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Analysis of a Urinary Biomarker Hydroxyproline for Clinical Assessment in Osteoporosis Patients

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The various degradation products derived from bone collagen are pyridinoline, deoxypyridinoline, N-telopeptides, and hydroxyproline are excreted in the urine. Among that hydroxyproline is an important biomarker used to correlate the osteoporosis condition. A simple method of reversed phased ultra-fast liquid chromatography (RP UFLC) with a PDA detector. Hydroxyproline, a UV inactive molecule, is derivatized with fluorenyl methyl chloroformate (FMOC) to form a UV active adduct. FMOC-hydroxyproline adduct and chlorthalidone as an internal standard were spiked to healthy adult urine and processed through strata phenomenex C18 cartridges. Extracts from cartridges were collected and analysed on Phenomenex C_{18} column (250 mm × 4.6 mm i.d., 5 µm particle size) as a stationary phase. The mobile phase was composed of acetonitrile and diethylamine buffer (DEA) pH 9.0, 50:50 v/v ratio with a flow rate of 0.8 ml min^{-1}. Elutes were analysed using a PDA detector at a wavelength of 265 nm. The proposed method was validated in accordance with the US-FDA guidance. In this study, the chromatographic peaks of hydroxyproline and chlorthalidone improved with a retention time of 2.5 and 6.4 min, respectively. Hydroxyproline exhibits excellent linearity with a correlation coefficient of 0.9994 (R^2). A new improved, validated, and easy bioanalytical method has been used for the clinical evaluation of hydroxyproline in biological fluids. It is used to quantify the biomarker concentration (hydroxyproline) in a urine sample and correlate to disease condition (osteoporosis).

Keywords: Hydroxyproline, Fluorenyl methyl chloroformate (FMOC), RP-UFLC, Bioanalytical method

INTRODUCTION

In osteoporosis, there is a loss of bone mass and destruction of bone architecture caused by the degradation of bone collagen [1-3]. In several products of bone collagen degradation like pyridinoline, deoxypyridinoline, N-terminal-telopeptides, and hydroxyproline are excreted in the urine [4-5]. It was found that the concentration of hydroxyproline (HYP) has significantly more in urine with postmenopausal osteoporosis women when compared with postmenopausal non-osteoporosis women [6]. Hence, they are used as a biomarker for the treatment or diagnosis of bone resorption sicknesses. Among such biomarkers used for bone disorders, hydroxyproline has been the most frequently used one [7]. Bone collagen contains 13% of HYP released during bone breakdown and, consequently, urinary estimation of HYP is predominantly used for indicating bone collagen turnover in normal and pathological conditions [8-9]. For the detection of this condition, both un-hydrolysed prolyl hydroxyproline (Pro-HYP) and a total 4-hydroxyproline (HYP) in urine have been suggested as disease biomarkers. Therefore, HYP act as a urinary marker that helps in identifying bone deterioration in osteoporotic patients. However, increased excretion of hydroxyproline in urine is coupled with the

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breakdown of connective tissue owing to the disease process, and only 4% of total urinary hydroxyproline is extracted in the free form in urine [10-13].

Due to the lack of chromophoric group in the HYP molecule, it was determined by derivatisation technique using reagent 7-chloro-4-nitrobenzofurazan (NBD-Cl) [14] in meat sample and 9-fluorenylmethoxycarbonyl chloride (FMOC) [15] in the collagen sample by an HPLC instrument with a fluorescence detector. In another report, HYP was determined by colorimetrically using reagent chloramine T followed by extracting with the ninhydrin reagent in serum samples [16] and rat skin [17]. The colorimetric method was reported in 24 h of urinary hydroxyproline estimation by treating urine samples with copper sulphate, NaOH, H_2O_2, and Ehrlich's reagent (4-dimethylamino benzaldehyde) and the results were expressed in mg/24 h [18-19]. These developed methods were less sensitive and tedious extraction procedures. A modified assay was developed to analyse HYP in tissue with the help of an aldehyde perchloric acid reagent [20] at a wavelength of 550 nm. In this developed method, analysis time, consuming more than 3 h. A nonderivatized HYP determination was carried out by the ion-exchange HPLC method in the rabbit model [21]. The developed method utilizes more than 40 min of run time for the quantification of HYP. In the present study, we developed a simple and sensitive chromatographic method to quantify the biomarker HYP concentration in the urine sample derivatized with Fluorenyl methyl chloroformate (FMOC) reagent to form UV adduct analysed by HPLC with a UV detector. Then this bioanalytical method is used for the clinical assessment of HYP in urine samples of postmenopausal woman osteoporosis patients against non-menopausal women osteoporosis.

EXPERIMENTAL

Materials and Instrumentation

A Shimadzu LC-2010 reverse phased ultra-performance liquid chromatography equipped with a PDA detector, and LC solution software was used for analysis. A phenomenex Kinetex C_{18} column was used for analysis. A mixture of acetonitrile and 0.2% DEA in the water was taken as a mobile phase to estimate hydroxyproline in the ratio of 50:50 (v/v), and the pH was adjusted to 9.0. The instrument flow rate was 0.8 ml min^{-1}, and the wavelength was set at 265 nm. The separated compound was detected by using a PDA detector with an injected volume of 10 µl.

Chemicals and Reagents

Acetonitrile, boric acid, potassium hydroxide, diethylamine (Merck), chlorthalidone (received as gift sample from Hetero Drugs Pvt Ltd., Hyderabad), fluorenyl methyl chloroformate (Sigma Aldrich), hydroxyproline (Sigma Aldrich), Millipore water (Direct Q). The standard stock solutions of HYP were prepared (100 mg) in 100 ml of Millipore water to get the desired concentration of 1000 mg l^{-1}. The derivatising reagent 0.015 M fluorenyl methyl chloroformate [FMOC] was prepared by dissolving 0.199 g of FMOC in 50 ml of acetone. The 0.05 M borate buffer pH 8.5 was prepared using 0.305 g of boric acid in 100 ml Millipore water, and the pH was adjusted to 8.5 with 1 M potassium hydroxide. The internal standard solution (SI) was prepared to weigh 50 mg of chlorthalidone in 50 ml of HPLC acetonitrile (1000 mg l^{-1}).

Patient Population and Study Design

The present work was carried out in the department of orthopedic, JSS Medical College & Hospital, Mysore, with a total number of 20 volunteers. Among them, 10 members are considered a control group, and the rest is considered a study group. The control group consists of 10 premenopausal women's in the age group of 25-45 years, and the study group consists of 10 postmenopausal women's in the age group of 46-65 years. In the study, only non-alcoholic and non-smokers participants were included, and they were free from fracture history last year.

Inclusion Criteria

Premenopausal (25-45 yrs), postmenopausal women (46-65 yrs), and who visits the outpatient orthopaedic department of JSS Medical College & Hospital, Mysore.

Exclusion Criteria

Pregnant women, breastfeeding, patients with anticonvulsant medications, and hormone replacement therapy are excluded from the study. In addition, patients with the second type of osteoporosis, liver disease, renal

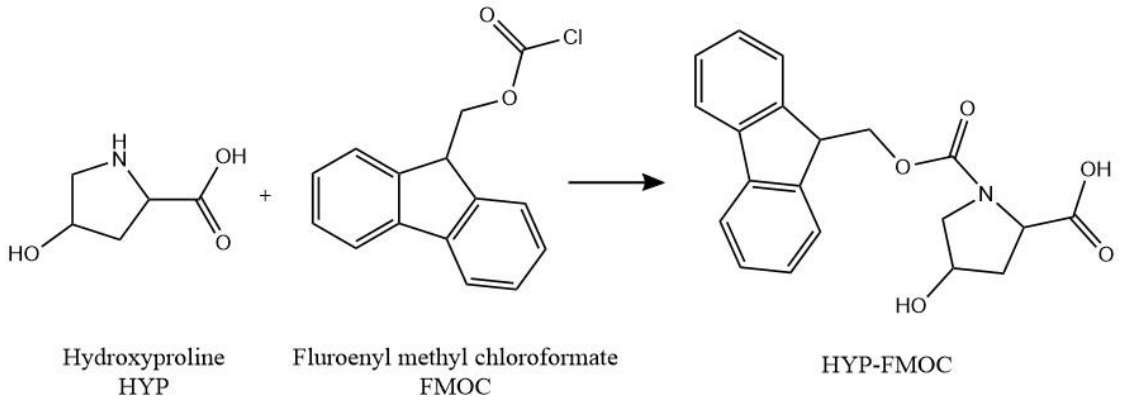

Fig. 1. Chemical reaction between hydroxyproline (HYP) and fluorenyl methyl chloroformate (FMOC).

disease, metastatic bone disease, and chronic, debilitating illness (cancer, AIDS) are also not considered for the study.

Ethical Clearance

The study was approved by the Institutional Ethics Committee of JSS Medical College & Hospital, Mysore, India (JSSMC/IEC/231220), and informed consent was obtained from every study participant. Under aseptic conditions, fasting urine samples were only collected from both groups at the baseline level.

Solid-phase Extraction

Firstly, the cartridges (Phenomenex C18 strata) were activated with 0.5 ml of acetonitrile and then equilibrated with 0.5 ml of water. Then, 0.5 ml of spiked urine sample was loaded and washed the cartridges with water to eliminate the matrix, metabolites, and other interference compounds. The analyte of interest was eluted with acetonitrile: diethylamine pH 9.0 (50:50 v/v).

Pre column Derivatization

HYP a UV inactive molecule, is derivatized with fluorenyl methyl chloroformate (FMOC) to form an active UV adduct. FMOC-hydroxyproline adduct is analysed by the UFLC method, as shown in Fig. 1. FMOC-hydroxyproline adduct and chlorthalidone as internal standard were spiked to healthy adult urine and processed through strata phenomenex C_{18} cartridges. Extracts from cartridges were collected and analysed on the reverse-phase column. The mobile phase was composed of acetonitrile and diethylamine (DEA) buffer pH 9.0 in the ratio 50:50 v/v at a flow rate of 0.8 ml min^{-1}. Elutes were analysed using a PDA detector at a detection wavelength of 265 nm. The proposed method was validated as per guidelines given in the US-FDA [20].

Spiking of Derivatized Hydroxyproline to Urine

Before analysing urine samples for hydroxyproline estimation, it was subjected to hydrolysis process by treating with 1 N HCl to avoid interference of other compounds like proteins and followed by neutralization with 0.1 N NaOH. After that, 600 µl of the pre-treated urine sample and 200 µl of each concentration of fluorenyl methyl chloroformate hydroxy proline complex along with 200 µl of 50 mg $l^{-1}$of the chlorthalidone internal standard was taken into a centrifuged tube. The detailed chemical reaction between HYP and reagent was shown in Fig. 1. The mixture was passed through Phenomenex C_{18} strata Solid Phase Extract (SPE) cartridges. The eluates from C_{18} SPE cartridges were filtered through 0.22 µm PTFE filters. 20 µl of each solution with a concentration range of 3-18 mg l^{-1} was injected in triplicate into the column, and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimization of HPLC Analysis

In the method development process, various trials are

Table 1. Chromatographic Parameters of Hydroxyproline and Internal Standard

Drug	Retention time (min)	Peak area	Tailing factor (Tf)	Theoretical plates (N)
Hydroxyproline	2.47	14695	0.945	4412
Internal standard	6.23	25843	1.245	3104

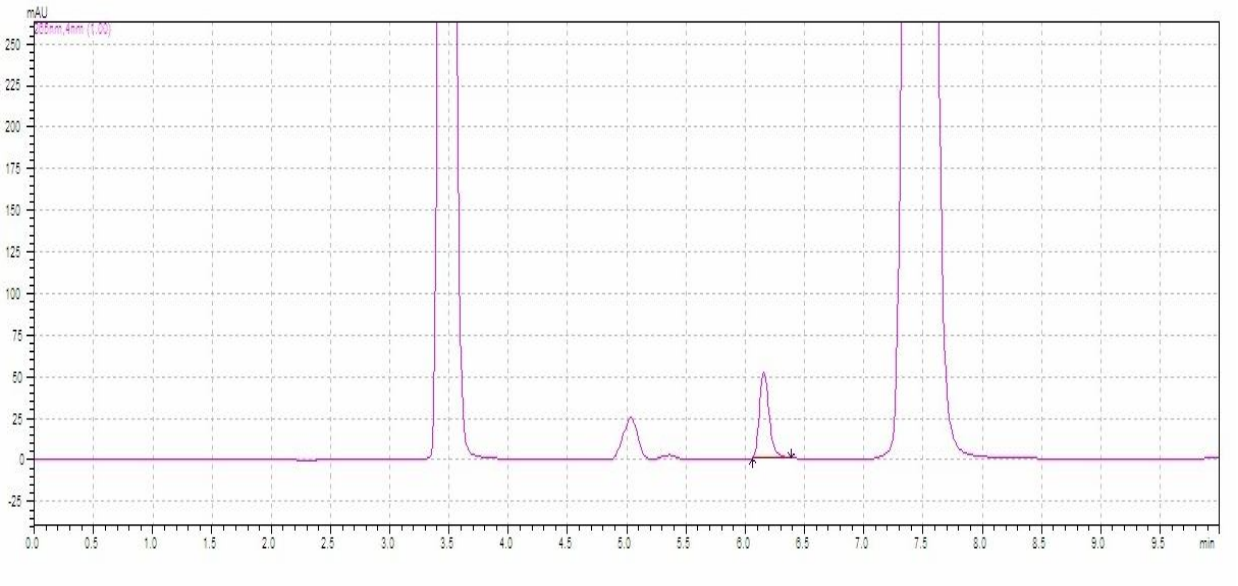

Fig. 2. Blank chromatogram (blank urine and internal standard Rt 6.4 min)

performed to get an optimized result. Initially, the method was started by using a mobile phase of acetonitrile: 0.2% DEA in water [pH 7.0] (80:20 v/v). Both hydroxyproline and unreacted FMOC reagent peaks were not sharp, and there was a merging of the peaks. So, this method was found to be not suitable. To get an optimized chromatogram, the pH of the mobile phase was changed to 8.0 (Acetonitrile: 0.2% DEA in water (80:20 v/v)). However, both hydroxyproline and unreacted FMOC reagent peaks were detected with few interfering peaks. To minimize various interfering peaks, the pH of the mobile phase was changed to 8.5. In that case, the hydroxyproline peak was broad, and the number of theoretical plates was less than 2000. To get a sharp derivatised hydroxyproline peak with better retention time and satisfactory theoretical plates, the pH of the mobile phase was changed to 9.0. The flow rate of the instrument was fixed with 0.8 ml min^{-1} and the wavelength at 265 nm. The separated compound was detected by using a PDA detector. The chromatographic parameters of the hydroxyproline and internal standard were provided in Table 1. The blank chromatogram, standard LLOQ chromatogram of HYP were shown in Figs. 2 and 3, respectively. Similarly, the chromatogram of HYP present in osteoporotic female patient's serum samples was shown in Fig. 4.

Selectivity

The method can show the response of a single analyte. It

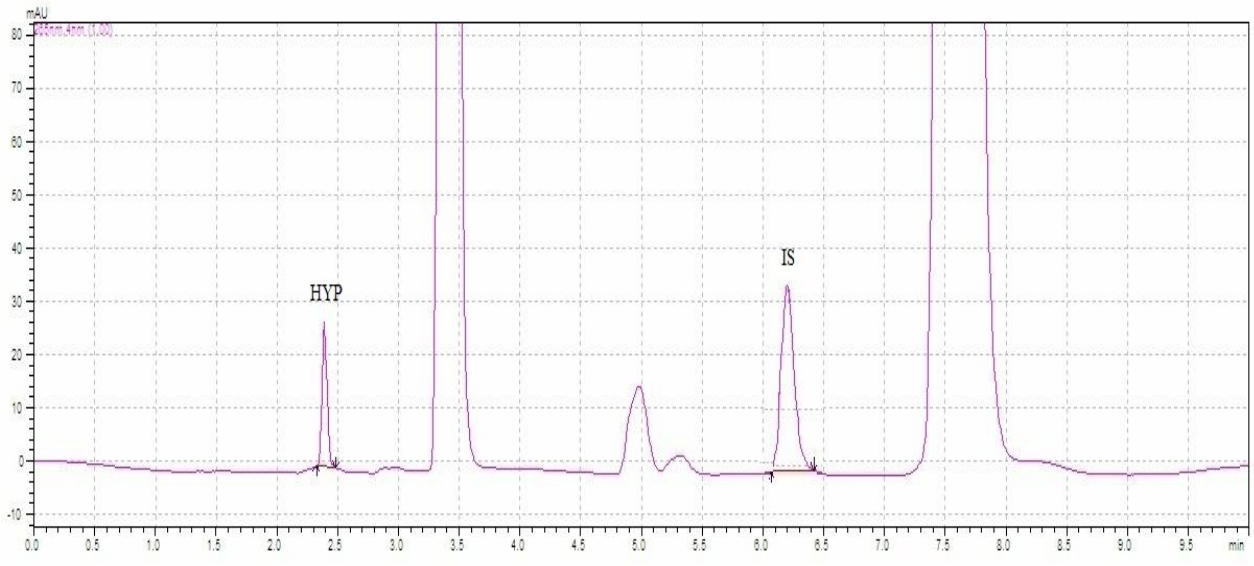

Fig. 3. Standard chromatogram of Hydroxyproline (HYP) (18 µg ml^{-1}) with Rt 2.5 min.

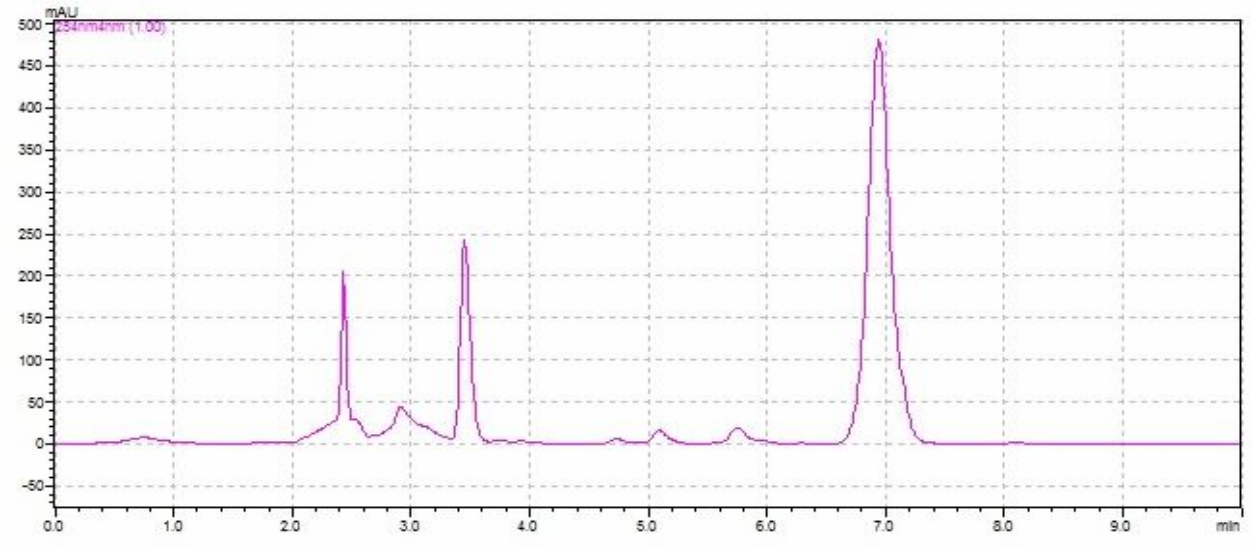

Fig. 4. Chromatogram for hydroxyproline (HYP) osteoporotic patients**.**

is ensured at the LLOQ level. Spiked samples of LLOQ were injected six times, along with six injections of blank plasma samples. It is found that all chromatograms were analyzed and found that the HYP and the IS peaks did not interfere with any endogenous components separated in the column. It confirms that the method was selective for the analysis of HYP of the extracted samples.

Calibration Curve

Various concentrations of hydroxyproline in the range

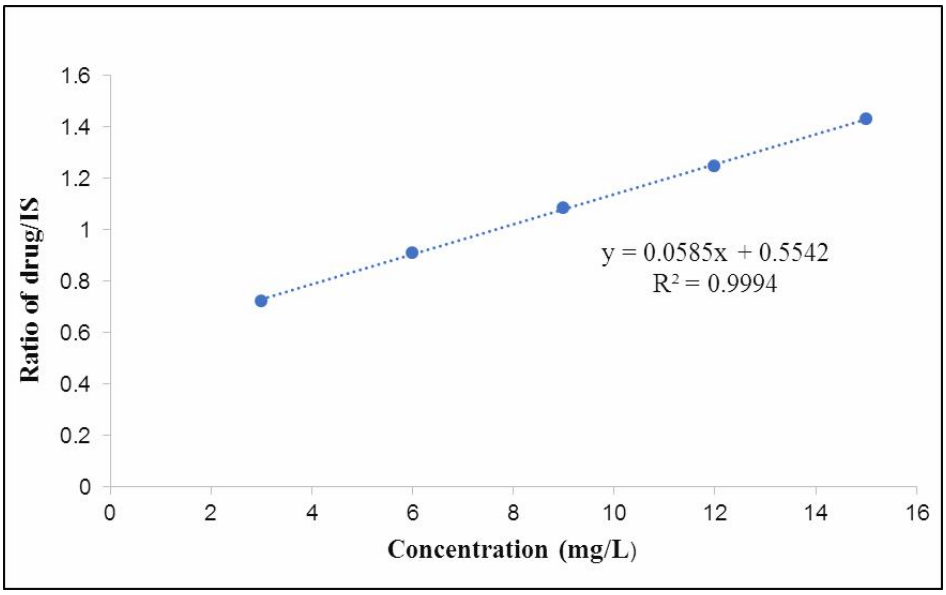

Fig. 5. Linear curve of hydroxyproline (HYP).

Table 2. Calibration Study of Hydroxyproline

The concentration of hydroxyproline (mg l^{-1})	Peak area of hydroxyproline	Peak area of internal standard (50 mg l^{-1})	The ratio of drug/IS
3	40580	55985	0.724
6	51023	55983	0.911
9	60896	55981	1.087
12	69851	55984	1.247
15	80241	55986	1.433
18	91234	55982	1.629

of 30-180 mg l^{-1} were prepared from a stock solution of 300 mg l^{-1}. Then 1 ml of each concentration was pipetted into a different 10 ml volumetric flask. Now add 0.8 ml FMOC and 0.8 ml of borate buffer pH 8.5 into each volumetric flask and made up to the mark with diluent. The solutions were kept aside for 20 min filled into vials by filtering through a 0.22 µm PTFE filter. Next, 10 µl of each solution was injected in triplicate into the column, and chromatograms were measured. Finally, the linear relation was obtained by establishing a calibration curve between the ratio of drug/IS and the concentration (Fig. 5).

Linearity

The linearity of the method was performed as per the

Table 3. Regression Parameters of Hydroxyproline

Parameter	Values
Range	3-18 mg l^{-1}
Regression equation	Y = 0.0585x + 0.5542
Correlation coefficient	0.9994
Slope	0.0585
Intercept	0.5542

Table 4. Recovery of Hydroxyproline (n = 6)

Concentration (mg l^{-1})	Hydroxyproline extracted from biological matrix	Unextracted authentic hydroxyproline	Mean recovery (%)
LQC (6)	51211	52134	98.22956
MQC (12)	70089	71189	98.45482
HQC (18)	91108	91990	99.0412

Table 5. Intra-day Accuracy and Precision Data of Hydroxyproline (n = 5)

Theoretical concentration (mg l^{-1})	Mean measured concentration (mg l^{-1})	Precision %RSD	Accuracy (%)
LLOQ (3)	2.97	1.28	99.11
LQC (6)	6.05	1.65	100.88
MQC (12)	11.89	0.88	99.12
HQC (18)	18.04	0.48	100.25

US-FDA guidelines by spiking six concentrations in the range of 3-18 mg l^{-1} of hydroxyproline. The calibration data and regression parameters of HYP and as shown in Table 2 and Table 3, respectively. The regression analysis was carried out, and the coefficient of correlation was determined, and it is well between the acceptance criteria, *i.e.*, the regression coefficient is 0.9994.

Recovery

The efficiency of the extraction procedure employed was determined by comparing the peak area of the analyte extracted from the replicated QC samples (n = 6) by a solid-phase extraction process with the peak area of the analyte from pure standards at equivalent concentrations. Recovery rates of hydroxyproline were obtained by taking three

Table 6. Inter-day Accuracy and Precision Data of Hydroxyproline (n = 5)

Theoretical concentration (mg l^{-1})	Mean measured concentration (mg l^{-1})	Precision (%RSD)	Accuracy (%)
LLOQ (3)	2.903	1.46	96.77
LQC (6)	5.99	1.16	99.83
MQC (12)	11.89	0.606	99.08
HQC (18)	17.89	0.525	99.98

Table 7. Stability of Hydroxyproline in Human Urine (n = 3)

Concentration (mg l^{-1})	Mean concentration found (mg l^{-1})	Precision (%RSD)	Accuracy (%)
Freeze thaw stability			
LQC (6)	5.97	1.08	99.611
HQC (18)	17.97	0.57	99.85
Short term stability			
LQC (6)	5.96	1.15	99.38
HQC (18)	17.83	0.141	98.92
Long term stability			
LQC (6)	5.81	1.03	96.83
HQC (18)	17.71	0.11	98.38

different concentrations such as 6, 12 and 18 mg l^{-1}, and the percentage of recovery was calculated and reported in Table 4. Recoveries were observed to range from 98.22-99.04% of HYP. The acceptance criteria for the developed method as per the US-FDA the acceptance criteria are ± 15% of nominal concentrations except LLOQ. Therefore, the results are within the acceptance criteria.

Precision

In the precision study, the determination of intra and interday precision was carried out in six replicates of the same concentration were injected into the chromatography instrument on three different non-consecutive days. The concentrations of these samples were determined by a calibration graph and the percentage of the coefficient of variation (CV) was calculated. The values are within the standard range and confirm the precision of the method as shown in Tables 5 and 6.

Stability Studies

It is part of the validation study to monitor the stability of hydroxyproline in urine samples. The tests were carried under different conditions at low and high concentrations in the linear range. In addition, stock solution stability (6 h), benchtop stability (12 h), freeze-thaw stability (three cycles), and the short-term stability (20 ± 10 °C for 25 days)

Table 8. Amount of Hydroxyproline in Urine Samples of Osteoporotic Patients

Patient No.	Amount of hydroxyproline in urine samples (mg g^{-1} creatinine)	
	Premenopausal women (Control group)	Postmenopausal women (Osteoporotic patients)
1	14.0	27
2	17.2	25
3	12.0	30
4	16.0	19
5	11.0	29
6	15.1	32
7	10.6	18
8	17.4	20
9	13	21
10	15.3	34
	14.16 ± 0.7748	25.50 ± 1.821[a]

The statistical method used to compare data was the Z test and paired t-test.
[a]P\0.001 highly significant.

were tested. As can be seen from Table 7, the results of the stability of hydroxyproline were found to be stable in human urine.

Reference Range

Collagen stability is mainly depending on the hydroxyproline content. Increased concentrations of hydroxyproline in urine/serum are usually associated with the degradation of connective tissue [21]. Testing of hydroxyproline in the serum and the urine is common. It was found that the amount of hydroxyproline in urine samples from osteoporosis patients was in the range of 18-34 mg l^{-1}. Table 8 shows that urinary hydroxyproline level was significantly increased compared with premenopausal women. According to the previous reports, the urinary hydroxyproline concentration of premenopausal females was found to be 17 mg g^{-1} creatinine [22-24]. Based on the standard values, it shows that the selected patient urine samples contain hydroxyproline levels were more than 17 mg g^{-1} creatine and concluded that they are suffering from osteoporosis.

CONCLUSIONS

The bio-analytical procedures were adopted to quantify hydroxyproline in urine as per the standard guidelines of USFDA. Based upon the nature of the drug sample, proper selection of the stationary phase and the mobile phase was performed. Hydroxyproline is a polar drug, and it is readily soluble, and is analysed by the reverse-phase column. A Phenomenex Kinetex C18 column was used to separate hydroxyproline in appropriate isocratic conditions. A mixture of acetonitrile and diethylamine was chosen as the mobile phase by adjusting the pH to 9.0 using a 0.05 M borate buffer (pH 8.5). Chlorthalidone was used as an internal standard to improve the accuracy, precision, and other parameters of the bioanalytical method. The parameters like system suitability, linearity, accuracy, precision, sensitivity, LOD and LOQ of the biological matrix were assessed and found within limits. All the stability studies were performed, and results were found within limits. Urine samples were taken from the 10 osteoporosis patients and the amount of hydroxyproline was estimated at mg g^{-1} creatinine. The quantity of HYP found in the patient's urine sample concentration is above standard values. So the selected patients had osteoporosis. The developed method can be used economically and efficiently to quantify the concentration of biomarkers in urine and diagnose osteoporosis, even during spine deformity and spinal surgery. This non-invasive technique can be used for screening and early detection of postmenopausal women's osteoporotic changes.

ABBREVIATIONS

HYP-Hydroxyproline
Pro-HYP: Propyl hydroxyproline
RP UFLC-Reverse Phase Ultra-Fast liquid chromatography
NBD-Cl: 4-Chloro-7-nitrobenzofurazan
FMOC: Fluorenyl methyl chloroformate
PDA: Photodiode array detector
FMOC-Cl: Fluorenyl methoxycarbonyl chloride

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Conflict of Interest

No conflicts to declare

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