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TECHNICAL NOTE

Practical evaluation of liquid chromatography/tandem mass spectrometry and enzyme immunoassay method for the accurate quantitative analysis of prostaglandins

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The accurate and robust measurement of prostaglandins (PG) concentration could help to understand the many physiological functions. The present study revealed that liquid chromatography/tandem mass spectrometry method for the PGs analysis can satisfy the requirements for both qualitative and quantitative performance as compared to competitive enzyme immunoassays.

[**Key words:** Prostaglandins; Liquid chromatography/tandem mass spectrometry; Enzyme immunoassay; Quantitative accuracy; Immunological cross-reaction]

Prostaglandins (PG) are lipid mediators derived from arachidonic acid that regulate many physiological and pathophysiological functions. PGD_2 and PGE_2, both of which are positional isomers produced by cyclooxygenases (COX1 and COX2), are representative prostanoids. PGE_2 is a potent stimulant and functions in inflammation, gastric and intestinal secretion, regulation of blood pressure, platelet aggregation, and so on (1). PGD_2, which is secreted by mast cells, Th2 cells, and dendritic cells, has long been implicated in allergic inflammation (2). In addition, PGD_2 is well known as one of the most potent sleep-inducing substances in the central nervous system of mammals (3,4). Since important biological events such as inflammation, mediating pain, and fever are indeed finely modulated by the levels of PGD_2 and PGE_2 at low concentration, the analyses of these metabolites require a highly sensitive and accurate quantitative methodology.

Bioassay methods such as competitive enzyme immunoassay (EIA) are the most commonly used quantitative procedure for the measurement of individual PG due to their sensitivity and specificity, which is a consequence of the unique ligand-antibody binding (4). However, the antibody preparation required for this technique is rather laborious, and cross-reactivity of the antibody with other compounds including prostaglandin analogs may be inevitable. Meanwhile, mass spectrometry-based techniques, especially liquid chromatography/tandem mass spectrometry (LC/MS/MS) under multiple-reaction monitoring (MRM), have attracted attention in the quantification of PGs owing to their selectivity (5,6). To date, no previous evaluation of EIA and LC/MS/MS method for the PGD_2 and PGE_2 analyses in terms of sensitivity and quantitative accuracy has been reported. The accurate measurement of PGs concentration could help to understand the underlying mechanisms and possibility of therapeutic interventions.

The aim of the present study is to examine the applicability of two commercially available EIAs to the measurement of PGD_2 and PGE_2, both of which are secreted by rat mast cells stimulated with dinitrophenyl-BSA, along with comparison to an LC/MS/MS assay (Fig. 1).

Rat mast cell line (RBL-2H3 cells) was purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). RBL-2H3 cells were cultured in Minimum Essential Medium (MEM, Sigma–Aldrich Co., St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum, 100 units mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin sulfate. After the sensitization of RBL-2H3 cells with 50 $ng\ mL^{-1}$ monoclonal anti-dinitrophenyl IgE, the cells were stimulated with 20 $ng\ mL^{-1}$ dinitrophenyl-BSA, and culture medium was picked up at 1, 3, 5, 10, and 15 min after the stimulation. Cultured medium was purified with Sep-Pak C18 Plus cartridge with 360 mg sorbent (Waters Corp., Miliford, MA, USA). Briefly, medium samples were diluted with ethanol (final ethanol concentration of 15% v/v) containing the radioisotope labeled standards of $[^3H_7]PGE_2$ and $[^3H_7]PGD_2$ (60 Bq for each per assay) (Perkin-Elmer Inc., Boston, MA, USA) for EIA as tracers for estimation of the recovery, or the stable isotope labeled standards of $[^2H_4]PGE_2$ and $[^2H_4]PGD_2$ (10 pmol) (Cayman Chemical Co., Ann Arbor, MI, USA) for LC/MS/MS analysis with the standard isotope-dilution method. The cartridges were conditioned with 10 mL of ethanol and allowed to equilibrate with 10 mL of Milli-Q water. Each sample was loaded on a cartridge, and then the cartridge was washed with 20 mL of hexane. The PGs-mixture was eluted with 5 mL of ethyl acetate to glass tube. The eluates were dried by evaporation under a stream of dry nitrogen. For the quantitative analysis of PGD_2 and PGE_2 with LC/MS/MS, the dried sample was reconstituted in 100 μL of water/methanol/acetic acid (90/10/0.05, v/v/v).

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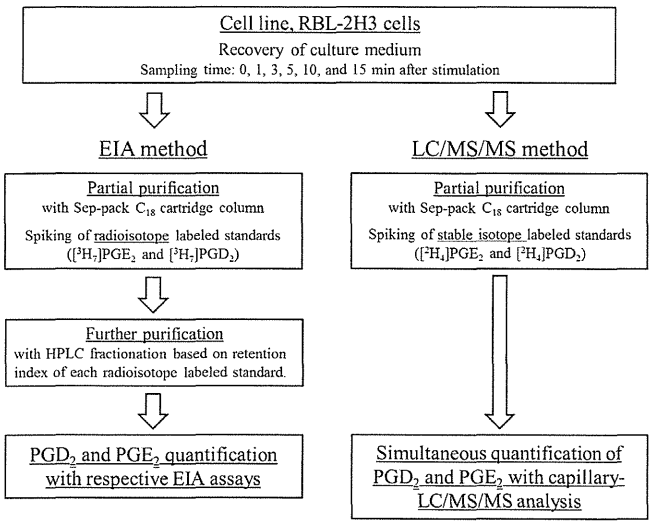

FIG. 1. Overview of EIA and LC/MS/MS protocols for the determination of PGD_2 and PGE_2.

In the case of EIA, the further purification of PGs was performed by HPLC fractionation (7). Finally, the quantification of PGD_2 and PGE_2 was performed with respective EIA kits (Cayman Chemical Co.) according to the methods describing in the instructions. LC/MS/MS system was composed of an LC Packings Ultimate equipped with a Switchos loading pump and a Famos autosampler (Dionex Corp., Sunnyvale, CA, USA) and an Esquire 3000 plus ion-trap mass spectrometer fitted with an ESI ion source (Bruker Daltonics Inc., Billerica, MA, USA). The LC/MS/MS analysis was performed under the following conditions: trapping column, Inertsil ODS-3 C18 column (0.3 × 5 mm, particle size of 5 μm, Dionex Corp.); mobile phase for sample loading, 0.05% (v/v) acetic acid in water; flow rate for sample loading, 10 μL min^{-1}; separation column, Inertsil ODS-3 C18 column (0.3 × 150 mm, particle size of 3 μm, GL Sciences Inc., Tokyo, Japan); sample loading time, 5 min; mobile phase for PGs separation, water/acetonitrile/acetic acid (95/5/0.05, v/v/v) (A) and acetonitrile/water/acetic acid (95/5/0.05, v/v/v) (B); flow rate: 4 μL min^{-1}; gradient curve, 40% B at 0 min, 50% B at 15 min, 90% B at 17 min, 90% B at 22 min, 40% B at 23 min, and 40% B at 30 min; injection volume, 10 μL; mode of mass analysis, negative ion mode; nebulizer flow, 9.0 psi; dry gas flow rate of 4.0 L min^{-1}; dry gas temperature, 250°C; capillary voltage, 4.5 kV; compound stability, 100%, trap drive level, 50%; target count, 10,000; maximum accumulation time, 100 ms; and spectral average, 7. The MS/MS spectra were analyzed in the auto MS/MS mode at fragmentation amplitude of 1.0 V. MRM transitions from the precursor ions to the most abundant product-ions were recorded. The optimized MRM transitions are as follows: PGD_2 and PGE_2, 350.8 > 314.8; and $[^2H_4]PGD_2$ (d_4-PGD_2) and $[^2H_4]PGE_2$ (d_4-PGE_2), 354.8 > 318.8.

The present study has evaluated the analytical capabilities (i.e., sensitivity, quantitative accuracy, and sample throughput) between LC/MS/MS and EIAs method based on the practical protocols for the PGs quantification (Fig. 1). EIAs assay is a very useful tool for the individual PG analysis in terms of economical, high throughput performance, and highly sensitive. However, EIAs may not provide specificity sufficient for the determination of PGs if immunological cross-reactions occur, since the antibodies used in EIA sometimes recognize not only the target molecule, but also structurally related molecules. Therefore, in our practical EIA protocol, after the partial purification of PGs with Sep-pack C_{18} cartridge, the further purification of each targeted PG was performed using HPLC fractionation based on retention index of each corresponding radioisotope labeled standard (7). On the other hand, in the LC/MS/MS analysis, the PGD_2 and PGE_2 were quantified by a standard isotope-dilution. The standard isotope-dilution method refers to the addition of the stable isotopomers of the target compounds prior to partial purification, enables the accurate quantification by compensating for both losses due to the sample pretreatment step and the nonspecific matrix effects caused by co-eluting components (8). In addition, it is well known that the sensitivity of LC/MS/MS can be increased by lowering the flow rate of the mobile phase, which can be achieved by using narrow diameter LC columns (9). Thus, we selected to the capillary LC system for PGs analysis.

The detection sensitivity of the LC/MS/MS system developed in this study was compared to commercially available EIA kits with PGD_2 and PGE_2 standard solutions. The limits of detection (LOD) of LC/MS/MS was estimated based on $S/N = 3$ in the LC/MS/MS separation, whereas the LOD of EIAs was calculated with 80% B/B_0 (% bound/maximum bound: ratio of the absorbance of a particular sample or standard well to that of the maximum binding B_0 well) value. The detection limits of LC/MS/MS were 1.5 nM (PGD_2) and 0.70 nM (PGE_2), while the LODs of EIAs were 0.15 nM (PGD_2) and 0.10 nM (PGE_2). Thus, the sensitivity of EIA was approximately 10-fold higher than that of LC/MS/MS, indicating that EIA measurement will lead to a minimization of starting material than the LC/MS/MS system used.

Fig. 2A shows the LC/MS/MS (MRM) chromatograms of deuterium-labeled internal standards and PGs released from RBL-2H3 cells stimulated with dinitrophenyl-BSA. The LC/MS/MS assay was able to separate the PGD_2, PGE_2, and unidentified metabolite (PGX) and detect them with same MRM transition. Next, the performance of quantitative accuracy of LC/MS/MS and EIAs was compared (Fig. 3). Based on the statistical analysis, the LC/MS/MS method for PGD_2 has no significant fault and can produce results compatible with EIA measurement (Fig. 3B). However, significant difference in the quantification of PGE_2 was observed between the LC/MS/MS and EIA strategies (Fig. 3A). The level of PGE_2 estimated using EIA was 3–8 times higher than that estimated with LC/MS/MS.

Here, in order to investigate the cause of analytical error of quantitative values between the two methodologies, we focused on the peak of the PGX, which represents the same MRM transition as PGD_2 and PGE_2 but different elution time (Figs. 2A and 3C). By means of product-ion scan mode in the ion-trap mass spectrometer, the MS/MS spectra of the PGE_2, PGD_2, and PGX were acquired by equal collision-induced dissociation parameter (Fig. 2B–D). The dissociation pattern of PGX was very similar to that of PGD_2 and PGE_2 (especially PGD_2). Previous study has revealed that a series of PG-like compounds termed isoprostanes are formed *in vivo* from the free radical-catalyzed peroxidation of arachidonate independent of COX (10). In addition, Brose et al. reported previously that the optimized LC/MS/MS method allows for the PGs (PGD_2 and PGE_2) and their stereoisomers, namely isoprostanes including entPGE_2, 8-isoPGE_2, 11β-PGE_2, and 15(R)-PGD_2 to be separated (6). Therefore, this unidentified peak, PGX is probably attributed to the stereoisomer of PGD_2 or PGE_2, or their mixtures. Considering our present findings and previous reports, the quantitative values of PGE_2 with EIA protocol will contain a part of PGX (Fig. 3C). Conceivably, PGE_2 fraction would be contaminated with PGX in the process of HPLC fractionation based on retention index of $[^3H_7]PGE_2$. Furthermore, antibody used in immunoassay for PGE_2 would recognize not only the PGE_2, but also structurally related molecule, PGX. Thus, these results raise the possibility that PGs might be overestimated by EIA compared with LC/MS/MS method, especially in a trace amount of metabolite such as PGE_2 in the present case.

Taking all of the present results into account, LC/MS/MS method is suitable than EIAs for PGs analysis in terms of quantitative accuracy, which would be useful for the underlying mechanisms of biological functions including sleep, central nervous system injury,

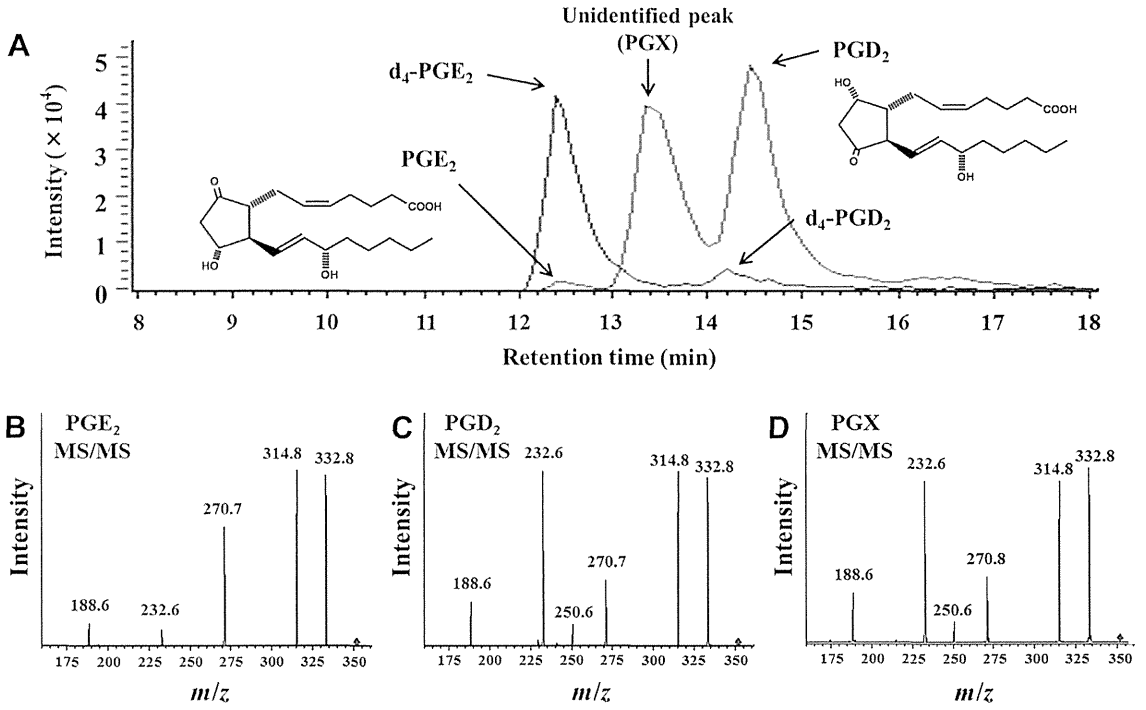

FIG. 2. LC/MS/MS (MRM) chromatograms of PGs released from RBL-2H3 cells with dinitrophenyl-BSA stimulation (A). Fragmentation patterns of PGE_2 (B), PGD_2 (C), and unidentified PGX (D) by means of product-ion scan.

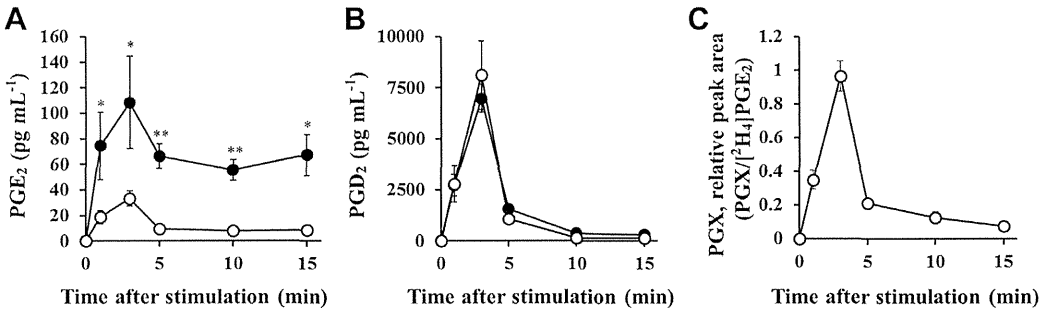

FIG. 3. Quantitative estimation of time-dependent production of PGE_2 (A), PGD_2 (B), and PGX (relative peak area) (C) in RBL-2H3 cells using LC/MS/MS (open circles) and EIA (closed circles). Error bars indicate standard deviations of five biological replicates. Equality of variances of each data point between LC/MS/MS and EIA results was judged by *F*-test at the 99% confidence level ($P > 0.01$, Student's *t*-test; $P < 0.01$, Welch's *t*-test). Statistical significance was then determined using Student's or Welch's *t*-test ($^{*}P < 0.01$, $^{**}P < 0.001$).

inflammation, and cancer, as well as the possibility of therapeutic treatments. In addition, more recently, the improvement of an LC/MS/MS analytical system enables the comprehensive and quantitative analysis of PGs and their related metabolites with high throughput (11). Accordingly, we predict that LC/MS/MS approach will be widely used as a standard method for the precise, robust, and simultaneous profiling of PGs.

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