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Full Length Research Paper

Stability indicating high performance liquid chromatographic assay for the pharmacokinetics of cyclooxygenase (COX-2) inhibitor etoricoxib in rats

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An accurate, sensitive and simple high-performance liquid chromatography (HPLC) assay with UV detection has been developed and validated for the simultaneous determination of etoricoxib and its internal standard (IS) flurbiprofen in plasma has been developed. After plasma samples clean up by protein precipitation, followed by solvent evaporation and reconstitution with the mobile phase, an aliquot of the resulted solution were injected into the chromatograph. Peaks were eluted from a Novapak-C8 column using a mobile phase consisting of acetic acid: triethylamine: acetonitrile: water (0.02: 0.01: 41: 59.97, v/v), pH 4.0 at flow rate of 1 ml/min. The detection wavelength was 245 nm with a limit of detection of 50 ng/ml, with a coefficient of variation of 9.8%. Small volumes of plasma were required (200 µL) for etoricoxib determination. The run time was 10 min with etoricoxib and IS eluted in 3.8 and 7.2 min, respectively. The standard curve for the drug was linear in the range of 100 - 5,000 ng/ml for etoricoxib with correlation coefficient > 0.997. This method has been fully validated and shown to be specific, accurate and precise. The assay was selective, where the drug peak was well resolved with no interference from different drugs which could be given concomitantly. The extraction procedure was simple and rapid producing good recovery and a clean baseline, and providing excellent resolution and peak shape for all analytes. The assay was applied to determine the pharmacokinetics of etoricoxib in rats after 15 mg/kg oral dose. Etoricoxib plasma concentrations time profile follows two-compartmental open model with fast distribution and slow elimination phases. This assay is being utilized in determining etoricoxib pharmacokinetics in animals to monitor its interactions with other drugs or food supplements in our laboratory.

Key words: Etoricoxib, COX-2, HPLC, pharmacokinetics, flurbiprofen, rats.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the symptomatic treatment of acute and chronic inflammatory diseases and pain (Rao et al., 1995; Reddy et al., 1996). The beneficial anti-inflammatory and analgesic effects of these drugs have been linked to the inhibition of the inducible cyclooxygenase-2 (COX-2) whereas their side effects such as gastrointestinal ulcers are believed to be mainly due to inhibition of the constitutive cyclooxygenase isoform (COX-1) (Simmons et al., 2004). In recent years, efforts have been made to find potent and selective COX-2 inhibitors that could reduce pain and inflammation without affecting the cytoprotective action of COX-1 (Hawley, 1999). Therefore, COX-2 selective inhibitors such as celecoxib, rofecoxib, valdecoxib, etoricoxib (EXB), and lumiracoxib have been developed for the long term treatment of patients suffering from chronic pain and inflammation [Penning et al., 1997; Prasit et al., 1999; Chan et al., 1999; Chauret et al., 2001). The incidences of upper gastrointestinal clinical events have been shown to besignificantly less with COX-2 selective inhibitors than traditional non-steroidal anti-inflammatory drugs (NSAIDs) in randomized gastro-

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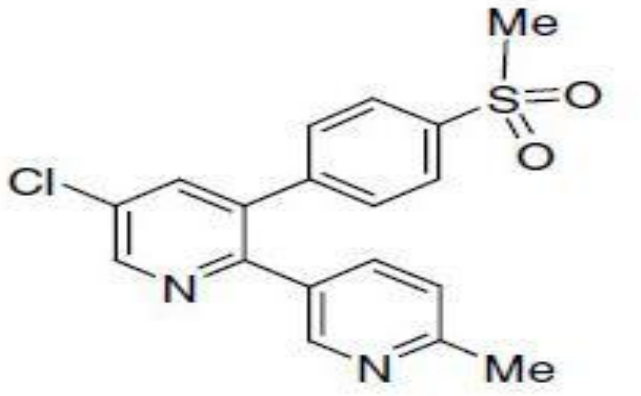

Figure 1. Chemical structure of etoricoxib.

gastrointestinal outcomes trials of 12 weeks to 12 months duration (Bombardier et al., 2000; Schnitzer et al., 2004; Singh et al., 2006; FDA , 2007).

EXB (Figure 1) is (5-choloro-3-(4- methanesulphonyl-phenyl) -6`-methyl-(2, 3`) bipyridinyl), a second-generation COX- 2 having higher *in vitro* selectivity compared to other drugs marketed currently. Merck, USA has deve-loped this drug for the treatment of osteoarthritis (Friesen et al., 1998; Riendeau et al., 2001; Patrignani et al. 2003).

Etoricoxib does not inhibit prostaglandin synthesis in the gastric mucosa, even at doses above the clinical dose range of 60 - 120 mg (Dallob et al., 2003). It is rapidly absorbed after oral ingestion with a bioavailability of > 80% and peak plasma concentration (Cmax) was achieved after (Tmax) 1 - 1.5 h (Cochrane et al., 2002; Stichtenoth and Frolich, 2003) . At steady state (reached within 7 days), Cmax of 3.6 µg/ml was reached with once-daily administration of etoricoxib 120 mg in fasted adults (Cochrane et al., 2002). Etoricoxib pharmacoki-netics are linear at clinically relevant doses and the pharmacokinetic half-life (t1/2, approximately 22 h), and pharmacological response, supports oral once-a-day dosing (Agrawal et al., 2001)).

Since this drug is used for a long term therefore, a need for a sensitive, simple and specific assay of EXB was indispensable. Being a latest molecule, very few HPLC methods were reported for etoricoxib so far in the literature. Liquid chromatography using mass/mass detection (LC - MS/MS) with atmospheric pressure chemical ionization (APCI), where a stable isotope of EXB was utilized as the IS, was reported and validated over a concentration range of 0.5 - 250 ng/ml (Rose et al., 2002). In another Electron spray ionization (ESI) LC - MS/MS study, phenazone, a structurally unrelated moiety, was used as an internal standard and validated over the concentration range of 0.2 - 200 ng/ml (Brautigam et al., 2003). Matthews et al. have developed an analytical method for the determination of EXB in human plasma and urine using solid phase extraction (SPE) followed by HPLC with photochemical cyclization and fluorescence detection. The limit of quantification was found to be 5 ng/ml (Matthews et al., 2001). Abrahim and Hartmann group has developed a derivitization method coupled with reversed phase HPLC for determination of enolate and its intermediates, and a stability indicating method for the quantitative analysis of impurities (Abrahim et al., 2002; Hartmann et al., 2003). (Werner et al., 2005) 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. However, the LC - MS/MS machine is quite expensive and is not readily available in most clinical, bio-analytical research laboratories. Recently, two HPLC were reported for the quantitation of EXB in human plasma using liquid-liquid extraction (Ramakrishna et al., 2005; Pavan et al., 2006). These methods required liquid-liquid extraction procedures or used large volume of plasma and are time consuming. None of them shows the effect of stress testing on EXB stability. Therefore, the objective of this study was to develop a reproducible, specific, sensitive, isocratic, reverse phase HPLC method with UV detection for the determination of etoricoxib in biological fluids. We also demonstrated the applicability of this method for preclinical pharmacokinetic studies in rats. Additionally, this method provides information about the stability of EXB under drastic conditions.

EXPERIMENTAL

Chemicals and reagents

Etoricoxib was kindly supplied by local pharmaceutical firm as standard certified samples and flurbiprofen was purchased from Sigma Chemical Company (ST. Louis, MO, USA). All other reagents and chemicals were analytical grade, and were used as received.

Instrumentation

An HPLC Waters' system was used in this study. It was equipped with Waters 484 variable UV absorbance detector and Waters 717 Plus autosampler. Waters 515 solvent delivery system was used to operate the isocratic flow. The analytical column was a Novapak C-8 (15 cm × 4.6 mm × 5 µm), with a C8 guard column (Waters Assoc., Milford, USA) . The data were collected with an Empower Pro Chromatography Manager Data Collection System, utilizing Centrino® computer connected to a laser jet printer.

Chromatographic conditions

The mobile phase consisted of acetic acid: triethylamine: acetonitrile: water (0.02: 0.01: 41: 59.97, v/v), pH 4.0, filtered through 0.22 µm millipore filter and degassed by sonication. The detection wavelength was 245 nm with a limit of detection of 20 ng/ml. Separation was carried out isocratically, at ambient temperature (22 ± 1°C), and a flow rate of 1.0 ml/min. The injection volume was 50 µl.

Preparation of standard solutions

Stock solutions of EXB and its IS were prepared by dissolving 1 mg/ml of each drug in methanol and stored in 4 ml amber glass vials at -80°C until used. Flurbiprofen was used as the assay internal standard (IS). Calibration standards were prepared daily by serial dilution of the stock standard with the mobile phase to give EXB concentrations in the range of 0.05 - 5.0 µg/ml with a constant concentration of the IS (2.0 µg/ml). EXB solutions were stable for at least 6 months when stored at - 80°C, and no evidence of degradation of the analytes was observed on the chromatograms during this period.

Plasma standards containing EXB for the daily calibration curve were prepared by adding an appropriate volume from the stock solutions to drug-free plasma to obtain final concentrations equivalent to 0.05 - 5 µg/ml and stored at - 80°C until assay. Internal controls were prepared by specific dilution of the stock solution in drug-free plasma to obtain 200 ng/ml.

Determination of Etoricoxib in spiked rat plasma

200 µL of rat plasma samples were spiked with 20 - 50 µ of EXB stock solutions and 20 µL of the IS in 1.8 ml disposable Eppendorf tubes. The tubes were vortexed for 30 s after each addition. The solution was mixed with 600 µL of acetonitrile, vortexed at high speed for 1 min, and centrifuged at 20,000 rpm for 15 min. The supernatant was transferred to a 5 ml centrifuge tube and evaporated to dryness under a stream of nitrogen at 37°C.

The residue was reconstituted in 100 µL of the mobile phase and 50µL volume was injected into HPLC. Each concentration was prepared at least in triplicate.

Calibration curve and calculation procedures

The nominal value of EXB concentration (µg/ml) in plasma was plotted as a function of the peak area ratio obtained of EXB and its IS. A linear regression line obtained and the estimated linear correlation coefficient was applied to the calibration curve prepared in triplicate (equation: $Y = a + bX$, where Y is the area under the peak (AUP) ratio of the drug to the internal standard, a is the intercept, b is the slope, and X is the concentration of EXB). The day curve was accepted, if the coefficient of variation (CV%) was 12% for all the tested concentrations (high, medium and low).

Limit of detection and limit of quantification

The limits of detection (LOD) and of quantification (LOQ) were determined based on the analysis of 6 replicates. The LOQ was defined as the lowest drug plasma concentration of the calibration daily curve which can be determined with an accuracy of 85 - 115% and precision < 12%. The LOD was defined as 0.5 times of the limit of quantification. In addition, the LOD presents a peak signal to noise of baseline ratio equivalent to 3:1, while the LOQ shows a ratio of 6:1.

Accuracy and precision

Accuracy was evaluated by analysis of multiple replicates (n = 6) of EXB in rat plasma. The intra-day accuracy was assessed by analysis of 6 replicates of the high, medium and low concentrations, respectively, 5000, 500, 100 ng/ml of EXB.

The inter- day accuracy was determined by the analysis of 6 repli-cates of the high, medium and low concentrations of EXB in 3 diffe-rent days within a month. Accuracy of the method expressed in term of bias (percentage deviation from the nominal concentration).

Precision of a quantitative method is the degree of agreement among individual tests, when the procedure is applied repeatedly to analyze multiple replicates in three different occasions. The intra-day precision was evaluated by analysis of the calibration curves of 6 replicates of different concentrations of EXB within the same day. The inter -day precision was determined by the analysis of 6 replicates of different concentrations of EXB in 3 different days. The overall precision of the method expressed as the coefficient of variation (CV%).

Recovery

Absolute recovery of EXB was assessed by extracting plasma specimens spiked with low, medium and high concentrations, respecttively, 100, 500, 5000 ng/ml of EXB. The area under the peak (AUP) was then compared with the AUP for mobile phase standards containing equivalent amounts of the drug without extraction. The efficiency of relative recovery was estimated by the peak area ratio integrated for EXB in plasma related to its internal standard, assayed accordingly to the procedure versus the peak area ratio integrated after direct injection of unprocessed reference solutions of the same concentration.

Specificity and selectivity

The specificity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. Therefore, randomly selected six blank, drug-free, plasma samples were analysized to determine the extent to which endogenous plasma components may contribute to interference with EXB or the IS. The results were compared with LOQ (20 ng/ml).

The selectivity of the method was investigated by testing several samples obtained from previous in vivo studies of rats received different drugs as acetaminophen, ketorolac, ketoconazole, fluconazole, flurbiprofen, naproxen, simvastatin, acyclovir, warfarin, piroxicam, amlodipine, meloxicam, tramadol, diclofenac, pipracillin and mefenamic, ketamine, voriconazole, indomethacin (Table 1).

Stability-indicating study

Short-term stability was performed at room temperature by repetition of several times of a sequence of injections up to 24 h; the study was done by testing a sequence of microvials on the rack of the autosampler containing plasma extracts in three different concentrations determined on the basis of a day curve. Biological matrices spiked blank plasma, were analyzed after three thawing cycles by HPLC in the same sequence after the cleanup of plasma samples as detailed above, using three different concentrations (n = 3) during three consecutive periods.

Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the procedure. Therefore, the drug was mixed with hydrogen peroxide solution, and boiled in water, in 1 N HCL and in 1N NaOH solutions. After completing degradation treatments, samples were allowed to cool at room temperature for 24 h and prepared according to assay sample solution without the internal standard. Samples were analyzed against a control sample in mobile phase without any treatment.

Robustness

The robustness of the method was determined, by using two different chromatographic system connected to two different Novapak C-8 columns, small changes in the acetonitrile ratio in the mobile phase and in the flow rate. The study was developed using

Table 1. Retention times of different NSAIDs and other possible concomitant drugs.

Drug	Retention Time, min	Drug	Retention Time, min
Etoricoxib	3.8	Tramadol	2.2
Ketorolac	2.1	Ketamine	ND
Flurbiprofen	7.2	Ketoconazole	6.1
Naproxin	4.3	Voriconazole	3.5
Piroxicam	2.7	Fluconazole	ND
Diclofenac	8.3	Pipracillin	1.6
Indomethacin	8.7	Amlodipine	4.8
Meloxicam	2.4	Acyclovir	1.3
Mefenamic acid	>15	Simvastatin	1.2
Acetaminophen	1.5	Warfarin	6.3

two different concentrations (n = 3).

Pharmacokinetics of EXB in rats

Animals handling and dosing Scheme: The animal handling was fully complied with our institutional policies. Sixteen male Wistar rats (194 ± 18 gm) were used in this study. Rats were randomly divided into 2 groups (n = 8) for different sampling time. Each group was marked and housed in one cage. Water was available *ad libitum* at all times during the experiment. After grinding, the content of one tablet was suspended in sterile water for injection (SWFI) containing 20% acacia mucilage. Each rat received an oral dose of 15 mg/kg. Immediately before each administration the sus-pension was vortexed for few seconds. Blood samples (0.5 ml) were collected in microtainer (a 600 µL plastic tube containing lithium heparin) at 0.25, 1, 3, 6 h and at 0.5, 2, 8 and 24 h from the first and the second groups, respectively, after drug administrations. Blood samples were collected from the orbital venous plexus, under light halothane anesthesia. Four blood samples were collected from each rat per day to avoid any damage to the eye. Therefore, each data point is the mean of 8 replicates. Plasma samples were separated by centrifugation at 4,000 rpm for 15 min and stored in Eppendorf tubes at - 80°C till assayed.

Data and statistical analysis

All results were expressed as the mean ± SD of 3 - 8 replicates. The student t -test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%. Pharmacokinetic parameters were estimated using compartmental and model-independent methods (Gibaldi and Perrier, 1982) using in-house developed program to estimate the pharmacokinetic parameters of etoricoxib. The area under the concentration time curve (AUC, µg ml-1 h) to the last data point was calculated by the linear trapezoidal rule and extrapolated to time infinity by the addition of CLast/λn where, CLast is the concentration of the last measured serum sample and λn is the terminal elimination rate constant. The area under the first moment (AUMC, µg ml-1 h2) was determined using the same rules followed for AUC calculation.

The mean residence time (MRT) was estimated from MRT = AUMC / AUC and disposition half-life (t1/2) was calculated from the terminal elimination rate constant using the formula t1/2 = 0.693 /λn. The apparent body clearance (Cl/F) was calculated using Cl/F = Dose/ AUC.

RESULTS AND DISCUSSION

The validation of the HPLC assay was assessed using the following parameters, namely, interference from other drugs or endogenous substances in rat plasma, linearity, LOQ, LOD, stability, accuracy, precision, and recovery. Various combinations of organic solvents (methanol and acetonitrile) and water with and without triethylamine or acetic acid were evaluated as mobile phase components. Finally, acetic acid: triethylamine :acetonitrile:water (0.02: 0.01: 41: 59.97, v/v) pH 4.0, mobile phase was selected as one that yielded sufficient resolution in a reasonable time, retention time less than 4 min. Flow rate of 1.0 ml/min was selected since an acceptable chromatography was obtained. Depending on the mobile phase, flow rate, and chromatographic system used the retention time ranged from 3 - 5 min. Six different waters analytical columns (C8 and C18) were also tested during method development and the Novapak-C8 column found to produce acceptable chromatography. The detection wavelength was 245 nm with a limit of detection of 50 ng/ml, with a coefficient variation of 9.8%.

Selectivity and Specificity

The selectivity of the method was investigated by testing several samples obtained from previous in vivo studies of rats received different drugs. The assay was selective, where the drug peak was well resolved with no interference from different drugs, which could be given concomitantly, except voriconazole's peak shows a partial overlap with EXB as listed on Table 1 and shown in Figure 2. Typical chromatograms obtained from extracts of drug-free plasma and a plasma sample obtained from a rat 2 h after a single oral dose of 15 mg/kg etoricoxib are presented in Figure 3. No endogenous plasma components elute at the retention time of etoricoxib or internal standard, which proves the assay specificity. Etoricoxib and IS eluted in 3.8 and 7.2 min, respectively.

Linearity, LOQ and LOD

Excellent linear relationships (r = 0.998) were demonstrated between AUP, of EXB to the internal standard, (Y) versus analyte concentration (µg/ml) in spiked plasma samples (X). The calibration curves were linear for con-

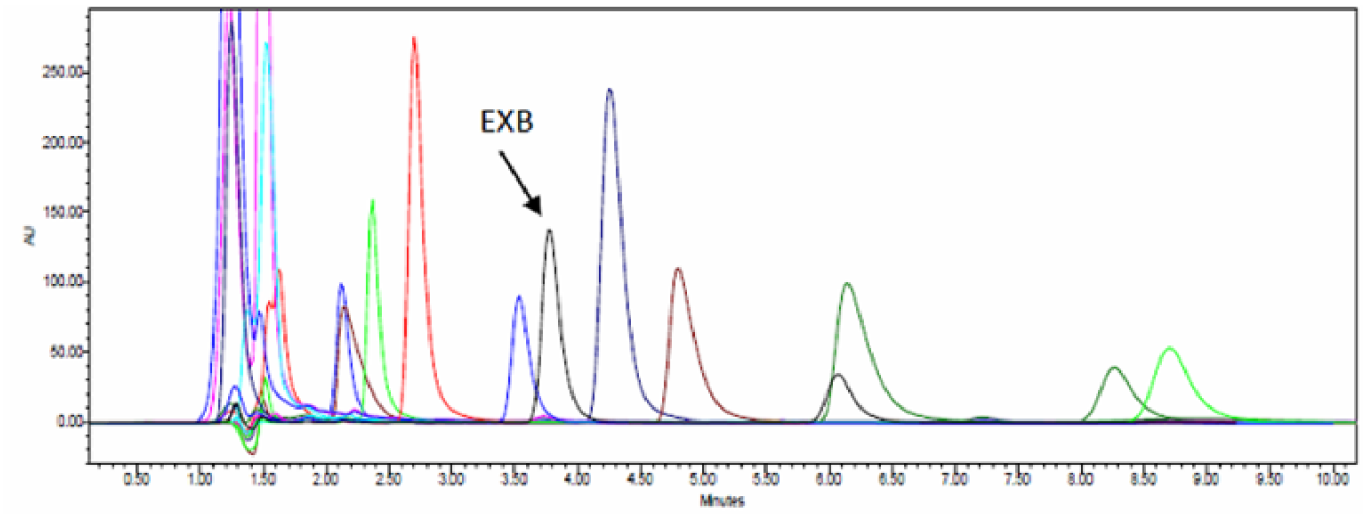

Figure 2. Overlay chromatograms of the different concomitant drugs listed in Table 1.

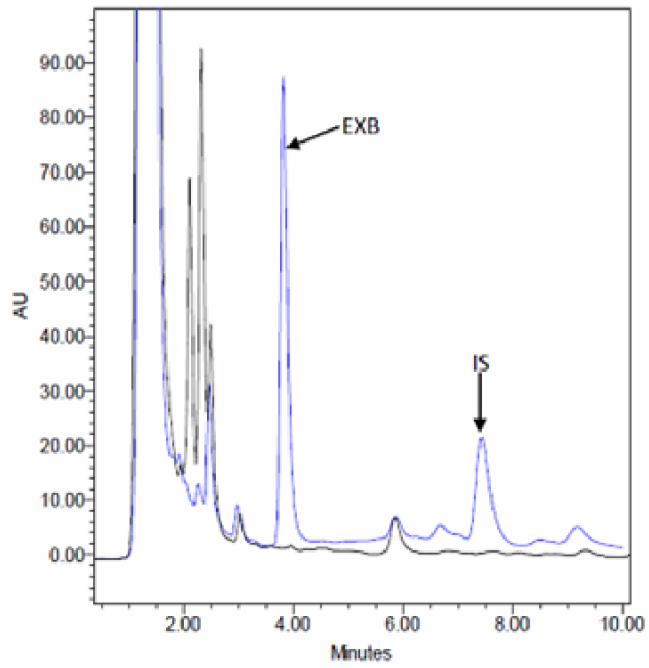

Figure 3. Overlay chromatograms of extracted drug-free blank rat plasma (black) and plasma samples (Blue), spiked with 2µg/ml of IS, withdraw from a rat after 2 h of oral dose administration of 15 mg/kg etoricoxib.

centrations ranging from 50 - 5000 ng/ ml. A typical calibration curve had the regression equation of Y = 0.0201 + 0.514 X with a correlation coefficient (r) of 0.998. Calibration curves were established on each day of analysis. The LOQ of this assay was 50 ng/ml in rat plasma, with a CV of 9.5% while the LOD was 20 ng/ml, with CV of 9.8%.

Precision and accuracy

Assay precision for etoricoxib was 7.2% based on CVExcellent linear relationships (r = 0.998) were values of 9.5, 4.8 and 7.4% for samples containing 100, 500 and 5000 ng/ml, respectively. Assay accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 97% (Table 2). The within and between day precision (CV%) calculated following replicate analysis (n = 6) of etoricoxib was < 10% over a wide range of concentrations.

Recovery and reproducibility

The mean percentage recovery of 0.1 to 5 g/ml of EXB was 91.6, 10.8 with CV% of 11.8% while Table 2 lists the recoveries of 3 different concentrations of EXB. The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared at three different days over a three week period. The mean correlation coefficient was > 0.997 of the slopes of the three standard calibration curves with CV% of 1.9%.

Analysis of variance of the data indicated no significant difference (p > 0.05) in the slopes, within-day and between-day, of the calibration curves. The results confirmed the reproducibility of the assay method.

Stability of EXB samples

There was no significant effect of day/fluorescent light was observed on the peak area ratios on drug solution left over the counter for a week (n = 6), therefore, throughout the study no light protection of the drug solutions were needed. No significant change in the EXB concentration was observed during plasma and mobile

Table 2. Summary of accuracy and precision during method validation at low, medium and high concentrations (*n* = 6).

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Intra-day* precision CV (%)	Inter-day** precision CV (%)
100	91	-9.0	4.5	9.5
500	487	-2.6	3.5	4.8
5000	5060	1.2	3.1	7.4

*The analyses were performed in the same day; CV: coefficient of variation.** The analyses were performed in 3 different days within three months.

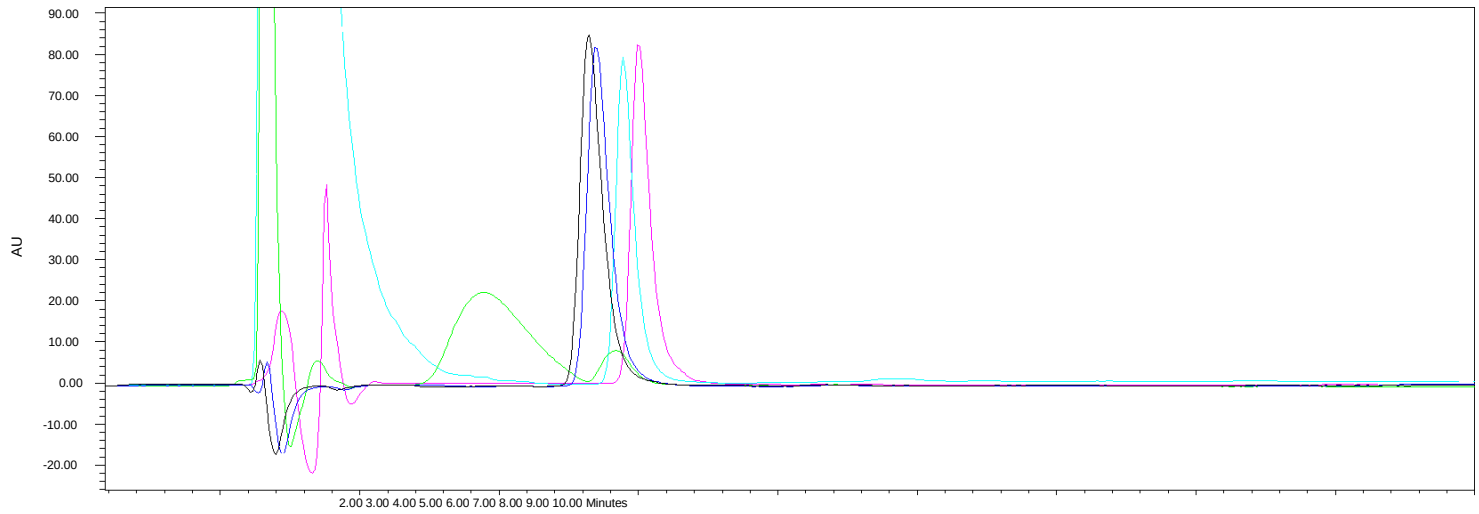

Figure 4. Overlay chromatograms of forced degradation of etoricoxib in water, as control, (Black), in hydrogen peroxide (Aqua blue) and boiled in 1 N HCL (Green), in water (Blue), and in 1 N NaOH (Fuchsia).

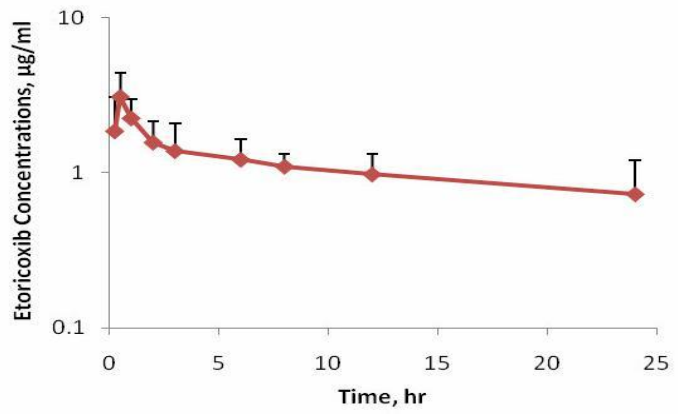

Figure 5. Mean plasma concentrations (+ SD) time profile of etoricoxib in rats after receiving oral dose of 15 mg/kg (n = 8).

phase short-term stability experiments. EXB in plasma samples or mobile phase were stable for at least 6. Etoricoxib was also stable even when subjected to drastic environments of mixing with hydrogen peroxide or boiling in water or alkali. However, it is significantly degraded, > 90%, when boiled in acidic condition, with the degraded peaks eluted earlier as seen in Figure 4.

Pharmacokinetics of etoricoxib in rats

Since etoricoxib is prescribed for a long term therapy, the possibility of its interactions with other drugs or food supplements is great. Therefore, this assay was developed to determine the hazards or safety of these concomitant administrations. The present assay was applied to determine the plasma concentrations of EXB to follow its pharmacokinetic in rats. Figure 5 shows the mean ± standard deviation of the mean (SD) plasma concentration–time profile of EXB. Following oral ad-ministration of EXB, a rapid absorption and a slow disposition was observed. Pharmacokinetic analysis was per- formed using non-compartmental methods (Gibaldi and Perrier, 1982). The pharmacokinetic parameters of EXB of 8 rats following oral administration of 15 mg/kg are summarized in Table 3. EXB concentration up to 24 h, about one half-life, was not below the LOQ. Therefore, the assay could be used to measure EXB for longer period of time after administration for adequate description of its pharmacokinetics.

Conclusion

This paper describes a simple, rapid, sensitive, specific,

Table 3. Mean pharmacokinetics of etoricoxib after a single oral administration of 15 mg/kg to rats.

Pharmacokinetic Parameters	Mean
C_{max}, µg/ml	3.1
T_{max}, hr	0.5
AUC_{0-t}, µg ml^{-1} hr	26.0
$AUC_{0-\infty}$, µg ml^{-1} hr^2	52.7
$t_{1/2}$, hr	25.4
MRT, hr	37.9
Cl/F, ml/min/kg	4.7

accurate and precise assay for the determination of etoricoxib, suitable for the analysis of large numbers of plasma samples. The method was completely validated and it offers significant advantages over those previously reported methods in terms of robustness, selectivity and sensitivity, guaranteeing stability, precision and accuracy. The results were used to generate profiles and to calculate the pharmacokinetic parameters etoricoxib. The proposed method is useful for the application to etoricoxib pharmacokinetics and interactions. This assay will be utilized in further investigations of etoricoxib with other drugs or food supplements in rats and human in the near future.

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