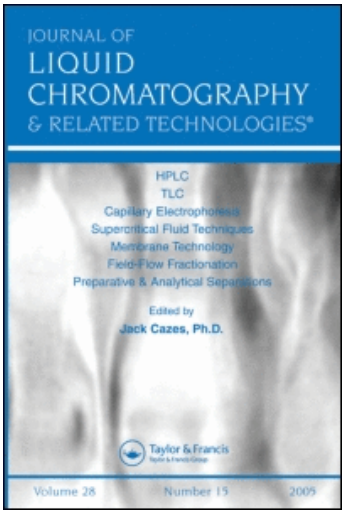

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Validation of an LC-Tandem MS/MS Method for the Determination of Etoricoxib in Human Plasma and Pharmaceutical Formulations

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Abstract: An analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for the determination of etoricoxib in spiked human plasma and pharmaceutical dosage forms. Etoricoxib and piroxicam (internal standard) were extracted from the plasma by liquid–liquid extraction using tert-butyl methyl ether as extraction solvent and separated on a C_{18} analytical column (50 mm × 3.0 mm I.D.). The mobile phase consisted of acetonitrile: water (95:5)/0.1% acetic acid (90:10, v/v). Detection was carried out by positive electrospray ionization (ESI+) in multiple reaction monitoring (MRM) mode. The chromatographic separation was obtained within 2.0 min and was linear in the concentration range of 1–5000 ng/mL. The mean extraction recoveries of etoricoxib and piroxicam from plasma were 96.09 and 95.54%, respectively. Method validation investigated parameters such as the linearity, precision, accuracy, specificity, and stability, giving results within the acceptable range. Moreover, the proposed method was successfully applied for routine quality control analysis of pharmaceutical products and the results compared with those obtained by the RP-HPLC method, showing significant correlation ($P > 0.05$).

Keywords: LC-MS/MS, Etoricoxib, Liquid–liquid extraction, Pharmaceutical analysis, Validation

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INTRODUCTION

Etoricoxib (5-chloro-6′-methyl-3-[4-(methylsulfonyl)phenyl]-2,3′-bipyridine) represents a second-generation of COX-2 inhibitors that has been developed for the treatment of many inflammatory diseases such as rheumatoid arthritis, osteoarthritis, pain relief, and acute gout, causing fewer gastrointestinal complications than conventional NSAIDs.[1–3]

Orally administered etoricoxib is well absorbed with a bioavailability of approximately 100%. Following 120 mg once daily dosing to steady state (reached within 7 days), the peak plasma concentration (geometric mean $C_{max} = 3.6\,\mu g/mL$) was observed at approximately 1 h (t_{max}) after administration to fasting adults. Etoricoxib pharmacokinetics is linear at clinically relevant doses and the pharmacokinetic half-life ($t_{1/2}$), approximately 22 h.[4–6]

An analytical HPLC method, with photochemical cyclisation and fluorescence detection for the quantitation of etoricoxib in human plasma and urine, was published using a structural analogue as internal standard and solid phase extraction (SPE).[7] A LC-MS/MS method with atmospheric pressure chemical ionization (APCI) was validated over the concentration range of 0.5–250 ng/mL for the determination of etoricoxib in human plasma, with a stable isotope as internal standard, and the run time of 8 min.[8] The LC-MS/MS method with electrospray ionization (ESI) for the determination of etoricoxib in human plasma, after extraction by SPE, was also developed over the concentration range of 0.2–200 ng/mL.[9] Liquid chromatography coupled to ion trap mass spectrometry, with APCI for quantitation of both etoricoxib and valdecoxib in human plasma, was also performed in the linear range of 10–2500 ng/mL.[10] Another liquid chromatography method for the quantitation of etoricoxib in human plasma was validated, with a run time of 10 min.[11] The LC coupled to MS detection has been used for both the identification and quantification of drugs at low concentrations in raw materials, in various pharmaceutical formulations, and biological fluids.[12] For the etoricoxib, there is no published method for the evaluation in pharmaceutical formulations.[13]

The aim of the present work was to validate a simple, fast, precise, and accurate LC-MS/MS method to be applied to the quantitative analysis of etoricoxib in human plasma, using a liquid-liquid extraction, improving the current published procedures, and demonstrating the applicability of the method for the potency evaluation of etoricoxib in pharmaceutical dosage forms.

EXPERIMENTAL

Chemicals and Reagents

Etoricoxib reference standard was generously supplied by Merck Research Laboratories (Rahway, USA) and piroxicam reference standard (Lot: 2a)

was purchased from European Pharmacopoeia. Arcoxia® tablets, containing 60, 90, and 120 mg of etoricoxib were obtained from commercial sources within their shelf life period. HPLC-grade acetonitrile, methanol, tert-butyl methyl ether, formic, phosporic, and acetic acid were purchased from Tedia (Fairfield, USA). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water (Labconco, Kansas City, USA) filtered through a 0.22 μm membrane filter was used.

Apparatus and Analytical Conditions

The LC-MS/MS method was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, LC-10 AD_{VP} pump, DGU-14A degasser, CTO-10 AD_{VP} column oven, and SPD-M10A_{VP} photodiode array (PDA) detector. A triathlon autosampler (Spark, Emmen, Holland) was used. The peak areas were integrated automatically by computer using a Masslynx software program. The experiments were carried out on a reversed phase Phenomenex (Torrance, USA) Luna C_{18} column (50 mm × 3.0 mm I.D., with a particle size of 3 μm and pore size of 100 Å). A security guard holder (4.0 mm × 3.0 mm I.D.) was used to protect the analytical column. The HPLC system was operated isocratically at controlled temperature (40°C) using a mobile phase of acetonitrile : water (95:5)/0.1% acetic acid (90:10, v/v). This was filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and run at a flow rate of 0.4 mL/min. The injection volume was 20 μL for both standard and samples. The triple quadrupole mass spectrometer (Waters, Milford, MA, USA), model Quattro LC, equipped with an electrospray source using a crossflow counter electrode run in positive mode (ESI+), was set up in multiple reaction monitoring (MRM) mode, monitoring the transitions 359.3 > 280 and 332 > 95, for etoricoxib and piroxicam (IS), respectively. For the optimization of mass spectrometer conditions, a mixed standard solution (1000 ng/mL) containing etoricoxib and IS was directly introduced and the following parameters were selected: nebuliser gas (nitrogen), cone gas, and desolvation gas set at 80, 74, and 480 L/h, respectively. Capillary voltage, extractor voltage, RF lens voltage, and source temperature were 3.2 kV, 3 V, 0.2 V, and 120°C, respectively. The dwell time was set at 0.5 seconds, the collision gas pressure (argon) was 2.3×10^{-3} mbar. The cone voltage was 62 and 32 V and the collision energy 30 and 20 eV, respectively for etoricoxib and IS. Data acquisition and analysis were performed using the software Masslynx (v 3.5) running under Windows 2000 on a workstation Compaq PC.

The RP-HPLC analysis was carried out on a reversed phase Phenomenex Synergi fusion C_{18} column (150 mm × 4.6 mm I.D., with a particle size of 4 μm and pore size of 80 Å). A security guard holder (4.0 mm × 3.0 mm I.D.) was used to protect the analytical column. The Shimadzu HPLC

system was operated isocratically at controlled ambient temperature (25°C) using a mobile phase of phosphoric acid 0.01 M, pH 3.0 adjusted with sodium hydroxide 3 M/acetonitrile (62:38, v/v), run at a flow rate of 1.0 mL/min using a photodiode array (PDA) detector at 234 nm. The injection volume was 10 μL of solution containing 100 μg/mL for both standard and samples.

Procedure

Preparation of Stock Solutions

The stock solution of etoricoxib was prepared by weighing 10 mg of reference material into a 10 mL volumetric flask and diluting to volume with acetonitrile, obtaining a concentration of 1 mg/mL. Piroxicam stock solution was also made at a final concentration of 1 mg/mL using acetonitrile. The prepared stock solutions were stored at 2–8°C and protected from light.

Preparation of Calibration Standards and Quality Control Samples

The stock solution of etoricoxib was diluted with acetonitrile to obtain calibration standard solutions with the concentrations of 10000, 1000, 100, and 10 ng/mL. The corresponding volume taken of the standard solutions were evaporated under nitrogen stream while immersed in a 40°C water bath, and the residues were reconstituted in 0.5 mL of blank plasma to prepare the calibration standards containing from 1 to 5000 ng/mL (1, 10, 20, 100, 200, 500, 1000, 3000, and 5000 ng/mL). The quality control (QC) samples were prepared in pooled plasma, with the concentrations of 3 (low), 2500 (medium), and 4000 ng/mL (high), and then divided in aliquots that were stored at −80°C until analysis.

Plasma Extraction Procedure

A total of 500 μL of the spiked plasma was transferred to a 15 mL glass tube, followed by addition of "50 μL of internal standard solution (500 ng/mL of piroxicam in acetonitrile) and 50 μL of formic acid. All samples were mixed by vortex agitation for 30 s. Then, a 4 mL aliquot of extraction solvent, tert-butyl methyl ether, was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The tubes were vortex mixed for 60 s, and then centrifuged for 5 min at 3000 rpm. The organic layer was filtered through a Millex GV 0.45 μm filter unit (Millipore, Bedford, MA, USA) into 15 mL conical glass tubes and evaporated under a nitrogen stream while immersed in a 40°C water bath. Each sample was reconstituted with 500 μL of acetonitrile:water (1:1, v/v) and vortex mixed for 30 s. The

samples were transferred to autosampler vials and 20 μL was injected into the LC-MS/MS system.

Validation of the Bioanalytical Method

The method was validated by the determination of the following parameters: specificity, linearity, range, recovery, accuracy, precision, lower limit of quantitation (LLOQ), and stability studies.

Specificity

Randomly selected six blank human plasma samples, which were collected under controlled conditions, were carried through the extraction procedure and chromatographed to determine the extent to which endogenous plasma components could contribute to interference with the analyte or the internal standard. The results were compared with LLOQ (1 ng/mL).

Calibration Curve

The calibration curves were constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS), and nine concentrations of etoricoxib including the LLOQ, ranging from 1 to 5000 ng/mL. The peak area ratio of the drug to the IS against the respective standard concentrations was used for plotting the graph and the linearity evaluated by a weighted (1/x) least squares regression analysis. The acceptance criteria for each calculated standard concentration was not more than 15% deviation from the nominal value, except for the LLOQ which was set at 20%.

Recovery

The analytical recovery was calculated by comparing chromatographic peak areas from unextracted standard samples and from extracted standard samples at three different concentrations (3, 2500, and 4000 ng/mL) for the etoricoxib and 25 ng/mL for the IS.

Accuracy and Precision

To evaluate the inter-day precision and accuracy, the quality control samples were analysed together with one independent calibration standard curve for 3 days, while intra-day precision and accuracy were evaluated through analysis of validation control samples at three different concentrations in six replicates in the same day. Inter- and intra-day precision was expressed as relative standard deviation (RSD). The accuracy was expressed as the percent ratio between the experimental concentration and the nominal concentration for

each sample. The evaluation of precision was based on the criteria[14] that the deviation of each concentration level should be within ±15%, except for the LLOQ, for which it should be within ±20%. Similarly for accuracy, the mean value should not deviate by ±15% of the nominal concentration, except the LLOQ, where it should not deviate by ±20% of the nominal concentration.

Lower Limit of Quantification (LLOQ) and Limit of Detection (LOD)

The lowest standard concentration on the calibration curve should be accepted as the limit of quantification if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compared to blank response and analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

Stability

The concentration of etoricoxib after each storage period was related to the initial concentration as zero cycle (samples that were freshly prepared and processed immediately). The samples were considered stable if the deviation (expressed as percentage bias) from the zero cycle was within ±15%.

Freeze-thaw Stability

The freeze-thaw stability of etoricoxib was determined at low, medium, and high QC samples (n = 3), over three freeze-thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analysed and the results compared with that of zero cycle.

Short-term Stability

Three aliquots each of the low, medium, and high unprocessed QC samples were kept at room temperature (25 ± 5°C) for 12 h. After 12 h the samples were analysed and the results compared with that of zero cycle.

Long-term Stability

Three aliquots each of the low, medium, and high QC samples were frozen at −80°C for 60 days. The samples were analysed and the results were compared with that of zero cycle.

Processed Sample Stability

Six aliquots, each one of the low, medium, and high QC samples were processed and placed into the autosampler at room temperature. Three sets

were analysed after 24 h and three sets after 48 h. The results were compared with that of zero cycle.

Analysis of Pharmaceutical Tablet Dosage Forms

For the preparation of the linearity curve, calibration standards were added with "50 μL of IS (500 ng/mL of piroxicam in acetonitrile) and the volume made up to 0.5 mL with the acetonitrile:water (1:1, v/v) to get the linearity range of 1 to 5000 ng/mL. Concentrations of 3, 2500, and 4000 ng/mL were taken as quality control samples. An aliquot of 20 μL of these solutions were injected for the analysis and the peak area ratio of the drug to the IS was considered for plotting the linearity graph. For the quantitation in the dosage forms, twenty tablets of each sample containing respectively, 60, 90, and 120 mg of etoricoxib, were separated, accurately weighed, and crushed to a fine powder. An appropriate amount of each concentration was transferred into an individual 50 mL volumetric flask, diluted to volume with acetonitrile, and sonicated for 20 min, obtaining the concentration of 1 mg/mL (stock solution). Working sample solutions were prepared daily and added with "50 μL of IS, and the volume made up to 0.5 mL with the acetonitrile:water (1:1, v/v) to get 1000 ng/mL of etoricoxib. An aliquot of 20 μL was injected for the analysis and the amount of etoricoxib per tablet calculated from the linear regression equation.

RESULTS AND DISCUSSION

To obtain the best chromatographic conditions, different columns and mobile phases consisting of acetonitrile-water or methanol-water were tested to provide sufficient selectivity and sensitivity in a short separation time. Modifiers such as formic acid, acetic acid, and ammonium acetate were tested. The best signal was achieved using acetonitrile:water (95:5)/0.1% acetic acid (90:10, v/v) with a flow rate of 0.4 mL/min in a C_{18} analytical column. The low flow rate and the short run time resulted, comparatively, in lower consumption of the mobile phase solvents with a better cost effective relation. The protonated molecular ions $[M + H]^+$ of etoricoxib and IS, observed on the full scan mass spectra, were m/z 359.3 and 332. Moreover, the collision energy in Q2 produced significant fragments. The MS/MS transition $359.3 > 280$ and $332 > 95$ were selected since the ion scan product with m/z 280 and 95 presented a higher abundance and stability for the etoricoxib and IS, respectively. The coupling of LC with MS/MS detection in the MRM mode showed high specificity, because only the ions derived from the analytes of interest were monitored.

The linearity was determined by six determinations of nine concentrations in the range of 1–5000 ng/mL. The value of the determination coefficient

($r^2 = 0.999$, $y = 0.812231 + 0.457897x$) indicated significant linearity of the calibration curve for the method. The LLOQ was calculated as 1 ng/mL and LOD was found to be 0.1 ng/mL. Comparison of the chromatograms of the blank and spiked human plasma (1 ng/mL) indicated that no interferences were detected from endogenous substances. A typical chromatogram obtained by the proposed LC-MS/MS method, with the resolution of the symmetrical peak corresponding to etoricoxib and piroxicam, is shown in Figure 1. The low retention times of 0.79 and 0.97 min allow a rapid determination of the drug, which is an important advantage for the routine analysis.

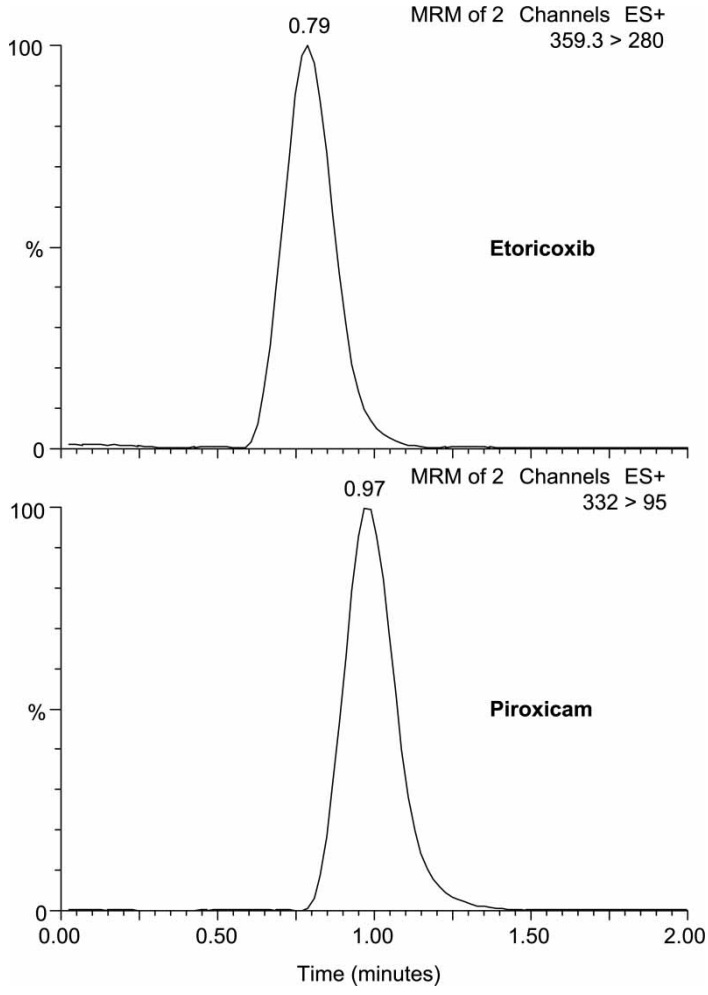

Figure 1. Representative LC-MS/MS chromatogram of low QC plasma sample containing etoricoxib (3 ng/mL) and IS (25 ng/mL).

Table 1. Recovery of etoricoxib and piroxicam after the extraction procedure

Etoricoxib concentration (ng/mL)	Recovery (%) (mean ± RSD%)	
	Etoricoxib[a]	Piroxicam[a]
3	97.22 ± 7.26	98.93 ± 7.22
2500	92.74 ± 4.04	93.72 ± 5.34
4000	98.32 ± 4.01	93.97 ± 5.00

[a]Mean of six replicates.

The results of liquid–liquid extraction method developed, using tert-butyl methyl ether as extraction solvent with 50 μL of formic acid, allowed high mean recoveries of etoricoxib (96.09%) and IS (95.54%) at the specified concentration levels, confirming the suitability of the method for the plasma samples (Table 1). For the extraction, different organic solvents and mixtures were also evaluated, including ethyl acetate, diethyl ether, and dichloromethane. The liquid–liquid extraction described in the literature[10,11] showed recoveries of etoricoxib in human plasma of 82.9 and 76.1%, respectively, lower than that reported in the present work. Solid phase extraction (SPE) procedures were also reported to extract the etoricoxib from plasma with recoveries $<90\%$,[7,9] but for routine analysis SPE methods are expensive and not available in most of the laboratories.

The intra-day accuracy of the method was between 100.30 and 108.33% with a precision of 4.18–6.33% (Table 2). The inter-day accuracy was between 98.34 and 103.67% with a RSD of 3.85–9.41% (Table 3). The data show that the method possesses adequate repeatability and reproducibility.

As shown in Table 4, the plasma samples were stable for at least 60 days at -80°C (long-term) and also after three freeze-thaw cycles, demonstrating that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Etoricoxib was stable in neat plasma for up to

Table 2. Intra-day precision and accuracy for the determination of etoricoxib in human plasma

Nominal concentration (ng/mL)	Mean concentration found (ng/mL)[a]	RSD (%)	Accuracy (%)
3	3.25	6.33	108.33
2500	2512.84	4.18	100.51
4000	4011.99	4.78	100.30

[a]Mean of six replicates.

Table 3. Inter-day precision and accuracy for the determination of etoricoxib in human plasma

Nominal concentration (ng/mL)	Day	Mean concentration found (ng/mL)[a]	Mean[b]	RSD (%)	Accuracy (%)
3	1	3.23			
	2	3.32	3.11	9.41	103.67
	3	2.79			
2500	1	2496.6			
	2	2383.4	2458.6	3.85	98.34
	3	2495.7			
4000	1	4020.0			
	2	3908.8	4048.4	6.09	101.21
	3	4216.5			

[a]Mean of five replicates.
[b]Mean of three days.

Table 4. Summary of stability of etoricoxib in human plasma

Stability	Zero cycle concentration (ng/mL)[a]	Concentration found after storage (ng/mL)[a]	RSD (%)	Bias[b] (%)
Long term	3.23	2.89	9.04	−10.52
	2528.06	2655.28	3.48	5.03
	4069.91	3818.29	3.93	−6.18
Short term	3.23	2.86	6.32	−11.45
	2528.06	2725.22	1.44	7.80
	4069.91	3581.62	1.67	−11.99
Autosampler 24 h	3.23	2.85	3.17	−11.76
	2528.06	2506.27	5.50	−0.86
	4069.91	4110.22	6.38	0.99
Autosampler 48 h	3.23	2.90	4.34	−10.21
	2528.06	2494.30	9.96	−1.33
	4069.91	4117.65	8.75	1.17
Three freeze-thaw cycles	3.23	2.90	5.31	−10.21
	2528.06	2662.05	7.38	5.30
	4069.91	4428.65	3.65	8.81

[a]Mean of three replicates.
[b]Bias = (measured concentration − nominal concentration/nominal concentration) × 100.

Table 5. Determination of etoricoxib in pharmaceutical dosage forms by the RP-HPLC and LC-MS/MS methods

Theoretical amount		Experimental amount[a]					
		RP-HPLC[b]			LC-MS/MS[b]		
Sample	mg per tablet	mg	%	RSD (%)	mg	%	RSD (%)
1	60	61.63	102.72	0.23	61.86	103.10	0.25
2	90	90.87	100.97	0.31	91.23	101.37	0.27
3	120	122.15	101.79	0.20	122.70	102.25	0.33

[a]$P > 0.05$ non-significant.
[b]Mean of three determinations.

12 h at room temperature (short-term). The results demonstrated that extracted samples could be analysed after keeping in the autosampler for at least 48 h with an acceptable precision and accuracy.

The LC-MS/MS method validated in this paper was also used for the potency evaluation of etoricoxib in tablet dosage forms as shown in Table 5, together with the results obtained from the analysis of the pharmaceutical formulations carried out by the RP-HPLC method, previously validated in the laboratory (data not shown). The values obtained from the two methods were compared statistically by the Student's *t*-test showing non significant difference ($P > 0.05$) between the experimental results. The proposed method can be used for the determination of etoricoxib without prior separation of the excipients of the formulation, with the advantage of very short time of analysis (<2 min), representing also an improvement for the quality control of pharmaceuticals as the technique is highly selective and sensitive.

CONCLUSION

A simple and fast LC-MS/MS method for the determination of etoricoxib in human plasma and pharmaceutical formulations was developed and validated. This method involves a single step liquid–liquid extraction procedure, using piroxicam, a commercially available substance, as internal standard. The short run time of 2 min and the relatively low flow rate (0.4 mL/min) allows the analysis of a large number of samples with less mobile phase that proves to be cost-effective. The data validation shows that the optimized LC-MS/MS method possesses specificity, sensitivity, linearity, precision, and accuracy over the entire range of significant therapeutic plasma concentrations. Therefore, the proposed method can be used for clinical and pharmacokinetic

studies, and for the routine quantitative analysis of etoricoxib in pharmaceutical dosage forms.

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