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# Bioanalytical Method Development and Validation of Ibuprofen Using RP-HPLC

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## ABSTRACT

An accurate, simple, precise and sensitive HPLC method with UV detection was developed and validated to separate and detect ibuprofen in human plasma using Nimesulide as an internal standard. Ibuprofen and Nimesulide were extracted from human plasma using acetonitrile protein precipitation and HPLC analysis was performed using Waters 515 Series pumps combined with a Waters PDA 2998 series photo diode array detector (DAD). The column used was Agilent C18 column (150mm×4.6mm, particle size 5-micron Agilent, USA). Analysis was isocratic at 1.5 ml/min flow rate with ACN: Buffer (0.025M Potassium dihydrogen ortho phosphate) pH 4.5 (55:45, v/v) as mobile phase. The mobile phase was premixed, filtered through a 0.2 µm nylon membrane filter to remove any particulate matter and degassed by sonication before use. The elution was detected at 230 nm. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was measured. The retention times of Ibuprofen 2.24 min and for I.S. 1.72 min respectively. The method was validated over the range of 0.5-8.0 µ/ml. The limit of detection was 0.06µg/ml and the limit of quantification was 0.193µg/ml for ibuprofen. Inter-day as well intra-day replicates of Ibuprofen, gave % R.S.D. below 2.07 and 2.001 respectively The absolute recovery of ibuprofen was greater than 90% were achieved. This method of analysis for Ibuprofen determination using RP-HPLC was applied for determination of Ibuprofen in plasma.

**Keywords**: Bio-analytical method, Plasma Extraction, Ibuprofen, Nimesulide, HPLC, ICH.

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## INTRODUCTION

The 2-arylproprionic acid derivative, Ibuprofen [RS-2-(4-isobutyl-phenyl)propionic acid], is one of the most potent orally active antipyretic, analgesic and nonsteroidal anti-inflammatory drug (NSAID)[1]. It is used extensively in the treatment of acute and chronic pain, osteoarthritis, rheumatoid arthritis and related conditions. This compound is characterized by a better tolerability compared with other NSAIDs[2].

Ibuprofen contains a chiral carbon atom on the propionic acid side-chain; therefore it exists as two enantiomers. It is usually marketed as a 50:50 mixture of the S-(+) and R-(-) enantiomers, even if it is known that the pharmacological activity is due almost exclusively to the S-(+) enantiomer (eutomer)[3, 4].

The mechanism of action of ibuprofen is the inhibition of cycloxygenase activity and therefore the synthesis of prostaglandins. The rank order of potency of NASIDs as inhibitors of prostaglandins synthesis in vitro trends to reflect their anti-inflammatory potency in vivo[5].

Ibuprofen is metabolized by oxidation in the liver to produce two major metabolites: 2-hydroxy- and 2-carboxy-ibuprofen. The conjugated and the unconjugated forms of these metabolites, as well as up to 10% of ibuprofen, are excreted in urine[6]. Ibuprofen appears to exert its pharmacologic actions by inhibiting cyclooxygenase and thus blocking the first step in the synthesis of prostaglandins[7].

Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use[8]. Due to presence of chiral carbon in Ibuprofen, it has easily studied by many spectroscopic techniques[9]. Several high-performance liquid chromatographic (HPLC) methods with ultraviolet detection for the determination of ibuprofen in biological fluids have been published on reversed phase columns[10, 11, 12]. The chiral columns are very expensive, less flexible and less durable[13]. Other methods for ibuprofen quantification include LC-MS[14, 15].

This paper describes the development and validation of a sensitive, specific, rapid, simple and economic HPLC bioanalytical method for ibuprofen quantification in human plasma. This report represents a one-step sample preparation using methanol that simplifies the analysis of ibuprofen in human plasma. Sample handling and chromatographic run times were minimized to provide fast quantitative results while maintaining the specificity accuracy and precision required for evaluation of ibuprofen.

# MATERIALS AND METHODS

## Experimental

### Solvents and chemicals

Ibuprofen and Nimesulide were supplied by Panacea Biotech Pvt. Ltd. (Himachal Pradesh, India). All the solvents were of analytical grade and used without further purification. Acetonitrile and methanol were of HPLC grade and obtained from J. T. Bakers. Dichloro Methane, Diethyl ether, Acetic acid and Ammonium Acetate were obtained from Merck (Worli, Mumbai, India). Water was deionized, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore. Drug free and Healthy human plasma was obtained from Tapovan blood bank, Sri Ganganagar, India where blood was collected from volunteers in tubes containing K2EDTA. After centrifugation, the plasma was transferred into polypropylene tubes and stored at or below -18°C.

### Instrumentation

HPLC analysis was performed using Waters 515 Series pumps combined with a Waters PDA 2998 series photo diode array detector (DAD). The column used was Agilent C18 column (150mm×4.6mm, particle size 5-micron Agilent, USA).

### Chromatographic condition

Analysis was isocratic at 1.5 ml/min flow rate with ACN: Buffer (0.025M Potassium dihydrogen ortho phosphate) pH 4.5 (55:45, v/v) as mobile phase. The mobile phase was prepared freshly every day. The mobile phase was premixed, filtered through a 0.2 µm nylon membrane filter to remove any particulate matter and degassed by sonication before use. The elution was detected at 230 nm. The substance was quantified using its peak area ratio of Ibuprofen to IS (Internal Standard). Prior to injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was measured.

### Protein Precipitation

The protein precipitation method was used for extraction of ibuprofen from plasma using acetonitrile as protein precipitant. 100 µL of blank plasma was spiked with 100.0 µL of standard ibuprofen from 100 µg/mL dilution of ibuprofen. This spiked plasma was vortexed for 2 min. and 800 µL Ibuprofen was added to precipitate plasma proteins. The mixture was further vortexed for 2 min and centrifuged at 10000 rpm for 10 min. After centrifugation, 100 µL of the supernatant was collected and 100 µL of I.S (nimesulide) was added of required concentration of

and diluted to 1.0 mL with acetonitrile. A 20.0 µL aliquot of final preparation was injected into the HPLC system.

**Validation parameters**

**Calibration curve (linearity)**

Five different concentrations of ibuprofen with constant IS concentration were spiked to the blank human plasma and calibration curve was constructed in the specified concentration range (0.5 µg/ml to 8.0 µg/ml). The calibration plot (peak area ratio of ibuprofen to IS versus ibuprofen concentration) was generated by replicate analysis (n = 6) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

**Precision**

Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows. Solutions containing lowest, intermediate and highest quantification concentrations (LQC MQC and HQC) of the calibration curve, i.e. 0.5 µg /ml, 2.0 µg /ml, 8.0 µg/ml were prepared. Six injections at each of the specified concentration levels were injected within the same day for repeatability, and over a period of 3 days (6 injections / day) for reproducibility. Mean and relative standard deviation were calculated and used to judge accuracy and precision of the method. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Intra-day precision and inter-day precision for the developed methods were measured in terms of % R.S.D. which was taken for conclusion.

**Accuracy**

For determining the accuracy of the proposed method, different quality control (QC) levels of drug concentrations in plasma [lower quality control samples (LQC) 0.5 µg/ml, medium quality control samples (MQC) 2.0 µg/ml, and higher quality control samples (HQC) 8.0 µg/ml] were prepared independently and analyzed.

**Recovery**

The absolute recovery of ibuprofen and I.S were determined by comparison of the peak areas from non-extracted and extracted samples in triplicate. The recovery was calculated as the relative standard deviation of the mean (R.S.D.) with R.S.D.

(%) = (standard deviation of the mean/mean) ×100.

**Detection and quantitation limits (LOD & LOQ)**

The limits of detection and quantitation were calculated by decreasing drug concentration till the ratio of peak area to noise is 3:1 for LOD and 10:1 for LOQ.

**Sensitivity**

In order to determine LOQ, three independent plasma samples containing 0.1µg/ ml of Ibuprofen were prepared (lowest possible measurable concentration) and analyzed. The peaks were integrated and concentrations were calculated using calibration equation. Mean concentration and % RSD for these three values were determined.

**Specificity**

The specificity of the method was determined by comparing the chromatograms obtained from the samples containing ibuprofen and IS with those obtained from blank plasma. Five blank plasma samples from six lots of human plasma were processed with and without the internal standard to evaluate presence of interfering peaks[16].

**Stability**

Blank plasma was spiked with the known amount of ibuprofen to achieve the concentration of LQC, MQC and HQC of ibuprofen (n = 3) and stored at −4 °C. The stability of these samples was checked up to 24 hrs to find out short term stability. Further, the freeze–thaw (20 °C / room temperature) stability of the ibuprofen spiked plasma samples was measured for three cycles.

**System suitability parameters**

System suitability tests are an integral part HPLC method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.

**Plate number or number of theoretical plates (n)**

This is a measurement of the sharpness of the peaks and therefore the efficiency of the column.

$$n = 16 (t / w_b)^2$$

Where , t is retention time of peak,

$W_b$ is peak width at base)

**Capacity factor (K)**

This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

$$K = t_R - t_M / t_M$$

Where $t_M$ is unrestrained peak retention time,

$t_R$ is retention time of peak of interest.

**Separation Factor (α)**

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the

capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

$$\alpha = K_B / K_A$$

Where, $K_B$ is capacity factor of peak B,

$K_A$ is capacity factor peak A

**Resolution Factor ($R_S$)**

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima to the mean value of the peak width at base (Wb).

$$R_S = 2(t_{R2} - t_{R1}) / w_{b1} + w_{b2}$$

Where, $t_{R1}$ and $t_{R2}$ are retention time of two adjacent peak, $w_{b1}$

$w_{b2}$ are peak width of respective peak.

**Peak Asymmetric Factor ($A_f$ ) and Peak Tailing Factor (T)**

The deviation from symmetry is measured by the Asymmetry Factor, $A_f$ or Tailing Factor T.

Asymmetric factor, $A_f$ is described by the following equation:

$$A_f = A_{10\%\,h} / B_{10\%h}$$

Where, A and B are sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height

**Tailing factor**, T is described by following equation:

$$T = (A_{5\%h} + B_{5\%h}) / 2A_{5\%h}$$

**Ruggedness**

This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents. Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test condition. The ruggedness of the method was studied by changing the experimental condition such as Changing to another column of similar type or Different operation in the same laboratory.

## RESULTS AND DISCUSSION

**Validation Parameters**

**Calibration curve**

The method was found to be linear in the range of 0.5 – 8.0 µg/ml (correlation coefficient $r^2$=0.9871. The peaks of plasma, Ibuprofen and IS are given in the chromatogram (Figure 1). All the validation parameters are given in Table 1 (Validation Parameters of RP-HPLC Bio-

analytical Method for Ibuprofen Estimation).

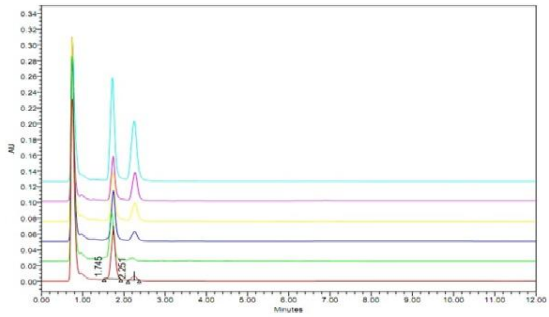

**Figure 1 Overlay spectra of blank plasma and blank plasma with Ibuprofen and I.S.**

**Table 1 Validation Parameters of RP-HPLC Bio-analytical Method for Ibuprofen Estimation.**

| Parameters | Results |
|---|---|
| Linearity | 0.5 – 8.0 µg/ml |
| Regression equation | y =0.1032x + 0.024 |
| Slope (m) | 0.1032 |
| Intercept (c) | 0.024 |
| Correlation coefficient ($r^2$) | 0.9871 |
| LOQ | 0.193 (µg/ml) |

**Accuracy and Precision**

Accuracy data in the present study is 89.61 % indicates that there was no interference of endogenous plasma components in Ibuprofen determination. Inter-day as well intra-day replicates of Ibuprofen, gave % R.S.D. below 2.001 and 2.070 % respectively (should be less than 15 % according to CDER guidelines for bio-analytical method validation), revealed that the proposed method is highly precise.

**Table 2 Precision Data for Bio-analytical Method**

| Levels | Precision | |
|---|---|---|
| | Intra-day (% RSD) | Inter-day (% RSD) |
| LQC | 1.990 | 2.289 |
| MQC | 1.187 | 1.121 |
| HQC | 2.827 | 2.801 |
| Mean | 2.001 | 2.070 |

Intra-day precision and inter-day variation of method were determined using three replicate injections of three concentration levels and analyzed on same day for three times and three different days. The results of inter-day and intra-day precision are explained in Table 2 **(**Precision Data for Bio-analytical Method).

**Recovery**

The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery of the drug and the internal standard was calculated and it was 89.55% and

90.11% respectively.

**Sensitivity**

The lowest measurable concentration was found to be 0.06 µg/ml i.e. LOQ for the developed bio-analytical method was 0.193 µg/ml. When this method is applied to animal plasma samples, its sensitivity was adequate for pharmacokinetic studies. Figure 2 shows the chromatogram of Plasma, drug and ibuprofen.

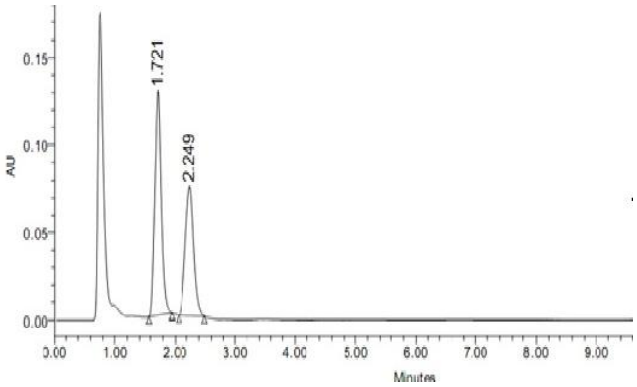


**Figure 2 Chromatogram of Plasma, Drug and Internal standard**

**Stability**

The plasma samples were stored at −4 °C, the short term stability and freeze thaw stability were checked. The results of short term stability are described in Table 3 **(**Short Term Stability Data for Bio-analytical Method)

**Table 3 Short Term Stability Data for Bio-analytical Method.**

| Level | 0 hr (%RSD) | 3 hr (%RSD) | 6 hr (%RSD) | 12 hr (%RSD) | 24 hr (%RSD) |
|---|---|---|---|---|---|
| **LQC** | 3.54 | 2.18 | 2.97 | 3.46 | 4.41 |
| **MQC** | 2.84 | 3.26 | 2.99 | 4.19 | 2.75 |
| **HQC** | 3.39 | 2.88 | 3.41 | 2.04 | 4.94 |

and freeze thaw stability data are explained in Table 4 **(**Freeze Thaw Stability Data for Bio-analytical Method).

**Table 4 Freeze Thaw Stability Data for Bio-analytical Method.**

| Level | 1st cycle (%RSD) | 2nd cycle (%RSD) | 3rd cycle (%RSD) |
|---|---|---|---|
| **LQC** | 8.269 | 7.912 | 9.269 |
| **MQC** | 7.085 | 9.452 | 7.183 |
| **HQC** | 9.006 | 7.849 | 8.413 |

**System Suitability Parameters**

The system suitability parameters such as asymmetric factor, tailing factor, theoretical plates and plate numbers were measured. The values found for these parameters are described in Table 5 **(**System suitability parameters of bio-analytical method). All the system suitability parameters found to be according to the acceptable limits of the bio-analytical methods.

**Table 5 System suitability parameters of bio-analytical method**

| Parameters | Results | | Acceptable limits |
|---|---|---|---|
| | Ibuprofen | Internal Standard | |
| Asymmetry | 1.02 | 0.99 | < 1.5 |
| Tailing Factor | 1.037 | 1.032 | < 2 |
| Plate no. | 3486 | 3393 | > 2000 |
| Resolution | 2.58 | 1.98 | > 1.5 |
| Capacity factor | 2.76 | 2.12 | > 2 |

**Ruggedness**

The ruggedness of the method was carried out by changing column and by different analyst in the same laboratory. The percentage CV of the HQC and LQC were calculated and were passed.

## CONCLUSION

The bio-analytical method developed is simple and shows good accuracy, specificity and reproducible. It can be used for the estimation of Ibuprofen in bio-fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of ibuprofen without any interference. The developed method could be applied in bioequivalence, pharmacokinetic and toxic-kinetic studies.

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