Calcif Tissue Int (1999) 64:40–44

Calcified
Tissue
International

Age-related Changes in Body Composition, Hydroxyproline, and Creatinine Excretion in Normal Women

M. Worsfold, M. W. J. Davie, M. J. Haddaway

Charles Salt Research Centre, Robert Jones & Agnes Hunt Hospital, Oswestry, Shropshire, UK SY10 7AG

Received: 30 July 1997 / Accepted: 9 July 1998

Abstract. We have made a cross-sectional study of relationships among age, whole body bone mineral content (WBBMC), and non-bone lean body mass (NBLBM) measured by dual energy X-ray absorptiometry (DXA), and daily excretion of hydroxyproline (OHP) and creatinine (Cr) in a group of normal women. WBBMC fell with age from the 6th decade, whereas NBLBM was almost constant. Creatinine excretion fell with age from the 5th decade until the 9th, to a much greater degree than NBLBM, reaching a nadir in the 8th decade. Daily excretion of hydroxyproline showed a peak in the 6th decade and fell moderately thereafter. The greater fall of creatinine compared with hydroxyproline resulted in rising OHP/Cr ratios with advancing age, in contrast to the pattern of hydroxyproline excretion. The use of creatinine as a correction for urine dilution or for lean body mass (LBM) in assays for markers of bone turnover must therefore be viewed with caution.

Key words: Hydroxyproline — Creatinine — Lean body mass — Bone mineral content — Normal women.

The excretion of hydroxyproline in urine has long been used as an index of bone turnover rates in humans, despite some drawbacks. The interpretation of the test is complicated by the nonquantitative excretion of hydroxyproline, due to partial processing in the liver and reabsorption of most free hydroxyproline by the kidney [1]. Some hydroxyproline may be released during collagen formation [2]. The relative contributions of alternative metabolic pathways are controversial and hard to quantify in humans. Their practical importance can perhaps best be gauged by clinical experience. Dietary hydroxyproline, mostly derived from meat and animal products, particularly gelatin, may contribute to the urinary pool [3], but this can easily be countered by controlling the diet for 2 or 3 days prior to and during the test. Many of the assay methods that have been proposed are either very tedious or unsuitable [4]. We have been using a simplified method, adapted to automated analysis, for more than 10 years.

The rate of creatinine excretion in urine has significance at two levels in the clinical chemistry of bone disease. At a practical level, creatinine concentrations are often used to correct for urine dilution as a substitute for 24-hour collection in studies of urinary excretion of bone metabolites. This assumes an unvarying relationship between creatinine concentration and urine dilution over the range of conditions relating to the application in question, and either a constant rate of excretion or a relationship that can be understood and does not detract from the aims of the study. At a more fundamental level, creatinine excretion reflects aspects of body composition and metabolism which are relevant to skeletal homeostasis. It is often used as an index of lean body mass (LBM). These two applications of creatinine excretion are interrelated, and to some extent the second conflicts with the first.

DXA is increasingly used to measure body composition. We wished to see how creatinine excretion is related to DXA estimates of LBM. We have used hydroxyproline excretion as a measure of bone turnover in relation to age, and examined the effect of creatinine use as a correction for urine dilution or as an index of lean mass.

Subjects and Methods

Women were recruited as volunteers mostly from local women's organizations [5]. Each was screened by a questionnaire administered by a member of staff at each visit for bone density measurement, when blood was obtained and 24-hour urine samples, collected at home the previous day, were acquired. Subjects were excluded from the study if there was evidence of metabolic bone disease or if they had received any form of therapy that might be expected to affect bone metabolism. Disqualifying therapies included oral steroids, bisphosphonates, and bone marrow active compounds. Use of thiazides, thyroxine at less than 200 mg/day or calcium supplements up to 1000 mg per day, were not excluded. Women who had used estrogen replacement therapy or oral contraceptives for more than 6 months before or during the study, or had undergone hysterectomy before the natural menopause or before 52 years of age, were also identified and are not included in this study. No subject was excluded because of low bone density. No recommendation for therapy was made, and there was no observable tendency for such women to be prescribed hormone replacement therapy (HRT) and hence to become excluded from the study. There were no exclusions on the basis of fractures or vertebral deformity. The data for 201 women between 40 and 93 years old were included in our analysis of urinary hydroxyproline and creatinine excretion. Whole body bone density and lean mass measurements were made on a subset of 75 women between 50 and 80 years old.

Methods

Subjects scheduled for 24-hour urine collections followed a collagen-free diet for 2 days prior to the start of the collection. These collections were made into polyethylene containers of 2.3 liter

Correspondence to: M. Worsfold

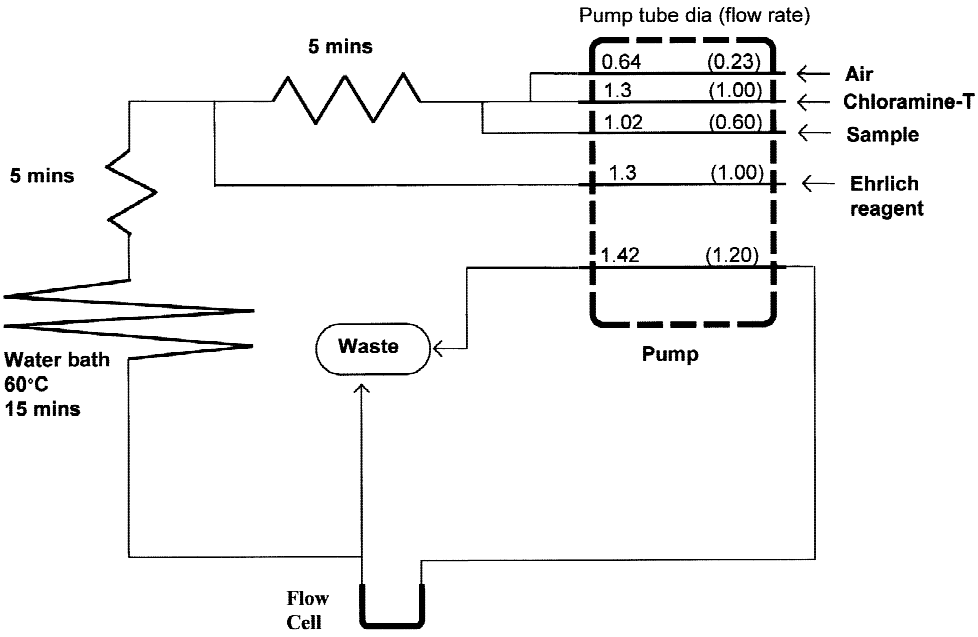

Fig. 1. Flow diagram of segmented-flow automatic analysis of hydroxyproline. See Methods for concentrations of reagents.

capacity containing 15 ml of 6 mol/liter HCl. The volume was measured at the end of the 24 hours and a sample was taken for storage at −20°C pending analysis. If the output was more than the capacity of the container, a second one was used and the contents of the two containers was mixed before sampling.

In order to free peptide-bound hydroxyproline, urine was first hydrolyzed by adding 0.5 ml of concentrated hydrochloric acid (SG = 1.33, approximately 12 mol/liter) to an equal volume of urine in a 7-ml polypropylene vial (Sarstedt, Leicester, UK) which was securely closed with a screw cap fitted with a nitrile rubber ''O'' ring. The vials were put into an oven at 110°C for 16 hours, removed, and allowed to cool. Two types of internal standards were added to extra vials prepared from two of the urines to be hydrolyzed each day—25 μl of either hydroxyproline (1 mg/ml) or a gelatin solution (5 mg/ml) (BDH). The first was to control for losses including those due to over-hydrolysis, and the second was a check on the completeness of hydrolysis. The hydrolysate was partially neutralized by adding 4.0 ml of sodium hydroxide (1 mol/liter) and the vials were centrifuged at about ×1000 *g* for 10 minutes. The clear supernatant was aspirated and stored at room temperature until used for assay of hydroxyproline.

Hydroxyproline was measured colorimetrically by an automated modification method of Bergman and Loxley [6] using a segmented flow system. Oxidation with chloramine-T was followed by the Ehrlich reaction with p-dimethylaminobenzaldehyde (pDAB) at 60° in a strongly acidic oxidizing medium with n-propanol as the organic solvent. Nonspecific color production was corrected by running full blanks without chloramine-T and subtracting the blank peak heights. The details are shown in the flow diagram (Fig. 1). The oxidation buffer was made up by adding 600 ml n-propanol to 330 ml of distilled water and 390 ml of a citrate/acetate buffer, pH 6.0. The citrate/acetate buffer contained 68 g NaOH, 68 g citric acid monohydrate, and 240 g of sodium acetate trihydrate per liter, adjusted to pH 6.0 with acetic acid. The oxidation medium consisted of 1.2 g chloramine-T freshly dissolved in 200 ml of the oxidation buffer, and kept in a dark reservoir. The Ehrlich's reagent contained 15 g pDAB (AR grade, Park Scientific, Northampton, UK) dissolved in a mixture of 130 ml n-propanol and 40 ml of 50% perchloric acid. This was also made up fresh each day and kept in a dark bottle, but is not as unstable as the chloramine-T.

A stock standard solution of 1.0 mg/ml hydroxyproline (BDH, Poole, UK) in 10 mmol/liter HCl was kept frozen in small portions and thawed as required to make working standards of 2, 5, 8, 10, and 12 μg/ml, also in 10 mmol/liter HCl. The working standards were kept at 4°C for up to 1 month. Two sets of standards were run at the start of each working day, in both rising and falling sequence, with an additional standard at intervals of about 20 samples to check the stability of the run. Recovery of added hydroxyproline was 73% ± 13.4.

Creatinine was measured routinely on an Ektachem analyzer, which uses a specific creatinine oxidase method.

WBBMC and LBM were measured on a Hologic QDR 1000/W densitometer. The lean mass exported as a database by the Hologic software (but not as printed in individual reports) includes bone mass. We therefore subtracted the exported WBBMC from the lean mass totals to derive the non-bone lean body mass (NBLBM). Statistical analysis was done with Statgraphics v5.0, using Student's *t* test for comparison of group data. Local ethics committee approval was obtained for this study.

Results

Figure 2 shows the excretion of hydroxyproline and creatinine and the non-bone lean body mass and whole body BMC in healthy women. The exclusion of oral contraceptive users meant that the numbers in the younger cohorts, especially younger than 50 years, were small, since oral contraceptive use nowadays is the normal condition for women of childbearing age in the developed world.

Daily creatinine excretion fell with age from the 5th decade until the 9th, when there was a small rise. The most marked fall was between the 7th and 8th decades ($P = 0.001$).

Hydroxyproline excretion showed a sharp rise between the 5th and 6th decades (Fig. 2), and fell again in old age. The ratio of hydroxyproline to creatinine, however, continued to rise until the 8th decade (Fig. 3).

Whole body bone mineral content fell throughout life from the 6th decade in our subjects. Non-bone lean body mass, as measured by DXA, hardly changed after the 6th decade, which is the youngest decade for which we have a

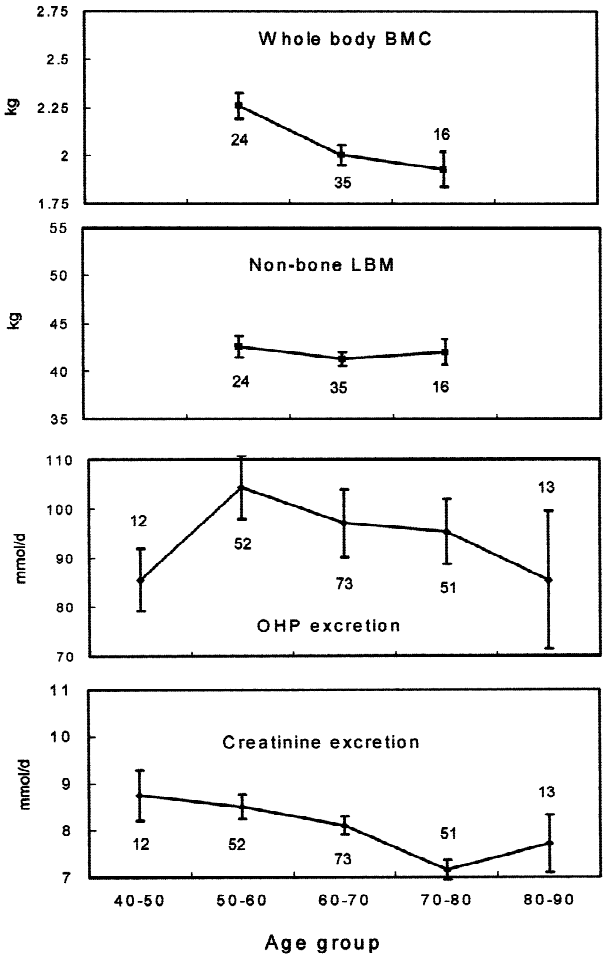

Fig. 2. Cross-sectional profiles of whole body BMC, non-bone lean body mass, daily hydroxyproline excretion, and daily creatinine excretion with age in healthy women who had not taken hormone replacement therapy, oral contraceptives, or had had a hysterectomy before the natural menopause or the age of 52. Each graph has been scaled to show the same (upper) proportion of the Y axis. Numbers adjacent to individual data points are the numbers of subjects averaged for this point. Bars are standard errors.

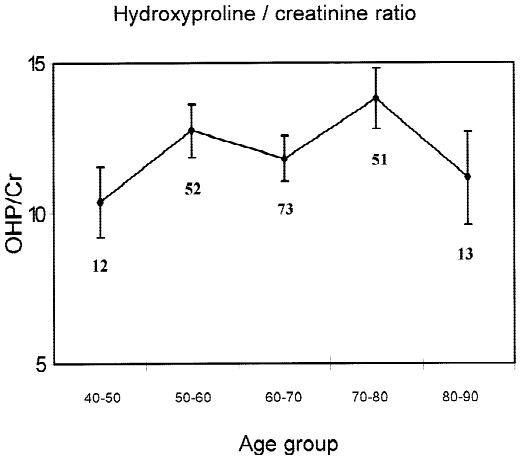

Fig. 3. Urinary hydroxyproline/creatinine ratios (μmol/mmol) of the same group of women as in Figure 2. Numbers adjacent to individual data points are the numbers of subjects averaged for this point. Bars are standard errors.

useful amount of data. The changes in NBLBM were much less than those of hydroxyproline, creatinine, and WBBMC.

There was only a small correlation between hydroxyproline excretion and NBLBM ($r = 0.25$), although in older women (>70 years) there was a tendency towards a slight negative relationship which was not statistically significant. Similarly, hydroxyproline excretion did not generally correlate with weight or whole body BMC, whereas creatinine excretion correlated quite well all these parameters (Cr versus NBLBM, $r = 0.56$; Cr versus WBBMC, $r = 0.40$; Cr versus wt, $r = 0.52$). This did not lead to a meaningful negative correlation of the OHP/Cr ratio with the same parameters, however.

Discussion

The fall in urinary creatinine excretion with increasing age has been observed before [7, 8, 9, 10], and the general pattern of OHP excretion after the menopause is also not novel [11, 12]. Most of the markers of bone turnover show a fall after puberty which reaches a nadir in the mid- or late 20s, followed by a rise peri- or immediately postmenopausally [11]. The excretion of both OHP [3, 12] and creatinine [13] may be influenced by diet, and it is therefore possible *a priori* that the profile of excretion with age could be influenced by dietary change at this time of life. In the case of OHP, adherence to a collagen-free diet for 1 or 2 days, prior to the collection should obviate this problem. A gelatin-free diet will generally also be meat free and hence mainly free of creatine and creatinine. Even so, it may take a week or more for the dietary contribution to creatinine excretion to subside [14] presumably because of the large body pool of creatine.

It must also be recognized that our subjects were volunteers at large and we had not direct control over their compliance with the protocol. This is a cross-sectional study and the usual caveats apply to inferences about longitudinal changes. In particular, the data for women in the oldest age group must be considered to be especially vulnerable to cohort survival effects. This may well be the most likely explanation of the apparent rise in creatinine excretion in the 9th decade. On the other hand, many of the reports of the use of creatinine as an index of urine volume, or indeed of muscle mass, are also cross-sectional, and our findings are relevant to them.

Since lean body mass does not follow the same age-related profile as creatinine excretion, our results show that creatinine excretion is not a reliable index of lean body mass, at least as measured by DXA. The overall correlation of these two measures is also not very close. Closer correlations with lean body mass estimated by total body potassium ($r = 0.99$) [15] and exchangeable potassium ($r = 0.9$) [16] have been reported, using a wide range of subjects, but total body water by antipyrine volume ($r = 0.65$) [17] and surface area ($r = 0.53$) [14] gave results similar to ours. Stewart et al. [18] reported a correlation ($r = 0.57$) of creatinine excretion with fat-free mass estimated by DXA which is similar to our figure, even though their subjects included a wide range of body sizes, but Rosenfalck et al. [19] found a much better correlation between these two measures ($r^2 = 0.85$ for normal adults). The range of body mass and creatinine excretion in our study is not very wide

(data not shown), being confined to mature healthy women, and so it is not surprising that our correlation coefficient is lower than in studies where the range is much wider because of the inclusion of children and adults of both sexes or of extremes of stature [15]. However, a well controlled comparison of creatinine excretion (averaged over 3 days) by healthy men on a meat-free diet, with total muscle mass measured by computerized axial tomography, gave a correlation coefficient of 0.92 [20]. It has been suggested by Heymsfield et al. [13] that the proportion of NBLBM that is functional muscle falls in older people, but there is little direct evidence for this, and the study quoted [21] does not in fact support this suggestion. A recent study using magnetic resonance imaging (MRI) as well as creatinine excretion and ^{40}K counting [22] has suggested that the fall in LBM with age is due mostly to loss of muscle mass, but this was based on the use of creatinine excretion as the definition of muscle mass, which correlated well with the cross-sectional areas of the thigh and upper arm muscles on MRI ($r = 0.88, 0.85$). Whatever the reason for the change in creatinine excretion with age, it has serious implications for the use of urine creatinine as a surrogate for 24-hour urine collections in estimating excretion rates of other metabolites. Our hydroxyproline/creatinine results show that a fall in creatinine excretion can generate a rise in a metabolite/creatinine ratio which is not seen if daily excretion rates are used. This might account for the relatively sharp postmenopausal peak seen by Hodgkinson and Thompson [23].

Creatinine excretion is said to fluctuate according to the menstrual cycle [24] which would tend to confound studies of urinary metabolites in relation to the menstrual phase [25]. Furthermore, daily creatinine excretion was found to fall by 17% after at least 1 year of estrogen-progestogen therapy [26] suggesting that at least some of a rise in urinary metabolite/creatinine ratios [27] might really have reflected changes in creatinine. Lueken et al. [28] reported urinary pyridinoline cross-links and hydroxyproline as creatinine ratios in a study of the effects of bedrest, but ignored the effect of bedrest on creatinine excretion [29]. Daily creatinine excretion is known to be variable [30, 31] and there is diurnal cycle that is also variable [32, 33]. There is a more marked diurnal variation in the tubular secretion rate [34] which is in opposition to the cycle of glomerular filtration rate [35]. This might dissociate creatinine output further from that of collagen metabolites. The position is made more complicated by recent evidence of tubular secretion of pyridinoline cross-links [36] which also shows a diurnal variation [37]. Other factors that can affect creatinine excretion have been reviewed [13, 38].

The small correlations between OHP excretion and body weight, WBBMC, or NBLBM are a little surprising. One would expect large people to excrete larger amounts of every metabolite, and both WBBMC and NBLBM are strongly linked to body size and hence weight. In particular, the size of the metabolizable bone collagen pool might be expected to be directly related to WBBMC, even allowing for the possibility of slight osteomalacia in some subjects, and should therefore be reflected in total OHP excretion.

Conclusions

Whole body BMC falls with age from the 6th decade, whereas non-bone lean body mass is almost unchanged throughout adult life. Creatinine excretion falls with age from the 5th decade to the 7th, to a much greater degree than NBLBM, the fall being most rapid between the 7th and 8th decades, and would seriously distort the pattern of OHP excretion if this were expressed as a ratio with creatinine. Hydroxyproline excretion tended to fall with age after the 6th decade. Using a creatinine correction for either urine volume or muscle mass is unsatisfactory.

References

1. Kivirikko KI (1970) Urinary excretion of hydroxyproline in health and disease. Int Rev Connect Tissue 5:93–163
2. Bienkowski RS (1984) Intracellular degradation of newly synthesised collagen. Coll Relat Res 4:399–412
3. Gasser A, Celled A, Courvoisier B, Depierre D, Hulme PM, Rinsler M, Williams D, Wooton R (1979) The clinical measurement of urinary total hydroxyproline excretion. Clin Chim Acta 95:487–491
4. Mitchell AD, Taylor IEP (1970) The spectrophotometric determination of hydroxyproline: an analytical investigation. Analyst 95:1003–1011
5. Haddaway MJ, Davie MWJ, McCall IW (1992) Bone mineral density in healthy normal women and reproducibility of measurements in spine and hip using dual-energy X-ray absorptiometry. Br J Radiol 65:213–217
6. Bergman I, Loxley R (1970) The determination of hydroxyproline in urine hydrolysates. Clin Chim Acta 27:347–349
7. Worsfold M, Sharp CA, Davie MWJ. Collagen metabolites in healthy women. In: Ring EFJ, Elvins DM, Bhalla AK, editors. Current research in osteoporosis and bone mineral measurement III. Proceedings of the Fourth Bath Conference on Osteoporosis and Bone Mineral Measurement; 1994 June 20–24; Bath, UK. British Institute of Radiology; 1994. p. 12–13
8. Siersbaeck-Nielsen K, Hansen JM, Kampmann J, Kristensen M (1971) Rapid evaluation of creatinine clearance. Lancet i:1133–1134
9. Cockcroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. Nephron 6:31–41
10. Blumenkrantz N, Asboe-Hansen G (1977) Urinary creatinine is unsuitable as a reference value for collagen metabolite output. Clin Biochem 10:109–110
11. Anderson J, Bannister DW, Tomlinson RWS (1965) Total urinary hydroxyproline excretion in normal human subjects. Clin Sci 29:583–587
12. Saleh AEC, Coenegracht JM (1968) The influence of age and weight on the urinary excretion of hydroxyproline and calcium. Clin Chim Acta 21:445–452
13. Heymsfield SB, Arteage C, McManus C, Smith J, Moffitt S (1983) Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine method. Am J Clin Nutr 37: 478–494
14. Bleiler RE, Schedl HP (1962) Creatinine excretion: Variability and relationships to diet and body size. J Lab Clin Med 59:945–955
15. Forbes GB, Bruining GJ (1976) Urinary creatinine excretion and lean body mass. Am J Clin Nutr 29:1359–1366
16. Muldowney FP, Crooks J, Bluhm MM (1957) The relationship of total exchangeable potassium and chloride to lean body mass, red cell mass and creatinine excretion in man. J Clin Invest 36:1357–1381
17. Edwards KDG, Whyte HM (1959) Creatinine excretion and body composition. Clin Sci 18:361–367
18. Stewart SP, Bramley PN, Heighton R, Green JH, Horsman A, Losowski MS, Smith MA (1993) Estimation of body composition from bioelectrical impedance of body segments: comparison with dual-energy X-ray absorptiometry. Br J Nutr 69: 645–655
19. Rosenfalck AM, Almdal T, Gotfredsen A, Hojgaard LL, Hilsted J (1995) Validity of dual X-ray absorptiometry scanning for determination of body composition in IDDM patients. Scand J Clin Lab Invest 55:691–699
20. Wang ZM, Gallagher D, Nelson ME, Matthews DE, Heymsfield SB (1996) Total-body skeletal muscle mass: evaluation

of 24-h urinary creatinine excretion by computerised axial tomography. Am J Clin Nutr 63:863–869
21. Tomlinson BE, Walton JN, Rebeiz JJ (1969) The effects of ageing and of cachexia upon skeletal muscle. J Neurol Sci 9:321–346
22. Welle S, Thornton C, Totterman S, Forbes G (1996) Utility of creatinine excretion in body-composition studies of healthy men and women older than 60 years. Am J Clin Nutr 63:151–156
23. Hodgkinson A, Thompson P (1982) Measurement of the fasting urinary hydroxyproline: creatinine ratio in normal adults and its variation with age and sex. J Clin Pathol 35:807–811
24. Smith OW (1942) Creatinine excretion in women: data collected in the course of urinalysis for female sex hormones. J Clin Endocrinol 2:1–12
25. Gorai I, Chaki O, Nakayama M, Minaguchi H (1995) Urinary biochemical markers for bone resorption during the menstrual cycle. Calcif Tissue Int 57:100–104
26. Jensen J, Christiansen C, Rodbro P (1986) Oestrogen-progestogen replacement therapy changes body composition in early post-menopausal women. Maturitas 8:209–216
27. Prestwood KM, Pilbeam CC, Burleson JA, Woodiel FN, Delmas PD, Deftos LJ, Raisz LG (1994) The short-term effects of conjugated estrogen on bone turnover in older women. J Clin Endocrinol Metab 79:366–371
28. Lueken SA, Arnaud SB, Taylor AK, Baylink DJ (1993) Changes in markers of bone formation and resorption in a bed rest model of weightlessness. J Bone Miner Res 8:1433–1438
29. Zorbas YG, Petrovskiy VN, Popescu DT (1990) Creatinine excretion in urine of man under hypokinesia and physical exercise. Mater Med Pol 4:295–299
30. Scott PJ, Hurley PJ (1968) Demonstration of individual variation in constancy of 24-hour urinary creatinine excretion. Clin Chim Acta 21:411–414
31. Edwards OM, Bayliss RIS, Millen S (1969) Urinary creatinine excretion as an index of the completeness of 24-hour urine collections. Lancet 1165–1166
32. Pasternack A, Kuhlback B (1971) Diurnal variations of serum and urine cretine and creatinine. Scand J Clin Lab Invest 27:1–7
33. Bollen A-M, Martin MD, Leroux BG, Eyre DR (1995) Circadian variation in urinary excretion of bone collagen cross-links. J Bone Miner Res 10:1885–1890
34. Dodge WF, Travis LB, Daeschner CW (1967) Comparison of endogenous creatinine clearance with inulin clearance. Am J Dis Child 113:683–692
35. van Acker BAC, Koomen GCM, Koopman MG, Krediet RT, Arisz L (1992) Discrepancy between circadian rhythms of inulin and creatinine clearance. J Lab Clin Med 120:400–409
36. Colwell A, Eastell R (1996) The renal clearance of free and conjugated pyridinium cross-links of collagen. J Bone Miner Res 11:1976–1980
37. Schlemmer A, Hassager C, Jensen SB, Christiansen C (1992) Marked diurnal variation in urinary excretion of pyridinium cross-links in premenopausal women. J Clin Endocrinol Metab 74:476–480
38. Boeniger MF, Lowry LK, Rosenberg J (1993) Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am Ind Hyg Assoc J 54:615–627