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

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10 **Keywords**: COX inhibitor, LC-MS/MS, lipids, TXB_2, PGE_2, whole blood assay

11 **1.Abstract**

12 **Objectives:** A high-throughput LC-MS method for TXB_2 and PGE_2 was developed for
13 human whole blood assay for COX inhibition. **Methods:** A surrogate analyte approach
14 was used for the quantitation of TXB_2 and PGE_2 by LC-MS. Fifty microliters plasma was
15 processed using solid-phase extraction. TXB_2-d_4 and PGE_2-d_4 were used as surrogate
16 analytes. The calibration curves were established for TXB_2 from 0.1 to 500 ng/mL and
17 for PGE_2 from 0.05 to 500 ng/mL. TXB_1 was used as internal standard. **Results:** The
18 response factor and parallelism between surrogate and authentic analyte were
19 verified. Heparinized whole blood assay for COX inhibition was optimized for sample
20 pretreatment, stimulant concentration and incubation time. **Conclusion:** The LC-MS
21 assay was successfully used to analyze inhibitory activity of four commercially
22 available COX inhibitors. The presented method offers a sensitive, high throughput
23 and low-cost alternative to ELISA for human whole blood assay for COX inhibition.

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25 **2. Introduction**

26 Prostaglandin-endoperoxide synthases, commonly known as cyclooxygenase (COX),
27 are responsible for the formation of thromboxanes and prostaglandins from
28 arachidonic acid[1,2]. COX enzymes exist in two isoforms, COX-1 and COX-2. The
29 former is expressed constitutively in many tissues, while the latter is generally induced
30 in cells in response to inflammatory cytokines, growth factors and toxins. Inhibition of
31 COX enzymes is widely studied for treatment of inflammation, pain relief, and cancer.
32 Traditional non-steroidal anti-inflammatory drugs (NSAIDs) show an increased risk of
33 gastrointestinal complications, likely due to inhibition of COX-1. Selective inhibitors of
34 COX-2 were subsequently developed. However, several of them were withdrawn from
35 market due to associated cardiovascular risk[3,4]. Two decades later, the therapeutic
36 and adverse effects of both selective and nonselective NSAIDs are still being widely
37 studied[5,6]. It has been shown that the differences in pharmacokinetic and
38 pharmacodynamic characteristics, as well as the dosage level, are more important in
39 evaluating individual risk and benefit of a drug candidate than simply looking at class
40 effects[3].

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41 Various *in vitro* assays can be used to characterize a compound's inhibitory activity
42 and selectivity for COX-1 and/or COX-2. These assays have been developed using
43 purified recombinant enzymes, enzyme expression in cultured cells, or enzyme
44 present from human whole blood[7,8]. Enzyme assays using purified protein can
45 reveal important kinetic parameters of inhibition mechanism. However, they cannot
46 account for the effects of plasma protein binding, cellular permeability, and other
47 interactions between an inhibitor and the complex biomolecules within a cell. While

48 cell-based assays account for some of these parameters, it still does not resemble the
49 physiological condition as many blood components are neglected. Therefore, assays
50 developed to examine inhibitor activity in human whole blood, which closely mimics
51 the *in vivo* environment, is the ideal system to evaluate compounds of interest in
52 developing new COX inhibitors.

53 In the COX enzymatic pathway, arachidonic acid is released from cell membrane
54 phospholipids by phospholipase A_2. As shown in Figure 1, cyclooxygenase enzymes
55 metabolize arachidonic acid to prostaglandin H_2, which can be further converted to
56 thromboxanes and prostaglandins. It has been shown that adding calcium ionophore
57 can stimulate release of arachidonic acid from cellular membrane *in vitro*, leading
58 COX-1 enzyme to generate thromboxane A_2 (TXA_2) which is immediately converted to
59 thromboxane B_2 (TXB_2). Also, lipopolysaccharide (LPS) can induce COX-2 activity,
60 where prostaglandin E_2 (PGE_2) level is elevated[8]. Therefore, TXB_2 and PGE_2 levels
61 correlate to COX-1 and COX-2 activities stimulated by calcium ionophore and LPS,
62 respectively. In the human whole blood assay, various concentrations of COX
63 inhibitors are added after COX-1 or COX-2 stimulation *in vitro*. Plasma samples are
64 collected from the experiment. Inhibition curves of COX-1 and COX-2 activity can be
65 plotted based on the concentrations of TXB_2 and PGE_2 production to calculate the
66 corresponding half maximal inhibitory concentrations (IC_{50}). Thus, sensitive and high
67 throughput assays for TXB_2 (COX-1 activity) and PGE_2 (COX-2 activity) in plasma are
68 needed. Commercial enzyme-linked immunosorbent assay (ELISA) kits are available
69 for both compounds. However, plasma samples from whole blood assay need to be
70 analyzed separately using different ELISA kits for PGE_2 and TXB_2. While the sensitivity

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71 of PGE_2 and TXB_2 detection by the kits is good, the calibration ranges are relatively
72 narrow (from 0.015 to 2 ng/mL). Furthermore, the binding antibodies suffer cross
73 reactivity with other analytes[9]. Multiple LC-MS/MS methods can be found in the
74 literature for detection of PGE_2 and related compounds. Excellent sensitivity below 10
75 pg/mL has been achieved by utilizing chemical derivatization[10]. Direct measurement
76 using LC-MS/MS can also have good sensitivity. However, some methods only cover
77 limited analytes including prostaglandins but not thromboxanes[11], and some
78 methods chromatographically separated a wide range of eicosanoids but require a
79 relatively long run time[12]. Recently, Gandhi et al. utilized a quick protein crash
80 method to analyze five lipids for tissue samples but not in plasma samples[13]. To our
81 best knowledge, this is the first work to report a sensitive and high-throughput LC-
82 MS/MS method for the simultaneous measurement of TXB_2 and PGE_2 for human
83 whole blood assay.

84 In the literature, whole blood assays for measurement of COX-2 activity are usually
85 similar to the experiment reported by Patrignani *et al.*[14]. However, for COX-1 whole
86 blood assays, there are three different procedures, including platelet assays, blood
87 clotting assays, and heparinized whole blood assays. While the platelets isolated from
88 freshly collected whole blood represent most of the COX-1 enzyme activity in blood,
89 platelet assays are not true whole blood assays. Young *et al.* developed a heparinized
90 blood assay that measures COX-1 and COX-2 activities from the same blood samples
91 by monitoring TXB_2[15]. Esser *et al.* simplified the heparinized blood procedure by
92 splitting samples into COX-1 and COX-2 inhibition after incubation with COX
93 inhibitors[16]. These experiments resemble physiological conditions and have less

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94 individual variation as the same blood samples were used for both COX-1 and COX-2
95 assays. The measured COX inhibition and selectivity are believed to be more relevant
96 in drug design and development. In this work, we further optimized the heparinized
97 whole blood procedure for both COX-1 and COX-2 assays. Several nonselective and
98 selective NSAIDs were evaluated for their COX-1 and COX-2 inhibitory activities in
99 human whole blood. The calculated IC_{50} from LC-MS/MS data were compared with
100 those calculated from ELISA analysis.

101

102 **3. Experimental**

103 **3.1 Chemicals and reagents**

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

115 **3.2 Instrumentation**

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116 For the LC-MS/MS method, all analyses were conducted on a Triple Quad 6500+ mass
117 spectrometer from Sciex (Foster City, CA) with a Turbo Ionspray interface. The system
118 was controlled by Analyst 1.6 software. The LC system used was a Shimadzu LC-30AD
119 pump coupled with a Nexera X2 SIL-30AC autosampler (Kyoto, Japan). Plasma samples
120 were extracted using Oasis MAX 96-well µElution plate from Waters (Milford, MA). A
121 Kinetex C18 column (2 x 50 mm, 2.6 µm) from Phenomenex (Torrance, CA) was used
122 for chromatographic separation. For the ELISA method, the plate reader used was a
123 SpectraMax 250 from Molecular Devices (San Jose, CA) controlled by SoftMax Pro
124 5.4.5 software.

125 **3.3 LC-MS/MS and ELISA Methods**

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

136 The Kinetex C18 column was maintained at ambient temperature. The mobile phase
137 A was 0.1% acetic acid in water, and the mobile phase B was acetonitrile/isopropanol
138 90:10. The gradient at an initial flow rate of 0.4 mL/min was ramped from 30% B to

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139 35% B between 0 and 3.2 min. From 3.3 to 3.9 min, the column was forward washed
140 at 0.6 mL/min and 90% B. After the flow rate was dropped back to 0.4 mL/min, the
141 column was re-equilibrated at 30% from 3.91 to 4.4 min. Mass spectrometric
142 detection using negative electrospray ionization (ESI) was carried out in selected
143 reaction monitoring (SRM) mode. The ion spray voltage was -4500 V. The ion source
144 temperature was 600 °C and the entrance potential (EP) was -10 V. Curtain gas, gas 1
145 and gas 2 were 30, 70 and 35 psi. Collision-activated dissociation (CAD) gas was set to
146 10 psi. For the transition of each analyte, declustering potential (DP), collision energy
147 (CE), and collision cell exit potential (CXP) were optimized as listed in Table 1. A
148 weighed linear $1/x^2$ regression was used to generate the calibration curve. Data
149 analysis was carried out with Sciex MultiQuant 2.1.1.

150 For the ELISA method, plasma samples were analyzed for TXB_2 and PGE_2 using TXB_2
151 Express and PGE_2 Express ELISA kits, respectively, according to the manual. All
152 calibration standards (15 – 2000 pg/mL) and samples were prepared in duplicates.
153 Sample concentrations were calculated using a four-parameter fit calibration curve
154 with SoftMax Pro 5.4.5.

155 **3.4 Method qualification and parallelism assessment**

156 Method qualification and stability

157 The method qualification was carried out in accordance to internal bioanalytical
158 guideline. The qualification batch contained a calibration curve, six replicates of QCs,
159 benchtop and freeze-thaw stability QCs. The linearity of the calibration curve was
160 determined using a linear $1/x^2$ weighted regression model. Calibration standards in

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161 human plasma were prepared fresh daily with concentration of 0.05, 0.1, 0.5, 2.5, 10,
162 50, 250, 400, and 500 ng/mL. The lower limit of quantitation (LLOQ) of TXB_2 and PGE_2
163 were determined to be 0.1 and 0.05 ng/mL (S/N ratio ≥ 5:1), respectively. Four levels
164 of quality control (QC) samples were prepared at concentrations of 0.15, 0.3, 20, and
165 375 ng/mL. The intraday accuracy and precision were assessed by analyzing six
166 replicates of QC samples per level. The interday assay precision was not evaluated for
167 method qualification. Assay accuracy and precision acceptance criteria were within
168 100% ± 20% according to internal bioanalytical guideline. Standards and QCs were
169 prepared using surrogate analyte (TXB_2-d_4 and PGE_2-d_4) in authentic matrix. Stability
170 of the surrogate (d_4) and the authentic analyte (d_0) in human plasma were assessed
171 for three freeze-thaw cycles and four hours at room temperature. A percent deviation
172 from of ± 20% was deemed acceptable to consider the sample stable under the
173 storage condition tested.

174 Response Factor and parallelism

175 For this surrogate analyte method, the mass spectrometry response of the surrogate
176 analyte should be comparable to that of the authentic analyte. The response factor
177 was determined by separately injecting neat solutions of authentic and surrogate
178 analyte in the presence of the internal standard. If the mean percent difference is
179 greater than 5%, the response factor should be applied for quantitation using the
180 surrogate analyte standard curve. A parallelism assessment was performed to
181 determine how well calibration standards prepared with the surrogate analyte track
182 the response of the authentic analyte in the biological matrix[17]. Six replicates of
183 plasma spiked with solvent-only or with authentic analyte at three concentrations

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184 (0.3, 20 and 375 ng/mL) were quantified using surrogate analyte curve. Theoretical
185 concentrations of the spiked QC were calculated from the endogenous level plus the
186 spiked amount. The precision and accuracy of the QCs were evaluated to establish
187 parallelism across the calibration range[18].

188 **3.5 COX-1 and COX-2 whole blood assay**

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

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

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207 COX-2, 10 µL of LPS stock solution in H_2O was added to blood samples resulting a final
208 concentration of 10 µg/mL. After mixing, the plate was incubated at 37 °C with 5%
209 carbon dioxide for 24 hours. Plasma were collected by centrifuging at 1100 xg for 10
210 minutes.

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

216

217 **4. Results and discussion**

218 **4.1 LC-MS/MS method development and qualification**

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

227 The chromatograms of the surrogate and authentic analytes as well as the internal
228 standard are shown in Figure 2. While mobile phases with formic acid or acetic acid

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229 were used in previous publications for TXB_2 and PGE_2, 0.1% acetic acid was found to
230 achieve the best sensitivity[20]. Different reversed phase columns were tested. The
231 peak shape of PGE_2 was generally good on most columns. However, interference
232 peaks were observed at a slightly earlier retention time for TXB_2 and TXB_2-d_4. Kinetex
233 C18 column provided the best separation of the interference peak. Gandhi et al.
234 reported that TXB_2 frequently had peak tailing at elevated column temperature (40
235 °C), so the column temperature should be kept at 20 ~ 25 °C[13]. We found that adding
236 10% isopropanol to mobile phase B also reduced the peak tailing for TXB_2. No
237 interference peak was observed for the internal standard, TXB_1. Prostaglandin D_2 and
238 E_2 are isobaric compounds and must be chromatographically resolved. Using an
239 analytical flow of 0.4 mL/min and a column-wash flow of 0.6 min/mL, we achieved
240 baseline separation using only 4.4 minutes of HPLC time. As shown in Figure 2(i), PGD_2
241 and PGE_2 were eluted at 2.95 min and 3.26 min, respectively. To establish parallelism
242 of the surrogate and authentic analytes in human plasma, endogenous levels of TXB_2
243 and PGE_2 were determined using surrogate analyte curve. The measured TXB_2 and
244 PGE_2 concentrations using 6 replicates of blank human plasma were 0.701 and 0.339
245 ng/mL, respectively. The same lot of human plasma was used to make three levels of
246 QCs by spiking in authentic analyte solution at 0.3, 20 and 375 ng/mL. The addition of
247 these QCs was determined by the same surrogate analyte curve. The relative error of
248 these QC was within 10.3% for both TXB_2 and PGE_2.

249 The LC-MS/MS method was qualified in one accuracy and precision batch with matrix
250 stability test. Calibration curves were regressed by plotting the analyte-to-IS peak area
251 ratio versus the concentration. Linear with $1/x^2$ weighing was determined to be the

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252 best curve fit for the range of 0.1 – 500 ng/mL for TXB_2-d_4 and 0.05 – 500 ng/mL for
253 PGE_2-d_4. The correlation coefficients for both curves were ≥ 0.99. Calibration
254 standards were within 88.6% and 106% of their nominal values for TXB_2-d_4 and within
255 82.3% and 114% for PGE_2-d_4. The assay accuracy was determined by calculating the
256 ratios of the calculated concentrations to their nominal values, and the intraday
257 precision was determined using a one-way analysis of variance (ANOVA). As presented
258 in Table 2, the overall assay accuracies were within 100% ± 9.7% and within 100% ±
259 10.8% for TXB_2-d_4 and PGE_2-d_4, respectively. The precisions were less than 9.69% CV
260 for TXB_2-d_4 and less than 9.04% CV for PGE_2-d_4. The freeze-thaw stability was accessed
261 at three QC concentrations. After three cycles, the stability for both TXB_2-d_4 and PGE_2-
262 d_4 was above 83.7%. After 4 hours at room temperature, the stability for the surrogate
263 analyte was above 81.5%. The matrix stability for the authentic analytes, TXB_2 and
264 PGE_2, was tested at 0.3 and 20 ng/mL. Stability samples going through 3 freeze-thaw
265 cycles or 4 hours on benchtop were compared against the samples stored at -80 °C
266 immediately after preparation. Freeze-thaw and benchtop stability for TXB_2 were
267 above 97.2% and 97.9%, respectively, and were above 96.9% and 94.3% for PGE_2,
268 respectively.

269 **4.2 Whole blood assay optimization and application**

270 The design of whole blood assay

271 For the research of COX inhibitors, *in vitro* screening assays, utilizing purified enzymes,
272 cell culture and human whole blood, have been developed. While enzyme assay is fast,
273 it only measures the effect of the drug on the enzyme itself. Cell assay can reflect drug-
274 cell interactions, but still neglects plasma protein binding of the drug and other blood

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275 compounds. Thus, we are particularly interested in human whole blood assays. COX-
276 1 human blood assay described in the literature falls into three general categories:
277 freshly isolated platelet assay, blood clotting assay, and heparinized whole blood
278 assay. Platelets constitutively express COX-1. Patrono *et al.* showed that TXB_2
279 production represents platelet COX-1 activity in response to endogenously formed
280 thrombin[21]. Thus, COX-1 potency can be calculated by measuring TXB_2
281 concentration from platelet assay. However, purified platelet does not resemble
282 physiological conditions, so its results can hardly be conferred to the human organism.
283 Patrignani *et al.* used unheparinized human whole blood and measured TXB_2
284 production for COX-1 inhibition after spontaneous blood clotting[14]. Brideau *et al.*
285 further demonstrated that TXB_2 production in spontaneously clotted blood reached a
286 plateau after 60 min. While the TXB_2 level was close to the baseline within the first
287 few minutes, at as early as 30 min it got to ~50% of the level at 60 min[7]. As a result,
288 to accurately access the compounds' COX-1 inhibition, testing compounds at
289 concentrations covering a full inhibition curve should be added to sub-aliquoted blood
290 as soon as the blood was collected. With the set up and blood handling requirement
291 of our on-site clinic and our lab, we had difficulty executing the blood clotting COX-1
292 assay to achieve proper result.

293 For the third option, Young *et al.* and Glaser *et al.* described methods using
294 heparinized human whole blood with calcium ionophore incubation for COX-1
295 inhibition[15,22]. Esser *et al.* modified the method by having blood samples for COX-
296 1 and COX-2 incubated together with testing compounds[16]. The advantage of this
297 approach is to have testing compound incubated in whole blood before splitting into

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298 COX-1 and COX-2 assays, minimizing the variations between the inhibited samples
299 going into next steps. On the other hand, COX-1 platelet and unheparinized blood
300 assays need to be processed separately from COX-2 assays because the blood used for
301 COX-2 must contain anti-coagulant to allow for the incubation with LPS. We used a
302 slightly modified approach for the heparinized blood assay as demonstrated in Figure
303 3. The blood samples used for COX-1 were split from those of COX-2 after incubation
304 with the testing compounds, which kept only one set of samples in the process until
305 immediately before stimulants were added.

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

318 Whole blood assay optimization

319 After the freshly collected heparinized human blood was incubated with the test
320 compound for an hour, the samples were divided into two aliquots. To determine

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321 COX-1 activity, calcium ionophore was added to one of the aliquots. After 1 hour of
322 incubation at 37 °C, plasma was harvested for the measurement of TXB_2. Calcium
323 ionophore concentrations ranging from 5 to 25 µM were tested. As shown in Figure
324 4(a), while TXB_2 production level increased as the concentration of the stimulant
325 increased, it leveled off and trended lower at 20 and 25 µM. At even higher calcium
326 ionophore concentration, e.g. at 50 µM, various degrees of hemolysis were observed
327 in collected plasma samples. We chose 15 µM calcium ionophore concentration for
328 our experiment. We also tested calcium ionophore incubation time from 15 minutes
329 to 3 hours. As expected, TXB_2 was released very rapidly upon platelet activation, and
330 no obvious trend was noted. An incubation time of 1 hour was selected.

331 The second aliquot of the drug-inhibited blood sample was used to determine COX-2
332 potency with LPS stimulation. Various concentrations of LPS at 0.05, 0.1, 0.5, 1, 5, 10,
333 50, and 100 µg/mL were tested for COX-2 stimulation. The resulted PGE_2
334 concentration didn't show any obvious trend. This was evidenced by the wide range
335 of LPS concentrations in previously reported COX-2 whole blood experiments. We
336 chose 10 µg/mL as the LPS incubation concentration for our experiment. PGE_2
337 productions from various LPS incubation time at 2, 4, 7, 24 and 30 hours, were plotted
338 in Figure 4(b). PGE_2 concentration increased more than 10 folds from 2 h to 24 h and
339 then dropped down at 30 hours. While we cannot rule out the possibility that the most
340 optimum incubation time occurs at the middle point of our tested time profile. The
341 overall increase from 7 to 24 hours incubation was not significant, so we only
342 investigated time frames in which the experiment could be finished within the same

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343 day of blood collection or on the following day. As a result, 24 hours LPS incubation
344 was chosen for our experiment.

345 **4.3 Comparison of LC-MS/MS and ELISA results**

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

358 COX-1 and COX-2 inhibition curves generated by two different analytical methods are
359 shown in Figure 5. While the shapes of the inhibition curve were similar, the absolute
360 levels of the quantitation results were quite different. For both TXB_2 and PGE_2 ELISA
361 assays, the polyclonal binding antibodies were known to have cross-reactivity with
362 various metabolites, resulting in 100%~200% overestimation compared to the more
363 specific LC-MS/MS method. As shown in Table 3, the calculated IC_{50} values from both
364 analytical methods were comparable for all four compounds except for that of COX-1
365 inhibition of etoricoxib. The results from ELISA (85.6 µM) and LC-MS/MS (149 µM)

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366 were both close to the upper limit of concentration range (1.2 pM - 200 µM). For highly
367 selective COX-2 inhibitors like etoricoxib, higher compound concentrations are
368 needed to complete its COX-1 inhibition curve at its fully inhibited side (Figure 5a).

369

370 **5. Conclusion**

371 In the present study, an LC-MS/MS method for the quantitation of TXB_2 and PGE_2 for
372 the plasma samples of COX inhibition human whole blood assay was developed using
373 surrogate analyte approach. The LC-MS/MS method eliminates the cross-reactivity
374 with metabolites found in ELISA kits. The assay is sensitive and high-throughput. The
375 response factor and parallelism between surrogate and authentic analyte were
376 investigated. A response factor was not applied when determining analyte
377 concentration from the surrogate analyte calibration curve since the difference was
378 less than 5%. Whole blood COX inhibition assay was optimized for sample
379 pretreatment, stimulant concentration and incubation time. The qualified TXB_2/PGE_2
380 combo assay was used to measure the COX-1 and COX-2 IC_{50} of four COX inhibitors.
381 The IC_{50} values were comparable between the LC-MS/MS and ELISA methods. In the
382 future, the method can also be used in plasma and tissue samples of animal species,
383 such as rat and mouse, that contain antibodies interfering with commercial ELISA kits.

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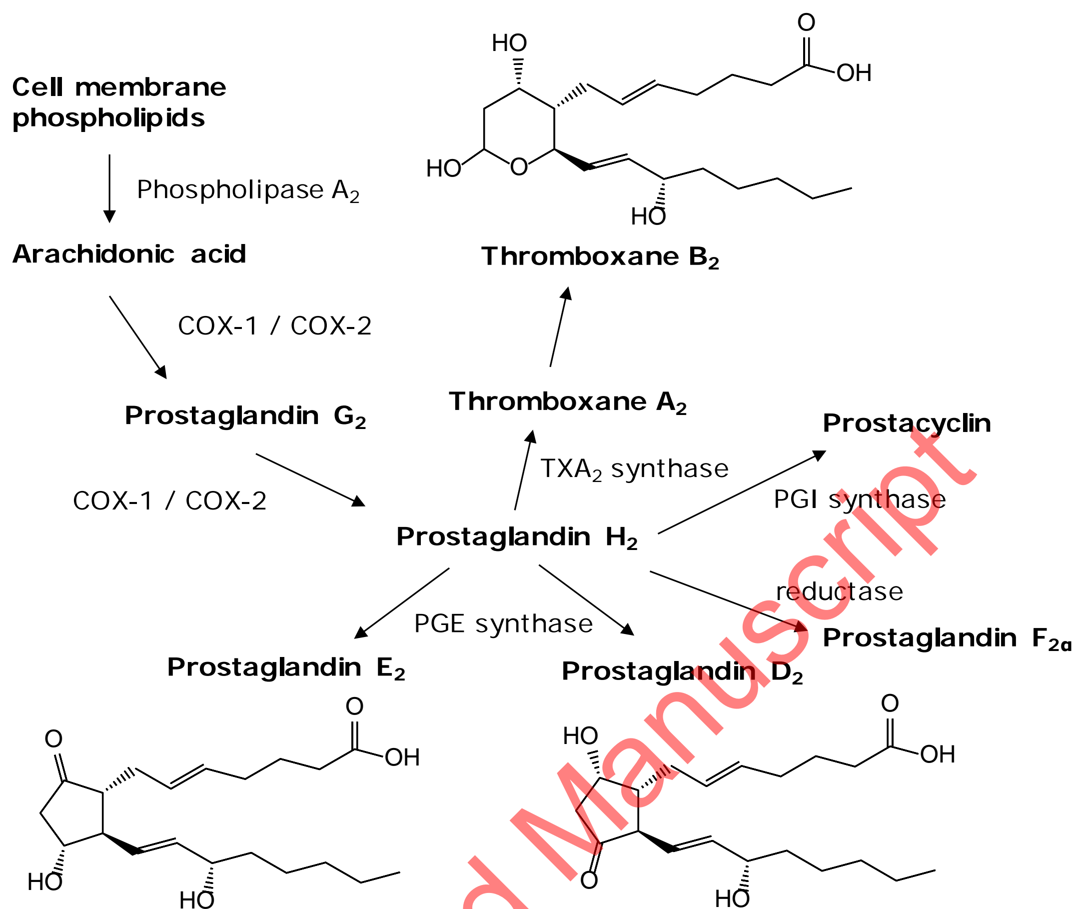
385 Figure 1. Overview of the arachidonic acid cascade through the COX pathway.



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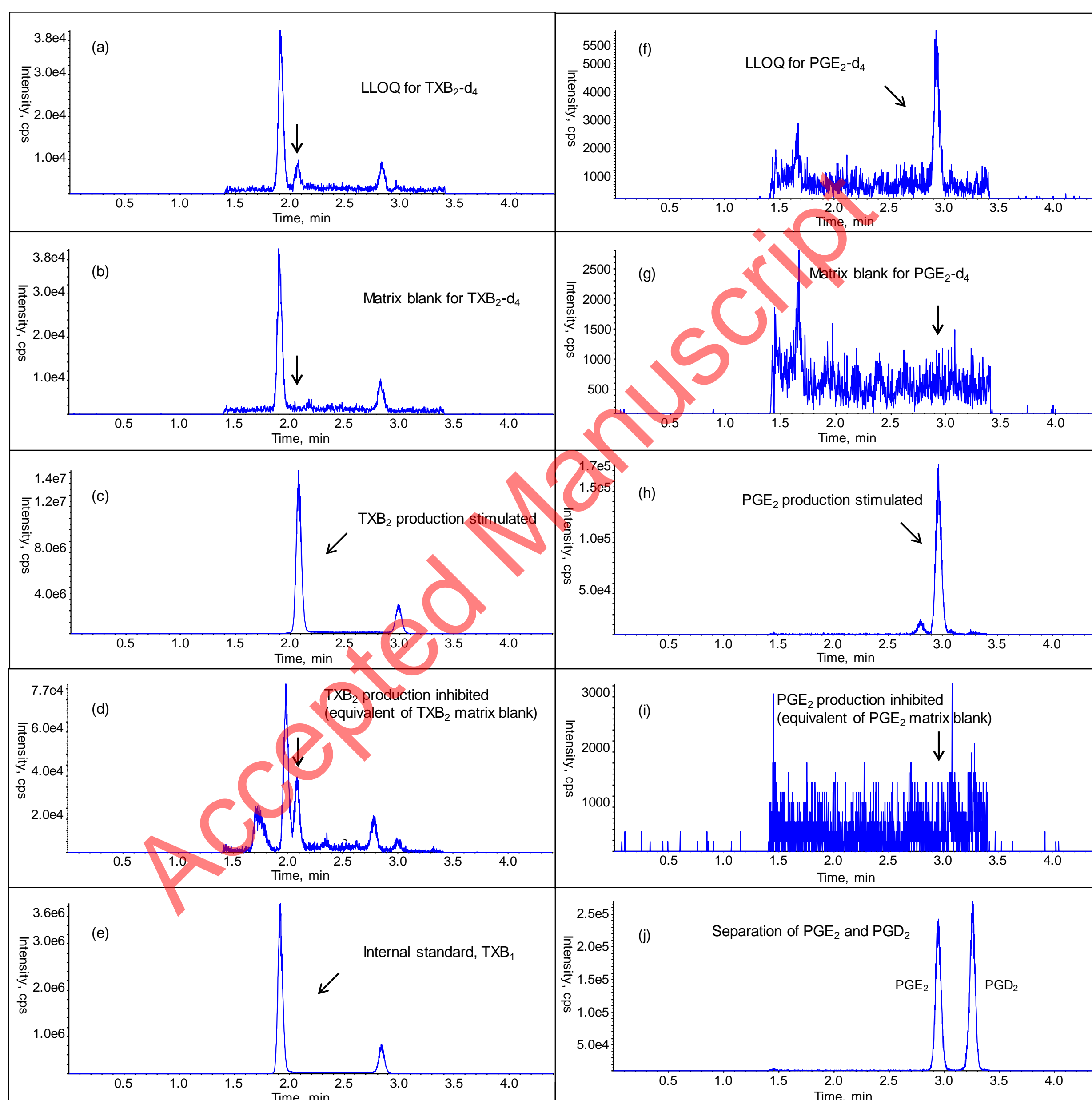
387 Figure 2. Chromatograms of (a) TXB_2-d_4 at LLOQ, (b) matrix blank for TXB_2-d_4, (c) TXB_2
388 production stimulated, (d) TXB_2 production inhibited, (e) internal standard TXB_1, (f)
389 PGE_2-d_4 at LLOQ, (g) matrix blank for PGE_2-d_4, (h) PGE_2 production stimulated, (i) PGE_2
390 production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



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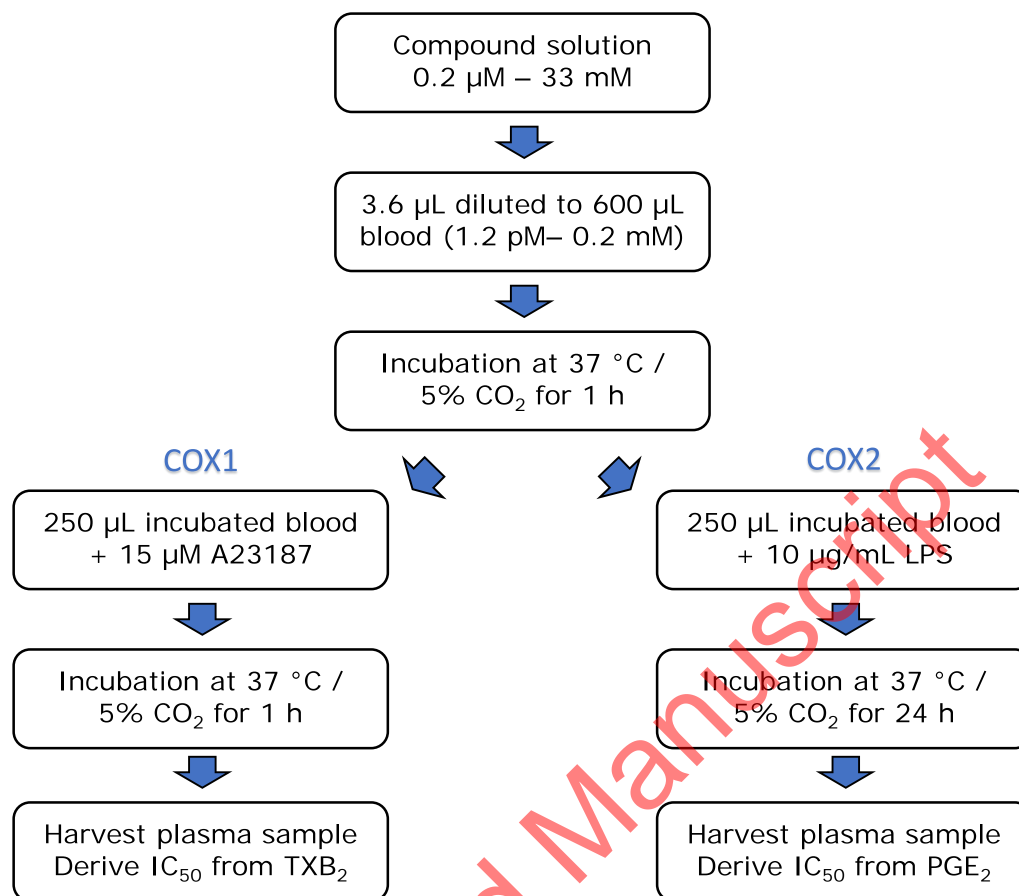
392 Figure 3. Optimized COX-1 and COX-2 whole blood assay procedure



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394 Note: A23187 = calcium ionophore; LPS = lipopolysaccharide

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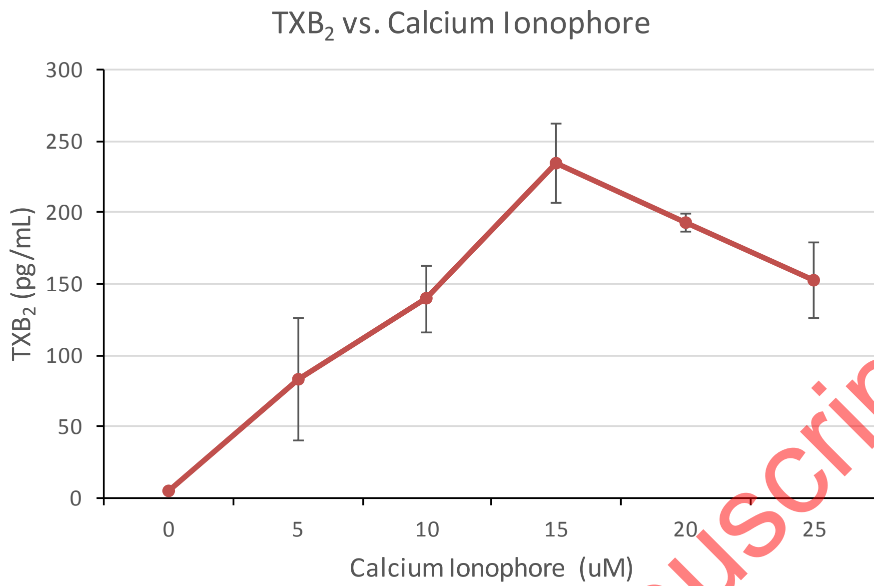
396 Figure 4(a). COX-1 assay TXB_2 production stimulated with different calcium ionophore

397 concentration



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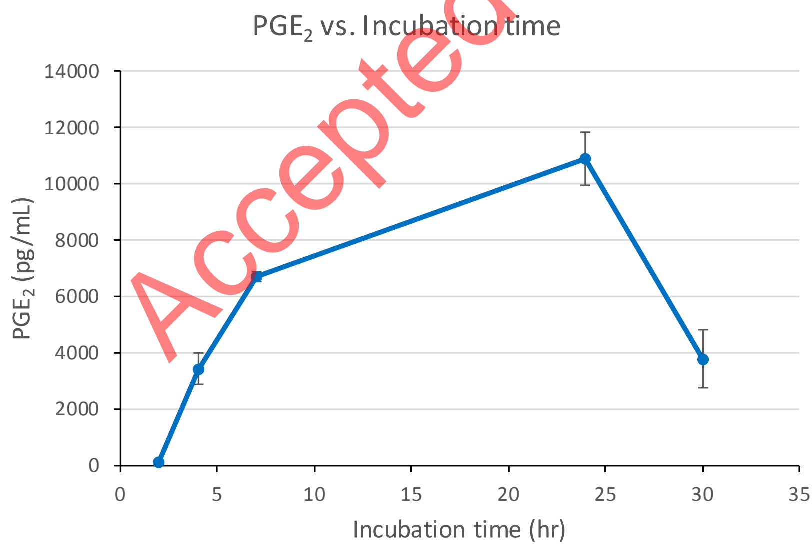
399 Figure 4(b). COX-2 assay PGE_2 production with different incubation time



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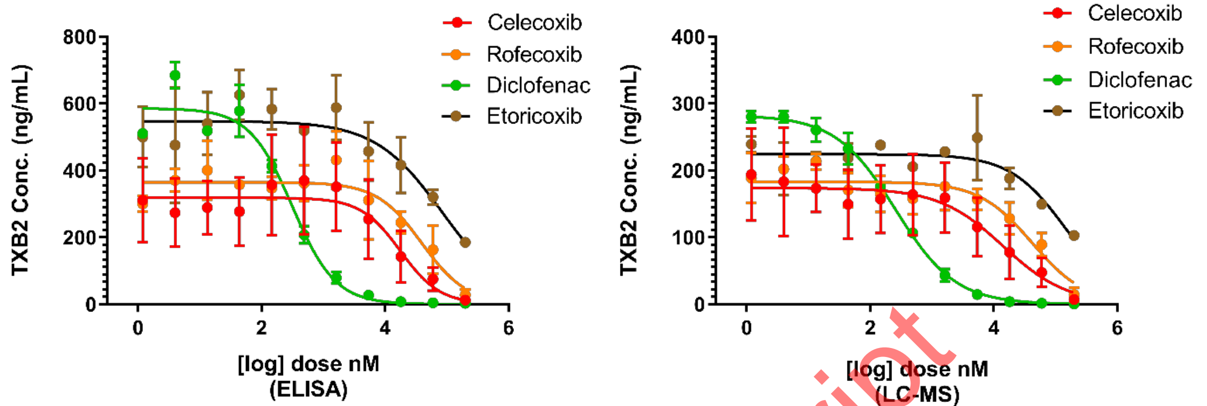
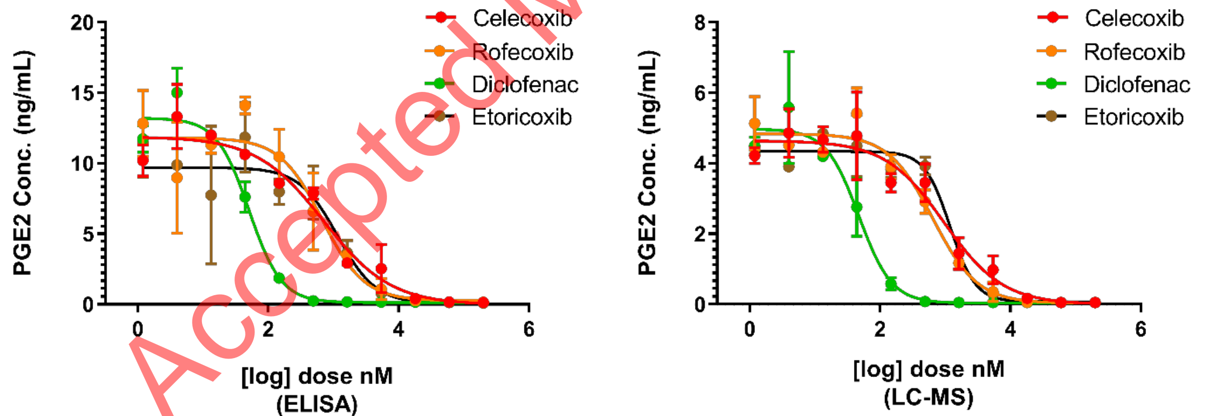
402 Figure 5(a). COX-1 inhibition curves of celecoxib, rofecoxib, diclofenac and etoricoxib
403 using data generated with ELISA and LC-MS/MS methods



404

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



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410 Table 1. Method characteristics including ion transitions, MS parameters and
411 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

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414 Table 2 (a). Accuracy, precision and stability in plasma for TXB_2-d_4 and plasma stability

415 for TXB_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		LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
Mean		0.251	18.6	350
Stability (%)		83.7	93.2	93.3
Benchtop		LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
Mean		0.244	18.1	344
Stability (%)		81.5	90.6	91.8
Number of replicates (n)	6	6	6	6
TXB_2 stability	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

416 Note: The LLOQ's of TXB_2-d_4 and PGE_2-d_4 were 0.1 and 0.05 ng/mL, respectively. As a

417 result, two LQC's, 0.15 and 0.3 ng/mL, were tested for accuracy and precision. For

418 freeze-thaw and benchtop stability of TXB_2, TXB_2-d_4, PGE_2 and PGE_2-d_4, only LQC2 (0.3

419 ng/mL) was evaluated.

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422 Table 2 (b). Accuracy, precision and stability in plasma for PGE_2-d_4 and plasma stability

423 for PGE_2

424

PGE_2-d_4	LQC1 (0.15 ng/mL)	LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
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		LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
Mean		0.335	20.3	333
Stability (%)		112	102	88.8
Benchtop		LQC2 (0.3 ng/mL)	MQC (20 ng/mL)	HQC (375 ng/mL)
Mean		0.316	19.8	337
Stability (%)		105	99.0	89.8
Number of replicates (n)	6	6	6	6

PGE_2 stability	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

425 Note: The LLOQ's of TXB_2-d_4 and PGE_2-d_4 were 0.1 and 0.05 ng/mL, respectively. As a

426 result, two LQC's, 0.15 and 0.3 ng/mL, were tested for accuracy and precision. For

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427 freeze-thaw and benchtop stability of TXB_2, TXB_2-d_4, PGE_2 and PGE_2-d_4, only LQC2 (0.3

428 ng/mL) was evaluated.

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432 Table 3 (a). COX-1 inhibition in human whole blood assay measured by ELISA and LC-

433 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

434 Note: IC_{50} values for etoricoxib were estimated based on partial inhibition curves.

435 COX-1 activities were not fully inhibited with 200 µM of etoricoxib.

436

437 Table 3 (b). COX-2 inhibition in human whole blood assay measured by ELISA and LC-

438 MS/MS

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

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441 **References**

442

443 1. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*,
444 294(5548), 1871-1875 (2001).
445 2. Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An
446 integrated omics analysis of eicosanoid biology. *J Lipid Res*, 50(6), 1015-1038 (2009).
447 3. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences
448 of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest*, 116(1), 4-
449 15 (2006).
450 4. Abraham NS, El-Serag HB, Hartman C, Richardson P, Deswal A. Cyclooxygenase-2
451 selectivity of non-steroidal anti-inflammatory drugs and the risk of myocardial
452 infarction and cerebrovascular accident. *Aliment Pharmacol Ther*, 25(8), 913-924
453 (2007).
454 5. Beales ILP. Time to reappraise the therapeutic place of celecoxib. *Ther Adv Chronic*
455 *Dis*, 9(5), 107-110 (2018).
456 6. Barcella CA, Lamberts M, McGettigan P *et al.* Differences in cardiovascular safety with
457 non-steroidal anti-inflammatory drug therapy-A nationwide study in patients with
458 osteoarthritis. *Basic Clin Pharmacol Toxicol*, 124(5), 629-641 (2019).
459 7. Brideau C, Kargman S, Liu S *et al.* A human whole blood assay for clinical evaluation of
460 biochemical efficacy of cyclooxygenase inhibitors. *Inflamm Res*, 45(2), 68-74 (1996).
461 8. Gierse JK, Zhang Y, Hood WF *et al.* Valdecoxib: assessment of cyclooxygenase-2
462 potency and selectivity. *J Pharmacol Exp Ther*, 312(3), 1206-1212 (2005).
463 9. Willenberg I, Meschede AK, Schebb NH. Determining cyclooxygenase-2 activity in
464 three different test systems utilizing online-solid phase extraction-liquid

Accepted Manuscript

465 chromatography-mass spectrometry for parallel quantification of prostaglandin E(2),
466 D(2) and thromboxane B(2). *J Chromatogr A*, 1391, 40-48 (2015).

467 10. Zhu H, Zhuang X, Liu S *et al.* Ultrahigh-performance liquid chromatography/tandem
468 mass spectrometry method for evaluating enzyme activity and screening inhibitors of
469 cyclooxygenase-2. *Rapid Commun Mass Spectrom*, 28(16), 1792-1800 (2014).

470 11. Cao H, Yu R, Tao Y, Nikolic D, van Breemen RB. Measurement of cyclooxygenase
471 inhibition using liquid chromatography-tandem mass spectrometry. *J Pharm Biomed*
472 *Anal*, 54(1), 230-235 (2011).

473 12. Shinde DD, Kim KB, Oh KS *et al.* LC-MS/MS for the simultaneous analysis of arachidonic
474 acid and 32 related metabolites in human plasma: Basal plasma concentrations and
475 aspirin-induced changes of eicosanoids. *J Chromatogr B Analyt Technol Biomed Life*
476 *Sci*, 911, 113-121 (2012).

477 13. Gandhi AS, Budac D, Khayrullina T, Staal R, Chandrasena G. Quantitative analysis of
478 lipids: a higher-throughput LC-MS/MS-based method and its comparison to ELISA.
479 *Future Sci OA*, 3(1), FSO157 (2017).

480 14. Patrignani P, Panara MR, Greco A *et al.* Biochemical and pharmacological
481 characterization of the cyclooxygenase activity of human blood prostaglandin
482 endoperoxide synthases. *J Pharmacol Exp Ther*, 271(3), 1705-1712 (1994).

483 15. Young JM, Panah S, Satchawatcharaphong C, Cheung PS. Human whole blood assays
484 for inhibition of prostaglandin G/H synthases-1 and -2 using A23187 and
485 lipopolysaccharide stimulation of thromboxane B2 production. *Inflamm Res*, 45(5),
486 246-253 (1996).

487 16. Esser R, Berry C, Du Z *et al.* Preclinical pharmacology of lumiracoxib: a novel selective
488 inhibitor of cyclooxygenase-2. *Br J Pharmacol*, 144(4), 538-550 (2005).

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Accepted Manuscript

489 17. Jones BR, Schultz GA, Eckstein JA, Ackermann BL. Surrogate matrix and surrogate
490 analyte approaches for definitive quantitation of endogenous biomolecules.
491 *Bioanalysis*, 4(19), 2343-2356 (2012).

492 18. Liu L, Cui Z, Deng Y, Dean B, Hop CE, Liang X. Surrogate analyte approach for
493 quantitation of endogenous NAD(+) in human acidified blood samples using liquid
494 chromatography coupled with electrospray ionization tandem mass spectrometry. *J*
495 *Chromatogr B Analyt Technol Biomed Life Sci*, 1011, 69-76 (2016).

496 19. Jian W, Edom RW, Weng N. Important considerations for quantitation of small-
497 molecule biomarkers using LC-MS. *Bioanalysis*, 4(20), 2431-2434 (2012).

498 20. Lubin A, Geerinckx S, Bajic S *et al.* Enhanced performance for the analysis of
499 prostaglandins and thromboxanes by liquid chromatography-tandem mass
500 spectrometry using a new atmospheric pressure ionization source. *J Chromatogr A*,
501 1440, 260-265 (2016).

502 21. Patrono C, Ciabattoni G, Patrignani P *et al.* Clinical pharmacology of platelet
503 cyclooxygenase inhibition. *Circulation*, 72(6), 1177-1184 (1985).

504 22. Glaser K, Sung ML, O'Neill K *et al.* Etodolac selectively inhibits human prostaglandin
505 G/H synthase 2 (PGHS-2) versus human PGHS-1. *Eur J Pharmacol*, 281(1), 107-111
506 (1995).

507

508

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