) was developed. The method was validated using artificial plasma and urine and linearity range, intra- and inter-day precision and accuracy, recovery of analytes, relative and absolute matrix effect and stability of analytes were determined. The use of artificial biofluids improved the method sensitivity as it eliminated the contribution of endogenous metabolites present in mice plasma and urine to validation procedure. The newly developed and validated method allowed to quantify 6-keto-$PGF_{1\alpha}$ and TXB_2 in mice plasma as well as 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 in urine samples with high sensitivity and accuracy. The calibration range was established from 0.1 to 100 ng/mL for all analytes using artificial biofluids and the recoveries were greater than 89.9%. All validated parameters met the criteria of acceptance specified in FDA and EMA guidance. This method was successfully employed for profiling of the changes in PGI_2 and TXA_2 generation in NO-deficient mice. This work demonstrated that NO-deficiency induced by L-NAME, evidenced by a fall in nitrite in plasma and urine, was associated with platelet activation, robust increase in TXB_2 and mild increase in 6-keto-$PGF_{1\alpha}$ concentration in plasma. Changes in 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 concentration in urine were less evident suggesting that the measurements in plasma better reflect modest changes in PGI_2/TXA_2 homeostasis than measurements in urine.

1. Introduction

Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) synthesized from arachidonic acid are physiological antagonists exerting their biological actions *via* membrane PGI receptor (IP) and TX receptor (TP), respectively. Arachidonic acid liberated from phospholipids e.g. by phospholipase A_2 (PLA_2), is metabolized by cyclooxygenases (COX-1 or COX-2) and then by prostacyclin synthase (PGIS), thromboxane A synthase (TXS), or other enzymes [1,2]. Although PGI_2 and TXA_2 released by diverse types of cells have been implicated in a variety of biological processes, platelet-derived TXA_2 and endothelium-derived PGI_2 play an important role in the regulation of cardiovascular homeostasis.

Endothelium-derived PGI_2 is a vasodilator, inhibitor of platelet activity, a potent vasoprotective agent maintaining vascular wall

Abbreviations: PGI_2, prostacyclin; TXA_2, thromboxane A_2; IP, PGI receptor; TP, TX receptor; PLA_2, phospholipase A_2; PGIS, prostacyclin synthase; TXS, thromboxane A synthase; UHPLC/MS/MS, ultra high-performance liquid chromatography coupled to mass spectrometry; TXB_2, thromboxane B_2; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; IP-KO, IP receptor knockout mice; NO, nitric oxide; ACN, acetonitrile; EtOAc, ethyl acetate; L-NAME, Nω-nitro-L-arginine methyl ester; AA, acetic acid; FA, formic acid; EtOH, ethanol; IS, internal standard; UFLC, ultra-fast liquid chromatography; MRM, multiple reaction monitoring; FDA, Food and Drug Administration; EMA, European Medicines Agency; CE, collision energy; DP, declustering potential; EP, entrance potential; CXP, collision cell exit potential; LLOQ, lower limit of quantification.

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integrity and thromboresistance, whereas TXA_2 causes vasoconstriction, platelets aggregation and contributes to vascular inflammation [1,3]. There are number of reports supporting that alterations in the balance of PGI_2/TXA_2 contribute to the development and progression of endothelial dysfunction and atherosclerosis [4,5], systemic and pulmonary hypertension [2], renal dysfunction [6] as well as diabetes [2,7]. Indeed, deficiency of PGI_2 in endothelium may lead to the excessive stimulation of TP receptors in endothelium and vascular smooth muscle cells by TXA_2 resulting in subsequent vasoconstriction and vascular inflammation [8]. On the other hand, when biosynthesis of TXA_2 by platelets increases, endothelium accommodate to this situation by a concomitant increase in PGI_2 production to maintain homeostatic balance. For example a parallel increase in biosynthesis of platelet-derived TXA_2 and vascular PGI_2 were observed in two experimental mice models of atherosclerosis [9]. Studies with genetically modified mice have clearly demonstrated consequences of the lost equilibrium in homeostatic regulation between PGI_2 and TXA_2. In IP receptor knockout mice (IP-KO), smooth muscle proliferative response to wire-induced vascular injury was augmented, along with a concomitant rise in TXA_2 biosynthesis [5]. When TP receptors were additionally deleted in these mice, proliferation of smooth muscle cells was abolished, suggesting that the vascular proliferative response in this model was mediated by the unopposed action of TXA_2 [5]. Importantly, altered PGI_2/TXA_2 ratio is frequently associated with the impairment of endothelial NO production, but the influence of NO on the PGI_2/TXA_2 balance was less thoroughly studied.

In biological fluids both PGI_2 and TXA_2 are unstable, act locally in an autocrine and paracrine manner, and their production can be assessed by the measurement of their stable metabolites such as 6-keto-$PGF_{1\alpha}$ and TXB_2 in plasma or 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 in urine [10].

In this work, a new, fast, sensitive, reliable and accurate UHPLC/MS/MS method for simultaneous quantification of 6-keto-$PGF_{1\alpha}$ and TXB_2 using artificial plasma and 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 applying artificial urine was developed and validated. Using this method, alterations in PGI_2 and TXA_2 biosynthesis in plasma and urine induced by L-NAME treatment in mice in relation to changes in NO metabolites in plasma and urine and platelet activation assessed by whole blood *ex vivo* assay were analysed.

2. Experimental

2.1. Chemicals and materials

All standard substances, salts and organic solvents used in this study were described in *Supplementary Data.*

2.2. UHPLC/MS/MS conditions

The quantitative analysis of 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, TXB_2, 2,3-dinor-TXB_2 was performed using UFLC Nexera system (Shimadzu, Kyoto, Japan) coupled to the triple quadrupole mass spectrometer QTrap 5500 (ABSciex, Framingham, Massachusetts, USA) equipped with Turbo V^{TM} ion source. The best separation of analytes was achieved applying Acquity UPLC BEH C18 (3.0 × 100 mm, 1.7 μm, Waters, Milford, Massachusetts, USA) analytical column. The mobile phases consisted of pure ACN (A) and 0.1% NH_4OH in water (v/v) (B) were delivered in gradient elution at the flow rate of 250 μL/min. Gradient elution was as follows: 85% B for 0.5 min, 85%-70% B from 0.5 to 2.0 min, 70%-30% B from 2.0 to 5.0 min, hold 30% B from 5.0 to 5.5 min, 30%-85% B from 5.5 to 6.0 min and 2.0 min for column equilibration at 85% B. The total time of analysis was 8.0 min, including 2.0 min of column equilibration. The sample injection volume was 5 μL. The data acquisition was carried out in multiple reaction monitoring mode (MRM) in the negative electrospray ion mode for all analytes and deuterated internal standards. The ion transitions selected for eicosanoids quantification as well as collision energy (CE), declustering potential (DP), entrance potential (EP) and collision cell exit potential (CXP) were listed in **Supplemental Table S1**. The operating parameters for mass spectrometer were as follows: spray voltage: −4000 V, source temperature: 500 °C, curtain gas: 25 psi, ion source gas 1: 40 psi, ion source gas 2: 50 psi.

2.3. Preparation of artificial biological fluids, standards solutions and samples

Preparation of artificial biological fluids, standards solutions and samples was described in *Supplementary Data.*

2.4. Method validation

The developed method for simultaneous quantification of 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, TXB_2 and 2,3-dinor-TXB_2 was validated consistent with FDA and EMA guidelines for bioanalytical methods validation [11,12] including the following parameters: linearity range, intra- and inter-day precision and accuracy, recovery of analytes, relative and absolute matrix effect and stability of analytes. The linearity range, accuracy and precision of the method as well as recovery of analytes were estimated using artificial plasma for 6-keto-$PGF_{1\alpha}$ and TXB_2 or artificial urine for 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, whereas matrix effects and stability of analytes after one freeze-thaw cycle were assessed utilizing mice plasma and urine. The lower limit of quantification (LLOQ) for validated method was estimated at 0.1 ng/mL for all analytes. Application of artificial biofluids allowed to eliminate the influence of endogenous concentration of analytes on validation procedures. Moreover, this novel approach allowed to achieve satisfactory results of method validation and application to mice samples.

2.5. Method application

The method was used to quantify stable metabolites of PGI_2 and TXA_2 in plasma and urine in NO-deficient mice. 16-week-old female C57Bl/6 mice purchased from Center of Experimental Medicine Medical University of Bialystok (Bialystok, Poland) were housed in colony cages (groups of 6/cage) under light/dark cycle in room with controlled temperature and humidity conditions receiving intact AIN–93 G diet and tap water *ad libitum* throughout experimental period (2 weeks).

Animals were divided into following groups (n = 6): control group fed with AIN–93 G diet and L-NAME group received L-NAME in tap water or diet at a daily dose of 100 mg/kg b.w for two weeks. After 2 weeks mice were sacrificed under anesthesia condition using ketamine (100 mg/kg) and xylazine (10 mg/kg) *via* intraperitoneal injection. Blood was taken from right ventricle using syringe contained heparin as anticoagulant and equipped with plastic tip. After blood centrifugation (664*xg*, 4 °C, 12 min) plasma was collected and stored at −80 °C for further analysis.

To collect urine specimens, mice were individually placed in specially-designed metabolic cages (Tecniplast). Twenty-four-hour urine samples were collected from control group and mice treated with L-NAME (for 2 weeks). Urine was clarified by centrifugation (10000 × *g*, 10 min) and stored at −80 °C for further measurements.