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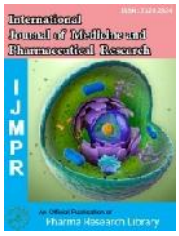

**Research Article** **Open Access**

# Bioanalytical Method Validation for Determination of Aceclofenac in $K_2$EDTA Human Plasma by LC-MS/MS

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**A B S T R A C T**

A simple reverse phase liquid chromatographic and mass spectroscopic analytical method has been developed and validated for estimation of Aceclofenac in plasma. The separation was carried out on Hypersil Gold C18,100 X 2.1 mm, 1.9 µ as Stationary phase, Mobile Phase: Methanol: 10mm ammonium acetate buffer pH 6.8 Elution mode : Isocratic A: B= 80:20% v/v Flow rate: 350 µL/min. Diclofenac was used as internal standard. The Aceclofenac and Diclofenac showed retention factor of 0.8 min ± 0.2 min respectively. The injection volume was 5µL and the total run time was 2 min. The method shows selectivity and linearity. The described LC-MS/MS method was linear over a concentration range of 38.673 to 19800.385ng/mL. The extraction recoveries for Aceclofenac and Diclofenac were found to be between 69.11 and 72.15%. The method shows to be stable for the studied parameters. The stability of the drug spiked human plasma samples during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state. The results of the study showed that the proposed LC-MS/MS method is simple, rapid, precise and accurate, which is useful for the estimation of Aceclofenac in bulk fluids and biological plasma sample analyte with accuracy and reproducibility.

**Keywords:** Aceclofenac, LC/MS/MS method, Diclofenac

**A R T I C L E I N F O**

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## 1. Introduction

Aceclofenac (ACF), {2-[2-(2, 6-dichlorophenyl) amino] phenyl acetoxyacetic acid} is a new phenyl acetic acid derivative with potent analgesic and anti-inflammatory properties and improved gastric tolerance. A white /almost white crystalline powder. Practically insoluble in water, freely soluble in acetone and soluble in Alcohol.

Aceclofenac is an orally administered NSAID which is a phenyl acetic acid derivative. It inhibits the prostaglandin synthesis. It is a potent inhibitor of the enzyme Cox which is involved in the synthesis of prostaglandins. It is used in the management of Osteo arthritis, Rheumatoid arthritis and Ankylosing spondylitis. The usual dose of Aceclofenac is 100mg twice a day. In patient with hepatic impairment an initial daily dose of 100mg can be used [1-6]. Literature survey revealed that Aceclofenac is estimated by High-performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS), Spectrophotometric, Spectrofluorometric, High-performance liquid chromatography with amperometric detection, HPLC and Chemometrically-Assisted Spectrophotometric Estimation, liquid chromatography/UV diode array detection/ atmospheric pressure chemical ionization mass spectrometry. Several methods have been reported for quantification of aceclofenac in plasma as mentioned above. The present investigation reports a simple, rapid, sensitive, and reproducible LC MS method for analysis of aceclofenac in plasma, using diclofenac as internal standard (IS) [7-18].

The Plan of the present study is as follows: Optimization of chromatographic conditions were proposed to be developed and optimized like selection of Ionization, selection of initial separation conditions, nature of the stationary phase, nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flow rate) and selection of internal standard. The developed method were also proposed to be validated using the various validation parameters such as, Accuracy, Precision, Linearity and Range, Limit of detection (LOD) / Limit of quantitation (LOQ), Selectivity / specificity, Stability and System suitability as per FDA guidelines [16, 19]. The Aceclofenac present in the biological fluid was proposed to be estimated.

## 2. Materials and Methods

Samples were separated on a reversed phase Hypersil Gold C18, 100 X 2.1 mm, 1.9 µ in isocratic mode. Mobile phase was 10 mM ammonium acetate/Methanol (20/80, v/v) at a constant flow rate of 350 µL/min. The column temperature was kept constant at 40˚C. The injection volume was 5µL and the total run time was 2 min. Aceclofenac and Diclofenac were ionized via electrospray ionization (ESI) in positive ion mode. The electrospray source parameters were fixed as follows: electrospray capillary voltage 3500 V, source temperature 260˚C. Nitrogen was used in the electrospray ionization source. The sheath and auxilary gas flows were 15 and 15 L respectively. The detection of the ions was performed in the selected reaction monitoring (SRM) mode, monitoring the transition of the precursor ion at m/z 354.2 to the product ion at m/z 250.0 for Aceclofenac, and the transition of the precursor ion at m/z 296.0 to the product ion at m/z 214.2 for Diclofenac.

**Solution Preparation:**

**Mobile Phase A** [**10mm Ammonium acetate buffer pH 6.8]:** Weighed accurately 0.07708 gm of Ammonium Acetate and added to 100 ml HPLC grade water in a 500 ml measuring cylinder and mixed well. Filtered the resulting solution using 0.22µ filter and transferred to 500ml reagent bottle, sonicated and labelled with three days of expiry from date of preparation.

**Mobile Phase B: [Methanol]**

Methanol was used as Mobile phase B. A volume of 500ml of Methanol was transferred to 500ml reagent bottle and labelled with three days of expiry from date of preparation.

**Precipitation Solvent: [Acetonitrile]**

Added 250ml Acetonitrile to reagent bottle, and labelled with three days of expiry from date of preparation.

**Diluent: [Methanol: Water (50:50 v/v)]**

Added 500 mL of methanol and 500 mL of water in a 1000 mL reagent bottle,mixed well and labelled with three days of expiry from date of preparation.

**Preparation of Calibration Standards and Spiked Calibration Standards in Plasma**

9.926 mg of Aceclofenac was weighed and transfered into a pre-labeled clean and dry 5 mL volumetric flask. Dissolved the contents with 0.5 mL of methanol and made up to 5.0 mL with methanol. The CC stock and working solutions were stored at 2°C to 8°C. The final concentration achieved upon purity correction was 1980.038 µg/mL

**Preparation of Quality Control Samples and Spiked Quality Control Samples:**

9.924 mg of Aceclofenac was weighed and transfered into a pre-labeled clean and dry 5 mL volumetric flask. Dissolved the contents with 0.5 mL of methanol and made up to 5.0 mL with methanol. The QC stock and working solutions were stored at 2°C to 8°C. The final concentration achieved upon purity correction was 1979.640 µg/mL.

**Preparation of Internal Standard Stock and Working Solution:** 10.231 mg of Diclofenac was weighed and transferred into a pre-labeled clean and dry 10 mL volumetric flask. Dissolved the contents with 0.5 mL of methanol and made up to 10.0 mL with methanol. The QC stock and working solutions were stored at 2°C to 8°C. The final concentration achieved upon purity & salt correction was 951.743 µg/mL.

**Sample Preparation**

The frozen QC samples and blank plasma were retrieved from deep freezer and thawed at room temperature. Fresh CC standards were prepared using the CC spiking scheme. The STD blank and STD zero were prepared by adding 20µL of diluent and 980 µL of blank plasma. All (CC, QC & STD Blank) samples were vortexed for homogeneity. Into a prelabelled poly propylene vial 200 µL of sample was aliquoted and added with 50.0 µl of ISTD (50 µg/mL) other than STD Blank sample and mixed well. Followed by 100.0 µl of Ammonium Acetate buffer was added and mixed for homogenity. The mixture was precipitated by addition of 600 µL Acetonitrile and vortexed. The samples were centrifuged at 14000 rpm and at 10 degrees for 10 mins and 0,5mL of supernatant was transferred to auto sampler vials, loaded into auto sampler and analysed.

**Data processing and calculations**

Chromatograms acquired using the Thermo LCQuan 3.0 software version supplied by thermo. The calibration curve was constructed by using a suitable linear regression analysis of the peak area ratio (Drug/ISTD) vs. the concentration of drug. The concentration of the Quality

control samples were calculated from following equation using regression analysis of spiked plasma calibration curve standard.

Y = m X + C,
X = Concentration in µg/mL
Y= Peak area ratio of drug to ISTD
m = Slope
C= Intercept.

**Method Validation:**

The method was validated for system suitability, auto sampler carryover test, selectivity, matrix effect, linearity, accuracy, precision, recovery, stability according to the principles of the FDA industry guidance

**System suitability:**

System suitability of the instrument for analysis was performed by injecting six replicates of neat MQC concentration samples of Aceclofenac with internal standard (Diclofenac) in mobile phase.

**Autosampler carryover test**

Autosampler carryover test was performed by injecting the processed blank sample following the highest calibration standard (STD-10). No significant interference at the retention time of analyte or internal standard was observed during the period of validation.

**Selectivity:**

The selectivity of this method was performed by analyzing blank plasma samples obtained from 6 healthy subjects, a lipemic sample and a hemolyzed sample. In order to test the interference at the retention time of Aceclofenac at quantification limit and Diclofenac (IS) at working concentration, the blank plasma samples were spiked with Aceclofenac at LLOQ and added with IS were analyzed according to the methodology

**Matrix Effect**

Matrix Factor was established in six individual plasma lots obtained from individual donors. Each lot was spiked with LQC and HQC samples and analyzed under the calibration curve.

**Linearity:**

The linearity of calibration curve for Aceclofenac was assessed at ten concentration levels in the range of 38.673 to 19800.30 ng/mL in plasma samples. Peak area ratios for each level against its corresponding concentration were measured and the calibration curve was obtained from the least-squares linear regression presented with their correlation coefficient.

**Extraction Recovery:**

The extraction recovery of analyte at three QC samples was determined by comparing the peak area responses from plasma samples spiked with analyte before extraction with those from drug-free plasma samples extracted and spiked with same concentration of analyte after extraction. The recovery of Aceclofenac and Diclofenac were determined using six replicates. The extraction recovery at low, medium and high levels of QC samples was obtained according Equation:

$$R(\%) = (PSbe/PSae) \times 100\%$$

where: R is extraction recovery, PSbe is the mean value of the peak area responses obtained from plasma samples spiked with analyte before extraction and PSae is the mean value of the peak area responses obtained from plasma samples spiked with analyte after extraction.

**Accuracy and Precision**

The intra-day data reflects the precision and accuracy of the method under the same conditions within one day. Intra-day accuracy and precision were obtained by analyzing six replicates of four QC samples (LLOQ, LQC, MQC and HQC). Accuracy was determined by the regressed (measured) concentration represented as a percentage of the target (nominal) concentration. The percent relative standard deviation (% RSD) of the regressed (measured) concentrations was used to report precision. The inter-day precision and accuracy were verified by repeating the above procedure at three different occasions.

**Stability:**

Stability of Aceclofenac in plasma was performed using six replicates of two QC samples at low and high levels. Samples were prepared by spiking drug-free plasma with appropriate volumes of Aceclofenac standard solutions. The stability was evaluated with six studies; stability in bench top stability, freeze-thaw, auto sampler, short-term and long-term stability as well as standard solution stability, according to described in subsequent sections.

## 3. Results and Discussion

**Sample Preparation and LC–MS/MS Analysis:**

The main aim of this work was to develop a rapid, selective and sensitive analytical method including an efficient and reproducible sample clean-up step for quantitative analysis of aceclofenac in human plasma. Based on our previous experience on optimization of analyses in plasma, sodium hydroxide was added to plasma samples in order to increase extraction efficiency, because weak bases as aceclofenac and diclofenacare in an undissociated form at neutral or alkaline pH values, resulting in higher extraction efficiency. Subsequently, a simple and inexpensive extraction procedure that could be implemented in monitoring laboratories provided an assay well suited for real time analyses. In optimizing the chromatographic conditions, the ammonium acetate buffer solution was adopted in the mobile phase of the HPLC in order to suppress the tailing phenomena of chromatographic peaks of aceclofenac and diclofenac. Besides, the concentration of the ammonium acetate buffer was investigated and the concentration of 10 mM ammonium acetate made the chromatographic peaks sharp and symmetric.

The acceptable retention and separation of aceclofenacanddiclofenac was obtained by using an elution system of 10 mM ammonium acetate/methanol (20/80v/v) as the mobile phase. The LC/MS/ MS method described here satisfies the requirement of routine analyses since it has a short run time (2 min), which has advantages over other methods described in the literature.

The MS optimization was performed by direct injection of aceclofenac and diclofenac into the mass spectrometer. The mass parameters were optimized to obtain better ionization of aceclofenac and diclofenac molecules. The full scan spectrum was dominated by protonated molecules [M+H]+

m/z 354.2 and 296.0 for aceclofenac and diclofenac molecules, and the major fragment ions observed in each product spectrum were at m/z 250.0 and 214.2 respectively.

**System suitability:**

System suitability of the instrument for analysis was performed by injecting six replicates of neat MQC concentration samples of Aceclofenac with internal standard (Diclofenac) in mobile phase. The CV% for area ratio of Analyte /Internal standard during system suitability for the method validation period was < 2.0%. The system suitability was performed prior to initiating any experiment on daily basis and found satisfactory

**Autosampler carryover test**

Autosampler carryover test was performed by injecting the processed blank sample following the highest calibration standard (STD-10). No significant interference at the retention time of analyte or internal standard was observed during the period of validation. Thus the method has no carry over related issues and the rinsing solution cleans the injector appropriately.

**Selectivity & Sensitivity**

Selectivity was established by using six plasma lots obtained from individual donors. Each individual plasma lot was analyzed as Blank, Blank+ISTD and LLOQ+ISTD. All lots met the acceptance and no significant interference was observed in the any of the individual lots.

**Matrix Effect**

Matrix Factor was established in six individual plasma lots obtained from individual donors. Each lot was spiked with LQC and HQC samples and analysed under the calibration curve. All lots met the acceptance of ±15% to the nominal concentration. Hence the method does not have any matrix interferences using the method designed.

**Recovery**

The recovery of Aceclofenac from matrix (at low, middle and high QC concentrations) was evaluated by comparison of area with extracted plasma samples to that of the neat samples prepared at the same quality control level concentration.

**Linearity**

The linearity of the method was established by analyzing three calibration curve of the validation runs. The method was linear through the range of 38.673 to 19800.385ng/mL. The $r^2$ value was above 0.98 for the entire calibration curve analyzed in the validation.

**Precision and Accuracy**

The Precision and Accuracy of the QC samples were analyzed from 3 PA runs. The inter and intra run precision (%CV) and Accuracy (% Bias) of the QC's were calculated within the batch and between the batch. All samples met the acceptance of ± 20% (%CV & % Bias) for LLOQ and ± 15% (%CV & % Bias) for LQC, MQC and HQC.

**Stabilities:** Stability of Aceclofenac was established under the below categories, which involved preparation of quality control samples LQC and HQC and analyzed as per the analytical method.

**I) Pre – processing stability**

a) **Bench top stability**

Quality control samples in $K_2$EDTA human plasma (n = 6 at low and high QC concentrations) were thawed on a bench at room temperature for 6 h 22 min prior to sample preparation. Aceclofenac was found to be stable in human plasma for at least 6 h 22 min on a bench at room temperature before analysis. Results of the analysis met acceptance criteria

**b. Freeze thaw stability**

Quality control samples (n = 6 at low and high QC concentrations) in K2EDTA Human plasma were subjected to three freeze-thaw cycles consisting of thawing on a bench at room temperature for at least 60 minutes, vortexing, and then refreezing (-60oC to -80oC) for at least 12 h. After three freeze-thaw cycles the samples were analyzed using freshly spiked calibration standards. Results of the analysis met acceptance criteria

**c. Long term stability**

Quality control samples LQC and HQC were stored frozen for 35 days -70±10°C prior to bioanalysis. Acceptable stability for Aceclofenac was demonstrated in $K_2$EDTA human plasma for 35 days. Results of the analysis met acceptance criteria.

**II) Post - processing stability**

**A. Auto sampler stability**

Quality control samples LQC and HQC were processed and stored in auto sampler for 27 hrs at 10°C and analysed under the CC. Acceptable stability for Aceclofenac was demonstrated in $K_2$EDTA human plasma for 27hrs . Results of the analysis met acceptance criteria.

**B. Stock and working solution stabilities**

**I) Stock solution short term**

The stock solution (0.200 mL) of Analyte and ISTD was kept on bench for 7 hrs 11 mins at room temperature and compared with the same stock stored at 2-8°C. The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%.

**II) Stock solution long-term**

The stock solutions of Analyte and ISTD were stored at 2-8°C for 45 days and compared with fresh stock. The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%. The stability was corrected using the correction factor for the difference between the fresh and the stored stock.

**III) Working solution short term**

The working solution (0.200 mL) of Analyte at MQC and ISTD 50 µg/mL was kept on bench for 6 hrs 30 mins at room temperature and compared with the same working solutions stored at 2-8°C. The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%.

**IV) Working solution long-term**

The working solution of Analyte and ISTD were stored at 2-8°C for 45 days and compared with fresh working solutions. The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%. The stability was corrected using the correction factor for new stock and stability stock used for preparing the working solutions.

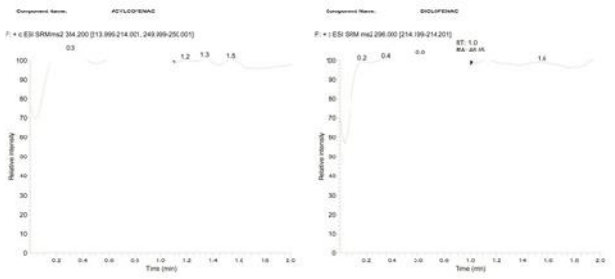

**Figure 1:** chromatogram for blank

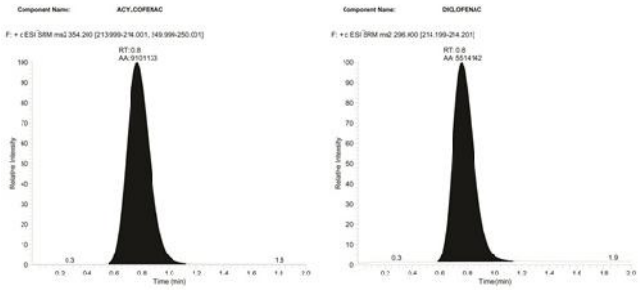

**Figure 2:** chromatogram for HQC of aceclofenac and diclofenac

**Table 1:** Data of validation parameters for Aceclofenac

| Parameters | Aceclofenac |
|---|---|
| System Suitability | |
| Analyte | 10672421 |
| Internal Standard | 5548176 |
| Area ratio | 1.926 |
| Auto Sampler Carryover test | No significant interference at the retention time of analyte or internal standard was observed during the period of validation |
| Selectivity and Specificity | All lots met the acceptance and no significant interference was observed in the any of the individual lots |
| Matrix effect | The method does not have any matrix interferences |
| Recovery studies | |
| LQC | 71.68% |
| MQC | 69.11% |
| HQC | 72.15% |
| ISTD | 69.98% |
| Linearity and Range | 38.673 to 19800.385ng/mL |
| Slope | 0.000104 |
| Standard deviation | 0.00037 |
| Correlation co-efficient | 0.9996 |
| Precision and Accuracy | |
| LLQC | 40.297ng/mL |
| LQC | 128.102 ng/mL |
| MQC | 7979.767 ng/mL |
| HQC | 15430.439 ng/mL |
| Stability | |
| Bench top stability | |
| LQC (% RE) | -3.10% |
| HQC (% RE) | 0.52% |
| Freeze thaw stability | |
| LQC (% RE) | -3.15% |
| HQC (% RE) | 0.23% |
| Long term stability | |
| LQC (% RE) | 0.02% |
| HQC (% RE) | -0.52% |
| Auto sampler stability | |
| LQC (% RE) | -1.55 |
| HQC (% RE) | 1.09 |
| Solution stability | |
| Stock solution | |
| Short term (analyte, ISTD %) | 100.37%, 100.61% 96.83%, 97.08% |
| Long term (analyte, ISTD%) | 104.47%, 105.66% |
| Working solution | 97.72%, 96.00% |
| Short term(analyte, ISTD %) | |
| Term (analyte, ISTD in %) | |

## 4. Conclusion

An alternative LC-MS/MS method for quantification of aceclofenac in human plasma has been successfully developed and validated. A simple and inexpensive precipitation extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real time analyses. The method exhibited excellent performance in terms of system suitability, selectivity, matrix effect, linearity, accuracy, precision, recovery and stability. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for therapeutic drug monitoring of aceclofenac and can be used in pharmacokinetic or bioequivalence studies of this drug.

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