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Validated liquid chromatographic ultraviolet method for the quantitation of Etoricoxib in human plasma using liquid–liquid extraction

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Abstract

A simple, sensitive and specific HPLC method with UV detection (284 nm) was developed and validated for quantitation of Etoricoxib in human plasma, the newest addition to the group of nonsteroidal anti-inflammatory drugs—a highly selective cyclooxygenase-2 inhibitor. Following a single-step liquid–liquid extraction with diethyl ether/dichloromethane (70/30, v/v), the analyte and internal standard (Zaleplon) were separated using an isocratic mobile phase of water/acetonitrile (58/42, v/v) on reverse phase Waters symmetry® C_{18} column. The lower limit of quantitation was 5 ng/mL, with a relative standard deviation of less than 20%. A linear range of 5–2500 ng/mL was established. This HPLC method was validated with between- and within-batch precision of 4.1–5.1% and 1.1–2.4%, respectively. The between- and within-batch bias was −3.8–4.7% and −0.6–9.4%, respectively. Frequently coadministered drugs did not interfere with the described methodology. Stability of Etoricoxib in plasma was >90%, with no evidence of degradation during sample processing (autosampler) and 30 days storage in a freezer. This validated method is sensitive and simple with between-batch precision of <6% and was used in pharmacokinetic studies.

Keywords: Etoricoxib; Liquid–liquid extraction; Quantitation; Human plasma

1. Introduction

Etoricoxib {5-chloro-3-(4-methanesulfonylphenyl)-6′-methyl-[2,3′]-bipyridinyl} (Fig. 1) is the newest addition to the group of nonsteroidal anti-inflammatory drugs (NSAIDs) known as selective cyclooxygenase-2 inhibitors (e.g., Celecoxib, Rofecoxib and Valdecoxib) [1–3]. This drug has been launched in 38 countries worldwide in Europe, Latin America and the Asia Pacific region. Recently Merck & Co. Inc., has submitted a new drug application (NDA) for ARCOXIA (etoricoxib) to the U.S. Food and Drug Administration for the treatment of osteoarthritis, rheumatoid arthritis, chronic low back pain, acute pain, dysmenorrheal, acute gouty arthritis and ankylosing spondylitis [2,4].

The NSAIDs exert their anti-inflammatory analgesic and antipyretic activities through the inhibition of cyclooxygenase (COX), a key enzyme for prostanoid synthesis. The enzyme exists as two isoforms: a constitutive form, COX-1, and an inducible form, COX-2 [5]. COX-1 is involved in prostaglandin synthesis and inhibition of this enzyme by nonselective NSAIDs is thought to be responsible for damage to the gastric mucosa and for antiplatelet activity, increasing the risk of bleeding. In contrast, COX-2 primarily synthesizes prostaglandins involved in inflammation. Selective inhibition of COX-2, while preserving COX-1 function, suppresses inflammation without causing the gastric adverse effects on increasing the risk of bleeding [1,2,6]. The development of COX-2 inhibitors with improved biochemical selectivity (such as etoricoxib and valdecoxib) over that of commercially available coxibs has been driven by the potential advantage of safety using higher coxib doses for increased efficacy.

Recently, clinical investigations have demonstrated a link between use of the sulfone COX-2 inhibitor, rofecoxib, and

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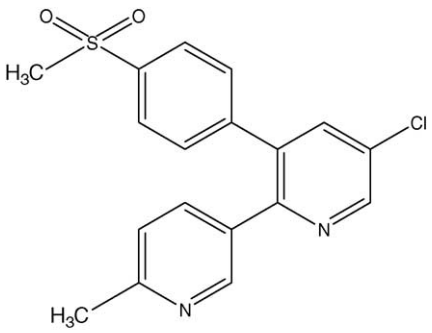
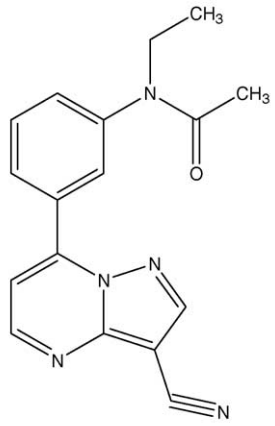


Etoricoxib



Zaleplon (I.S.)

Fig. 1. Chemical structures of Etoricoxib and Zaleplon.

increased risk for atherothrombotic events. This increased risk was not observed for a sulfonamide COX-2 inhibitor (celecoxib), indicating a potential non-enzymatic mechanism for rofecoxib. Walter et al. [7] demonstrated that sulfone COX-2 inhibitors (rofecoxib and etoricoxib) increase the susceptibility of biological lipids to oxidative modification through a non-enzymatic process. These findings may provide mechanistic insight into reported differences in cardiovascular risk for COX-2 inhibitors.

Etoricoxib does not inhibit prostaglandin synthesis in the gastric mucosa, even at doses above the clinical dose range of 60–120 mg [8]. It is rapidly absorbed after oral ingestion with a bioavailability of >80% and a t_{max} of 1–1.5 h [1,6]. At steady state (reached within 7 days), peak plasma concentration (C_{max}) of etoricoxib of 3.6 μg/mL was reached with once-daily administration of etoricoxib 120 mg in fasted adults [6]. Etoricoxib pharmacokinetics are linear at clinically relevant doses and the pharmacokinetic half-life ($t_{1/2}$, approximately 22 h), and pharmacological response, supports oral once-a-day dosing [9].

An analytical method for the quantitation of Etoricoxib in human plasma and urine using solid phase extraction (SPE) and HPLC with photochemical cyclisation and fluorescence detection with a structural analogue as internal standard has been published. The limit of quantification determined by Matthews et al. [10] was 5 ng/mL. Rose et al. [11] reported a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method with atmospheric pressure chemical ionization (APCI) for determination of Etoricoxib in human plasma with a stable isotope of Etoricoxib as internal standard. The runtime of this method was 8 min and it was validated over the concentration range 0.5–250 ng/mL. Recently, Bräutigam et al. [12] reported a LC–MS/MS method with electrospray ionization (ESI) for the determination of Etoricoxib in human plasma using SPE. However, the LC–MS machine is quite expensive and is not readily available in most clinical, bio-analytical research laboratories. To date, no simple HPLC method with commonly used ultraviolet absorbance detection has been reported for Etoricoxib quantitation at therapeutic concentrations in plasma. This paper describes a simple, selective and sensitive HPLC/UV method for quantitation of the plasma concentrations of Etoricoxib using liquid–liquid extraction. Additionally, this method provides information about the stability of Etoricoxib in plasma and during sample processing (autosampler). This method has been successfully used for clinical Etoricoxib pharmacokinetic studies.

When the manuscript is under revision, Werner et al. [13] presented a liquid chromatography–mass spectrometry method for the quantification of both etoricoxib and valdecoxib in human plasma. The method was validated over a linear range 10–2500 and 5–1000 ng/mL using the other substrate as internal standard.

2. Experimental

2.1. Chemicals

Etoricoxib drug substance and Zaleplon (internal standard, IS) were obtained from Cadila Healthcare Limited (Ahmedabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol, LiChrosolv acetonitrile, diethyl ether and dichloromethane were from Merck (Darmstadt, Germany). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Chromatography

The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100 μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The separation of compounds was made on a Waters symmetry® C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) at 30 °C temperature. The mobile phase was a mixture of water/acetonitrile (58/42, v/v) pumped at a flow-rate of 1.2 mL/min. Detection

was set at a wavelength of 284 nm. Peak quantitation was done by peak area ratio method. All regressions and figures presented in this study were generated by Shimadzu Class VP v 6.12 software.

2.3. Sample processing

A 1 mL volume of plasma was transferred to a 15 mL glass test tube, and then 50 μL of IS working solution (50 μg/mL) was spiked. Next a 5-mL aliquot of extraction solvent, diethyl ether/dichloromethane (7/3), was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 5 min using a Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The sample was then centrifuged using Multifuge 3S-R (Kendro Laboratory Products, Sorvall-Heraeus, Germany) for 3 min at 800 × *g*. The organic layer (4 mL) was quantitatively transferred to a 6 mL glass tube and evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 250 μL of water/methanol (50/50, v/v; diluent) and a 100 μL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Working solutions for calibration (0.1, 0.3, 1, 2, 4, 10, 20 and 50 μg/mL) and controls (0.1, 0.3, 25 and 40 μg/mL) were prepared from the stock solution by an adequate dilution using diluent (water/methanol, 50/50, v/v). The IS working solution (50 μg/mL) was prepared by diluting stock solution with diluent. Fifty microlitres of working solutions were added to 950 μL of drug-free plasma to obtain Etoricoxib concentrations of 5, 15, 50, 100, 200, 500, 1000 and 2500 ng/mL. Five percent working solutions of quality controls were added to 95% of drug-free plasma in pool, to obtain etoricoxib concentrations of 5 ng/mL (LLOQ), 15 ng/mL (low), 1250 ng/mL (medium) and 2000 ng/mL (high), as a single batch at each concentration. The quality control pools were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at −70 °C until analysis.

Each validation run consisted of a double control, system suitability sample, blank samples (a plasma sample processed without an IS), a zero sample (a plasma processed with IS), calibration curve consisting of eight non-zero samples covering the total range (5–2500 ng/mL) and QC samples at three concentrations ($n = 6$, at each concentration). Such validation runs were generated on six consecutive days. Calibration samples were analyzed from low to high at the beginning of each validation run and other samples were distributed randomly through the run. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. At least 67% of non-zero standards should meet the above criteria, including acceptable LLOQ and upper limit of quantitation.

2.4.2. 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 may contribute to interference with the analyte or the internal standard. The results were compared with LLOQ (5 ng/mL).

2.4.3. Recovery

Recovery of Etoricoxib was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six unprocessed reference solutions. Recovery of IS was evaluated by comparing the mean peak areas of ten extracted quality control samples to mean peak areas of ten unprocessed reference solutions of the same concentration.

2.4.4. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by repeated analysis of Etoricoxib in human plasma. The run consisted of a calibration curve plus six replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and six replicates of LLOQ, low, medium and high quality control samples for Etoricoxib on three separate occasions. During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate and plasma samples to be determined.

The overall precision of the method expressed as relative standard deviation and accuracy of the method expressed in terms of bias (percentage deviation from true value).

2.4.5. Stability

Twenty-four hour stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h for each cycle. Autosampler stability of Etoricoxib was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 24 h. Stability of Etoricoxib in human plasma was tested after storage at approximately −70 °C for 30 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of Etoricoxib after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

3. Results and discussion

3.1. Separation

Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with Etoricoxib at 1000 ng/ml and at LLOQ (5 ng/ml), and plasma sample obtained from a healthy subject after 36 h following an oral 120 mg dose of Etoricoxib. The analytes were well separated from co-extracted material under the described chromatographic conditions at retention times of 7.8 and 5.2 min, respectively. The peaks were of good shape, completely resolved one from another at therapeutic concentrations of Etoricoxib. No interference with constituents from the plasma matrix was observed.

3.2. Linearity and sensitivity of the assay

The peak area ratio of Etoricoxib to IS in human plasma was linear with respect to the analyte concentration over the range 5–2500 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least-squares linear regression. The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a $1/x^2$ weighing factor. The mean linear regression equation of calibration curve for the analyte was $y = 0.675(\pm 0.0365) \times -0.540\ (\pm 1.2861)$, where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The correlation coefficient (r) for Etoricoxib was above 0.999 over the concentration range used. Table 1 summarizes the calibration curve results for the analyte. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between- and within-batch validations.

The lower limit of quantification (LLOQ), the lowest concentration in the standard curve, which can be measured with acceptable accuracy and precision for the analyte from normal human plasma was established as 5 ng/mL. The mean response for the analyte peak at the assay sensitivity limit

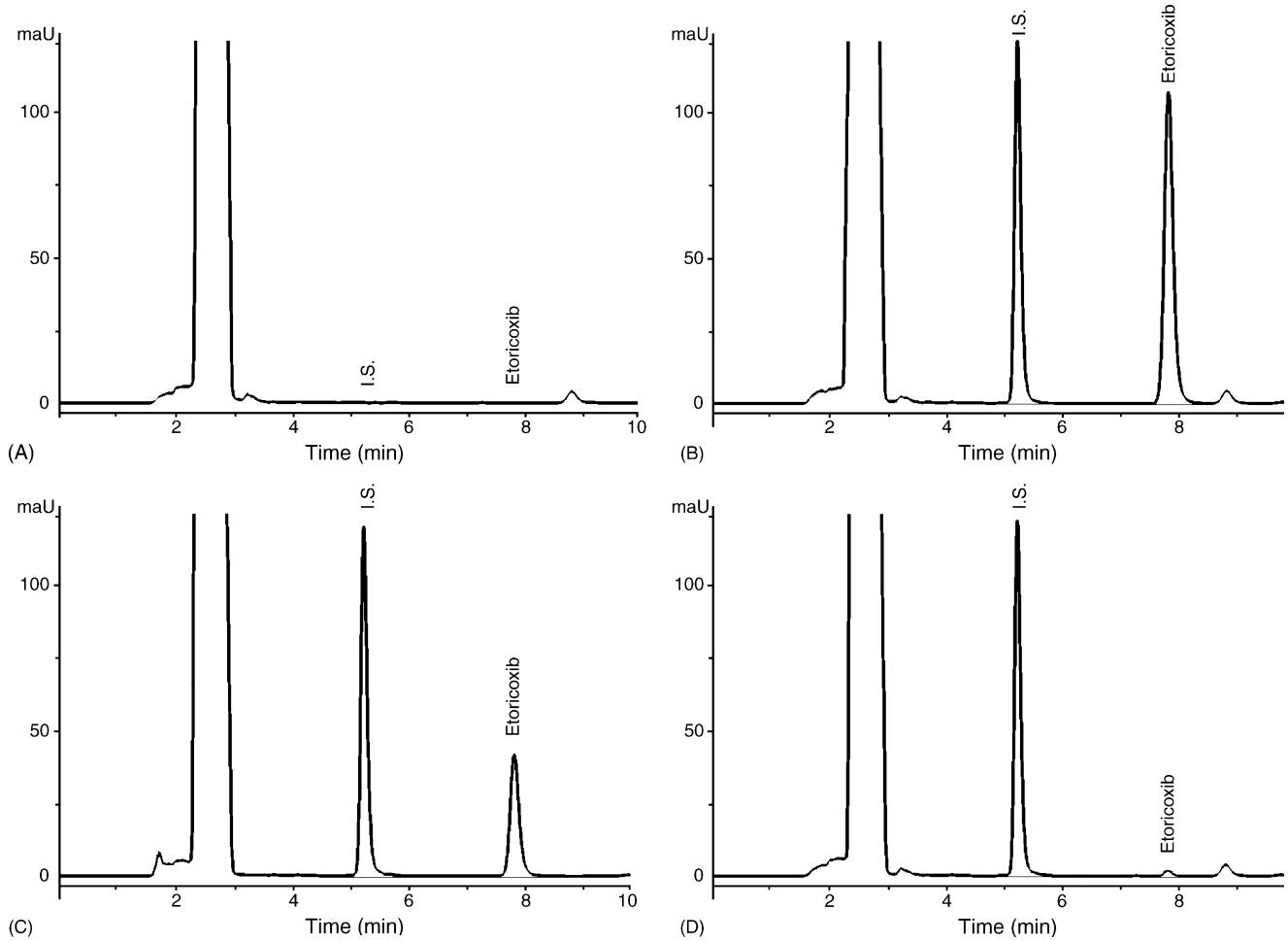


Fig. 2. Chromatograms of (A) blank human plasma; (B) human plasma sample spiked with 1000 ng/mL of Etoricoxib and IS; (C) plasma sample from a healthy subject after 36 h following a 120 mg oral dose of Etoricoxib, the plasma concentration was determined to be 479.96 ng/mL for Etoricoxib; (D) spiked human plasma sample at LLOQ (5 ng/mL). Approximate retention times: Etoricoxib = 7.8 min; IS = 5.2 min.

Table 1
Precision and accuracy data of back-calculated concentrations of calibration samples for Etoricoxib in human plasma

Concentration added (ng/mL)	Concentration found (mean ± S.D. $n = 6$) (ng/mL)	Precision (%)	Bias (%)
5	5.22 ± 0.22	4.3	4.4
15	15.08 ± 0.46	3.1	0.5
50	51.56 ± 2.87	5.6	3.0
100	98.00 ± 4.79	4.9	−2.0
200	192.26 ± 10.57	5.5	−3.8
500	490.22 ± 36.99	7.5	−1.9
1000	942.06 ± 58.96	6.3	−5.8
2500	2410.51 ± 144.92	6.0	−3.5

(5 ng/mL) was ≈8.12-fold greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte.

3.3. Extraction

In the previous studies, all the publications described a solid phase extraction method (using either Oasis HLB or 96-well plate equipped with C8 material) to extract the Etoricoxib from plasma; the extraction recovery was >70% [10–12]. For routine clinical analysis, a high-throughput method with expensive SPE is always not advantageous as such equipment/techniques are not available in most of the laboratories. We were looking for alternative simple methods. The extraction method should also be suitable for an internal standard that is commercially available without being a stable isotope or a structural isomer of Etoricoxib. In order to develop a single step liquid–liquid extraction procedure with sufficient recovery, we investigated a large range of extraction solvents. The absolute recovery of Etoricoxib after single extraction from plasma using chloroform, ethyl acetate and hexane were all <50%; however, when the diethyl ether/dichloromethane (7/3) was used, the absolute recovery was quite high (>75% for both Etoricoxib and IS). The commercially available substance Zaleplon was chosen as the IS for several reasons. First, Zaleplon physicochemical properties are very well in match with Etoricoxib with regard to this bioanalytical method. Second, the chromatographic and extraction properties are similar to Etoricoxib.

The extraction recovery of Etoricoxib at low, medium and high quality control samples was 76.1 ± 2.1%, 75.6 ± 2.3% and 76.6 ± 2.4%, respectively. It indicates that extraction recovery of Etoricoxib is independent of concentration. The recovery of IS was 75.9% at the concentration used in the assay (50 μg/mL).

3.4. Specificity

There were no interfering peaks present in six different randomly selected samples of drug free human plasma used for analysis at the retention times of either analyte or IS There was no interference of Etoricoxib and IS analysis by other potentially co-administered drugs such as paracetamol, nicotinamide, ibuprofen, caffeine, aspirin, ampicillin, amoxicillin, loratadine, desloratadine, atorvastatin, clopidogrel, metformin, glimepiride, celecoxib, rofecoxib, valdecoxib, naproxen and nimuselide.

3.5. Accuracy of the assay

The accuracy values for between- and within-batch studies at the LLOQ and at low, medium and high concentrations of Etoricoxib in plasma were within acceptable limits ($n = 3$) (Table 2).

3.6. Precision of the methods

3.6.1. Within-batch variability of the assay

The results shown in Table 2 indicate that the assay method is reproducible for replicate analysis of Etoricoxib in human plasma within the same day.

3.6.2. Between-batch variability of the assay

The results shown in Table 2 indicate that the assay method is reproducible on different days.

Table 2
Accuracy and precision of the HPLC method for determining Etoricoxib concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision ($n = 6$)			Between-batch precision ($n = 3$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Bias (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Bias (%)
5	5.23 ± 0.11	2.2	4.7	5.17 ± 0.21	4.1	3.4
15	16.42 ± 0.17	1.1	9.4	15.71 ± 0.75	4.8	4.7
1250	1242.26 ± 29.30	2.4	−0.6	1201.56 ± 55.64	4.6	−3.8
2000	2092.72 ± 27.92	1.3	4.6	1986.74 ± 100.78	5.1	−0.6

Table 3
Stability of the samples

Sample concentration (ng/mL)	Concentration found immediately (ng/mL)	Concentration found after storage (ng/mL)	Precision (%)	Bias (%)
Short term stability for 24 h ($n = 6$) in plasma				
15	14.75	14.98	2.8	1.5
2000	1956.42	2000.58	4.5	2.2
Three freeze and thaw cycles ($n = 6$)				
15	14.75	14.73	7.6	−0.1
2000	1956.42	1800.24	2.9	−7.9
Autosampler stability for 24 h ($n = 6$) (after extracting and reconstitution)				
15	14.75	15.77	1.9	6.9
2000	1956.42	2014.88	4.7	2.9
30-days stability at −70 °C ($n = 6$)				
15	15.19	14.56	6.9	−4.1
2000	2015.63	1864.28	5.1	−7.5

3.7. Stability

Stock solutions of Etoricoxib (1 mg/mL) and IS (1 mg/mL) were prepared separately in methanol. The weight of analytes was corrected for purity. The solutions were stable for at least 6 months when stored under light-protected conditions at 4 °C. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. These were performed as described in Section 2.4.5. All stability results are summarized in Table 3. Three freeze–thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that Etoricoxib was stable in human plasma under these conditions. QC samples were stable for at least 30 days if stored frozen at approximately −70 °C. Testing of autosampler stability of quality control samples (Table 3) indicated that Etoricoxib is stable when kept in the autosampler for up to 24 h.

3.8. Dilution integrity

The dilution integrity was also conducted to assess whether the upper concentration limit (2500 ng/mL) can be extended. Quality control samples (in six replicates) at concentration 4000 ng/mL were diluted by two times with blank plasma, and the assay precision and accuracy were determined in a similar manner as described in Section 2.4.4. For Etoricoxib, the concentration found was 4015 ± 3.7% ng/mL and bias was 3.2%. The results suggested that samples whose concentrations were greater than the upper limit of the standard curve could be re-analyzed by appropriate dilution.

3.9. Application to clinical study

The present HPLC method was for the first time employed to determine the pharmacokinetic parameters of Etoricoxib in subjects' plasma samples of clinical studies. After a single oral dose of 120 mg Etoricoxib tablet in 12 healthy subjects, concentration versus time profiles were constructed for up to 96 h for Etoricoxib quantitation. Fig. 3 shows the representative concentration–time profiles of Etoricoxib in one subject following a 120 mg oral dose of Etoricoxib under fasting condition. The maximum Etoricoxib plasma concentration was 2074 ng/mL, t_{max} was 1.5 h, and $t_{1/2}$ in the terminal elimination phase was 22.2 h. The parameter values are in good agreement with those reported previously [9].

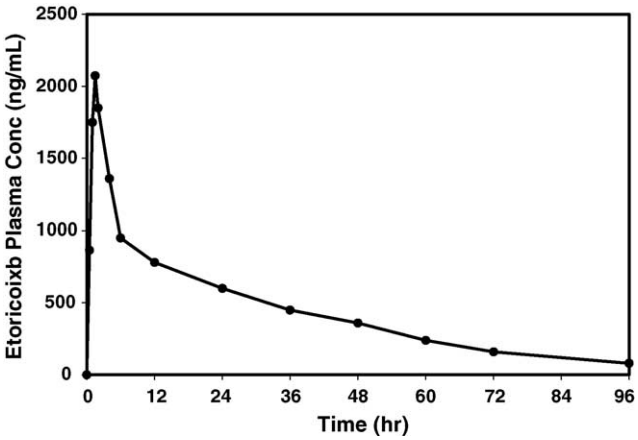


Fig. 3. Concentration versus time profiles over 96 h of Etoricoxib in human plasma from a subject receiving a single 120 mg dose of Etoricoxib.

4. Conclusions

The developed HPLC/UV method employing liquid–liquid extraction for sample preparation is very simple and convenient for the quantitation of Etoricoxib in human plasma samples. The previously reported methods for the analysis of Etoricoxib in biological fluids [10–13] were not too satisfactory because all of them were too expensive. The validation data also demonstrate good precision, accuracy and high extraction efficiency. The validated method allows quantification of Etoricoxib in the 5–2500 ng/mL range. In addition, this method has a short turnover time (<10 min) and is suitable for clinical pharmacokinetic studies. In conclusion,

this paper describes a very simple and sensitive HPLC method for the quantitation of Etoricoxib suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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