

Research Article
DEVELOPMENT AND VALIDATION OF A SIMPLE HIGH
PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD
FOR DETERMINATION OF KETOPROFEN IN HUMAN PLASMA

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ABSTRACT

The present study was aimed to develop and validate a simple reversed-phase high performance liquid chromatographic method with UV detection at 260 nm for the determination of ketoprofen in human plasma. The sample preparation involved a simple protein precipitation method using perchloric acid along with acetonitrile as the deprotenizing agents. Chromatographic separation was achieved isocratically by using C-18 column (5 μ m, 250 x 4.6 mm ID). The mobile phase was comprised of 0.05M disodium hydrogen phosphate buffer and acetonitrile (70:30, v/v) adjusted to pH 6.5 with glacial acetic acid. Analysis was run at a flow rate of 1.0 mL/min with 50 μ L injection volume and the samples were quantified using peak height measurement. The calibration curve was linear in the range of 156.25-20000 ng/mL with correlation coefficient (r) of 0.9999 (\pm 0.0001). The within-day accuracy ranged between 83.80 and 112.56% with precision between 3.41 and 7.07%. The between-day accuracy ranged between 91.57 and 99.84% with precision between 1.62 and 16.06%. The recovery ranged between 96.45 and 102.92% with precision between 2.90 and 9.92%, respectively. The values of accuracy, precision and recovery obtained were within the acceptable limits as proposed by USFDA Bioanalytical Guidelines. In conclusion, the developed and validated method can be used to estimate plasma ketoprofen concentration after oral administration of Pharmaceutical dosage forms.

KEY WORDS: Ketoprofen, Pharmaceutical analysis, HPLC, Human plasma.

1. INTRODUCTION

Ketoprofen is a potent non-steroidal anti-inflammatory drug (NSAID) used for the treatment of a wide range of painful and inflammatory illnesses¹⁻⁴. Like most NSAIDs, ketoprofen is advantageous because it lacks addictive potential and does not result in sedation or respiratory depression. Ketoprofen is a white or almost white crystalline powder having empirical formula C₁₆H₁₄O₃ with molecular weight of 254.3 and melting point 94° to 97°C. It has pKa of 5.94. It is practically insoluble in water, freely soluble in alcohol, acetone, and dichloromethane⁵. Having low solubility and high permeability, ketoprofen is a class II drug according to Biopharmaceutics Classification System (BCS)⁶⁻⁷. The chemical name of ketoprofen is [2-(3-benzoylphenyl)-propionic acid]⁸⁻⁹ and belongs to the aryl propionic acid class. The chemical structure of ketoprofen is represented in Figure 1.

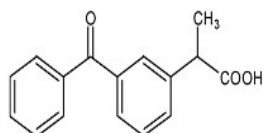


Figure 1 Chemical structure of ketoprofen

Several reversed-phase HPLC methods for determining ketoprofen concentration in human plasma have been reported in the literature¹⁰⁻¹⁵. However, these methods involved liquid-liquid or solid phase extraction techniques, which are often tedious, time consuming and also expensive.

Low drug recovery was obtained from plasma samples containing ketoprofen, subjected to direct protein precipitation with acetonitrile, in a ratio of 2:1 (ACN : plasma, v/v). In order to increase ketoprofen recovery, [Adriaenssens and Prescott, 1978](#); [Susan et al., 1987](#) used 20 μ L of 30% perchloric acid as the deprotenizing agent for protein precipitation¹⁶⁻¹⁷. However, gradient elution, which requires gradient pump, was as used for drug analysis.

Hence, the aim of this part of the study was to develop and validate a simple and rapid HPLC method, with isocratic elution, for the determination of ketoprofen in human plasma after direct deprotenization.

2. MATERIAL AND METHODS

2.1 Materials

The materials used in this study were; ketoprofen supplied by Hovid Pharmaceutical Company (Hovid Inc, Ipoh, Malaysia). Disodium hydrogen phosphate and methanol obtained from BDH (Dorset, England) and Merck (Darmstadt, Germany) respectively. Acetonitrile (ACN) and glacial acetic acid were purchased from J. T. Baker (Phillipsburg, NJ, USA). All solvent and chemical used were of analytical reagent or HPLC grade.

2.2 Instrumentation and chromatographic conditions

The high performance liquid chromatography (HPLC) system used consisted of an isocratic pump Jasco PU-980 (Jasco, Japan), a Gilson® 119 UV detector (Gilson, USA) equipped with a Hitachi D-2500 Chromo-integrator (Hitachi, Tokyo, Japan)

and a Rheodyne 7725 (Cotati California, USA) sample injector fitted with a 50 μ L sample loop. The detector was operated using a sensitivity range of 0.005 AUFS and wavelength of 260 nm.

A Thermo C-18 column from Thermo Electron Corporation (California, USA) (5 μ m, 250 x 4.6 mm ID) fitted with a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA) packed with Perisorb RP-18, 30-40 μ m pellicular stationary phase (Upchurch Scientific, Oak Harbour, WA, USA) was used for chromatographic separation. The mobile phase comprised 0.05M disodium hydrogen phosphate buffer and acetonitrile (70:30, v/v) adjusted to pH 6.5 with glacial acetic acid. Analysis was run at a flow rate of 1.0 mL/min and the samples were quantified using peak height measurement.

2.3 Preparation of stock and working standard solutions

Standard stock solution of ketoprofen was prepared by dissolving 50 mg of ketoprofen in 100 mL of methanol to give a concentration of 500 μ g/mL, and was stored at 4°C. A series of working standard solutions of ketoprofen were prepared by appropriate serial dilution of the stock solution in mobile phase, giving final concentrations of 20000, 10000, 5000, 2500, 1250, 625, 312.5, and 156.3 ng/mL.

2.4 Preparation of calibration standards

Calibration plasma standards were prepared in a concentration range of 156.25–20000 ng/mL by spiking blank human plasma with a known concentration of ketoprofen. This was carried out by first spiking 1 mL of standard stock solutions consisting of ketoprofen (500 μ g/mL) into 25 mL of blank plasma, to give a final concentration of 20 μ g/mL. Then, subsequent concentrations were prepared by serially diluting this plasma sample with blank plasma to obtain concentrations of 20000, 10000, 5000, 2500, 1250, 625, 312.5, and 156.3 ng/mL.

2.5 Sample preparation procedure

Prior to analysis, the plasma samples were treated using the following method: A 250 μ L aliquot of plasma was pipetted accurately into a 1.5 mL eppendorf micro centrifuge tube and deproteinized by adding the deprotenizing agents; 10 μ L of 70% perchloric acid and 500 μ L acetonitrile (ACN). The mixture was vortexed for 90 seconds, and then centrifuged (Eppendorf, Hamburg, Germany) at 12800 g for 20 min. A 50 μ L aliquot of the supernatant was then injected into the column.

2.6 Bio-analytical method validation

Five points of the standards which represented the low, medium, and high concentrations (156.3, 312.5, 1250, 5000, and 20000 ng/mL), were used to

determine the within-day and between-day precision and accuracy of the method (n = 6).

2.6 (a) Linearity

The linearity is defined as the ability of the analytical method, within a given range, to obtain results that are directly proportional to the concentration of the analyte in the sample. The linearity is expressed in terms of the correlation coefficient (r). The linearity of the method was evaluated using freshly prepared spiked plasma samples with ketoprofen in the concentration range of 156.3 to 20000 ng/mL. Six linearity curves were analyzed. The samples were quantified using the peak height of analyte. The peak height was plotted against plasma concentration and the standard curves were modeled by the linear regression equation: $y = mx + c$. A correlation coefficient of more than 0.999 is desirable for all the calibration curves.

2.6 (b) Limit of detection and limit of quantification

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample which can be detected, but not necessarily quantified under the stated experimental conditions. It can be determined at a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample which can be determined with acceptable accuracy and precision under the stated experimental conditions of the method. Like LOD, LOQ is expressed as a concentration, with the precision and accuracy of the measurement also reported.

2.6 (c) Specificity

Drug-free plasma samples obtained from six subjects were processed and injected into HPLC system to assess if the endogenous components in the plasma might interfere at the retention time of ketoprofen.

2.6 (d) Accuracy and precision

Accuracy is the measure of how close the experimental value is to the true value. It is measured as the percentage of analyte recovered by assay spiking samples. While, precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. It is normally expressed as the percentage relative standard deviation (Coefficient of variance).

The within-day and between-day assay were used to validate the accuracy and precision of the assay by determining the standard samples of ketoprofen in human plasma. The accuracy was expressed as the percentage recovery of measured concentration of the samples and was calculated using the equation below.

% Accuracy=

Measured value/ Theoretical value X 100 %

...(Eq. 1)

The precision is denoted as percentage coefficient of variation and calculated using the following equation

% C.V =

Standard deviation /Mean value X 100 % .(Eq. 2)

For within-day accuracy and precision validation, six sets of spiked plasma samples at five different drug concentrations were assayed within a single day with one standard calibration curve on the same run. On the other hand, for between-day accuracy and precision validation, six sets of spiked plasma samples at five different concentrations were evaluated on a single sample of each concentration on the daily basis over six different days, with one standard calibration curve constructed on each day of the analysis. The evaluation of precision and accuracy was based on the criteria of United States Food and Drug Administration guidelines¹⁸, which stated that the percent coefficient of variation for each concentration should not be more than $\pm 15\%$ except for the limit of quantification where it

should not be exceed $\pm 20\%$. Similarly for accuracy, it should be within 85-115% of the each concentration except for the limit of quantification where it is allowed up to 80-120%¹⁹

2.6 (e) Recovery

The recovery of ketoprofen was evaluated by comparing the mean peak height of 156.25, 312.50, 1250, 5000, and 20000 ng/mL of ketoprofen in plasma with that of aqueous standards of corresponding concentration. According to the acceptance criteria, the recovery of the analyte does not need to be 100%, but should be consistent, precise and reproducible¹⁸

3. RESULTS AND DISCUSSION**3.1 Linearity**

The mean standard calibration curve, presented in Figure 2, was obtained by analyzing plasma samples spiked with ketoprofen at the concentration range of 156.25 to 20000 ng/mL. The calibration curve exhibited a good linearity over the concentration range of 156.25 to 20000 ng/mL of ketoprofen. The mean linear regression equation from six replicates of calibration curves was $y = 6.8739 (\pm 0.6503) x - 145.9904 (\pm 288.9831)$, with a correlation coefficient (r) of 0.9999 (± 0.0001).

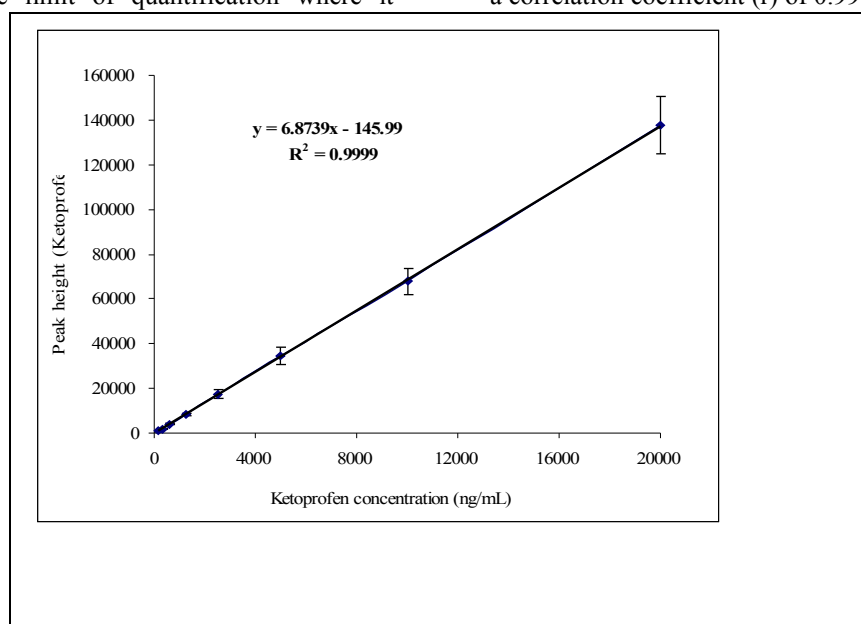


Figure 2 Mean standard calibration curve of ketoprofen in human plasma.

Mean \pm SD, N = 6.

Table 1 Summary of the data of ketoprofen standard calibration curve in human plasma.

Mean \pm SD, N = 6.

Theoretical concentration (ng/mL)	Ketoprofen peak height (Mean \pm SD, N = 6)	Accuracy (% ACC)	Precision (% CV)
156.3	862 \pm 126.0	93.2	18.6
312.5	1918 \pm 150.3	96.3	11.4
625	4157 \pm 514.8	100.1	5.8
1250	8383 \pm 368.3	99.7	6.7
2500	17474 \pm 1766.4	102.6	4.4
5000	34494 \pm 4046.1	100.6	3.9
10000	67828 \pm 5996.7	98.9	1.4
20000	137599 \pm 12918.4	100.2	0.3

3.2 Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined by spiking blank plasma with decreasing concentrations of ketoprofen. The LOQ and LOD were determined by injecting 50 μ L of plasma sample. LOD obtained for ketoprofen was 78.1 ng/mL at a signal to noise ratio of 3:1, whereas LOQ was 156.3 ng/mL with injection volume of 50 μ L. To determine the accuracy and precision of the obtained LOQ value, the plasma samples containing 156.3 ng/mL of ketoprofen were injected 6 times within one day. The accuracy values of ketoprofen were found to be 93.2%, and precision expressed as %CV was 18.6% (Table 1).

3.3 Specificity

No interference from the endogenous compounds was observed at the retention time of ketoprofen, based on the chromatograms of drug-free plasma and plasma spiked with ketoprofen and subject plasma obtained at 5 hour after oral administration of the test formulation of ketoprofen. The retention time of ketoprofen was 5.50 minute, as shown in Figure 3.

3.4 Accuracy and precision

The within-day and between-day reproducibility of HPLC assay for determination of ketoprofen in plasma was expressed as accuracy (%ACC) and precision (%CV). The results are summarized in Table 3.2. The within-day accuracy ranged between 83.8 and 112.6% with precision between 3.4 and 7.1%. The between-day accuracy ranged between 91.6 and 99.8% with precision between 1.6 and 16.1%. The values of accuracy and precision obtained were within the acceptable limits as proposed by USFDA guidelines¹⁸ These values show a considerable degree of reproducibility of HPLC assay for the within-day and between-day analytical runs.

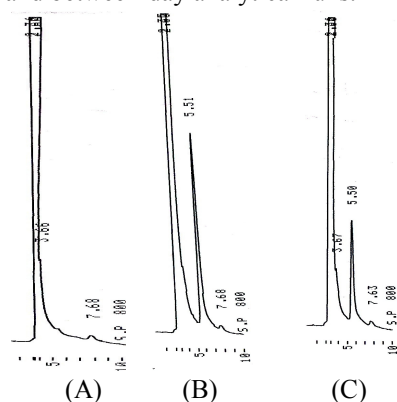


Figure 3 Chromatogram for ketoprofen in human plasma. (A) Blank human plasma, (B) Human plasma spiked with 5000 ng/mL, and (C) Subject plasma obtained 5 hour after oral administration of ketoprofen. The retention time of ketoprofen 5.50 minutes.

3.5 Recovery

The recoveries of ketoprofen from human plasma at low, medium and high concentrations (156.3, 312.5, 1250, 5000, and 20000 ng/mL) were between 96.5 and 102.9% with a CV of 2.9 and 9.9% respectively (Table 3).

Table 3 Recovery of ketoprofen from human plasma during sample preparation. Mean \pm SD, N = 6.

Theoretical concentration (ng/mL)	%Recovery	Precision (%CV)
156.3	96.5 \pm 2.8	2.9
312.5	90.5 \pm 7.6	8.6
1250	97.0 \pm 9.6	9.9
5000	99.4 \pm 8.0	8.0
20000	102.9 \pm 3.7	3.6

4. CONCLUSIONS

In conclusion, a simple, accurate, precise, sensitive, and specific isocratic RP-HPLC method was developed and validated for the determination of ketoprofen in human plasma. The chromatographic assay showed good characteristics and fulfilled our analytical validation criteria. The simplicity of the method involves a simple one step deproteinization, allows for numerous samples to be processed in a relatively short period of time and reduced workload. Another advantage in this method uses cheap and readily available C18 column. Hence, this HPLC method is suitable for determination of ketoprofen concentration in human plasma for *in vivo* pharmacokinetic/bioavailability studies of the drug.

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