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DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE DETERMINATION OF ASPIRIN IN PLASMA

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ABSTRACT

The current study focuses on developing a specific, sensitive, precise, and accurate liquid chromatography-mass spectrometry (LC-MS) method for measuring the concentration of aspirin in plasma. This study successfully separated aspirin using a Symmetry C18 (250mm x 4.6mm; 5m) column, 0.2% v/v acetic acid in acetonitrile (5:95% v/v) as mobile phase, and a flow rate of 0.800 mL/min. The results show that the aspirin retention time was 4.2 minutes, with linearity ranging from 1 to 5000 ng/mL. The detection concentration was less than 2 ng/mL, with relative errors and standard deviations below ±15%. Thus, the proposed method exhibits exceptional sensitivity, accuracy, and specificity, making it suitable for pharmacokinetic studies.

INTRODUCTION:

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) that is frequently prescribed because of its anti-inflammatory [1], analgesic [2] and antipyretic [1] qualities. In addition to inhibiting cyclooxygenase, aspirin functions as an antiplatelet agent and can inhibit smooth muscle proliferation at high doses while inducing angiogenesis and endothelial proliferation.[3] Moreover, aspirin's potential for dentin and periodontal ligament regeneration has been highlighted by recent studies.[4,5] Precise measurement of aspirin concentrations in biological samples is necessary to track drug levels, comprehend pharmacokinetics, and guarantee patient safety. A very precise and sensitive bioanalytical method is LC-MS/MS. It makes it possible to quantify medications and their metabolites in a variety of biological samples, including tissues or bodily fluids from important nonclinical PK studies, as well as blood, plasma, and serum.[6] Moreover, LC-MS/MS is cost-efficient and only needs a small sample quantity, which enables the quantification of medications even at extremely low concentrations (pg to ng). Proving that the method is repeatable and suitable for its intended use in assessing drug levels in

biological matrices is a step in the method development and validation process.[7,8] Once confirmed, this technique will be a useful tool for maximizing drug concentrations in biological samples. The main goal of this work is to use LC-MS/MS to develop and validate a new, easy-to-use, sensitive, and precise method for measuring aspirin in rabbit plasma.

MATERIALS AND METHODS

Chemicals and reagents: LCMS grades of acetic acid and acetonitrile were procured from Merck Mumbai. HPLC-grade water was purchased from SD Fine Chemicals, Chennai, India. Aspirin was gift sample from was gift sample from Alta Labs, Mumbai and Furoseimide (internal standard) was procured from Sigma Aldrich, Mumbai. The animal studies on rabbits were carried out at Krupanidhi College of Pharmacy, Bengaluru, approved by the Institutional Ethical Committee with reference no- KCP/IAEC-570/2021.

Instrument: LC-MS/MS instrument consist of Prominence LC-20AT, Shimadzhu, Japan combined with an MS/MS API 4000 Triple Quadruple, SCIEX, USA furnished with electro Spray Ionization as the source of ion.[7]

Optimized LC-MS parameters: The study utilized Prominence LC-20AT (Shimadzu Corporation, Kyoto, Japan) with the mass spectrometer API 4000, Triple Quadruple (SCIEX, USA), and Analyst 1.5 software (Applied Biosystems Sciex, Toronto, Canada). Liquid chromatography consists of a binary pump, a vacuum degasser, and an autosampler. A mobile phase consisting of acetonitrile and 0.2% acetic acid (5:95% v/v) was employed to differentiate between eluted aspirin and furosemide using a symmetric c18 HPLC column. The flow rate was 0.8 mL/min, and samples were stored at 10°C and 40°C in the autosampler and column, respectively. We used the electrospray ionization positive mode ionization technique for spectroscopic analysis. Table 1 shows the optimized mass spectra parameters.

Standard solutions:

The aspirin was dissolved in a solution of 0.2% acetic acid in acetonitrile to prepare a stock solution of 1 mg/mL. In order to attain the desired concentrations, this solution was subsequently diluted. Each reaction was initiated by combining 100 µL of blank rabbit plasma with 1.2% formic acid in pre-labeled tubes. The final concentration was achieved by enriching each tube with 150 µg of potassium fluoride. Each tube was supplemented with aspirin standard solutions of varying concentrations, ranging from 1 to 5000 ng/mL. Each tube containing the calibrant concentrations was filled with 1 ml of TBME solvent containing 1 µg/ml of furosemide as an internal standard after being thoroughly mixed. The samples were then centrifuged at a speed of 10,000 revolutions per minute at 4 °C for 10 minutes after the contents of all tubes were vigorously mixed for 10 minutes at room temperature. Consequently, an equivalent volume of liquid from each tube was transferred to new tubes and subjected to nitrogen gas evaporation. Ultimately, 100 µl of acetonitrile (ACN) was added to each tube to restore the precipitate.

Method validation: The LC-MS/MS method has been validated in accordance with the strict Bioanalytical Method Validation Guidelines established by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA)[9,10].

Table 1: Optimized Mass Spectra Parameters

Parameters	Optimized value
Collision Gas	Nitrogen
Curtain Gas	25 psi
Declustering Potential	-41 eV
Ionspray Voltage	-4200 KV
Entrance Potential	-10.00 eV
Temperature (Source)	500°C
Collision energy	-8.00 eV
Ionsource gas 1	40 psi
Ionsource gas 2	35 psi
Collision Cell Exit Potential	-12.00eV

Table 2: System suitability results

Injection no.	Peak area
1	5359982
2	5256987
3	5158991
4	5139782
5	5367782
6	5245678
Average	5254867
%RSD	1.39

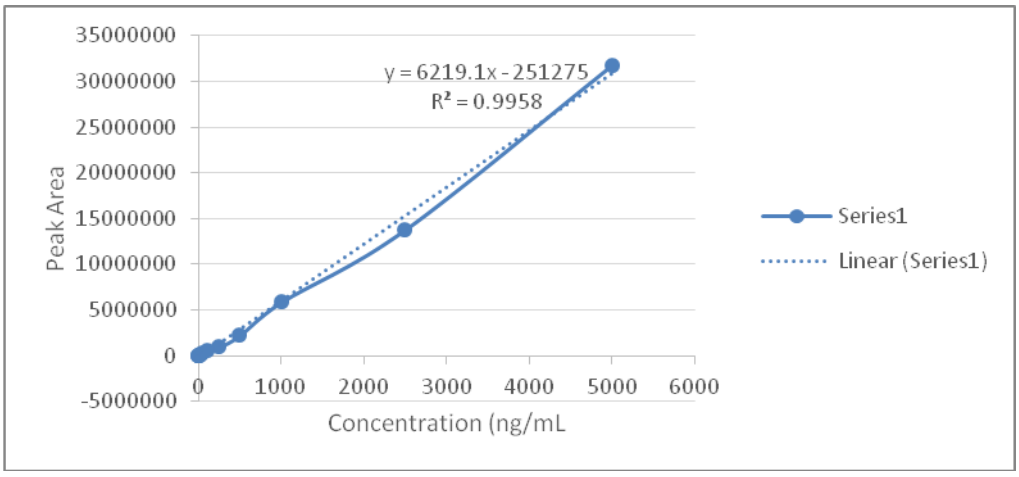

*Figure 1: Calibration curve of Aspirin in Plasma, *Mean±SD, n=6*

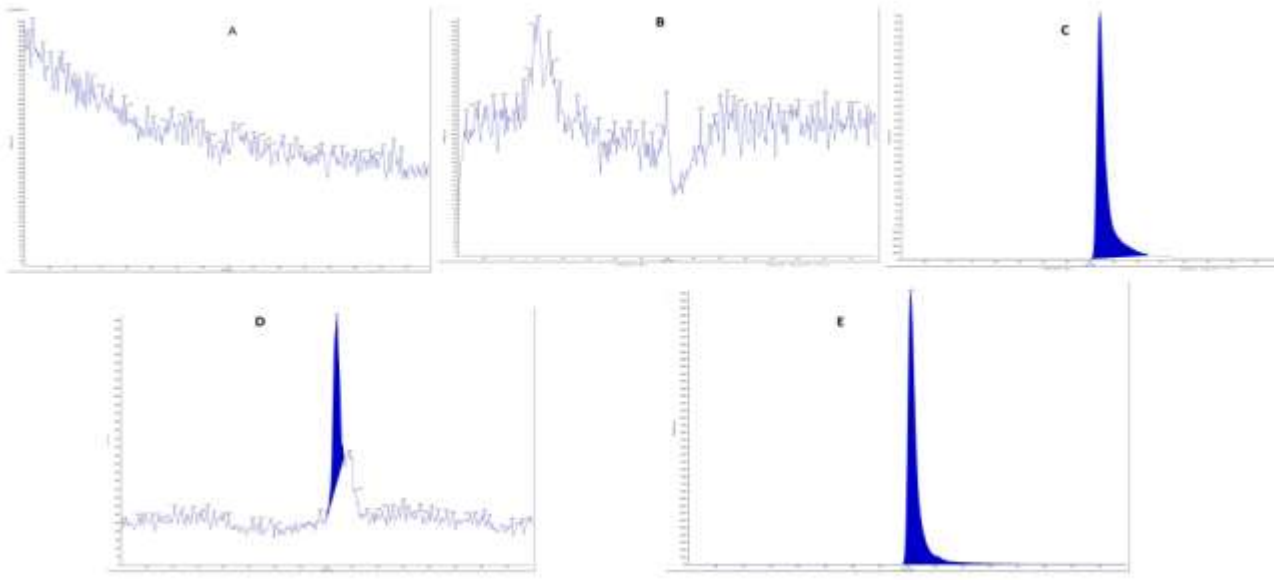

Fig 2: Chromatogram A) Mobile Phase B) Plasma C) LLOQ D) Aspirin after 8h of oral administration E) IS

Table 3: Precision and Accuaracy

Concentration (ng/mL)	Interday			Intraday		
	Observed Concentration (ng/mL)	Precision RSD (%)	Accuracy RE (%)	Observed Concentration (ng/mL)	Precision RSD (%)	Accuracy RE (%)
10	9.83±0.23	2.33	1.7	9.86±0.49	4.96	1.4
100	102.67±1.2	1.168	2.6	102.89±1.08	1.04	2.89
1000	978.67±10.3	1.05	2.13	979.45±21.56	2.2	2.05

Mean±SD, n=6

System suitability: The LCMS system's suitability with the approach was assessed by injecting a blank solution and a 100% standard aspirin solution into it six times. The system's suitability was assessed by calculating the percentage relative standard deviation (% RSD).[9, 10]

Sensitivity and linearity: To verify linearity, calibration standards were developed and analyzed over three days. The peak area ratio (analyte/IS) has been plotted against the spiking aspirin concentrations to construct calibration curves.[9,10]

Specificity: Six blank plasma samples from rabbits were prepared and analyzed to assess potential interference from endogenous compounds. The blank plasma samples were then compared to the LLOQ, plasma after oral administration (8h) and IS-spiked blank plasma. [9, 10]

Accuracy and precision: Accuracy was assessed by determining six replicates of the low, medium, and high QC samples on three consecutive days.[9,10]

Limit of Detection and Limit of Quantification: Limit of Detection (LOD) and Limit of Quantification (LOQ) where determined using the formula

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} (2)$$

Where σ = the standard deviation of the response S = the slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte.

RESULTS AND DISCUSSION: A highly effective method was developed as a result of the optimization of the LC-MS method through a process of trial and error. This method employed a Symmetry C18 column (250 mm x 4.6 mm; 5 µm) and a mobile phase that contained 0.2% acetic acid in acetonitrile. The flow rate was 0.8 mL/min, and the injection volume was 10 µl. The autosampler and column temperatures were maintained at 5°C and ambient, respectively. In the optimized chromatogram, aspirin was eluted at 4.2 min, with the molecular ion and base peak detected at 178.82 in the mass spectrum. In the meantime, furosemide was detected at 4.05 min, and the molecular ion and base peak were eluted at 392.1 in the mass spectrum.

System compatibility: The level of system suitability is an essential component of bioanalysis. The system's effectiveness is facilitated by its suitability, which results in the production of consistent and precise data. This procedure includes evaluations of instrument response, signal stability, and carryover. The acceptance criteria were met by the results (Table 2).

Calibration Curve: The calibration curves for aspirin showed a linear relationship over a concentration range of 1 to 5000 ng/mL is represented in figure 1. The regression equation was y = 6219.1x - 251275. Here, y represents the peak area of aspirin, and x represents the aspirin concentration in ng/mL of plasma samples. The correlation coefficient (R2) for all calibration curves was 0.9958.

Specificity: Figure 2 shows the typical chromatograms of the blank plasma, the blank plasma spiked with aspirin and IS, and the plasma after oral administration(8h). A comparison of the blanks and spiked blanks demonstrated the method's high specificity. The aspirin fragmentation pattern did not affect the mobile phase, the pure matrix, or the endogenous component in any way, as their peaks did not change. Thus, the method is highly precise [9].

Accuracy and Precision: The precision and accuracy were assessed at concentrations of 10, 100, and 1000 ng/mL for the low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC), respectively. Table 3 displays the results, the intraday % RE and %RSD ranged from 1.7% to 2.6% and 1.05% to 2.33% respectively. Whereas, % RE and %RSD for interday was 1.4% to 2.05% and 1.04% to 4.96% respectively. The RSD and RE percentages were within ±15%. The obtained results confirm the developed method's accuracy, repeatability, and specificity.

Limit of Detection and Limit of Quantification

The LOD and LOQ of aspirin were found to be 1.06 ng/mL and 3.21 ng/mL, respectively, using the calibration curve method. These results indicate that aspirin can be detected at concentrations below 2 ng/mL.

CONCLUSION

An LC-MS/MS method has been developed to accurately measure the concentration of aspirin in plasma using electrospray ionisation in positive mode. The

results demonstrate that the method exhibits accuracy, precision, and specificity. The method demonstrated high sensitivity, with a detection limit of 1.06 ng/mL. Therefore, the validated method has great potential for conducting pharmacokinetic studies.

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Conflict of interest: The authors declare no conflicts of interest.

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