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Validation of a Sensitive LC-MS/MS Method for Quantifying Imrecoxib and Its Two Metabolites in Human Plasma: Application in a Pharmacokinetic Study

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Abstract

A sensitive LC/MS/MS method was developed and validated for the identification and quantification Imrecoxib, its hydroxyl metabolites M1 and its carboxyl metabolites M2 in human plasma. Chromatographic separation was performed on a Welch Ultimate XB C18 column (2.1 mm × 50 mm, 5 μm) with a gradient elution of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer operated in the multiple reaction monitoring (MRM) mode with the transitions of m/z 370.1→236.1 for Imrecoxib, m/z 386.4→326.4 for hydroxyl metabolites M1, m/z 400.3→236.0 for carboxyl metabolites M2 and m/z 244.2→185.1 for agomelatine (internal standard, IS). The total run time was 3.5 min. Standard curve concentrations ranged from 0.5-60 ng/mL for Imrecoxib, 1 to 100 for M1, and 2-800 ng/mL for M2 in plasma. Selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, stability, matrix effect and recovery and carry-over effect were evaluated for all analytes. The validated method was applied to support a pharmacokinetic study of simultaneous determination of Imrecoxib and M1 and M2 in 12 Chinese healthy volunteers.

Keywords: LC-MS/MS: Imrecoxib; Pharmacokinetic; Human plasma

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most frequently prescribed group of drugs worldwide and are mainly used for the treatment of pain, inflammation and fever in clinical therapeutics [1,2]. The therapeutic efficacy as well as the toxicity of NSAIDs is generally attributed to the blockade of prostaglandin synthesis by inhibition of cyclooxygenase (COX) enzymes [3-5]. Nowadays, it is well established that there are at least two COX isozymes, COX-1 and COX-2 [6]. Traditional NSAIDs act primarily by inhibiting COXs [7]. Inhibition of COX-1 by NSAIDs leads to heavy gastrointestinal toxicity. New COX-2 selective inhibitors, such as celecoxib (Celebrex) and rofecoxib (Vioxx) [8] have fewer gastrointestinal side effects comparing with traditional NSAIDs. However, an increased risk of myocardial infarction and cardiovascular thrombotic events associated with the use of highly selective COX-2 inhibitors were subsequently observed [6], therefore, design of NSAIDs that preferentially inhibit COX-II with moderate selectivity seems more promising. Imrecoxib, 4-(4-methylsulfonyl-phenyl)-1-propyl-3-(p-tolyl)-3-pyrrolin-2-one, is a novel and moderately selective COX-2 inhibitor. Imrecoxib has been in the phase III of clinical trials in China for the treatment of acute and chronic inflammatory diseases [6]. The absorption of Imrecoxib is rapid, with the mean C max occurring approximately 2 hours after oral dosing, Imrecoxib is completely metabolized with CYP2C9 and the major metabolites in plasma are hydroxyimrecoxib (M1) and carboxyimrecoxib (M2). The elimination half-life of Imrecoxib is about 20 hours [9]. Up to now, there are few pharmacokinetic studies about imrecoxib [10-14]. We first report a highly selective and sensitive LC–MS/MS method for the simultaneous determination of imrecoxib (M0), hydroxyl imrecoxib (M1) and carboxy imrecoxib (M2) in human plasma. This new method has been fully validated in terms of selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, stability, matrix effect and recovery. It has been successfully applied in a bioequivalence study of M0, M1 and M2 in ten healthy humans.

Experimental

Chemicals and reagents

Imrecoxib (99.60% purity), M1 (100% purity), M2 (100% purity), agomelatine (internal standard, IS, 94% purity) were generously supplied by Jiangsu hengrui medicine co., LTD. Methanol and acetonitrile of HPLC grade were purchased from Merck KGaA (Darmstadt, Germany). Analytical grade formic acid was purchased from Thermo Fisher Scientific and ammonium acetate was purchased from Dima Technology Inc. (Guangzhou, China). Ultrapure water (Chengdu Ultra Technology Co., Ltd., Chengdu, China) was used throughout the study. Blank human plasma was obtained from healthy volunteers and stored at -80°C before use.

Instrumentation and conditions

Liquid chromatography: A Shimadzu liquid chromatography system (Shimadzu Corporation, Japan) equipped with a high-pressure pump (LC-20AD), online degasser (DGU-20A-3R), an autosampler (SIL-20AC) and a column oven (CTO-20A) was used. Chromatographic separation was performed on a Welch Ultimate XB C18 column (2.1 mm × 50 mm, 5 μm) (Welch Materials, Inc. China) with a gradient elution of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The flow rate for the gradient elution was 500 μL/min. The run time was 3.5 min. The gradient started from 50% solvent B for the first 0.3 minute, then was decreased to 5% until the 1.2 min and kept at 5% until the 2.2 min. Between the 2.2 and 2.3 min, the solvent B was increased to 50% and kept there until the 3.5 min to allow the column to equilibrate. The autosampler was conditioned at +4°C and the column oven was conditioned at +35°C. The injection volume was 5 μL.

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Mass spectrometry: Detection of the analytes and internal standard was achieved with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with an ESI Turbo ion spray. The instrument was operated in the positive ionization multiple reaction monitoring (MRM) mode. Data were acquired and processed using Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA). The detecting ions were as follows: m/z 370.1→236.1 for Imrecoxib, m/z 386.4→326.4 for M1, m/z 400.3→236.0 for M2, m/z 244.2→185.1 for agomelatine. The capillary voltage and source temperature of the mass spectrometer was set at 5500 V and 550°C, respectively. Collision activated dissociation gas (CAD) was set at 10, the curtain gas (CUR) at 30 and nebulizer and heater gas (GS1 and GS2) were fixed at 30 and 45, respectively.

Preparation of calibration standards and quality control (QC) samples: The appropriate amount of Imrecoxib, M1, M2 and agomelatine was exactly weighed and dissolved in methanol to prepare stock standard solution respectively. Each analyte (Imrecoxib, M1, and M2) was prepared two stock solutions and they should be inspected before they were used. One stock solution was diluted with methanol to standard work solutions at concentrations of 30, 60, 180, 360, 720, 1800 and 3600 ng/mL for Imrecoxib, 60, 120, 360, 720, 1500, 3000 and 6000 ng/mL for M1 and 120, 600, 2400, 6000, 12000, 24000 and 48000 ng/mL for M2. The other was diluted with methanol to QC solutions at concentrations of 30, 90, 1200, 3000 and 3600 ng/mL for Imrecoxib, 60, 180, 1800, 4800 and 6000 ng/mL for M1, 120, 360, 9000, 39000 and 48000 ng/mL for M2. Take the same amount of the three analytes at same concentration level of the work solution and mix to get a series of mix work solution. The IS work solution was prepared by dissolving agomelatine in methanol to 50 ng/mL. The standard and QC samples were consisted of 5% mix work solution and 95% human plasma.

Sample processing: 0.2 ml of plasma and 30 µL of IS (50 ng/ml) were added to a 2 ml polypropylene tube. Then 200 µL of 0.2 M phosphoric acid water, and 1 mL ethyl acetate was added to the tube. The sample was vortex-mixed for 2 min, and centrifuged at 15,000 rpm for 3 min under +4°C. After centrifugation, 700 µL supernatant fluid was transferred into a new polypropylene tube and evaporated to dryness at 40°C. The dried extract was reconstituted with 150 µL of methanol, vortex-mixed and centrifuged again. Then supernatant fluid was transferred to a clean autosampler vial. 5 µL of this solution was injected into the LC-MS/MS system.

Method validation

Selectivity: In order to evaluate selectivity, 6 different lots of blank matrix (one replicate for each lot) prepared and extracted without addition of analytes or internal standard. No significant peak should present in the blank extracts at the retention time of the analytes or the internal standard and in the relevant mass channels at a level greater than 20.0% of the mean LLOQ calibrator peak signal or by more than 5.0% of the mean internal standard peak signal of LLOQ calibrators.

Linearity and lower limit of quantification (LLOQ): Calibration curve was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the y=bx+c using weighing factor (1/x2). The correlation coefficient (r) was used to evaluate the linearity of the calibration curve and it should be no less than 0.98. The LLOQ is defined as the lowest concentration level which can be measured precisely and accurately. Six samples of LLOQ should be detected. The accuracy of single LLOQ should be within ± 20% and the amount of unqualified was no more than 1/3 and the relative standard deviation (RSD) was below 20%.

Precision and accuracy: The intraday accuracy and precision were evaluated with QC samples at four concentration levels by analyzing 6 independently prepared samples at each level in one analytical run

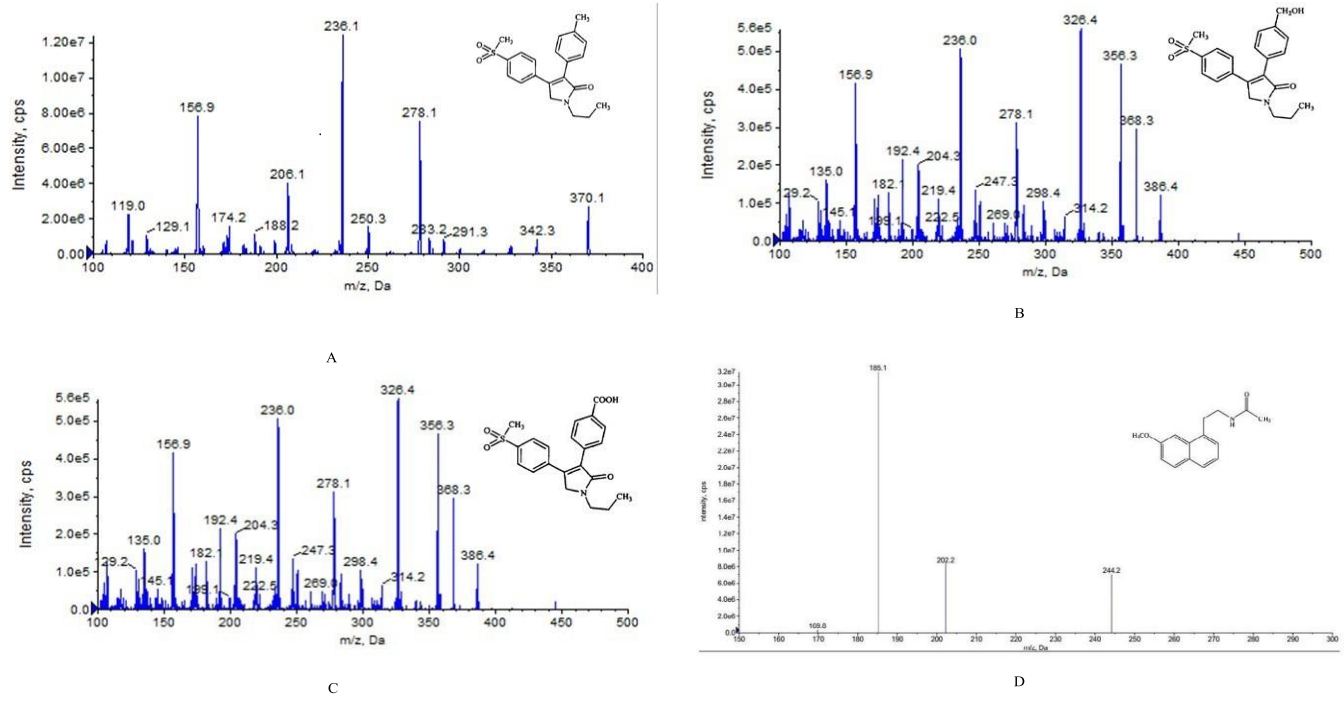


Figure 1: Ion pairs of M0(A), M1(B), M2(C), IS(D).

and the inter-day accuracy and precision were in three analytical runs for three days. Accuracy was evaluated and reported by calculating the relative error (RE %) from the nominal concentration. Precision was determined by calculating the relative standard deviation (RSD %) of replicates within one sample run (intraday) and between samples run (inter-day). Adequate accuracy and precision was defined as ≤15%, except for the LLOQ, where it was ≤20%.

Recovery: Extraction recovery will be calculated by comparing the area of analyte/internal standard obtained from individual regular QC to the mean area of analyte/internal standard from blank extracts post-spiked with analyte and internal standard at the same nominal concentrations. Extraction recovery is evaluated at three QC concentrations (low, medium, and high) with 6 replicates for the analytes and at the working concentration used in the method for internal standard.

Matrix effect: The matrix effect is given as the ratio of the peak area of analyte/internal standard obtained from analysis of extracted blank matrix (from 6 different lots) samples post-spiked with the analytes at three concentrations (low, medium, and high) in singlet and internal standard at the working concentration, relative to the area ratio of analyte/internal standard obtained from corresponding neat solutions analyzed in 6 replicates (n=6). For analytes and the IS, the matrix factor will be calculated for each lot of matrix, by comparing the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte and IS), to the mean peak area in absence of matrix (pure solution of the analyte and IS). The IS-normalized matrix factor will also be calculated by dividing the matrix factor of the analyte by the matrix factor of the IS. The %RSD of the IS-normalized matrix factor calculated from the 6 lots of matrix should not be greater than 15.0%.

Stability: The bench-top stability at room temperature for 8 h, freeze-thaw stability after three freeze–thaw cycles, post-preparative stability at room temperature for 8 h, long-term stability at -80°C for 90 days of sample were examined to make sure the stability of analytes during operation.

Carry-over: Carry-over was assessed by injecting blank samples after the upper limit of quantification (ULOQ) for three times. The peak in the blank sample following the ULOQ should not be greater than 20% of the lower limit of quantification (LLOQ) and 5% for the internal standard.

Application to a pharmacokinetic study: The method was used to analyze plasma samples containing Imrecoxib and M1 and M2 in healthy Chinese volunteers. The study was approved by the Ethics Committee and followed the principles of the Declaration of Helsinki. All subjects gave written informed consent. Subjects took a single dose of 50 mg Imrecoxib tablets after fasted for 10 h. Blood samples were taken before and then at 25 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h and 48 h post-dose. Blood was drawn into a heparin anticoagulant tube at various time points, and then centrifuged, with the supernatant plasma stored at −80°C until analysis. The main pharmacokinetic parameters of Imrecoxib and M1 and M2 were calculated by non-compartmental model using Drug and Statistics Software version 3.0 (Mathematical Pharmacology

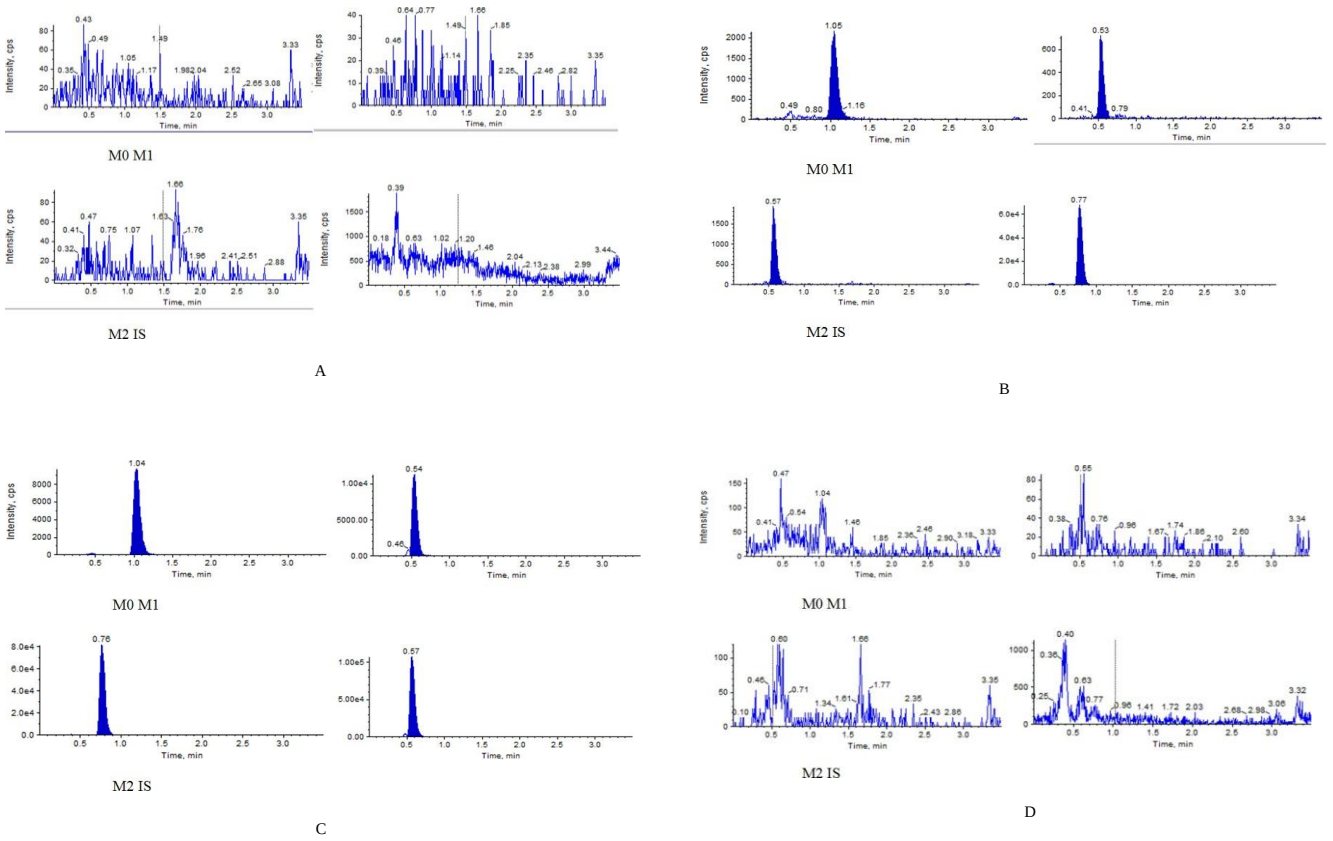


Figure 2: Chromatograms of M0, M1, M2, IS. (A) Blank plasma sample (B) LLOQ sample (C) Plasma sample of 1.5 h after administration (D) Carry-over sample.

Analyte	Nominal concentration (ng/ml)	Intra-day (n=6) Mean ± SD	RSD (%)	RE (%)	Inter-day (n=18) Mean ± SD	RSD (%)	RE (%)
Imrecoxib	0.5	0.48 ± 0.02	4.15	-4.43	0.48 ± 0.03	5.39	-4.94
	1.5	1.50 ± 0.06	3.77	-0.22	1.47 ± 0.06	4.18	-2.11
	20	19.68 ± 0.39	1.97	-1.58	19.77 ± 0.40	2.04	-1.14
	50	50.17 ± 0.52	1.03	0.35	50.31 ± 6.29	12.5	0.61
M1	1	0.97 ± 0.04	4.03	-2.7	0.95 ± 0.04	4.17	-5.33
	3	2.86 ± 0.06	2	-4.56	2.91 ± 0.07	2.26	-3
	20	19.38 ± 0.62	3.22	-3.08	19.30 ± 0.47	2.44	-3.5
	80	75.83 ± 0.75	0.99	-5.21	73.92 ± 1.66	2.25	-7.6
M2	2	2.22 ± 0.07	3.15	11	2.00 ± 0.18	9.01	-0.23
	6	6.21 ± 0.12	1.97	3.54	6.05 ± 0.23	3.84	0.78
	150	153.09 ± 5.15	3.37	2.06	152.86 ± 5.68	3.71	1.91
	650	647.96 ± 8.92	1.38	-0.78	658.16 ± 23.46	3.56	1.26

Table 1: Precision and accuracy data for the determination of Imrecoxib, M1 and M2 (3 days with six replicates per day).

Analyte	Nominal concentration (ng/ml)	Recovery		Matrix effects	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
Imrecoxib	1.5	94.07 ± 4.78	5.08	1.0962 ± 0.0241	2.2
	20	96.03 ± 3.91	4.07	1.0186 ± 0.0195	1.92
	50	93.98 ± 2.21	2.35	1.0299 ± 0.0331	3.21
M1	3	96.59 ± 4.35	4.51	1.0556 ± 0.0161	1.53
	20	96.16 ± 3.28	3.41	1.0603 ± 0.0336	3.17
	80	95.99 ± 2.01	2.09	1.0742 ± 0.0942	8.77
M2	6	94.35 ± 3.67	3.89	1.0321 ± 0.0610	5.92
	150	96.34 ± 2.09	2.17	1.0457 ± 0.0513	4.91
	650	95.14 ± 2.57	2.71	1.0651 ± 0.0400	3.75

Table 2: Recovery and matrix effects data of three analytes (Six replicates per concentration).

Analyte	Nominal concentration (ng/ml)	Short-term		Long-term		Freeze-thaw		Extract-left	
		Actual concentration (ng/ml)	RE (%)	Actual concentration (ng/ml)	RE (%)	Actual concentration (ng/ml)	RE (%)	Actual concentration (ng/ml)	RE (%)
Imrecoxib	1.5	1.59 ± 0.04	6.11	1.30 ± 0.09	-13.11	1.50 ± 0.05	-0.22	1.47 ± 0.07	-2
	50	49.62 ± 1.40	-0.76	46.60 ± 1.36	-6.81	46.74 ± 2.24	-6.53	49.55 ± 0.50	-0.9
M1	3	2.84 ± 0.17	-5.44	2.68 ± 0.08	-10.56	3.08 ± 0.03	2.56	2.79 ± 0.07	-6.89
	80	78.06 ± 1.78	-2.43	74.00 ± 1.76	-7.5	78.56 ± 1.52	-1.81	76.28 ± 2.18	-4.65
M2	6	6.37 ± 0.12	3.29	6.20 ± 0.03	6.33	6.93 ± 0.65	4.96	5.86 ± 0.27	-3.51
	650	669.93 ± 26.64	5.05	658.91 ± 24.78	2.19	672.76 ± 22.20	4.39	655.83 ± 19.38	3.04

Table 3: Stability of three analytes under different storage conditions (Six replicates per concentration for each stability term).

Professional Committee of China), respectively.

Results, Discussion and Conclusion

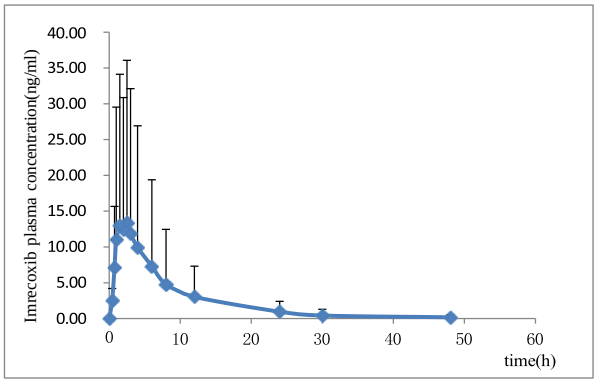
Method development

The metabolite M2 contains a hydroxyl group. To determined three analytes in the same condition, we scanned them and IS in positive mode with ESI Turbo ionspray first. We got a m/z 370.3/236.1 for quantification of Imrecoxib. Two ion pairs were selected for M1 at m/z 386.4/236.0 and m/z 386.4/326.4 and two for M2 at m/z 400.3/236.0 and m/z 400.3/340.4. And we chose m/z 386.4/236.0 for M1 and m/z 400.3/236.0 for M2 because they have higher S/N values in solvents. The final ion pairs were shown in (Figure 1). To choose a better separation condition and get a higher signal and a better peak, we tried several columns and different mobile phase. We find that ammonium acetate and formic acid in water may reduce the Signal of three analytes and affect the retain time of M1 and M2. However, M2 had a bad peak without formic acid in water. So, we finally chose acetonitrile as the organic phase and 0.1% formic acid in water as the water phase. There was no significant difference among different C18 columns. To get a good and quickly separation, we choose a 50 mm C18 column and a 500 μl/min rate. All analytes had proper retain time and good peak on the Welch Ultimate XB C18 column (2.1 mm × 50 mm, 5μm), this is why we choose the column.

Method validation

Selectivity: The result of selectivity was shown in (Figure 2). Imrecoxib, M1, M2 and IS were retained at 1.05 min, 0.53 min, 0.56 min and 0.77 min. Matrix had no contribution to analyte and internal standard in 6 blank samples.

Linearity and LLOQ: The calibration curves of analytes were linear within the concentrations ranging from 0.5 to 60 ng/ml for Imrecoxib and 1 to 100 ng/ml for M1 and 2 to 800 ng/ml for M2. The back-calculated concentrations of calibration standards were within 12.0% for all levels of nominal values for Imrecoxib、M1 and M2.



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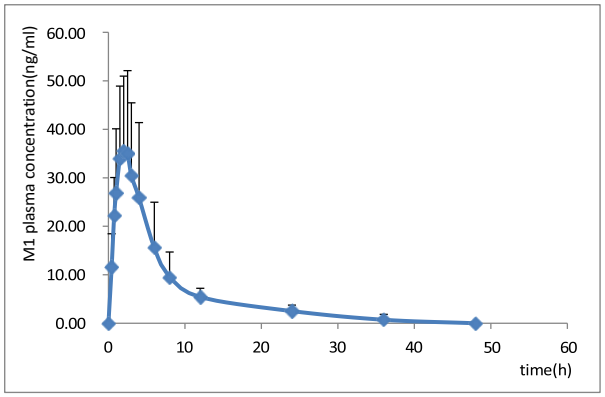

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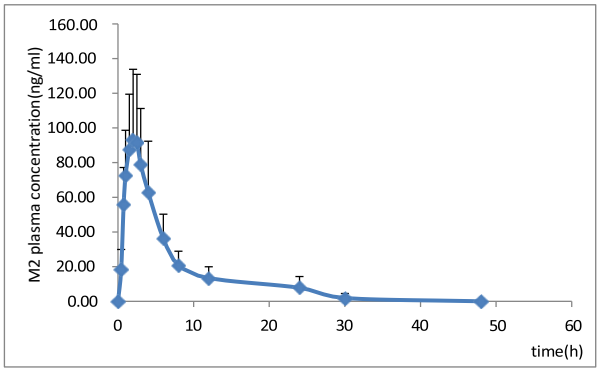

C

Figure 3: Mean plasma concentration-time curves of Imrecoxib (A) M1 (B) M2 (C) After a single oral administration of 50 mg of Imrecoxib tablets to healthy Chinese volunteers (n=9).

The standard curve regression equation were y=0.0761x+0.00426, y=0.00619x+0.000811 and y=0.00903x+0.00161 and the correlation coefficient were 0.9943, 0.9967, and 0.9976 for Imrecoxib, M1 and M2, respectively. All calibration standards at each concentration met the acceptance criteria. %RE of the mean of 6 LLOQ samples from nominal concentration was -4.43%, -5.62%, and -8.45%, %RSD of replicate results was 4.15%, 6.20%, and 7.63% for Imrecoxib, M1 and M2, respectively.

Precision and accuracy: The results of intra- and inter-day accuary and precision were shown in Table 1. All samples of each concentration level of each analyte met the acceptance criteria. The results show that the method was precise and accurate for the determination of all analytes in human plasma.

Parameters	Imrecoxib (mean ± SD)	M1 (mean ± SD)	M2 (mean ± SD)
C_{max} (ng/mL)	14.4 ± 22.51	39.20 ± 14.96	101.62 ± 39.07
T_{max} (h)	1.86 ± 0.55	1.97 ± 0.71	1.53 ± 0.62
AUC_{0-t} (ng.h/mL)	117.69 ± 191.58	259.34 ± 117.64	455.03 ± 174.73
$AUC_{0-\infty}$ (ng.h/mL)	126.47 ± 193.34	291.69 ± 118.12	479.01 ± 182.45
$t_{1/2}$ (h)	8.96 ± 5.73	9.17 ± 5.13	5.98 ± 6.46

Table 4: The pharmacokinetic parameters of imrecoxib (M0), M1 and M2 in plasma following a single dose administration of 50mg imrecoxib tablet in healthy subjects (Mean ± SD, n=9).

Recovery and matrix effect: The results were shown in Table 2. The extraction recoveries for Imrecoxib, M1 and M2 at all tested levels were between 93.98% to 96.07%, 95.99% to 96.59% and 94.35% to 96.34%, respectively. The %CV of recovery for all analytes at all levels is no more than 5.08%. The extraction recoveries for IS was 98.31% and the %CV of recovery for IS was 3.25%. The matrix factor (MF) ranged from 1.0186 to 1.0962, 1.0556 to 1.0742 and 1.0321 to 1.0651 for Imrecoxib, M1 and M2, respectively with an overall %CV of 8.77%. The matrix factor (MF) of IS was 1.0564 and the %CV of IS-normalized matrix factor among all 6 lots was no more than 2.2%.

Stability: The stability results in each condition are shown in Table 3, which indicating that all analytes in the samples are stable when stored under these conditions.

Pharmacokinetic study: The validated LC-MS/MS method was successfully applied to a bioequivalence study after a single oral dose of 50 mg of Imrecoxib to nine healthy male volunteers. The plasma concentration-time profiles of Imrecoxib, M1 and M2 are shown in (Figure 3) and the related pharmacokinetic parameters are summarized in Table 4.

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