# Circadian Variation of Urinary Excretion of Elastin and Collagen Crosslinks (44291)

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*Abstract.* **Urinary levels of collagen- and elastin-crosslink amino acids have been used as biologic markers for degradation of collagen and elastin in the body. Circadian variation of collagen-crosslink amino acids is well known. The current study was undertaken to determine whether there is also circadian variation in excretion of elastin-crosslink amino acids. We used an isotope dilution-HPLC assay to measure the elastin-crosslink amino acids, desmosine (DES) and isodesmosine (IDES), and the collagen-crosslink amino acids, hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP), in urine. Sixteen apparently healthy subjects collected urine from 5:00 to 7:00 AM, and from 5:00 to 7:00 PM. Mean urinary excretion of DES and IDES in women was 56% and 41% higher ($P < 0.001$), respectively, in AM versus PM specimens when normalized by the creatinine content of the urine specimen. For men, the corresponding values were 11% and 13% higher (not statistically significant). Mean urinary excretion of HP and LP in women was 61% and 71% higher ($P < 0.001$), respectively, in AM versus PM specimens. For men, the corresponding values were 11% and 19% higher (not statistically significant). Differences were not found in the AM versus PM rates of excretion of creatinine in men or women. These findings demonstrate the occurrence of circadian variation in HP, LP, DES and IDES in women but not in men. We conclude that the time of collection of urine specimens, especially from women, must be taken into consideration in using the urinary levels of these crosslink amino acids as biologic markers for collagen or elastin degradation.** [P.S.E.B.M. 1998, Vol 218]

Collagen and elastin are synthesized as discrete monomeric units that are crosslinked to their polymeric state following the action of lysyl oxidase on epsilon amino groups of lysyl or hydroxylysyl residues (1). Once crosslinked, collagen and elastin usually exhibit long half-lives *in vivo* (2–4). The crosslink amino acids characteristic of collagen include hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP). The characteristic crosslink amino acids of elastin include desmosine (DES) and isodesmosine (IDES). These four crosslink amino acids are not metabolized, and their presence in a normal diet does not affect their urinary levels normalized by creatinine content (5–9). Accordingly, HP and LP, and DES and IDES, have been used as biologic markers of degradation in the body of collagen and elastin, respectively (10–12).

A number of investigators have found marked diurnal variation in urinary excretion of HP and LP in pre- and postmenopausal women (13–15) with a lesser effect in men (16). The purpose of the present study was to determine if the urinary excretion of DES and IDES also exhibits a circadian pattern. Using an isotope dilution-HPLC procedure to measure urinary DES, IDES, HP, and LP, we found that these four crosslink amino acids show similar diurnal patterns of excretion with significantly higher levels in the morning in premenopausal women but not in men.

## Methods

**Subjects and Study Protocol.** Sixteen healthy adult lifetime never-smokers (8 premenopausal women, mean age ± 1 SD of 34 ± 12 yr; 8 men, 39 ± 12 yr), were recruited from among the employees of Boston University Medical Center and their relatives, after Institutional Review Board approval. Volunteers were instructed to empty their bladder at 1700 (5:00 PM) without collecting the speci-

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men, record the time, and, at 1900 (7:00 PM) to void again, collect and freeze the specimen, and record the time. The following morning, the subjects were to awaken at 0500 (5:00 AM), empty their bladder without collecting the specimen, record the time, return to bed, awaken at 0700 (7:00 AM), void again, collect and freeze the specimen, and record the time. The time intervals were chosen as those approximating times of minimum and maximum rates of excretion of collagen crosslinks, respectively (15).

**Processing of Urine Specimens.** Urine specimens were brought into the laboratory for processing, and their volumes were measured. Measurement of DES, IDES, HP, and LP in urine was performed using a method developed in our laboratory and described in detail elsewhere (10, 17). Aliquots were removed for creatinine determination using a kit (Sigma Diagnostics, St. Louis, MO).

[$^{14}$C]DES (500 dpm, 0.05 nmol) and [$^{14}$C]HP (300 dpm, 0.03 nmol) were added to aliquots of urine. The urine samples were combined with an equal volume of 12 *N* HCl, refluxed under $N_2$ at 110°C to hydrolyze peptide bonds, dried, and subjected to gel filtration steps. Finally, fractions that were highly enriched in the relatively high-molecular-weight DES, IDES, HP, and LP crosslink amino acids were analyzed by HPLC methodology. DES and HP eluting from the HPLC were collected for liquid scintillation spectrometry to determine recovery. Values for DES and HP were calculated using an isotope dilution calculation and normalized for the creatinine content of the aliquot of urine that was analyzed. Values for IDES and LP were calculated using the same dilution factors as found for DES and HP, respectively, since we have found that recoveries of DES and IDES are not different, and HP and LP are not different (10, 17). DES and IDES values were expressed as μg/g creatinine, and HP and LP were expressed as nmol/mmol creatinine in accordance with the literature.

The creatinine content of each specimen was divided by the time elapsed between the initial (discarded) and final (collected) voids, to calculate the rate of creatinine excretion. A ratio of the creatinine excretion rate for the AM period divided by that for the PM period was calculated for each subject to assess the effect of time of day on creatinine excretion.

**Statistical Analysis.** Urinary excretion of the elastin-specific crosslink amino acids, DES and IDES, and the collagen-specific amino acids, HP and LP, were examined in relation to time of day (AM or PM) and gender by full repeated measures ANOVA with a time by gender interaction term, using the SAS software package (SAS Institute, Cary, NC). Analysis of the effect of time of day on crosslink excretion and creatinine excretion, stratified by gender, was performed using paired *t* tests. A *P* value < 0.05 was considered significant. Values given are mean ± 1 SD.

## Results

Considering all 16 subjects, urinary excretion of each of the four crosslink amino acids, DES, IDES, HP, and LP, was significantly higher in AM than PM specimens ($P < 0.001$ for each comparison, Table I). However, for each crosslink amino acid, the difference between AM and PM values was greater in women than in men ($P < 0.01$ for each gender × time interaction). This is also shown in Figure 1. There was no significant difference between AM and PM creatinine excretion rates in either men or women (Table I).

For women, mean urinary excretion of the elastin degradation products, DES and IDES, were 56% and 41% higher ($P < 0.001$ for each), respectively, in specimens collected from 5:00 to 7:00 AM than from 5:00 to 7:00 PM (Table I). For men, the corresponding values were 11% and 13% higher ($P = 0.21$ and $P = 0.07$, respectively). Mean urinary excretion of the collagen degradation products, HP and LP, in women were 61% and 71% higher ($P < 0.001$ for each), respectively, in specimens collected from 5:00 to 7:00 AM than from 5:00 to 7:00 PM (Table I). For men, the corresponding values were 11% and 19% higher ($P = 0.23$ and $P = 0.11$, respectively).

## Discussion

In this study we found that women excreted 41% to 71% more HP, LP, DES, and IDES from 5:00 to 7:00 AM than from 5:00 to 7:00 PM (all $P < 0.001$). In men, the urinary excretion of these amino acids was only 11%–19% greater in the morning than in the evening (all, NS, Table I). The diurnal variation of collagen-derived crosslink amino acids in healthy premenopausal women has been described previously, but this is the first report of diurnal variation of elastin-derived crosslink amino acids in women.

Since the rates of crosslink excretion are normalized by the creatinine content of the urine aliquot, we considered the

**Table I.** Differences in excretion of crosslink amino acids based upon time of day

| Variable | Men | Women |
|---|---|---|
| desmosine (μg/g creatinine) | | |
| AM | 5.68 ± 1.86 | 6.84 ± 1.51 |
| PM | 5.10 ± 1.49 | 4.39 ± 1.06 |
| *P* value | 0.21 | <0.001 |
| isodesmosine (μg/g creatinine) | | |
| AM | 4.65 ± 1.23 | 5.53 ± 0.93 |
| PM | 4.10 ± 1.11 | 3.93 ± 0.92 |
| *P* value | 0.07 | <0.001 |
| hydroxylysylpyridinoline (nmol/mmol creatinine) | | |
| AM | 26.65 ± 6.45 | 39.25 ± 11.15 |
| PM | 23.91 ± 4.70 | 24.44 ± 6.53 |
| *P* value | 0.23 | <0.001 |
| lysylpyridinoline (nmol/mmol creatinine) | | |
| AM | 5.78 ± 1.85 | 7.83 ± 2.70 |
| PM | 4.86 ± 1.45 | 4.59 ± 1.60 |
| *P* value | 0.11 | <0.001 |
| creatinine (g/24 hr) | | |
| AM | 2.21 ± 0.63 | 1.39 ± 0.58 |
| PM | 1.97 ± 0.72 | 1.23 ± 0.22 |
| *P* value | 0.51 | 0.47 |

*Note.* *P* values are for paired *t* tests of the AM versus PM values within gender.

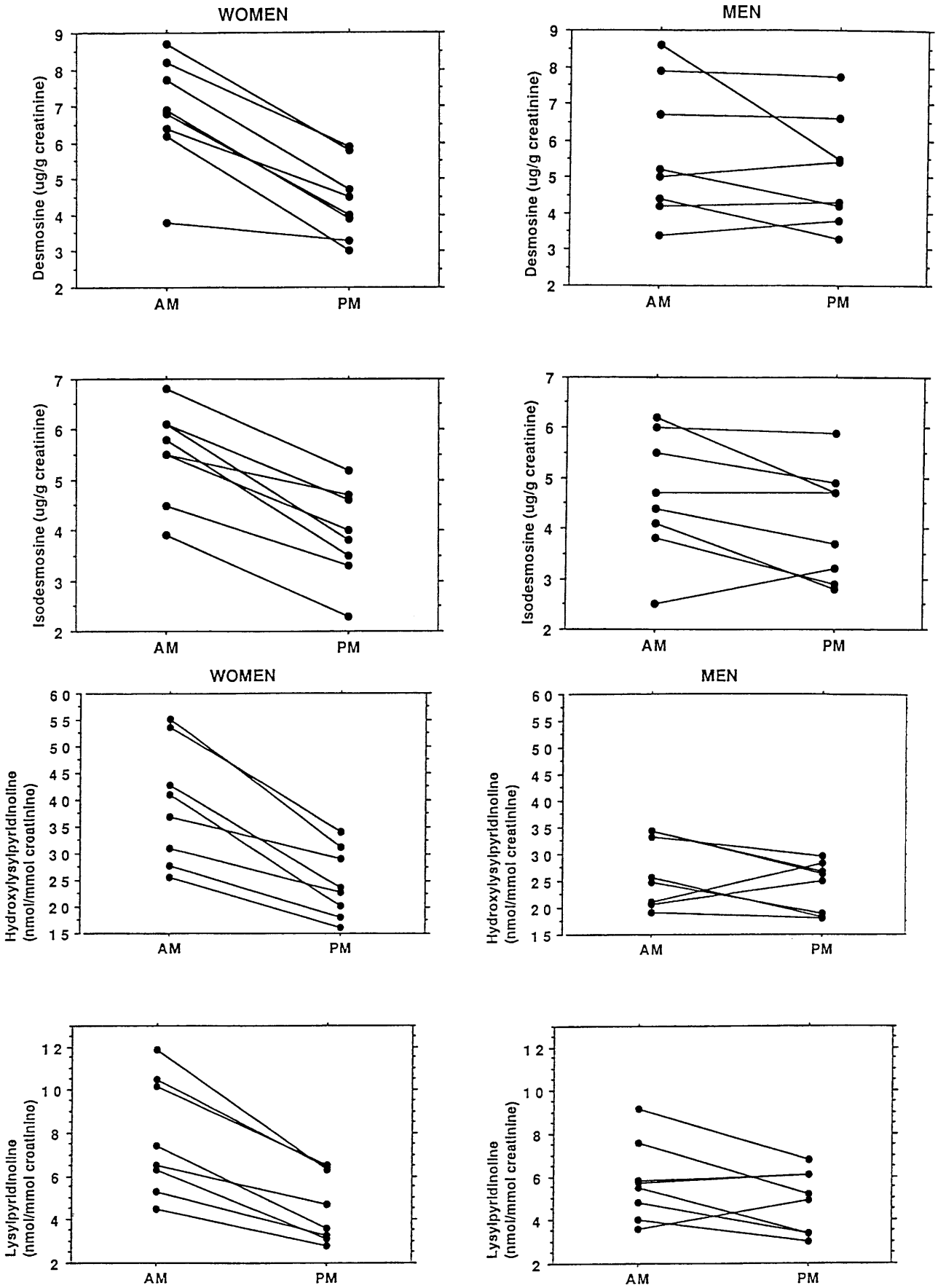


**Figure 1.** The AM and PM values of DES, IDES, HP, and LP for individual subjects grouped by gender.

possibility that the observed diurnal variation might be due to a lower rate of creatinine excretion from 5:00–7:00 AM than from 5:00–7:00 PM. However, there were no significant differences in creatine excretion rates between the 5:00–7:00 AM and 5:00–7:00 PM periods for either women or men. This is in accord with one published study that found creatinine excretion to be constant throughout the day (18), although another recent study found diurnal variations in creatinine excretion (19).

Bone collagen resorption is thought to be the major source of urinary HP and LP (20). Bone collagen exhibits an HP/LP ratio of approximately 3.5, as compared with soft tissue collagen in which the HP/LP ratio is usually greater than 10 (20). If increased bone resorption were the major source of the increased HP and LP, then the HP/LP ratio of the AM specimen should match the HP/LP ratio of bone collagen more closely. On the other hand, if the major source of the increase were soft tissue collagen, the HP/LP ratio should rise. For example, we reported that the HP/LP ratio in the urine of women between Weeks 1 and 5 following parturition frequently exceeded 10, as the uterus underwent postpartum involution (21). This high HP/LP ratio compared with a usual value for nonpregnant, healthy women of approximately 5. We found a small and statistically insignificant decrease in the HP/LP ratio for the AM as compared with the PM period (5.18 ± 0.90 vs 5.49 ± 0.75, respectively) suggesting little diurnal variation in the tissue origins of these excreted crosslinks. However, the number of subjects is too small to identify subtle changes in HP/LP ratio.

The tissue source(s) for increased elastin crosslinks in women at 5:00–7:00 AM is not known. In a recent study in hamsters, we identified a number of tissue pools of DES besides lung, large arteries, and skin (4). These additional tissue pools have not been examined in humans.

Bone collagen resorption is known to be controlled by the osteoclast, a monocyte-derived cell (22). The cells that are sources of elastolytic enzymes and could contribute to elastin degradation include macrophages, which are also monocyte-derived, as well as neutrophils, smooth muscle cells, and platelets (23). Circadian variation in cytokine levels might similarly influence osteoclasts and cells capable of elastin degradation although gender differences in the circadian variation of cytokine levels have not been reported.

One practical implication of these findings is to show that the time of collection of the urine specimen, especially from women, can affect the values obtained for DES, IDES, HP, and LP and should be considered when using them as biologic markers for elastin or collagen degradation. Differences in DES excretion for men and women have not been found in earlier studies although urine specimens were collected at times during the day when maximum elevation of urinary DES levels from women were not likely to be present (10).

Finally, one may speculate on the effect of using the circadian variation in collagen and elastin degradation to affect collagen and elastin degradation therapeutically. Schlemmer and coworkers have reported that neither bedrest, age, menopause, nor osteopenia influence the circadian variation (14). Calcium supplementation at 2300 hr greatly attenuated the circadian rhythm of bone resorption in women, as measured by two markers (LP and $NT_x$), abolished the nighttime increase in levels of parathyroid hormone, and reduced the overall daily bone resorption in healthy premenopausal women (24). Morning calcium supplementation was less effective. The effect of calcium supplementation on the circadian pattern of excretion of the elastin crosslink amino acids, DES and IDES, is not known. If DES excretion were to exhibit a similar circadian pattern under pathologic conditions as it does in healthy premenopausal women, administration of therapeutic agents, such as elastase inhibitors, might be most efficacious in the late evening.

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