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A Fast One Step Extraction and UPLC-MS/MS Analysis for E_2/D_2 Series Prostaglandins and Isoprostanes

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Abstract

Prostaglandins (PG) and isoprostanes (iso-PG) may be derived through cyclooxygenase or free radical pathways and are important signaling molecules that are also robust biomarkers of oxidative stress. Their quantification is important for understanding many biological processes where PG, iso-PG, or oxidative stress are involved. One of the common methods for PG and iso-PG quantifications is LC-MS/MS that allows a highly selective, sensitive, simultaneous analysis for prostanoids without derivatization. However, the currently used LC-MS/MS methods require a multi-step extraction and a long (within an hour) LC separation to achieve simultaneous separation and analysis of the major iso-PG. The developed and validated for brain tissue analysis one-step extraction protocol and UPLC-MS/MS method significantly increases the recovery of the PG extraction up to 95%, and allows for a much faster (within 4 min) major iso-PGE_2 and -PGD_2 separation with 5 times narrower chromatographic peaks as compared to previously used methods. In addition, it decreases the time and cost of analysis due to one-step extraction approach performed in disposable centrifuge tubes. All together, this significantly increases the sensitivity, and the time and cost efficiency of the PG and iso-PG analysis.

Keywords

Prostaglandin; isoprostane; brain lipids; prostanoid analysis; ischemia; microwave irradiation; ultra high performance liquid chromatography; tandem mass spectrometry

INTRODUCTION

Prostaglandins (PG) are critically important signaling molecules derived from polyunsaturated fatty acids. In the brain, PG regulate a variety of normal processes such as synaptic plasiticity due to modulation of adrenergic, noradrenergic, glutamatergic neurotransmission and membrane excitability [1–4], and are critical for instantaneous regulation of cerebral blood flow in response on changes in neuronal activity [5–7]. PG also play a critical role in many pathophysiological conditions including inflammation, cancer, neurodegeneration, neuropsychiatric conditions, and central nervous system injury [8–15]. Inhibitors of PG biosynthesis are widely used as treatment for pain, fever, and inflammation indicating the importance of PG in these physiological processes.

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PG and isoprostanes (iso-PG) are produced either enzymatically or non-enzymatically. The enzymatic pathway is COX 1/2 dependent while the non-enzymatic pathway uses a free radical non-enzymatic mechanism for polyunsaturated fatty acid oxidation esterified on phospholipids [16–20]. There are four positional isoforms of isoPG. PGE_2 and PGD_2 which are COX1/2 derived belong to the 15- series. The other isoforms are the 5-, 8-, and 12-series [21]. Iso-PGE_2 and -D_2 have four chiral centers which result in 16 chiral isoforms within each series. The most thermodynamically stable of the 15-series PG are PGE_2, PGE_2 enantiomer (ent-PGE_2), 8iso-PGE_2, 11β-PGE_2, 15(R)-PGE_2, 5trans-PGE_2, PGD_2, and Δ12-PGD_2 all of which are abundant in tissues [17; 22–24]. 15-series PG are not only stable and robust oxidative stress biomarkers [25; 26], but might also possess biological activity [24; 27]. Upon tissue stimulation, platelet activating factor acetylhydrolase II might release esterified PGE_2 and iso-PG from phospholipids [28; 29]. This rapid increase in PG upon initial stimulation has numerous downstream effects [30–32] which may play a critical role in later phases of tissue response. Because PG and iso-PG not only play critical roles in normal and pathophysiological process, but also are biomarkers for oxidative stress, their quantification is important in understanding these processes.

Several methods have been developed for quantification of esterified iso-PGE_2 and –PGD_2 in tissues including enzyme immunoassay, radioimmunoassay, high performance liquid chromatography (HPLC) with UV or fluorescence detectors, and gas chromatography with MS or flame ionization detection. The most specific and least laborious technique is liquid chromatography with tandem mass spectrometer detection (LC-MS/MS) [33; 34]. While HPLC-MS/MS has been used to separate iso-PGE_2 species [17; 23; 33; 35–41], ultra performance liquid chromatography-MS/MS offers increased resolution and sensitivity with a shorter run time. However, UPLC-MS/MS has only been utilized for the separation of isoPGF_2 species [42–44] not for the separation of iso-PGE_2 and/or -PGD_2.

In order to quantify tissue PG levels by LC-MS/MS, the PG must first be extracted from the tissue. There are a variety of methods used for PG extraction including hexane/2-propanol [45], ether [46], acetone/chloroform [33; 47], and methanol followed by solid-phase extraction [35; 48; 49]. These techniques require multiple sample transfers and large solvent volumes which decrease extraction efficiency and MS sensitivity due to sample loss and increased chemical background [33]. In addition, they are expensive in terms of both time and supplies.

To address these limitations, we developed a one-step methanol extraction protocol followed by quantification by UPLC-MS/MS. This method allows for the simultaneous analysis of all the major PGE_2/D_2 and iso-PGE_2 and -PGD_2 species with an increased extraction efficiency and sensitivity while reducing both time and cost of the analysis.

MATERIALS AND METHODS

Chemicals

9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGE_2), 9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid (entPGE_2), 9-oxo-11β,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (11β-PGE_2), 9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid (8-*iso*PGE_2), 9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic-3,3,4,4-d_4 acid (PGE_2-d_4), 9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (PGD_2), 9α,15R-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (15(R)-PGD_2), 9α,15S-dihydroxy-11-oxo-prosta-5Z,12E-dien-1-oic acid (Δ12PGD_2), 9-oxo-11α,15R-dihydroxy-prosta-5Z,13E-dien-1-oic acid (15(R)-PGE_2), 9-oxo-11α,15S-dihydroxy-prosta-5E,13E-dien-1-oic acid (5trans-PGE_2) were purchase from Cayman Chemical Co.

(Ann Arbor, MI). All solvents used for LC were Optima grade from Fischer Scientific (Fair Lawn, NJ).

Animals

All animal use was approved by the University of North Dakota IACUC (Protocol #0903-1 and #08061) and is in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals 8th Addition. Male CD-1 mice were provided food and water *ad libitum.* The ages of mice used in this study were between 3–4 months.

Tissue Prostaglandin Extraction and Sample Preparation for Mass Spectrometry

To measure basal PG levels, mice were anesthetized with isofluorane (3%) and euthanized by head focused microwave irradiation (3 kW for 1.5 s) to heat denature enzymes to prevent post-mortem PG formation [33; 36]. To model brain global ischemia, mouse brains were analyzed 5 min after decapitation [33; 50–52]. Proteins were heat denatured in a boiling water bath for 5 min prior to analysis to prevent further post-mortem PG production. The brains were pulverized into a powder under liquid nitrogen temperatures.

Acetone extraction of brain tissue was performed as described previously [33; 36; 53]. Briefly, approximately 10 mg tissue was homogenized in a Tenbroeck tissue grinder containing 1 mL saline, 2 mL acetone. PGE_2-d_4 (1ng) was used as an internal standard. The homogenate was transferred to silanized with Sigmacote reagent (Sigma-Aldrich, St. Louis, MO, USA) glass tubes and centrifuged at 2000xg for 10 min. The supernatant was washed 3 times with 2 mL hexane, acidified to pH 3.5 with 30 μL 2M formic acid, and extracted with 2 mL chloroform. The chloroform layer was cooled at −80 °C for 15 min to separate any remaining upper phase which was removed after the samples were allowed to warm to room temperature.

To perform the Bligh and Dyer extraction [54], approximately 10 mg of tissue was homogenized Tenbroeck tissue grinder containing 1 ng PGE_2-d_4 in 190μL saline, 250 μL chloroform, and 500 μL methanol give a one-phase system. The homogenate was transferred to silanized glass tubes and centrifuged at 2000xg for 10 min. The supernatant was separated into two phases by the addition of 250 μL chloroform and 250 μL saline. The samples were vortexed and centrifuged at 2000xg for 10 minutes. The chloroform phase was collected.

For both Bligh and Dyer and acetone extracts, the chloroform phase was evaporated under nitrogen and transferred to silanized microinserts (Agilent, Santa Clara, CA USA) using two rinses of 150 μL chloroform with 10% methanol. The solvent in the microinserts was evaporated under nitrogen and re-dissolved in 100 μL methanol.

The methanol extract was performed by weighing approximately 10 mg of tissue into 90 μl methanol containing 1 ng PGE_2-d_4 in a disposable microcentrifuge tube. Higher tissue mass might be used with increased methanol volume while tissue to methanol ratio is maintained at 1 to 9. Lower methanol volume (up to 50% tested) resulted in the same extraction efficiency (93±6%, n=3) but the analysis variability (relative standard deviation) was gradually increased up to 20±5% at 50% methanol. For cell culture media or plasma extraction, the ratio might be decreased to 1 to 7.5 without altering variability that was at the 5.1±0.1% level and dropped to 21±5% at 50% methanol. The sample was sonicated 2 cycles, 7 sec each with power output of 50J (Model 150 Sonic Dismembrator, Fisher Scientific), vortexed for 5 minutes, and centrifuged at 10,000xg for 15 minutes at 4 °C. The supernatant was transferred to silanized microinserts. The samples were placed at −80 °C for at least 10 min to precipitate additional proteins. If additional precipitate was formed after warming the samples, they were centrifuged at 1000xg for 10 minutes and the supernatant

was transferred to new microinserts. If an increase in sensitivity was needed, the samples were concentrated by drying under nitrogen and re-dissolved in a smaller volume of methanol.

UPLC iso-PG separation

The LC system consisted of a Waters ACQUITY UPLC pump with wellplate autosampler (Waters, Milford, MA). Samples were separated on an ACQUITY UPLC HSS T3 column (1.8 μM, 100 Å pore diameter, 2.1×150mm, Waters, Milford, MA) with an ACQUITY HSS T3 Vanguard precolumn (1.8 μM, 100 Å pore diameter, 2.1×5mm, Waters, Milford, MA). The column temperature was 55 °C. Ten microliters of extract was injected onto the column using an autosampler maintained at 8 °C.

The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) with a flow rate of 0.45 mL/min. Solvent B was initially held at 39% for 0.5 minutes, then increased to 40.5 % over the next 6.88 minutes, and increased to 98% over 0.2 minutes to flush the column. Seven minutes later solvent B was returned to the initial levels over 0.2 minutes and maintained for 2 minutes. The iso-PG separation was achieved during first 4 min.

Because the tissue methanol extracts contain a number of very hydrophobic lipids including cholesterol esters and triacylglycerols that are not eluted from the column with acetonitrile, we tested if the retained lipids might change PGE_2 retention time. The retention time was consistent (within 0.01 min) after about 200 brain samples analyzed. However, to increase the column life we recommend flashing the column with 90–95% 2-propanol in acetonitrile for 30–40 min at 0.3 ml/min flow rate followed by equilibration with acetonitrile/water for 20–30 min at the end of analysis or after 30–40 samples analyzed.

Mass Spectrometry and Method Validation

To quantify PG, a quadrupole time-of-flight mass spectrometer (Q-TOF, Synapt G2-S, Waters, Milford, MA) with electrospray ionization ion source was used. MassLynx V4.1 software (Waters) was used for instrument control, acquisition, and sample analysis. The source was operated in negative ion mode with an cone voltage of 20V. The capillary voltage was 1.51 kV. The source temperature was 110 °C and the desolvation temperature was 350 °C. The analyzer was operated at 10,000 resolution (fwhm at m/z 554) with extended dynamic range and an acquisition time of 0.1 s. Data was acquired in MS^E mode where the transfer T-Wave element was alternated between low energy (2V) and high energy states where the voltage applied to the transfer T-wave element was from 10–25V [55]. The cone and desolvation gas flow rates were 10 L/h and 1000 L/h, respectively with the nebulizer gas at 6 Bar. The lock spray for mass correction was leucine enkephalin (400 pg/μL, ACN:water, 50:50) infused at a rate of 10 μL/min. iso-PGE_2 were monitored in the high energy channel (MS/MS channel) at 189.1279 m/z with a mass window of 0.02 that is the most specific product ion for 15 series iso-PGE_2 [36], and in the low energy channel (MS channel) at 351.2171. The internal standard PGE_2-d_4 was also monitored in the high energy channel at 275.2391 as the most abundant and specific product ion, and in the low energy channel 355.2391.

The PG were quantified using a stable isotope dilution approach against PGE_2-d_4 internal standard [36; 53; 56]. The standard curves were generated using a constant concentration of PGE_2-d_4 (100pg on column) and variable concentrations of PGE_2 and PGD_2 (1 pg to 10 ng on column). The slopes of the generated standard curves were similar to the previously observed values [36; 53; 56].

The limit of quantification (LOQ) was defined as the concentration which gives a S/N of 9. The dynamic range was determined as the range of analyte concentration resulted in a linear response ($r^2 > 0.95$).

The matrix effect was determined by spiking PGE_2 into a methanol extract of microwaved tissue and the response was compared to PGE_2 alone. In these experiments, approximately 30 mg of tissue was weighed into 270 μL methanol and extracted as described above. Prior to injection on column, 90 μL of the extract was spiked with either 1 ng PGE_2 in 10 μL methanol or with 10 μL methanol alone to correct for endogenous PGE_2. One nanogram of PGE_2 in 100 μL methanol was analyzed as a separate sample.

iso-PG Determination

Because most of iso-PG are found esterified onto phospholipids [17; 27; 57–59], we released iso-PG from phospholipids using $sPLA_2$ [36]. Approximately 100 mg of fixed with microwave irradiation brain tissue were sonicated in 100 μL buffer (80 mM HEPES, pH 7.4, with 300 mM sodium chloride, 20 mM $CaCl_2$, 8 mM Triton X-100, 60% glycerol, and 2 mg/mL BSA) with 10 ng PGE_2-d_4 as internal standard. The samples were incubated in the presence of sPLA2 (0.9 μmol/min total activity, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Following incubation, 900 μL methanol was added and the samples were extracted as described above.

Statistics

Statistical comparisons were performed using a two-way, unpaired Student's t-test using a GraphPad Prism 5 software (GraphPad, San Diego, CA). Statistical significance was defined as <0.05. All values are expressed as mean ± SD.

RESULTS AND DISCUSSION

iso-PG separation by UPLC

Previously, we have developed an HPLC method for the major isobaric iso-PGE_2 separation [36]. However, the drawback for this method is a long 1 h separation time and wide chromatographic peaks that decreases signal to noise ratio when quantified using MS, thus limiting the sensitivity and increasing time of the analysis. To improve the run time and peak shape, we developed a new UPLC method for the major isobaric iso-PGE_2 separation that allows for a fast 4 min separation with 5 times sharper chromatographic peaks (Fig. 1). Although UPLC approach has been applied for PG analysis in the past [42–44; 60], to the best of our knowledge, it has not been used for iso-PGE_2 and -PGD_2 separation.

A number of reverse phase UPLC columns have been tested including ACQUITY (Waters, Milford, MA, USA) BEH HILIC (1.7 μm, 2.1×100 mm), BEH C_{18} (1.7 μm, 2.1×150 mm), CSH C_{18} (1.7 μm, 2.1×150 mm), HSS C_{18} (1.8 μm, 2.1×150 mm), and HSS T_3 (1.8 μm, 2.1×150 mm) with acidified methanol/water, methanol/2-propanol/water, and acetonitrile/water gradients (data not shown). The best separation was achieved using HSS T_3 column with acetonitrile/water acidified with 0.1% formic acid gradient that allowed for the separation of all commercially available PGE_2- and PGD_2 like iso-PG, as well as brain endogenous iso-PG with the exception for 8iso-PGE_2/15R-PGE_2 and PGE_2/ent-PGE_2, (Fig. 1). The achieved separation was similar to the previously reported method [36], however it is 15 times shorter and results in 5 times sharper chromatographic peaks. This improvement increases the signal to noise ratio, thus allowing for a lesser sample volume, or less sensitive but higher resolution and wider mass range MS detectors to be used for analysis.

Tissue extraction and sample preparation

Next, we aimed to improve a previously reported extraction and sample preparation protocol [33; 36; 61] to increase extraction recovery, reduce chemical background noise, and decrease preparation time. Although a number of PG extraction and purification protocols are currently used including methanol extraction followed by solid-phase extraction [35; 48; 49; 62], extraction with diethyl ether [46], hexane/2-propanol [45], and acetone followed by chloroform/methanol [33; 36; 47; 51; 63], the best results were achieved using a liquid/liquid extraction with acetone/chloroform when evaluated for LC-MS/MS analysis [33]. We modified our previously reported liquid/liquid extraction protocol [33; 36] by substituting acetone/chloroform with methanol that is widely used for eicosanoid extraction [35; 49; 62], using a smaller volume for extraction solvents (90 uL of methanol for 10 mg of tissue samples), and replacing tissue homogenization in glass homogenizer with sonication in disposable centrifuge tubes as described in the Methods section.

Extraction efficiency—Previously, we have demonstrated that acetone/chloroform extraction protocol provides superior performance over liquid extraction followed by solid phase purification or over hexane/2-propanol extraction when used for MS analysis [33]. We compared our new extraction protocol to the acetone/chloroform method. In addition, we compared it to the chloroform Bligh and Dyer extraction method [54] which has not been previously compared to other methods for MS analysis. The modified single-step methanol extraction resulted in a much higher (96.7±9.9%) recovery of internal standard (Table 1). These results are consistent with high (within 90%) recovery of other single step extraction protocols [64] and at least 20% higher as compared to multiple-step extraction approaches (Table 1 and [33]). The improved extraction efficiency might be the result of elimination of analyte loss with transfer/evaporation steps used in the previous protocols.

To validate the extraction efficiency of endogenous prostanoids, we compared quantification results for the endogenous PGE_2 and PGD_2 levels in ischemic brain tissue using new and previously validated extraction methods (Table 2). PG were quantified against deuterium labeled internal standard as described in the Methods section. To model brain global ischemia, mouse brains were analyzed 5 min after decapitation [33; 50–52]. Proteins were heat denatured in boiling water bath for 5 min before analysis to prevent further post-mortem PG production. The results were not different between all three methods tested, indicating that the single-step methanol extraction method allows for the same quantitative results as compared to other commonly used methods, and the efficiency of endogenous PG and internal standard extraction was the same for all methods tested.

To further validate the methanol single-step extraction method, we tested the effect of sonication time on PG extraction efficiency. The results presented in the Table 3 indicate that one sonication cycle (7 sec) was sufficient to extract brain tissue PG.

Because we did not concentrate the tissue extract, we injected only 10% (10 μL out of 100 μL of extract that corresponds to 1mg of tissue) of the extracted sample sufficient for ischemic brain sample analysis using Q-TOF MS. The higher injected volume resulted in altered chromatographic peak shape (data not shown). This might potentially limit the method use when lower PG concentrations are present in the sample. Thus, we validated the sample concentration protocol that would allow to up to 90% of sample injection. The samples spiked with internal standard were dried under a nitrogen stream in silanized microinserts and re-dissolved in 11 μL of 90% methanol as described in the Methods section. The precipitate formed was removed by centrifugation. This protocol allowed for 4.1 ± 0.3 fold signal to noise ratio increase with consistent endogenous PG levels calculated as compared to non-concentrated samples (Table 4). The PG signal levels might be further increased by increasing the sample mass extracted followed by concentration protocol.

Matrix Effect, Variability, LOQ, Dynamic Range—The elimination of purification steps from the extraction protocol and shortening the LC separation time might result in the co-elution of other molecules with PG from the matrix with increased complexity, thus resulting in the ion suppression also called a matrix effect. To validate the matrix effect, we spiked PGE_2 and PGE_2d_4 brain extract and compared the peak area of spiked standards versus standards alone as described in the Methods section. The peak area of spiked PGE_2 standard was corrected for the endogenous PGE_2 levels. The peak areas were not different between spiked versus non-spiked standards (Table 5), indicating a limited matrix effect of our method.

Importantly, the new extraction and sample preparation protocols resulted in a significant improvement of the signal to noise ratio (Table 1). The increased signal to noise ratio might be the result of improvement of a number of factors. This might include an increase in signal response because of a better recovery and 5 times sharper peaks, and lower chemical noise as a result of decreased number of steps and decreased reagent volume used for the sample preparation.

To further validate the method, we determined the intra- and inter-day variability by analyzing the same fixed brain tissue. The results were not different between different sets, and the relative standard deviation was between 5–7% for brain PG analyzed (Table 4). Importantly, the relative standard deviation was dramatically increased up to 45% when non-fixed brain tissue was analyzed (data not shown), indicating the necessity for brain tissue fixation by enzyme heat denaturing to prevent PG formation during tissue extraction and analysis [33].

The LOQ were within 1.0±0.5 pg on column for PGE_2 and PGD_2 when Waters G2-S Q-TOF instrument was used. However, these values might be significantly improved when more sensitive triple quadrupole mass analyzers are used in MRM (SRM) mode. The linear dynamic range, which is also an instrument dependent function, was between 1 pg to 50 ng on column.

In summary, the improved method for PG analysis significantly increases the recovery of the PG extraction up to 95%, and the new UPLC method allows for a much faster (15 times) major iso-PGE_2 separation with 5 times narrower chromatographic peaks as compared to previously used methods. It also allows for the use of disposable low-cost centrifuge tubes instead of reusable glass silanized tubes and glass homogenizers, thus decreasing cross-contamination of glassware and eliminating glassware silanization and wash procedures. In addition, the new protocol eliminates two centrifugation and phase aspiration steps, and eliminates the need for multiple transfer/evaporation steps [33; 36]. All together, this significantly increased the signal to noise ratio, and decreased the sample preparation and analysis time and cost.

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Abbreviations

PG	prostaglandins
iso-PG	isoprostanes

PGE_2	9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid
11β-PGE_2	9-oxo-11β,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid
8iso-PGE_2	9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid
Ent-PGE_2	9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid
15(R)-PGE_2	9-oxo-11α,15R-dihydroxy-prosta-5Z,13E-dien-1-oic acid
5trans-PGE_2	9-oxo-11α,15S-dihydroxy-prosta-5E,13E-dien-1-oic acid
PGE_2-d_4	9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic-3,3,4,4-d_4 acid
PGD_2	9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid
15(R)-PGD_2	9α,15R-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid
Δ12PGD_2	9α,15S-dihydroxy-11-oxo-prosta-5Z,12E-dien-1-oic acid
PL	phospholipids
PLA_2	phospholipase A_2
sPLA_2	secreated phospholipase A_2
20:4n-6	arachidonic acid
UPLC	ultra high performance liquid chromatography
LC-MS/MS	LC with tandem mass spectrometer detection
COX	cyclooxygenase

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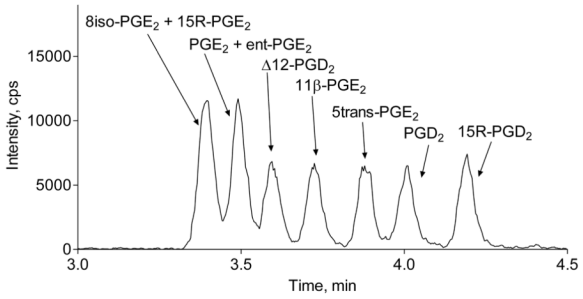
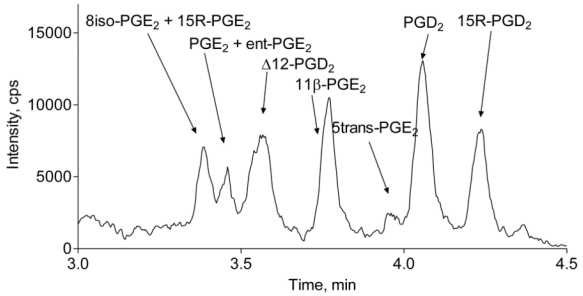
A. Standard mixture



B. Brain endogenous iso-PG from fixed brain



C. Brain endogenous PG from ischemic brain

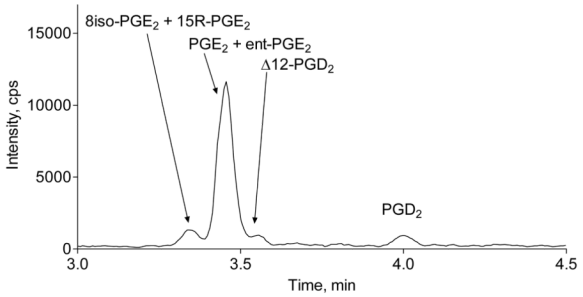


Figure 1. UPLC-MS/MS extracted ion chromatogram for major iso-PGE_2 and iso-PGD_2 species

Extracted ion chromatograms for 189.128 m/z product ion were generated using 0.02 mass window extracted from high energy (MS/MS) channel. Samples were analyzed using UPLC-MS method as described in the Methods section. **Panel A**: A standard iso-PGE_2 and iso-PGD_2 mixture (50 pg each) was dissolved in 10 μL of methanol and injected onto UPLC column. **Panel B**: Mouse brains were fixed using head-focused microwave irradiation to prevent post-mortem PG formation and subjected to bee venom $sPLA_2$ treatment to release esterified endogenous iso-PG. 100mg of tissue was extracted and samples were concentrated and analyzed as described in the Methods section. **Panel C**: Mouse non-fixed (ischemic) brains were incubated for 5 min at room temperature to increase endogenous PG levels and subjected to heat-denaturing in boiling water bath for 5 min to prevent further PG formation during tissue analysis. Ten mg of tissue was extracted with 90 μL of methanol and 10 μL of the extract was analyzed as described in the Methods section.

Table 1

Extraction efficiencies and signal to noise ratios for different extraction protocols

	Single-step methanol	Acetone/chloroform	Bligh and Dyer
	Mean ± SD %	Mean ± SD %	Mean ± SD %
PGE_2d_4	96.7 ± 9.9	76.0 ± 5.1	10.0 ± 1.0
PGE_2	92.9 ± 12.1	77.3 ± 4.9	12.0 ± 1.1
Signal to noise	421 ± 47	289 ± 45	61 ± 31

Standards (1ng of PGE_2d_4 and PGE_2) were analyzed before or after extraction with 10 mg of fixed with head focused microwave irradiation brain tissue. The peak areas for PGE_2 standard extracted with tissue were corrected for the endogenous PGE_2 in brain extracts. The recovery (extraction efficiency) was calculated as percent of peak area of standards extracted with tissue versus standards alone. Signal to noise ratios were calculated for 100pg of PGE_2d_4 extracted with 1mg of brain tissue. n=4

Table 2

Endogenous PG levels quantified in ischemic brain tissue using different extraction protocols

	Single-step methanol		Acetone/chloroform		Bligh and Dyer	
	Mean ± SD ng/gww	**RSD %**	**Mean ± SD ng/gww**	**RSD %**	**Mean ± SD ng/gww**	**RSD %**
PGE_2	5.07 ± 0.33	6.6	4.77 ± 0.76	15.9	5.27 ± 1.36	25.8
PGD_2	1.02 ± 0.06	5.9	1.10 ± 0.19	17.2	0.90 ± 0.18	19.5

Mouse brains were analyzed 5 min after decapitation to model global brain ischemia as described in the Method section. Brain tissue was fixed before analysis by protein heat denaturing for 5 min in a boiling water bath and extracted using different extraction protocols as described in the Method section. One ng of PGE_2d_4 was used as internal standard for PG quantification by UPLC-MS/MS as described in the Method section. n=4

Table 3

Effect of sonication on brain endogenous PG levels

	1 sonication cycle		2 sonication cycles		5 sonication cycles	
	Mean ± SD ng/gww	**RSD %**	**Mean ± SD ng/gww**	**RSD %**	**Mean ± SD ng/gww**	**RSD %**
PGE_2	4.69 ± 0.55	11.6	4.97 ± 0.35	7.0	4.72± 0.28	5.9
PGD_2	1.13 ± 0.09	7.7	1.16 ± 0.11	9.8	1.21 ± 0.07	5.5

Ischemic brain tissue was prepared and analyzed as described in the Table 2. Each sonication cycle was performed for 7 sec with power output of 50J (Model 150 Sonic Dismembrator, Fisher Scientific), n=4

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Table 4

Intra- and inter-day precision for brain endogenous PG analysis:

	Day one, not concentrated		Day one, concentrated 5x		Day two, not concentrated	
	Mean ± SD ng/gww	**RSD %**	**Mean ± SD ng/gww**	**RSD %**	**Mean ± SD ng/gww**	**RSD %**
PGE_2	4.83 ± 0.29	6.1	4.85 ± 0.26	5.4	4.76 ± 0.23	4.7
PGD_2	1.05 ± 0.08	7.7	1.08 ± 0.07	6.5	1.10 ± 0.07	6.7

Global brain ischemia was modeled for 5 min as described in the Method section. Brain tissue was fixed by protein heat denaturing for 5 min in a boiling water bath and 10mg extracted using the one-step methanol extraction protocol as described in the Method section. One ng of PGE_2d_4 internal standard was used as internal standard for PG quantification by UPLC-MS/MS as described in the Method section. n=4

Table 5

Matrix effect for single step methanol extraction followed by UPLC-MS/MS analysis

	Standard dissolved in methanol	Standard spiked with brain extract
	Mean ± SD peak area	Mean ± SD peak area
PGE_2d_4	2520 ± 191	2483 ± 136
PGE_2	2826 ± 366	2780 ± 410

Standards (1ng of PGE_2d_4 and PGE_2) were analyzed with or without spiking with 100 μL of extracts from fixed with head focused microwave irradiation brain tissue, and 10 μL of the sample was loaded on the UPLC column. The peak areas for PGE_2 standard in spiked samples were corrected for the endogenous PGE_2 areas in non-spiked brain extracts. n=4