SN Computer Science (2024) 5:884
https://doi.org/10.1007/s42979-024-03237-x

ORIGINAL RESEARCH

Bioanalytical Method Development and Validation for the Estimation of Hydroxyproline in Urine Samples of Osteoarthritic Patients Using LC–MS/MS Technique

Anil Kumar Tallam[1] · Konatham Teja Kumar Reddy[2] · Uttam Prasad Panigrahy[3] · Alapati Sahithi[4] · S. Prema[5] · Jeetendra Kumar Gupta[6] · D. Jeslin[7] · Aparajita Neogi[8] · Vinod Thomas[9] · R. S. Chandan[10]

Received: 24 April 2024 / Accepted: 16 August 2024

Abstract

Osteoarthritis (OA) is a prevalent disorder among the elderly, which is characterized by the degradation of articular cartilage, leading to joint erosion and pain. The release of hydroxyproline, a major component of collagen, into body fluids is a marker of cartilage degradation. Still, an increasing number of people are studying bioanalytical methods and validation for the estimation of hydroxyproline. Yet, there are still no consistent conclusions about OA patients. Blood contains a massive number of metabolites that can potentially become biomarkers, but the metabolome coverage of current analytical techniques remains insufficient. Clear knowledge related to this important issue would definitely facilitate a better understanding and validation of the hydroxyproline. Therefore, this study aims to develop and validate a reliable LC–MS/MS method for estimating hydroxyproline levels in urine samples of osteoarthritic patients. The method demonstrated high sensitivity, selectivity, and precision, with a linear range of 10 ng–320 ng ml^{-1} and a correlation coefficient (r^2) of 0.9993. Sample preparation involved hydrolyzing urine samples and optimizing chromatographic conditions for effective separation. The method validation followed ICH M10 guidelines, ensuring accuracy, precision, and stability. The results indicated that hydroxyproline is a significant biomarker for assessing cartilage degradation severity, thus aiding in the effective management of OA. The developed LC–MS/MS method provides a tool for diagnosing and monitoring OA, thus providing sensitivity to disease progression and treatment efficacy. This method's high-throughput capability is essential for large-scale clinical studies, enhancing the understanding of OA and improving patient outcomes.

Keywords LC–MS/MS · Bioanalytical method · Hydroxyproline · Osteoarthritis · Validation · ICH M10 guidelines

Abbreviations

LC–MS/MS	Liquid chromatography–mass spectroscopy/mass spectroscopy
ACN	Acetonitrile
CN	Cyano
RSD	Relative standard deviation

Introduction

Osteoarthritis is a disorder of the bone joints, especially the synovial joints, wherein the articular cartilage loss causes abrasion and the weakening of the joints [2]. The cartilage acts as a cushion between the joints, which helps to absorb shock as well as prevent friction between the same [2, 7]. Due to the breakdown of the cartilage, friction occurs between the connected bones as they rub against each other with every movement. This leads to swelling, inflammation, stiffness, and mild pain [7].

Apart from 70 percent of water and chondrocytes, the bone cartilage matrix is composed of various components [12]. The most abundant component present are collagens, followed by proteoglycans, which are complex glycoconjugates that contain protein cores to which glycosaminoglycan can chain and the N-linked and O-linked oligosaccharides are attached covalently [4]. Thus, the N-linked oligosaccharides are attached to the nitrogen atom of asparagine side chains, while O-linked oligosaccharides are attached to the oxygen atom of serine or threonine side chains. These glycosidic linkages play crucial roles in protein folding, stability, and cell signaling.

Hydroxyproline (Hyp) is a major component of collagen and constitutes around 12–14% of the total amino acid

Extended author information available on the last page of the article

Published online: 17 September 2024

content of mature collagen. It contributes to collagen stability along with proline [4, 12]. Thus, hydroxyproline is selected as a biomarker for osteoarthritis due to its abundance in collagen, constituting about 12–14% of the total amino acid content. During cartilage degradation, hydroxyproline is released into the body fluids, metabolized in the liver, and excreted in urine. This makes hydroxyproline a reliable marker for assessing the extent of cartilage erosion, thus providing insights into the severity of osteoarthritis. The production of hydroxyproline takes place in the lumen of the endoplasmic reticulum, and ninety percent of the Hyp released in the course of the bone collagen's degradation is initially metabolized in the liver [7, 12]. Then, it is excreted in the urine, where it may be detected either as free or protein-bound Hyp by bioanalytical techniques [13, 14]. Therefore, Hyp in urine can be considered as a biomarker for cartilage degradation [10]. Calculating hydroxyproline levels in osteoarthritic patients helps in:

- Diagnosing osteoarthritis: Identifying early stages of cartilage degradation.
- Monitoring disease progression: Tracking changes in hydroxyproline levels to assess disease severity.
- Evaluating treatment efficacy: Measuring the impact of therapeutic interventions on cartilage preservation and repair.

Developing advanced analytical methods like LC–MS/MS is important for diagnosing osteoarthritis due to their enhanced precision and sensitivity. These methods enable accurate identification and quantification of biomarkers, such as hydroxyproline, and provide disease progression and treatment efficacy. This study aims to effectively identify and estimate hydroxyproline concentration as a urinary biomarker for osteoarthritis diagnosis and treatment. Examining the complexity of advanced methods like LC–MS/MS is important for a more comprehensive understanding of osteoarthritis. To achieve this, a highly sensitive LC–MS/MS approach was established, optimized, and validated by following ICH M10 guidelines. Background literature for the study is provided in the bibliography (5–19).

Materials and Methods

Rationale for LC-MS/MS Selection

LC–MS/MS was chosen as the analytical method for several reasons. LC–MS/MS is renowned for its high sensitivity and selectivity and is essential for detecting low concentrations of biomarkers in complex biological samples. The appeal of these LC–MS/MS assays is that the limitations of other techniques, including cross-reactivity, low analytical specificity, and limited dynamic range, can be tackled. Numerous potential advantages for clinical LC–MS/MS assays include:

- Shorter assay development and optimization time compared with immunoassays
- Ability to include multiple analyte analyses in a single method
- Wide applicability to various analytes, including drugs, hormones, small molecules, carbohydrates, nucleic acids, and proteins.

This technique provides excellent reproducibility and accuracy that are crucial for robust method validation according to regulatory guidelines like ICH M10. Moreover, LC–MS/MS enables simultaneous analysis of multiple analytes, facilitating comprehensive biomarker assessment and novel insights into disease mechanisms. Its capability for rapid analysis and high-throughput screening is particularly beneficial for large-scale osteoarthritis studies. Firstly, LC–MS/MS offers higher sensitivity as well as selectivity, allowing for the detection and quantification of lower concentrations of analytes in complex biological samples like urine. This is particularly advantageous for biomarker analysis in diseases like osteoarthritis, where accurate measurement of biomarkers, such as hydroxyproline, is more important. Additionally, LC–MS/MS provides excellent reproducibility and accuracy essential for robust method validation according to regulatory guidelines, such as the ICH M10. The LC–MS/MS methods were optimized by selecting appropriate chromatographic columns, mobile phase compositions, and gradient programs to reduce analysis times while maintaining high resolution and sensitivity. The method also employed efficient sample preparation techniques, thus minimizing processing time and enabling high-throughput screening of clinical samples that are essential for large-scale osteoarthritis research. Moreover, LC–MS/MS allows for simultaneous analysis of multiple analytes, enabling a comprehensive assessment of disease biomarkers and potentially exposing novel observations into disease mechanisms. Finally, LC–MS/MS methods can be optimized to achieve rapid analysis times, facilitating high-throughput screening of clinical samples, which is needed for large-scale studies in osteoarthritis research. High-throughput screening of clinical samples is crucial in large-scale osteoarthritis studies as it allows for the rapid analysis of numerous samples, thereby increasing the efficiency and productivity of the research. This capability is essential for timely data collection and analysis, enabling researchers to draw statistically significant conclusions and identify potential biomarkers quickly. LC–MS/MS offers several advantages over other analytical techniques, such as:

- Higher sensitivity and selectivity: Capable of detecting low concentrations of hydroxyproline with minimal interference.
- Simultaneous analysis: Allows for the simultaneous quantification of multiple analytes.
- High throughput: Suitable for large-scale studies due to rapid analysis times.
- Enhanced accuracy and precision: Provides reliable and reproducible results that are essential for clinical applications.

Overall, LC–MS/MS was chosen as the preferred analytical method owing to its superior performance characteristics and suitability for addressing the specific requirements of biomarker analysis in osteoarthritis.

Chemicals, Materials, and Reagents

LC–MS/MS grade acetonitrile was obtained from J.T. Baker, Avantor, along with analytical grade 0.3% formic acid, which was purchased from Loba Chemie. Millipore water used in the study was of LC–MS grade, and it was acquired from the Millipore Direct-Q® 3 UV water purification system. The Millipore Direct-Q® 3 UV water purification system is designed to produce ultrapure water with low levels of organic and inorganic contaminants. It uses a combination of filtration, UV radiation, and deionization to remove impurities, ensuring that the water used in the analysis does not introduce any interference, which is crucial for high-sensitivity techniques like LC–MS/MS. Millipore water was chosen for this study due to its high purity and consistency, which are essential for minimizing background noise and interference in LC–MS/MS analysis. Unlike distilled or deionized water, Millipore water undergoes additional purification processes to remove organic and inorganic contaminants, ensuring the reliability and accuracy of the analytical results. The pure drug Hydroxyproline, analytical grade 0.3% formic acid, and 6 N HCl were procured from Loba Chemie. Urine samples for bioanalytical purposes were acquired from JSS Ayurveda Hospital, Mysuru.

Instrumentation and Analytical Conditions

The instrumentation and analytical conditions for the liquid chromatography (LC) and mass spectrometry (MS/MS) components of the analysis are detailed below:

Liquid chromatography (LC):

- Instrument: Ultra-fast liquid chromatograph (UFLC) (Shimadzu, Japan)
- UFLC offers advantages in terms of speed and resolution compared to traditional HPLC. However, it requires specific techniques and instrumentation for presenting the overall method's complexity. The UFLC employed in this study offers several advantages over traditional HPLC, including higher speed and resolution. The UFLC system (Shimadzu, Japan) allows rapid separation of analytes, thereby significantly reducing overall analysis time. It is equipped with advanced pumps and detectors that enhance sensitivity and reproducibility, crucial for accurate biomarker quantification.
- Column: Cyano column (4.6 × 150 mm, 5 µm; Shimadzu, Japan). The Shimadzu Shim pack CN Column was chosen for its optimal balance of hydrophobic and polar interactions, which enhances the separation of hydroxyproline from other urinary components. The column's stability and reproducibility under varying conditions make it ideal for robust biomarker analysis.
- Column temperature: 50 °C
- Mobile phases:
  - Pump A: 0.3% formic acid
  - Pump D: acetonitrile (ACN)
- Flow rate: 0.5 ml/min
- Injection volume: 10 µl
- Run time: 16 min
- Gradient elution for pump D: (specified in the passage)

Mass spectrometry (MS/MS):

- Instrument: API 4000 LC–MS/MS system (SCIEX)
- Ionization mode: positive
- Multiple reaction monitoring (MRM): Used to selectively detect hydroxyproline
- Precursor ion (Q1) mass: 1203
- Product ion (Q3) mass: 1185
- Dwell time: 100 ms

The source conditions are adjusted as: collision gas—4; curtain gas: 30; ion source gas 1—50; ion source gas 2—60. The compound conditions were maintained as: declustering potential—117 V; entrance potential—10 V; collision energy—30 eV; collision cell exit potential—53 V. During the development and optimization of the LC–MS/MS methodology, several challenges and details were perceived. One significant challenge was optimizing the chromatographic conditions to achieve optimal separation of hydroxyproline from other matrix components present in urine samples. Therefore, optimizing chromatographic conditions is challenging due to the need to balance various parameters, such as mobile phase composition, flow rate, column temperature, and gradient program. Each parameter can significantly impact the resolution, retention time, and peak shape. Achieving the optimal conditions requires iterative testing and fine-tuning to ensure accurate separation and detection

of hydroxyproline in the presence of other urinary components. This required careful selection of the chromatographic column, mobile phase composition, and gradient program to ensure adequate resolution and peak shape. Additionally, optimizing the mass spectrometry parameters, such as ionization mode, collision energy, and fragmentor voltage, presented another challenge to achieving optimal sensitivity and selectivity for hydroxyproline detection. Furthermore, establishing robust sample preparation techniques, including hydrolysis of urine samples and extraction of hydroxyproline, required particular optimization to validate high recovery and minimal matrix interference. Moreover, maintaining consistency and reproducibility across multiple analytical runs during method validation posed another complexity, thereby requiring severe control of experimental variables and a thorough assessment of method performance parameters. Overall, addressing these challenges and complexity required a systematic and iterative approach for method development and optimization, ultimately leading to the establishment of a reliable LC–MS/MS methodology for hydroxyproline quantification in urine samples for osteoarthritis research.

Preparation of Standard Solutions and Quality Control Samples

10 mg of standard hydroxyproline drug was accurately weighed and transferred into a 10 ml volumetric flask. It was then dissolved with Millipore water and made up to mark to get a concentration of 1000 µg ml^{-1}. Serial dilutions were performed to achieve the working standard solution of a concentration of 1 µg/ml.

From the standard working solution of 1 µg/ml, 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml, 1.6 ml, and 3.2 ml aliquots were taken into six 10 ml volumetric flasks, respectively. After that, Millipore water was used to fill the flasks up to the mark. Finally, a concentration range of 10–320 ng was achieved.

Preparation of Sample Solutions

Urine samples from five individuals, suffering from osteoarthritis, were gathered and stored at 20 °C till investigation. Later, 0.1 ml of urine was taken from each sample container and transferred into five test tubes separately, followed by the addition of 6 N HCl to each test tube. It was left overnight at 70–80 °C to undergo hydrolyzation. This process breaks down the peptide bonds, releasing free hydroxyproline. The hydrolyzed samples were then filtered and prepared for LC–MS/MS analysis, thus ensuring efficient recovery of hydroxyproline for accurate quantification. 1 ml of the hydrosylate was filtered out from each test tube using a 1 ml syringe (Phenomenex) equipped with a 0.2 µm Whatman disposable filter. In addition, the solution is taken into five Eppendorf tubes (Phenomenex) separately.

1 ml of Millipore water was added to each tube containing 1 ml of hydrosylate and resuspended. The sample solutions were then filtered out into vials using 0.2 µm Whatman disposable filters and stored in the refrigerator until analysis.

Method Validation

The method validation was performed according to the ICH M10 guidelines. The validation ensured that the bioanalytical method developed for the estimation of hydroxyproline was well-grounded, valid, and served the intended purpose. Statistical analysis for method validation included calculating the mean, standard deviation (SD), and relative standard deviation (%RSD). Linearity was assessed using regression analysis, with the correlation coefficient (r^2) indicating the linear relationship between concentration and peak area. Techniques for assessing a bioanalytical method's accuracy, precision, sensitivity, and specificity are,

- Accuracy: Assessed by comparing the measured concentration to the nominal concentration and expressed as a percentage of the nominal value.
- Precision: Evaluated by calculating the relative standard deviation (%RSD) of repeated measurements within a run (within-run) and across different runs (between-run).
- Sensitivity: Determined by the lowest concentration of hydroxyproline can be accurately and precisely quantified (LLOQ).
- Specificity: Confirmed by the absence of interference from other components in the urine samples, ensuring the selective detection of hydroxyproline.

Selectivity

In analyzing the standards, the developed bioanalytical approach proved to be highly selective. The blank and standard solutions were analyzed to assess selectivity. Selectivity was assessed by analyzing blank urine samples and hydroxyproline standards under the same chromatographic conditions. The absence of interfering peaks in the blank samples at the retention time of hydroxyproline confirmed the method's selectivity. The chromatogram for the standard showed a peak at 3.2. There was no interference peak, where the analyte peaks should have a good intensity of more than $5e^4$. An analyte peak intensity of more than 5e4 indicates a strong signal-to-noise ratio, which is essential for accurate quantification. High peak intensity ensures that the detected

signal is significantly above the background noise, reducing the likelihood of false positives or negatives. This strength is critical for reliable detection and quantification of hydroxyproline, especially at low concentrations.

Accuracy and Precision

The samples are evaluated by considering within-run and between-run parameters at each concentration level for at least 5 runs. Within-run parameters refer to the consistency of measurements within a single analytical run, whereas between-run parameters assess consistency across different runs. Both parameters were evaluated to ensure the method's reliability, with within-run accuracy and precision measured over five replicates and between-run assessed across different days. Accuracy was found to be within limits according to ICH M10 guidelines. The approach was found to be accurate as the analyzed samples' accuracy was found to be within $\pm$ 15% of the nominal concentration.

Precision was evaluated in terms of %RSD, where, the developed method proved to be precise enough and resulted in values within limits. The % RSD was found to be much less than 20%, as required.

% Recovery was evaluated to measure the response of the spiked sample to the analytical method compared to the response of the non-spiked standard to the method. The value was found to be more than 70% and thus, the method was recognized to be effective for the estimation of hydroxyproline.

Sensitivity

The lowest level of concentration [LLOQ] was considered for analyzing the sensitivity of the method in identifying and estimating hydroxyproline concentration at different time slots. Five analytical runs were performed for the lowest concentration for each time of analysis. The method was found to be highly sensitive in the determination of hydroxyproline.

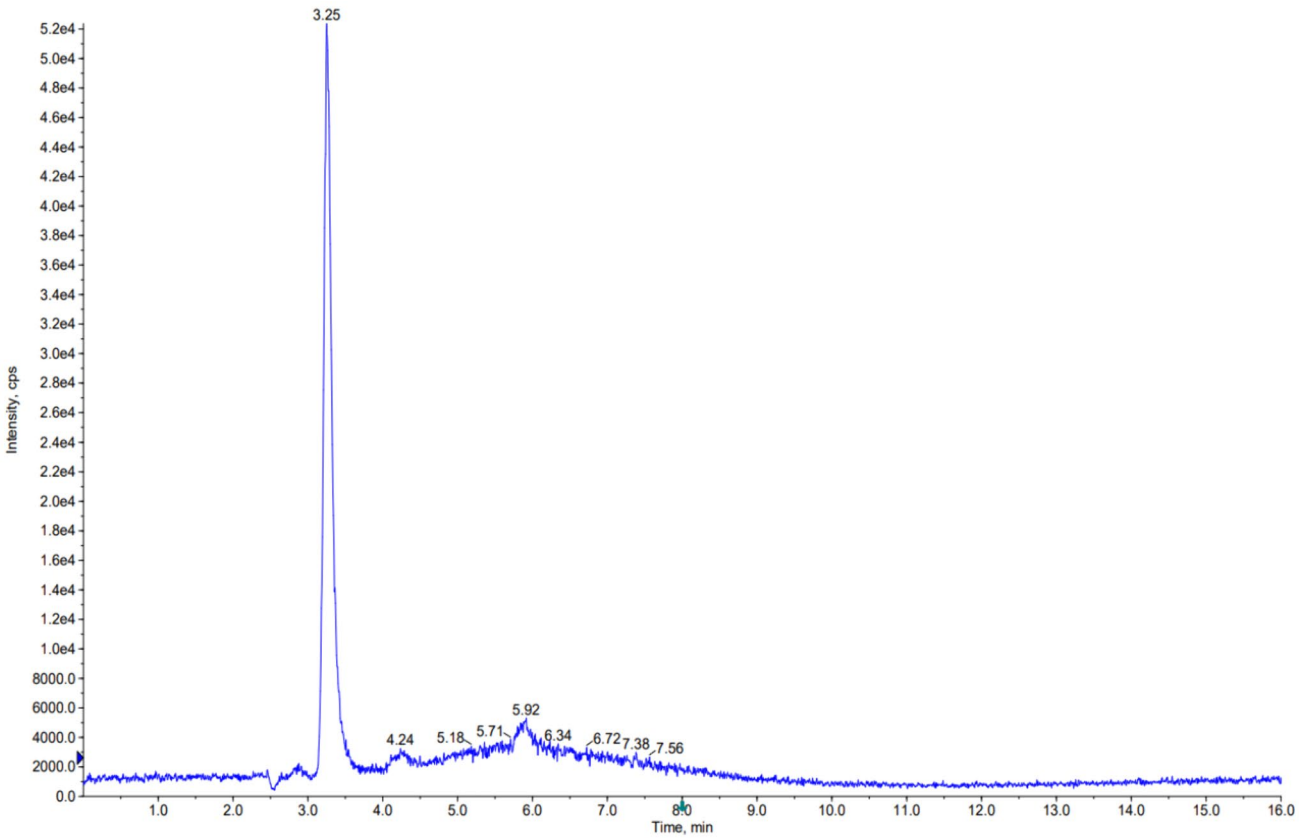

Fig. 1 Chromatogram for standard

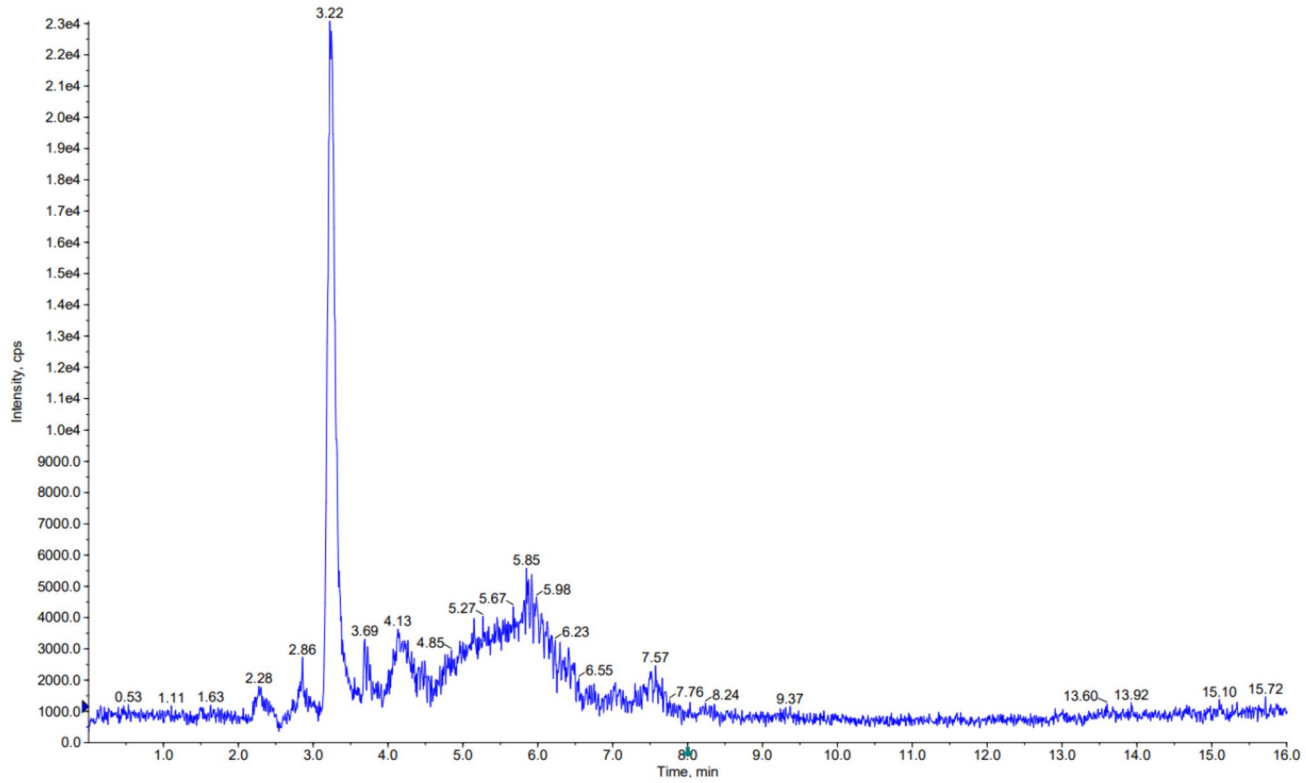

Fig. 2 Chromatogram for sample

Stability

To analyze the stability of the analyte at various conditions, low and high concentration levels were considered to carry out freeze–thaw, Benchtop, and long-term stability studies. The mean recovered concentration for all three tests was found to be less than 10% within limits.

The freeze–thaw stability was conducted to ensure that the analyte remains stable when repeatedly taken out from the frozen condition. The analyte was stored for 12 h prior to the thawing process. The stability of the analyte at both low and high concentrations was found to be within limits.

The bench-top stability study is a short-term test, which ensures that the analyte remains stable under laboratory ambient conditions. The low and high concentrations are kept on a bench top at the same temperature and the mean recovered concentration is measured to check stability. In bench-top stability studies, the mean recovered concentration is measured by comparing the analyte's concentration in samples stored at ambient conditions with the freshly prepared samples. The recovery is calculated by analyzing multiple replicates and averaging the results, ensuring that the analyte remains stable throughout the test, thus validating the method's robustness. The stability was observed to be within limits for both concentrations.

A long-term stability test is conducted to check whether the analyte retains its stability over a longer period of time when stored in the refrigerator at temperatures between – 70 and 20 °C. The low and high concentrations of the analyte showed stability for the long-term test.

Calibration Curve

The calibration curve is depicted by the linear relationship of standard concentrations with that of their peak areas. The establishment of a linear relationship between standard concentrations and peak areas is fundamental for quantifying hydroxyproline accurately. The linearity ensures that the detector's response is proportional to the analyte concentration, facilitating precise and reliable measurements. This relationship is critical for creating a calibration curve that can be used to determine unknown sample concentrations based on their peak areas. The range of concentrations taken here for plotting the calibration curve is 10–320 ng ml^{-1}. The standard concentrations established a linear relationship with peak area, wherein it was found that the response increased with the increase in standard concentration. The correlation coefficient (r^2) was measured from the calibration curve to determine the linearity. The primary factors contributing to the calibration curve's

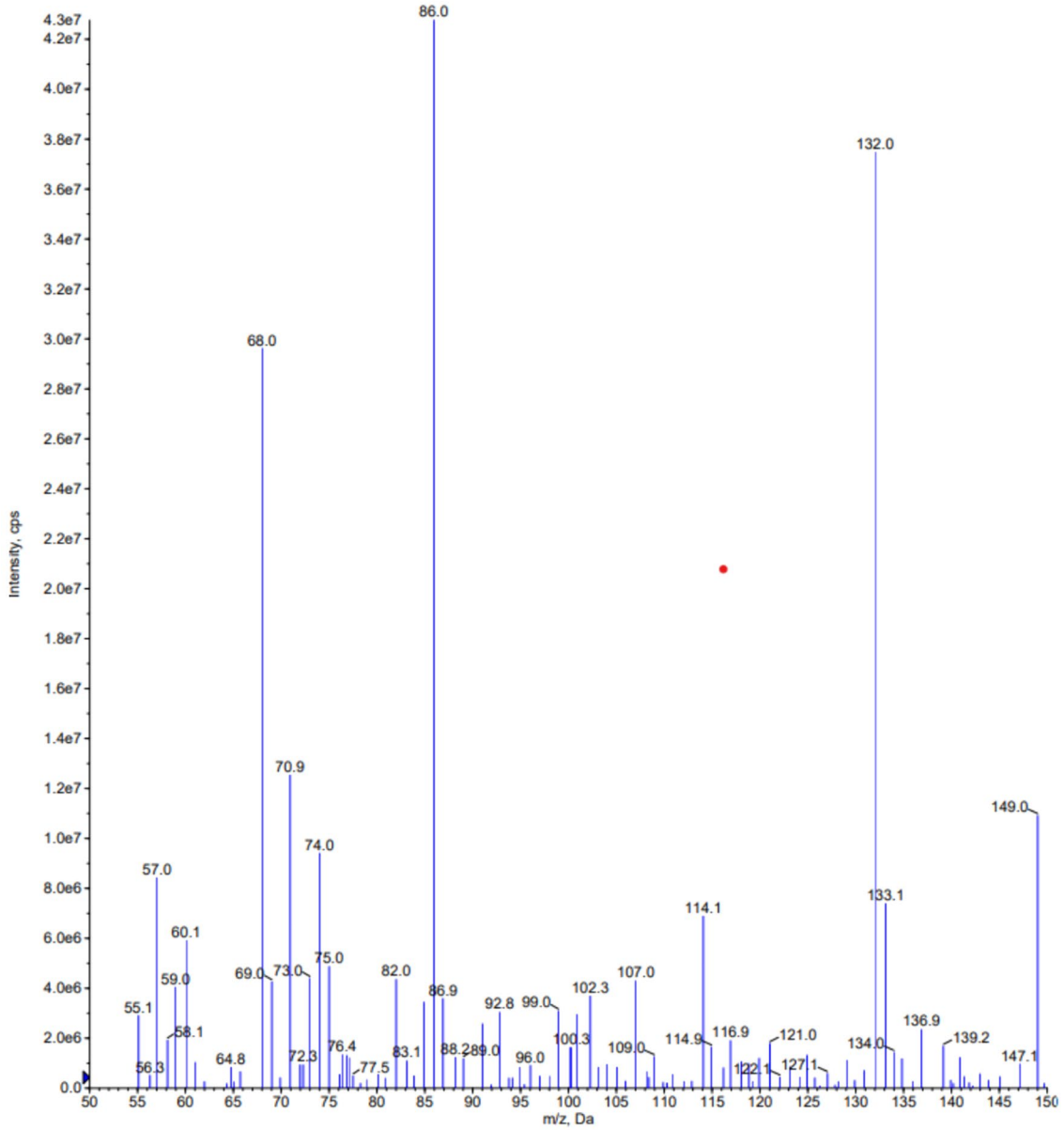

Fig. 3 Q3 fragmentation

excellent linearity include the precision of standard preparation, the consistency of sample injection volumes, and the stability of the analytical system. A high correlation coefficient indicates that the method can reliably measure hydroxyproline across a wide range of concentrations, ensuring the accuracy of the analytical results. The approach was estimated to be linear as the r^2 value was found to be more than 0.99.

Specificity

The specificity of the LC–MS/MS method was validated by analyzing blank and spiked urine samples. The chromatographic conditions were optimized to achieve distinct separation of hydroxyproline from other urine components. The use of multiple reaction monitoring (MRM) in positive ionization mode further ensured selective detection of hydroxyproline without interference from other compounds.

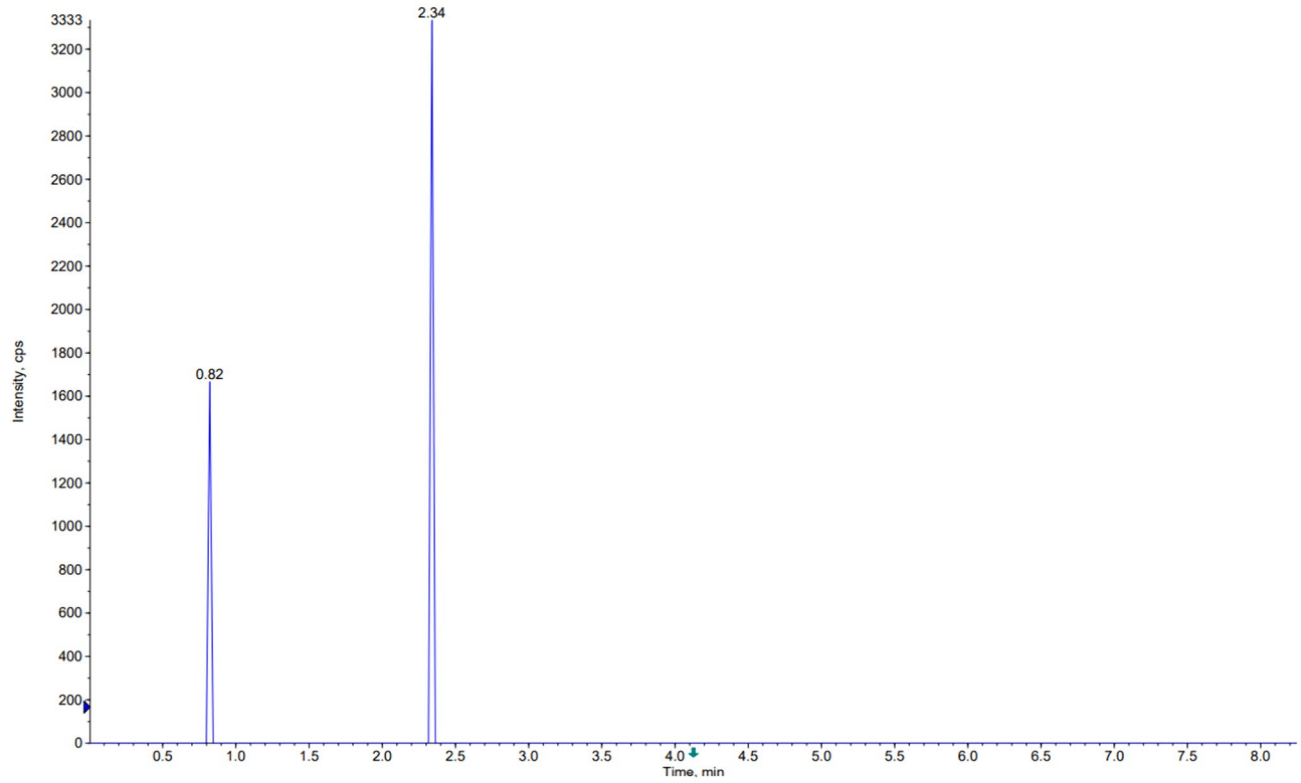

Fig. 4 Blank chromatogram

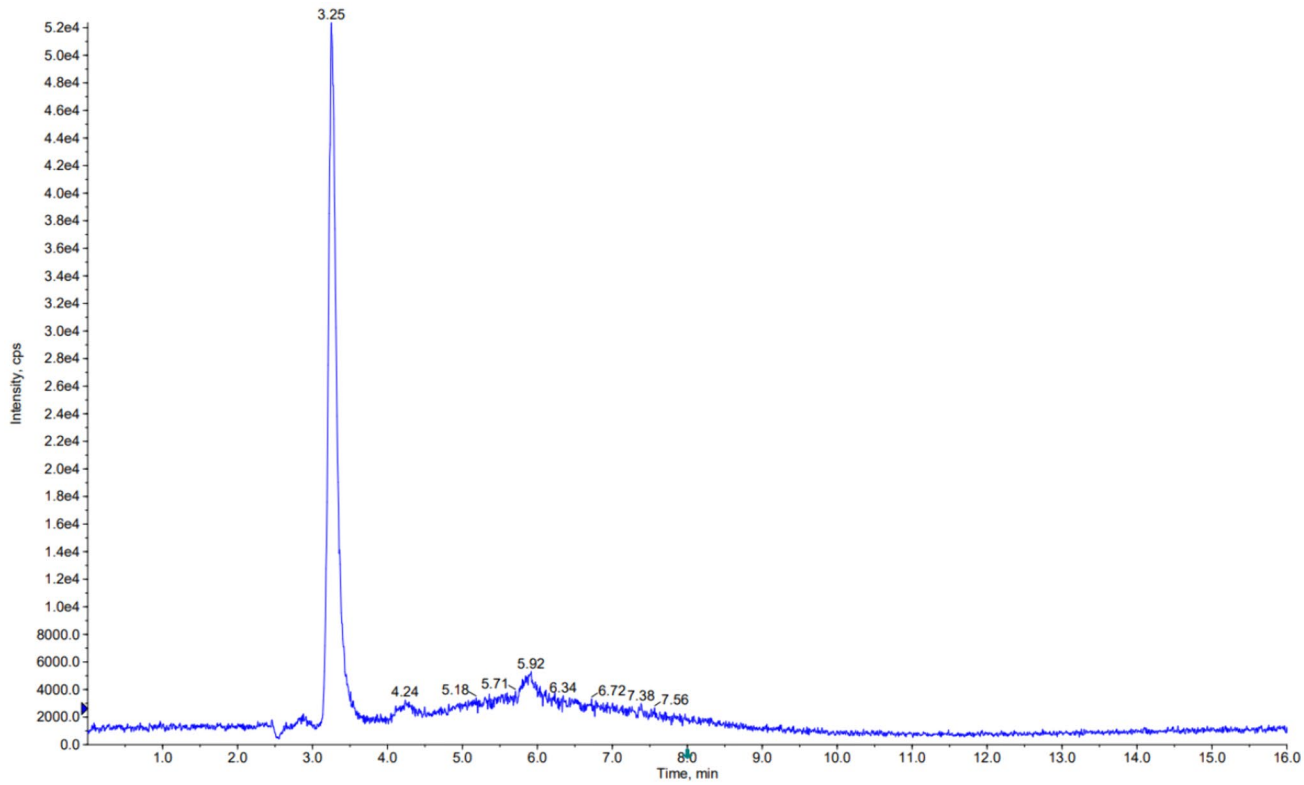

Fig. 5 Standard chromatogram

Table 1 Results for accuracy and precision: within run

Concentration (ng ml^{-1})	Peak area	Actual concentration	Mean concentration	SD	% accuracy	Nominal concentration	%RSD
10	84,362	10.366	10.253	0.192	102.535	− 2.535	1.878
	84,632	10.467					
	84,268	10.007					
	84,423	10.327					
	84,236	10.098					
20	96,364	20.158	20.987	1.201	104.938	− 4.938	1.765
	97,264	21.275					
	96,426	20.235					
	96,472	20.292					
	98,634	22.977					
40	113,496	41.432	40.195	0.763	100.488	− 0.488	1.900
	112,479	40.169					
	112,468	40.156					
	111,837	39.372					
	112,217	39.844					
160	207,627	158.325	158.983	3.081	99.364	0.635	1.937
	208,639	159.581					
	211,567	163.217					
	208,324	159.190					
	204,628	154.600					
320	334,529	315.912	314.125	1.595	98.164	1.835	0.507
	332,648	313.576					
	331,449	312.087					
	332,564	313.472					
	334,258	315.575					

Results and Discussion

Method Development

The complexity of LC–MS/MS significantly enhances the overall diagnostic capabilities for osteoarthritis by providing a comprehensive and detailed analysis of biomarkers, such as hydroxyproline, associated with the disease. LC–MS/MS's multi-stage process, involving liquid chromatography and mass spectrometry, allows for precise identification and quantification of biomarkers in complex biological samples like urine, which is important for accurate diagnosis. This intricate technique enables researchers to detect low concentrations of biomarkers, providing a more sensitive diagnostic approach for early disease detection and monitoring. Additionally, LC–MS/MS's ability to simultaneously analyze multiple analytes allows for a comprehensive assessment of various biomarkers associated with osteoarthritis, potentially finding new understandings of disease mechanisms and progression. Next, the criteria for selecting optimal conditions for LC–MS/MS analysis of hydroxyproline include:

- Sensitivity and selectivity: Ensuring the method could detect hydroxyproline at low concentrations with minimal interference.
- Resolution: Achieving clear separation of hydroxyproline from other urine components.
- Reproducibility: Consistent results across multiple runs and different samples.
- Stability: Hydroxyproline remains stable under various storage and analysis conditions.

Moreover, the advanced instrumentation and sophisticated data analysis techniques employed in LC–MS/MS minimize background noise and matrix effects, improving the reliability and accuracy of diagnostic results. The core steps involved in developing the bioanalytical method for estimating hydroxyproline in urine include:

1. Selection of analytical technique: LC–MS/MS was chosen for its high sensitivity and selectivity.
2. Preparation of standard and sample solutions: Standard solutions were prepared by serial dilutions of hydroxy-

Table 2 Results for accuracy and precision: between run

Concentration (ng ml^{-1})	Peak area	Actual concentration	Mean concentration	SD	%Accuracy	Nominal concentration	% RSD or % CV
10	87,999	9.770	9.515	0.487	95.154	4.845	1.7
	87,896	9.642					
	87,989	9.758					
	87,095	8.647					
	87,989	9.758					
20	98,230	22.475	22.493	0.076	112.467	− 12.467	0.340
	98,155	22.382					
	98,264	22.517					
	98,326	22.594					
	98,248	22.497					
40	112,538	40.243	39.871	0.347	99.678	0.321	1.82
	112,366	40.029					
	111,826	39.358					
	112,368	40.032					
	112,095	39.693					
160	210,568	161.977	162.497	0.876	101.560	− 1.560	0.539
	211,255	162.830					
	210,826	162.297					
	212,056	163.825					
	210,228	161.554					
320	336,567	318.443	319.343	0.872	99.794	0.205	0.273
	337,642	319.778					
	337,524	319.631					
	336,568	318.444					
	338,158	320.418					

proline, and urine samples were hydrolyzed to release hydroxyproline.

3. Optimization of chromatographic conditions: Parameters, such as column type, mobile phase composition, and gradient program, were optimized for effective separation.
4. Mass spectrometry parameter optimization: Ionization mode, collision energy, and fragmentor voltage were adjusted for optimal detection.
5. Method validation: The method was validated for selectivity, accuracy, precision, sensitivity, and stability according to ICH M10 guidelines.

Table 3 Results for % recovery

Concentration (ng ml^{-1})	Analytical peak area	Bioanalytical peak area	% Recovery
10	107,665	84,297	78.29563925
20	127,676	98,222	76.93066825
40	130,762	113,325	86.66508619
160	195,783	143,816	73.45683742
320	251,743	212,170	84.28039707

The developed chromatographic method identified the conditions for effective elution with respect to proper peak shape, retention time, and greater intensity within a run time of 16 min. Thus, effective elution and proper peak shape in chromatography are achieved by optimizing various parameters, such as the choice of the column, mobile phase composition, and gradient program. These elements influence the retention time, resolution, and symmetry of the analyte peaks, ensuring clear separation from other components. Proper peak shape is essential for accurate quantification and identification of hydroxyproline in complex biological matrices. Some of the challenges perceived during the development and optimization of the LC–MS/MS method are,

- Matrix interference: Optimized sample preparation and chromatographic conditions to minimize interference.
- Peak resolution: Adjusted mobile phase composition and gradient program to achieve clear separation.

Table 4 Result obtained for sensitivity

Concentration (ng ml^{-1})	Peak area	Actual concentration	Mean concentration	SD	% Accuracy	Nominal concentration	%RSD
10	87,935	9.691	9.710	0.051	97.104	2.895	1.7
	87,996	9.766					
	87,923	9.676					
	87,993	9.763					
	87,906	9.655					
10	87,839	9.571	9.694	0.078	96.947	3.052	0.806
	87,963	9.725					
	87,916	9.667					
	87,973	9.738					
	87,999	9.770					
10	87,919	9.671	9.678	0.074	96.783	3.216	0.770
	87,959	9.720					
	87,990	9.759					
	87,926	9.679					
	87,830	9.560					

Table 5 Results of stability studies

Stability	Concentration (ng ml^{-1})	Peak area	Actual concentration	Mean recovered concentration
Freeze–thaw	20	98,034	22.232	22.352
		98,235	22.481	
		98,123	22.342	
	160	211,467	163.093	162.682
		210,573	161.983	
		211,369	162.971	
Bench-top	20	98,252	22.502	22.487
		98,243	22.491	
		98,223	22.466	
	160	211,035	162.557	162.359
		211,235	162.805	
		210,358	161.716	
Long-term	20	98,235	22.481	22.478
		98,237	22.484	
		98,226	22.470	
	160	210,536	161.937	162.208
		210,358	161.716	
		211,369	162.971	

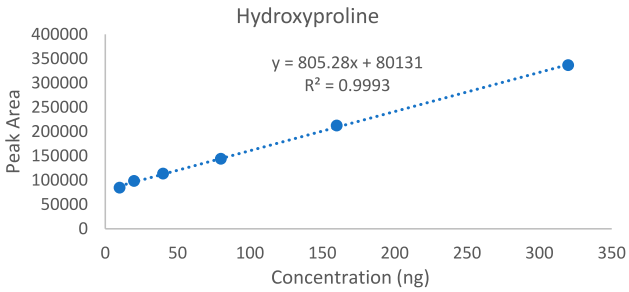

Fig. 6 Calibration curve

Table 6 Linearity results of hydroxyproline

Concentration (ng ml^{-1})	Peak area
10	84,297
20	98,222
40	113,325
80	143,816
160	212,170
320	336,283
Regression equation	y = 805.28x + 80,131
Correlation coefficient (r^2)	0.9993
Slope	805.28
Intercept	80,131

- Sensitivity optimization: Fine-tuned the mass spectrometry parameters to enhance the detection of hydroxyproline.
- Stability validation: Conducted stability studies to ensure consistent results under various conditions.

The separation was done using a Shimadzu Shim pack CN Column (4.6 × 150 mm, µm) with a flow rate of 0.5 ml/min. Mobile phases were pumped from Pump A and Pump D, containing 0.3% Formic acid and ACN, respectively. The Low-pressure Gradient method was adopted by keeping solvent D concentration at 2% and solvent A at 98%. The

low-pressure gradient method was employed to achieve a gradual change in solvent composition, optimizing the separation of hydroxyproline from other urinary components. This approach enhances peak resolution and reproducibility, which are crucial for accurate quantification in complex biological samples. The method's effectiveness was reflected in the clear, well-resolved chromatographic peaks observed during the analysis. Challenges encountered during the method's development phase included:

- Matrix effects: Interference from other compounds in urine samples was minimized by optimizing sample preparation and chromatographic conditions.
- Chromatographic separation: Achieving optimal separation required fine-tuning the column type, mobile phase composition, and gradient program.
- Mass spectrometry parameters: Balancing sensitivity and selectivity was addressed by adjusting ionization mode, collision energy, and fragmentor voltage.
- Sample stability: Ensuring hydroxyproline stability involved validating the method under various conditions, such as freeze–thaw cycles and long-term storage.

The column temperature was maintained at 50 °C, and the analyte injection was carried out at a volume of 10 µl. The chromatograms obtained for the standard and sample have been displayed in Figs. 1 and 2, respectively. The detection of the analyte was done by mass spectrometer [electron channel multiplier]. The Q3 fragmentation data of hydroxyproline is shown in Fig. 3.

Peaks for daughter ions were obtained at 68 and 86 as shown above.

Method Validation

In the method validation process, the developed LC–MS/MS method exhibited robustness and reliability in quantifying hydroxyproline levels in urine samples. The method's selectivity was illustrated by the nonexistence of interference peaks in chromatograms, ensuring accurate detection of hydroxyproline. Accuracy and precision assessments confirmed that the method provided reliable quantification of hydroxyproline concentrations within acceptable limits as per the ICH M10 guidelines. The ICH M10 guidelines provide a framework for the validation of bioanalytical methods to ensure that they are reliable, reproducible, and suitable for their intended purpose. These guidelines cover various aspects, such as selectivity, accuracy, precision, sensitivity, linearity, and stability. Validation according to ICH M10 ensures that the method can produce consistent and accurate results across different laboratories and conditions. Sensitivity testing revealed the method's ability to accurately detect hydroxyproline even at low concentrations, further highlighting its diagnostic utility. Stability studies demonstrated the method's reliability over various storage and handling conditions, ensuring consistent and reproducible results. Additionally, the calibration curve displayed superior linearity with a higher correlation coefficient (r^2), indicating the method's accuracy in quantifying hydroxyproline across a wide concentration range. Collectively, these validation results underscore the effectiveness of the LC–MS/MS method in accurately measuring hydroxyproline levels, thus enhancing its diagnostic potential for osteoarthritis.

Selectivity

The developed bioanalytical approach has proved to be highly selective in analyzing the standards. The blank and standard solutions were analyzed to assess selectivity. The chromatogram for the standard showed a peak at 3.2. There was no interference peak, where the analyte peaks should have a good intensity of more than $5e^4$. This high selectivity can be attributed to the involved nature of LC–MS/MS, particularly the meticulous optimization of chromatographic separation. By achieving optimal separation of hydroxyproline from other matrix components present in urine samples, LC–MS/MS provides accurate identification and quantification of the target analyte. The chromatograms for blank and standard are shown in Figs. 4 and 5, respectively.

Accuracy and Precision

In accordance with ICH M10 guidelines, the technique was validated for accuracy in which the analyzed samples' concentrations were found to be within ± 15% of the nominal values.

The % RSD was found to be much less than 20% as required.

% Recovery was found to be more than 70% and thus the method was proven to be effective in estimating hydroxyproline. The results for accuracy and precision are shown in Tables 1 and 2. Data for percentage recovery has been enlisted in Table 3.

Sensitivity

Five analytical runs were performed for the lowest concentration for each time of analysis. Detecting hydroxyproline at low concentrations is feasible due to the high sensitivity of the LC–MS/MS technique, which employs MRM mode for selective and precise detection. The optimized sample preparation and chromatographic conditions minimize matrix effects and enhance the analyte's signal, allowing for accurate detection even at low levels. The method was found

to be highly sensitive in the determination of hydroxyproline, and the data for the same has been provided in Table 4. The method's high sensitivity at the lowest concentration level is attributed to the optimized LC–MS/MS parameters, including the use of MRM mode that selectively detects hydroxyproline with minimal interference. This sensitivity is crucial for detecting low levels of hydroxyproline in early-stage osteoarthritis, facilitating early diagnosis and monitoring of the disease progression.

Stability

The mean recovered concentration for all three tests was found to be less than 10% within limits.

The stability of the analyte, when freeze-thawed, at both low and high concentrations was found to be within limits. The stability tests at low and high concentrations ensure that the method is robust under different conditions. The low concentrations test the method's sensitivity and limit of detection, while the high concentrations assess the method's capacity to handle upper-range values. The stability within limits indicates that the analyte does not degrade significantly, maintaining the accuracy and precision of the measurements across the tested range. The stability was observed to be within limits for both low and high concentrations in the case of bench-top stability.

The low and high concentrations of the analyte were found to show stability for the long-term stability test. Hydroxyproline stability was assessed under various conditions, including freeze–thaw, bench-top, and long-term storage. Stability studies confirmed that hydroxyproline remained stable, with mean recovered concentrations within ± 10%. This stability ensures precise and consistent measurements that are critical for reliable biomarker quantification in clinical samples. The results for stability have been displayed in Table 5.

Calibration curve

The range of concentrations taken here for plotting the calibration curve is 10 ng–320 ng ml^{-1}. The correlation coefficient (r^2) from a calibration curve quantifies the degree of linear relationship between the concentration of the analyte and its corresponding peak area. A high r^2 value close to 1 indicates a strong linear relationship, validating the method's ability to produce consistent and reliable results over the calibration range. The standard concentrations established a linear relationship with the peak area in which the correlation coefficient (r^2) was measured and found to be more than 0.99. The plot obtained is shown in Fig. 6. The peak areas obtained for each concentration have been enlisted in Table 6 along with the values for linearity.

Calibration curve details:

- Regression equation: $y = 805.28x + 80{,}131$
- Correlation coefficient (r^2): 0.9993
- Slope: 805.28
- Intercept: 80,131

Conclusion

In conclusion, the complexity of LC–MS/MS significantly contributes to the effectiveness of the developed bioanalytical method, enabling precise identification and quantification of biomarkers like hydroxyproline in urine samples of osteoarthritic patients. This method offers rich and detailed data, enhancing diagnostic accuracy and facilitating early detection and treatment monitoring of osteoarthritis. The bioanalytical method developed for estimating hydroxyproline in urine is primarily applied in diagnosing and monitoring osteoarthritis. It can also be used in research to study cartilage degradation and the effectiveness of treatments that are aimed at slowing or reversing this process. Additionally, this method could be extended to other conditions in which hydroxyproline is a relevant biomarker. The method exhibited a linear relationship of standard hydroxyproline concentrations with peak area, and an r^2 value of 0.999 was obtained. The optimized method was able to produce a good response in terms of good peak shape and intensity of more than 5 e^4. The approach was demonstrated to be accurate and precise with accuracy, and the RSD was expressed well in % within limits. The method was also highly sensitive in detecting and estimating hydroxyproline at LLOQ. The analyte was also observed to be stable under various applied conditions. Therefore, the developed bioanalytical method was proficient in estimating Hydroxyproline and can serve as an excellent tool in the diagnosis and treatment of osteoarthritis.

Author contributions All author is contributed to the design and methodology of this study, the assessment of the outcomes and the writing of the manuscript.

Funding No funds, grants were received by any of the authors.

Data availability All data generated or analysed during this study are included in the manuscript.

Code availability Not applicable.

Declarations

Conflict of interest There is no conflict of interest among the authors.

References

1. Biondi PA, Chiesa LM, Storelli MR, Renon P. A new procedure for the specific high-performance liquid chromatographic determination of hydroxyproline. J Chromatogr Sci. 1997;35(11):509–12. https://doi.org/10.1093/chromsci/35.11.509.
2. Jones BQ, Covey CJ, Sineath MH Jr. Nonsurgical management of knee pain in adults. Am Fam Physician. 2015;92(10):875–83 (**PMID: 26554281**).
3. Kindt E, Gueneva-Boucheva K, Rekhter MD, Humphries J, Hallak H. Determination of hydroxyproline in plasma and tissue using electrospray mass spectrometry. J Pharm Biomed Anal. 2003;33(5):1081–92. https://doi.org/10.1016/s0731-7085(03)00359-5.
4. Kivirikko KI. Urinary excretion of hydroxyproline in health and disease. Int Rev Connect Tissue Res. 1970;5:93–163. https://doi.org/10.1016/b978-0-12-363705-5.50008-7.
5. Langrock T, García-Villar N, Hoffmann R. Analysis of hydroxyproline isomers and hydroxylysine by reversed-phase HPLC and mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2007;847(2):282–8. https://doi.org/10.1016/j.jchromb.2006.10.015.
6. Legido-Quigley C, Stella C, Perez-Jimenez F, Lopez-Miranda J, Ordovas J, Powell J, van-der-Ouderaa F, Ware L, Lindon JC, Nicholson JK, Holmes E. Liquid chromatography-mass spectrometry methods for urinary biomarker detection in metabonomic studies with application to nutritional studies. Biomed Chromatogr. 2010;24(7):737–43. https://doi.org/10.1002/bmc.1357.
7. Manek NJ, Lane NE. Osteoarthritis: current concepts in diagnosis and management. Am Fam Physician. 2000;61(6):1795–804.
8. McLafferty FW. Mass spectrometric analysis. Molecular rearrangements. Anal Chem. 1959;31:82–7.
9. Mei H, Hsieh Y, Nardo C, Xu X, Wang S, Ng K, Korfmacher WA. Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. Rapid Commun Mass Spectrom. 2003;17(1):97–103. https://doi.org/10.1002/rcm.876.
10. Reed P, Holbrook IB, Gardner ML, McMurray JR. Simple, optimized liquid-chromatographic method for measuring total hydroxyproline in urine evaluated. Clin Chem. 1991;37(2):285–90.
11. Rydziel S, Canalis E. Analysis of hydroxyproline by high performance liquid chromatography and its application to collagen turnover studies in bone cultures. Calcif Tissue Int. 1989;44(6):421–4. https://doi.org/10.1007/BF02555971.
12. Smith R. Collagen and disorders of bone. Clin Sci (London, England: 1979). 1980;59(4):215–23. https://doi.org/10.1042/cs0590215.
13. Tiwari G, Tiwari R. Bioanalytical method validation: an updated review. Pharm Methods. 2010;1(1):25–38. https://doi.org/10.4103/2229-4708.72226.
14. Zhou S, Song Q, Tang Y, Naidong W. Critical review of development, validation, and transfer for high throughput bioanalytical LC-MS/MS methods. Curr Pharm Anal. 2005;1:3–14.

Authors and Affiliations

Anil Kumar Tallam[1] · Konatham Teja Kumar Reddy[2] · Uttam Prasad Panigrahy[3] · Alapati Sahithi[4] · S. Prema[5] · Jeetendra Kumar Gupta[6] · D. Jeslin[7] · Aparajita Neogi[8] · Vinod Thomas[9] · R. S. Chandan[10]

✉ Anil Kumar Tallam
anil.tallam312@outlook.com

Konatham Teja Kumar Reddy
teja.konatham1704@gmail.com

Uttam Prasad Panigrahy
uttampanigrahy@gmail.com

Alapati Sahithi
sahithireddy14@gmail.com

S. Prema
premspharm@gmail.com

Jeetendra Kumar Gupta
jk.gupta@gla.ac.in

D. Jeslin
Jeslin.123@gmail.com

Aparajita Neogi
aparajitaneogi@gmail.com

Vinod Thomas
Vinothomas066@gmail.com

R. S. Chandan
rschandan@jssuni.edu.in

[1] Department of Pharmacy, Shri Venkateshwara University, Rajabpur, India

[2] Department of Pharmaceutical Analysis, University College of Technology, Osmania University, Hyderabad, India

[3] Department of Pharmaceutical Analysis, Faculty of Pharmaceutical Sciences, Assam Down Town University, Guwahati, India

[4] Department of Pharmaceutical Analysis, Nalla Narasimha Reddy Education Society's Group of Institutions, Hyderabad, India

[5] Department of Pharmaceutical Chemistry, Crescent School of Pharmacy, B.S. Abdur Rahman Crescent Institute of Science and Technology, Peerakankaranai, India

[6] Department of Pharmacology, Institute of Pharmaceutical Research, GLA University, Chaumuhan, India

[7] Department of Pharmaceutics, Sree Balaji Medical College and Hospital Campus, Bharath Institute of Higher Education and Research, Chennai, India

[8] Department of Pharmaceutical Chemistry, JSS Academy of Higher Education and Research, Mysore, India

[9] Department of Pharmacy Practice, National College of Pharmacy, Shimoga, India

[10] Department of Pharmaceutical Chemistry, JSS Academy of Higher Education & Research, Mysore, India