ISSN: 2455-7072

Contents lists available at http://www.albertscience.com

ASIO Journal of Analytical Chemistry (ASIO-JAC)

Volume 1, Issue 1, 2015, 01-05

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS OF CELECOXIB IN BLOOD PLASMA BY HPLC TECHNIQUE

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ARTICLE INFO

Research Article History

Received: 12 August, 2015
Accepted: 25 October, 2015

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ABSTRACT

The objective of the present work involved the development of simple, rapid, sensitive and cost effective RP-HPLC method for the estimation and quantification of celecoxib in rabbit plasma to evaluate their linearity, accuracy, precision, and recovery studies. The spiked plasma serum samples used for the preparation of calibration curve and it was prepared by adding required volumes of standard celecoxib solutions and volume made up to 2 ml by pooled blank rabbit plasma serum to yield final concentrations of 250-5000 ng mL-1. Each sample was run one by one by injecting 20 µL into the injecting port; after washing the HPLC machine, initially with methanol followed by mobile phase, in a flow rate of 1.25 ml/min, detection was carried out at 250 nm and washing time with mobile phase itself was given to 30 min. Good linearity was obtained for calibration curves. By plotting the peak height vs. the celecoxib concentration (ng ml^{-1}), the following regression equations were found: Y=58.848X + 420.54 (250 nm; concentration range: 250-5000 ng ml^{-1}; r^2 =0.9992).The precision of the method was estimated by calculating the RSD values for the results obtained at two different plasma concentrations. The LLOQ was found to be 81.46 $ngmL^{-1}$. Intra-day precision ranged from 1.72% to 4.08%. Inter-day precision ranged from 1.09% to 7.76%. The percentage of intra and inter-day accuracy was in the range of 100.88-102.81% and 99.06-99.85%, respectively. The assay method demonstrated high degree of accuracy and precision.The analyte was found to be stable in rabbit plasma when stored at -20°C. The method was simple, rapid, highly sensitive, accurate, precise, and cost effective for celecoxib after rabbit plasma was simply extracted by liquid-liquid extraction method. Also, this method was extended for determination of celecoxib presence in blood plasma serum.

***Keywords*:** Celecoxib, RP-HPLC method, rabbit plasma serum, quantification.

1. INTRODUCTION

A systematic literature survey has revealed that a limited number of analytical methods have been reported for analysis of celecoxib (CXB) [1]. An ultraviolet (UV) spectrophotometric method based on absorption at 251 nm was used for assay of CXB in bulk drugs and capsules [2]. The method was unsuitable for assay of CXB in blood plasma serum. It was therefore, thought worth-while to develop a chromatographic method for analysis of CXB in blood plasma serum. Chromatographic methods using acetonitrile and buffer as mobile phase have been reported for assay of CXB in biological fluids [3-5], bulk drugs [6-9], and pharmaceutical dosage forms [10] but only two methods are available for analysis of CXB in pharmaceutical dosage forms in which methanol–water (85:15) were used as mobile phase [10,11]. In one of these methods a mass spectrophotometer was used as detector.

The principal objective of this study was therefore, to develop a new, simple, economical, selective, precise, reproducible, and stability-indicating high-performance liquid chromatographic (HPLC) method with a wide linear range and good sensitivity for assay of CXB in the bulk drug and in rabbit plasma using UV detection. Hence, the objective of this work was to develop a simple, sensitive, rapid and reliable RP-HPLC method for the quantifications of celecoxib in rabbit plasma.

Celecoxib is not included in any of the pharmacopoeias. The method was validated in accordance with International Conference on Harmonization (ICH) guidelines [12].

dids no.: 03.2016-28554435, dids Link: http://dids.info/didslink/03.2016-51528642/

2. MATERIALS & METHODS

Chemicals and reagents used:
Celecoxib was obtained from Jubilant Pharmaceuticals, Noida, India. Methanol and HPLC grade water were purchased from Merck, Mumbai, India. Water was glass-double distilled and further purified from Milli Q water purification system.

Instruments:
HPLC analysis was performed with LC-2010 CHT liquid chromatography, Shimadzu, Japan. Data acquisition was performed with LC solution software, Shimadzu, Japan.

DEVELOPMENT OF ANALYTICAL METHODS:
For analysis of plasma celecoxib, high performance liquid chromatography technique was used according to a modified version of some earlier reported methods [13-15].

Chromatographic conditions:
Mobile phase: 750 ml methanol mixed with 250 ml mili Q water.
Flow rate: 1.25 ml/min.
Column: Hypersil BDS C18 (Thermo Technologies, USA).
Column size: 250 x 4.6 mm, silica size: 5 µm, porous size: 0.45 µm.
Column temperature: Ambient (24°C).
Detector: UV detector (wave length 250 nm).
Injection volume: 20 µl.
Total run time: 20 min.

Preparation of standard stock solutions:
The preparation of standard curve was modified as reported from some earlier methods [10-12]. Preliminary stock solution was prepared by transferring accurately weighed 10 mg of celecoxib into a 100 mL volumetric flask, dissolved in 50 mL of methanol and final volume was made up with methanol to yield concentration of 100 µg mL^{-1}. Final stock solution was prepared by further diluting the first stock solution to make final concentration 10 µg mL^{-1}. The solution was stored in a refrigerator below 8°C and bought to room temperature before use.

Preparation of standard solutions:
The spiked plasma serum samples used for the preparation of calibration curves; by adding required volumes of standard celecoxib solutions and volume made up to 2 ml by pooled blank rabbit plasma serum to yield final concentrations of 250-5000 ng/ml.
Standard samples were prepared to yield final concentrations of 250, 500, 1000, 2000, 4000 & 5000 ng/ml. Each sample was run one by one by injecting 20 µL into the injecting port after washing the HPLC machine, initially with methanol followed by mobile phase, in a flow rate of 1.25 ml/min. Detection was carried out at 250 nm and washing time with mobile phase itself was given to 20 min. Calibration curves were constructed by plotting peak area ratio (y) of celecoxib to the internal standard versus celecoxib concentrations (x). Linear regression was used to quantitative plasma drug concentrations in samples through determination of the peak area of celecoxib.

Accuracy and precision:
Inter-day precision and accuracy of the assay was evaluated by running three validation batches on three separate days. Each batch consisted of three replicates of quality control (QC) samples at low, medium, and high concentration. The intra-day precision and accuracy was also consisted of three replicates of quality control (QC) samples at low, medium, high concentration. Precision was expressed as percentage of relative standard deviation (%R.S.D.). The precision determined at each concentration level should not exceed 15% of R.S.D.

Method validation:
Validation of the proposed method included an evaluation of the following characteristics selectivity, specificity, linearity, precision, accuracy, and analyte stability.
Each and every standard solution of celecoxib in the range of 250-5000 ng mL^{-1} of samples were showing retention time in 9.361 min i.e., peak time. The run samples were plotted and to make a standard curve and to see the linearity of the areas vs. concentrations, which is checked by linear regression coefficient value (R^2) 0.9992. These helps later to identify the celecoxib concentration in rabbit's serum sample in respect to standard plotted graph.
The Linearity and LLOQ, specificity, accuracy and precision, extraction recovery. stability study, Limit of detection (LOD) and Limit of Quantitation (LOQ) were performed and calculated [14-17] as specified as follows.

Linearity:
To establish linearity, a series of calibration standards were prepared by adding a known concentration of celecoxib to drug free human plasma and analyzed. Linearity was analyzed by weighted least-squares linear regression ($1/x^2$) of calibration curves based on peak area.

Limit of Detection (LOD) and Quantification (LOQ) Limits:
LOD and LOQ were determined by the standard deviation ($S_{y/x}$) method [3-6]. Blank samples were injected in triplicate and the peak area of this blank was calculated. LOD and LOQ were determined from the slope; S, of the calibration plot and the standard deviation of the response for the blank sample; $S_{y/x}$, by using of the formula:

$$\mathbf{LOD = 3.3 \times S_{y/x}/S \text{ and } LOQ = 10 \times S_{y/x}/S} \quad \text{.....11.1}$$

Specificity:
Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The specificity of the method was evaluated by the analysis of rabbit blank plasma samples.

Extraction recovery:
The extraction recovery of the celecoxib from the plasma was evaluated by comparing the peak areas of QC samples at low, medium, and high concentrations with peak areas of corresponding standard solutions of same concentration dissolved in the supernatant of the processed blank rabbit plasma.

Stability study:
The stability of celecoxib in blood plasma was assessed by analyzing three replicates of low, medium, and high QC samples under different temperature and time conditions.

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Freeze-thaw stability was performed by subjecting un-extracted QC samples to freezer (-20°C) for seven days. QC samples were stored at -20°C for seven days and ambient temperature at 35±5°C for 24 hrs stability of drug. All stability testing QC samples were determined by using calibration curve of freshly prepared standards. The concentrations obtained were compared with the actual values of the QC samples.

3. RESULTS AND DISCUSSION

Method development of chromatographic condition:
Selection of best solvent system is the critical step in HPLC method development to get adequate chromatographic separation. In the preliminary experiments, methanol and acetonitrile in various proportions were tested. The chromatograms could efficiently proceed by mixing only with 750 ml methanol and volume made up to 1000 ml with mili Q water.

Validation of the method:
The validation of the analytical HPLC method was carried out calculating linearity, specificity, inter-day and intra-day precision and accuracy, extraction recovery and stability. Good linearity was obtained for calibration curves. By plotting the peak height vs. the celecoxib concentration (ng ml^{-1}), the following regression equations were found: Y=58.848X + 420.54 (250 nm; concentration range: 250-5000 ng ml^{-1}; r^2 =0.9992).The precision of the method was estimated by calculating the RSD values for the results obtained at two different plasma concentrations. Representative chromatograms of rabbit blank plasma and plasma spiked with the drug are shown in **figure 1 and 2** respectively.

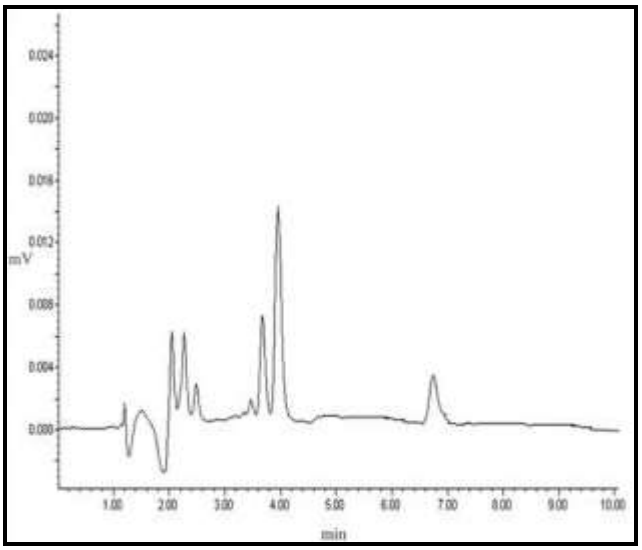

Figure 1: Chromatogram of blank (drug free) rabbit plasma.

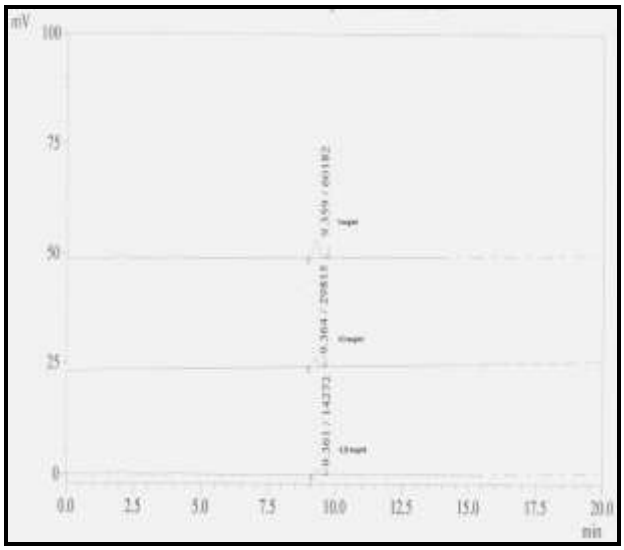

Figure 2: Shows typical chromatograms of blank plasma spiked with drug at various concentrations.

Linearity:

Linearity was studied by preparing solutions at different concentration levels when the concentration of celecoxib and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship (r^2=0.9992) was observed between the in the concentration range of 250-5000 ng mL^{-1}, for standard solution of drug with blank blood plasma, with a regression equation Y=58.848X +420.54, where Y is the peak area (AUC) and X is the concentration of celecoxib in ng/ml. The correlation coefficient was 0.9992, over these concentration ranges as shown in **figure 3**. The LLOQ was found to be 81.46 ng mL^{-1}.

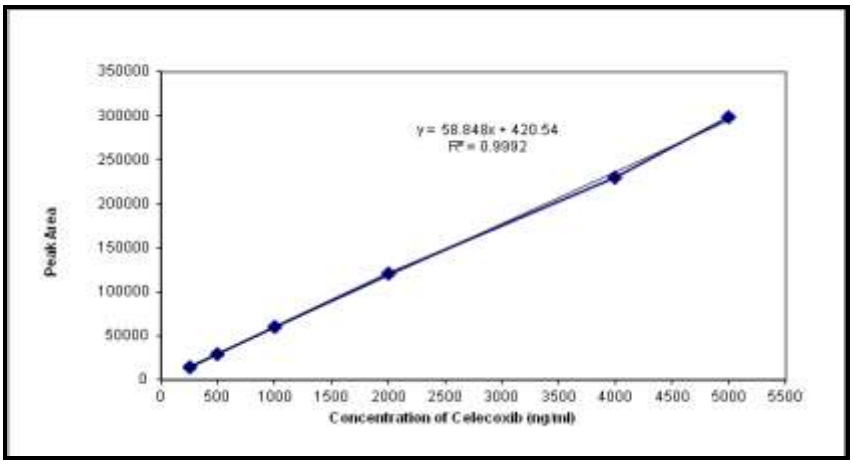

Figure 3: Calibration curve of rabbit plasma spiked with celecoxib.

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Specificity:
The specificity of the method was investigated by comparing chromatograms of three different sources of rabbit plasma. No significant peaks were observed at the retention times of drug in rabbit blank plasma. Representative chromatograms of rabbit blank plasma and plasma spiked with the drug are shown in **figure 1 and 2** respectively.
An optimum flow rate of 1.25 ml/min for the mobile phase resulted in the retention times of 9.361 min for celecoxib. No interfering peaks were observed in the chromatogram of blank rabbit plasma. For the sample preparation in analysis of celecoxib in biological fluids several extraction procedures have been described [16-21].
Analytical method validation:
The retention time of celecoxib was 9.361 min.

respectively. Linear relationship between peak area ratio and celecoxib concentration was observed ($r^2 > 0.9992$) in a concentration range from 250-5000 ng/ml, **figure 3.** Recoveries of the analyte varied between 90-110% for high, medium and low concentration (5000, 1000 and 250 ng/ml respectively). The inter-day and intra-day precision were in the acceptable range (%CV < 15%).
Table 1 summarizes the mean values of accuracy and precision for both intra and inter-day assays. Both precision and accuracy were within the acceptable ranges for bio-analytical purpose. Intra-day precision ranged from 1.72% to 4.08%. Inter-day precision ranged from 1.09% to 7.76%. The percentage of intra and inter-day accuracy was in the range of 100.88-102.81% and 99.06-99.85%, respectively. The assay method demonstrated high degree of accuracy and precision.

Table 1: Intra and inter-day precision and accuracy for celecoxib (*n* = 3)

QC sample (ng mL^{-1})	Intra-day variation			Inter-day variation		
	Mean ± S.D.	%R.S.D.*	Accuracy %	Mean ± S.D.	%R.S.D.*	Accuracy %
250	255.37 ± 10.43	4.08	102.14	247.64 ± 19.22	7.76	99.06
1000	1028.13 ± 22.76	2.21	102.81	994.44 ± 19.95	2.01	99.44
5000	5044.18 ± 86.74	1.72	100.88	4992.35 ± 54.55	1.09	99.85

*% Relative standard deviation (R.S.D.) = (standard deviation/mean) × 100

Extraction recovery:
Recovery of the celecoxib from the extraction procedure was examined by comparing the detector response obtained from the extracted sample and the detector response obtained for direct injection of standard solution. Recovery experiment was performed at three concentration levels (low, medium and high) with three replicates. Recovery results (**table 2)** presented that maximum recovery was achieved with 94.44%. The extraction recovery was found to be satisfactory as it was consistent, precise, and reproducible. Thus single step liquid-liquid extraction procedure used in this method proved to be efficient and simple enough to extract drug simultaneously from rabbit plasma.

Table 2: Extraction recovery of celecoxib (*n* = 3).

Analyte	QC sample (ng mL^{-1})	%RSD *	Extraction recovery %
celecoxib	250	4.06	90.22
	1000	2.14	91.67
	5000	1.34	94.44

*% Relative standard deviation (R.S.D.) = (standard deviation/mean) × 100

Stability:
The solution stability of celecoxib was carried out by leaving the test solutions in a tightly capped volumetric flask at -20°C & 35±5°C. The same sample solutions were assayed for a predetermined time interval up to the study period against freshly prepared solutions. **Table 3** summarizes the results of stability study carried out under various conditions. The analyte was found to be stable at ambient temperature (35±5°C) for at least 24 h in rabbit plasma. The percentage of accuracy obtained was more than 97.84%. The analyte remained unaffected at -20°C for seven days and the percentage of accuracy was found to be more than 98.66% even after seven days. Stability results indicated that rabbit plasma sample could be thawed and refrozen without compromising the integrity of the samples.

Table 3: Stability summary of celecoxib (*n* = 3)

Stability	QC sample (ng mL^{-1})	Mean ± S.D.	R.S.D.%	Accuracy %
Freeze-thaw stability (seven days)	250	246.66 ± 11.64	4.71	98.66
	1000	991.43 ± 27.65	2.78	99.14
	5000	4987.45 ± 88.77	1.78	99.75
Ambient temperature (35±5°C) (24 h)	250	244.59± 9.76	3.99	97.84
	1000	979.56 ± 28.22	2.88	97.96
	5000	4964.38 ± 96.54	1.94	99.29

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4. CONCLUSIONS

In the present study, a RP-HPLC method was developed and validated according to ICH guidelines [12] for the determination of celecoxib in rabbit plasma. The analyte was found to be stable in rabbit plasma when stored at -20°C. The method was simple, rapid, highly sensitive, accurate, precise, and cost effective for celecoxib after rabbit plasma was simply extracted by liquid-liquid extraction method. Also, the method was extended for determination of celecoxib presence in blood plasma serum.

ACKNOWLEDGEMENTS

The generosity of Jubilant Pharmaceuticals, Noida, India, is gratefully acknowledged for providing the gift samples of celecoxib. The authors are sincerely thankful to Dr. Subas Kumar and Mr. P. Lohani, Oasis Lab., Jaipur, Rajasthan, India, for supporting to carry out the HPLC analysis.

AUTHORS STATEMENT

The authors state no conflict of interest and no payment have been received for preparation of this manuscript.

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