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Measurement of cyclooxygenase inhibition using liquid chromatography-tandem mass spectrometry

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Abstract

Because cyclooxygenases (COX) convert arachidonic acid into pro-inflammatory cyclic endoperoxides, inhibition of these enzymes and especially the inducible COX-2 form is an important therapeutic approach to manage inflammatory diseases and possibly prevent cancer. Due to side effects of existing non-selective and COX-2 selective non-steroidal anti-inflammatory agents, the discovery of new COX inhibitors continues to be an area of active investigation. Since existing assays are slow or lack specificity, a liquid chromatography-tandem mass spectrometry (LC-MS-MS) based COX inhibition assay was developed and validated for the rapid and accurate quantitative analysis of the COX product prostaglandin-E₂. The assay was validated using four COX inhibitors, celecoxib, indomethacin, resveratrol, and diclofenac that exhibit different selectivities towards COX-1 and COX-2. The IC₅₀ values of celecoxib and resveratrol for ovine and human COX-2 were compared, and the K_m values were determined. Since considerable inter-species variation was observed, human COX-2 should be used for the discovery of COX inhibitors intended for human use. This sensitive and accurate LC-MS-MS based assay is suitable for the rapid screening of ligands for COX-1 and COX-2 inhibition and for IC₅₀ determinations.

Keywords

LC-MS-MS; COX-1; COX-2; cyclooxygenase; prostaglandin E₂

1. INTRODUCTION

Cyclooxygenase (COX)-1 and/or COX-2 are the targets of widely used non-steroidal anti-inflammatory drugs (NSAIDs). Expressed constitutively in all tissues, COX-1 is essential for such physiological processes as maintenance of the gastrointestinal tract, renal function and fever [1]. COX-2 is normally undetectable in most tissues but is induced during inflammatory, degenerative, and neoplastic processes. COX-1 and COX-2 catalyze the conversion of arachidonic acid to the endoperoxide prostaglandin H₂ (PGH₂) (see Figure 1) which is then metabolized to form prostaglandins, thromboxanes and prostacyclin by non-rate limiting enzymes [2]. In aqueous solution, unstable PGH₂ can rearrange non-enzymatically to form PGD₂ and PGE₂. By inhibiting COX-1 and/or COX-2, NSAIDs prevent the enzymatic conversion of arachidonic acid to pro-inflammatory cyclic

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endoperoxides. Based on the assumption that selective inhibition of COX-2 might reduce the side effects of NSAIDs, the discovery of selective COX-2 inhibitors has become an important area of pharmaceutical research.

To facilitate the discovery of new COX inhibitors, in vitro screening assays have been developed that utilize cells in culture [3] or purified enzymes. By carrying out enzyme assays using COX-1 and COX-2, the selectivity of inhibitors may be determined based on the ratio of their IC₅₀ values (IC₅₀ is the concentration of a compound that inhibits enzyme activity by 50%) [4]. Since assays using purified enzymes are faster, more convenient and less expensive than cell-based assays, purified COX-1 and COX-2 were used in this investigation.

Several functional COX assays using purified or recombinant enzymes have been reported and include an oxygen consumption assay [5], a peroxidase co-substrate oxidation assay [5], a radiolabeled chemical inhibition assay [6], and an enzyme-linked immunosorbent assay (ELISA) [5]. The oxygen consumption assay uses a relatively insensitive O₂ sensor and requires more COX than most other assays. The peroxidase co-substrate oxidation assay lacks accuracy since it can respond to various free radical intermediates formed during COX catalysis or might fail to respond when the test inhibitor is an antioxidant [7]. Alternatively, COX inhibition activity can be determined by assessing PGE₂ production using an ELISA or using radiolabeled arachidonic acid and HPLC with radioactivity detection [8]. The ELISA requires almost 2 days per assay and lacks selectivity since it might respond to several prostaglandins, and the radioisotope method requires specialized licensing, training, handling, and disposal of wastes.

To overcome the limitations of previous assays, we developed an in vitro COX inhibition assay based on the selective and rapid LC-MS-MS quantitative analysis of PGE₂. Although similar to existing ELISA and radioisotope methods and therefore not novel, this assay has technical advantages that include more selective measurement of PGE₂ than either the ELISA or radioisotope assays due to the combination of HPLC separation and tandem mass spectrometric detection, faster analysis than ELISA (less than 1 h total including extraction, reaction and analysis), and no need for radioisotopes. The assay was validated by using a range of potent and weak inhibitors of COX-1 and COX-2 inhibitors including celecoxib, indomethacin, resveratrol, and diclofenac.

2. EXPERIMENTAL

2.1. Materials

Ovine COX-1 (oCOX-1), ovine COX-2 (oCOX-2), human recombinant COX-2 (hCOX-2), arachidonic acid, PGE₂, d₄-PGD₂, and d₂-PGE₂ (labeled with deuterium atoms at positions 3 and 4, see Figure 1) were purchased from Cayman Chemicals (Ann Arbor, MI). The cofactors (-)-epinephrine and hematin, and the COX inhibitors indomethacin, resveratrol and diclofenac were purchased from Sigma-Aldrich (St. Louis, MO). Celecoxib was purchased from 3B PharmaChem International (Wuhan, China). All organic solvents were HPLC grade or better and were purchased from Thermo Fisher (Hanover Park, IL). Formic acid was purchased from EMD Chemicals (San Diego, CA). Purified water was prepared by using a Millipore Milli-Q purification system (Millipore, Billerica, MA). All other chemicals and solvents were ACS reagent grade, unless stated otherwise.

2.2. COX inhibition assay

In an Eppendorf tube, 146 µL of 100 mM Tris.HCl (pH 8.0) buffer, 2 µL of 100 µM hematin (co-factor) and 10 µL of 40 mM L-epinephrine (co-factor) were mixed at room temperature. Next, 20 µL of Tris.HCl (pH 8.0) buffer containing 0.2 µg COX-2 or 0.1 µg COX-1

(approximately 1 unit of enzyme; 1 unit COX utilizes 1 nmol O₂/mg/min at 37 °C) was added, and the solution was incubated at room temperature for 2 min. A 2 µL aliquot of the COX inhibitor in DMSO was added to the enzyme solution and preincubated at 37 °C for 10 min. Negative controls were identical except that 2 µL aliquots of DMSO without inhibitor were used instead.

Each COX reaction was initiated by adding 20 µL of arachidonic acid in Tris.HCl (pH 8.0) buffer to give a final concentration of 5 µM; and the reaction was terminated after 2 min by adding 20 µL of 2.0 M HCl. The surrogate standards d₄-PGE₂ and d₄-PGD₂ (10 µL aliquot of 50 ng/mL solution in methanol) were added to correct for errors or degradation during sample handling and for variation in injection volume or instrument response during LC-MS-MS. After 30 min, PGE₂, PGD₂ and their surrogate standards were extracted from each incubation mixture using 800 µL hexane/ethyl acetate (50:50, v/v). The organic phase was removed, evaporated to dryness, and reconstituted in 100 µL methanol/water (50:50, v/v) for analysis using LC-MS-MS (see details in the next section).

The concentration of PGE₂ in each sample was measured using LC-MS-MS, and the percent of COX inhibition by each test solution was determined by comparing the amount of PGE₂ produced in the experiment with that produced in the negative control incubation. The formation of PGD₂ was measured for quality control purposes, since the levels of PGD₂ should be proportional to those of PGE₂. For IC₅₀ value determination, 12 different concentrations of each inhibitor were assayed three times. The IC₅₀ value of each inhibitor toward COX-1 or COX-2 was determined by plotting and analyzing the inhibition curve data using Graph Pad Prism 5 software (Mountain View, CA). The selectivity of each inhibitor towards COX-2 was calculated as the ratio of the IC₅₀ values (COX-2/COX-1). Using 7 concentrations of arachidonic acid from 0 to 32 µM, the initial rates of formation of PGE₂ were determined for ovine COX-1, ovine COX-2 and human COX-2 using LC-MS-MS. From these data, Michaelis-Menten curves were plotted, and the K_m values were determined using SigmaPlot 9 software (Systat Software; San Jose, CA).

2.3. LC-MS-MS

Negative ion electrospray tandem mass spectrometric measurement of PGE₂ was carried out using an Applied Biosystems (Foster City, CA) API 4000 triple quadrupole mass spectrometer equipped with a Shimadzu (Columbia, MD) Prominence HPLC system based on the method of Cao *et al.* [9]. A Waters (Milford, MA) XTerra MS C₁₈ analytical column (2.1×50 mm, 3.5 µm) was used for HPLC separations with an isocratic mobile phase consisting of acetonitrile/aqueous 0.1% formic acid (35:65; v/v) at a flow rate of 200 µL/min. The deprotonated molecules of *m/z* 351 and *m/z* 355 corresponding to PGE₂ and the surrogate standard d₄-PGE₂, respectively, were selected for collision-induced dissociation at a collision energy of -23 eV. The abundant product ions of *m/z* 271 and *m/z* 275, corresponding to the [M-H-2H₂O-CO₂]- product ions of PGE₂ and d₄-PGE₂, respectively, [9] were measured using selected reaction monitoring.

3. RESULTS AND DISCUSSION

3.1. Assay optimization

Beginning with the COX incubation conditions cited in the literature [10], multiple parameters were optimized including the enzyme level, the substrate concentration, the reaction time, and the post-reaction extraction time. A preincubation of at least 10 min was found to be necessary before adding the arachidonic acid substrate because potent COX-2 inhibitors such as celecoxib are often time-dependent and require several minutes of interaction with the enzyme to reach full inhibition potency [11]. The optimum amount of

COX per incubation was approximately 1 unit which varied from 0.1 μg to 0.2 μg according to the specific activity of the enzyme preparation. A reaction time of 2 min was selected because the production of PGE_2 was linear up to 2 min after which the reaction rate declined. Under the conditions of the COX incubation, the half-life of PGH_2 was determined to be ~ 5 min. Therefore, samples were extracted after 30 min to allow time for spontaneous, non-enzymatic formation of PGE_2 and PGD_2 from the enzymatic product PGH_2 .

Since Marnett *et al.* [12] reported that substrate concentration can affect the selectivity of COX inhibition, this variable was investigated using the COX-2 selective inhibitor celecoxib. We confirmed that as the concentration of arachidonic acid was increased, the COX-2 selectivity of celecoxib also increased. For example, using celecoxib at 10 μM , and arachidonic acid concentrations of 1 μM , 5 μM or 100 μM , the selectivity of celecoxib for ovine COX-2 increased from 0.99 to 2.52 and then to $>1,000$, respectively. This observation confirmed the report of Marnett *et al.* [12].

The K_m values of ovine COX-1, ovine COX-2 and human COX-2 were determined to be $4.67 \pm 0.56 \mu\text{M}$, $1.94 \pm 0.39 \mu\text{M}$ and $3.66 \pm 0.51 \mu\text{M}$, respectively. The Michaelis-Menten curves for these determinations are shown in Figure 2. These values are similar to those determined previously using radiolabeled arachidonic acid for ovine and human COX-1 of $3.7 \pm 0.5 \mu\text{M}$ [13] and 2.9 μM [14], respectively. Based on this information, an arachidonic acid concentration of 5 μM was selected for use in all subsequent COX reactions.

Marnett *et al.* [12] predicted that if the COX reaction mixture is static and is not stirred or shaken, higher COX-2 selectivity might be observed for a COX inhibitor than when the reaction mixture is agitated. This prediction was based on the hypothesis that a selective COX-2 inhibitor might dissociate rapidly from COX-1 but remain tightly bound to COX-2, resulting in a longer duration of action against COX-2 than COX-1. Using celecoxib and ovine COX, we found that gently shaking the COX reaction mixture during incubation produced more reproducible results than those obtained using static reaction conditions. However, the IC_{50} values and ovine COX-2 selectivity of celecoxib were identical for both groups regardless of agitation. Therefore, all subsequent COX reactions were carried out using gentle shaking to mix the samples and thereby enhance the reproducibility of the measurements.

3.2. COX inhibition assay validation

During LC-MS-MS, the chromatographic peaks corresponding to PGE_2 and PGD_2 were separated completely with retention times of 3.0 min and 3.5 min, respectively (see LC-MS-MS chromatograms in Figure 3). Since an isocratic mobile phase was used, samples could be injected onto the LC-MS-MS system at least every 5 min. The surrogate standards $\text{d}_4\text{-PGE}_2$ and $\text{d}_4\text{-PGD}_2$ co-eluted with the corresponding unlabeled prostaglandins providing correction factors for errors during sample preparation or fluctuations in mass spectrometer response. Since the concentration of PGE_2 always exceeded that of PGD_2 (Figure 3), PGE_2 levels were used instead of PGD_2 levels for the determination of COX inhibition and IC_{50} values. The ratio of PGE_2 to PGD_2 remained a constant value of 4.6 for these reaction conditions. Any deviation in this ratio would be an indication of assay problems such as contamination of the samples with PGE_2 or PGD_2 , contamination of COX with prostaglandin synthases, or co-eluting substances interfering with the LC-MS-MS analysis.

The calibration curve for the measurement of PGE_2 was linear ($r^2 > 0.999$) over the range 0.10 to 500 ng/mL. The limit of detection and limit of quantitation were 20 pg/mL and 100 pg/mL, respectively. The intraday and interday accuracies of PGE_2 measurement were from 99.4 to 100.8% and the precision was within 3.2% for concentrations of 500 pg/mL to 10 ng/

mL. For additional details concerning the measurement of PGE₂ using LC-MS-MS, see Cao *et al.* [9].

The IC₅₀ values for the inhibition of COX by the NSAIDs celecoxib, indomethacin, diclofenac, and the natural product resveratrol are shown in Table 1, and the inhibition curves used to calculate the IC₅₀ values of these compounds are shown in Figures 4 and 5. The inhibition of ovine COX-1, ovine COX-2 and human COX-2 were evaluated, but no human COX-1 was available from commercial sources for this study. Diclofenac is a potent, nonselective inhibitor of COX that exhibits a time-dependent two-step mechanism of inhibition [15]. The IC₅₀ values for diclofenac inhibition of ovine COX-1, ovine COX-2 and human COX-2 were 0.06 μ M, 0.79 μ M and 0.40 μ M, respectively, and the IC₅₀ ratio (ovine COX-1/human COX-2) was 0.15. Diclofenac did not show any species differences when comparing the data for ovine COX-2 and human COX-2. Dannhardt *et al.* [16] reported similar results with IC₅₀ values for COX-1 and COX-2 that were 0.01 μ M and 0.03 μ M, respectively, and an IC₅₀ ratio (COX-1/ COX-2) of 0.33. Please note that Dannhardt *et al.*, used bovine aortic coronary endothelial cells for the evaluation of COX-1 and COX-2 inhibitors instead of purified enzymes.

Indomethacin is a time-dependent COX inhibitor with slightly higher affinity for COX-1 than for COX-2 [17]. Previously, IC₅₀ values of 0.08 μ M and 1 μ M were reported for ovine and human COX-1 and COX-2, respectively [17]. In this investigation, the IC₅₀ value of indomethacin for ovine COX-1 was 0.42 μ M, and the IC₅₀ value for human COX-2 was 2.75 μ M, which are similar to the literature values. Indomethacin did not show any significant species differences between inhibition of ovine COX-2 and human COX-2 (Table 1).

The only natural product evaluated in this study, resveratrol, has been reported to non-selectively inhibit COX-1 and COX-2 with IC₅₀ values of 0.83 ± 0.44 μ M and 0.99 ± 0.40 μ M, respectively [18]. In that paper, the authors did not mention whether ovine, human or other form of COX was used. As shown in Table 1, we found similar IC₅₀ values of 0.86 ± 0.70 μ M for ovine COX-1 and 3.06 ± 2 μ M for human COX-2. Resveratrol showed species selectivity of ~20-fold for inhibition of ovine COX-2 vs. human COX-2 (Table 1).

Celecoxib is a time-dependent, high affinity COX-2 selective inhibitor [17], that inhibited ovine COX-1 and human COX-2 with IC₅₀ values of 30 μ M and 50 nM, respectively (Table 1 and Figure 5). As expected, celecoxib showed high COX-2 selectivity (600:1) when comparing the ratio of the IC₅₀ values for human COX-2 to ovine COX-1 (Table 1). However, celecoxib inhibited ovine COX-2 with an IC₅₀ value of only 3.6 μ M. Our data are consistent with the results of Penning *et al.* who reported celecoxib IC₅₀ values of 40 nM toward COX-2 and 15 μ M to COX-1 in *in vitro* COX-1 and COX-2 assays [19]. Also, the 600-fold selectivity of celecoxib measured in our functional assay is similar to the previously reported 300-fold selectivity of celecoxib for COX-2 compared to COX-1 [12,17].

Despite examining COX inhibition data for celecoxib and other compounds in the literature, we could find no other examples of large species differences in selectivity between ovine COX-2 and human COX-2. The other NSAIDs that we investigated did not show IC₅₀ species differences for COX-2 inhibition as large as those observed for celecoxib (Table 1). Since celecoxib showed the highest affinity for human COX-2 among the inhibitors evaluated in this study, its high species selectivity might be related to a unique fit in the human COX-2 active site that cannot occur in the structurally different ovine COX-2 active site. Because of the possibility of strong species selectivity of COX-2 ligands, we

recommend using human COX-2 instead of ovine COX-2 for the discovery and optimization of new COX-2 inhibitors.

Although this LC-MS-MS COX assay is based on the formation of PGE₂, the COX-catalyzed formation of prostaglandins from arachidonic acid involves first, a cyclooxygenase reaction, and second, a peroxidase reaction (Figure 1). Therefore, drugs discovered using this assay might inhibit the cyclooxygenase and/or the peroxidase reactions. For newly discovered COX inhibitors, the specific mechanism of inhibition would need to be investigated using additional experiments.

4. CONCLUSIONS

A COX inhibition assay based on the selective measurement of PGE₂ using LC-MS-MS has been developed, optimized and validated by analyzing known COX inhibitors. This sensitive assay uses small amounts of enzyme that are comparable to ELISA-based assays. The assay is also fast and requires less than 5 min per sample for LC-MS-MS analysis. When comparing the IC₅₀ values of well characterized COX inhibitors for ovine COX-2 vs. human COX-2, significant species differences were observed for some inhibitors such as resveratrol and celecoxib. In view of this species selectivity for some COX inhibitors, the use of human COX-2 is recommended whenever possible.

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Figure 1.

Arachidonic acid metabolism through the COX pathway. Cyclooxygenases COX-1 and COX-2 convert arachidonic acid to the intermediate prostaglandin PGG₂ and then to PGH₂ which either spontaneously decomposes to form primarily PGE₂ and PGD₂ or is converted by other enzymes to thromboxane-A₂, prostacyclin-GI₂, or prostaglandins such as PGE₂, PGD₂ and PGF₂. (* indicates sites of deuterium labeling in the surrogate standards d₄-PGE₂ and d₄-PGD₂)

Figure 2. Michaelis-Menten curves for A) ovine COX-1; B) ovine COX-2; and C) human COX-2. Using these data, K_m values were determined to be $4.67 \pm 0.56 \mu\text{M}$, $1.94 \pm 0.39 \mu\text{M}$ and $3.66 \pm 0.51 \mu\text{M}$, respectively.

Figure 3.

Examples of LC-MS-MS selected reaction monitoring (SRM) data showing the detection of PGE₂ (solid line, SRM m/z 351 $\rightarrow$ m/z 271, retention time 3.0 min) in reaction mixtures after incubation of COX with arachidonic acid and (A) no inhibitor (control); (B) 50 nM celecoxib producing 50% inhibition of PGE₂ formation; and (C) 33 μ M celecoxib producing >90% inhibition of PGE₂ formation. The surrogate standards [d_4]-PGE₂ and [d_4]-PGD₂ (dashed line, SRM m/z 355 $\rightarrow$ m/z 275) were added at a constant level to each sample.

Figure 4. Determination of IC_{50} values for the inhibition of COX-1 and COX-2 by indomethacin, resveratrol and diclofenac. These data were obtained using the new LC-MS-MS based assay. The IC_{50} values of these compounds for human COX-2, ovine COX-2 and ovine COX-1 that were determined from these data are shown in Table 1.

Figure 5.

Determination of the IC₅₀ values of celecoxib, a COX-2 selective inhibitor, for the inhibition of purified ovine COX-1, purified ovine COX-2 and human recombinant COX-2. These data were obtained using the LC-MS-MS based assay. Note that human COX-2 was more sensitive to inhibition by celecoxib than was ovine COX-2.

Table 1

IC₅₀ values and selectivity of standard COX inhibitors determined using LC-MS-MS quantitative analysis of PGE₂.

COX inhibitor	Measured IC ₅₀ (μM) (N=3; ± std. dev.)		Selectivity	
	ovine COX-1	ovine COX-2	ovine COX1 / ovine COX2	ovine COX1 / human COX2
Resveratrol	0.86 ± 0.10	0.15 ± 0.06	5.57	0.28
Celecoxib	30.0 ± 15.3	3.16 ± 2.16	9.49	600
Indomethacin	0.42 ± 0.21	1.32 ± 0.49	0.32	0.16
Diclofenac	0.06 ± 0.02	0.79 ± 0.21	0.08	0.16