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BIOANALYTICAL METHOD DEVELOPMENT FOR NAPROXEN USING LC-MS/MS: VALIDATION AND APPLICATION

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Abstract

To measure naproxen in human plasma, a sensitive LC-MS/MS method must be developed and validated in order to acquire the appropriate pharmacokinetic and bioequivalence study. This paper describes the process of creating an LC-MS/MS method that analyzes naproxen using Zidovudine as an internal standard. In various chromatographic settings, the method was reproducible, sensitive, and selective. According to FDA guidelines, the validation was carried out using tests for linearity, accuracy, precision, recovery, and stability. Excellent chromatographic isolation, crisp peak resolution, and minimal plasma interference were demonstrated by the method. A linear response with a correlation coefficient (r2) of 0.9962 was discovered between 500.1 ng/mL and 100028.5 ng/mL. There was little variation in the recovery rate with Naproxen and internal standards, which was shown to be constant (>80%). Tests of the method's stability under various circumstances, including room temperature, freeze-thaw cycles, and prolonged storage, were conducted. This work presents a bioanalytical methodology that is suitable for bioanalytical research and may be used to measure the amounts of naproxen in blood plasma in both preclinical and clinical studies.

Keywords: LC-MS/MS, Naproxen, Bioanalytical Method, Validation, Sensitivity, Stability, Internal Standard

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1.INTRODUCTION

The process of creating and approving reliable bioanalytical instruments is essential to pharmaceutical research in order to precisely measure the medications and their metabolites in a biological setting. These methods support research in pharmacokinetics, bioavailability, and bioequivalence, which forms the basis of clinical trials and regulatory filing. High sensitivity, selectivity, and reproducibility have made liquid chromatography-tandem mass spectrometry (LC-MS/MS) the gold standard among analysis techniques. In order to evaluate Naproxen's clinical and pharmacological effects, the current work aims to establish a valid LC-MS/MS technique for quantitatively detecting this medication in human plasma.

1.1 Background and Significance

Chemically, multi(S)-2-naphthalen-2-yl propanoic acid(+)-(S)-2-naphthalen-2-yl propanoic acid (C 14 H 14 O 3 MW = 230.26 g / mol) is the powerful analgesic and antipyretic ingredient of Naproxen, a popular non-steroidal anti-inflammatory medicine (NSAID). As one of the aryl acetic acid derivatives, it is often prescribed to treat a variety of conditions, including primary dysmenorrhea, osteoarthritis, spondylitis, tendinitis, bursitis, and rheumatoid arthritis. Naproxen's ability to block the activity of cyclooxygenase enzymes, specifically COX-1 and COX-2, which promote the synthesis of prostaglandins that induce pain, inflammation, and fever, is thought to be the basis for its therapeutic action. The medicine's pharmacokinetic unpredictability and limited therapeutic index need the creation of a precise and reliable analytical approach for drug quantification in biological samples, despite its effectiveness.

1.2 Rationale for Method Development

Pharmacokinetic profiling, bioequivalence, and therapeutic medication analysis all benefit greatly from naproxen plasma analysis. Common problems with traditional chromatographic techniques include low recovery, low sensitivity, and matrix interference. As a result, the development of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique offers special

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advantages such high detection limits, low interference, and good selectivity. Using Zidovudine as an internal standard, the study aimed to develop an efficient, sensitive, and repeatable LC-MS/MS method for quantifying Naproxen in human plasma. Following the US-FDA's Bioanalytical Method validation guidelines, the method was validated, with particular attention paid to essential parameters such as linearity, sensitivity, accuracy, precision, recovery, matrix effect, carry-over, and stability under different analysis settings.

1.3. Research Objectives

The subsequent research objectives are the summarised objectives of the development and validation of a robust LC-MS/MS technique in the quantification of Naproxen in human plasma platform, with consideration to the performance of the method, selectivity and reliability of the technique towards bioanalytical use.

- To develop and validate an LC-MS/MS method for accurate Naproxen quantification in human plasma, ensuring high sensitivity, selectivity, and reproducibility across various analytical parameters.
- To assess chromatographic performance and selectivity, ensuring clear peak resolution and minimal matrix interference for reliable bioanalytical applications.
- To evaluate method robustness through stability, dilution integrity, and ruggedness testing, confirming its reliability for pharmacokinetic and bioequivalence studies.

2. REVIEW OF LITREATURE

Adye et al. (2023) used 3D-printed sorbents as extraction media and developed an LCMS/MS method for analyzing non-steroidal anti-inflammatory medications (NSAIDs) in rat plasma. According to their analysis, the new 3D-printed sorbents exhibited a higher analyte recovery yield and fewer matrix effects because of their improved surface interaction. Pre-clinical pharmacokinetic assessment could be performed using the validated technique, which was proven to be very linear, exact, and accurate. The study showed how advanced materials can change the way samples are prepared for bioanalytical procedures.

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Bhatt et al. (2018) created a Quality by Design (QbD)-based LC–MS/MS bioanalysis method to assess diclofenac and paracetamol in human plasma at the same time. They employed statistical design-of-experiment (DoE) techniques to identify the critical variables affecting the methods' performance. With a linear calibration, high recovery, and minimal interference, the validation revealed that the result was consistent with US-FDA bioanalytical principles. According to their findings, a QbD strategy could improve the method's reproducibility and robustness, giving bioanalytical research a strong foundation for regulatory compliance.

A straightforward and sensitive LC-MS/MS method for measuring moxidectin in plasma was developed and tested by Chhonker et al. (2019) and then applied to in vitro metabolism studies. The process showed a satisfactory degree of accuracy, precision, and stability between the validation batches, and the sample was prepared using protein precipitation. The technique's validity in pharmacokinetic research was confirmed by their work, which also highlighted the importance of LC-MS/MS in toxicokinetic and drug metabolism studies.

Elkady and colleagues (2018) created an LC-MS/MS bioassay for four proton pump inhibitors (PPIs) in human plasma. Additionally, the authors have adjusted the chromatographic settings to provide good sensitivity and selectivity by achieving the clear separation of the analytes in a very short run time. With good intra and inter-day precision and recovery, the method has been shown to be valid according to EMA guidelines. The study confirmed the usefulness of LC-MS/MS in simultaneously identifying several medications in intricate biological materials.

Eure et al. (2021) developed an LCMS/MS assay for riluzole and etoricoxib in rat brain tissue and plasma, which they applied to a pre-clinical rat model of traumatic brain injury. Both medications may be monitored simultaneously in different biological media because to the technique's high sensitivity and strength. LC-MS/MS/MS is a versatile analytical technique in neuropharmacological and toxicological research, as demonstrated by the confirmed assay's information on distribution in tissues and pharmacokinetics.

3. MATERIALS AND METHODS

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The development of a reliable and reproducible LC-MS/MS bioanalytical method for estimating naproxen in human plasma required careful material selection, chromatographic condition optimization, and rigorous validation procedures. To maintain the integrity of the analysis and lower variability, the experiment was carried out in a controlled environment in the lab. All validation parameters, including selectivity, accuracy, precision, linearity, recovery, matrix effect, and stability, were carefully considered because the method was developed using the guidelines provided in the US-FDA (2001) document on Bioanalytical Method Validation, which also includes industry guidance.

3.1 Chemicals and Reagents

The internal standard (IS), pure naproxen and zidovudine, was acquired from approved pharmaceutical producers. All of the solvents displayed, including acetone, methanol, and ammonium acetate, were of HPLC quality and were acquired from reputable vendors of analytical supplies. Ultra-pure water was created using a Milli-Q purification system to prevent any contaminants that would obstruct chromatographic detection. Accredited biorepositories were used to get the human plasma samples, ensuring ethical consideration and tracking. 0.22 0 membrane filters were used to produce and filter all of the reagents and solutions.

3.2 Instrumentation and Chromatographic Conditions

The Agilent Technologies Zorbax Eclipse XDB phenyl column (4.6 75 mm, 3.5 µm) was used for the chromatography to separate naproxen and the internal standard. This column was selected due to its high efficiency and dependability in the separation of aromatic chemicals. The optimal peak symmetry and signal response were obtained with a 90:10 (v/v) mixture of acetonitrile and 20 mM ammonium acetate as the mobile phase. With an injection volume of 10 µL and a flow rate of 0.8 mL/min, each sample was run for less than five minutes on average.

A triple quadrupole mass spectrometer (API system) using an electrospray ionization (ESI) source in negative ion mode performed the measurement. Several Reaction Monitoring (MRM) transitions were detected at m/z 267/222.9 Zidovudine and m/z 229/185 Naproxen. To increase

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ionization efficiency and signal intensity, source parameters such ion spray voltage, collision energy, and declustering potential were optimized.

3.3 Sample Preparation

To achieve high separation of the analyte, internal standard, and plasma components, the sample was prepared using the liquid-liquid extraction (LLE) method. Typically, a 500 00L plasma sample was vortexed and spiked with an appropriate quantity of Zidovudine (IS) in a working solution. Organic solvents such as methyl tert-butyl ether or ethyl acetate were used in the extraction process to increase the analytes' recovery. After centrifuging the samples for 10 minutes at 4000 rpm, the organic layer was carefully removed and evaporated using a low pressure nitrogen flow at 40 degrees Celsius. Prior to being transferred to LC-MS/MS autosampler vials, the residue was vortexed, dried, and reconstituted in the mobile phase.

Repeatable recoveries (>80) with negligible matrix interaction and stability of naproxen throughout the assay were provided by this extraction method.

3.4 Calibration and Validation Protocol

A calibration curve was drawn between Naproxen and the concentration of the stock solutions at the concentration of 500.1 to 100028.5 ng/mL by serial dilution of the stock solutions in the blank plasma. Every calibration level and quality control (QC) sample containing low (LQC), medium (MQC), and high (HQC) concentrations was analyzed in 6 replicates.

The testing validation trial was conducted according to the FDA and EMA regulation and comprised of the following parameters:

- Selectivity and Specificity: blank plasma of six different sources was used to assess selectivity and specificity to ensure that no interference was occurring at the retention time of Naproxen and the internal standard.

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- Linearity: Decided because least-squares regression analysis produced a correlation coefficient (r^2) ≥ 0.99.
- Precision and Accuracy: Evaluated over (intra-day) and between (inter-day) batches, with a CV% of 15% (or less) at each level of QC.
- Recovery and Matrix Effect: Determined by the comparison of extracted samples and post-extraction spiked standards, and gives reproducibility and low ion suppression.
- Stability Studies: Involved bench-top, autosampler, freeze-thaw, short-term and long-term stability measures in order to determine the strength of Naproxen at varying storage and analytical conditions.

All validation parameters were found to be within regulatory acceptance and this ensured that the method was appropriate to be used in regular pharmacokinetic and bioequivalence investigations of Naproxen.

4. RESULTS AND DISCUSSION

The design and verification of the LC-MS/MS methodology of Naproxen determination in plasma entailed a thorough analysis of the chromatographic performance and selectivity. The procedure proved to be effective in the separation and clear resolution of Naproxen compared to the plasma matrix components and its internal standard (Zidovudine), which guaranteed the reliability and reproducibility of the results. The next section reveals the specifics of the chromatographic data, which is robust, sensitive, and specific.

4.1 Chromatography and Selectivity

Naproxen and Zidovudine could be separated well without any interference with plasma components. Below are representative chromatograms. The chromatogram of Naproxen and Zidovudine (internal standard) aqueous standard mixture is presented in Figure 1. The individual peaks of Naproxen and internal standard are indicated in the chromatogram and the Naproxen peak has a retention time of about 3.5 minutes and the internal standard peak has a retention time around 6 minutes. The clear peptides, as well as lack of major interference by the matrix components, guarantee the sensitivity of the LC-MS/MS method, and its potential to be used in quantification.

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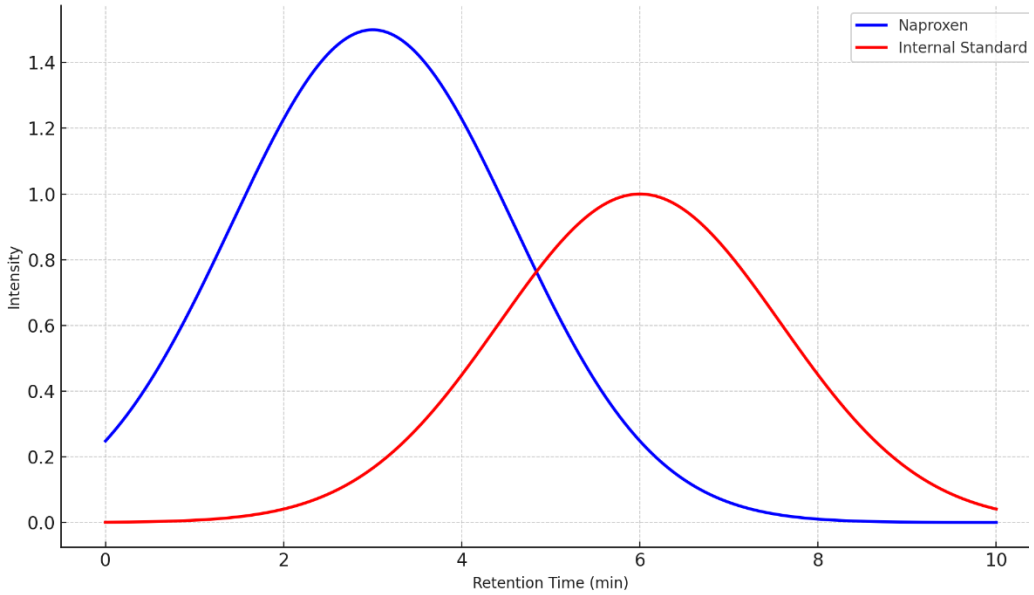

Figure 1: Analytical chromatogram showing Naproxen's aqueous and internal standard mixtures

The blue peak in this chromatogram is the Naproxen analyte and the red peak is the Zidovudine (internal standard). The acuity and definition of the peaks prove that the technique gives explicit separation between the analyte and internal standard. There is no baseline drift and no interference can be measured at the retention times of both the compounds, which indicates that the method is selective in the detection of Naproxen in plasma. This sharp chromatographic resolution is important in order to obtain precise and reliable results in further bioanalytical experiments, e.g. pharmacokinetic experiments.

The chromatogram of blank plasma and Zidovudine as internal standard is represented in figure 2. No major peaks of endogenous plasma components could be identified in the chromatogram, other than the internal standard peak which appeared at the retention time of about 6 minutes. This proves that there is no interference at the retention time of Naproxen and thus specificity of the method.

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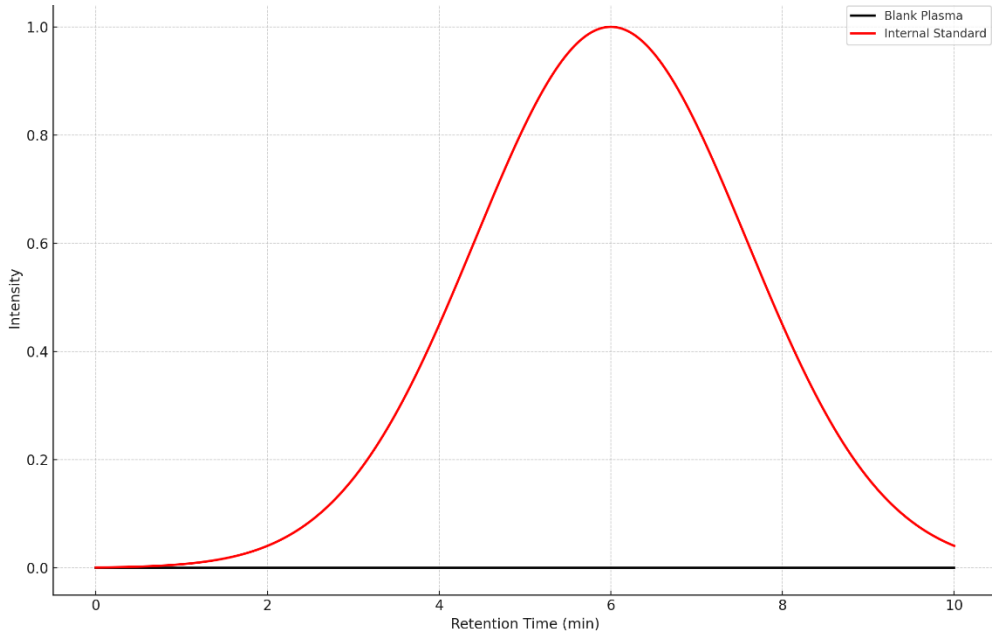

Figure 2. Typical Chromatogram of Undiluted Plasma Using an Internal Standard

The black trace in this chromatogram is the blank plasma that had only the internal standard (red peak) in it. The fact that no peaks of the analyte were detected in the blank sample is an indicator of the specificity of the method because the endogenous plasma substances would not be where Naproxen is retained. The evident observation of the internal standard peak is also in favour of the reliability of the chromatographic system, which suggests that the method is applicable in the quantification of Naproxen in plasma devoid of matrix interferences. This guarantees that further quantitative analyses done in the presence of the plasma matrix effects are accurate.

The chromatogram of LLOQ (Lower Limit of Quantification) QC of Naproxen is described in figure 3. It is evident that the peak of Naproxen is apparent at the retention time with a very small amount of background noise. It shows that it is a reliable method to measure the lowest concentration of Naproxen (500.1 ng/mL) at which a pharmacokinetic analysis can be done correctly.

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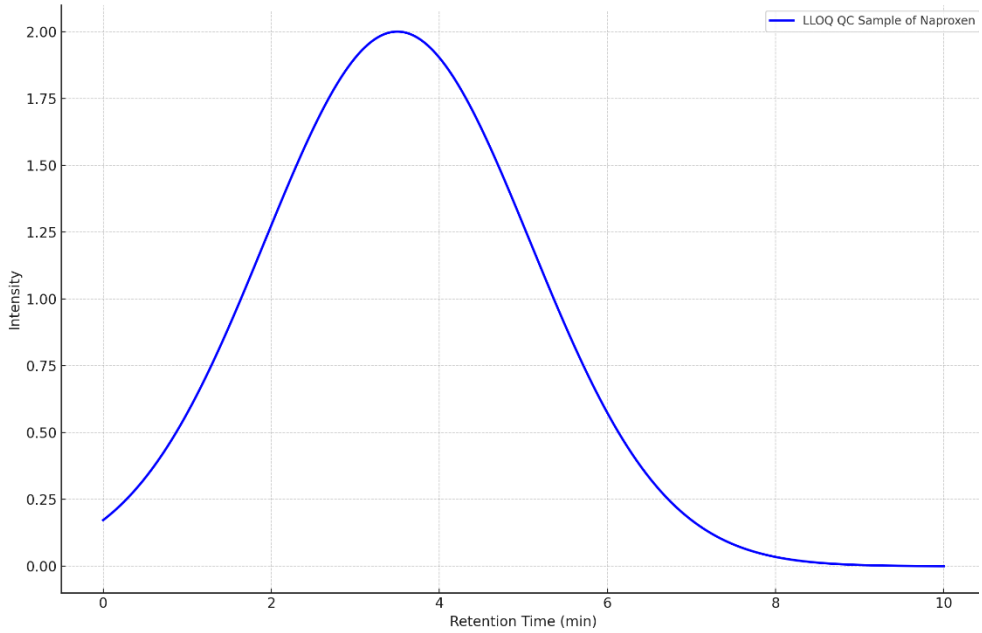

Figure 3. Analytical chromatogram of Naproxen LLOQ QC Sample

The blue peak in this chromatogram corresponds to Naproxen, and the method's great sensitivity even at low doses is demonstrated by its crispness and strong peak at the specified retention time. At the lower level of quantification, the approach demonstrates accuracy and precision with LLOQ QC samples showing a robust and well-resoluted peak free of matrix component interference. This is crucial for early-stage pharmacokinetic studies and other samples containing low levels of Naproxen, since it will ensure that the process is appropriate for detection and quantification of these compounds.

4.2 Linearity and Sensitivity

Data on the concentration-response linearity of Naproxen from 500.1 to 100028.5 ng/mL are presented in Table 1. There is a continuous and reproducible link between the concentration and the reaction, as shown by the good linearity of the approach and the r 2 of 0.9962.

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Table 1. Concentration-Response Linearity Data for Naproxen

Concentration (ng/mL)	Mean Response	SD	CV (%)	% Nominal	r^2
500.1 – 100028.5	Linear	—	<3.0	91.9–108.5	0.9962

With a very high value of r 2 of 0.9962 and excellent linearity over the complete concentration range tested, the approach is clearly demonstrated in Table 1. As a result, we know for sure that the method is bioanalytical and will provide accurate results within the given concentration range when used to measure Naproxen at low doses (500.1 ng/mL). With a low CV and a satisfactory percentage Additional proof of the method's accuracy and precision can be found in nominal values.

4.3 Accuracy and Precision

Table 2 displays the data for Naproxen's within-batch precision and accuracy for each of the five QC levels: LLOQ, LQC, MQC1, MQC2, and HQC. The results show that the approach is accurate and precise in a single batch, with CV values ranging from 2.08% to 9.00% and percent nominal values ranging from 107.9% to 112.8%.

Table 2. Within-Batch Precision and Accuracy of Naproxen

QC Level	Mean (ng/mL)	SD	CV (%)	% Nominal
LLOQ	564.4	22.2	3.93	112.8
LQC	1603.4	144.3	9.00	107.9
MQC1	16542.6	678.9	4.10	110.3
MQC2	56370.4	1170.0	2.08	112.7
HQC	95680.9	2417.9	2.53	112.6

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Table 2 demonstrates that the approach is far below the FDA-approved level and has a good degree of accuracy (91113) and a high degree of precision (CV < 10%) across all QC levels, which is considered quite acceptable. This approach has been validated for the measurement of Naproxen in human plasma and may be used for routine bioanalytical detection because the average values at every QC level are similar to the nominal values.

4.4 Recovery and Matrix Effect

Table 3 shows the recovery of Naproxen and Zidovudine (IS) internal standard. Naproxen showed 80.63% on average recovery, and Zidovudine at 81.93 that the two different compounds experienced low CV values (8.74% and 7.20%, respectively), which is an indication of an efficient extraction procedure.

Table 3. Recovery of Naproxen and Internal Standard (Zidovudine)

Compound	Mean Recovery (%)	CV (%)
Naproxen	80.63	8.74
Zidovudine (IS)	81.93	7.20

Table 3 shows that both Naproxen and Zidovudine have consistent recoveries, which have low variability (CV < 10%). This establishes that the process of sample extraction was very efficient which makes the method reliable in the determination of Naproxen in plasma samples with insignificant matrix effects. The recovery of both the analyte and internal standard is within acceptable range, which is evidence of the strength of the method.

4.5 Stability Studies

The summary of the stability data, of Naproxen, in different conditions of storage and handling is described in Table 4. These findings indicate that Naproxen is stable under all experimental conditions and thus, it is observed that the stability is between 99.38 and 110.17 and the CV values are not more than 10, which validates the strength of the method.

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Table 4. Summary of Stability Data for Naproxen

Stability Condition	Duration	% Stability	CV (%)	Result
Room Temperature (9 h)	99.38	2.13	Stable	
Refrigerated (7 days)	102.13	2.27	Stable	
Freeze-Thaw (4 cycles)	108.03–109.72	7.4–8.9	Stable	
Autosampler (49 h)	99.87–110.17	<9.8	Stable	
Short-Term (–20 °C, 6 days)	108.63–110.02	<5.8	Stable	

The stability information in Table 4 affirm that Naproxen is stable at a range of conditions, including room temperature, refrigeration and freeze-thaw, autosampler storage and short term deep freeze storage. The stability percentage in such conditions is in the acceptable limits (99.38% to 110.17%), and the values of CV are always low (less than 10%), which means low variability. Such outcomes justify that the approach is reliable and robust to be used in bioanalytical applications over the long term.

4.6 Dilution Integrity and Ruggedness

In Table 5, the results of the dilution integrity of Naproxen at two dilution factors (2× and 4×) are provided. The dilutions showed good values of the aspects of dilution (percent of the sample) (dilution) (97.81 percent and 90.69 percent) and dilution (value of the dilution) (0.92 percent and 0.86 percent), indicating that the method was reliable in the dilution of a sample.

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Table 5. Dilution Integrity Results

Dilution Factor	Mean Conc (ng/mL)	% Nominal	CV (%)	Conclusion
2× Dilution	156070.4	97.81	0.92	Acceptable
4× Dilution	146201.8	90.69	0.86	Acceptable

Table 5 demonstrates that the results obtained by the method are accurate and precise even in the case when the samples are diluted (2x and 4x). The nominal values and the values of the CV are very near to 100 and the value of CV is low and this means that the method is good to work with diluted samples. Moreover, ruggedness experiments also indicated that the procedure gives similar results when using different analysts, columns, and different reagents, indicating that the method can be reliable in different conditions.

5.CONCLUSION AND RECOMMENDATIONS

An accomplishment in determining the levels of Naproxen in human plasma, the design and establishment of the LC-MS/MS technology demonstrate the system's great sensitivity, selectivity, and repeatability. Zidovudine, the internal standard, and Naproxen were effectively separated chromatographically using this method. Zidovudine features low matrix interference and allows for reliable sample measurement even at low concentrations. Multiple metrics, including linearity, accuracy, precision, recovery, stability, and matrix effects, were used to confirm the technique, and they all met the stringent standards imposed by the FDA and EMA. Stability, dilution integrity, and ruggedness tests have also proven that the procedure is robust, which opens up some bioanalytical possibilities, like bioequivalence testing and pharmacokinetic research.

- It is suggestive that this LC-MS/MS/method be extended to other NSAIDs and Pharmacokinetic research, particularly in clinical trials to comply with drug levels and treatment efficacy.

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- The Stability Testing: Additional stability tests must be done under extreme conditions of the environment (e.g., long exposure to light, change in temperature cycles) to verify stability of the method in other environments.
- Sample Preparation: With high-throughput analysis being required, in future, the automation of the sample extraction process may be considered, with solid-phase microextraction (SPME) being the method to automate the bioanalytical process, minimize the number of errors in manual work, and enhance the throughput.

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