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A Simple and Sensitive Reverse-Phase High Performance Liquid Chromatographic Method for the Determination of Celecoxib in Rat Plasma

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A simple reversed-phase liquid chromatography method was developed for the quantitative determination of cyclooxygenase-2 inhibitor celecoxib in small volume of rat plasma. The method is simple, sensitive, and highly selective and involves single step extraction of plasma with methanol, with as low as 0.1 ml of rat plasma. The retention time of celecoxib was 5.4 min. The method has been validated for linearity precision, accuracy and inter- intra day variation. Recoveries of celecoxib were around 78 % over the calibration curve range. The method was found to be suitable in studying the pharmacokinetic aspects of celecoxib during the preliminary studies in rats.

Celecoxib, 4-[5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazol-1yl] benzene sulphonamide, is a 1,5-diaryl-substituted pyrazole with a pKa of 11.1 (fig. 1). Celecoxib was the first specific inhibitor of cycloxygenase-2 (COX-2) to be approved by US Food and Drug Administration (FDA), in 1998[1]. This clinical introduction of celecoxib has been the result of the important discovery of the COX isoenzymes and the subsequent search for molecules effective in selectively inhibiting COX-2 with little or no effect on COX-1. The major clinical goal was to produce an NSAID that had little or no effects on the gastrointestinal (GI) tract and kidney[1]. Celecoxib is used in the treatment of rheumatoid arthritis, osteoarthritis, and for the management of the pain of these conditions[2-4].

For pharmacokinetic studies, a method that allows an accurate measurement of low concentration of celecoxib is needed. Till now very few methods have been reported for the estimation of celecoxib in plasma using HPLC with UV detection[5,6]. The former has utilized solid phase extraction (SPE) procedures for sample purification. Others have utilized GCMS technique[7], Normal phase HPLC with column switching[8], or extraction of the plasma samples with chloroform and subsequent determination by liquid chromatography with fluorescence detection[9].

All these methods suffer from the drawbacks such as lengthy procedures, use of toxic solvents and use of sophisticated instruments like LCMS, normal phase HPLC with column switching and use of expensive and toxic solvents such as chloroform or isopropanol or iso octane. Present method is aimed at developing a simple and rapid reverse phase HPLC method for the determination of celecoxib in rat plasma employing UV detection with a small volume of plasma (0.1 ml).

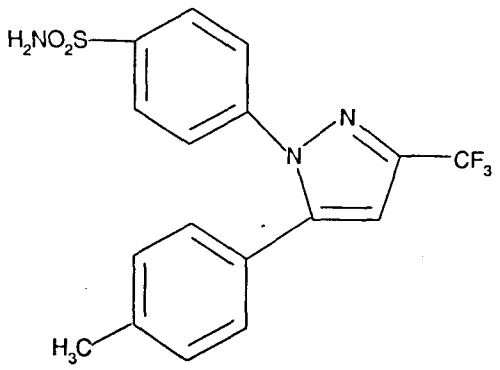

Fig. 1: Structure of celecoxib.

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MATERIALS AND METHODS

Celecoxib and ketoprofen were gift sample from Dr. Reddy's Research Foundation, Hyderabad. Acetonitrile (ACN) and methanol (MeOH) were of HPLC-grade (Thomas Baker, Mumbai). Glacial acetic acid and all other chemicals were of analytical grade (S. D. Fine Chemicals, Mumbai). Deionized water was used in the mobile phase (NANOpure Diamond, Barnstead, USA). Male Wistar rats (150-180 g) were obtained from National Institute of Nutrition, Hyderabad, India. Necessary approvals from Institutional Animals Ethics Committee (IAEC) were obtained for conducting this study in the animals.

Instrumentation and chromatography:

The HPLC system consisted of a model LC-10AT VP chromatographic pump (Shimadzu, Japan) equipped with 20-μl loop and a model SPD-10A VP UV/vis Detector (Shimadzu, Japan). Integration was achieved by using the software C-R8A CHROMATOPAC (Shimadzu, Japan). Separation was carried out on a YMC HPLC Column (150x4.6 mm I.D., 4 μm particle size), C18 reversed phase column (YMC, Inc. U.S.A.). The chromatographic analysis was performed at ambient temperature. The mobile phase consisted of acetonitrile and water (55:45 v/v); the pH of mobile phase was adjusted to 2.95 with glacial acetic acid. The flow rate was set to 1.5 ml/min. The prepared mobile phase was filtered through a 0.45 μm pore-size membrane filter and ultrasonically degassed prior to use. The UV detection was set at a wavelength of 254 nm and the peak area was recorded using chromatographic data system. The run time was set to 7 min.

Calibration and quality control sample:

The stock standard solutions of celecoxib and the internal standard (ketoprofen) were prepared in methanol. Working standard solutions were obtained by dilution of stock solution with methanol.

Calibration samples were compared by adding known amounts of celecoxib (20, 40, 80, 100, 200, 400, 800 and 1000 ng) to the blank serum. The samples were subjected to extraction procedure and HPLC analysis as described above. Standard curves (20-1000 ng) were obtained by plotting peak area ratio of celecoxib to internal standard on 'y' axis against different concentrations of celecoxib on 'x' axis. The calibration curve was evaluated by linear regression analysis and the concentration of celecoxib in unknown samples was calculated using the regression equation.

The assay precision and variability were determined by preparing quality control samples (20, 100, 400 and 1000 ng/ml) of celecoxib and adding them to blank serum. Each concentration was subjected to intra-day and inter-day precision and accuracy and extraction recovery in six replicates. These samples were stored at -20° until analyzed.

Intra-day and inter day precision and accuracy:

The intra-day precision and accuracy were evaluated on four quality control samples after extraction and subsequent HPLC estimation. Inter-day precision and accuracy were determined using these quality control samples which were extracted and analyzed on different days. Precision was expressed as coefficient of variation (% C.V). Accuracy was expressed as [(mean observed concentration/actual concentration) x100].

Extraction recovery:

The extraction efficiency with this present method was determined at four different concentrations (20, 100, 400 and 1000 ng/ml) of celecoxib and for internal standard (500 ng/ml). The extraction recovery was calculated by comparing the peak areas obtained from these extracted quality control standards in rat plasma to those obtained by the direct injection of the standard quality control samples.

Pharmacokinetic study in rats:

Rats were fasted overnight and were administered celecoxib at a dose of 20 mg/kg as a 1% gum acacia suspension. Blood samples were collected prior to celecoxib administration and at 0.5, 1, 2, 3, 4, 8, 12 and 24 h intervals after administration into heparinized tubes. Plasma was separated and stored at –20° until analyzed. The plasma samples were analyzed as mentioned above. To 100 μl of plasma, 10 μl of internal standard solution (50 ng/ml in methanol) was added and vortexed for 5 min. A volume of 150 μl of methanol was added and the mixture was vortexed for 15 min and centrifuged at 5000 rpm for 10 min. The supernatant was aspirated and injected into HPLC system after filtering through 0.2 μ nylon membrane filter.

RESULTS AND DISCUSSION

The present method was aimed at developing a simple HPLC method to determine celecoxib in rat plasma using the most commonly available HPLC system. Both celecoxib and ketoprofen were clearly separated with better selectivity. The retention times for these drugs were 5.4 and 2.3 minutes respectively. The volume of methanol was optimized

at 150 μl after initial pilot experiments. We also tried, in the pilot studies, evaporating the supernatant of methanol after extraction and subsequent reconstitution in mobile phase, but this method resulted in unclean extracts with more interfering peaks. Hence extraction of the plasma with 150 μl of methanol and direct injection of the supernatant into the HPLC system was found to yield more elegant and clean chromatograms. The results after recovery experiments for 4 concentrations viz. 20, 100, 400, and 1000 ng/ml are given in Table 1. The average recoveries for celecoxib and internal standard were found to be 77.9±4.1 and 91.1±2.8, respectively.

The specificity of the present method was evaluated with blank plasma and in about 25 samples tested no interference peaks in the plasma were observed. The chromatograms of blank plasma, of lowest concentrations (20 ng) in calibration curve and of plasma sample obtained 8 h after oral administration of 20 mg/kg of drug to rat has been presented in fig. 2. With this present method, the calibration curve was found to be linear over the range of 20-1000 ng, with correlation coefficient (r^2) of 0.99 with the following regression equation y=76.539x–64.394. Accuracy and precision were determined for the quality control samples viz. 20, 100, 400 and 1000 ng/ml in linearity range. The inter- and intra day assay variabilities were less than 10% with less than 10% error throughout the range were given in Table 2 and 3. Stability testing was done on the quality control samples by employing freeze thaw cycling. No degradation of celecoxib observed during 6 cycles of freezing and thawing. The extracted samples were found to be stable for at least 2 weeks when stored at 4°.

TABLE 1: EXTRACTION RECOVERIES OF CELECOXIB FROM RAT PLASMA AT VARIOUS CONCENTRATIONS.

	Concentration (ng/ml)	Recovery* (%)
Celecoxib	20	82.7±2.5
	100	81.2±2.3
	400	75.9±3.6
	1000	74.6±1.5
Internal Standard	Average	77.9±4.1
	500	91.1±2.8

*Each value is the mean ±standard deviation of 3 determinations.

The mean plasma concentration and time profiles of celecoxib in rats are presented in fig. 3. The pharmacokinetic parameters calculated are presented in Table 4. To conclude, the present method offers a simple and sensitive HPLC method for the estimation of celecoxib from rat plasma requiring only a small amount of plasma. This method is found suitable for studying the pharmacokinetics of new dosage forms of celecoxib during optimization studies.

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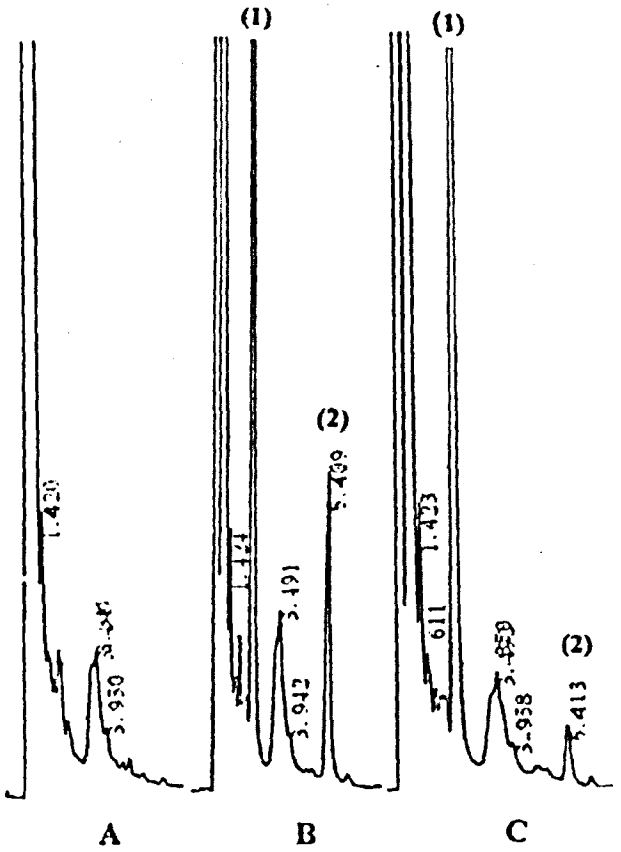

Fig. 2: Representative chromatograms of rat plasma, celecoxib and ketoprofen.

A represents blank rat plasma. B represents plasma spiked with ketoprofen (peak 1) and celecoxib (peak 2) and C represents plasma sample from a rat 3 h after administration of 20 mg/kg celecoxib, showing ketoprofen (peak 1) and celecoxib (peak 2).

TABLE 2: INTER-DAY PRECISION AND ACCURACY FOR THE DETERMINATION OF CELECOXIB IN RAT PLASMA.

Actual (ng/ml)	Observed (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
20	20.5±1.8	8.7	102
100	90.2±4.2	4.6	90.2
400	407±6.8	1.6	101.7
1000	996±1.3	0.1	99.6

[a]Expressed as relative standard deviation. [b]Expressed as (mean observed concentration/actual concentration)x100.

TABLE 3: INTRA-DAY PRECISION AND ACCURACY FOR THE DETERMINATION OF CELECOXIB IN RAT PLASMA.

Actual (ng/ml)	Observed (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
20	20.8±1.8	8.7	104.0
100	93.4±4.8	5.1	93.4
400	392±5.6	1.4	98.0
1000	992±1.5	0.1	99.2

[a]Expressed as relative standard deviation. [b]Expressed as (mean observed concentration/actual concentration)x100.

TABLE 4: PHARMACOKINETIC PARAMETERS OF CELECOXIB IN RATS.

Area (0-Inf) (ng/ml/h)	Terminal Half Life (h)	Mean Residence Time (h)	C_{max} (ng/ml)	T_{max} (h)
3206±284	4.33±0.23	9.09±0.35	240.6±5.62	8.00±0.00

Each value is mean±standard deviation of 3 determinations. Route of administration is oral and the dose used is 20 mg/kg.

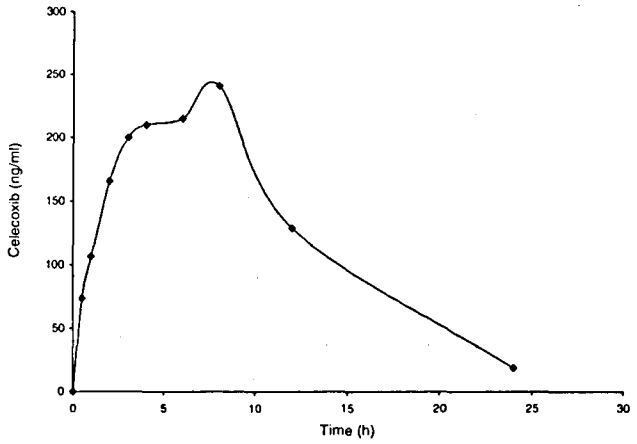

Fig. 3: Serum concentration-time curve of celecoxib in rats.

Rats were administered 20 mg/kg celecoxib orally and sample size is n=3.

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