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# Development and Validation of LCMS/MS Methods For Estimation of Bioavailability of Anti-Inflammatory Drugs in Human Plasma

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***Abstract:***

A simple and specific method for determination of bioavailability of Mefenamic acid/ Meloxicam/ Aceclofenac in Human Plasma by liquid chromatography-tandem mass spectrometry operated in positive ionization mode was developed and validated. The LC-MS/MS methods were highly sensitive and suitable for the detection of drug in plasma even in low concentrations. Solid phase extraction methodology was adopted in plasma sample preparation, which provides consistent extraction recovery with minimal endogenous interference and matrix effect. The method involves a rapid solid phase extraction from plasma, simple isocratic chromatographic conditions and mass spectrometric detection in the positive ionization mode using multiple reactions monitoring that enables detection down to low nanogram levels with a short run time. Different parameters such as linearity, range, precision, accuracy, ruggedness and robustness, limit of detection (LOD) and limit of quantification (LOQ) were used for full validation of the method. The results were found to be acceptable as per the guidelines of International Conference on Harmonization (ICH). The method found to be novel, rapid, linear, precise, accurate, robust and rugged and can be successfully applied for the routine analysis of Mefenamic acid / Meloxicam/ Aceclofenac with more sensitivity and covers wider range of quantitation. The method also found to be useful and economical.

***Keyword:*** *Human Plasma, liquid chromatography-tandem, mass spectrometry.*

## INTRODUCTION

In the bioanalytical arena, gas-chromatography (GC) has long been established as the “gold standard” for the separation of small molecules. However, with the recent developments in LC-MS, this approach has become increasingly important for robust and reliable assays in the fields of clinical and forensic toxicology and doping control. LC has become an attractive alternative to GC, with the ability to analyses hydrophilic, thermolabile and non-volatile analytes, which were not sufficiently covered by GC. Additionally, LC-MS offers a fast, sensitive and selective approach where the simple sample preparation is amenable to fast, high-throughput assays and limits potential sources of error. A part from that, small, polar molecules present formidable challenges during LC- MS/MS analysis, such as (a) separation of metabolites from intended analyte, (b) analysis of thermally degradable drugs during ionization, (c) separation of interfering/co- eluting peaks from the analyte and (d) elimination of the matrix effect. The above goals were

resolved in order to avoid biased results for pharmacokinetics analysis. The use of highly specific and sensitive analytical procedures is therefore required to achieve low limits of detection. The aim of this study was the development of sensitive and new analytical procedures for the accurate and specific determination of the selected drugs in human plasma, based on liquid chromatography-tandem mass spectrometry by overcoming above challenges.

## LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a powerful analytical technique for chemical analysis and related applications, with an ability to separate, analyses, and/or purify virtually any sample. Like all chromatographic techniques, it operates by separating the various chemical species in a mixture from each other based on the difference in their distribution coefficients. The first known chromatography is traditionally attributed to Russian botanist Mikhail Tswett who in the early 1900's used columns of calcium carbonate to separate plant compounds during his research of chlorophyll. Further, development of chromatography occurred in 1950's with the contribution of Nobel laureates Archer John Porter Martin and Richard Laurence Millington Synge who established the basics of partition chromatography and developed Plate theory.

The simplest forms of LC are based upon the separation of a sample components eitheron paper with a solvent, known as paper chromatography (PC) or on a thin layer of stationary phase coated onto a slide which is placed in solvent, thin-layer chromatography (TLC). Column chromatography-based LC, a much more powerful technique than the planar paper chromatography and TLC methods and with a greater sample capacity. Components within a mixture are separated in a column based on each component's affinity for the mobile phase. So, if the components are of different polarities and a mobile phase of a distinct polarity is passed through the column, one component will migrate through the column faster than the other. The technique known as HPLC was first coined by C. Horváth in 1970, who by combining high pressure pumps (~500 psi) and strengthened packed columns, created the high pressures and strengths of column necessary for the technique then known as high pressure LC. Tremendous leaps in technology were seen over the following decade with new instruments being produced that could develop ~6000 psi of pressure and with directly incorporated injector systems, detectors and columns. For column chromatography, flow of the mobile phase solvent is achieved in several ways. Gravity or a vacuum may be used where columns are not designed to withstand high pressures, as with the open glass columns employed by Tswett. Such columns employ large diameter solid phase particles (>50 µm), so that there is less resistance to flow and less chance of building up pressure [1].By employing smaller solid-phase particle sizes (<10 µm), improved chromatographic separation can be achieved due to the overall greater solid-phase surface area. However, due to smaller particles having a greater flow resistance, there is a need for greater pressures to drive the liquid mobile phase solvent through the column. Thus high pressure pumps and columns packed within metal casing are employed to create and withstand the high pressures necessary for the technique known as high performance liquid chromatography (HPLC).

By combining improved instruments with improved column chemistries (smaller particles and higher pressures) improved separations were achieved; therefore, the technique was re-coined as high-performance liquid chromatography, thus preserving the originalHPLC acronym. HPLC is now one of the most widely used tools in analytical chemistry, having been applied to the analysis of a huge range of samples in industry [2]. Almost any compound that dissolves in liquid can be analyzed, even in minute concentrations as low as parts per trillion. Traditional detectors for liquid chromatography include refractive index,

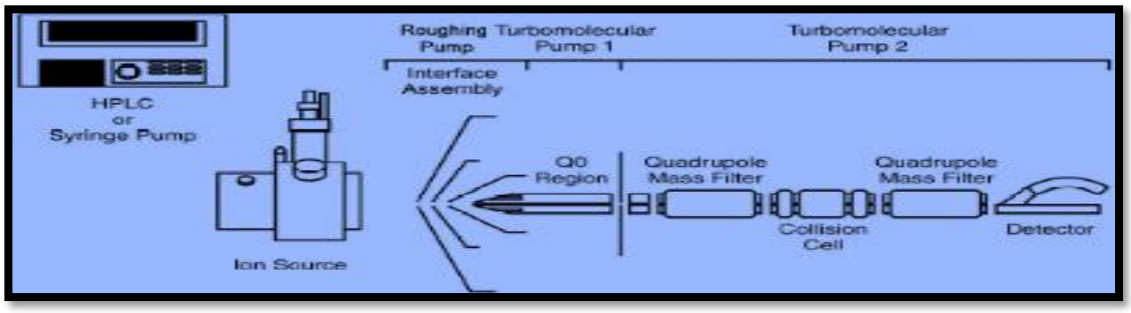
electrochemical, fluorescence and ultraviolet-visible (UV-Vis) detectors. Some of these generate two-dimensional data; that is, data representing signal strength as a function of time. Others, including fluorescence and photo diode array (PDA) UV-Vis detectors generate three dimensional data [3]. These latter methods can be extremely useful for detecting certain classes of compound that either absorbs UV or fluoresce (e.g. kaempferols, quercetins and spermidines); indeed PDA detectors have frequently been linked online to HPLC or UHPLC prior to MS in bioanalytical studies to provide a further dimension to aid in the characterization of the detected analytes [4-7]. However, to acquire the volume of information from an analyte required for full structural elucidation, it is a necessity to employ MS and or NMR spectroscopy detectors.

**MASS SPECTROMETRY**

In 1897, J. J. Thomson first used an apparatus to measure *e*/*m* (a symbol to report earliera charge-to-mass ratio rather than the present mass spectrometry (MS) standard of mass-to-charge; *m*/*z*). In 1906, he was awarded the Nobel Prize in Physics for simultaneously measuring e/*m* and *e* (mass of the electron) by building an instrument with the help of collaborators. Later, this instrument was recognized as a mass spectrometer to measure the masses of charged atoms. By 1940, MS equipment was commercially available and became a useful tool to the physicists and industrial chemists. In the period of 1918 to mid 90's different ionization techniques were developed to analyze the small molecules as well as proteins. McLafferty, Biemann and Djerassi significantly contributed to understand the fragmentation pattern of known molecular structures and also to the identification of the structures of unknown compounds using MS instruments [8] .A mass spectrometer is an instrument in which positive or negative gas-phase ions formed from sample molecules are analyzed according to their mass-to-charge ratio (*m/z*).Ionization is a key step since ions are far more easily manipulated than neutral molecules. Unfortunately differing analytes vary in their ionization efficiencies, *i.e*. the proportion of metabolite in solution converted to ions in the gas phase. Once ionized, the mass analyzer detects the ion abundance and *m/z*, which can be related back to the analytes absolute molecular weight. It should be able to form, separate and detect ions based on their *m/z*. Generally, it is a set of functional components including

(1) sample inlet
(2) ionization source
(3) mass analyzer
(4) detector and
(5) data recorder.

Samples can be introduced into the mass spectrometer through a controlled vacuum leak followed by ionization in the vacuum chamber or through direct infusion or liquid chromatographic introduction of an analyte solution and creation of ions at atmospheric pressure (atmospheric pressure ionization; API). Ion signals can be obtained under the scan of a magnetic or electric field at a value appropriate to the *m/z* ratio and with intensity proportional to the number of ions [9-10]. A data system (run on a personal computer) is responsible for the storage of the paired *m/z* and abundance values as well as their processing and display in a mass spectral format.




**Schematic diagram of Mass Spectrometer Outline of API mass spectrometryinstrumentation**

An API interface / source consists of five parts: sample introduction, ionization either by electro spray (ESI) or atmospheric-pressure chemical ionization (APCI), ion sampling aperture, atmospheric-pressure to vacuum interface and ion optical system to transport ions into the mass analyzer. The design of the last two parts is very important as it determines whether the high ionization efficiency that can be achieved in API solves the analytical problems or not [11] . The API mass spectrometry works as follows. The analyte in solution is introduced intoan atmospheric-pressure ion source region by nebulization. Further ions are generated either by ESI or APCI. The ions produced by the ion source are mainly formed by ionizing a neutral molecule through protonation, deprotonation or adduct formation. These ions are sampled by an ion sampling device into a first pumping stage and further sampled by a skimmer into a second pumping stage. During this process, ion beams havea tendency to diverge. Therefore, proper ion optics are necessary to minimize ion beam divergence. For this efficient transfer, several ion guiding systems were developed. The ion focusing and transfer device in the second pumping stage helps to transport the ions into the mass analyzer region. The mass analyzer is used to separate ions according totheir mass-to charge ratios based on their characteristic behavior in electric and/or magnetic fields [12].

## MATERIALS AND METHODS:

### Materials used:

| S.NO | STANDARDS USED | Manufacturer |
|---|---|---|
| **1** | Mefenamic acid | Smilax laborateries ltd, Hyderabad |
| **2** | Meloxicam | Smilax laborateries ltd, Hyderabad |
| **3** | Aceclofenac | Smilax laborateries ltd, Hyderabad |

**Chemicals and reagents used**

| S.NO | | Chemicals | Manufacturer | Grade |
|---|---|---|---|---|
| 1 | | Acetonitrile | Merck | HPLC |
| 2 | | Methanol | Lobachemie | HPLC |
| 3 | | Sodium Bicarbonate | Qualigens | Excelar |
| 4 | | Ethyl acetate | Qualigens | HPLC |
| 5 | | Diethyl amine | Qualigens | HPLC |
| 6 | | Ammonia | Qualigens | SQ |

Water of HPLC grade from Milli-Q RO system was used.

™ Blank plasma (K2 EDTA) procured from AZIDUS Laboratories

**Analytical Method for Estimation of Mefenamic acid in plasma [1]:**

**LC-MS/MS method:**

A summary of the chromatographic and mass spectrometric conditions is as follows:UPLC : WATERS

Mass spectrometer : API 2000
Ion source : Heated nebulizer
Polarity : Negative
Detection ions
Mefenamic acid : 238.4 * amu (parent), 198.0* amu (product)
Mefenamic acid –d4 : 242.2* amu (parent), 200.6 amu (product)
Column : Phenomenax, synergi 4 µ Polar- RP80 A, 4.6x75mm
Column oven temperature : 35° C
Peltier temperature : 10°C
Mobile phase : Ammonium formate: Acetonitrile (30:70)
Flow rate : 1ml/min.
Volume of injection : 10 µ L
Retention time : Mefenamic acid - 0.5 to 1.20 minutes
: ISTD-0.5 to 1.20 minutes
Run time : 2.00 minutes

1. **Extraction procedure**

**Step 1:** Blank, calibration curve standards and the subject samples were withdrawn from the deep freezer and allowed them to thaw. The thawed samples were vortexed to ensure complete mixing of contents. To 0.25 ml of plasma sample in a ria vial, 25ul of Mefenamic acid –d4 standard (75µg/ml) was added. To plasma blank, 25 ul of 60% Acetonitrile in water solution wasadded and vortexed the samples to ensure complete mixing of contents.

**Step 2:**

Add 2.5 ml of tertitary butyl methyl ether, place on a shaker for 15 minutes and centrifuge for 10 minutes at 4000rpm at 20$^{O}$C. Transfer supernatant (organic layer ) into the another rial vial. Evaporate this layer under a stream of nitrogen at 45 $^{O}$C. The residue was reconstituted with 0.5 ml of mobile phase and vortexed. The samples were transferred in to auto-injector vials and loaded the vials in to auto sampler. 10 ul of sample was injected in to LC-MS/MS system. Analyte Concentrations of stock dilutions of standard Mefenamic acid solution with plasma were shown.

## Estimation of Meloxicam in plasma:

**LC-MS/MS method[2]:**

**Chromatographic conditions**

A summary of the chromatographic and mass spectrometric conditions is as follows:UPLC : WATERS

Mass : API 2000
Ion source : Heated Nebulizer
Polarity : Positive ion mode.
Detection ions:
Meloxicam : 352.1*amu (parent), 115.1* amu (product)
Piroxicam : 332.2*amu (parent), 121. 0 *amu (product)

Column : Agilent,Zorbax Eclipse –XDB, 4.6xl50mm, 5µ
Column oven temperature: 35.0 °C
Peltier temperature : 20 °C
Mobile phase : 5mM Ammonium formate: Acetonitrile (30:70)Flow
rate : 1.000ml/min.
Volume of injection : 15 µl
Retention times : Meloxicam 0.80 to 1.50 minutes
Run time : 2.00 minutes

**Estimation of Aceclofenac in plasma [3]:**

**LC-MS/MS method:**

A summary of the chromatographic and mass spectrometric conditions is as follows:HPLC
: Agilent 1200
Mass spectrometer : API 4000 (MDS SCIEX LC-MS/MS)Ion
source : Turbo Ion Spray
Polarity : Negative ion mode
Column : Zorbax XDB C18, 2.1 x 50 mm, 5 µm
Column oven temperature : 30° C
Autosampler temperature : 5°C
Mobile phase : 0.1% Formic Acid (100 % v/v)

Flow rate : 0.6 mL/min

| | |
|---|---|
| Retention time | : Aceclofenac – 2.18 min |
| Set point (Horizontal) | : Celecoxib (ISTD) – 2.19 min<br>: 5.0 |
| Set point (Vertical) | : 5.0 |
| Injection Volume | : 5 µL |
| Run time | : 3.50 minutes |
| **MRM transitions:**<br>Aceclofenac | : 353.0 amu (parent), 75.0 amu (product) |
| Celecoxib (ISTD)<br>**MRM Conditions** | : 380.2 amu (parent), 316.2 amu (product) |
| Curtain Gas (CUR) | : 20.0 PSI |
| Collision Gas (CAD) | : 8.0 PSI |
| Temperature (TEM) | : 550.0 °C |
| Ion Spray Voltage (IS) | : -4500V |
| Ion Source Gas (GS1) | : 30.0 PSI |
| Ion Source Gas (GS1) | : 30.0 PSI |
| Polarity | : Negative |

| | |
|---|---|
| Resolution (Q1) | : Unit |
| (Q3) | : Unit |

**MRM parameters**

| | | |
|---|---|---|
| Aceclofenac | Declustering Potential (DP) | -40.0 |
| | Entrance Potential (EP) | -10.0 |
| | Collision Energy (CE) | -26.0 |
| | Collision Cell Exit Potential (CXP) | -6.0 |
| Celecoxib (ISTD) | Declustering Potential (DP) | -55.0 |
| | Entrance Potential (EP) | -10.0 |
| | Collision Energy (CE) | -30.0 |
| | Collision Cell Exit Potential (CXP) | -8.0 |

**Extraction procedure**

**Sample preparation procedure – Acetonitrile protein precipitation**

- Withdraw plasma sample and thaw at room temperature. Vortex for proper mixing. Pipette 0.045 mL of plasma sample into micro tube; add 5 µL of internal standard (500 ng/mL) andvortex for 30 sec. Then add 5 µL of respective working standard and vortex for proper mixing.
- To the above micro tube add 150 µL of Acetonitrile drop by drop while vortexing for 1.0 min. Centrifuge at 10000 rpm for about 10.0 min at 10°C.
- Transfer the clear solution into the labeled vials and inject 5 µL into chromatographic system.

**Pharmacokinetic evalution of Mefenamic acid or Meloxicam or Aceclofenac fast dissolving tablets 5.:**

**Dosage forms:** Pure drug and fast dissolving tablets prepared in the laboratory conditions and chosen on the basis of drug content, in-vitro release studies and stability conditions were chosen as dosage forms for administration.

**Subject selection:** Thirty Six New Zealand healthy rabbits with a mean age of 10±2 weeks and with a mean body weight of 3±0.2 kg were used in this study. Each group consisted of six rabbits (n=6) each and were subjected for overnight fasting, it was taken care that there was no stress on the animals. Rabbits were randomly divided into two groups for different sampling timeand each group was housed in one cage. Food and water were available ad libitum at all times during the experiment.

**Grouping of animals**

Group I - Rabbits treated with pure Mefenamic acid

Group II - Rabbits treated with Mefenamic acid fast dissolving tablets

Group III - Rabbits treated with pure Meloxicam

Group IV - Rabbits treated with Meloxicam fast dissolving tablets

Group V - Rabbits treated with pure Aceclofenac

Group VI - Rabbits treated with Aceclofenac fast dissolving tablets

The study was conducted in a crossover design with 2 weeks washout periods in between the two experiments. The animal dose of Mefenamic acid pure drug and its fast-dissolving tablets was calculated relevant to human dose by using the following formula [6].

**Blood sampling:** About 1 ml of blood samples were collected from the tracheal lobular vein of the rabbit using and the blood was stored in screw top heparinized plastic tubes, the sampling time for blood was done at 0 mins(predose), 1 hr, 2 hr, 4 hr , 6 hr, 8hr, 10 hr, 12 hr , 14 hr, 16 hr,18 hr, 20 hr, 24hr and 48 hr. The plasma was immediately separated by aspiration aftercentrifugation at 4000 rpm for 5 minutes and frozen at -20 ºC until analysed by LC-MS/MS method.

**Determination of Pharmacokinetic Parameters [7]:**

Various pharmacokinetic parameters such as peak plasma concentration ($C_{max}$), time at which peak occurred ($T_{max}$), area under the curve (AUC), elimination rate constant ($K_{el}$), biological half-life ($t_{½}$) and mean residence time (MRT) were calculated using the non-compartmental pharmacokinetics data analysis software PK Solutions 2.0™ (Summit Research Services, Montrose, CO, USA).

## RESULTS AND DISCUSSION:

Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play a significant role in evaluation and interpretation of pharmacokinetic data.For the successful conduct of pharmacokinetic study, the development of selective and sensitive bioanalytical methods playsan important role for the quantitative evaluation of drugs and their metabolites (analytes).To develop a method with a desired LLOQ and ULOQ and accuracy the LC–MS detection is best available option as compared with the another analytical techniques .The LC-MS/MS methods were highly sensitive and suitable for the detection of drug in plasma even in low concentrations .

The concentrations of the unknown samples were calculated from the equation using regression analysis of spiked plasma calibration standard with $1/x^2$ as weighting factor. $y = mx + c$

Where, y = Ratio of Mefenamic acid peak area and ISTD peak area (analyte area / ISTD area); x = Concentration of Mefenamic acid ;

m = Slope of the calibration curve; c = y-axis intercept value.

Linear regression analysis equation of stock dilutions of standard Mefenamic acid solution withplasma is, $y = 0.000129x + 0.000368$

Linear regression analysis equation of stock dilutions of standard Meloxicam solution withplasma is $y = 0.00232x – 0.00134$.

Linear regression analysis equation of stock dilutions of standard Aceclofenac solution withplasma is, $y = 0.0010x – 0.0003$

**Comparative *in vivo* evaluation of Mefenamic acid pure drug and its fast-dissolving tablets formulations in rabbits**

Pharmacokinetic parameters such as absorption rate constant, elimination rate constant, half-life, AUC, and MRT were calculated from the plot of time versus plasma concentration and subjected to statistical analysis. Plasma concentration of Mefenamic acid following oral administration of oral suspension and fast dissolving tablet formulations in rabbits at different times were calculated. The results indicated that the parameters significantly differed following optimized fast dissolving tablets administration, compared to pure drug administration. The highest mean $C_{max}$ value was observed for

optimized orodispersable tablets (68.33 ± 0.42 ng/ml) compared to pure drug (27.72 ± 0.31ng/ml). The mean time taken to peak plasma concentration for *(Tmax)* following administration of pure drug was 11.53 ± 0.011 hours, while it was 6.09 ± 0.072 hour following administration of selected optimized orodispersable tablets. The elimination rate constant (Kel) for pure drug and optimized. The AUC0-α values observed with optimized fast dissolving tablets 686.1.±2.07 ng hr/ml in compared to pure drug values 191 ± 1.43 ng hr/ml. Thus, the results of pharmacokinetic studies indicated rapid and higher oral absorption of Mefenamic acid when administered as its fast dissolving tablets. Both Ka and AUC were markedly increased by fast dissolving tablets.

**Comparative *in vivo* evaluation of Meloxicam pure drug and its fast dissolving tablets in rabbits**

Plasma concentration of Meloxicam following oral administration of oral suspension and optimized microspheres formulations in rabbits at different times were calculated. The results indicated that the parameters significantly differed following optimized Fast dissolving tablets administration, compared to pure drug administration. The highest mean Cmax value was observed for optimized Fast dissolving tablets (158.46 □ 0.15ng/ml) compared to pure drug (67.56 □ 0.43 ng/ml). The mean time taken to peak plasma concentration for *(Tmax)* following administration of pure drug was 11.42 □ 0.32 hours, while it was 6.15 □ 0.14 hour following administration of selected optimized Fast dissolving tablets . Thus, the results of pharmacokinetic studies indicated rapid and higher oral absorption of Meloxicam when administered as its Fast dissolving tablets. Both Ka and AUC were markedly increased by Fast dissolving tablets.

**Comparative *in vivo* evaluation of Aceclofenac pure drug and its fast dissolving tablets formulations in rabbits**

Plasma concentration of Aceclofenac at different times were calculated.The results indicated that the parameters significantly differed following optimized fast dissolving tablets administration, compared to pure drug administration. The highest mean Cmax value was observed for optimized fast dissolving tablets (59.20±1.56 ng/ml) compared to pure drug (31..93 ± 1.24ng/ml). The mean elimination halflife (t1/2) following administration of pure drug was 6.34 ± 0.011hours, while it was 2.23 ± 0.072hour following administration of selected optimized fast dissolving tablets. The elimination rate constant (Kel). The AUC0-α values observed with optimized fast dissolving tablets 1386.1.±2.07ng hr/ml in compared to pure drug values 392.7 ± 1.43ng hr/ml.Thus, the results of pharmacokinetic studies indicated rapid and higher oral absorption of **Aceclofenac** when administered as its fast dissolving tablets . Both Ka and AUC were markedly increased by fast dissolving tablets.

## SUMMARY AND CONCLUSION

The bioanalytical methodology described in this manuscript was specific, sensitive, accurate and precise. The method involved a simple sample preparation by liquid- liquid extraction followed by isocratic chromatographic separations. The method has been found to be reproducible by performing three Precision and Accuracy (P&A) batches consisting of one intra day batch and two inter day batches.A sensitive method that is precise and accurate . The LC-MS/MS method was capable of estimating low ng/ml of drug accurately in plasma withhigh degree of reproducibility. The developed method is simple, rapid and specific LC–MS/MS method for the determination of Mefenamic acid or Meloxicam or Aceclofenac in plasma. Use of stable labeled isotopes as internal standards helped us to obtain the consistent and reproducible results. Also, the method showed no matrix effect and limited variability in recovery between

analyte and IS. The method utilized only 100 µL of plasma for sample processing. A simple SPEtechnique with direct injection (avoids drying, evaporation and reconstitution steps) for sample preparation, thereby significantly reduces the sample processing time. The total run time per analysis of each sample is 3.0 min which allows analysis of more samples in a single day. The method showed suitability for pharmacokinetic studies in humans. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring withthe desired precision and accuracy.

## CONCLUSIONS

Based on the results obtained in this study, it is concluded that the present validated method can be successfully applied for the estimation of Mefenamic acid or Meloxicam or Aceclofenac in plasma.. The method for determination of Mefenamic acid or Meloxicam or Aceclofenac in plasma. using LCMS/MS with mass detection met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, recovery and dilution integrity. The procedure followed in this method has the least concentration i.e. LLOQ is very low than previously reported methods. The plasma sample required is very low for the analysis. The method was found to be sensitive, simple and inexpensive than earlier methods. The method validated is suitable for quantitative determination of Mefenamic acid or Meloxicam or Aceclofenac in plasma. The proposed method is less hazardous to human health and to the environment as well as being faster and more economic. This method will permit pharmacokinetic and pharmacodynamic studies of the drug in humans.

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