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Evaluation and comparison of bone resorption in premenopausal and postmenopausal women by estimating urinary crosslink biomarkers with high performance liquid chromatography

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ABSTRACT

A simple, precise and rapid reversed phase HPLC method was developed for the estimation of urinary crosslink Pyridinoline (PYD) and Deoxypyridinoline (DPD) in premenopausal and postmenopausal women. The method was carried out on a C-18 reverse phase column (LichroCART, 150 X 4mm, 5µm; Merck) using 2 mM Heptafluorobutyric acid: Acetonitrile (95:5) as the mobile phase and detection was carried out using Water[32] 2475 multi λ fluorescence detector (Excitation λ 295 nm, Emission λ 395 nm). The retention times for PYD and DPD were found to be 10.7 ± 0.2 minutes and 11.7 ± 0.2 minutes respectively. The % recovery for PYD and DPD in urine matrix was found to be 102.89 % and 108.33 % respectively. Form the study it was found that the postmenopausal women are at a higher risk of bone resorption due to high bone fragility as compared to the premenopausal women.

Keywords: HPLC, Pyridinoline (PYD), Deoxypyridinoline (DPD), Heptafluorobutyric acid, Acetonitrile.

INTRODUCTION

Osteoporosis is a debilitating metabolic bone disease characterized by low bone mass and architectural deterioration of bone tissue that leads to enhanced bone fragility and fracture typically at hip, vertebrae and distal forearm. In this disorder, the progressive decrease in bone mass leads to an increased susceptibility to fracture, which can result in substantial morbidity and mortality [1, 2].

This disease is caused by enhanced activity of osteoclasts, the bone resorbing cells as compared to osteoblasts, the bone forming cells. It is common in older individuals and specifically postmenopausal women due to deficiency of hormones mainly estrogen which controls the bone metabolism.

Early identification of risk of developing osteoporosis is important to prevent osteoporosis and associated morbidity. This can further reduce the heavy expenses involved in management of osteoporosis.

Optimal treatment and prevention of osteoporosis require modification of risk factors, particularly smoking cessation, adequate physical activity and attention to diet, in addition to pharmacologic intervention. Number of

pharmacologic option like estrogen replacement therapy, calcium and vitamin-D supplement [3], anabolic agents like fluoride, growth hormone and IGF-I, parathyroid hormone, statins and strontium ranelate [4] are found to useful in treatment of osteoporosis. Bisphosphonates inhibit bone resorption by reducing the recruitment and activity of osteoclasts and increasing apoptosis [5-7].

Various markers of bone metabolism serve as important tool in the diagnosis of osteoporosis. These include determination of bone mineral density (BMD), bone formation markers like serum osteocalcin, alkaline phosphatase and bone resorption markers like urinary crosslinks.

The measurement of bone resorption using urinary biochemical markers has been studied as a clinical tool to predict fracture risk in postmenopausal women [8, 9]. In prospective nested case-control analysis [10–12] and cohort studies [13–16], elevated bone resorption markers have been found to be associated with an increased risk of osteoporosis. For the identification of high-risk individuals, combination strategies, using bone markers, and bone mineral density (BMD) have been shown to increase test specificity without any loss in sensitivity [17].

Urinary crosslinks, Pyridinoline (PYD) and Deoxypyridinoline (DPD) are one of the earliest markers used in the diagnosis of osteoporosis. When collagen is degraded as a part of regular tissues turnover or because of disease-induced increased collagen degradation, these compounds can be found in blood and are excreted in urine in peptide bound form or as a free molecule. The amount of pyridium cross-Links in the blood or urine are well established indicators of bone resorbtion since bone collagen has highest turnover as compared to other tissues [18,19].

Measurement of the urinary excretion of pyridinium crosslinks has advantages over other markers of collagen breakdown, such as urinary hydroxyproline [20], because the crosslinks occur only in mature fibrils and are not released from precursors or intermediate forms of collagen.

However, most excreted pyridinoline are in peptide bound form, so that an acid hydrolysis step is needed to generate the free form [21]. They are mainly used to investigate bone metabolism and to manage bone diseases [22-24]. Other condition for which PYD and DPD have been suggested as biomarkers for arthritis [25] and certain type of cancer [26,27]. The ease of collection of urine makes the ultimate choice for the analysis of these markers in urine. Also non invasive nature of sample collection provides further advantage. With respect to metabolic bone diseases, measurement of pyridinium cross-links can be used to identify people with elevated bone resorption, increase bone formation or otherwise altered bone metabolism to predict response to antiresorptive therapy in postmenopausal women and to assess risk of osteoporosis where treatment may include antiresorptive or selective estrogen receptor modulator.

A variety of chromatographic and immunological methods to measure either free pyridinium crosslinks or the sum (total) of free and peptide-bound pyridinium cross-links have been developed. The chromatographic methods are HPLC [28,29]; LCMS [30] assays that simultaneously measure PYD and DPD, whereas the immunological methods measure either one pyridinium cross-link only or the sum of PYD and DPD. Most assays measure these compounds in urine with only a few assays using serum [31,32]. Various clinical studies have been performed in different population to assess the correlation between urinary crosslink levels and risk of osteoporosis. The technique, with some modifications in the HPLC separation, has now been used extensively in monitoring collagen degradation in a wide range of arthritic diseases [33] and metabolic bone disorders [34,35]. However no such comparative study has been carried out in Indian female population.

MATERIALS AND METHODS

Chemicals and reagents

Heptafluorobutyric acid was purchased from Sigma Chemical Co. St. Louis, USA. HPLC grade acetonitrile and tetrahydrofuran were obtained from Thomas Baker (Chemical) Pvt. Ltd., Mumbai, India. n-butanol and hydrochloride acid were from E-Merck (India) Ltd., Mumbai. Glacial acetic acid AR obtained from Sd-fine chem. Ltd., Mumbai. Triple distilled water (TDW) was prepared in lab using distillation assembly. Urinary calibration standard of PYD and DPD, Internal standard and solid phase extraction (SPE) cartridges containing CF-1 grade cellulose were purchased from Chromsystem GMBH, Germany.

Apparatus
The experiment was carried out with Water- Millennium [32] HPLC system equipped with Water 1525 Binary HPLC pump and Water 2475 multi λ fluorescence detector. The column used was C-18 reverse phase (LichroCART, 150 X 4mm, 5µm; Merck)

HPLC conditions
2mM Heptafluorobutyric acid: Acetonitrile (95:5 v/v) were used as a mobile phase and detection were carried out at Excitation λ 295 nm, Emission λ 395 nm using 50 µL injection volume and 1.2 mL/ min flow rate.

EXPERIMENTAL PREPARATIONS
Buffers
Wash buffer : n-Butanol: Glacial acetic acid AR: TD water (4:1:1)
Extraction buffer : Glacial acetic acid AR: Acetonitrile (2: 3)
Elution Buffer : Heptafluorobutyric acid (pH - 4.2; 2 mM)

Urine collection
First void urine samples were collected from 50 premenopausal and 50 post menopausal female volunteers.

Urine calibration standard (UCS)
Lyophilized urine calibration standard was dissolved in 1.0 mL of TDW and the vial was kept (with occasional gentle shaking) for about 10 min. until fully reconstituted. Then it was stored in fridge at 2-8^{o}C. The PYD and DPD concentrations in UCS were reported to be 1277 pmol/mL and 260 pmol/mL respectively.

Hydrolysis of the urine samples
100 µL urine or UCS, 20 µL internal standard and 100 µL concentrated hydrochloric acid were taken into appropriate labeled hydrolysis vessels. The vessels were carefully sealed with the temperature stable screw caps and incubated overnight for 18 hr at 110^{0}C. After hydrolysis the samples were cooled slowly to room temperature.

Quality control standard (QC).
The Urine calibration standard with known concentrations i.e. QC-1 at low (PYD-28 pmol/mL and DPD-5.7 pmol/mL), QC-2 at medium (PYD-652 pmol/mL and DPD-132.7 pmol/mL) and QC-3 at high (PYD-1277 pmol/mL and DPD-260 pmol/mL) concentration were analyzed in triplicates for six different days. Recovery, accuracy and precision were determined by estimating concentration of QC.

Sample preparation
2.0 mL of washed buffer was applied to each SPE cartridges and centrifuged at about 1000 rpm until buffer has passed through completely and the collected buffer was thrown away. Then the extraction buffer (2.0 mL) was added to each hydrolyzed urine sample and mixed by vortexing. The entire sample volume was loaded onto the conditioned cartridge and centrifuge at about 1000 rpm, and the collected elute was thrown away. Wash buffer (2.5 mL X 3) was applied to each SPE cartridges and centrifuged at 2000 rpm and the collected elute was thrown away. Then tetrahydrofuran (1.0 mL) was applied to each SPE cartridges and centrifuge (2000 rpm) so as to remove any traces of n-butanol, and the SPE cartridges were air dried. Finally, the crosslinks were eluted in fresh tube with 1.0 mL of 2 mM heptafluorbutyric (pH 4.2) acid and 50 µL of elute was directly injected onto HPLC.

Accuracy and Precision
The quantitation of PYD and DPD was done by single point calibration method. Accuracy and precision of the assay method were determined at low, medium and high concentration levels. One set of samples consisting of low, medium and high concentrations of QC samples were processed and assayed in triplicates for six days. Intra- and inter-batch accuracy was determined by calculating the % bias from the theoretical concentration. Precision, in terms of relative standard deviation (RSD) was obtained by subjecting the data to one-way analysis of variance (ANOVA).

Recovery
Recovery study was carried out by standard addition method and the drug was calculated bycomparing the peak area response at low, medium and high QC samples.

DISCUSSION

A simple and selective fluorometric HPLC assay method was developed for total Pyridinoline (PYD) and Deoxypyridinoline (DPD) in urine by using Water-Millennium [32] HPLC software. The PYD and DPD were isocratically separated on a C_{18} reverse phase column using 2 mM heptafluorobutyric acid:acetonitrile (95: 5 v/v) as mobile phase. The detection was carried out at excitation λ 295 nm and emission λ 395 nm. The retention times for PYD and DPD were found to be 10.7 ± 0.2 and 11.7 ± 0.2 respectively (Fig. 1).

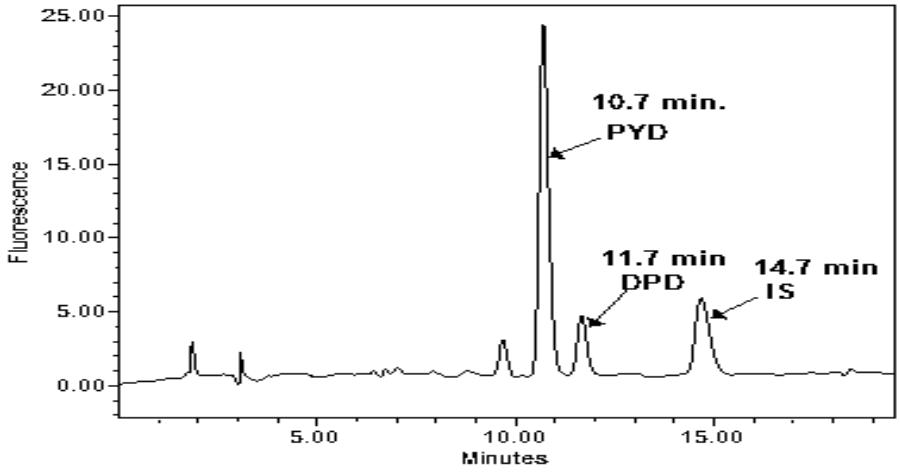

FIG 1. Representative chromatograms of PYD and DPD with internal standard

The method was further validated with respect to accuracy and precision. All the validated parameters were within acceptable range and shown in Table 1 and 2. The mean % recovery for PYD and DPD in urine matrix was found to be 102.89 % and 108.33 % respectively as shown in Table 3. The analysis was carried out on 50 premenopausal as well as on 50 postmenopausal women volunteers for comparison of bone resorption by estimating content of PYD and DPD. As seen from Table 4, the PYD levels in post menopausal women were significantly higher than that of premenopausal women (mean 139.44 ± 10.98 vs. 73.13 ± 4.10, $p<0.05$). The DPD which is highly specific for bone was also significantly higher in postmenopausal women as compared to premenopausal women (mean 39.34 ± 4.98 vs. 14.31 ± 0.84, $p < 0.05$).

Table 1- results of accuracy of urinary markers

Analyte	Parameter	% Bias		
		QC-1	**QC-2**	**QC-3**
		PYD-28 pmol/ml DPD-5.7 pmol/ml	PYD-652 pmol/ml DPD-132.7 pmol/ml	PYD-1277pmol/ml DPD-260 pmol/ml
PYD	Inter- assay variability	4.02	4.51	5.22
	Intra- assay variability	4.03	4.45	2.32
DPD	Inter- assay variability	13.16	6.66	9.26
	Intra- assay variability	17.29	6.45	7.89

Table-2 Results of precision Study of Urinary markers

Analyte	Parameter	% RSD		
		QC-1	**QC-2**	**QC-3**
		PYD-28 pmol/ml DPD-5.7pmol/ml	PYD-652 pmol/ml DPD-132.7 pmol/ml	PYD-1277 pmol/m DPD-260 pmol/ml
PYD	Inter-assay	5.5	9.1	6.93
	Intra-assay	6.71	3.53	5.57
DPD	Inter-assay	14.3	4.85	4.6
	Intra-assay	16.75	3.44	2

Table 3- Results of recovery study of urinary Markers

Analyte	% Recovered*		
	QC-1 PYD-28 pmol/ml DPD-5.7 pmol	**QC-2** PYD-652 pmol/ml DPD-132.7 pmol/ml	**QC-3** PYD-1277 pmol/ml DPD-260 pmol/ml
PYD	102.28± 3.52	104.51 ± 5.49	101.87 ± 4.08
DPD	109.86 ± 8.20	107.78 ± 2.86	107.36 ± 3.01

** Mean of six observations, ± Standard Deviation*

Table IV. PYD and DPD in premenopausal and postmenopausal volunteers

Subjects	PYD (pmol/mmol)		DPD (pmol/mmol)	
	Premenopausal	**Postmenopausal**	**Premenopausal**	**Postmenopausal**
1	37.27	106.88	12.11	28.25
2	63.35	322.55	17.39	44.11
3	66.64	77.98	8.65	17.35
4	76.97	130.12	12.62	20.82
5	131.27	69.24	16.89	40.51
6	70.77	85.88	14.52	22.08
7	116.32	81.39	21.59	25.79
8	87.37	236	21	42.79
9	48.21	141.74	12.1	158.04
10	39.52	142.57	4.95	30.35
11	42.58	112.09	17.34	23.02
12	68.66	327.76	22.62	38.88
13	71.95	83.19	13.88	25.12
14	82.28	135.33	17.85	15.59
15	136.58	74.45	22.12	35.28
16	76.08	91.09	19.75	16.85
17	121.63	86.6	26.82	20.56
18	92.68	241.21	26.23	138.21
19	53.52	146.95	17.33	37.58
20	44.83	147.78	10.18	25.12
21	34.21	137.36	6.88	30.35
22	42.9	136.53	12.16	46.21
23	82.06	230.79	3.42	19.45
24	111.05	76.18	7.39	22.92
25	65.46	80.67	11.66	42.61
26	125.96	64.03	8.29	24.38
27	71.66	124.91	16.36	27.89
28	61.33	72.77	15.77	44.89
29	58.04	317.34	6.87	106.14
30	31.96	101.67	5.23	32.45
31	39.69	110.28	14.21	115.94
32	65.77	325.95	19.49	26.15
33	69.06	81.38	10.75	42.01
34	79.39	133.52	14.72	15.25
35	103.69	72.64	18.99	18.72
36	34.85	89.28	16.62	38.41
37	60.93	84.79	23.69	19.98
38	64.22	239.4	23.1	23.69
39	72.55	145.14	14.2	40.69
40	128.85	145.97	7.05	28.24
41	74.01	103.48	10.01	29.28
42	119.55	319.15	15.29	44.11
43	90.59	74.68	6.55	17.35
44	51.44	126.72	10.52	20.82
45	42.75	65.84	14.79	40.51
46	36.29	82.48	12.42	22.08
47	44.98	77.99	19.49	25.79
48	84.14	232.6	18.9	42.79
49	113.09	138.34	10.12	30.35
50	67.45	139.17	4.56	121.23
Mean	**73.13**	**139.44**	**14.31**	**39.34**
SEM	**4.1**	**10.98**	**0.84**	**4.48**
p-value	**0.0207**		**0.0342**	
CI	**95%**			

CONCLUSION

Determination of total PYD and DPD in urine is an important aspect in early diagnosis of osteoporosis. Various clinical studies have been performed in different population to assess the correlation between urinary crosslinks levels and risk of osteoporosis. No such study has been carried out in Indian female population. Therefore in proposed work urinary total PYD and DPD were measured in 100 Indian female volunteers. The concentrations of these markers were normalized with urine creatinine values. From the analytical data of 100 female volunteer it is concluded that the postmenopausal women are more susceptible for bone resorption, osteoporosis and higher risk of fracture in comparison with premenopausal women.

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