Assessment and Recommendations on Factors Contributing to Preanalytical Variability of Urinary Pyridinoline and Deoxypyridinoline


Background: Pyridinoline (PYD) and deoxypyridinoline (DPD) are two of the most extensively characterized biochemical bone markers, but the interpretation of results is hampered by biologic and other preanalytical variability. We reviewed factors contributing to preanalytical variation of pyridinium cross-links in urine.

Methods: We searched four databases for English-language reports on PYD and/or DPD in urine. Searches were restricted to humans, except for studies of stability, when the search was expanded to other species. The 599 identified articles were supplemented with references from those articles and with articles known to the authors.

Results: The mean reported within-day variability was 71% for PYD (range, 57–78%) and 67% for DPD (range, 53–75%). The mean interday variability was 16% for both DPD and PYD (range for PYD, 12–21%; range for DPD, 5–24%). The mean intersubject variabilities across studies were 26% for PYD (range, 12–63%) and 34% for DPD (range, 8–98%) for healthy premenopausal women and 36% (range, 22–61%) and 40%, (range, 27–54%) for postmenopausal women, respectively. Specimen instability and errors in creatinine measurements were additional sources of variability.

Conclusions: Intra- and intersubject variability can be reduced by collecting specimens at a specific time of the day and by maintaining similar patient status at each specimen collection regarding factors such as medications and dietary supplements.

Biochemical bone markers can provide a valuable tool in the management of metabolic bone diseases. Their most recognized application in clinical practice is for monitoring treatment for osteoporosis as an adjunct to bone mineral density measurements. Other applications that have been investigated include their use as a diagnostic tool for bone diseases other than osteoporosis and as predictive markers for bone loss and the risk of bone fracture (1–3). Biochemical markers are available to assess both bone formation and bone loss (resorption). Because most metabolic bone diseases are characterized by an increase in bone resorption, these particular biochemical markers are of special interest. Our focus in this review will be exclusively on the pyridinium cross-links pyridinoline (PYD), hydroxylysylypyridinoline and deoxypyridinoline (DPD; lysylpyridinoline) because they are part of an extensive laboratory standardization program at the CDC designed to improve the measurement of biochemical markers and risk factors associated with selected chronic diseases. A description of other bone markers, including other resorption markers, such as type I collagen telopeptide breakdown products, is provided in several reviews (4–7).

One of the main issues hampering the interpretation of PYD and DPD results for clinical use is preanalytical and analytical variation (8). The effects of analytical variability can be minimized through standardization of results of

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laboratory measurements by controlling imprecision through the use of good laboratory practices and by validating proper method calibration through appropriate traceability to reference methods, using suitable reference materials. These strategies have already demonstrated their benefit for other analytes, such as cholesterol, and are currently being implemented for PYD and DPD, as stated above, as well as for other bone markers (9).

Despite extensive reports on preanalytical variability for various analytes, no overview has been published to date that addresses in detail this source of variability and its contribution to unreliable PYD and DPD measurements. Furthermore, there has been no previous effort to establish guidelines for minimizing or controlling the effects of preanalytical variability on PYD and DPD results. Preanalytical variability for other biochemical bone markers is acknowledged, but the expert panel convened to consider this topic focused exclusively on preanalytical variability in pyridinium cross-links measurements, with the primary objective of presenting guidelines that can facilitate comparability and interpretation of PYD and DPD results. Evaluating the clinical applicability or performance of these markers and defining clinical decision levels is beyond the scope of this review and have been addressed in other reviews (3, 10). Likewise, comparing the clinical utility of the various formation and resorption markers will not be addressed here. No recommendation of pyridinium cross-links measurements over other resorption markers is implied.

Preanalytical variability includes variation from specimen collection and handling, as well as biologic variation of the individual being tested. Biologic variation is composed of an intraindividual (within-subject, both within- and among-day) component and an interindividual (between-subjects) component. Recognizing and controlling factors that contribute to preanalytical variability is crucial for the interpretation of bone marker results. Identifying true biologic change by taking into consideration an individual’s biologic variability is the basis for the concept of least significant change for monitoring patients during treatment, as proposed by Hannon et al. (11). To compare data between patients or assess patient data within a reference interval requires that factors affecting the biologic variability be identified and controlled or minimized consistently.

Most data have been generated using urine, and only limited results are available from other specimen types (12–15). Therefore, because of the paucity of reviewed literature on specimens other than urine, this review will address only urinary PYD and DPD. Pyridinium cross-links can be measured as total PYD and DPD (sum of free and peptide bound) after acidic hydrolysis or as free, non-peptide-bound molecules only. HPLC methods and immunoassays that measure free as well as total pyridinium cross-links in urine have been developed and are reported to correlate well (16, 17). Differences in the response to medications or other events have been observed between free pyridinium cross-links and total cross-links (18–22). However, the observed differences are generally only in magnitude or in the time point of the change, rather than suggesting different clinical interpretations. PYD and DPD are reported to change in parallel in various conditions with the exception of arthritis, in which there is a relatively greater increase in PYD (23). Thus, for the purpose of this review, distinctions between free and total pyridinium cross-links, or between PYD and DPD, were made only if they were regarded as necessary for general understanding.

LITERATURE SEARCH
We searched the literature for scientific articles published until January 2001 that dealt with PYD and/or DPD (keywords used: PYD, DPD, PYR, DPYR, pyridinoline, deoxypyridinoline, lysylpyridinoline, and hydroxypyridinoline). We limited the search to English as the language, urine as specimen, and humans as subjects, except in the case of stability, when the search was expanded to other species. A second search was performed on the topic of creatinine measurement. Both searches were done in MEDLINE, Current Contents, The Cochran Database of Systematic Reviews, and CANCERLIT (1969–2000). A total of 599 articles were identified as potentially relevant, and additional references from these articles were examined. We also included additional literature that had not been identified by the electronic search but was found to be appropriate.

Assessment of Biological Variability

INTRAINDIVIDUAL VARIABILITY

Circadian cycle. Both PYD and DPD measured in urine follow a circadian or diurnal cycle with a peak in the early morning and nadir in the late afternoon to early evening. The magnitude of the diurnal change, i.e., nadir concentration divided by peak concentration, expressed as a percentage is listed in Table 1. The median change was 73% for PYD (range, 57–78%) and 70% for DPD (range, 53–75%). The authors of one study (24) observed a night/day difference of greater magnitude (nadir/peak ratio of 57% for PYD and 54% for DPD) than the others; in that study, nine specimens were collected over 24 h. The authors of another study, which involved one nighttime and one daytime collection, observed a night/day difference in peptide-bound DPD but not in free DPD (25). The magnitude of the daily cycle is expected to be higher as the number of collections during the cycle increases; fewer but longer collection intervals will smooth changes during each collection and not detect the full amplitude. Because sources of variability other than diurnal variation are reported in terms of distribution (CV), comparisons of diurnal variation with other sources of variation are difficult. One study (26) used a statistical package to convert amplitude to distribution. The authors found that a 70% (nadir/peak) amplitude corresponded to a CV of 10.4%. The mean of nadir/peak values for PYD and DPD...
was 69%. Therefore, the proportion of variability attributable to diurnal variation can be assumed to approximate a CV of 10%.

Although the excretion of cross-links in urine varies by condition, the nadir/peak ratio seems to be reasonably consistent (24, 27). Postmenopausal and elderly osteopenic women demonstrated similar relative amplitudes in their diurnal patterns. The diurnal cycle is reportedly unaffected by hormonal changes during the menstrual cycle (24). Although differences in amplitude of the diurnal cycle between men and women have been reported, the differences were not substantial (28). Children demonstrated a diurnal pattern similar to that of adults (29). After 5 days of bed rest, the total cross-link excretion was reported to increase, but the relative amplitude of the cycles remained the same (24). Consistent with the large increases from afternoon to early morning, wake/sleep cycle (nyctohermal) differences are expected; in fact, daytime concentrations were reported to be ~78% of night concentrations (30).

*Day-to-day variability.* Even with morning collections at the same time each day for each person, longer-term biologic variability contributes to the overall variation. The reported day-to-day variation (Table 2) averaged 15.7% for PYD (range, 12–21% in five collection conditions/studies) and 17.4% for DPD (range, 5–24% in six combinations). Weekly variation was similar, averaging 15.8% for PYD and 17.1% for DPD. One study with three collection conditions was eliminated from the DPD series because the reported variations were much lower than those in the other studies, perhaps because of the small number of collections (two in 2 weeks) (31). In another study, the monthly variation in DPD monitored over 5 months in both males and females averaged 16.7%. The reported variations for DPD were consistently higher than those for PYD. One collection per day over 5 days and up to 21 days showed approximately the same variability as collections made weekly over 5 weeks. Similar results were obtained when samples were collected monthly over 5 months. Consequently, collection of daily specimens over 5 days seems a reasonable approximation of biologic variation. In fact, one study reported that three collections over 3–5 days captured the full range of biologic variation (32).

### INTERINDIVIDUAL VARIABILITY

The only study specifically intended to assess intersubject variability of urinary pyridinium cross-links reported intersubject variabilities of 14.2% (PYD) and 13.0% (DPD) for postmenopausal healthy women and 18.7% (PYD) and 37.8% (DPD) for postmenopausal osteopenic women (8). This study included only a small number of postmenopausal women. To obtain information across different studies and groups of subjects, we calculated the biologic variability based on the following equation:

\[
CV_B^2 = CV_I^2 - CV_A^2
\]

In which \( CV_I \) is the total variability calculated from mean concentrations and standard deviations, and \( CV_A \) is the analytical variability (intraassay variability) reported for each study. We considered only studies using first morning void (FMV), second morning void (SMV), or 24-h urines. If the analytical variability was missing, we assumed a variability of 10%, which is the average analytical variability mentioned by the authors cited in Table 3. On the basis of the obtained biologic variability, the intersubject variability (\( CV_{\text{inter}} \)) was estimated using the following equation:

\[
CV_{\text{inter}}^2 = CV_B^2 - CV_{\text{intra}}^2
\]

with an intraindividual variation (\( CV_{\text{intra}} \)) of 16% as discussed previously.

As shown in Table 3, the average interindividual variability was highest in children [DPD, 48% (range, 23–82%); PYD, 35% (range, 10–40%)], followed by postmenopausal women [DPD, 40% (range, 27–54%); PYD, 36% (range, 22–61%)] and premenopausal women [DPD, 34% (range, 8–98%); PYD, 26% (range, 12–63%)]. This estimate is not applicable to men, for whom negative values were obtained with the available data. Within the group of children, the highest variability was observed between the ages of 10 and 18 years. Within the group of menopausal and postmenopausal women, no correlation

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**Table 1. Diurnal variation in PYD and DPD.**

<table>
<thead>
<tr>
<th>PYD, %</th>
<th>DPD, %</th>
<th>Population</th>
<th>Assay</th>
<th>Reference</th>
</tr>
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<tbody>
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<td>78b</td>
<td>75b</td>
<td>9 young, healthy adults</td>
<td>HPLC</td>
<td>(209)</td>
</tr>
<tr>
<td>57</td>
<td>54</td>
<td>18 premenopausal women</td>
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<td>(113)</td>
</tr>
<tr>
<td>78</td>
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<td>9 healthy premenopausal women</td>
<td>HPLC</td>
<td>(24)</td>
</tr>
<tr>
<td>74</td>
<td>70</td>
<td>9 healthy postmenopausal women</td>
<td>Immunoassay</td>
<td>(115)</td>
</tr>
<tr>
<td>72</td>
<td>65b</td>
<td>31 young adult women</td>
<td>Immunoassay</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38 healthy adults</td>
<td>Immunoassay</td>
<td>(161)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 postmenopausal women</td>
<td>HPLC</td>
<td>(211)</td>
</tr>
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</table>

Median: 73%  Median: 70%

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\* Nadir/peak × 100%.

\* Nadir-to-peak amplitudes estimated from plots.
between age groups and variability was found. There were substantial differences in the variability as well as in the mean concentrations in the different studies even when data were grouped for methods and collection mode.

Factors Affecting Urinary PYD and DPD Excretion

The following factors are reported to affect urinary PYD and DPD excretion and therefore may contribute to the inter- and intrasubject variability.

INFLUENCE OF AGE, SEX, GEOGRAPHIC EFFECTS, AND RACE

Of the three studies investigating premenopausal women, the changes in the urinary excretion of PYD and DPD in women 20–50 years of age were small and reached a maximum of 10–15% at age 50 compared with age 20, with the highest excretion rates at age 20–29 (33–35). When this age range was excluded, no significant changes were observed in the age group 30–50. Usually menopause is defined as an absence of menses for at least 12 months. However, before menopause there is a poorly defined period of perimenopausal status, which could last several months or years and is characterized by irregular menses and subtle estrogen deficiency associated with increased follicle-stimulating hormone. The only study investigating the influence of the perimenopausal status on urinary pyridinoline cross-links excretion reported no differences between premenopausal women and perimenopausal women (36). Two cross-sectional studies compared total PYD and DPD concentrations of premenopausal women and very recently menopausal women of similar ages. They showed 30–55% higher pyridinium cross-links excretion rates in postmenopausal women than in premenopausal women, with a larger increase in DPD vs PYD (36, 37). One study suggested that there was no change in free DPD excretion through menopause (36).

These data have been confirmed by a longitudinal study of women who became postmenopausal during follow-up (38). After menopause, changes in the excretion rates of urinary PYD appear small (33) with a slight increase of ~8–15% during age 50–80 in the urinary excretion of total PYD (33, 39), but not of total DPD (33). For urinary free PYD and DPD, the increase is reported to be larger (49–70% for free PYD and 50% for free DPD from age 50 to age 80). This increase does not seem to be related to

| Day-to-day | 16 | 24 | 21/21 days | 24-h | 3 postmenopausal women | HPLC | (209) |
| Week-to-week | 21 | 22 | 5/5 weeks | 24-h | 6 men and women | HPLC | (162) |
| Month-to-month | 16 | 8/5 months | FMV | Men | Immunoassay | (161) |

<table>
<thead>
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<th>Population</th>
<th>Assay</th>
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<td>(42)</td>
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<td>(16)</td>
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<td>Men</td>
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<td>17 men and women</td>
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<td>Month-to-month</td>
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<td>FMV</td>
<td>Men</td>
<td>Immunoassay</td>
</tr>
</tbody>
</table>

Mean 16 13 (17)

Non-peptide-bound cross-links measured by HPLC.

Week-to-week mean CV deleting outliers from one study (31).

| Day-to-day | 16 | 24 | 21/21 days | 24-h | 3 postmenopausal women | HPLC | (209) |
| Week-to-week | 21 | 22 | 5/5 weeks | 24-h | 6 men and women | HPLC | (162) |
| Month-to-month | 16 | 8/5 months | FMV | Men | Immunoassay | (161) |

Mean 17

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decreased creatinine excretion resulting from decreased muscle mass (35).

The available studies on urinary pyridinium cross-links in men report concordant results with high excretion rates in young men at age 20, which decrease and reach a nadir at age 50–60 (34, 40, 41). After the age of 60, the urinary excretion of free PYD and free and total DPD increased slightly with age, by ~20–35%.

Pyridinium cross-link concentrations in children are reported to be 5- to 10-fold higher than in adults and to change drastically from birth to cessation of bone growth at 20–25 years of age. The pattern of change during childhood is described consistently among different investigators, with the highest concentrations reported in newborns and relatively constant or slightly decreasing concentrations from early childhood (age 3–5 years) to start of puberty. No information is given whether these small changes are statistically significant (42–45). Increasing pyridinium cross-link concentrations are observed with progressing pubertal stage, leading to a peak followed by a decrease to normal adult excretion rates (29, 42, 44, 46–49).

Higher concentrations of total and free pyridinium cross-links have been observed in preterm newborns than in term newborns (50–52). The high concentrations observed in newborns declined in the first 4–8 weeks after birth (51). During puberty, concentrations increase to a peak and then decrease to adult concentrations (42, 43, 45, 53). In studies in which the pubertal stage was assessed by categorizing participants in Tanner stages, for girls the highest concentrations were observed at Tanner stages 1–3, and in boys the highest concentrations were observed at Tanner stage 3–5 (18, 30, 46, 54, 55). No sex-specific differences have been reported in children before puberty.

<table>
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<tr>
<th>Group</th>
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<th>24-h sample</th>
<th>Age range or mean age, years</th>
<th>n</th>
<th>PYD, nmol/mmol Cre</th>
<th>DPD</th>
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<td>146&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>22</td>
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<sup>a</sup> Cre, creatinine; IA, immunoassay; NA, not analyzed.
<sup>b</sup> nmol·L<sup>–1</sup>·24 h<sup>–1</sup>.
<sup>c</sup> CV<sub>inter</sub> < CV<sub>intra</sub>.
<sup>d</sup> Data not available.
GEOGRAPHIC EFFECTS AND EFFECTS OF RACE
No study exists investigating urinary pyridinium cross-links concentrations in different countries with assays performed in the same laboratory, using the same method and the same type of urine samples. Therefore, a meta-analysis (one-sided ANOVA) of reported concentrations of healthy premenopausal women from different regions [England (30), Germany (43), France (34, 56), Sweden (57), Japan (37, 58–60), Australia (36), South Africa (61), Taipei (62), and Italy (63)] was performed. The studies were grouped according to type of sample collection, and a recently reported interlaboratory variability (64) was taken into consideration as analytical variability. Significant differences were found only between Japan and all other countries.

The available studies investigating the effect of race on urinary PYD and DPD excretion rates compare African and Caucasian individuals only (26, 61, 65, 66). The largest study performed in premenopausal and postmenopausal women from South Africa (61) observed nonsignificant differences in urinary free PYD in native African women compared with Caucasian women (2% and 9% lower for premenopausal and postmenopausal, respectively) (61). Similar findings are reported in a recent study for DPD in Caucasian and Afro-Caribbean men (65). Other studies, with fewer participants, reported slightly but significantly lower urinary PYD in African-American compared with Caucasian-American women (26, 66).

INFLUENCE OF THE MENSTRUAL CYCLE, CONTRACEPTION, PREGNANCY, AND LACTATION
Of the three studies investigating changes during menstrual cycle of apparently healthy menstruating women, two found no significant changes in urinary DPD (67, 68). The third study observed a slight increase in urinary DPD excretion rates of ~5% from early follicular phase to the luteal phase peak before falling back to baseline during luteal phase (69). Investigations in women with abnormal cycles described controversial findings. Authors reported either increased concentrations (25% for DPD; PY not analyzed) in the case of hypothalamic amenorrhea (70) or decreased concentrations (20% PYD; 35% DPD) in amenorrheic women compared with eumenorrheic women and sedentary controls (71). The hypothalamic amenorrheic women in both studies had low estrogen concentrations. No data are available on urinary DPD and PYD in oral contraceptive users. During pregnancy, pyridinium cross-links increase substantially, especially during the third trimester, with concentrations generally being two or more times higher than in nonpregnant women and in the first trimester (72–74). DPD concentrations are reported to increase further with uterine involvement in the first weeks postpartum (75). During lactation, PYD gradually returns to prepregnancy concentrations (74).

SEASONAL VARIATIONS
Of the studies investigating seasonal variations (76, 77), one study was cross-sectional, with relatively large group sizes of elderly men and women; the second study was a longitudinal study of only 20 women, using a crossover design complicated by supplementation with vitamins D and K; and the third study was performed cross-sectionally in groups of young women (78). Urinary excretion of pyridinium cross-links was higher in the winter (October 1 to April 30) than in the summer. There were, however, differences in the magnitude of these changes, which varied from ~5% to 25%; in many cases, these were not statistically significant. In addition, the changes were not always synchronized with seasonal changes in the concentrations of vitamin D metabolites and parathyroid hormone.

INFLUENCE OF PHYSICAL ACTIVITY
As indicated in one study, the type of exercise seems to influence urinary cross-links concentrations (79). Increased excretion rates (significant and nonsignificant) have been reported by authors investigating the effect of running (80–83). In other types of sports [gymnastics (84), endurance exercise (85), aerobic exercise (86), weight lifting (87), resistance exercise (88, 89)] or physical activity [occupational walking (90, 91)], either no differences or decreased excretion rates (significant and nonsignificant) were reported, with changes ranging from 8% to 40%. Studies investigating the effect of immobilization from prolonged bed rest (4 days or longer) consistently found increased concentrations for urinary PYD and DPD (92–94). One study with bed rest over 20 days found increasing concentrations with a peak after 10 days followed by a decrease. Another study reported excretion rates that remained increased 5 days after bed rest was terminated. The reported changes ranged from 20% to 44% for PYD and 27% to 44% for DPD. Weightlessness as experienced during space flight has been reported to increase pyridinium cross-links significantly (95, 96).

INFLUENCE OF DIET
Extreme fasting over 4 days had no significant effect on pyridinium cross-links concentrations (97). However, in severely malnourished children, concentrations were decreased to approximately one-third of those after recovery from malnourishment (98). One cross-sectional study investigated the influence of different diets in women 45–55 years of age with regard to fruit and vegetable intake, using a food-frequency questionnaire. The potassium, magnesium, and phosphate intake was negatively correlated with PYD excretion, and potassium, magnesium, β-carotene, and fiber intake was negatively correlated with DPD excretion. Statistical calculations indicated that magnesium intake accounted for 12% of the variability (99). Another study investigated high sodium intake (250 mmol/day) and found increased DPD excretion rates (27%) in postmenopausal women (100). How-
ever, other studies investigating the effect of sodium intake, using different diets with lower sodium concentrations, found no significant effects on excretion rates of pyridinium cross-links (101, 102). No effects were reported for phosphate supplementation (1500 mg (103)), high protein intake (2.7 g protein per kg of body weight per day (104)), zinc supplementation (105), and increased milk consumption (586 mL/day (106)). Diets low in copper (0.7 mg/day) significantly increased excretion rates in DPD and PYD (30% and 25%, respectively) compared with a medium copper diet (107). Vitamin D deficiency resulting from vitamin D-deficient diets or malabsorption reportedly caused increased pyridinium cross-links excretion rates (108–111). Concentrations were approximately two- to threefold higher in deficient postmenopausal women than in healthy controls.

Calcium supplementation (≥600 mg/day) decreased urinary pyridinium cross-links excretion rates in men and women (premenopausal and postmenopausal). The degree of observed changes as well as the time point in which significant changes were observed differed for immunoreactive free and total pyridinium cross-links (111, 112). Changes up to ~33% as a result of calcium supplementation have been reported (104). The effects of calcium supplementation on diurnal variability are reportedly controversial. Evening calcium administration, but not morning calcium administration, seems to suppress the nocturnal increase in cross-links concentrations (113, 114). However, another study described no effect of evening administration on the nighttime increase in PYD (115). Increased pyridinium cross-links have been reported in alcoholics and abstainers even 5 years after alcohol withdrawal (116). No association between smoking and pyridinium cross-links excretion rates and no difference in PYD and DPD excretion rates in smokers were found regardless of fast or slow loss in lung function (117, 118).

INFLUENCE OF DISEASES AND MEDICATION

Evidently, urinary pyridinium cross-links are affected by metabolic disorders of bone, such as osteoporosis, and change with treatments to cure these disorders (11, 119–122). However, several other diseases, conditions, and drugs are known to affect urinary PYD and DPD excretion rates. Some of these conditions affect bone metabolism directly, whereas others may affect the clearance of pyridinium cross-links or affect other cross-links containing tissue rather than bone. A profound increase in bone turnover (concentrations 100% or higher than in healthy controls) occurs in patients with hyperthyroidism (123–126), hyperparathyroidism (127), Paget disease (128), Ehlers–Danlos syndrome (129, 130), multiple myeloma, hypercalcemia of malignancy, and certain cancers, particularly if they are associated with bone metastases (14, 131–133). In addition, fractures are known to cause increased PYD and DPD concentrations that remain increased up to 1 year after occurrence of fracture (134–136). More modest or inconsistent observations are reported for other diseases, such as diabetes mellitus (137–141) or arthritis (142, 143). Other diseases and conditions that are reported to cause increased pyridinium cross-links concentrations are liver dysfunction (144), renal osteodystrophy (153), Camurati–Engelmann disease (146), spinal cord injury (147), bone marrow transplantation (148), gastrointestinal diseases related to nutrition and mineral metabolism (149), cystic fibrosis (150), scleroderma (151), growth hormone/receptor deficiencies and other growth disorders, growth hormone treatment (152), hyperprolactinemia in amenorrheic patients with estrogen deficiency (153), myelomeningocele (154), and serno-negative spondylarthropathy (155). Decreased pyridinium cross-links concentrations are reported in fibromyalgia (156), severe burns (157), and acute lymphoblastic leukemia in children (158). Excretion rates may vary with disease stage and severity or drug dosage (111). Although this listing is not exhaustive, it shows the range of conditions in which PYD and DPD excretion can be altered. In this context it should be mentioned that the drug sulphasalazine has been reported to interfere with DPD in a HPLC assay (159).

STABILITY OF CALIBRATORS AND SAMPLES. PYD and DPD dissolved in water are very sensitive to ultraviolet (UV) light. The rate of degradation depends on the pH of the solution and the wavelength of the light source used for exposure. The highest degradation rate is at the wavelength of maximum absorbance. When UV light was used at wavelengths of 254 and 365 nm, the degradation rate was higher at high pH (163). Complete degradation of the aqueous calibrator was observed within 6 h of exposure to a UV lamp (164). Pyridinium cross-links in urine are much less sensitive to UV light (163, 165). A 1-cm layer of urine absorbs >99% of UV light (163). No effect on
urinary cross-links was observed with laboratory light (fluorescent lights and filtered daylight) (164, 166). In addition, no effect of daylight was found on urine stored in a large container (850 mL) for a whole day during the summer. However, a small amount of degradation was observed if the urine was stored in a small (2 mL) container (163). The effect of UV light on the degradation of cross-links was greater for free PYD and DPD than for total pyridinolines (free and peptide bound) with a decrease of 80% in free DPD and a decrease of 60% in total DPD after 3 days of exposure.

The degradation in urine was also pH dependent, with greater degradation at higher pH (163, 164, 166). The half-life of pyridinium cross-links was ~1 h at 50 °C and 6 h at 37 °C (167). No degradation was observed at or below 20 °C (down to −70 °C) for up to 9 months (165, 167). Findings concerning freeze–thaw stability are controversial. The authors of one study found no degradation of cross-links in urine after 10 freeze–thaw cycles (167), whereas the authors of another study found degradation after 5 freeze–thaw cycles (166).

**Creatinine measurement.** Urinary concentrations of PYD and DPD are frequently expressed as molar ratios with the creatinine concentration. Considering the importance of creatinine in defining cross-links excretion rates, examining factors affecting creatinine concentrations in urine is essential. Several endogenous and exogenous compounds influence assay results. These effects vary depending on the type of creatinine assay used (alkaline picric acid assay, enzymatic assay, or HPLC assay). The presence of high salt concentrations; fluorescein (168); carbonyl compounds (169), in particular acetoacetic acid and ketoacids (170); glucose (171); and several drugs (172), including dopamine (173), cephalosporins (174, 175), and tri-methoprim (176), cause a moderate to high increase in creatinine concentration. In contrast, high concentrations of bilirubin (177) produce negative interference. Several lifestyle and pathologic conditions influence the creatinine concentration, including strenuous exercise [30–50% vs baseline (178)], stress [5–10% vs control (179)], dietary intake of meat or polyunsaturated fat [10–30% vs control (180)], time of menstrual cycle [10–15% vs baseline (181)], pregnancy [5–20% vs baseline (182)], age, infection, trauma, and renal insufficiency [20–100% vs control (183, 184)].

Daily excretion of creatinine follows a circadian rhythm with a 14% higher value in the late afternoon (185), compared with the 24-h mean. When creatinine is measured in 24-h urine, the intraday variation is generally 11–15% (186–192), whereas it is 29–31% for FMV samples (190, 192) and 36–45% in random urine samples (190, 192, 193). In these studies, the intersubject variability was 13–28% for 24-h urine samples, 26% for FMV, and 33–36% in random urine. The 24-h creatinine output in men is significantly higher than in women (194).

Relatively few studies have described the stability of creatinine in urine specimens, and most of them are based on the short-term stability of creatinine in urine stored 1–30 days. Creatinine is generally stable in urine stored at 4 °C for at least 5–7 days (195, 196), although some studies indicated no significant change in creatinine concentration when urine was stored refrigerated for 30 days (197). Data on creatinine stability in urine stored after the addition of acid or alkali are controversial. The authors of one study found some loss of creatinine when acidified urine was stored frozen (−15 °C) (198), whereas the authors of another study found no significant change in creatinine under these conditions (195). No data are available about the effect of freeze–thaw cycles on the creatinine concentration. In our hands, up to five–freeze thaw cycles did not significantly change the creatinine concentration (A.K. Srivastava, unpublished observation).

**Discussion**

Urinary PYD and DPD concentrations can be affected substantially by several preanalytical factors. These factors need to be recognized and controlled before collecting a specimen to minimize variability and facilitate data interpretation. Minimalized variability leads to consistent signal-to-noise ratios and reference intervals, and consequently to constant limits of detection for abnormal pyridinium cross-links excretion rates or significant changes in the excretion rates.

The within-day or diurnal variability of pyridinium excretion, as one of the important sources of variability, is ~10%, and the among-day variation for DPD and PYD is 16% within an individual. On the basis of these data, specimen collections made at random times during the day would be expected to increase the variation from 16% to ~19% compared with serial collections made at the same time of day. Therefore, collecting serial urine specimens from a patient as close as practical to the same time each day could eliminate the within-day component of variation. To reduce the among-day variability, multiple collections (e.g., three over 3–5 days) analyzed in duplicate (as individual samples or pooled) may improve the reliability of clinical decision-making, as has been shown in a similar approach to minimize the effect of biologic variability in blood lipid testing (199). Among the different types of urine collection, the 24-h collection offers the advantage of allowing assessment of an integrated daily excretion, but it is complex and inconvenient. A SMV or FMV collection may provide the best signal-to-noise ratio because of the high pyridinium cross-links concentrations in the morning. The SMV might have some advantage in clinical practice in that patients who urinate frequently during the night might better accommodate the SMV and may even provide it during the office visit.

The average among-subject biologic variability was 35% for premenopausal women, 42% for postmenopausal women, and 45% for children (average of PYD and DPD excretion rates) with a high variability between different
studies. The observed high inconsistency in the biologic variability across studies is probably attributable to the heterogeneity of the individuals investigated in the studies (age ranges, definition of menopausal status, different exclusion criteria). However, as indicated by some studies using well-characterized participants, biologic variation as low as 14–18% in healthy individuals can be achieved. To minimize biologic variability, individuals need to be grouped according to their age and gender, which are two factors that have a profound affect on biologic variability.

Because of the rapid changes during childhood, children of the same age or within a narrow age range (2 years) should be used for comparison purposes. In case of pubertal children, it might be more suitable to compare children of the same pubertal status (i.e., categorized in Tanner stages) than using children of the same age. In adult premenopausal women, the smallest variability was observed from age 30 until the start of menopause. The available data on perimenopausal women and on menopausal transition are not sufficient to make any general statements about variability in PYD and DPD excretion rates. For postmenopausal populations, it would be suitable to express postmenopausal excretion rates per age group rather than by postmenopausal years because age is an easier and more reliable variable to collect than years since menopause. Until the start of puberty, no sex-specific differences have been reported. The most pronounced differences between men and women are observed at the start of menopause. Therefore, concentrations for both genders need to be assessed and interpreted separately.

Profound changes are also observed during the third trimester of pregnancy (73,200,201). In addition, certain diseases and medications, such as fractures, hyperparathyroidism, hyperthyroidism, or certain types of cancers, substantially impact urinary PYD and DPD concentrations. To properly interpret results obtained from cross-links measurements, a detailed characterization of the patient and exact diagnosis of all diseases and conditions are necessary. Other factors affecting biologic variability with only modest effects or inconsistent findings reported are menstrual cycle, physical activity, diet, seasonal variation, and geographic differences. These factors seem to be important when small differences in urinary pyridinium cross-links excretion rates need to be detected.

The small changes in urinary PYD and DPD concentrations during the menstrual cycle with lower concentrations in the follicular phase and increased concentrations in the luteal phase are consistent with findings obtained with other bone markers (202). The available data on the impact of irregular menstrual cycles indicate that the excretion rates of pyridinium cross-links in women with abnormal cycles might be different from the excretion rates in women with regular cycles. No data are available on the impact of oral contraceptives. However, studies with other bone markers point to possibly lower pyridinium cross-links excretion rates in oral contraceptive users than in nonusers (203). Data available on the impact of physical activity are somewhat scattered, with most changes being nonsignificant. The study designs are too different to find any correlations across the studies. However, the reported magnitudes of changes (up to 40%) are substantial and need to be confirmed in further studies. Data on immobilization consistently show increases in PYD, DPD, and creatinine concentrations with prolonged bed rest. Consequently, excretion rates of immobilized individuals may need to be considered separately from those for nonimmobilized individuals.

Long-term effects of diets or impaired dietary behavior have been reported in situations such as severe malnutrition or anorexia nervosa, with decreased excretion rates in the first and increased excretion rates in the latter case. In supplementation studies, significant effects were reported with vitamin D, calcium, and copper supplementation. For calcium, it was shown that the time of supplementation affects diurnal variation. Patients’ diets should be carefully observed, not only in view of the effect on pyridinium cross-links concentrations but also in view of creatinine concentrations, which are known to be affected by diet. Therefore, nutritional status and use of dietary supplements need to be assessed carefully. The small number of relevant published reports in the area of seasonal variation is insufficient to clearly assess its impact. The reported magnitude of changes, 10–20%, might be significant and needs to be confirmed in further studies. The available data on geographic differences and differences attributable to race are very limited and cannot completely be separated from those of lifestyle or nutrition. However, studies using other bone markers and more appropriate study designs found geographic differences and differences attributable to race (204,205).

In addition to factors that affect the biologic variability of urinary PYD and DPD measurement, other components of preanalytical variability, such as handling of samples and calibrators and creatinine correction, are of importance. The reported susceptibility of pyridinium cross-links to light requires special precautions when handling samples and calibrators. Because urinary PYD and DPD concentrations usually are corrected for creatinine, variability in creatinine measurements impacts pyridinium cross-links results. The available data indicate that a considerable part of variability can derive from the biologic variability in creatinine excretion. Creatinine measurements should not be used to adjust cross-links concentrations in patients with renal insufficiency, acute infection, early phase of injury, or trauma because creatinine output is not proportional to muscle mass under these conditions. In this context, it needs to be noted that creatinine correction in children can have several limitations (206–208) and therefore needs to be considered carefully.
CONCLUSION/RECOMMENDATIONS

Variability in urinary PYD and DPD measurements from preanalytical factors contributes substantially to problems in data interpretation. However, this variability can readily be minimized and/or standardized by applying the following suggested recommendations:

1. Specimens should be collected at a specific time of day to avoid diurnal variability. SMV urine seems to be the most practical urine type and therefore should be used for routine measurements, except for special applications or situations requiring a different type of urine.

2. Excretion rates from the same type of urine collection should be used for data comparisons.

3. Samples and calibrators should not be exposed to direct sunlight.

4. Collected samples should be stored at 2–6 °C if they are analyzed on the same day or frozen at −20 °C if they are analyzed after >24 h. No preservatives should be added to the sample, except for 24-h urines. Here, the sample should be stabilized with a weak acid (e.g., boric acid) and/or other chemicals to avoid changes from microbial contamination. In such a case, specifications of the assay in use need to be taken into consideration to avoid loss of assay performance.

5. Reference intervals should be established using well-characterized, healthy premenopausal women after assessment of menses and plasma follicle-stimulating hormone concentrations. The age range of 30–45 years would probably be the most adequate.
   a. Perimenopausal women should not be included in the population used to establish reference intervals for urinary pyridinium cross-links.
   b. For men, separate reference intervals should be defined.
   c. For children, reference intervals should be reported by age group not older than 2 years; during puberty, Tanner stages should be mentioned.

6. Abnormalities in menstrual cycle should be identified, and results should be interpreted carefully in such patients.

7. Factors and conditions affecting creatinine excretion should be recognized and included in data interpretation.

8. Use of dietary supplements should be assessed, not only in view of vitamin D and calcium intake, but also in view of other vitamins and minerals.

9. Current and previous diseases and conditions need to be taken into consideration. Data obtained from immobilized patients should be interpreted with caution. Data from immobilized patients probably should be compared with data from ambulatory individuals rather than with healthy individuals described under number 5.

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