ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research 2 (5) 2013, 01-06

RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS OF INDOMETHACIN IN RABBIT BLOOD PLASMA BY RP-HPLC TECHNIQUE

*Bankim Chandra Nandy[1, 2], A. K. Gupta[2], B. Mazumder[1]

[1] Dibrugarh University, Dibrugarh, Assam, India

[2] Jayoti Vidyapeeth Women's University, Jaipur, Rajasthan, India

Received 02 August 2013; Revised 20 August 2013; Accepted 4 September 2013

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 indomethacin 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 indomethacin 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 1.5 ml/min, detection was carried out at 240 nm and washing time with mobile phase itself was given to 30 min. Good linearity was obtained for calibration curve. By plotting the peak height vs. the indomethacin concentration (ng ml^{-1}), the following regression equation was found: Y=39.72X-563.29 (detected at 240 nm; concentration range: 250-5000 ng ml^{-1}; r^2 =0.9998). The precision of the method was estimated by calculating the RSD values for the results obtained at two different plasma concentrations. At 1000 & 250 ng ml^{-1}, the within day reproducibility was 2.18 & 3.34% (*n* = 3) respectively, while the between day reproducibility was 2.37 & 3.75% (*n* = 3) respectively. Inter-day precision ranged from 1.38% to 3.75%. The percentage of intra and inter-day accuracy was in the range of 100.1-100.54% and 99.85-99.97%, respectively. 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 indomethacin after rabbit plasma was simply extracted by liquid-liquid extraction method. Also, this method was extended for determination of indomethacin presence in blood plasma serum.

INTRODUCTION:

The Indian Pharmacopoeia [1] uses a titration method for the determination of indomethacin. The USP 26 method for indomethacin substance determination [2] recommends using of (4mm×30 mm, 5µm) analytical column containing L1 packing (ODS chemically bonded to silica). There are also many other methods published in scientific journals as well. The oldest methods for indomethacin assay determination are even from the 1980s of last century. They usually include indomethacin; eventually another anti-inflammatory drugs determination in various biological fluids by means of RP-HPLC [1-5].

The objective of the present work involved development of simple, rapid, sensitive, and cost effective RP-HPLC method for the estimation and quantification of indomethacin in rabbit plasma and the proposed method was validated as per International Conference of Harmonization (ICH) guidelines [4] to evaluate their linearity, accuracy, precision, and recovery studies. The method developed was validated and applied to determine the pharmacokinetic studies of indomethacin.

2. MATERIALS & METHODS:

Chemicals and reagents used:

Indomethacin was obtained from Jubilant Pharmaceuticals, Noida, India. Methanol and o-phosphoric acid 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 indomethacin, high performance liquid chromatography technique was used according to a modified version of some earlier reported methods [2-6].

*Corresponding author: Bankim Chandra Nandy | Email: talktobankim@gmail.com

Chromatographic conditions:

Mobile phase: 0.8 ml phosphoric acid mixed with 600 ml methanol and volume made up to 1000 ml with mili Q water.
Flow rate: 1.5 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^{o}C).
Detector: UV detector (wave length 240 nm).
Injection volume: 20 µl.
Total run time: 30 min.

Standard stock solution:

The preparation of standard curve was modified as followed as reported from some earlier methods [2-5]. Preliminary stock solution was prepared by transferring accurately weighed 10 mg of indomethacin into a 100 mL volumetric flask, dissolved in 10 mL of methanol and final volume was made to yield concentration of 100 µg mL^{-1}. Second stock solution was prepared by further diluting the first stock solution to make final concentration 10µg mL^{-1}. The solution was stored in a refrigerator below 8^{o}C and bought to room temperature before use.

Calibration curves:

The spiked plasma serum samples used for the calibration curves were prepared by adding required volumes of standard indomethacin solutions and volume made up to 2 ml by pooled blank rabbit plasma serum to yield final concentrations of 250-5000 ng mL^{-1}. Calibration curves were plotted with peak area of drug on *Y*-axis and concentration on *X*-axis.

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 1.5 ml/min, detection was carried out at 240 nm and washing time with mobile phase itself was given to 30 min.

Method validation:

The method was validated for selectivity, linearity, precision, accuracy, and stability according to ICH guidance [4] for validation of bio analytical methods. Each and every standard solution of indomethacin in the range of 250-5000 ng mL^{-1} of samples were showing retention time in 13.83 min i.e., peak time. The run samples were plotted to see the linearity of the areas vs. concentrations, which was checked by linear regression coefficient value (R^2) 0.9998. These helps later to identify the indomethacin concentration in rabbit's serum sample in respect to standard plotted graph.

Linearity:

To establish linearity, a series of calibration standards were prepared by adding a known concentration of indomethacin 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 use of the formulae **LOD = 3.3 × $S_{y/x}$/S and LOQ = 10 × $S_{y/x}$/S**.

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.

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.

Extraction recovery:

The extraction recovery of the indomethacin 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 indomethacin in blood plasma was assessed by analyzing three replicates of low, medium, and high QC samples under different temperature and

time conditions. 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 DISCUSSIONS:

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 ammonium acetate in various proportion were tested. When ammonium acetate was added in the mobile phase, the response of the analyte was distinctly decreased.

The chromatograms could efficiently proceed by mixing with 0.8 ml phosphoric acid in 600 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 curve. By plotting the peak height vs. the indomethacin concentration (ng ml^{-1}), the following regression equations were found: Y=39.72X-563.29 (detected at 240 nm; concentration range: 250-5000 ng ml^{-1}; r^2 =0.9998).

The precision of the method was estimated by calculating the RSD values for the results obtained at two different plasma concentrations. At 1000 & 250 ng ml^{-1}, the within day reproducibility was 2.18 & 3.34% *(n* = 3) respectively, while the between day reproducibility was 2.37 & 3.75% *(n*=3) respectively. Representative chromatograms of rabbit blank plasma and plasma spiked with the drug are shown in **figures 1 and 2** respectively.

Linearity:

Linearity was studied by preparing solutions at different concentration levels when the concentration of indomethacin and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship (r^2=0.9998) was observed in the concentration range of 250-5000 ng mL^{-1}, for standard solution of drug with blank blood plasma, with a regression equation Y=39.72X-563.29, where Y is the peak area (AUC) and X is the concentration of indomethacin in ng/ml. The correlation coefficient was 0.9998, over these concentration ranges as shown in **figure 3**. The LLOQ was found to be 60.70 ng mL^{-1}.

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 **figures 1 and 2** respectively.

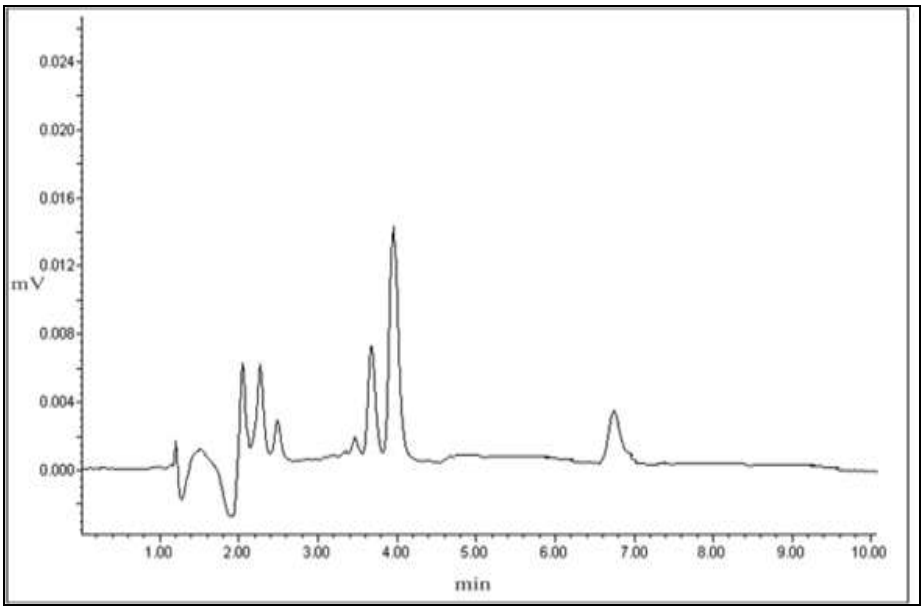

Figure 1: Chromatogram of blank (drug free) rabbit plasma

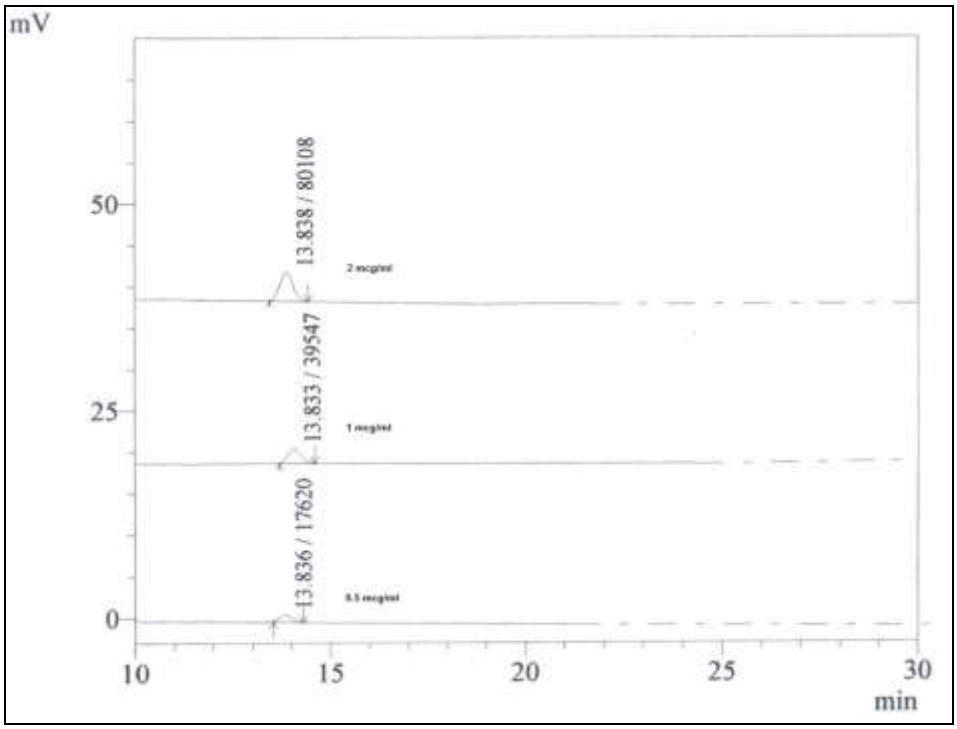

Figure 2: Chromatogram of rabbit plasma spiked with indomethacin.

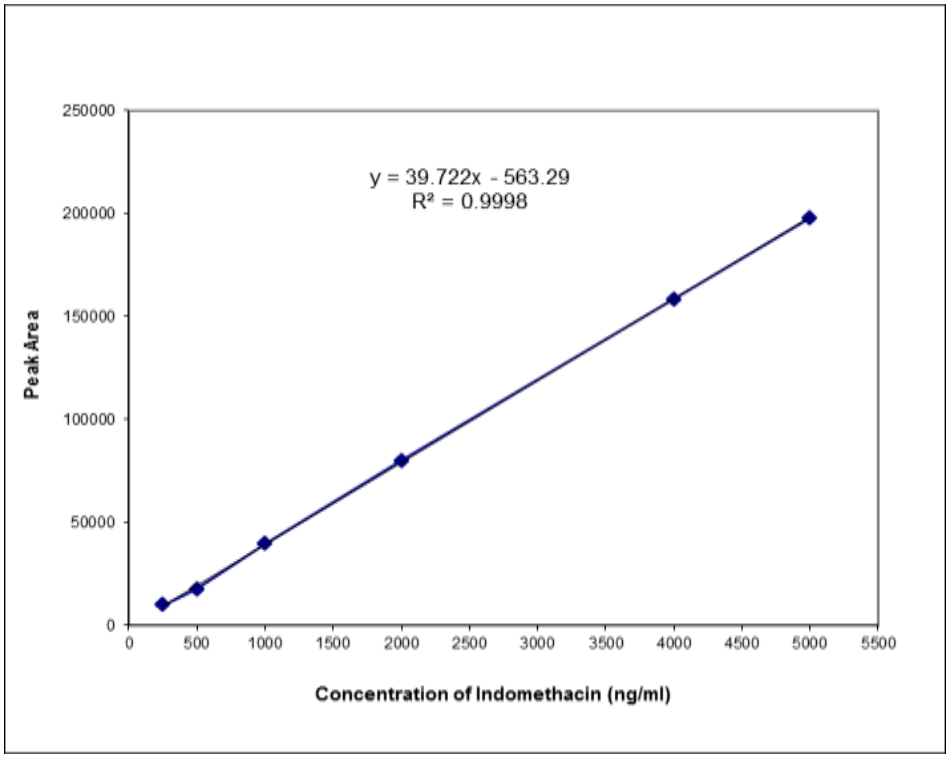

Figure 3: Calibration curve of rabbit plasma spiked with indomethacin.

Accuracy and precision:

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 1.13% to 3.34%. Inter-day precision ranged from 1.38% to 3.75%. The percentage of intra and inter-day accuracy was in the range of 100.1-100.54% and 99.85-99.97%, respectively. The assay method demonstrated high degree of accuracy and precision.

Table 1: Intra and inter-day precision and accuracy for indomethacin (*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	251.37 ± 8.42	3.34	100.54	249.81 ± 9.38	3.75	99.92
1000	1002.13 ± 21.94	2.18	100.21	998.51 ± 23.75	2.37	99.85
5000	5005.18 ± 56.75	1.13	100.10	4998.74 ± 69.48	1.38	99.97

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

Extraction recovery:

Recovery of the indomethacin 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 presented that maximum recovery was achieved with 93.36%.

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

Analyte	QC sample (ng mL^{-1})	%RSD *	Extraction recovery %
Indomethacin	250	2.85	90.73
	1000	2.97	88.64
	5000	4.45	93.36

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

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.

Stability:

The solution stability of indomethacin was carried out by leaving the test solutions in a tightly capped volumetric flask at -20^{o}C & 35±5^{o}C. The same sample solution was 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^{o}C) for at least 24 h in rabbit plasma. The percentage of accuracy obtained was more than 95.14%. The analyte remained unaffected at -20^{o}C for seven days and the percentage of accuracy was found to be more than 99.34% 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 indomethacin (*n* = 3)

Stability	QC sample (ng mL^{-1})	Mean ± S.D.	R.S.D.%	Accuracy %
Freeze-thaw stability (seven days)	250	248.34 ± 10.71	4.31	99.34
	1000	994.45 ± 24.68	2.48	99.44
	5000	4993.55 ± 85.58	1.71	99.87
Ambient temperature (35±5^{o}C) (24 h)	250	237.86 ± 7.62	3.20	95.14
	1000	986.65 ± 24.14	2.44	98.66
	5000	4975.67 ± 97.43	1.95	99.51

4. CONCLUSIONS:

In the present study, a RP-HPLC method was developed and validated according to ICH guidelines [4] for the determination of indomethacin in rabbit plasma. The analyte was found to be stable in rabbit plasma when stored at -20^{o}C. The method was simple, rapid, highly sensitive, accurate, precise, and cost effective for indomethacin after rabbit plasma was simply extracted by liquid-liquid extraction method. Also, this method was extended for determination of indomethacin presence in blood plasma serum.

1. ACKNOWLEDGEMENTS

The generosity of Jubilant Pharmaceuticals, Noida, India, is gratefully acknowledged for providing the gift samples of indomethacin. 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.

2. AUTHORS STATEMENT

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

3. REFERENCES:

1. Indian Pharmacopoeia commission government of India Ministry of Health & Family Welfare, Inida, 2007, (1), 609-611.
2. The United States Pharmacopoeia 28 NF, Asian edition, United States pharmacopoeial convention, 2005, pp 184.
3. Hubert PH, Renson M, Cromment J. A fully automated high-performance liquid chromatographic method for the determination of indomethacin in plasma. Journal of Pharmaceutical & Biomedical Analysis, 1989, 7 (12), pp. 1819-1827.
4. ICH, Q2 (R1) Validation of Analytical Procedure: Text and Methodology, International Conference on harmonization, 2005.
5. Novakov L, Matysov L, Havlikov L, Solicha P. Development and validation of HPLC method for determination of indomethacin and its two degradation products in topical gel. Journal of Pharmaceutical and Biomedical Analysis, 2005, 37, 899–905.
6. Florey K, Indomethacin, In: Analytical Profiles of drug substances, Rajkamal Electric Press, Delhi, India, Vol-13, 1984, pp 211-235.

