Journal of
Applied Bioanalysis

openaccess

http://dx.doi.org/10.17145/jab.20.014

ORIGINAL RESEARCH

LC-MS/MS assay for the simultaneous quantitation of thromboxane B_2 and prostaglandin E_2 to evaluate cyclooxygenase inhibition in human whole blood

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Citation:
Shi Y, Murrey HE, Ahn K, Weng N, Patel S. LC-MS/MS assay for the simultaneous quantitation of thromboxane B2 and prostaglandin E2 to evaluate cyclooxygenase inhibition in human whole blood. J Appl Bioanal 6(3), 131-144 (2020).

Editor:
Mark Shapiro, MCS Pharma Consulting LLC, Stokesdale NC, USA.

Received: May 01, 2020.
Revised: June 14, 2020.
Accepted: June 16, 2020.

Funding & Manuscript writing assistance:
The authors have no financial support or funding to report and they declare that no writing assistance was utilized in the production of this article.

Financial & Competing interests:
The authors are employees of Jansen R & D and they have declared that no financial and competing interest exist.

OBJECTIVES: A high-throughput LC-MS method for TXB_2 and PGE_2 was developed for human whole blood assay for COX inhibition.

METHODS: A surrogate analyte approach was used for the quantitation of TXB_2 and PGE_2 by LC-MS. Fifty microliters plasma was processed using solid-phase extraction. TXB_2-d_4 and PGE_2-d_4 were used as surrogate analytes. The calibration curves were established for TXB_2 from 0.1 to 500 ng/mL and for PGE_2 from 0.05 to 500 ng/mL. TXB_1 was used as internal standard.

RESULTS: The response factor and parallelism between surrogate and authentic analyte were verified. Heparinized whole blood assay for COX inhibition was optimized for sample pretreatment, stimulant concentration and incubation time.

CONCLUSION: The LC-MS assay was successfully used to analyze inhibitory activity of four commercially available COX inhibitors. The presented method offers a sensitive, high throughput and low-cost alternative to ELISA for human whole blood assay for COX inhibition.

KEYWORDS: COX inhibitor, LC-MS/MS, lipids, TXB_2, PGE_2, whole blood assay.

INTRODUCTION

Prostaglandin-endoperoxide synthases, commonly known as cyclooxygenase (COX), are responsible for the formation of thromboxanes and prostaglandins from arachidonic acid [1,2]. COX enzymes exist in two isoforms, COX-1 and COX-2. The former is expressed constitutively in many tissues, while the latter is generally induced in cells in response to inflammatory cytokines, growth factors and toxins. Inhibition of COX enzymes is widely studied for treatment of inflammation, pain relief, and cancer. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) show an increased risk of gastrointestinal complications, likely due to inhibition of COX-1. Selective inhibitors of COX-2 were subsequently developed. However, several of them were withdrawn from market due to associated cardiovascular risk [3,4]. Two

decades later, the therapeutic and adverse effects of both selective and non-selective NSAIDs are still being widely studied [5,6]. It has been shown that the differences in pharmacokinetic and pharmacodynamic characteristics, as well as the dosage level, are more important in evaluating individual risk and benefit of a drug candidate than simply looking at class effects [3].

Various *in vitro* assays can be used to characterize a compound's inhibitory activity and selectivity for COX-1 and/or COX-2. These assays have been developed using purified recombinant enzymes, enzyme expression in cultured cells, or enzyme present from human whole blood [7,8]. Enzyme assays using purified protein can reveal important kinetic parameters of inhibition mechanism. However, they cannot account for the effects of plasma protein binding, cellular permeability, and other interactions between an inhibitor and the complex biomolecules within a cell. While cell-based assays account for some of these parameters, it still does not resemble the physiological condition as many blood components are neglected. Therefore, assays developed to examine inhibitor activity in human whole blood, which closely mimics the *in vivo* environment, is the ideal system to evaluate compounds of interest in developing new COX inhibitors.

In the COX enzymatic pathway, arachidonic acid is released from cell membrane phospholipids by phospholipase A_2. As shown in **Figure 1**, cyclooxygenase enzymes metabolize arachidonic acid to prostaglandin H_2, which can be further converted to thromboxanes and prostaglandins. It has been shown that adding calcium ionophore can stimulate release of arachidonic acid from cellular membrane *in vitro*, leading COX-1 enzyme to generate thromboxane A_2 (TXA_2) which is immediately converted to thromboxane B_2 (TXB_2). Also, lipopolysaccharide (LPS) can induce COX-2 activity, where prostaglandin E_2 (PGE_2) level is elevated [8]. Therefore, TXB_2 and PGE_2 levels correlate to COX-1 and COX-2 activities stimulated by calcium ionophore and LPS, respectively. In the human whole

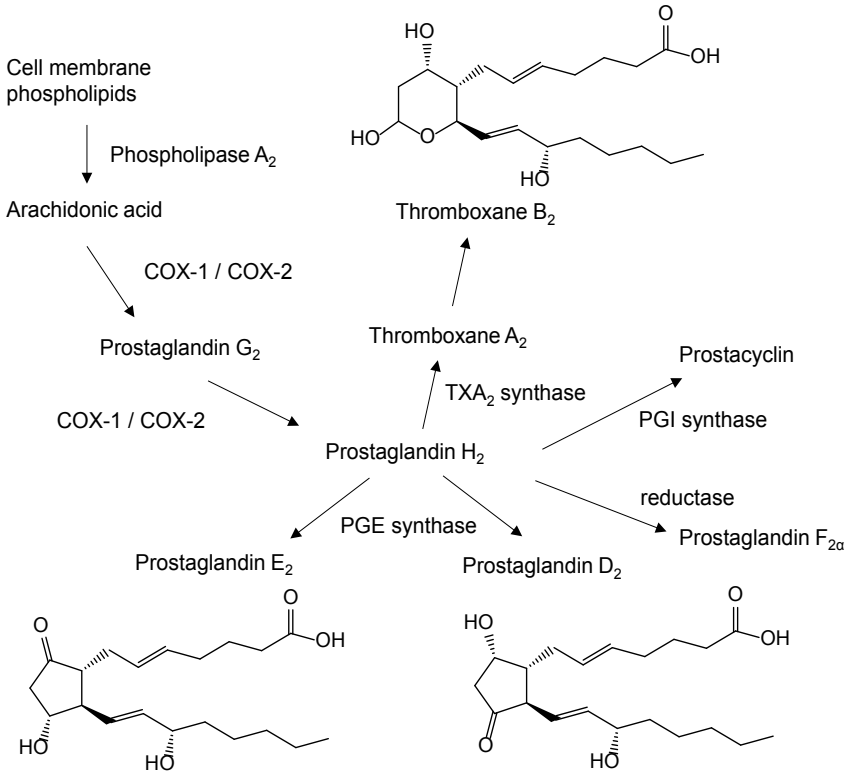

Figure 1. Overview of the arachidonic acid cascade through the COX pathways.

blood assay, various concentrations of COX inhibitors are added after COX-1 or COX-2 stimulation *in vitro*. Plasma samples are collected from the experiment. Inhibition curves of COX-1 and COX-2 activity can be plotted based on the concentrations of TXB_2 and PGE_2 production to calculate the corresponding half maximal inhibitory concentrations (IC_{50}). Thus, sensitive and high throughput assays for TXB_2 (COX-1 activity) and PGE_2 (COX-2 activity) in plasma are needed. Commercial enzyme-linked immunosorbent assay (ELISA) kits are available for both compounds. However, plasma samples from whole blood assay need to be analyzed separately using different ELISA kits for PGE_2 and TXB_2. While the sensitivity of PGE_2 and TXB_2 detection by the kits is good, the calibration ranges are relatively narrow (from 0.015 to 2 ng/mL). Furthermore, the binding antibodies suffer cross reactivity with other analytes [9]. Multiple LC-MS/MS methods can be found in the literature for detection of PGE_2 and related compounds. Excellent sensitivity below 10 pg/mL has been achieved by utilizing chemical derivatization [10]. Direct measurement using LC-MS/MS can also have good sensitivity. However, some methods only cover limited analytes including prostaglandins but not thromboxanes [11], and some methods chromatographically separated a wide range of eicosanoids but require a relatively long run time [12]. Recently, Gandhi et al. utilized a quick protein crash method to analyze five lipids for tissue samples but not in plasma samples [13]. To our best knowledge, this is the first work to report a sensitive and high-throughput LC-MS/MS method for the simultaneous measurement of TXB_2 and PGE_2 for human whole blood assay.

In the literature, whole blood assays for measurement of COX-2 activity are usually similar to the experiment reported by Patrignani et al. [14]. However, for COX-1 whole blood assays, there are three different procedures, including platelet assays, blood clotting assays, and heparinized whole blood assays. While the platelets isolated from freshly collected whole blood represent most of the COX-1 enzyme activity in blood, platelet assays are not true whole blood assays. Young et al. developed a heparinized blood assay that measures COX-1 and COX-2 activities from the same blood samples by monitoring TXB_2 [15]. Esser et al. simplified the heparinized blood procedure by splitting samples into COX-1 and COX-2 inhibition after incubation with COX inhibitors [16]. These experiments resemble physiological conditions and have less individual variation as the same blood samples were used for both COX-1 and COX-2 assays. The measured COX inhibition and selectivity are believed to be more relevant in drug design and development. In this work, we further optimized the heparinized whole blood procedure for both COX-1 and COX-2 assays. Several non-selective and selective NSAIDs were evaluated for their COX-1 and COX-2 inhibitory activities in human whole blood. The calculated IC_{50} from LC-MS/MS data were compared with those calculated from ELISA analysis.

EXPERIMENTAL

Chemicals and reagents

PGE_2, PGD_2, TXB_2, PGE_2-d_4, TXB_2-d_4, and TXB_1 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Methanol and acetonitrile of HPLC grade were obtained from EMD Millipore (Billerica, MA, USA). Formic acid (reagent grade), LPS from *E. coli*, calcium ionophore, celecoxib, rofecoxib, etoricoxib, and diclofenac were purchased from Sigma-Aldrich (St. Louis, MD, USA). Deionized water was purified via Milli-Q system from EMD Millipore, and cell culture grade water was from Mediatech, Inc (Manassas, VA, USA). TXB_2 and PGE_2 Express ELISA kits, which contained analyte standards, antibodies, tracer, coated plates, Ellman's reagent, wash and dilution buffers, were purchased from Cayman Chemical. Human blood from donors who had received no NSAIDs for at least 10 days was collected into vacutainers containing heparin and used within 1 hour of collection.

Instrumentation

For the LC-MS/MS method, all analyses were conducted on a Triple Quad 6500+ mass spectrometer from Sciex (Foster City, CA, USA) with a Turbo Ionspray interface. The

Table 1. Method characteristics including ion transitions, MS parameters and retention time.

Analyte	Parent ion (m/z)	Fragment ion (m/z)	DP (V)	CE (V)	CXP (V)	Retention time (min)
PGE_2	351	271	-50	-25	-10	2.95
PGE_2-d_4	355	275	-50	-25	-10	2.92
PGD_2	351	271	-40	-23	-10	3.26
TXB_2	369	169	-40	-25	-20	2.09
TXB_2-d_4	373	173	-40	-25	-20	2.06
TXB_1	371	171	-50	-28	-15	1.92

DP: declustering potential; CE: collision energy; CXP: collision cell exit potential.

system was controlled by Analyst 1.6 software. The LC system used was a Shimadzu LC-30AD pump coupled with a Nexera X2 SIL-30AC autosampler (Kyoto, Japan). Plasma samples were extracted using Oasis MAX 96-well µElution plate from Waters (Milford, MA). A Kinetex C18 column (2 x 50 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA) was used for chromatographic separation. For the ELISA method, the plate reader used was a SpectraMax 250 from Molecular Devices (San Jose, CA, USA) controlled by SoftMax Pro 5.4.5 software.

LC-MS/MS and ELISA Methods

To simultaneously measure TXB_2 and PGE_2 using LC-MS/MS, 50 µL of plasma sample was mixed with 20 µL of internal standard solution (200 ng/mL TXB_1 in water/methanol 50:50), and 500 µL water. An Oasis MAX µElution plate was conditioned with 200 µL of methanol and 200 µL of acetonitrile/water 25:75. After vortex mixing, the samples were loaded to SPE plate and washed with 200 µL of acetonitrile/water 25:75 and then 200 µL of freshly prepared 5% ammonia in water. The plate was dried with positive pressure for 1 minute. Analytes were eluted with 40 µL of 1% formic acid in acetonitrile/water 50:50 and 40 µL of 1% formic acid in acetonitrile. The samples were vortex-mixed well and centrifuged for 1 minute. Five microliters sample extract was injected onto the LC-MS/MS system for analysis.

The Kinetex C18 column was maintained at ambient temperature. The mobile phase A was 0.1% acetic acid in water, and the mobile phase B was acetonitrile/isopropanol 90:10. The gradient at an initial flow rate of 0.4 mL/min was ramped from 30% B to 35% B between 0 and 3.2 min. From 3.3 to 3.9 min, the column was forward washed at 0.6 mL/min and 90% B. After the flow rate was dropped back to 0.4 mL/min, the column was re-equilibrated at 30% from 3.91 to 4.4 min. Mass spectrometric detection using negative electrospray ionization (ESI) was carried out in selected reaction monitoring (SRM) mode. The ion spray voltage was -4500 V. The ion source temperature was 600 °C and the entrance potential (EP) was -10 V. Curtain gas, gas 1 and gas 2 were 30, 70 and 35 psi. Collision-activated dissociation (CAD) gas was set to 10 psi. For the transition of each analyte, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized as listed in **Table 1**. A weighed linear $1/x^2$ regression was used to generate the calibration curve. Data analysis was carried out with Sciex MultiQuant 2.1.1. For the ELISA method, plasma samples were analyzed for TXB_2 and PGE_2 using TXB_2 Express and PGE_2 Express ELISA kits, respectively, according to the manual. All calibration standards (15–2000 pg/mL) and samples were prepared in duplicates. Sample concentrations were calculated using a four-parameter fit calibration curve with SoftMax Pro 5.4.5.

Method qualification and parallelism assessment

Method qualification and stability

The method qualification was carried out in accordance to internal bioanalytical guideline. The qualification batch contained a calibration curve, six replicates of QCs, benchtop and freeze-thaw stability QCs. The linearity of the calibration curve was determined using a linear $1/x^2$ weighted regression model. Calibration standards in human plasma were prepared fresh daily with concentration of 0.05, 0.1, 0.5, 2.5, 10, 50, 250, 400, and 500 ng/mL. The lower limit of quantitation (LLOQ) of TXB_2 and PGE_2 were determined to be 0.1 and 0.05 ng/mL (S/N ratio ≥ 5:1), respectively. Four levels of quality control (QC) samples were prepared at concentrations of 0.15, 0.3, 20, and 375 ng/mL. The intra-day accuracy and precision were assessed by analyzing six replicates of QC samples per level. The interday assay precision was not evaluated for method qualification. Assay accuracy and precision acceptance criteria were within 100 ± 20% according to internal bioanalytical guideline. Standards and QCs were prepared using surrogate analyte (TXB_2-d_4 and PGE_2-d_4) in authentic matrix. Stability of the surrogate (d_4) and the authentic analyte (d_0) in human plasma were assessed for three freeze-thaw cycles and four hours at room temperature. A percent deviation from of ± 20% was deemed acceptable to consider the sample stable under the storage condition tested.

Response Factor and parallelism

For this surrogate analyte method, the mass spectrometry response of the surrogate analyte should be comparable to that of the authentic analyte. The response factor was determined by separately injecting neat solutions of authentic and surrogate analyte in the presence of the internal standard. If the mean percent difference is greater than 5%, the response factor should be applied for quantitation using the surrogate analyte standard curve. A parallelism assessment was performed to determine how well calibration standards prepared with the surrogate analyte track the response of the authentic analyte in the biological matrix [17]. Six replicates of plasma spiked with solvent-only or with authentic analyte at three concentrations (0.3, 20 and 375 ng/mL) were quantified using surrogate analyte curve. Theoretical concentrations of the spiked QC were calculated from the endogenous level plus the spiked amount. The precision and accuracy of the QCs were evaluated to establish parallelism across the calibration range [18].

COX-1 and COX-2 whole blood assay

Fresh blood was collected in heparinized vacutainers from volunteers with consent. Protocol for Employee Volunteer Participation in Sample Donation for Research was under the governance of Quorum Institutional Review Board (reference Pro00035367). The subjects had not taken any NSAIDs for at least 10 days prior to blood collection. The first and highest compound spiking solution was prepared at 33.3 mM in DMSO. Ten additional spiking solutions were subsequently diluted 3.33x from the previous solution. The lowest solution was 0.2 µM. A blank DMSO solution completed the 12-point inhibition curve. The final compound concentrations ranged from 1.2 pM to 0.2 mM (167,000 folds). COX inhibitors were added within one hour of blood collection. All compounds, celecoxib, rofecoxib, diclofenac and etoricoxib, were tested in duplicates. The samples were pipetted mixed 10 times prior to incubation in a humidified 37 °C incubator with 5% carbon dioxide for 1 hour. The samples were then divided equally into two aliquots for COX-1 and COX-2 experiments.

For COX-1, 2 µL of calcium ionophore in DMSO was added to each well resulting in a final concentration of 15 µM. After pipette mixing of the samples, the plate was incubated at 37 °C for one hour with continuous shaking at 200 rpm. The reactions were terminated by quickly chilling the plates in an ice batch, centrifuging at 1100 x *g* for 10 minutes to separate the plasma and storing the separated plasma at –80 °C. For COX-2, 10 µL of LPS stock solution in H_2O was added to blood samples resulting a final concentration of

10 µg/mL. After mixing, the plate was incubated at 37 °C with 5% carbon dioxide for 24 hours. Plasma were collected by centrifuging at 1100 x *g* for 10 minutes.

Plasma samples from the whole blood assay were analyzed with both ELISA and LC-MS/MS methods. TXB_2 and PGE_2 concentration data were analyzed using Prism 7.0 (GraphPad Software, San Diego, CA) and fitted to a four-parameter logistic function using nonlinear regression analysis. IC_{50} values of COX-1 and COX-2 inhibition were calculated based on the inhibition curve.

RESULTS AND DISCUSSION

LC-MS/MS method development and qualification

Two strategies were commonly used for analyzing endogenous compound concentration in biological matrix: surrogate analyte in authentic matrix and authentic analyte in surrogate matrix [19]. We used the first approach in which the mass spectrometry response of authentic and surrogate analyte must be investigated. Six replicates of TXB_2 and PGE_2 neat solution at 5 ng/mL were injected into LC-MS/MS system with the presence of internal standard TXB_1. The analyte-to-IS peak area ratio were compared to that of TXB_2-d_4 and PGE_2-d_4. The mean response factor was 0.99. Therefore, the response factor was not applied during sample analysis.

The chromatograms of the surrogate and authentic analytes as well as the internal standard are shown in **Figure 2**. While mobile phases with formic acid or acetic acid were used in previous publications for TXB_2 and PGE_2, 0.1% acetic acid was found to achieve the best sensitivity [20]. Different reversed phase columns were tested. The peak shape of PGE_2 was generally good on most columns. However, interference peaks were observed at a slightly earlier retention time for TXB_2 and TXB_2-d_4. Kinetex C18 column provided the best separation of the interference peak. Gandhi et al. reported that TXB_2 frequently had peak tailing at elevated column temperature (40 °C), so the column temperature should be kept at 20 ~ 25 °C [13]. We found that adding 10% isopropanol to mobile phase B also reduced the peak tailing for TXB_2. No interference peak was observed for the internal standard, TXB_1. Prostaglandin D_2 and E_2 are isobaric compounds and must be chromatographically resolved. Using an analytical flow of 0.4 mL/min and a column-wash flow of 0.6 min/mL, we achieved baseline separation using only 4.4 minutes of HPLC time. As shown in **Figure 2** (i), PGD_2 and PGE_2 were eluted at 2.95 min and 3.26 min, respectively. To establish parallelism of the surrogate and authentic analytes in human plasma, endogenous levels of TXB_2 and PGE_2 were determined using surrogate analyte curve. The measured TXB_2 and PGE_2 concentrations using 6 replicates of blank human plasma were 0.701 and 0.339 ng/mL, respectively. The same lot of human plasma was used to make three levels of QCs by spiking in authentic analyte solution at 0.3, 20 and 375 ng/mL. The addition of these QCs was determined by the same surrogate analyte curve. The relative error of these QC was within 10.3% for both TXB_2 and PGE_2.

The LC-MS/MS method was qualified in one accuracy and precision batch with matrix stability test. Calibration curves were regressed by plotting the analyte-to-IS peak area ratio versus the concentration. Linear with $1/x^2$ weighing was determined to be the best curve fit for the range of 0.1 – 500 ng/mL for TXB_2-d_4 and 0.05 – 500 ng/mL for PGE_2-d_4. The correlation coefficients for both curves were ≥ 0.99. Calibration standards were within 88.6% and 106% of their nominal values for TXB_2-d_4 and within 82.3% and 114% for PGE_2-d_4. The assay accuracy was determined by calculating the ratios of the calculated concentrations to their nominal values, and the intraday precision was determined using a one-way analysis of variance (ANOVA). As presented in **Table 2**, the overall assay accuracies were within 100 ± 9.7% and within 100 ± 10.8% for TXB_2-d_4 and PGE_2-d_4, respectively. The precisions were less than 9.69% CV for TXB_2-d_4 and less than 9.04% CV for PGE_2-d_4. The freeze-thaw stability was accessed at three QC concentrations. After three cycles, the stability for both TXB_2-d_4 and PGE_2-d_4 was above 83.7%. After 4 hours at room temperature, the stability for the surrogate analyte was above 81.5%. The

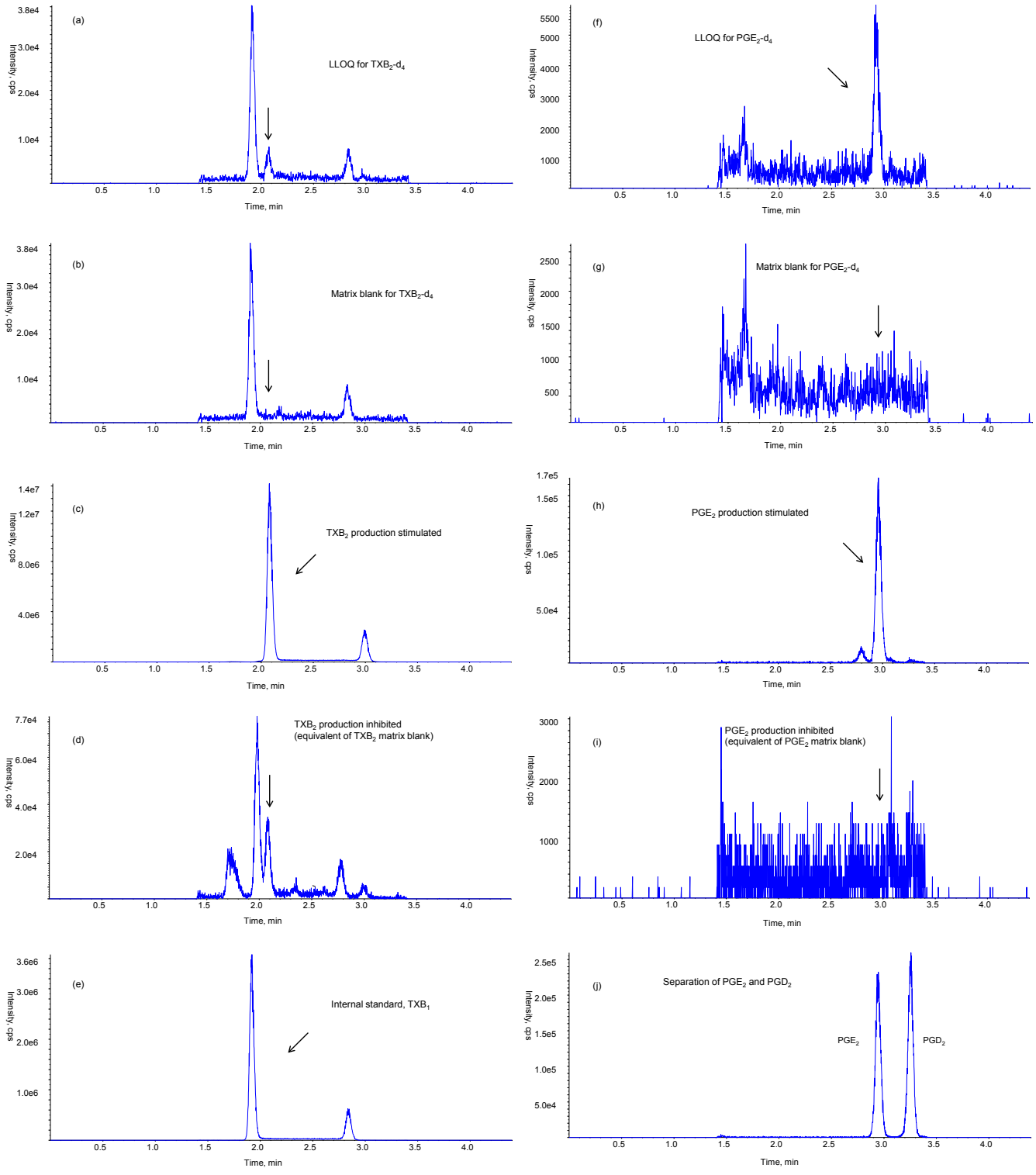

Figure 2. Chromatograms of (a) TXB_2-d_4 at LLOQ, (b) matrix blank for TXB_2-d_4, (c) TXB_2 production stimulated, (d) TXB_2 production inhibited, (e) internal standard TXB_1, (f) PGE_2-d_4 at LLOQ, (g) matrix blank for PGE_2-d_4, (h) PGE_2 production stimulated, (i) PGE_2 production inhibited, and (j) separation of PGE_2 and PGD_2.

Table 2. Accuracy, precision and stability in plasma for TXB_2-d_4, PGE_2-d_4 and plasma stability for TXB_2 and PGE_2.

TXB_2-d_4	LQC1 (0.15 ng/mL)	LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
Mean	0.136	0.283	18.9	349
Accuracy (%)	90.3	94.3	94.6	92.9
Precision (%CV)	9.69	5.33	1.81	3.41
		Freeze-thaw		
Mean		0.251	18.6	350
Stability (%)		83.7	93.2	93.3
		Benchtop		
Mean		0.244	18.1	344
Stability (%)		81.5	90.6	91.8
Number of replicates (n)	6	6	6	6
TXB_2	LQC2 F/T	MQC F/T	LQC2 BT	MQC BT
Stability (%)	103	97.2	112	97.9
Number of replicates (n)	3	3	3	3
PGE_2-d_4				
Mean	0.156	0.300	20.7	335
Accuracy (%)	104	99.9	104	89.2
Precision (%CV)	9.04	6.72	4.00	3.11
		Freeze-thaw		
Mean		0.335	20.3	333
Stability (%)		112	102	88.8
		Benchtop		
Mean		0.316	19.8	337
Stability (%)		105	99.0	89.8
Number of replicates (n)	6	6	6	6
PGE_2	LQC2 F/T	MQC F/T	LQC2 BT	MQC BT
Stability (%)	108	102	94.3	96.9
Number of replicates (n)	3	3	3	3

The LLOQ's of TXB_2-d_4 and PGE_2-d_4 were 0.1 and 0.05 ng/mL, respectively. As a result, two LQC's, 0.15 and 0.3 ng/mL, were tested for accuracy and precision. For freeze-thaw and benchtop stability of TXB_2, TXB_2-d_4, PGE_2 and PGE_2-d_4, only LQC2 (0.3 ng/mL) was evaluated.

matrix stability for the authentic analytes, TXB_2 and PGE_2, was tested at 0.3 and 20 ng/mL. Stability samples going through 3 freeze-thaw cycles or 4 hours on benchtop were compared against the samples stored at -80 °C immediately after preparation. Freeze-thaw and benchtop stability for TXB_2 were above 97.2% and 97.9%, respectively, and were above 96.9% and 94.3% for PGE_2, respectively.

Whole blood assay optimization and application

The design of whole blood assay

For the research of COX inhibitors, *in vitro* screening assays, utilizing purified enzymes, cell culture and human whole blood, have been developed. While enzyme assay is fast, it only measures the effect of the drug on the enzyme itself. Cell assay can reflect drug-cell interactions, but still neglects plasma protein binding of the drug and other blood compounds. Thus, we are particularly interested in human whole blood assays. COX-1 human blood assay described in the literature falls into three general categories: freshly isolated platelet assay, blood clotting assay, and heparinized whole blood assay. Platelets constitutively express COX-1. Patrono et al. showed that TXB_2 production represents platelet COX-1 activity in response to endogenously formed thrombin [21]. Thus, COX-1 potency can be calculated by measuring TXB_2 concentration from platelet assay. However, purified platelet does not resemble physiological conditions, so its results can hardly be conferred to the human organism. Patrignani et al. used unheparinized human whole blood and measured TXB_2 production for COX-1 inhibition after spontaneous blood clotting [14]. Brideau et al. further demonstrated that TXB_2 production in spontaneously clotted blood reached a plateau after 60 min. While the TXB_2 level was close to the baseline within the first few minutes, at as early as 30 min it got to ~50% of the level at 60 min [7]. As a result, to accurately access the compounds' COX-1 inhibition, testing compounds at concentrations covering a full inhibition curve should be added to sub-aliquoted blood as soon as the blood was collected. With the set up and blood handling requirement of our on-site clinic and our lab, we had difficulty executing the blood clotting COX-1 assay to achieve proper result. For the third option, Young et al. and Glaser et al. described methods using heparinized human whole blood with calcium ionophore incubation for COX-1 inhibition [15,22]. Esser et al. modified the method by having blood samples for COX-1 and COX-2 incubated together with testing compounds [16]. The advantage of this approach is to have testing compound incubated in whole blood before splitting into COX-1 and COX-2 assays, minimizing the variations between the inhibited samples going into next steps. On the other hand, COX-1 platelet and unheparinized blood assays need to be processed separately from COX-2 assays because the blood used for COX-2 must contain anti-coagulant to allow for the incubation with LPS. We used a slightly modified approach for the heparinized blood assay as demonstrated in **Figure 3**. The blood samples used for COX-1 were split from those of COX-2 after incubation with the testing compounds, which kept only one set of samples in the process until immediately before stimulants were added.

COX-2 whole blood assays in the literature frequently have a preincubation step with aspirin. Constitutively expressed COX-1 in platelet can also generate PGE_2 which can be irreversible acetylated by aspirin. The rapid enzymatic hydrolysis of aspirin by plasma esterases ensured that no intact drug was present in whole blood by the time of LPS stimulation, so the LPS stimulated COX-2 activity is not affected. The final PGE_2 readouts reflects COX-2 inhibition of the testing compounds. However, PGE_2 production by platelets only accounts for 1~2% of cyclooxygenase products and its relative contribution to LPS stimulated whole blood PGE_2 production was marginal even without aspirin exposure *in vivo* or *in vitro* [14]. Furthermore, the expression of COX-1 stays steady after incubation with LPS for up to 24 hours. Thus, under the experimental conditions commonly used for COX-2 whole blood assay, it is not necessary to pre-incubate the blood with aspirin to inhibit COX-1 activity [7].

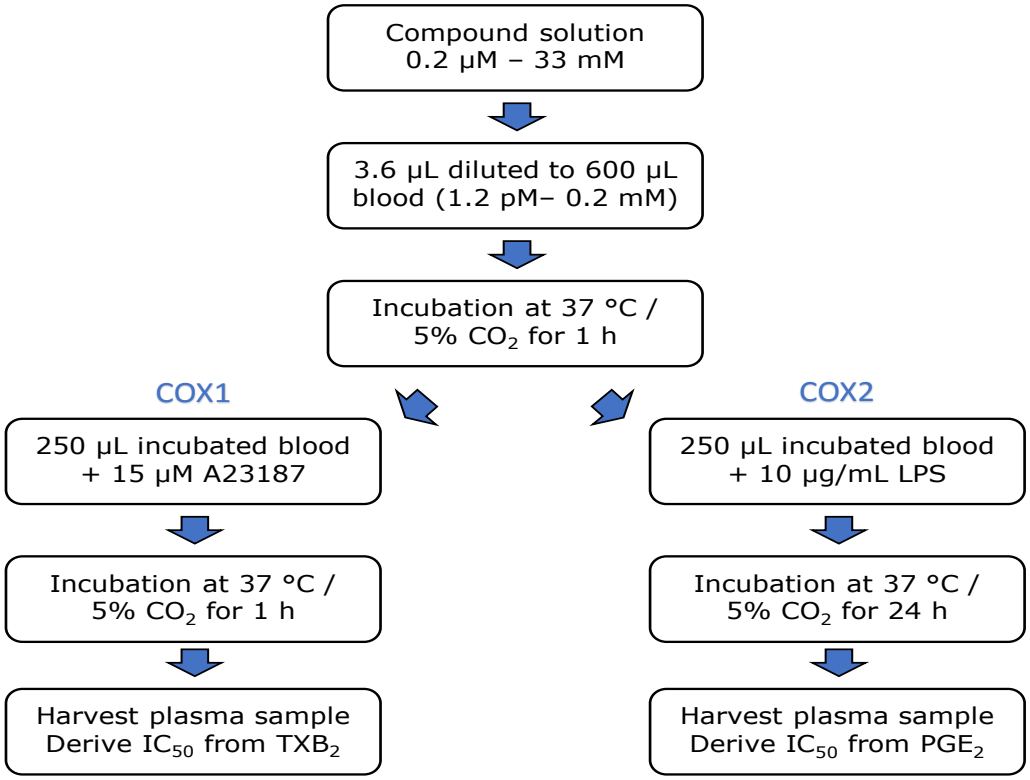

Figure 3. Optimized COX-1 and COX-2 whole blood assay procedure (A23187 = calcium ionophore; LPS = lipopolysaccharide).

Whole blood assay optimization

After the freshly collected heparinized human blood was incubated with the test compound for an hour, the samples were divided into two aliquots. To determine COX-1 activity, calcium ionophore was added to one of the aliquots. After 1 hour of incubation at 37 °C, plasma was harvested for the measurement of TXB_2. Calcium ionophore concentrations ranging from 5 to 25 µM were tested. As shown in **Figure 4a**, while TXB_2 production level increased as the concentration of the stimulant increased, it leveled off and trended lower at 20 and 25 µM. At even higher calcium ionophore concentration, e.g. at 50 µM, various degrees of hemolysis were observed in collected plasma samples. We chose 15 µM calcium ionophore concentration for our experiment. We also tested calcium ionophore incubation time from 15 minutes to 3 hours. As expected, TXB_2 was released very rapidly upon platelet activation, and no obvious trend was noted. An incubation time of 1 hour was selected.

The second aliquot of the drug-inhibited blood sample was used to determine COX-2 potency with LPS stimulation. Various concentrations of LPS at 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL were tested for COX-2 stimulation. The resulted PGE_2 concentration didn't show any obvious trend. This was evidenced by the wide range of LPS concentrations in previously reported COX-2 whole blood experiments. We chose 10 µg/mL as the LPS incubation concentration for our experiment. PGE_2 productions from various LPS incubation time at 2, 4, 7, 24 and 30 hours, were plotted in **Figure 4b**. PGE_2 concentration increased more than 10 folds from 2 h to 24 h and then dropped down at 30 hours. While we cannot rule out the possibility that the most optimum incubation time occurs at the middle point of our tested time profile. The overall increase from 7 to 24 hours incubation was not significant, so we only investigated time frames in which the experiment could

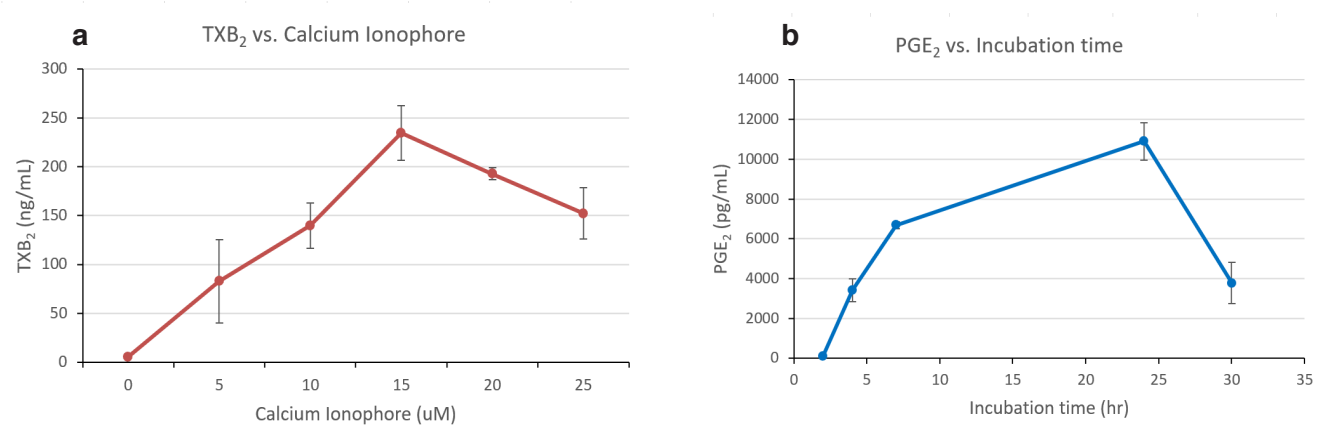

Figure 4. (a) COX-1 assay TXB_2 production stimulated with different calcium ionophore concentration; (b) COX-2 assay PGE_2 production with different incubation time.

be finished within the same day of blood collection or on the following day. As a result, 24 hours LPS incubation was chosen for our experiment.

Comparison of LC-MS/MS and ELISA results

Four COX inhibitors, celecoxib, rofecoxib, diclofenac and etoricoxib, were tested in duplicates using optimized whole blood assay conditions. Plasma samples collected from the whole blood assay were analyzed using both LC-MS/MS and ELISA methods. We developed the sensitive LC-MS/MS method to simultaneously analyze TXB_2 and PGE_2 in a large linear range. While the instrument running time was longer, the more labor-intensive sample process time was shorter and no over-curve re-assay was needed because of the large curve. Two ELISA kits were used to analyze COX-1 and COX-2 assay samples separately. For TXB_2, most of the samples were at or above 100 ng/mL which was significantly higher than the upper limit of quantitation at 2 ng/mL for the ELISA assay. Between the initial analysis and over-the-curve re-assay, four batches were needed for analyzing TXB_2 and PGE_2 samples using ELISA. With the LC-MS/MS method, all samples were analyzed in one batch.

COX-1 and COX-2 inhibition curves generated by two different analytical methods are shown in **Figure 5**. While the shapes of the inhibition curve were similar, the absolute levels of the quantitation results were quite different. For both TXB_2 and PGE_2 ELISA assays, the polyclonal binding antibodies were known to have cross-reactivity with various metabolites, resulting in 100%~200% overestimation compared to the more specific LC-MS/MS method. As shown in **Table 3**, the calculated IC_{50} values from both analytical methods were comparable for all four compounds except for that of COX-1 inhibition of etoricoxib. The results from ELISA (85.6 μM) and LC-MS/MS (149 μM) were both close to the upper limit of concentration range (1.2 pM - 200 μM). For highly selective COX-2 inhibitors like etoricoxib, higher compound concentrations are needed to complete its COX-1 inhibition curve at its fully inhibited side (**Figure 5a**).

CONCLUSION

In the present study, an LC-MS/MS method for the quantitation of TXB_2 and PGE_2 for the plasma samples of COX inhibition human whole blood assay was developed using surrogate analyte approach. The LC-MS/MS method eliminates the cross-reactivity with metabolites found in ELISA kits. The assay is sensitive and high-throughput. The response factor and parallelism between surrogate and authentic analyte were investigated.

Table 3. COX-1 and COX-2 inhibition in human whole blood assay measured by ELISA and LC-MS/MS

COX-1 IC_{50} (μM)	Celecoxib	Rofecoxib	Diclofenac	Etoricoxib
ELISA	17.8	39.6	0.316	~85.6
LC-MS/MS	14.2	42.2	0.259	~149
COX-2 IC_{50} (μM)	Celecoxib	Rofecoxib	Diclofenac	Etoricoxib
ELISA	0.705	0.670	0.0508	1.15
LC-MS/MS	0.965	0.646	0.0475	1.19

IC_{50} values for etoricoxib were estimated based on partial inhibition curves. COX-1 activities were not fully inhibited with 200 μM of etoricoxib.

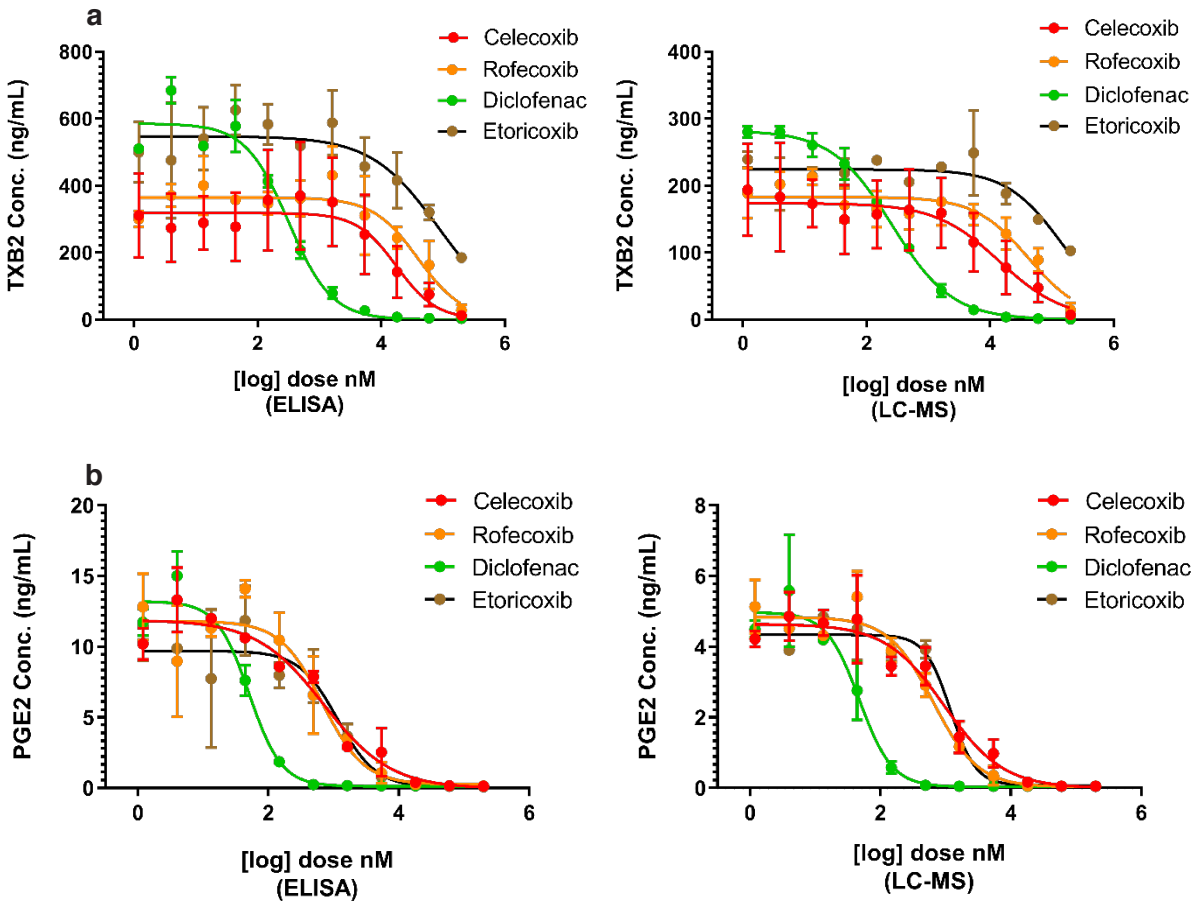

Figure 5. (a) COX-1 inhibition curves of celecoxib, rofecoxib, diclofenac and etoricoxib using data generated with ELISA and LC-MS/MS methods; (b) COX-2 inhibition curves of celecoxib, rofecoxib, diclofenac and etoricoxib using data generated using ELISA and LC-MS/MS methods.

A response factor was not applied when determining analyte concentration from the surrogate analyte calibration curve since the difference was less than 5%. Whole blood COX inhibition assay was optimized for sample pretreatment, stimulant concentration and incubation time. The qualified TXB_2/PGE_2 combo assay was used to measure the COX-1 and COX-2 IC_{50} of four COX inhibitors. The IC_{50} values were comparable between the LC-MS/MS and ELISA methods. In the future, the method can also be used in plasma and tissue samples of animal species, such as rat and mouse, that contain antibodies interfering with commercial ELISA kits.

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