Diurnal Variation of Biopyrrin Excretion in Random Urine Specimens Is Not Corrected by Creatinine

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Circulating bilirubin is thought to function as a physiological antioxidant. One of the decomposition products of this process is the biopyrrins, which include two regioisomers: biotripyrrin-a (1,14,15,17-tetrahydro-2,7,13-trimethyl-1,14-deoxy-3-vinyl-16H-triptyrin-8,12-dipropionic acid) and biotripyrrin-b (1,14,15,17-tetrahydro-3,7,13-trimethyl-1,14-deoxy-3-vinyl-16H-triptyrin-8,12-dipropionic acid). We measured biopyrrins in random urine specimens and investigated whether the biopyrrin values obtained were valid when expressed as a ratio of the creatinine (Cr) concentrations. All of the random urine specimens collected over 48 hr were from presumably healthy adults. We measured the biopyrrins by means of an enzyme-linked immunosorbent assay (ELISA) using an anti-bilirubin monoclonal antibody. When the values were expressed in terms of the ratio to Cr,

the within-day coefficient of variation (%CV) of the excretion of biopyrrins was reduced to $27\% \pm 10\%$ ($P < 0.05$) from $59\% \pm 27\%$. However, assay values on random or spot urine specimens were unreliable because of the large %CV. The biopyrrin concentrations only in the first-morning-urine specimens in terms of both absolute amounts and ratios to Cr significantly reflected those in a 24-hr urine specimen ($P < 0.001$). Concentrations in a random urine specimen voided at the second collection or later did not correlate with the concentration in a 24-hr urine specimen ($P > 0.05$), even if their values were corrected by Cr. The amounts of biopyrrins excreted in 24-hr urine specimens were significantly correlated with the 24-hr cortisol excretion ($P < 0.001$) but not to uropepsin ($P > 0.05$). *J. Clin. Lab. Anal.* 21:1–6, 2007. © 2007 Wiley-Liss, Inc.

Key words: antioxidant; bilirubin; cortisol; creatinine correction; first morning urine; 24-hour urine; physico-psychological stress; uropepsin

INTRODUCTION

For many years, bilirubin was generally regarded as a potentially toxic waste product formed during heme catabolism. However, more recent evidence suggests that it is a physiological antioxidant that may scavenge oxygen and peroxy radicals as efficiently as α -tocopherol (1,2). The bilirubin referred to here is unconjugated bilirubin bound to serum albumin—not bilirubin conjugated with glucuronic acid (3). The former is present in normal serum, and the latter is increased in serum from patients with hepatobiliary diseases. During oxidative injury or “stress,” the hepatic mRNA activity of heme oxygenase-1, a rate-limiting enzyme, is rapidly induced (4). Heme oxygenase-1 converts heme to

biliverdin, which is subsequently reduced to bilirubin by the cytosolic enzyme biliverdin reductase.

Recently, Yamaguchi and coworkers (5,6) isolated and identified oxidative metabolites of bilirubin from the urine of healthy humans by means of an enzyme-linked immunosorbent assay (ELISA) using an anti-bilirubin monoclonal antibody (no. 24G7) (7–9). This

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antibody has an epitope in the dipyrrole region of bilirubin, and reacts with oxidized metabolites of bilirubin, unconjugated bilirubin, and conjugated bilirubin. Unconjugated and conjugated bilirubins are undetectable or not present at all in the urine of normal subjects. Yamaguchi et al. (5,6) referred to the oxidative metabolites of bilirubin, including its two regioisomers, biotripyrrin-a (1,14,15,17-tetrahydro-2,7,13-trimethyl-1,14-deoxy-3-vinyl-16H-tripyrin-8,12-dipropionic acid) and biotripyrrin-b (1,14,15,17-tetrahydro-3,7,13-trimethyl-1,14-deoxy-3-vinyl-16H-tripyrin-8,12-dipropionic acid), as biopyrrins. Circulating bilirubin functions as a suicide antioxidant (i.e., a terminal antioxidant) and is thought to decompose to biopyrrins.

Recent studies observed elevated urinary excretion of biopyrrins in cases of sepsis (10,11), ischemic heart disease (12), congestive heart failure (13), atopic dermatitis (14), surgical stress (15,16), and psychological stress (17,18). All of these reports took urinary biopyrrins to be a ratio of creatinine (Cr) concentrations, based on the assumption that Cr correction can greatly reduce the variability of the results from random urine samples. The aim of this study was to investigate whether the urine biopyrrin-to-Cr ratio can correct for the diurnal variation of the voided volume. We also sought to determine whether Cr correction is useful for other established urine markers (e.g., uropepsin and cortisol) that responded to physico-psychological stress.

MATERIALS AND METHODS

Subjects

We collected two 24-hr urine samples from 11 Japanese adults. Seven were women (W1–W7, 22–60 years old) and four men (M1–M4, 23–50 years old), all of whom were presumed to be in good health. Written informed consent was obtained from all subjects, and our study complied with the rules for human experimentation in our institution. Some of the subjects completed the experiments two or three times. After urine volumes were measured, all specimens were stored at -80°C for later analyses of biopyrrins, uropepsin, urinary free cortisol, and Cr.

Methods

We measured the urinary concentrations of the biopyrrins by ELISA using an anti-bilirubin monoclonal antibody (no. 24G7; Shino-Test Corp., Kanagawa, Japan) (7,8). We calibrated the ELISA test with unconjugated bilirubin, and expressed the biopyrrin values in terms of the unconjugated bilirubin concentration. The ELISA test was linear from 0.1 to $3.2\mu\text{mol/L}$. The within-day assay for five consecutive replicates gave

a mean $\pm$ SD (and CV) of $0.66 \pm 0.039\mu\text{mol/L}$ (5.9%), and the between-day assay for 5 days gave a mean $\pm$ SD (and CV) of $0.57 \pm 0.040\mu\text{mol/L}$ (7.0%). We also measured the concentration of uropepsin, an aspartic proteinase found in urine and produced in the gastric mucosa as pepsinogen (19,20), using the Anson-Mirsky hemoglobin method (21). Also, we assayed the urinary concentrations of free cortisol by a specific radioimmunoassay technique (Cortisol assay kit, cat. #1841; Beckman Coulter-Immunotech Diagnostics, France). We determined the urinary Cr concentration with a Hitachi 7150 automated analyzer (Hitachi High-Technologies Corp., Tokyo, Japan) using reagents from Shino-Test Corporation.

Analysis of Data

We tested for statistically significant differences using the Wilcoxon matched-pairs signed-ranks test for non-Gaussian variables. We defined a statistically significant difference or agreement as $P < 0.05$.

RESULTS

24-hr Urinary Biopyrrin Excretion

On the first morning of the collections, the first urine sample was discarded. We then collected all urine voided for the next 24 hr, including the first from the next morning. We calculated the amounts of biopyrrin excreted in urine over 24 hr from the sum of the amounts separately obtained in random urine specimens. We found almost equal amounts of biopyrrins in the two 24-hr urinary biopyrrin excretions on days 1 and 2, and the same was true for Cr. Successive 2-day excretions of biopyrrins were not affected by the variation in 24-hr urine volumes (see Table 1). Also, no significant differences were observed between the two 24-hr excretions of uropepsin or cortisol. The urinary excretions of biopyrrins, uropepsin, and cortisol in the 24-hr urine specimens were significantly correlated with the amount of Cr excreted over 24 hr. The correlation coefficients with Cr were 0.596 for biopyrrins, $P < 0.01$; 0.535 for uropepsin, $P < 0.01$; and 0.819 for cortisol, $P < 0.001$. The amounts of biopyrrins excreted in 24-hr urine specimens were significantly correlated to those of cortisol ($P < 0.001$), but not to those of uropepsin ($P > 0.05$).

Within-Day Variation in Urinary Biopyrrin Excretion

Our subjects collected four to 12 random urine specimens (eight times on average) over 24 hr. The amounts of Cr, biopyrrins, uropepsin, and cortisol excreted in each of the random urine specimens varied

TABLE 1. Twenty-four-hr urine volume and urinary excretion of biopyrrins, uropepsin, or cortisol in successive two days*

Subjects	Age (year)	Day 1 (day 2)				
		Urine 24-hr volume (mL/day)	Cr excretion (mg/day)	BP excretion (μ mol/day)	UP excretion (mg/day)	UFC excretion (μ g/day)
W1	22	2658 (3063)	1110 (1229)	1.0 (0.9)	1.40 (1.58)	nd
W1	22	4319 (2823)	1284 (1059)	1.4 (0.9)	1.80 (1.58)	nd
W2	60	1435 (1348)	902 (827)	1.5 (2.2)	0.39 (0.39)	25 (22)
W3	30	1088 (1682)	1153 (1020)	1.1 (1.3)	0.43 (0.32)	35 (40)
W4	33	1485 (1370)	1156 (1146)	2.4 (2.2)	0.55 (0.40)	35 (40)
W5	34	454 (1600)	858 (896)	2.4 (1.8)	0.51 (0.74)	27 (51)
W6	31	1275 (600)	695 (671)	6.6 (7.1)	0.25 (0.20)	26 (32)
W7	39	850 (794)	1078 (865)	1.8 (1.9)	0.73 (0.50)	28 (31)
M1	53	1948 (1975)	1265 (1153)	1.6 (1.6)	0.99 (0.75)	nd
M1	53	2015 (1944)	1198 (1137)	1.8 (2.0)	0.68 (0.50)	nd
M1	54	1638 (1930)	1127 (1007)	1.3 (1.8)	0.94 (0.87)	32 (35)
M2	26	1444 (1351)	1484 (1258)	1.3 (1.6)	1.13 (0.97)	nd
M2	26	1234 (2015)	1608 (1360)	2.3 (2.2)	1.00 (1.56)	nd
M3	37	1410 (1548)	1856 (1779)	2.7 (2.9)	1.31 (1.41)	60 (43)
M4	41	2275 (4535)	1617 (1975)	5.4 (3.0)	0.61 (1.00)	55 (79)

*Fourty-eight-hr collections were made from two 24-h collections on "Day 1" and "Day 2", respectively. 48-hr collections were performed two times for W1 and three times for M1. No significant difference between two successive days was observed in urinary excretion of biopyrrins, uropepsin, and cortisol ($P > 0.05$).

Cr, creatinine; BP, biopyrrins; UP, uropepsin; UFC, urinary free cortisol; nd: not determined.

according to their urine volume. The within-day %CV was $53\% \pm 17\%$ for Cr (mean $\pm$ SD of tests, from 11 subjects); $59\% \pm 27\%$ for biopyrrins; $51\% \pm 17\%$ for uropepsin; and $77\% \pm 30\%$ for cortisol. When values of biopyrrins, uropepsin, and cortisol were expressed in terms of their ratio to Cr, only the %CV of biopyrrins was significantly reduced ($27\% \pm 10\%$, $P < 0.05$). The CV of uropepsin changed to $47\% \pm 21\%$ ($P > 0.05$), and that of cortisol increased to $79\% \pm 32\%$ ($P > 0.05$). This finding is probably due to the diurnal excretion of cortisol, which shows a maximum level in the morning and a nadir during the night. When the concentrations of biopyrrins in each random urine were compared between two successive days, the lowest value observed in one day with a higher 24-hr excretion was lower than the highest value observed on another day with a lower 24-hr excretion (W2, W4, W5, W6, W7, M1, and M4 in Table 2), even though they were expressed as the ratios to Cr. The same was true for uropepsin and cortisol.

When we compared the values of random urine specimens with those in a 24-hr urine specimen, we found that the first morning urine voided after a night's sleep (6 hr and 15 min, on average, in our subjects) reflected the concentrations in a 24-hr urine specimen. The concentrations of biopyrrins, uropepsin, and cortisol in terms of both absolute amounts and ratios to Cr in the first morning void significantly reflected those in a 24-hr urine specimen ($P < 0.001$; Fig. 1). The analyte concentrations in a random urine specimen voided at the second collection or later did not correlate

with the analyte concentration in a 24-hr urine specimen ($P > 0.05$). We conclude that the assay values of biopyrrins, uropepsin, and cortisol are reliable provided the specimens are collected at a fixed time of day (in this case, in the morning). The respective amounts of biopyrrins, uropepsin, and cortisol expressed as the fraction in the first morning void divided by the total over 24 hr were 25% for biopyrrins (range = 6–45%), 18% for uropepsin (8–35%), and 20% for cortisol (4–36%).

DISCUSSION

The metabolites described here were excreted in urine and determined in 24-hr collections. Because the collection of 24-hr specimens is fraught with problems, the use of random urine specimens and correction with the Cr concentration provide a convenient way to estimate the 24-hr excretion (22). For this reason, the aim of our study was to investigate whether the corrected values of biopyrrins in a random specimen (divided by Cr) would serve as well as a 24-hr collection. The within-day %CV of biopyrrins excreted in random urines was significantly reduced by correction with Cr. Also, the biopyrrins in a random first-morning-urine specimen collected after a night's sleep was significantly correlated with the biopyrrins in a 24-hr urine collection. We conclude that urinary excretion of biopyrrins in 24-hr urine specimens can be estimated from a first-morning-urine specimen corrected by the Cr excretion.

TABLE 2. Within-day variation in urinary excretion of biopyrrins, uropepsin, or cortisol*

Subjects		Voiding times	Excretion in 24-hr urine (and random urine) specimens		
			BP/Cr ($\mu\text{mol/g}$)	UP/Cr (mg/g)	UFC/Cr ($\mu\text{g/g}$)
W1	Day 1	10	0.9 (0.5: 0.4–1.5)	1.27 (0.68: 0.48–4.32)	nd
	Day 2	11	0.7 (1.1: 0.3–1.2)	1.28 (0.83: 0.55–2.42)	nd
W1	Day 1	12	1.1 (1.3: 0.3–1.7)	1.40 (0.94: 0.50–5.22)	nd
	Day 2	11	0.9 (1.0: 0.3–1.5)	1.49 (0.62: 0.57–6.62)	nd
W2	Day 1	7	1.6 (1.2: 1.2–2.4)	0.43 (0.19: 0.19–0.95)	27 (19: 2–62)
	Day 2	7	2.7 (2.8: 2.1–3.4)	0.47 (0.28: 0.07–1.25)	27 (19: 8–62)
W3	Day 1	6	0.9 (0.8: 0.8–1.1)	0.37 (0.41: 0.26–0.42)	30 (14: 9–96)
	Day 2	8	1.2 (0.8: 0.8–2.0)	0.31 (0.40: 0.18–0.43)	40 (43: 12–350)
W4	Day 1	8	2.1 (2.5: 1.2–2.9)	0.48 (0.54: 0.27–0.63)	30 (34: 12–83)
	Day 2	9	1.9 (1.2: 1.2–2.3)	0.35 (0.33: 0.13–1.02)	35 (18: 8–171)
W5	Day 1	6	2.8 (4.1: 2.1–4.1)	0.59 (0.45: 0.26–1.02)	32 (8: 7–82)
	Day 2	10	2.1 (1.9: 1.5–2.6)	0.83 (0.87: 0.30–1.60)	57 (81: 9–146)
W6	Day 1	6	9.6 (5.5: 5.5–16.3)	0.35 (0.26: 0.26–0.50)	37 (14: 14–137)
	Day 2	6	10.6 (7.6: 7.5–16.6)	0.30 (0.20: 0.16–0.59)	47 (27: 6–134)
W6	Day 1	7	nd (4.4: 0.8–6.8)	nd	nd
W7	Day 1	4	1.7 (1.6: 1.5–2.3)	0.68 (0.24: 0.24–1.19)	26 (14: 14–37)
	Day 2	5	2.1 (2.5: 1.7–3.2)	0.57 (0.69: 0.30–1.27)	36 (34: 5–56)
M1	Day 1	8	1.2 (1.0: 0.8–2.0)	0.78 (0.51: 0.51–1.12)	nd
	Day 2	8	1.4 (1.7: 0.8–2.5)	0.65 (0.49: 0.30–1.06)	nd
M1	Day 1	8	1.5 (1.1: 0.8–2.5)	0.57 (0.28: 0.28–0.93)	nd
	Day 2	8	1.8 (2.1: 1.4–2.1)	0.44 (0.28: 0.28–0.97)	nd
M1	Day 1	6	1.2 (1.2: 0.9–1.3)	0.83 (0.52: 0.52–1.82)	28 (29: 12–99)
	Day 2	7	1.8 (1.7: 1.1–2.8)	0.87 (0.61: 0.57–1.40)	35 (35: 17–69)
M2	Day 1	10	0.9 (1.1: 0.5–1.2)	0.76 (0.66: 0.48–1.55)	nd
	Day 2	8	1.2 (0.8: 0.8–2.1)	0.77 (0.80: 0.44–1.65)	nd
M2	Day 1	9	1.4 (1.5: 1.2–1.7)	0.62 (0.48: 0.47–0.90)	nd
	Day 2	11	1.6 (1.2: 0.8–2.2)	1.15 (0.79: 0.70–2.26)	nd
M3	Day 1	5	1.5 (1.3: 1.3–1.7)	0.70 (0.51: 0.51–0.88)	32 (16: 16–71)
	Day 2	5	1.6 (1.4: 1.4–1.9)	0.79 (0.54: 0.44–1.12)	24 (14: 9–41)
M4	Day 1	5	3.4 (4.5: 2.3–4.5)	0.38 (0.18: 0.18–1.31)	34 (35: 16–62)
	Day 2	8	2.6 (1.9: 1.9–3.3)	0.51 (0.48: 0.30–0.57)	40 (78: 22–78)

*Forty-eight-hr collections were made from two 24-hr collections on “Day 1” and “Day 2”, respectively. 48-hr collections were performed two times for W1 and three times for M1. For W6, one 48-hr collection and another 24-hr collection was performed. Values are excretions in 24-hr urine specimens (first-morning urine: variation range in random urine).

Cr, creatinine; BP, biopyrrins; UP, uropepsin; UFC, urinary free cortisol; nd: not determined.

Random urine specimens collected at times other than the first morning void may be misleading because of diurnal variation, even if their values are corrected by Cr. Based on a literature search, we believe this the first report to investigate the effectiveness of Cr correction for biopyrrins excreted in random urine specimens.

Shimoharada et al. (23) determined the biopyrrins-to-Cr ratio in first morning urine from 33 healthy adults (33 ± 18 years old) for 1 month, and concluded that the between-day variance was physiologically small and the values seldom fluctuated more than twice the mean value ($\pm$ SD) obtained in their volunteers ($0.82 \pm 0.29 \mu\text{mol/g}$ Cr for men; $0.74 \pm 0.11 \mu\text{mol/g}$ Cr for women). We determined the 95% distribution range (i.e., the reference range) for urinary biopyrrin excretion from our subjects after we performed a log transformation. The distribution of the values in terms of both absolute amounts and ratios to Cr were non-Gaussian. We found reference

ranges of $0.8\text{--}4.0 \mu\text{mol}$ (absolute amounts) in 24-hr urine specimens, $0.7\text{--}3.1 \mu\text{mol/g}$ Cr (ratio) in 24-hr urine, and $0.5\text{--}3.1 \mu\text{mol/g}$ Cr (ratio) in first-morning-urine specimens from both men and women.

However, W5 was excluded from this calculation because she showed the highest biopyrrin excretion we have ever encountered. Apart from having non-hemolytic unconjugated hyperbilirubinemia, she was a healthy woman. Her serum bilirubin concentrations were always in the range of $15\text{--}34 \mu\text{mol/L}$, and the conjugated bilirubin was below our detection limits. No increased concentrations of other antioxidants (e.g., ascorbic acid, α -tocopherol, uric acid, SH-groups, and β -carotene) were detected in her serum, but the plasma concentration of oxidized low-density lipoprotein (LDL) was extremely low at 1.52 U/mL (the reference range in our laboratory is $3\text{--}20 \text{ U/mL}$). Her urinary excretion of biopyrrins was within our reference range at

Fig. 1. Comparison of urinary excretion of biopyrrins, uropepsin, and cortisol in 24-hr and first-morning urine specimens. The plots above represent absolute amounts of biopyrrins, uropepsin, and cortisol excreted in urine, and the plots below represent values corrected by the Cr concentration in the same urine specimen.

10:00 a.m. and 12:00 a.m. (1.0 and $0.8 \mu\text{mol/g}$ Cr, respectively; Table 2), which also indicates that samples other than the first-morning-urine sample are unreliable. In previous studies the 24-hr excretion of uropepsin was reported to increase in patients with stress ulcers (24,25), while 24-hr excretion of cortisol was reportedly increased by psychological stress (26). Since the amounts of biopyrrins excreted in 24-hr urine specimens were significantly correlated to those of cortisol but not to uropepsin, we consider that the mechanism of excretion of urinary biopyrrins is more likely linked to the cortisol stress response.

In conclusion, in this study diurnal variation of biopyrrin excretion in random urine specimens was not corrected by Cr. However, the biopyrrin concentrations in the first-morning-urine specimens, in terms of both absolute amounts and ratios to Cr, were significantly correlated with those in a 24-hr urine specimen and were also usable in the estimation of 24-hr urinary biopyrrin excretion.

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REFERENCES

1. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043–1046.
2. Mayer M. Association of serum bilirubin concentration with risk of coronary artery disease. *Clin Chem* 2000;46:1723–1727.
3. Stocker R, Glazer AN, Ames BN. Antioxidant activity of albumin-bound bilirubin. *Proc Natl Acad Sci USA* 1987; 84:5918–5922.
4. Yamaguchi T, Terakado M, Horio F, Aoki K, Tanaka M, Nakajima H. Role of bilirubin as an antioxidant in an ischemia: reperfusion on rat liver and induction of heme oxygenase. *Biochem Biophys Res Commun* 1996;223:129–135.
5. Yamaguchi T, Shioji I, Sugimoto A, Komoda Y, Nakajima H. Chemical structure of a new family of bile pigments from human urine. *J Biochem* 1994;116:298–303.
6. Yamaguchi T, Horio F, Hashizume T, et al. Bilirubin is oxidized in rats treated with endotoxin and acts as a physiological antioxidant synergistically with ascorbic acid *in vivo*. *Biochem Biophys Res Commun* 1995;214:11–19.
7. Shimizu S, Izumi Y, Yamazaki M, Shimizu K, Yamaguchi T, Nakajima H. Anti-bilirubin monoclonal antibody: I. Preparation and properties of monoclonal antibodies to covalently coupled bilirubin-albumin. *Biochim Biophys Acta* 1988;967: 255–260.
8. Izumi Y, Yamazaki M, Shimizu S, Shimizu K, Yamaguchi T, Nakajima H. Anti-bilirubin monoclonal antibody: II. Enzyme-linked immunosorbent assay for bilirubin fractions by combination of two monoclonal antibodies. *Biochim Biophys Acta* 1988; 967:261–266.

9. Yamaguchi T, Shioji I, Sugimoto A, Komoda Y, Nakajima H. Epitope of 24G7 anti-bilirubin monoclonal antibody. *Biochim Biophys Acta* 1996;1289:110–114.
10. Otani K, Shimizu S, Chijiwa K, Yamaguchi K, Kuroki S, Tanaka M. Increased urinary excretion of bilirubin oxidative metabolites in septic patients: a new marker for oxidative stress *in vivo*. *J Surg Res* 2001;96:44–49.
11. Vitek L. Impact of serum bilirubin on human disease. *Pediatrics* 2005;115:1411–1412.
12. Morita Y, Takahashi H, Kamihata H, Yamamoto Y, Hara K, Iwasaki T. Urinary excretion of biopyrrins, oxidative metabolites of bilirubin, increases after spasm provocation tests in patients with coronary spastic angina. *Int J Cardiol* 2001;80:243–250.
13. Hokamaki J, Kawano H, Yoshimura M, et al. Urinary biopyrrins levels are elevated in relation to severity of heart failure. *J Am Coll Cardiol* 2004;43:1880–1885.
14. Tsukahara H, Shibata R, Ohshima Y, et al. Oxidative stress and altered antioxidant defenses in children with acute exacerbation of atopic dermatitis. *Life Sci* 2003;72:2509–2516.
15. Tsujinaka T, Fujita J, Morimoto T, et al. Increased urinary excretion of bilirubin metabolites in association with hyperbilirubinemia after esophagectomy. *Surg Today* 1998;28:1119–1123.
16. Kozaki N, Shimizu S, Chijiwa K, et al. Bilirubin as an antioxidant for surgical stress: a preliminary report of bilirubin oxidative metabolites. *HPB Surg* 1999;11:241–248.
17. Yamaguchi T, Shioji I, Sugimoto A, Yamaoka M. Psychological stress increases bilirubin metabolites in human urine. *Biochem Biophys Res Commun* 2002;293:517–520.
18. Miyaoka T, Yasukawa R, Yasuda H, et al. Urinary excretion of biopyrrins, oxidative metabolites of bilirubin, increases in patients with psychiatric disorders. *Eur Neuropsychopharmacol* 2005; 15:249–252.
19. de Azevedo WF Jr, Canduri F, Fadel V, Teodoro LG, Hial V, Gomes RA. Molecular model for the binary complex of uropepsin and pepstatin. *Biochem Biophys Res Commun* 2001; 287:277–281.
20. Canduri F, Teodoro LG, Fadel V, et al. Structure of human uropepsin at 2.45 Å resolution. *Acta Crystallogr D Biol Crystallogr* 2001;57(Pt 11):1560–1570.
21. Vennes JA, Rames ED, Simon W. Uropepsinogen in chronic emotional stress. *Gastroenterology* 1958;35:200–205.
22. Ginsberg JM, Chang BS, Matarese RA, Garella S. Use of single voided urine samples to estimate quantitative proteinuria. *N Engl J Med* 1983;309:1543–1546.
23. Shimoharada K, Inoue S, Nakahara M, et al. Urine concentration of biopyrrins: a new marker for oxidative stress *in vivo*. *Clin Chem* 1998;44:2554–2555.
24. Balibrea JL, Gomez J, Alemany JR, et al. Restraint-induced stress ulcer: I. Hypothalamic, urinary, and adrenal biochemical studies. *World J Surg* 1980;4:83–89.
25. Groza P, Ursea N, Vasilescu F, Munteanu A, Lungu D, Bolocan N. Changes in some digestive enzymes after a seven-day orbital flight. *Physiologie* 1983;20:27–33.
26. Junghanns K, Backhaus J, Tietz U, et al. Impaired serum cortisol stress response is a predictor of early relapse. *Alcohol Alcohol* 2003;38:189–193.