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Development and validation of an automated SPE-LC-MS/MS assay for valdecoxib and its hydroxylated metabolite in human plasma

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Abstract

A sensitive and specific liquid chromatography-tandem mass spectrometry assay was developed to quantitate valdecoxib (I) and its hydroxylated metabolite (II) in human plasma. The analytes (I and II) and a structurally analogue internal standard (IS) were extracted on a C_{18} solid phase extraction (SPE) cartridge using a Zymark RapidTrace™ automation system. The chromatographic separation was performed on a narrow-bore reverse phase Zorbax XDB-C_8 HPLC column with a mobile phase of acetonitrile:water (50:50, v/v) containing 10 mM ammonium acetate. The analytes were ionized using negative electrospray mass spectrometry, then detected by multiple reaction monitoring (MRM) with a tandem mass spectrometer. The precursor to product ion transitions of *m/z* 313 → 118 and *m/z* 329 → 196 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.5–200 ng/ml of I and II in human plasma with absolute recoveries from plasma at 91 and 86%, respectively. The lower limit of quantitation was 0.5 ng/ml for I and II. Acceptable precision and accuracy were obtained for concentrations over the calibration curve ranges (0.5–200 ng/ml). Sample analysis time for each injection was 5 min, a throughput of 70 human plasma standards and samples per run was achieved. The assay has been successfully used to analyze human plasma samples to support clinical phase I and II studies.

Keywords: LC-MS/MS; Automated solid phase extraction; Valdecoxib; COX-2 inhibitor

1. Introduction

Valdecoxib (I), 4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide, is a new anti-inflammatory drug that is highly selective for inhibition of the inducible form of cyclooxygenase (COX-2) [1]. This drug (BEXTRA®, Pfizer Inc.) has been approved by US FDA for the treatments of rheumatoid arthritis, osteoarthritis and primary dysmenrrhea [2–5]. Constitutive cyclooxygenase (COX-1) appears to be responsible for most of the physiological prostaglandin production associated with gastric lining cytoprotection. However, COX-2 is involved in the acute inflammatory response

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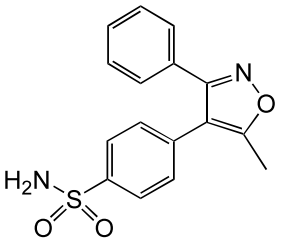
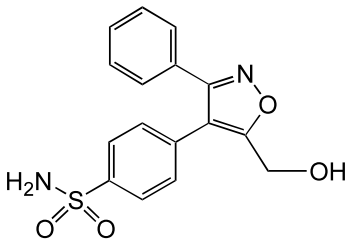
including joint inflammation. Thus, the selective inhibition of COX-2 while preserving COX-1 function provides an anti-inflammatory and analgesic effect without compromising the gastrointestinal tract [6–8]. In fact, clinical studies have demonstrated that COX-2 inhibitors lead to a significant reduction in joint pain and joint tenderness/pain and joint swelling with a statistically-significantly lower incidence of gastric ulceration [9,10]. Additionally, recent studies indicate that COX-2 inhibitors appear to provide some relief for preventing colon cancer and Alzheimer's disease [8,11–13]

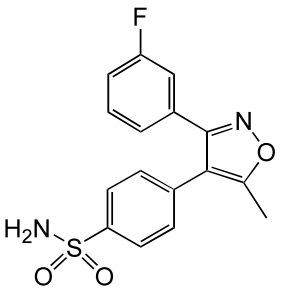
In order to fully evaluate the pharmacokinetics of valdecoxib and its pharmacological active hydroxylated metabolite in human plasma to support the clinical development, a sensitive and specific analytical method was required. Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is a proven analytical tool for the rapid, sensitive and specific determination of drugs in biological fluids [14–16]; therefore, the technique was applied in the assay development for valdecoxib and its hydroxylated metabolite. This paper describes the development and validation of a highly sensitive liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of valdecoxib (I) and its hydroxylated metabolite (II) (Fig. 1) in human plasma. The procedure consisted of an automated C_{18} solid phase extraction (SPE) of I, II and an internal standard (IS) (Fig. 1) from 0.4 ml of human plasma using a Zymark Rapid Trace™ automation system. After extraction, the samples were injected onto a reverse phase Zorbax XDB C_8 HPLC column for separation. The analytes were detected by mass spectrometry using negative ion electrospray ionization with MRM mode. The concentrations of I and II were calculated by peak area ratios of the analytes to their internal standard using calibration curves generated with weighted linear regression analysis. Acceptable precision and accuracy of the assay were achieved for quality controls, hemolyzed plasma samples, three freeze–thaw stability, dilution, long-term stability and sample processing stability. The assay was successfully used to support clinical studies in valdecoxib and parecoxib drug developments [17].



Valdecoxib (I)



Hydroxylated valdecoxib (II)



Internal standard (IS)

Fig. 1. Chemical structures of valdecoxib (I), hydroxylated valdecoxib (II) and their internal standard (IS).

2. Materials and methods

2.1. Materials

HPLC grade methanol and acetonitrile were purchased from Baxter Healthcare (Muskegon, MI). Ammonium acetate was obtained from Aldrich Chemical Corp. (Milwaukee, WI). Deionized water was generated from a Milli-Q water purifying system purchased from Millipore Corp. (Bedford, MA) and in-house high-purity nitrogen

was used for evaporation and mass spectrometer. I, II and IS reference standards as well as C-14 radiolabeled I and II were synthesized at Pfizer Inc. (Skokie, IL) with chemical purity $>99\%$. Human plasma was acquired from healthy human volunteers who did not take any medications for at least 1 month.

2.2. Preparation of standard and quality control samples

Stock solutions of I, II and IS (1 mg/ml) were separately prepared in 10 ml volumetric flasks with acetonitrile and water (50/50, v/v). These solutions were serially diluted with water to obtain the desired concentrations. The stock solutions were kept refrigerated (4 °C) and discarded 1 month after preparation. The plasma concentrations of the calibration standards were 0.5, 1, 2, 5, 10, 50, 100, 150 and 200 ng/ml. Plasma pools at 0.5, 1, 2, 50, 100, 200 and 1000 ng/ml were prepared as quality control (QC) and dilution QC samples. These standards and QC samples were prepared with appropriate volumes of I and II stock solutions in 50 ml volumetric flasks by diluting to the volumes with human plasma to achieve the desired concentrations. Then 500 μl of aliquots were transferred into 1.8 ml cryotubes (NuncInter Med. Roskilder, Denmark) that were capped and stored in a ≈ -20 °C freezer. Internal standard working solution (100 ng/ml) was prepared in a 100 ml volumetric flask by diluting 10 μg/ml of IS stock solution with water.

2.3. Extraction of samples

Frozen plasma samples were thawed in a water-bath at room temperature and centrifuged at $2000 \times g$ for 5 min at 4 °C. Aliquots of 400 μl supernatant of each human plasma standard and validation sample were placed in disposable glass tubes and 400 μl of IS working solution (100 ng/ml) was added. The plasma samples were vortexed and placed in the loading modules of a Rapid-Trace™ automatic solid phase extraction system (Zymark, Hopkinton, MA). C_{18} Bond Elut SPE cartridges (100 mg, 1 cc reservoir, Varian, Harbor City, CA) were conditioned with 2 ml of methanol and 2 ml of water, then the plasma samples were loaded onto the cartridges that were washed with 2 ml of water and eluted with 250 μl of acetonitrile. The solvent was removed under a stream of nitrogen on a TurboVap (Zymark) at room temperature to obtain residues that were reconstituted in 100 μl of the mobile phase and transferred into autosampler vials. Then 20 μl of the reconstituted samples was injected onto the LC-MS/MS system for analyses.

2.4. LC-MS/MS

LC-MS/MS analyses were performed using a system comprised of a Perkin-Elmer ISS 200 LC autosampler (Norwalk, CT), an Agilent 1050 HPLC pump (San Fernando, CA) and a PE Sciex, API-III-Plus quadrupole mass spectrometer (Concord, Ont.). The separation was carried out on a narrow-bore reverse phase Zorbax XDB-C_8 HPLC column (Chadds Ford, PA) with an isocratic mobile phase of acetonitrile:water (50:50, v/v) containing 10 mM ammonium acetate at a flow rate of 100 μl/min. The eluate from the HPLC was directly introduced into the mass spectrometer using electrospray ionization in negative ion mode. The electrospray interface and orifice voltages were set at -3700 V and -62 V, respectively. The nitrogen nebulizer gas was set at 50 psi with the nitrogen curtain gas adjusted to a constant flow rate of 1.8 l/min. The electrospray interface and mass spectrometer parameters were optimized to obtain maximum sensitivity at unit resolution. The MRM experiment was conducted by monitoring the precursor ion to product ion transitions from m/z 313 (Q1) to m/z 118 (Q3) for I, from m/z 329 (Q1) to m/z 196 (Q3) for II and from m/z 331 (Q1) to m/z 118 (Q3) for IS. The dwell time for each scan was 0.35 s. Argon was used as collision gas at gas thickness of 2.5×10^{15} molecules/cm^2 with a collision offset energy of 25 eV to induce fragmentation in the collision cell.

2.5. Method validation

For the between-run statistics, a calibration curve, a set of validation samples and human plasma blanks to simulate a routine analysis run

size of 70 were analyzed on 4 separate days. For the within-run statistics, a calibration curve, five sets of validation samples and human plasma blanks to simulate a routine analysis run size of 70 were analyzed on a fifth day. The first set of the validation samples in the within-run experiment was also used for the fifth day between-run calculation. The peak areas generated by the MRM of I, II and their internal standard (IS) were obtained from the MacQuan data system (PE Sciex, Concord, Ont.). The ratios of the peak areas of m/z 313 → m/z 118 to m/z 331 → m/z 118 and m/z 329 → m/z 196 to m/z 331 → m/z 118 were then calculated for I and II, respectively. Calibration curves were obtained by a weighted (1/concentration^2) least squares linear regression analysis. Concentrations of I and II in the samples were calculated using the equations from the appropriate calibration curves. The between-run and within-run precision and accuracy were determined by analyzing five sets of validation samples.

3. Results and discussion

3.1. Method development

An automated solid phase extraction (SPE) method was used to isolate and concentrate I and II from human plasma. Performing a SPE can be a labor intensive process, especially when large numbers of sample must be processed, thus the automation of the SPE allows samples to be processed unattended, often with improved precision and recovery. A seven-module Zymark Rapid Trace™ automated extraction system was selected to increase the throughputs of the extraction process by reducing the sample preparation time. This system is fully automated and provided high precision and reproducibility. The extraction process included conditioning 100 mg C_{18} Varian Bond Elut SPE columns, loading plasma samples, washing the column and eluting the analytes from the column. The total extraction time for 70 plasma samples with these seven modules was ≈ 1.5 h.

A narrow bore Zorbax XDB-C_8 HPLC column (50 × 2.1 mm, 5 μm, MAC-MOD Analytical, Chadds Ford, PA) was used to separate the analytes. The low flow rate of the narrow bore column was ideal for coupling to an electrospray mass spectrometer interface. Various mobile phase concentrations of acetonitrile and ammonium acetate buffer were evaluated to improve the LC separation and enhance sensitivity in mass spectrometry for I, II and IS. An isocratic system was selected with a mobile phase of acetonitrile:water (50:50, v/v) containing 10 mM ammonium acetate. The optimal flow rate for a narrow bore HPLC column (50 × 2.1 mm) would be 200–300 μl/min in order to achieve the best peak shape. However, during the validation a turbo-ionspray source was not available in our laboratory so that a lower flow rate of 100 μl/min was used to compromise the limitation of the interface. Under this condition, the retention times of I, II and IS were ≈ 3, 2 and 3.5 min, respectively. The total run time for each sample was 5 min.

Tandem mass spectrometric detection was used to provide a sensitive and selective assay for I and II in human plasma. Negative ionization was used to detect the two analytes because negative ion mass spectrometry provided at least five-fold improved signal to noise ratio for I and II than does positive ionization. The negative ion electrospray mass spectrum of I revealed a deprotonated molecular ion $[M-H]^-$ at m/z 313 with the collision-induced dissociation (CID) product ions at m/z 270, 248, 233, 206, 192, 172, 144, 80 and a base peak at m/z 118 (Fig. 2a). The negative electrospray mass spectrum of II has a $[M-H]^-$ ion at m/z 329 with the CID product ions at m/z 132, 117, 78 and a base peak at m/z 196 (Fig. 2b). The internal standard (IS) gives a $[M-H]^-$ ion at m/z 331 with the CID product ions at m/z 288, 266, 251, 236, 222, 209, 172, 144, 80 and a base peak at m/z 118 (Fig. 2c). MRM mode was used to detect I, II and IS by monitoring the transitions from the precursor ions to the most abundance product ions of m/z 313 → m/z 118, m/z 329 → m/z 196 and m/z 331 → m/z 118, respectively. The LC-MRM chromatograms of a human plasma blank with IS and 0.5 ng/ml of I and II with IS demonstrated the selectivity and specificity to the MRM techniques (Fig. 3a, b).

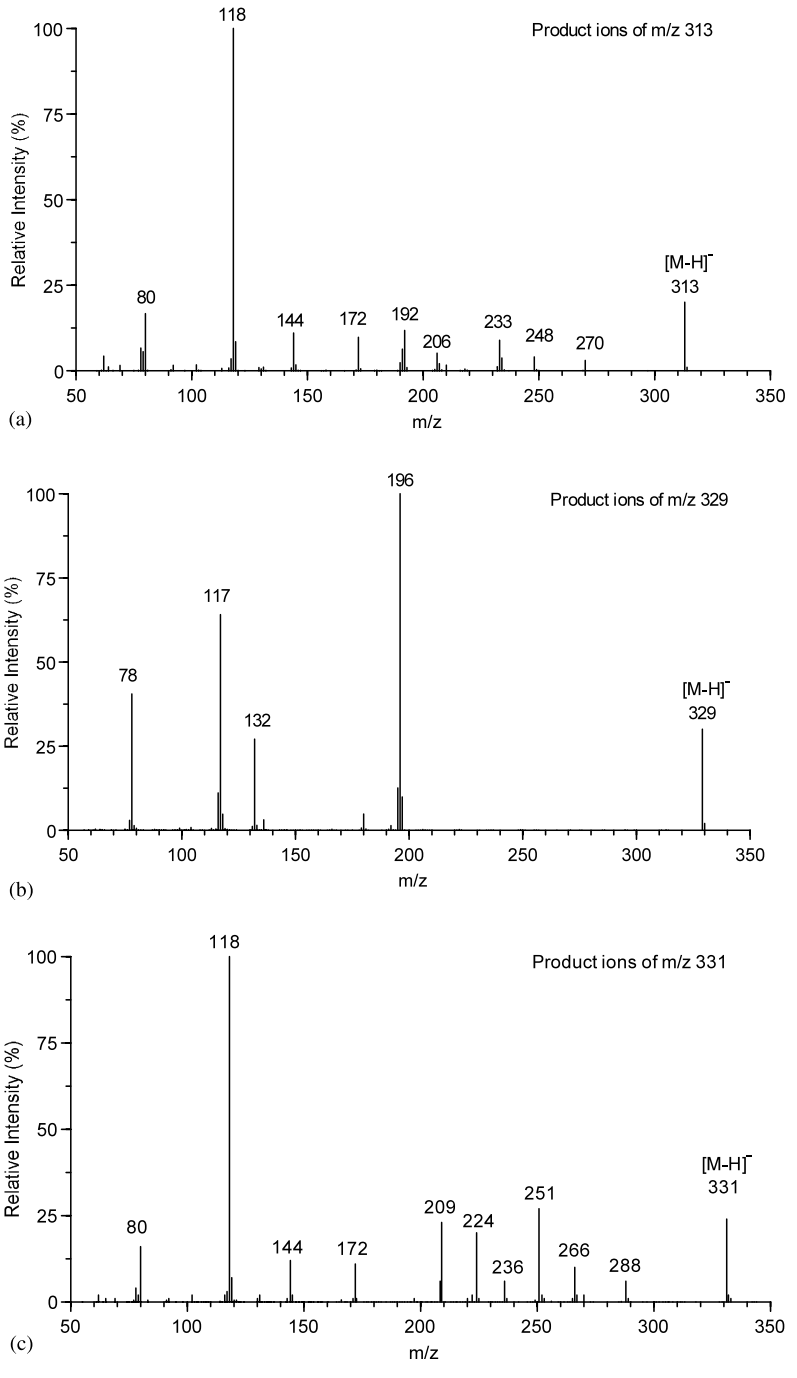


Fig. 2. CID spectra of (a) I (*m/z* 313), (b) II (*m/z* 329) and (c) IS (*m/z* 331).

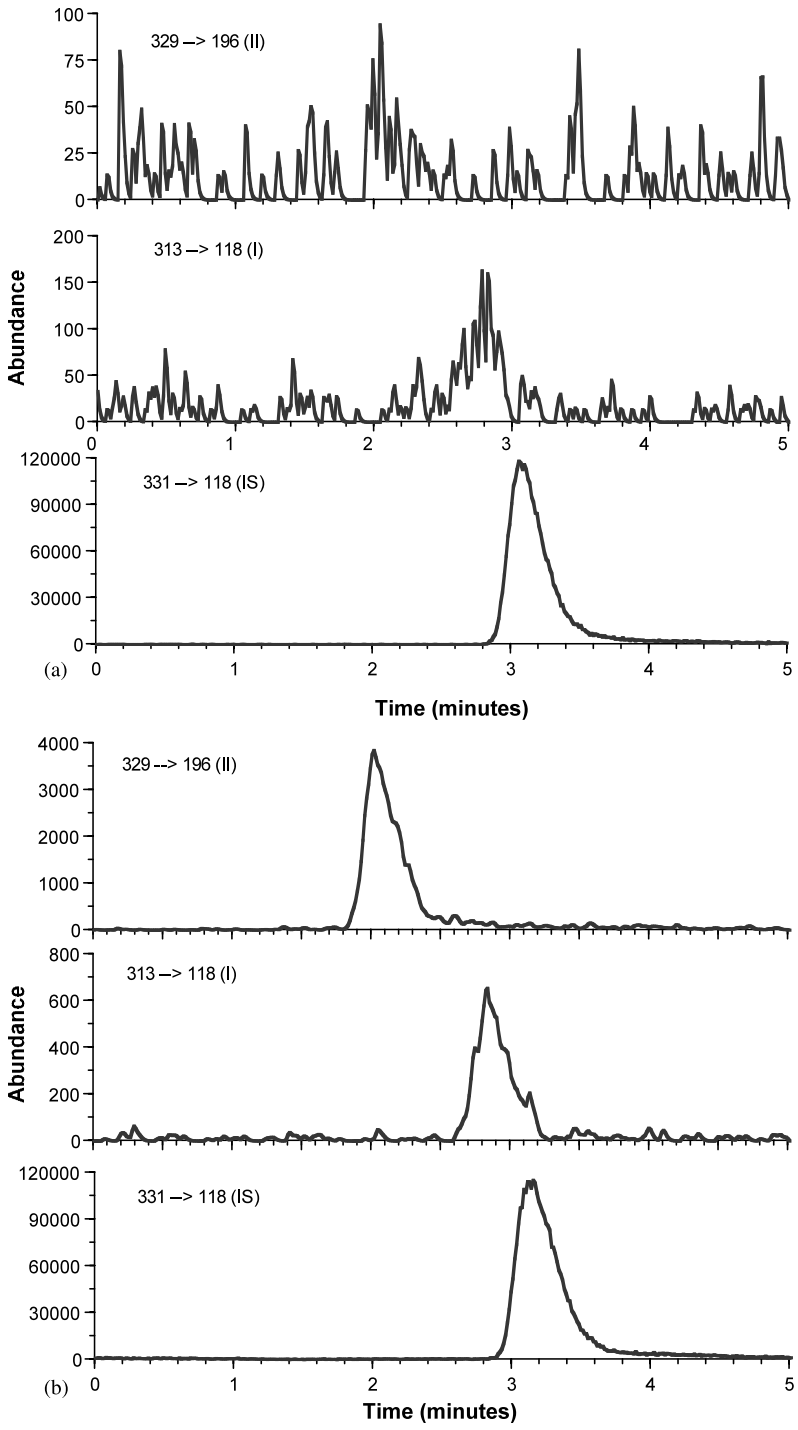


Fig. 3. Representative LC-MRM chromatograms of (a) blank human plasma spiked with IS (100 ng/ml) and (b) human plasma spiked with both I, II (0.5 ng/ml) and IS (100 ng/ml).

3.2. Extraction recovery

Blank human plasma was spiked separately with radiolabeled [^{14}C]–I and [^{14}C]–II at three different concentrations (0.5, 50 and 200 ng/ml) in triplicate and extracted by the automated SPE method described above. The radioactivity that eluted from the SPE cartridges was compared to the radioactivity spiked in the plasma before the extraction. The extraction recoveries for I and II were 90.7% ($n=9$) and 85.7% ($n=9$) throughout the concentration ranges.

3.3. Matrix effect

The blank plasma contribution in LC-MRM chromatograms at the elution regions of I, II and IS in male and female human plasma without addition of the analytes was evaluated to ensure the specificity of the method. The mean response for the peak I at the assay sensitivity limit (0.5 ng/ml) was $\approx$5.35- and 4.62-folds greater than the mean response for the peak seen in six male and female individual blank human plasma samples, respectively, at the retention time of I. The mean response for the peak II at the assay sensitivity limit (0.5 ng/ml) was $\approx$7.42- and 4.65-folds greater than the mean response for the peak seen in six male and female individual blank human plasma samples, respectively, at the retention time of II. The percent responses without and with IS at the retention time of IS in six male and six female individual blank human plasma samples were 0.389 and 0.229%, respectively. These results demonstrated that no considerable endogenous contribution from blank human plasma affected the measurement of the analytes. In addition, matrix ion suppression in the LC-MS/MS method was evaluated by comparison of the peak intensities of the analytes from the samples spiked with water or human plasma. The results showed that there was no significant difference for peak responses between these samples. This effect is most likely due to the sample cleanup with SPE extraction and the relatively longer HPLC run time.

3.4. Validation

3.4.1. Calibration curves

Calibration curves were linear over the concentration range of 0.5–200 ng/ml for both I and II. The nine point calibration curves gave acceptable results for both analytes and were used for all the calculations. The mean linear regression equations of standard curves for I and II were $y = 0.000990(\pm0.000127)+0.00411(\pm0.00083)x$ and $y = 0.00165(\pm0.00028)+0.00842(\pm0.00112)x$, respectively, where y was the peak area ratio of I to IS or II to IS and x was the concentration of I or II. The correlation coefficients of the weighted calibration curves generated during the validation ranged from 0.996 to 0.999 for I and 0.992–0.999 for II, respectively. The slope of the calibration curve of II was much greater than that of I. The difference in the slopes can be explained by the higher ionization efficiency of the hydroxylated metabolite versus the parent compound in negative ion mode mass spectrometry. The calibration curves obtained as described above were suitable for generation of acceptable data for the concentrations of I and II in the samples during the between-run and within-run validations. The validation samples were randomized daily, processed and analyzed in position either (a) immediately following the calibration curve, (b) in the middle of the run or (c) at the end of the run. The data are summarized in Table 1.

3.4.2. Lowest concentration

The lower limit of quantitation (LLOQ) for the human plasma assay was 0.5 ng/ml for both I and II. The between-run precision (expressed as coefficient of variation, CV (%)) was 6.51 and 12.0% for I and II, respectively. The between-run accuracy (expressed as analytical recovery, AR (%)) was 115 and 88.2% for I and II, respectively. The within-run precision was 6.32 and 10.8% and the accuracy was 112 and 106% for I and II, respectively.

3.4.3. Middle and upper concentrations

The middle and upper of quantitation levels ranged from 1 to 200 ng/ml of I and II in human plasma. For the between-run experiment, precision ranged from 3.66 to 10.2% and the accuracy

Table 1
Assay validation results obtained from between-run and within-run experiments for I and II in human plasma

Spiked conc. (ng/ml)	Between-run			Within-run		
	Mean conc. (ng/ml)	CV (%)	AR (%)	Mean con. (ng/ml)	CV (%)	AR (%)
Analyte I						
0.5	0.572	6.51	115	0.559	6.32	112
1	1.05	10.2	105	1.06	13.3	106
2	2.31	6.16	115	2.31	4.63	115
50	52.9	4.45	106	46.5	4.40	100
200	191	3.66	95.2	182	1.97	91.1
1000[a]	1000	8.82	100	999	2.42	99.9
$2_{f/t}$[b]	2.21	9.06	110			
$100_{f/t}$[b]	105	7.82	105			
50_{hemo}[c]	47.5	8.34	95.0			
Analyte II						
0.5	0.441	12.0	88.2	0.530	10.8	106
1	1.03	11.7	103	1.20	4.22	120
2	2.20	5.74	110	2.31	8.45	115
50	55.6	5.77	111	60.4	9.83	120
200	197	6.61	98.5	207	7.76	103
1000[a]	998	9.97	99.8	1040	3.85	104
$2_{f/t}$[b]	1.96	9.69	97.9			
$100_{f/t}$[b]	109	5.59	109			
50_{hemo}[c]	51.2	7.18	103			

[a] The sample was processed with 10-fold dilution.
[b] The sample was assayed after three freeze–thaw cycles.
[c] The sample was from hemolyzed plasma.

ranged from 95.2 to 115% for I. The precision ranged from 5.74 to 11.7% and the accuracy ranged from 98.5 to 111% for II. For the within-run experiment, the precision ranged from 1.97 to 13.3% and the accuracy ranged from 91.1 and 115% for I. The precision ranged from 4.22 to 9.83% and accuracy ranged from 103 to 120% for II.

3.4.4. Dilution

The upper concentration limits can be extended with acceptable precision and accuracy to 1000 ng/ml of I and II by a 10-fold dilution with control human plasma. For the between-run experiment, the precision was 8.82 and 9.97% and accuracy was 100 and 99.8% for I and II, respectively. For the within-run experiment, the precision was 2.42 and 3.85% and accuracy was 99.9 and 104% for I and II, respectively. The results suggested that samples whose concentrations are greater than the upper limit of the standard curve can be assayed to obtain acceptable data.

3.4.5. Freeze–thaw stability

The freeze–thaw stability of I and II was determined by measuring the assay precision and accuracy for the samples which underwent three freeze–thaw cycles. The stability data were used to support repeat analyses. The frozen plasma samples containing separated I and II were thawed at room temperature for 2–3 h, refrozen for minimum of 1 day, thawed for 2–3 h, refrozen for minimum 1 day, thawed and then analyzed. The results showed that I and II were stable in human plasma through three freeze-thaw cycles. The precision ranged from 7.82 to 9.06% and the accuracy ranged from 105 to 110% for I. The precision ranged from 5.59 to 9.69% and accuracy ranged from 97.9 to 109% for II. The results demonstrated that plasma samples could be

thawed and refrozen without compromising the integrity of the samples.

3.4.6. Effect of plasma hemolysis

Hemolyzed human plasma samples containing I and II were evaluated to ensure that the precision and accuracy are acceptable for analysis of such samples. The precision was 8.34% and the accuracy was 95.0% for I, while the precision was 7.18% and the accuracy was 103% for II. Thus, sample hemolysis did not interfere with the assay.

3.5. Assay specificity

To ensure the assay specificity, six human plasma samples from dosed subjects at times approximately equal to C_{max} and two half-lives later were analyzed using the validated method. Three ion transitions for I and II were monitored for the samples from dosed subjects and for standard solutions. The ratios of the ion transitions used in the human plasma assay to the two additional selected ion transitions from the dosed subjects were compared to the ion transition ratios from the standards of I and II. The ion transition ratios of *m/z* 313→118 to *m/z* 313→233 and *m/z* 313→118 to *m/z* 313→192 for I from the dosed subjects gave an acceptable CV of 11.8 and 12.4%. The relative ratios were 96.2 and 101% when compared to the ion transition ratios from the standard I (Table 2). The ion transition ratios of *m/z* 329→196 to *m/z* 329→132 and *m/z* 329→196 to *m/z* 329→117 for II from the dosed subjects gave an acceptable CV with 9.69 and 9.85%. The relative ratios were 92.0 and 94.4% when compared to the ion transition ratios from the standard II (Table 2). These data suggested that no unreported metabolites were produced that would interfere with the assay. Also, no endogenous substances were produced in response to administration of valdecoxib that would interfere with this assay.

Table 2
Assay specificity results for I and II in human plasma

Analyte I	Ion transition ratios (peak area)	
	(*m/z* 313→118)/(*m/z* 313→233)	(*m/z* 313→118)/(*m/z* 313→192)
Standard samples (n = 3)		
Mean	6.52	5.21
CV (%)	1.13	1.35
Dosed samples ($n = 6$)[a]		
Mean	6.27	5.25
CV (%)	11.8	12.4
Relative ratio (%)[b]	96.2	101
Analyte II	Ion transition ratios (peak area)	
	(*m/z* 329→196)/(*m/z* 329→132)	(*m/z* 329→196)/(*m/z* 329→117)
Standard samples (n = 3)		
Mean	4.99	1.60
CV (%)	6.68	1.17
Dosed samples (n = 6) [a]		
Mean	4.59	1.51
CV (%)	9.69	9.85
Relative ratio (%)[b]	92.0	94.4

[a] Equal samples ($n = 3$) were from the dosed human plasma at the time point of Cmax and at the time points of two half lives.

[b] Relative ratio (%) = (mean of dose samples/mean of standard samples) × 100.

3.6. Processed sample stability

Extracted validation samples at low, middle and high concentrations were kept standing at room temperature for over 24 h and then were reanalyzed and quantitated against calibration curves from freshly prepared standards. The precision and accuracy for I from these extracted and stored samples were 1.85–6.76% and 96.4–110%, respectively, while, the precision and accuracy for II were 4.34–9.13% and 104–117%, respectively (Table 3). The results demonstrated that extracted samples could be analyzed after standing at room temperature for at least 24 h with an acceptable precision and accuracy.

3.7. Sample collection and processing studies

In vitro studies were performed to determine whether I and II were lost and/or degraded in human blood during sample collection and processing. In Experiment A, I and II were separately

Table 3
Processed sample stability results for I and II after 24 h at room temperature

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	No. of replicas	CV (%)	Recovery (%)
Analyte I				
2	2.20	3	6.76	110
50	49.7	3	6.18	99.4
200	193	3	1.85	96.4
Analyte II				
2	2.16	3	4.34	108
50	58.6	3	9.13	117
200	209	3	5.12	104

incubated with freshly collected human blood for 15 min on ice followed by centrifugation to separate the plasma from the blood. In Experiment B, I and II were separately incubated with freshly collected human blood for 60 min on ice followed by centrifugation to separate the plasma from the blood. In Experiment C, I and II were separately incubated with freshly collected human blood for 60 min at room temperature followed by centrifugation and leaving the plasma fraction over packed erythrocytes for an additional 60 min at room temperature. Then, the plasma was separated by centrifuge and stored in a −20 °C freezer until analysis. The mean concentration of I from B (incubated on ice for 60 min) and C (incubated at room temperature for 60 min) gave CVs of 1.94 and 8.39% and relative recoveries of 96.4 and 97.9% when compared to the concentrations from control A (incubated on ice for 15 min). Similarly, the mean concentration of II from B and C gave CVs of 2.66 and 1.32% and relative recoveries of 117 and 80%, when compared to the concentrations from control A. It was thereby concluded that I and II were stable in human blood for at least 60 min at room temperature and in human plasma partitioned over erythrocytes for an additional 60 min at room temperature.

The applicability of the devices associated with the collection and processing of human plasma samples containing I and II was studied. An aliquot of the human plasma was passed through a Vacutainer needle during the transfer to a Vacutainer with a green stopper. The concentration of I from E (needle, Vacutainer, green stopper) gave a CV of 2.87% and a relative recovery of 101% when compared to the concentration from the control sample D (no collection device). Similarly, the concentration of II from E gave a CV of 6.73% and a relative recovery of 97.6% when compared to the concentration from the control sample D. These data suggested that the procedure used in the collection process to obtain the blood samples should not introduce any bias in the samples.

3.8. Long-term storage stability

The sample long-term storage stability at −20 °C was evaluated to establish acceptable storage conditions for clinical samples. Aliquots of human plasma samples spiked separately with I and II at concentrations of 2 and 100 ng/ml were analyzed on day one. Then the samples from the same pools were analyzed after storage at −20 °C for 70 or 75 days. The results indicated that I and II were stable when stored frozen at −20 °C for at least 70 days. The CV and AR for I on Day 70 ranged from 1.21 to 9.09% and 89.8–94.2%, respectively. For II on Day 75 the CV ranged from 2.13 to 5.14% and the AR was 103% (Table 4).

3.9. Length of run

A run was defined as a group of standards, validation and blank samples that were processed through automated solid phase extraction, analyzed by serial LC-MS/MS injections and calcu-

Table 4
Results for long-term frozen storage stability samples for I and II at −20 °C

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	Storage days	No. of replicates	CV (%)	Relative recovery (%)
Analyte I					
2	1.80	70	3	9.09	89.8
100	94.2	70	3	1.21	94.2
Analyte II					
2	2.06	75	3	5.14	103
100	103	75	3	2.13	103

lated from the standard curves. A total of 70 human plasma standards and samples can be analyzed per run with acceptable precision and accuracy.

4. Conclusions

An automated, high throughput and sensitive LC-MS/MS assay was developed to support valdecoxib clinical studies. Acceptable data were generated for both I and II using weighted linear regression (1/concentration^2) and full calibration curves for human plasma samples. The LLOQ was 0.5 ng/ml for both analytes. Acceptable precision and accuracy were obtained for concentrations above the sensitivity limit and within the calibration curve range of 0.5–200 ng/ml. The upper concentration limit can be extended with acceptable precision and accuracy to 1000 ng/ml by a 10-fold dilution with control human plasma into the calibration curves range. Hemolyzed human plasma can be analyzed with acceptable precision and accuracy. Collection devices for use in the collection of human blood was also evaluated and proved to be acceptable. I and II were stable for at least 60 min at room temperature in human blood and an additional 60 min at room temperature in plasma over packed erythrocytes. Extracts of I and II can be analyzed after standing at room temperature for at least 24 h with acceptable precision and accuracy. The validated SPE/LC-MS/MS human plasma assay is specific for I and II in human plasma and the samples can be stored frozen at −20 °C for at least 70 days. The assay is fast, rugged with a throughput 70 samples per run in 8 h and 140 human plasma samples on two analytical runs per day. The method was the first validated analytical assay for valdecoxib and was successfully applied in the determination of I and II in human plasma for clinical studies [15].

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