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Journal of Chromatography B, 785 (2003) 123–134
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of valdecoxib and its metabolites in human urine by automated solid-phase extraction–liquid chromatography–tandem mass spectrometry

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Received 18 June 2002; received in revised form 28 October 2002; accepted 28 October 2002

Abstract

A simple, sensitive and specific automated SPE–LC–MS–MS assay was developed and validated for determination of valdecoxib (I), its hydroxylated metabolite (II) and carboxylic acid metabolite (III) in human urine. The analytes (I, II and III) and a structural analogue internal standard (I.S.) were extracted on a C_{18} solid-phase extraction cartridge using a Zymark RapidTrace™ automation system. The chromatographic separation was performed on a narrow-bore reverse phase HPLC column with a mobile phase of acetonitrile–water (50:50, v/v) containing 10 m*M* 4-methylmorpholine (pH 6.0). The analytes were ionized using negative electrospray mass spectrometry, then detected by multiple reaction monitoring with a tandem mass spectrometer. The precursor to product ion transitions of m/z 313→118, m/z 329→196 and m/z 343→196 were used to measure I, II and III, respectively. The assay exhibited a linear dynamic range of 1–200 ng/ml for I and II and 2–200 ng/ml for III in human urine. The lower limit of quantitation was 1 ng/ml for I and II and 2 ng/ml for III. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. Run time of 5.5 min for each sample made it possible to analyze a throughput of 70 human urine samples per run. The assay has been successfully used to analyze human urine samples to support clinical phase I and II studies.

Keywords: Valdecoxib

1. Introduction

Valdecoxib (I) is a new anti-inflammatory drug that is highly selective for inhibition of the inducible form of cyclooxygenase (COX-2) [1]. This drug (Bextra, Pharmacia) has recently been approved by the US FDA for treatments of rheumatoid arthritis, osteoarthritis and pain [2–6]. Cyclooxygenase is responsible for prostaglandin synthesis. The enzyme exists as two isoforms: a constitutive form, COX-1, and an inducible form, COX-2 [7]. The constitutive COX-1 appears to be responsible for most of the physiological prostaglandin production associated with gastric lining cytoprotection. In contrast, the inducible COX-2 is involved in acute inflammatory response including joint inflammation. The selective inhibition of COX-2 while preserving COX-1 function provides an anti-inflammatory and analgesic effect without compromising the gastrointestinal tract

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00863-2

[8–10]. In fact, clinical studies have demonstrated that COX-2 inhibitors lead to a significant reduction in joint pain, joint tenderness/pain, and joint swelling with a statistically-significantly lower incidence of gastric ulceration [11,12].

Valdecoxib is metabolized primarily by cytochrome P450 2C9 and 3A4 to the pharmacological active hydroxylated metabolite (II) and the carboxylic acid metabolite (III) in humans [13–15]. A sensitive analytical method was requested for determination of the concentrations of Valdecoxib and these metabolites in human urine in order to understand their renal clearances in clinical studies. To date no high-performance liquid chromatography (HPLC) and liquid chromatography–tandem mass spectrometry (LC–MS–MS) methods have been reported for Valdecoxib. LC–MS–MS is widely used for determination of drugs in biological fluids [16–20]. The technique has advantages such as speed, sensitivity and specificity, which are required for assay development for Valdecoxib. Here, we report the development and validation of a sensitive LC–MS–MS method for the simultaneous quantitation of Valdecoxib (I) and its metabolites (II and III) (Fig. 1) in human urine. The procedure consisted of an automated C_{18} solid-phase extraction (SPE) of I, II, III and an internal standard (I.S.) (Fig. 1) from 0.5 ml of human urine using a Zymark RapidTrace™ automation system. After extraction, the samples were injected onto a reversed-phase Keystone Prism RP HPLC column for separation. The analytes were detected by tandem mass spectrometry using negative electrospray ionization with multiple reaction monitoring (MRM) mode. The concentrations of I, II and III were calculated by peak area ratios of the analytes to their internal standard using standard curves generated with weighted linear regression analysis. Acceptable precision and accuracy of the assay were achieved for quality control, freeze–thaw stability, dilution and long-term stability samples. The assay was successfully used to support clinical studies for Valdecoxib and its parenteral drug Parecoxib [14,15].

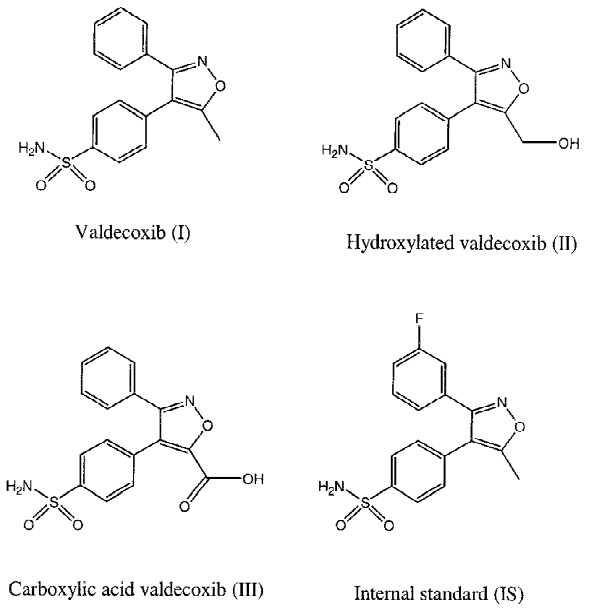

Fig. 1. Chemical structures of Valdecoxib (I), hydroxylated valdecoxib (II), carboxylic acid valdecoxib (III) and their internal standard (I.S.).

2. Experimental

2.1. Materials

All chemicals were of analytical grade and were purchased from the following suppliers: methanol and acetonitrile from Baxter Healthcare (Muskegon, MI, USA), ammonium acetate and 4-methylmorpholine from Aldrich (Milwaukee, WI, USA). High-purity water was obtained using a Millipore system (Milford, MA, USA). I, II, III and I.S. reference standards as well as ^{14}C-radiolabeled I, II and III were synthesized at Pharmacia (Skokie, IL, USA) with chemical purity greater than 99%. β-Glucuronidase (*Helix pomatia*), 0.2 M sodium acetate buffer (pH 5.0) and 0.2 *M* glycine buffer (pH 10) were purchased from Sigma (St. Louis, MO, USA). Human urine 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, III and I.S. (1 mg/ml) were separately prepared in 10-ml volumetric flasks with acetonitrile–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 urine concentrations of the calibration standards were 1, 2, 5, 10, 50, 100, 150 and 200 ng/ml. Urine pools at 1, 2, 5, 50, 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, II and III stock solutions in 100 ml volumetric flasks by diluting to the volumes with human urine to achieve the desired concentrations. Then 700-μl of aliquots were transferred into 1.8-ml cryotubes (NuncInter Med. Roskilder, Denmark) that were capped and stored in an approximately −20 °C freezer. The I.S. working solution (100 ng/ml) was prepared in a 100-ml volumetric flask by diluting 10 μg/ml of I.S. stock solution with 100 m*M* ammonium acetate buffer (pH 6.8).

2.3. Extraction of samples

Frozen urine samples were thawed in a water bath in a microwave oven for 60 s on defrost cycle. The thawed samples were vortexed and centrifuged at 2000 *g* at 4 °C for 5 min. Aliquots of 500 μl supernatant from each human urine standard and validation sample were placed in disposable glass tubes and 500 μl of I.S. solution (100 ng/ml) in 100 m*M* ammonium acetate buffer (pH 6.8) was added. The urine samples were vortexed and placed in the loading modules of a RapidTrace automatic SPE system (Zymark, Hopkinton, MA, USA). C_{18} Bond Elut SPE cartridges (100 mg, 1 cc reservoir, Varian, Harbor City, CA, USA) were conditioned with 2 ml of methanol and 2 ml of water. The urine samples were loaded onto the cartridges that were washed with 4 ml of water and eluted with 500 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 of acetonitrile–water (50:50, v/v, pH 6.0) containing 10 m*M* 4-methylmorpholine, and transferred into autosampler vials. Then 20 μl out of the 100 μl reconstituted samples was injected onto the LC–MS–MS system for analyses.

2.4. Hydrolyses of glucuronides

Enzymatic hydrolysis of glucuronide conjugate of II was conducted by adding approximately 100 units of β-glucuronidase in 0.5 ml of 0.2 *M* sodium acetate buffer (pH 5.0; 1:1, v/v) into 0.5 ml of human urine. The mixed samples were incubated at 37 °C for 16 h in a shaking waterbath. After the incubation 0.5 ml of I.S. solution (100 ng/ml) in 100 m*M* ammonium acetate buffer (pH 6.8) was added and the analytes were extracted by SPE as described above. The alkaline hydrolysis of glucuronide conjugate of I was carried out by reaction of 0.5 ml of human urine samples with 0.5 ml of 0.2 *M* glycine buffer (pH 10) at 37 °C for 16 h. After the reaction, the samples were acidified to neutral pH using formic acid and diluted with 0.5 ml of I.S. solution (100 ng/ml) in 100 m*M* ammonium acetate buffer (pH 6.8), then extracted with the SPE cartridges.

2.5. LC–MS–MS

LC–MS–MS analyses were performed using a system comprised of an ISS 200 LC autosampler (Perkin-Elmer, Norwalk, CT, USA), a 1050 HPLC pump (Hewlett-Packard, Wilmington, DE, USA) and an API-III-Plus quadrupole mass spectrometer (PE Sciex, Concorde, Canada). The separations were carried out on a narrow-bore reverse phase Keystone Prism RP HPLC column (50×2 mm, 5 μm, Keystone Scientific, Bellefonte, PA, USA) with an isocratic mobile phase consisting of acetonitrile–water (50:50, v/v, pH 6.0) containing 10 m*M* 4-methylmorpholine at a flow-rate of 100 μl/min. The column effluent was directly introduced into the mass spectrometer using electrospray ionization in negative mode. The electrospray interface and orifice voltages were set at −3700 and −52 V, respectively. The nitrogen nebulizer gas was set at 60 p.s.i. (1 p.s.i. = 6894.76 Pa) 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, from *m/z* 343 (Q1) to *m/z* 196 (Q3) for III and from

m/z 331 (Q1) to m/z 118 (Q3) for I.S. with 0.3-s dwell time. Argon was used as collision gas at gas thickness of 2.5×10^{15} molecules/cm^2 with collision offset energy of 25 eV to induce fragmentation in the collision cell.

2.6. Method validation

To compile between-run statistics, a urine calibration curve, a set of validation samples at different concentrations and human urine blanks to simulate a routine analysis run size of 70 were analyzed on 4 separate days. For the within-run statistics, a urine calibration curve, five sets of validation samples and human urine blanks to simulate a routine analysis run size of 70 were analyzed on a 5th 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, III and their I.S. were obtained from the MACQUAN data system (PE Sciex). The ratios of the peak areas of m/z 313$\rightarrow m/z$ 118 to m/z 331$\rightarrow m/z$ 118, m/z 329$\rightarrow m/z$ 196 to m/z 331$\rightarrow m/z$ 118 and m/z 343$\rightarrow m/z$ 196 to m/z 331$\rightarrow m/z$ 118 were then calculated for I, II and III, respectively. Calibration curves were obtained by a weighted (1/concentration^2) least squares linear regression analysis. Concentrations of I, II and III in the samples were calculated using the equations from the appropriate calibration curves. The between- and within-run precision and accuracy were determined by analyzing five sets of validation samples. The validation samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the run, or (c) at the end of the run. The validation criteria of the between- and within-run precision were 20% or better for the limit of quantitation and 15% or better for the rest of concentrations, and the accuracy was 100 ± 20% or better for the limit of quantitation and 100 ± 15% or better for the rest of concentrations.

3. Results and discussion

3.1. Automated sample extraction

SPE method was used to isolate and concentrate I, II and III from human urine. Performing sample extraction can be a labor-intensive process, especially when large numbers of sample must be processed. SPE method was widely used in pharmaceutical industries because it is easy to automate. In addition, the automation of the SPE allows samples to be processed unattended, often with improved precision and recovery. A seven-module Zymark RapidTrace automated extraction system was selected to increase the throughput of the extraction process by reducing the sample preparation time. This system was fully automated and provided high precision and reproducibility. The extraction process included conditioning 100 mg C_{18} Varian Bond Elut SPE columns, loading urine samples, washing the column, and eluting the analytes from the column. The total extraction time for 70 urine samples with these seven modules was approximately 1.5 h.

3.2. Selection of HPLC column and mobile phase

Various HPLC columns were evaluated to separate the analytes. A narrow-bore Keystone Prism RP HPLC column was selected because it provided the best separation for all four compounds. The low flow-rate for the narrow-bore column was ideal for coupling to an electrospray MS interface. Different mobile phases were evaluated to improve HPLC separation and enhance sensitivity in MS. An isocratic system using a mobile phase of acetonitrile–water (50:50, v/v, pH 6) containing 10 m*M* of 4-methylmorpholine was optimal for all four analytes with respect to peak shape and mass spectral response. Addition of 4-methylmorpholine to the mobile phase was used to enhance the response of the analytes in negative ionization MS [21]. The pH of the mobile phase was adjusted to 6.0 using glacial acetic acid to improve the peak shape. Under this condition, the retention times of I, II, III and I.S. were approximately 4, 2.5, 3.5 and 4.3 min, respectively. The total run time for each sample was 5.5 min.

3.3. Mass spectrometry

Electrospray MS–MS was used to analyze the compounds. Negative ionization was selected to detect the analytes because negative ion mass spectrometry provided at least five-fold improved signal-to-noise ratio for I, II and III over positive ionization.

The negative electrospray mass spectrum of I gave a deprotonated molecular ion at m/z 313. The collision-induced dissociation (CID) spectrum of m/z 313 revealed product ions at m/z 270, 248, 233, 206, 192, 172, 144, 80 and a base peak at m/z 118 (Fig. 2a). The fragmentation pathway of I in its CID spectrum is proposed as follows. The product ions of m/z 270 and 248 were formed by the loss of 43 ($COCH_3$) or 65 Da (SO_2H) from the m/z 313. The further loss of 64 (SO_2), or 78 Da (SO_2N) from the m/z 270 resulted in the product ions at m/z 206 and 192. The cleavage between the phenyl and the sulfonamide group yielded the product ions of m/z 80 and 233. The product ions at m/z 118, 144 and 172 corresponded to the fragments of $NH_2SO_2C_3H_2$, $NH_2SO_2C_5H_4$ and $NH_2SO_2C_7H_8$, respectively. The negative electrospray mass spectrum of II has a deprotonated molecular 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 base peak at m/z 196 was formed by the loss of $C_6H_5CNCH_2O$ via a five-member ring rearrangement mechanism on the isoxazole ring. The product ions at m/z 132 and 117 were generated from the loss of 64 (SO_2) and 79 ($NHSO_2$) Da from the ion at m/z 196, respectively. The product ion at m/z 78 corresponded to the fragments of NSO_2. The negative electrospray mass spectrum of III has a deprotonated molecular ion at m/z 343 with the same CID product ions as II (Fig. 2c). The base peak at m/z 196 was formed by the loss of C_6H_5CNCOO via a five-member ring rearrangement mechanism on the isoxazole ring. The I.S. gives a deprotonated molecular ion at m/z 331 with the CID product ions at m/z 288, 266, 251, 236, 224, 209, 172, 144, 80 and a base peak at m/z 118 (Fig. 2d). The fragmentation mechanism of I.S. in its CID spectrum is similar to that of I. The product ions of m/z 288 and 266 were formed by the loss of 43 ($COCH_3$) or 65 Da (SO_2H) from the m/z 331, respectively. The further loss of 64 (SO_2) or 78 Da (SO_2NH) from the m/z 288 resulted in the product ions at m/z 224 and 209. The cleavage between the phenyl and the sulfonamide group yielded the product ions of m/z 80 and 251. The product ions at m/z 118, 144 and 172 corresponded to the fragments of $NH_2SO_2C_3H_2$, $NH_2SO_2C_5H_4$ and $NH_2SO_2C_7H_8$, respectively.

LC–MRM is very powerful technique for pharmacokinetic studies since it provides sensitivity,

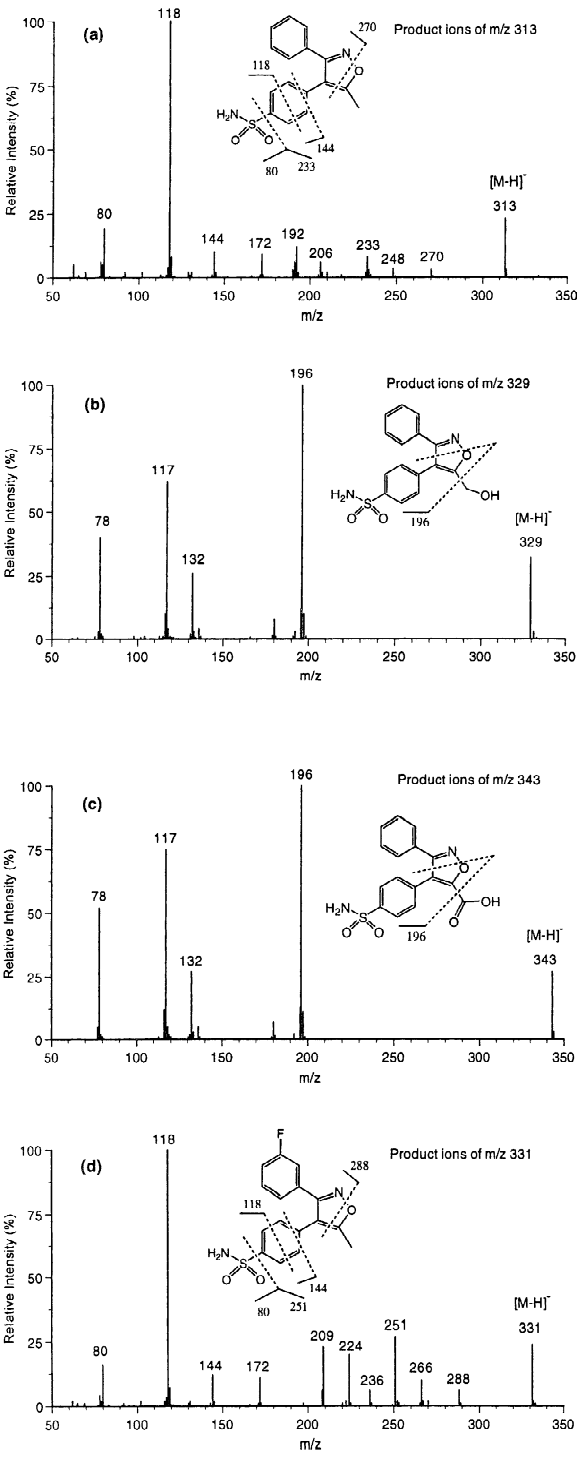

Fig. 2. CID spectra of (a) I (m/z 313), (b) II (m/z 329), (c) III (m/z 343) and (d) I.S. (m/z 331).

selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for all analytes. I, II, III and I.S. were measured by monitoring the transitions from the parent ions to the most abundance product ions of m/z 313→m/z 118, m/z 329→m/z 196, m/z 343→m/z 196 and m/z 331→m/z 118, respectively. The representative LC–MRM chromatograms of a human urine blank with I.S. and 2 ng/ml of I, II and III with I.S. are shown in Fig. 3. No significant urine matrix contribution was observed in the LC–MRM chromatograms, demonstrating the selectivity and specificity of the MRM technique.

3.4. Extraction recovery

Blank human urine was spiked separately with radiolabeled [^{14}C]-I, [^{14}C]-II and [^{14}C]-III 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 urine before the extraction. It was observed that the extraction recoveries for the analytes in human urine from different human subjects varied due to different pH and salt concentrations in individual human urine. To limit the variability of extraction efficiency, human urine samples were prediluted with equal volume of different buffer solutions to control and adjust the urine pH to 3, 5, 6.8 and 8. The results showed that the best extraction efficiency and lowest variability were achieved when the urine samples were diluted with equal volume of 100 m*M* ammonium acetate buffer (pH 6.8). Under this condition the extraction recoveries for I, II and III from human urine were 94.4±3.2% ($n=3$), 89.7±3.9% ($n=3$) and 90.3±4.7% ($n=3$). The extraction recovery for I.S. was not measured due to lack of a ^{14}C radiolabeled compound. I.S. is a structural analogue of the parent compound I, therefore, it is assumed that their extraction recoveries are similar.

3.5. Matrix effect

The blank human urine contribution in LC–MRM chromatograms at the elution regions of I, II, III and I.S. in male and female human urine without addition of the analytes was evaluated to ensure the specificity of the method. No peaks eluting at the retention times of I, II, III and I.S. were detected in six male and female individual blank human urine samples. These results demonstrated that no considerable endogenous contribution from blank human urine affected the measurement of the analytes. In addition, matrix 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 urine. The results showed that there was no significant difference for peak responses between these samples. This effect is most likely due to the sample clean-up with SPE extraction and the relatively longer HPLC run time.

3.6. Standard curves

Standard curves were linear over the concentration range of 1–200 ng/ml for I and II and 2–200 ng/ml for III. The seven or eight point standard curves gave acceptable results for all analytes and were used for all the calculations (Fig. 4). The mean correlation coefficients of the weighted standard curves generated during the validation were 0.998, 0.995 and 0.996 for I, II and III, respectively. The standard curves obtained as described above were suitable for generation of acceptable data for the concentrations of I, II and III in the samples during the validations.

3.7. Lowest concentration

The lower limit of quantitation (LLOQ) for the human urine assay was 1 ng/ml for both I and II. The LLOQ for III was raised to 2 ng/ml because the precision and accuracy of III at the concentration of 1 ng/ml did not meet the validation criteria ($<\pm20\%$). The between-run precision at the LLOQ—expressed as coefficient of variation (C.V.)—was 9.01, 13.3 and 10.7% for I, II and III, respectively. The between-run accuracy—expressed as analytical recovery (AR)—was 104, 95.4 and 104% for I, II and III, respectively (Table 1). The within-run precision was 6.42, 11.6 and 10.7% and the accuracy was 109, 97.4 and 109% for I, II and III, respectively.

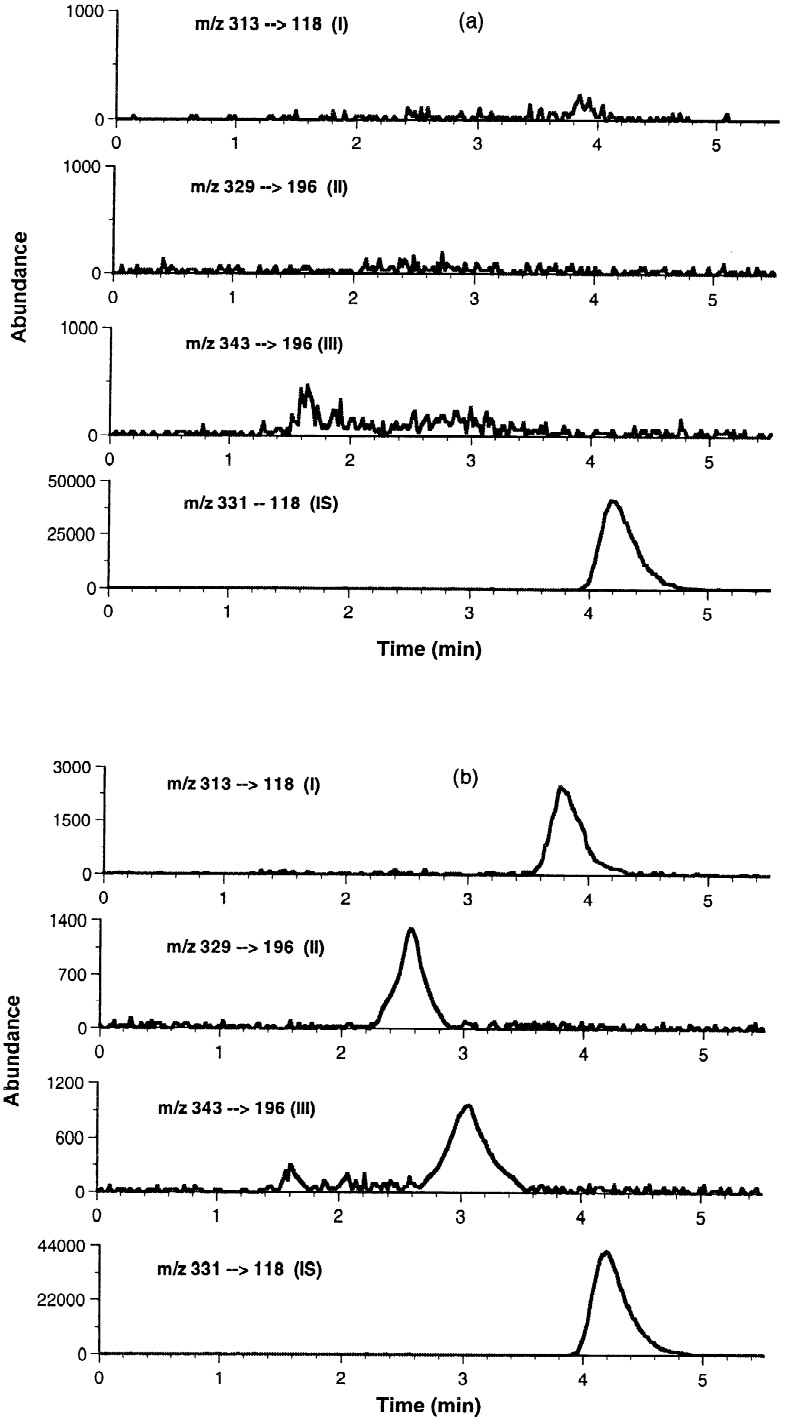

Fig. 3. Representative LC–MRM chromatograms of (a) blank human urine spiked with I.S. (100 ng/ml), and (b) human urine spiked with both I, II, III (2 ng/ml) and I.S. (100 ng/ml).

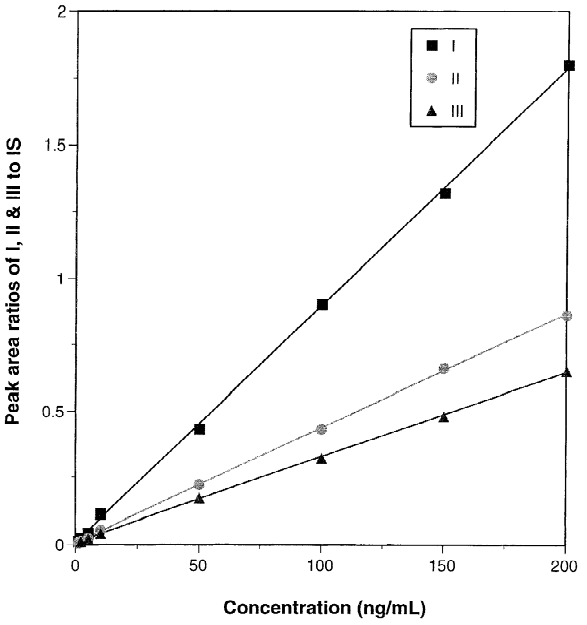

Fig. 4. Representative standard curves of I, II and III in human urine in the range of 1– or 2–200 ng/ml.

3.8. Middle and upper concentrations

The middle and upper quantitation levels ranged from 2 to 200 ng/ml for I and II and 5 to 200 ng/ml for III in human urine. For the between-run experiment, the precision ranged from 2.04 to 7.71% and the accuracy ranged from 94.9 to 105% for I; the precision ranged from 3.08 to 9.80% and the accuracy ranged from 97.8 to 101% for II; the precision ranged from 4.30 to 9.56% and the accuracy ranged from 94.9 to 105% for III. (Table 1). For the within-run experiment, the precision and accuracy for I, II, and III met the validation criteria ($<\pm 15\%$).

3.9. Dilution

The upper concentration limits can be extended with acceptable precision and accuracy to 1000 ng/ml by a tenfold dilution with control human urine. For the between-run experiment, the precision at 1000 ng/ml was 3.78, 7.60 and 4.10% and accuracy at 1000 ng/ml was 97.4, 97.8 and 96.5% for I, II and III, respectively (Table 1). For the within-run experiment, the precision was 2.59, 9.32 and 3.96% and accuracy was 99.5, 108 and 102% for I, II and III, respectively. These data suggested that samples whose concentrations are greater than the upper limit of the standard curves could be reanalyzed by dilution to obtain acceptable data.

3.10. Freeze–thaw stability

The freeze–thaw stabilities of I, II and III were 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 urine samples containing separated I, II and III 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, II and III were stable in human urine through three freeze–thaw cycles. The precision ranged from 4.09 to 6.82% and the accuracy ranged from 94.7 to 103% for I. The precision ranged from 5.16 to 18.5% and accuracy ranged from 95.6 to 99.6% for II. The precision ranged from 4.56 to 9.53% and accuracy ranged from 93.3 to 108% for III (Table 1). The results demonstrated that human urine samples could be thawed and refrozen without compromising the integrity of the samples.

3.11. Sample collection and processing studies

In vitro studies were performed to determine whether I, II and III were lost and/or degraded in human urine during sample collection and processing. In Experiment A, I, II and III were separately incubated in freshly collected human urine for 15 min on ice then stored in a $-20\,^{\circ}C$ freezer. In Experiment B, I, II and III were separately incubated in freshly collected human urine for 120 min at room temperature followed by storage in a $4\,^{\circ}C$ refrigerator for an additional 24 h, then stored in a $-20\,^{\circ}C$ freezer. The mean concentration of I, II and III from B gave precision of 2.6, 9.90 and 8.81% as well relative recoveries of 81.8, 96.9 and 95.4% when compared to the mean concentrations from control A (incubated on ice for 15 min). It was thereby concluded that I, II and III were stable in human urine for at least 120 min at room temperature and an additional 24 h at $4\,^{\circ}C$.

Table 1
Assay validation results obtained from between-run experiments for I, II and III in human urine

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	No. of replicates	Precision (%)	Accuracy (%)
Analyte I				
1	1.04	5	9.01	104
2	2.11	5	4.29	105
5	4.97	5	7.71	99.4
50	51.2	5	3.81	102
200	190	5	2.04	94.9
1000[a]	974	5	3.78	97.4
$2_{f/t}$[b]	2.06	5	4.09	103
$100^{b}_{f/t}$	94.7	5	6.82	94.7
Analyte II				
1	0.954	5	13.3	95.4
2	2.02	5	9.80	101
5	5.06	5	9.69	101
50	50.0	5	6.71	100
200	196	5	3.08	97.8
1000[a]	995	5	7.60	99.5
$2^{b}_{f/t}$	1.91	5	18.5	95.6
$100^{b}_{f/t}$	99.6	5	5.16	99.6
Analyte III				
2	2.08	5	10.7	104
5	5.23	5	9.56	105
50	48.6	5	6.07	97.2
200	190	5	4.30	94.9
1000[a]	965	5	4.10	96.5
$2^{b}_{f/t}$	2.16	5	9.53	108
$100^{b}_{f/t}$	93.3	5	4.56	93.3

[a] Sample was processed with ten-fold dilution.
[b] Sample was assayed after three freeze–thaw cycles.

3.12. 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 urine samples spiked separately with I, II and III at concentrations of 2 and 100 ng/ml were analyzed on day 1. Then the samples from the same pools were analyzed against standard curves from freshly prepared standards after storage at −20 °C for 133 days. The results indicated that I, II and III were stable when stored frozen at −20 °C for 133 days. The precision and accuracy for I on day 133 ranged from 6.60 to 12.3% and 93.0 to 96.4%, respectively. For II on day 133 the precision and accuracy ranged from 3.75 to 5.16% and 87.5 to 94.5%. The precision and accuracy for III on day 133 ranged from 2.41 to 11.7% and 95.3 to 97.2% (Table 2).

3.13. Hydrolyses of glucuronide conjugates of I and II

Based on the results of metabolism studies, I and II were excreted in human urine predominately as their glucuronide conjugates [13]. Therefore, the concentrations of total I and II were also measured after glucuronide hydrolysis. The deglucuronidation of the glucuronide conjugates of I and II was conducted by alkaline hydrolysis and enzymatic hydrolysis (β-glucuronidase), respectively. Under the experimental conditions used as described earlier, hydrolyses of the conjugate proceeded essentially to completion. To examine the impact of free I, II and III during alkaline and enzymatic hydrolyses, three sets of QC samples were treated with alkaline and enzymatic hydrolyses separately and analyzed against the standard curves generated from the

Table 2
Results for long-term frozen storage stability samples for I, II and III after 133 days at −20 °C

Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	No. of replicates	Precision (%)	Accuracy (%)
Analyte I				
2	1.93	3	12.3	96.4
100	93.0	3	6.60	93.0
Analyte II				
2	1.75	3	5.16	87.5
100	94.5	3	3.75	94.5
Analyte III				
2	1.94	3	11.7	97.2
100	93.6	3	2.41	95.3

samples that did not go through hydrolyses. After β-glucuronidase hydrolysis, the mean concentrations of I, II and III gave precisions of 9.80, 12.4 and 11.6% as well as analytical recoveries of 97.5, 92.3 and 85.7%, indicating that there was no impact of enzymatic hydrolysis to the assay. In contrast, after alkaline hydrolysis the mean concentrations of I, II and III gave precisions of 9.30, 18.9 and 24.5% as well analytical recoveries of 93.8, 75.4 and 51.2%, suggesting II and III were not stable under the alkaline hydrolysis. Based on these results, the concentration of total I was analyzed using alkaline analysis, while the concentrations of total II and III were assayed using enzymatic hydrolyses. In addition, the stability of glucuronide conjugates of I and II during the sample processing was examined to determine whether they degraded into free I and II. Carbon-14 radiolabeled glucuronide conjugates of I and II were isolated by HPLC from the human metabolism study [13] and spiked with human urine blank. The spiked samples were then extracted and analyzed using the validated automated SPE–LC–MS–MS assay to measure the concentrations of free I and II. The results showed that there was no measurable free I and II in these spiked samples. Further HPLC radiochromatogram profiling of these spiked samples after the extraction indicated that the ^{14}C-radiolabeled glucuronide conjugates of I and II were stable under the assay conditions. Furthermore, to examine precision and accuracy of this assay for measurements of I and II as their glucuronide conjugates, the spiked ^{14}C-radiolabeled glucuronides of I and II human urine samples were deglucuronidated separately by alkaline and enzymatic hydrolyses, then analyzed in triplicate for I and II using the validated automated SPE–LC–MS–MS assay. The results showed that the mean precisions and accuracies for I and II were 14.6, 10.8 and 87.6, 92.9%, respectively, indicating the assay was capable to accurately measure I and II as their glucuronide conjugates in human urine. The actual concentrations of I and II as their glucuronide conjugates were calculated based on their radioactive measurements [concentration = radioactivity × specific activity of the dose compound × (molecular mass of I or II)/molecular mass of the dose compound].

3.14. Clinical application

This method was applied to two clinical studies with over 1100 human urine samples analyzed in 22 analytical runs. Approximately 100 human urine samples were analyzed with standards and QCs in two runs per day. For each run, six QCs (duplicated at three concentration levels) and two dilution QCs (when analyzing for dilution samples) were randomly located among the clinical samples within a run. Approximately 10% of the analyzed samples exceeded the upper lever concentration of the standard curves and were reanalyzed by tenfold dilution. All the samples from −10–0 h predose, and 0–4, 4–8, 8–12, 12–24, 24–48, 48–72 h postdose were blinded during the runs. No Valdecoxib and its metabolites were detected in predose −10–0-h human urines for all human subjects. The QC statistic data from two clinical studies demonstrated that the precision and accuracy of QCs from these studies met the validation criteria (<20% and within100±20% for LLOQ,

<15% and within 100±15% for the rest of concentrations). Typical LC–MRM chromatograms from human urine after dosing with 2 mg of Valdecoxib are shown in Fig. 5. The mean urinary concentrations of free I, II and III for 0–24 h postdose after 2 mg single oral dose of Valdecoxib were 15.4, 4.92 and 10.2 μg/ml, respectively. Since Valdecoxib and its hydroxylated metabolite (II) were mainly excreted as the glucuronide conjugates in human urine, the total concentrations of I and II in human urine were further analyzed using this validated assay after treatment with β-glucuronidase or base. The pharmacokinetics parameters obtained from these studies will be reported in detail elsewhere.

4. Conclusions

We have developed and validated an automated and sensitive LC–MS–MS assay for Valdecoxib and its metabolites in human urine. Acceptable data were generated for I, II and III using weighted linear regression ($1/\text{concentration}^2$) and full standard curves for human urine samples. The LLOQ was 1

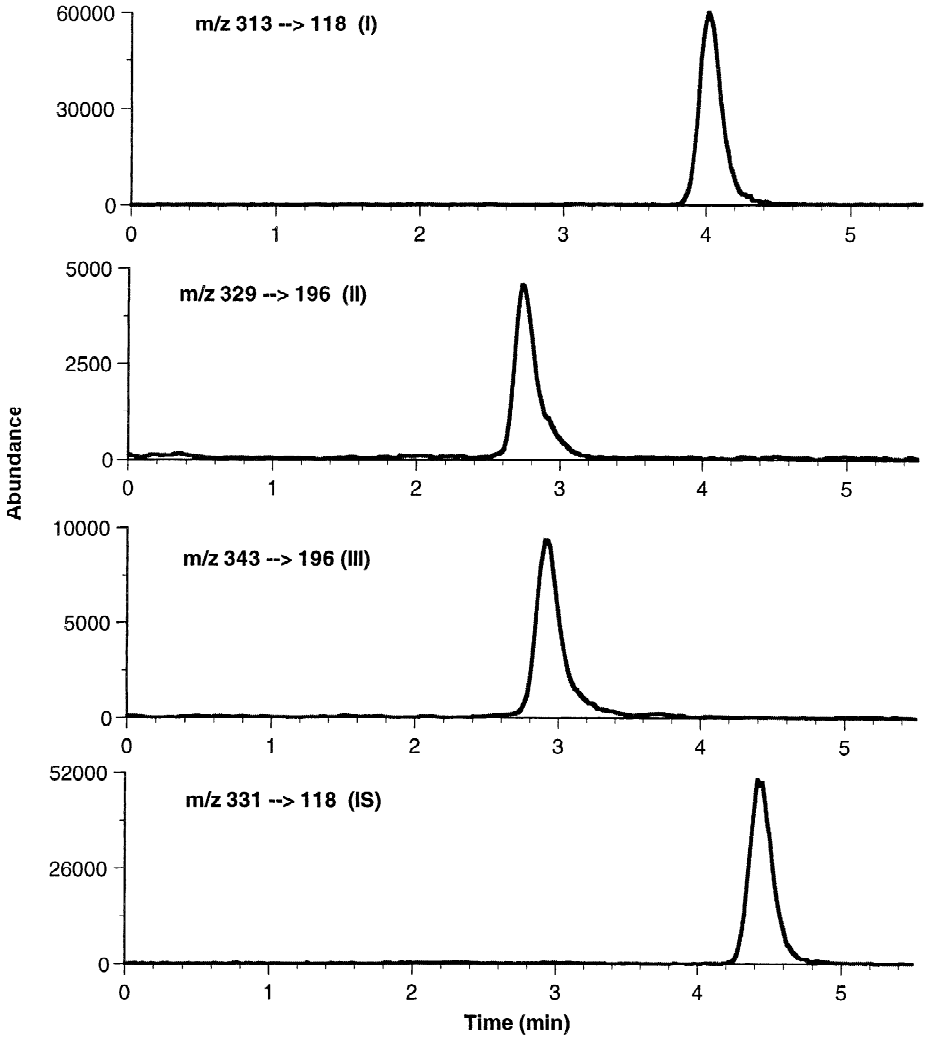

Fig. 5. Representative LC–MRM chromatograms of the human urine sample from a human subject after a dose of 2 mg of Valdecoxib.

ng/ml for I and II and 2 ng/ml for III. Acceptable precision and accuracy were obtained for concentrations above the sensitivity limit and within the standard curve range of 1– or 2–200 ng/ml. The upper concentration limit can be extended with acceptable precision and accuracy to 1 μg/ml by a tenfold dilution with control human urine. The validated SPE–LC–MS–MS human urine assay is specific for I, II and III in human urine and the samples can be stored frozen at −20 °C for at least 133 days. The method was the first validated analytical assay for Valdecoxib and its metabolites in urine and was successfully applied to the determination of urine concentrations of I, II and III in clinical studies.

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