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High Performance Liquid Chromatographic Determination of Etoricoxib in Human Plasma

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A simple, sensitive and reproducible high performance liquid chromatographic (HPLC) has been developed for the analysis of etoricoxib in plasma. This validated method is successfully applied for the estimation of etoricoxib in human volunteers. The HPLC analysis was performed using BDS-Hypersil C-18 (250 mm × 4.6 mm, 5 µm, Thermo electron corporation, USA) column. The mobile phase used was aqueous buffer (containing 0.3 mL triethyl amine (as peak modifier) and 0.4 mL orthophosphoric acid per liter) : acetonitrile (62:38, v/v). The analytes were detected at 284 nm. The total time for a chromatographic separation was *ca.* 10 min. Extraction of etoricoxib from plasma was carried out using diethylether:dichloromethane (6:4; v/v). The validated quantitation ranges of this method were 15-3200 ng/mL with coefficients of variation of 2.2-5.4 %. In between and within batch precision was 1.3-5.6 and 0.8-6.7 %, respectively. In between and within batch relative error was 0.6-(-4.8) and 0.9-4.7 %, respectively. Stability of etoricoxib in plasma was > 89 %, with no evidence of degradation during sample processing and 45 days storage in a deep freezer at -70ºC. This validated method is sensitive and simple with precision of < 6 % and can be used for pharmacokinetic studies and therapeutic drug monitoring.

Key Words: Etoricoxib, HPLC-UV, Plasma, Bioanalysis.

INTRODUCTION

Etoricoxib (Arcoxia®), [5-chloro-2-(6-methyl pyridine-3-yl)-3-(4-methyl-sulfonylphenyl)pyridine], is a new non-steroidal antiinflammatory drug with high selectivity in cyclooxygenase-2 inhibitory activity. It has been launched in more than 50 countries world wide. It is indicated to relieve the signs and symptoms of osteoarthritis, rheumatoid arthritis and

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acute pain, chronic back pain, dysmenorrhoea, ankylosing spondylitis and acute gouty arthritis[1].

Non-steroidal antiinflammatory drugs (NSAIDs) produce their antiinflammatory, analgesic antipyretic activites through the inhibition of cyclo-oxygenase (COX-1 and COX-2) enzymes for the prostanoid synthesis. The COX-1 enzyme is responsible for the prostaglandin synthesis and inhibition of this enzyme by non-selective NSAIDs is thought to be responsible for damage to gastric mucosa and for antiplatelets activity, increasing risk of bleeding. On the other hand, COX-2 enzyme primarily synthesizes prostaglandin involved in inflammation. Selective inhibition of COX-2 enzyme reduces the inflammation without causing the gastric irritation or bleeding. Etoricoxib does not inhibit prostaglandin synthesis even at doses above the therapeutic doses (60-120 mg). It is rapidly absorbed after oral ingestion with a bioavailability of > 80 % and t_{max} of 1-1.5 h. Etoricoxib exhibits linear pharmacokinetics over the therapeutic dose range. Based on the reported literature the peak plasma concentration (C_{max}) following administration of 120 mg dose of etoricoxib is 3.6 μg/mL[2-5].

A few HPLC methods using solid phase extraction (SPE), derivatization and detection using fluorescence[6], LC-MS/MS[7-9] or HPLC-UV[10] using other internal standard and column have been previously reported. For routine analysis, a high-throughput analysis with LC-MS/MS is always not advantageous as such equipment and techniques are not available in most of the laboratories. Therefore, new methods with both simple and sensitive and economical determination of etoricoxib are required which can detect at least 2.5 % of the C_{max}. This paper describes a simple, economical and sensitive HPLC method with ultraviolet detection for determination of etoricoxib in human plasma using a liquid-liquid extraction.

EXPERIMENTAL

Etoricoxib reference material (99.63 %) (Fig. 1) was obtained as gift from the Orchid Chemical and Pharmaceutical Ltd., Chennai, (India), whereas the rofecoxib (Internal standard, 99.25 %) was obtained from Aarti drugs (India). Acetonitrile and methanol of HPLC grade and orthophosphoric acid (85 % v/v) and triethylamine of analytical grade were purchased from Fisher Scientific (Fairlawn, New Jersey, USA). Double distilled deionized water was used for HPLC.

Chromatography and quantitation: For chromatographic analysis, Merck-Hitachi (Lachrome®) HPLC equipped with quaternary gradient pump (Lachrome® 7100), variable UV-Visible detector (model-7400), an Autosampler (L7200) with a rheodyne injector (Rheodyne 7125) holding 100 μL loop was used. The signals were acquired and analyzed using Windows 2000 based, D700 HSM chromatography data station software.

The separation was achieved by using a BDS-Hypersil-C18 column (250 × 4.6 mm, 5 µm, Thermoelectron corporation, USA) with mobile phase consisting of aqueous buffer (consist of 0.3 mL triethylamine and 0.4 mL orthophosphoric acid per liter) and acetonitrile (62:38, v/v). Mobile phase was filtered through 0.45 µm Millipore filter before use and degassed in an ultrasonic bath. The observed pH of the mobile phase was 2.95 ± 0.15. The mobile phase was delivered at a constant flow-rate of 1.25 mL/min. A peak area ratio method was used for quantitation. The etoricoxib concentration in human plasma samples was determined by a standard curve that was analyzed with weighted least-squares linear regression. The weighing factor was $1/x^2$.

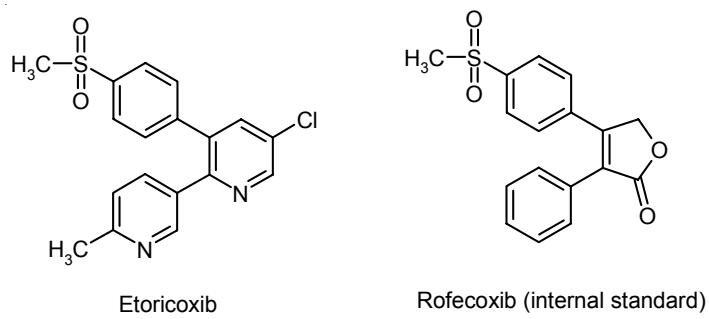


Fig. 1. Chemical structure of etoricoxib and internal standard (rofecoxib)

Sample processing: A 950 µL volume of plasma was transferred to a 15 mL polypropylene tube and the 50 µL of internal standard working solution (20 µg/mL) was added. Solution was vortexed and then 6 mL aliquot of extraction solvent diethylether: dichloromethane (6:4, v/v) was added using Ceramus® Classic Bottle top Dispenser (Hirsch Mann Laborgerate GmbH, Eberstadt, Germany). The sample was vortexed for 5 min using Vibrax Vortexer (Model VX-Z VXR Basic, IKAWerke GmbH & Co. Staufen, Germany) and centrifuged for 5 min at 1000 g. The organic layer was transferred to a 10 mL clean test tube using Pasteur pipette and evaporated to dryness using nitrogen stream. Then the residue was reconstituted using 300 µL of mobile phase. Aliquot of 100 µL were injected into HPLC.

Bioanalytical method validation

Stock solutions, calibration and control samples: Stock solutions of etoricoxib (1.00 mg/mL) and internal standard (1.00 mg/mL, rofecoxib) were prepared in a methanol-water (50:50, v/v) solution. An intermediate working solution (200 µg/mL) of etoricoxib was prepared for preparation of other working solution of lower concentration. Working solution for the

calibration (0.3, 0.6, 1.2, 7.2, 14.4, 25, 48, 64 µg/mL) and quality control sample (0.3, 0.9, 30 and 54 µg/mL) were prepared from the stock solution or intermediate working solution by an appropriate dilution using diluent (methanol: water, 50:50, v/v). The internal standard working solution 20 µg/mL was prepared by diluting stock solution with diluent. 50 µL of working standard solutions of etoricoxib were added to 950 µL of drug free plasma to obtain etoricoxib concentration of 15, 30, 60, 360, 720, 1250, 2400 and 3200 ng/mL. Similarly the quality control samples of etoricoxib as a single batch [of concentration 15 (LLOQ), 45 (low), 1500 (mid) and 2700 ng/mL (high)] were prepared by spiking the 5 % working solution to the 95% of pooled blank plasma. These solutions were aliquoted and stored in the deep freezer (-70ºC, Platinum 500V, Angelantoni Industrie S.p.a, Italy) till analysis. Before the spiking, all drug free plasma was tested to make sure that there was no endogenous interference at the retention times for etoricoxib and the internal standard. The quality control samples were extracted with the calibration standards to verify the integrity of the method.

A calibration curve was made from a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.) and eight non-zero samples covering the total range (15-3200 ng/mL), including lower limit of quantification (LLOQ). Such calibration curves were generated on six consecutive days. Linearity was assessed by a weighed ($1/x^2$) least squares regression analysis. The acceptance criterion for each back-calculated standard concentration was 15 % deviation from the nominal value except lower limit of quantification, which was set at 20 %.

Limit of quantitation, accuracy and precision: Quantitation was based on peak area measurement. The lower limit of quantitation (LLOQ) was defined as the lowest concentration, which can be determined with confidence (with a precision of less than 20 % and accuracy of 80-125 %) on a day-to-day basis. Within-batch accuracy and precision evaluation were performed by repeated analysis of etoricoxib in human plasma. The batch consist of a calibration standards, six replicates of LLOQ, low, mid, medium and high quality control samples, while between batch accuracy and precision were assessed by analysis of similar sequence of samples on three consecutive days. The overall precision of the method expressed as relative standard deviation and accuracy of the method expressed in term of relative error (bias).

Recovery: Recovery of etoricoxib was evaluated by measuring the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six neat reference solutions (unprocessed). Recovery of rofecoxib (I.S.) was evaluated by comparing the mean peak areas of six extracted quality control samples to mean peak areas of six neat reference solutions (unprocessed) of the same concentration.

Specificity: Randomly selected blank plasma samples, which were collected under controlled conditions, were carried through the extraction procedure and chromatographed to determine the extent which endogenous plasma component may contribute to interference with the analyte or the internal standard. The results were compared with the results to those obtained from with drug concentration nominally at 15 ng/mL of an extracted etoricoxib plasma sample.

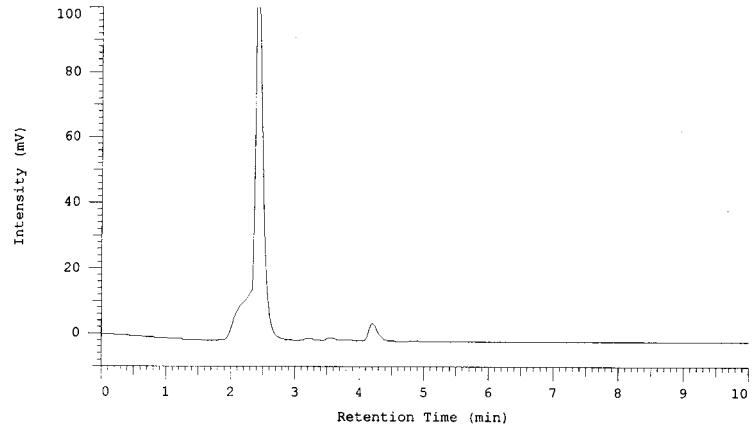
Sensitivity: The limit of detection of etoricoxib was defined, as analyte responses are at least six times than the response compared to blank response.

Stability: The short term stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for *ca*. 24 h. Freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2-3 h, refrozen for 12-24 h. Auto-sampler stability of etoricoxib was tested by analysis of processed and reconstituted low and high plasma quality control samples, which are stored in the auto-sampler tray for 24 h at 5 ± 1ºC. Stability of etoricoxib in human plasma was tested after storage at approximately -70ºC for 45 d. 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.

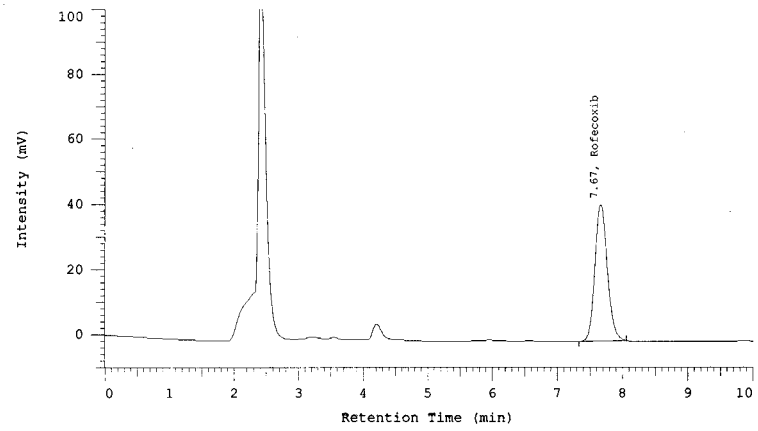
Stock solution stability: The working solution (1 µg/mL) of etoricoxib was repeatedly (n = 3) injected into the chromatograph immediately after preparation (time 0) and at 3, 6 and 9 h after bench top storage at room temperature and 4ºC. This injection protocol was repeated after 1, 3, 6, 8, 15, 27 and 45 d storage of this solution stored under light protected condition between 4-8ºC.

RESULTS AND DISCUSSION

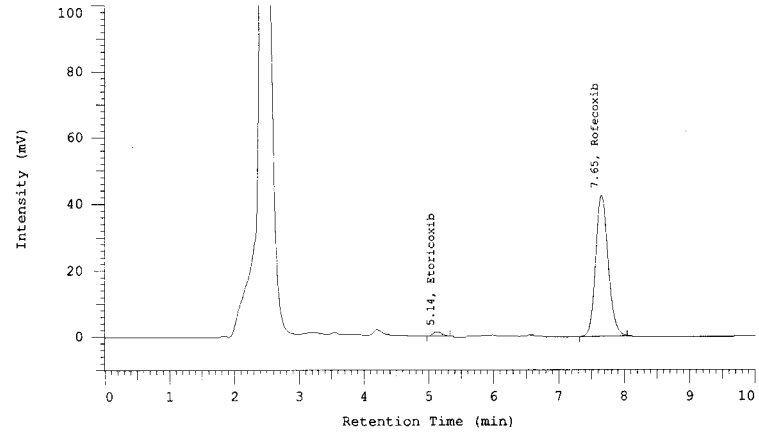
The representative chromatograms of A) blank plasma, B) blank plasma with internal standard, C) plasma spiked with etoricoxib at 15 ng/mL, D) plasma spiked with etoricoxib at 1250 ng/mL, E) plasma sample from volunteer 3.5 h after a single dose of 120 mg of etoricoxib are shown in Fig. 2. The analytes were well separated from one another under the described chromatographic condition. The tailing of this drug is reduced with the help of peak modifier triethylamine, which allows the use of BDS-Hypersil C-18 column easily. The one step liquid-liquid extraction with diethyl ether: dichloromethane (6:4, v/v) was sufficient to isolate the etoricoxib and rofecoxib from plasma without any interfering peaks. The method is specific


Intensity (mV)
Retention Time (min)

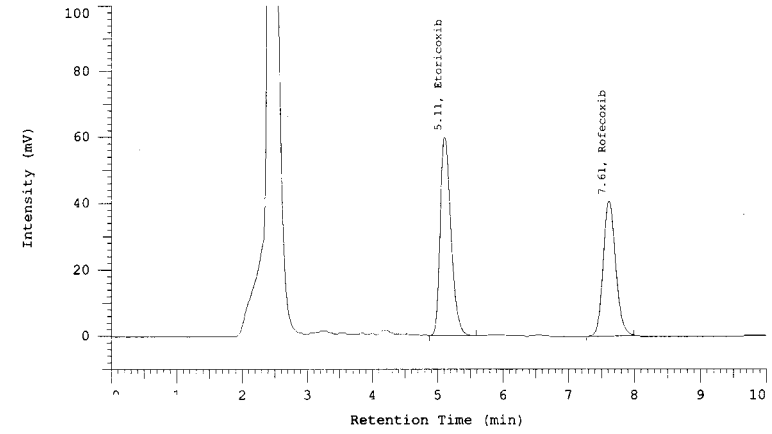
(A)


Intensity (mV)
7.67, Rofecoxib
Retention Time (min)

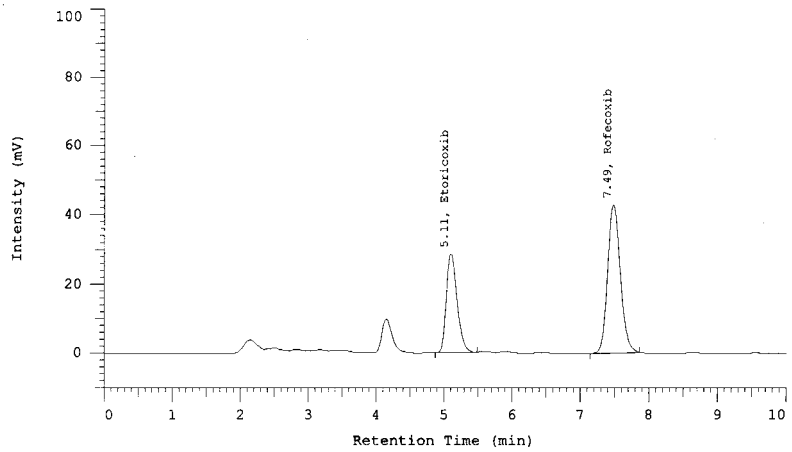
(B)


Intensity (mV)
5.14, Etoricoxib
7.65, Rofecoxib
Retention Time (min)

(C)



(D)



(E)

Fig. 2. Chromatograms of (A) blank plasma, (B) blank plasma with internal standard (1000 ng/mL), (C) plasma spiked with etoricoxib at 15 ng/mL (LLOQ), (D) plasma spiked with etoricoxib at 1250 ng/mL, (E) plasma sample from volunteer 3.5 h after a single dose of 120mg of etoricoxib. Approximate RT: Etoricoxib ~5.1; IS ~7.5 min

for etoricoxib and is more economical than the other reported method as it utilizes the common C-18 column instead of Waters Symmetry or YMC ODS-AQ column.

Limit of quantitation, linearity, precision and accuracy: Linear least square regression analysis of the calibration graph on six different days demonstrated, linearity between the response and the nominal concentration of etoricoxib over the range of 15-3200 ng/mL with an average

regression of 0.999 (or better). The representative calibration equation for this method was: y = 0.001263 (± 0.0001205)x + 0.000912 (± 0.000394). The limit of quantitation (LOQ) in human plasma for etoricoxib was 15 ng/mL. Within and between day precision and accuracy was evaluated by assaying quality control with different concentration of etoricoxib. Within and between days relative standard deviation (precision, % CV) were less than 6.7 and 5.6 %, respectively. Within and between day relative errors (bias, %) were less than 4.7 and -4.8% respectively (Tables 1 and 2).

TABLE-1
PRECISION AND ACCURACY (RELATIVE ERROR) OF BACK CALCULATED CONCENTRATION OF STANDARD SAMPLE FOR ETORICOXIB IN SPIKED PLASMA DURING 6 d

Spiked concentration (ng/mL)	Concentration found (mean ± SD, n = 6) (ng/mL)	Precision (%)	Relative error (Bias, %)
15	15.1 ± 0.5	3.5	0.7
30	31.6 ± 1.4	4.5	5.3
60	58.2 ± 3.2	5.4	-3.0
360	351.1 ± 10.2	2.9	-2.5
720	739.5 ± 28.2	3.8	2.7
1250	1235.4 ± 31.4	2.5	-1.2
2400	2416.0 ± 52.1	2.2	0.7
3200	3218.7 ± 88.2	2.7	0.6

TABLE-2
PRECISION AND ACCURACY (RELATIVE ERROR) FOR DETERMINATION OF ANALYTE IN SPIKED PLASMA

Spiked concentration (ng/mL)	Within assay precision (n = 6)		
	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Relative error (Bias, %)
15	15.7 ± 1.1	6.7	4.7
45	44.2 ± 1.8	4.1	-1.8
1500	1542.8 ± 40.2	2.6	2.9
2700	2725.0 ± 23.1	0.8	0.9
Between assay precision (n = 3)			
15	14.5 ± 0.8	5.6	-3.5
45	42.8 ± 1.7	4.0	-4.8
1500	1530.9 ± 55.1	3.6	2.1
2700	2716.4 ± 35.7	1.3	0.6

Recovery: Mean recoveries were 76.5-80.5 % with coefficient of variation of 3.7-6.3 % at three different concentrations range for etoricoxib (45, 1500 and 2700 ng/mL). The recovery of internal standard was 90.2 % at the concentration used in assay (1000 ng/mL). (Table-3).

TABLE-3
EXTRACTION RECOVERY OF ANALYTE FROM PLASMA (n = 6)

Analyte	Concentration added (ng/mL)	Recovery (%)	Precision (%)
Etoricoxib	45	80.5	3.7
	1500	79.8	5.4
	2700	76.5	6.3
Rofecoxib	1000	90.2	3.2

Specificity: There were no interfering peaks present in six different randomly selected samples of drug free plasma used for the analysis at the retention time of either analyte or internal standard.

Stability: The stability experiments were aimed to test, all possible conditions that the sample might experience after collecting and prior the analysis. All the stability results are given summarized in Table-4. Three freeze-thaw cycle and 24 h room temperature storage for low and high quality control samples indicated that etoricoxib was stable in human plasma

TABLE-4
STABILITY OF THE ETORICOXIB IN SAMPLES

Sample concentration (ng/mL)	Concentration found (ng/mL)	Precision (%)	Relative error (%)
Short term stability for 24 h (n = 6) in plasma			
45	44.7 ± 2.50	5.6	-0.7
2700	2769.0 ± 25.2	0.9	2.6
Freeze-thaw stability (n = 6)			
45	41.8 ± 1.30	3.1	-7.1
2700	2635.1 ± 35.2	1.3	-2.4
Auto-sampler stability (n = 6)			
45	43.3 ± 2.80	6.5	-3.8
2700	2728.2 ± 40.5	1.5	1.0
45 d stability at -70ºC (n = 6)			
45	45.7 ± 1.90	4.2	1.6
2700	2654.1 ± 55.4	2.1	-1.7

under these conditions. Testing of auto-sampler stability of quality control samples indicated that the etoricoxib is stable when kept in the auto-sampler for up to 24 h at 5 ± 1ºC. Evaluation was based on back-calculated concentrations. QC samples were stable for at least 45 d if stored frozen at *ca*. -70ºC. The stock solutions were stable for at least 2 months when stored at 4-8ºC.

Conclusion

The HPLC method described here is simple, sensitive, specific and fully validated as per guidelines[11]. The previously reported methods for the analysis of etoricoxib in plasma were expensive due to the expensive columns or equipment. The method has shown acceptable precision, accuracy, adequate sensitivity and linearity for etoricoxib (15-3200 ng/mL), which is suitable for the estimation of etoricoxib at different therapeutic dose levels for pharmacokinetic studies as well as therapeutic drug monitoring. The HPLC procedure described for determination of etoricoxib has been applied to pharmacokinetic studies.

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