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ABSTRACT

Background: Specific gravity (SG) may perform as well as creatinine (CR) correction for adjusting urinary hormone concentrations, as well as offer some advantages. We compared the two methods and applied them to US and Bangladeshi specimens to evaluate their use in different populations.

Methods: Pearson correlations between serum concentrations and SG, CR, and uncorrected urinary concentrations were compared using paired daily urine and serum specimens from one menstrual cycle from 30 US women. Corrected urinary estrone conjugate (E1C) and pregnanediol glucuronide (PDG) concentrations were compared with serum estradiol (E2) and progesterone (P4). Urine specimens across one menstrual cycle from 13 Bangladeshi women were used to evaluate the applicability of both methods to a non-industrialized population. Linear mixed effects models were used to compare CR and SG gravity values in the Bangladeshi vs. US specimens.

Results: There was no significant difference between SG corrected vs. serum and CR corrected vs. serum correlations for either assay. Usable CR results were obtained for all US specimens, but 37% of the Bangladeshi specimens were below the CR assay limit of detection. The Bangladeshi sample had significantly lower CR, and higher inter- and intra-subject CR variability, compared to the US sample.

Conclusions: SG is a useful alternative to CR correction for normalizing urinary steroid hormone concentrations, particularly in settings where CR values are highly variable or unusually low.

³Nonstandard Abbreviations: SG, specific gravity; CR, creatinine; B, Bangladeshi sample; E2, estradiol; IFMA, immunofluometric assay; LH, luteinizing hormone; P4, progesterone; EIA, enzyme immunoassay; PDG, pregnanediol glucuronide; E1C, estrone conjugates

Introduction

Urine specimens have several advantages over serum, saliva, and blood spots for monitoring reproductive hormone patterns. Urine collection is non-invasive, poses minimal infectious disease risk to participants and researchers, and provides sufficient volume for multiple assays and future research. Urine specimens are ideally suited for large studies because they can be collected and stored by participants, and compliance is high [1-3]. Urine also provides integrated hormone measures without the confounding effects of pulsatile secretion [4]. Finally, urinary levels of reproductive steroid hormone metabolites are generally higher than the circulating serum levels, providing better quantification of the lower end of the physiological scale in humans, which can be close to or below the limit of detection in serum RIAs [5]. Urinary hormone results must, however, be adjusted to account for the concentration of the specimen, which depends on a subject's hydration status and time since last urination.

We examined the performance of two methods of adjustment: specific gravity (SG)³ correction and creatinine (CR) correction. Urine SG is the ratio of the density of a urine specimen to the density of water [6]. Specific gravity increases with solute concentration and is most accurately measured by refractometry [6]. Creatinine, a byproduct of muscle activity, is cleared from the blood stream by the kidneys and excreted in urine [7]. Urinary creatinine concentrations are determined by colorimetric assay [8], and analyte concentrations are usually reported as a ratio of the analyte concentration to creatinine concentration.

Based on an early finding that daily individual creatinine excretion was fairly consistent [9], urinary creatinine became a common method of assessing kidney function in clinical settings and correcting for analyte concentrations, including reproductive hormones, in urine [4]. Using creatinine to normalize urinary analyte concentrations can be problematic, however, because

there is evidence that creatinine excretion is not consistent: numerous studies have found considerable inter- and intra-subject variability in creatinine values and dependence on sex, age, activity, and diet [10-18]. Population variation in creatinine excretion may also exist but to the best of our knowledge, this topic has not been investigated.

Specific gravity is an alternative method with several advantages over creatinine although it is not widely used, perhaps in part because of a lack of data evaluating its performance relative to creatinine. In this study we compared SG and CR correction methods on urinary hormone metabolite concentrations from healthy US women using serum hormone measurements as the standard. We then applied both methods to spot specimens from Bangladeshi women to evaluate their applicability in a nonindustrialized population.

Materials and Methods

Samples and Specimens

A total of 799 daily urine and serum specimens were collected over one menstrual cycle from 30 US women in 1997-1998. Thirteen women aged 20-25 years and 17 women 40-45 years old were recruited for a study on reproductive aging. Monetary compensation was provided, participants provided written informed consent, and all procedures were approved by the Institutional Review Board of the University of Washington. All participants were normally cycling, in good health, had a mean body mass index of 22.6 kg/m² (SD = 2.36, range 18.9 – 27.7), and were not using medications or hormones. Blood specimens were obtained by venipuncture, beginning with the first day of menstrual bleeding and continuing until the first day of menstrual bleeding of the subsequent cycle. Serum specimens were immediately assayed and all cycles were confirmed ovulatory by transvaginal ultrasound. Urine specimens were taken daily in the clinic, usually before noon, at the same time as serum collection and immediately

stored at -20°C . Urine specimens remained frozen until thawing two years later for aliquoting, assay, and measurement of specific gravity [19]. The specimens underwent 2 to 3 more freeze thaws cycles prior to creatinine assay in 2003.

For the Bangladeshi sample (B), 13 cycling women were selected randomly from a sample of subjects participating in a 9-month study of early pregnancy loss. All participants were married, noncontracepting residents in the nonintervention demographic surveillance region of the rural Matlab district in Bangladesh [20]. No monetary compensation was provided, all participants provided written informed consent, and all procedures were approved by the Institutional Review Boards of The Pennsylvania State University and the International Centre for Diarrhoeal Disease and Research, Bangladesh [20]. Spot urine specimens were collected by community health care workers every 3 or 4 days over the course of one menstrual cycle in 1993. The specimens were stored at the health care worker's home in a cooler with ice packs for 1 to 3 days until they were transported to a field hospital and stored at 4°C . One to three days later, the specimens were brought to room temperature and specific gravities were taken. The specimens were preserved with 0.17 g/mL boric acid solution, stored at -20°C , and transported to the US by frozen air freight. Specimens remained frozen until 1995 when they were thawed and assayed for creatinine and steroid hormone metabolites.

Assays

All serum specimens were assayed by RIA for estradiol (E2) and by immunofluometric assay (IFMA) for luteinizing hormone (LH), but serum progesterone (P4) was measured only in the luteal phase. The RIA for E2 (ICN Biomedicals) cross-reacts 20% with estrone, 1.5% with estriol, and $<1\%$ with all other steroids. The inter- and intra-assay CVs were 16% and 7%, respectively. The inter- and intra-assay CVs for the LH IFMA (Delphia) were 2.8% and 4.7%

respectively. The RIA for P4 (Diagnostic Systems Laboratories) cross-reacts <5% with all other steroids and the inter- and intra-assay CVs were 13% and 11% respectively.

Urine specimens were analyzed with competitive enzyme immunoassays (EIAs) for urinary steroid hormone metabolites. All US and Bangladeshi urine specimens were assayed for pregnanediol glucuronide (PDG) and estrone conjugates (E1C). The PDG and E1C EIAs have been described elsewhere [19]. Briefly, the PDG assay used the monoclonal antibody Q330 (Quidel Corporation) and reference calibrator 5 β -pregnane-3 α ,20 α -diol glucuronide (Sigma Catalog. No. P3635). The inter- and intra-assay coefficients of variation for the PDG assay were 10.3% and 9.2% respectively [19]. E1C was measured using the 155-B3 monoclonal capture antibody (Fortune Kohen, Weizmann Institute, Rehovet, Israel), and estrone- β -D-glucuronide reference calibrator (Sigma Catalog No. E1752). The inter- and intra-assay CVs were 10.9% and 7.3% [19]. The optical density for the EIAs was measured with a Dynatech MR7000 Plate Reader (test wavelength 405 nm, reference wavelength 570 nm). All specimens were run in duplicate on microtiter plates, and hormone concentrations were estimated from optical density using a four parameter logistic model in Biolinx 1.0 Software (Dynex Laboratories, Inc).

Urinary creatinine was measured by reaction with sodium hydroxide and picric acid using the method described by Jaffe [8] with calibrators purchased from Sigma (Catalog No. 925-11). The optical density of specimens was read with a Dynatech MR7000 Plate Reader (test wavelength 490 nm, reference wavelength 630 nm) and creatinine concentrations were estimated with a four parameter logistic model in Biolinx 1.0 Software. The inter- and intra-assay CVs for the creatinine assay were 1.6% and 13.8% respectively.

The creatinine correction formula applied to each sample was:

$$\text{creatinine corrected concentration (nmol/nmol)} = \frac{\text{raw hormone concentration (nmol / volume)}}{\text{creatinine (nmol /volume)}}.$$

Specific gravity measurements were taken with a hand-held urine specific gravity refractometer (Atago Uricon-PN, NSA Precision Cells, Inc). The specific gravity of Bangladeshi urine specimens was measured before freezing, whereas the specific gravity for the US specimens was measured after the first freeze-thaw cycle. The correction formula applied to each hormone result was:

$$\text{specific gravity corrected concentration} = \text{raw hormone concentration} \times \frac{(\text{specific gravity}_{\text{target}} - 1.0)}{(\text{specific gravity}_{\text{sample}} - 1.0)}$$

where $\text{specific gravity}_{\text{target}}$ is a population mean specific gravity [20]. The target specific gravity used was 1.020 [21] for US specimens and 1.015 [20] for Bangladeshi specimens.

Statistical Analyses

Pearson correlations between serum hormone values and raw, SG corrected, and CR corrected urinary hormone results were calculated using an optimum lag day. Individual cycles were aligned by day of midcycle serum LH peak (day 0) and a mean hormone value for each cycle day was calculated (n = 34 paired urine/serum cycle days from 30 cycles). The effect of time between appearance of hormones in serum and clearance into the urine was evaluated by correlating serum results with urine results lagged by -1, 0, 1, 2, and 3 days behind serum. The optimum lag day was selected on the basis of the highest correlation with serum results.

The correlations of serum E2 with urinary E1C, and serum P4 with urinary PDG, were weighted by the number of specimens available for each cycle day. The 95% confidence intervals for the difference between SG corrected urine vs. serum, CR corrected urine vs. serum, and uncorrected urine vs. serum average correlations were obtained using bootstraps. The hypothesis that two correlations were the same was rejected when the confidence interval did not include zero.

We used a linear mixed effects model to test for differences in mean CR or SG by population (US vs. B). Mixed effects models allow the significance of a fixed effect (in this case, the mean CR or SG difference between the US and B populations) to be tested while accounting for within subject repeated measures by modeling a random effect for each subject as follows:

$$Y = a + bX + c_i + e_{ji}$$

where Y is either a creatinine or specific gravity value, X is 0 for US and 1 for Bangladesh, a is the estimated CR or SG for the US sample, b is the estimated difference between CR or SG between Bangladesh and US, c_i is a random effect estimated for the i^{th} subject and e_{ji} is the residual error associated with the j^{th} specimen from the i^{th} subject. We also used linear mixed effects models to compare between and within subject (residual) variability for each population in separate models by estimating the standard deviation for the c_i term representing between subject variability and the e_{ji} term, the within subject variability.

Results

For both assays, the highest correlation between urine and serum values occurred on the same lag day for uncorrected, SG corrected, and CR corrected values (Table 1). PDG and E1C results lagged one day behind their respective serum values. When optimum lag day was used, weighted correlations between serum and SG corrected urinary hormone values were high for both assays, ranging from 0.94 to 0.97 (Table 2). Correlations were also high for CR corrected (0.93 to 0.98) and uncorrected urine results (0.92 to 0.93) (Table 2). There was no significant difference in the correlations between SG corrected urine vs. serum values and CR corrected urine vs. serum for either hormone (Table 3). In the progesterone assays, there was a significant difference between uncorrected urine vs. serum correlations and both SG corrected urine vs. serum and CR corrected urine vs. serum correlations (Table 3).

All US specimens had a usable creatinine result while 37% of Bangladeshi specimens had creatinine values below the assay limit of detection (0.002 nmol/L), even when assayed undiluted. In the linear mixed effects analyses, the US sample had significantly higher creatinine levels than the Bangladeshi sample (mean US = 11.58 nmol/L; mean B = 1.58 nmol/L; $p < 0.001$). Figure 1 shows the distribution of creatinine values for each US and Bangladeshi participant and illustrates the large difference in creatinine values between the two populations. US specimen inter-subject and intra-subject standard deviations were 4.8 nmol/L and 0.16 nmol/L respectively. In Bangladeshi specimens these measures were 1.14 nmol/L and 0.27 nmol/L, showing greater intra-subject variation in creatinine values. The ratio of within to between subject standard deviation for creatinine in the US sample was almost seven times smaller than the Bangladesh sample (0.033 for US, 0.24 for Bangladesh).

When individual Bangladeshi subject hormone profiles were corrected with CR, extremely low CR values resulted in spuriously high corrected hormone results (Figure 2). Very low CR values in the Bangladesh sample caused unreasonably poor concordance between uncorrected and CR corrected hormone results (Figure 3A). For example, if low (< 0.1 nmol/L) creatinine specimens are removed, the correlation between CR corrected and uncorrected PDG results increases (Figure 3B). However, all these specimens with unusable creatinine results give an acceptable correlation with uncorrected PDG values when adjusted by specific gravity (Figure 3C).

Figure 4 shows the distribution of specific gravity values for each US and Bangladeshi participant. Unlike for creatinine, the distributions of specific gravity values for the two populations were similar. The mean specific gravity values in the US (1.014) and Bangladesh (1.013) specimens did not significantly differ ($p = 0.50$). The ratio of within to between subject

standard deviation in specific gravity values was 1.52 for the US sample and 1.22 for the Bangladeshi sample, indicating a similar amount of variation in this measure for the two populations.

DISCUSSION

Our results show that specific gravity performs as well as creatinine in correcting steroid hormone metabolite results for urine concentration, in agreement with other studies comparing SG and CR normalization of urinary analyte concentrations [22, 23]. We demonstrated that SG adjustment is applicable in specimens with CR levels that are not sufficiently high to correct metabolite concentrations. We have also provided evidence of a population level difference in urinary CR values and concurrent population level similarities in SG measurements. Currently, CR correction is widely used to adjust for urine analyte concentration in spot and first morning specimens although several studies have concluded that it offers no advantage over unadjusted results [24-27]. Our results did indicate a small but significant improvement in PDG correlation with serum P4 values when adjusted by CR and SG. While CR and SG perform similarly, SG offers some advantages, particularly for large scale research on reproductive hormones.

Specific gravity is inexpensive; a handheld refractometer and transfer pipettes are the only equipment needed, while the creatinine assay is a microtiter plate based assay. The creatinine assay uses picric acid, a harsh physical irritant that is explosive when dry, so special use and storage conditions are necessary. Finally, even though multiple samples can be assayed simultaneously for creatinine, it does not offer a time advantage over specific gravity. An equal number of samples can be measured on the refractometer in the time needed to complete the creatinine assay.

Specific gravity refractometers measure urine density via the ratio of light refraction between air and a urine specimen; refraction increases with solute concentration of the specimen [28]. Urine density, however, varies with the total mass of solutes which depends not only on the number of particles present, but also on their molecular weight. Therefore, specific gravity is affected more by the presence of heavy molecules such as glucose, albumin, phosphates, sulfates, radiocontrast media, and heavy metals than it is by low molecular weight substances like sodium, chloride, and urea [6, 29, 30]. Thus, specific gravity correction may not be an appropriate method for subjects with diabetes mellitus and nephrotic syndrome which cause high levels of glucose and protein in urine, increasing specific gravity and underestimating urine analyte concentration [30]. Diabetes, starvation and dehydration produce ketones from fat metabolism. The ketones are excreted in urine and erroneously lower specific gravity readings because they are less dense than water [28]. Several authors have also indicated that urine solute concentrations given by specific gravity correction may be inaccurate if the urine specimen is very dilute or very concentrated [31, 32]. However, a minimum acceptable SG of 1.010 [16, 33] seems too high; 31% (247 of 799) of our specimens from healthy US women had SG values lower than this, and our averaged SG corrected urine vs. serum correlations were very high. Specific gravity readings are also affected by temperature fluctuations which make specimens expand or contract altering their density [16] and therefore readings should be taken at a consistent temperature.

Creatinine correction of urinary hormone values is problematic primarily because creatinine excretion exhibits inter- and intra-subject variations and is influenced by time of day, age, sex, diet, body mass, and activity level. This variation could complicate interpretations of analytes reported in ratio to creatinine. Because creatinine is a byproduct of muscle use, its production is

expected to vary with body composition and activity. Edwards [34] reported a lean body mass to urinary creatinine correlation of 0.65 from a group of 31 men and women. Bleiler and Schedl [35] found the same measure to be 0.47-0.48 in 11 women and 0.53-0.55 in 51 men and reported correlations between urinary creatinine and weight and body surface area in 24-hour specimens. Muscularity also contributes to observed sex differences in creatinine levels. Men produce more creatinine than women and have a higher clearance rate [17]. Kesteloot and Joosens [18] reported a mean creatinine clearance of about 101 mL/min in 2,075 men and 86.9 mL/min in 1,933 women. This and other studies [36, 37] also showed a decline in creatinine clearance with age resulting in increased serum and decreased urinary levels at older ages, making CR correction particularly questionable for adjusting urinary hormone values in research concerning aging, such as the transition to menopause.

Diets with substantial amounts of particular kinds of meat, such as beef, can also affect urinary creatinine levels. Meat contains creatine, the precursor of creatinine, which can accumulate in the body and lead to a gradual increase in creatinine excretion [7]. Cooking meat converts creatine to creatinine which is quickly excreted and can cause considerable short-term creatinine increases in the hours following ingestion [10, 15].

Population differences in activity, nutritional status, and body composition may account for the differences observed between the Bangladesh and US sample, and challenge the use of creatinine as a correction method for this population. Most women in Bangladesh suffer from chronic under-nutrition and infectious disease, and have limited access to health care [38]. They also have a very low BMI: the mean BMI for a large random sample of nonpregnant women aged 15-45 years of age in 1992 in Matlab, Bangladesh was 18.8 kg/m² (SD = 1.9) [38]. Thus, the low and variable creatinine levels in our Bangladesh sample may reflect these factors. It

should be noted that the effects of differences in specimen treatment conditions between the Bangladesh and US samples on detected creatinine was not tested and cannot be ruled out. However, several studies have shown creatinine to be very stable refrigerated and frozen [39, 40], and we did not find any effect of the specimen treatment conditions on urinary levels of E1C and PDG in a test of a range of treatment conditions [19].

Additionally, although the original use of creatinine was to check the completeness of 24-hour urine specimens, many studies have shown considerable intra-individual variation in daily creatinine excretion. Alessio *et al.* [16], using 4 consecutive 24-hour urine collections, observed variation ranging between 9.2% and 79.4% in the extreme values of 16 individuals. Similarly Greenblatt *et al.* [14] found a range of 63% to 244% in 24-hour collections from 8 subjects. In a summary of studies on 24-hour creatinine excretion, Curtis and Fogel [12] show some subjects had relatively consistent daily creatinine excretion with individual coefficients of variation around 5%, while other subjects were highly variable with individual CVs exceeding 20%.

Creatinine correction is particularly questionable when applied to spot samples because creatinine excretion over short intervals also shows considerable variation. Verstergaard and Leverett [11] showed subsequent 2 hour interval samples varying by over 100%, and several studies have reported spot sample creatinine variation to be several times higher than variation for 24 hour values [12, 13]. The inapplicability of creatinine correction to spot specimens presents a significant problem for large prospective studies on reproductive hormones. Frequent collections are needed to accurately characterize hormonal patterns and daily 24-hour urine collections are impractical, difficult to obtain, and would present storage and shipping problems.

This study supports the use of specific gravity as an alternative to creatinine for urinary hormone concentration correction. Both methods show high correlation with serum hormone

values. However, specific gravity correction offers several practical advantages over creatinine.

Given these advantages, we recommend specific gravity as an alternative to creatinine for adjusting urinary steroid hormone metabolite concentrations, particularly in populations with very low or highly variable urinary creatinine levels.

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Table 1

Pearson Correlations between serum and lagged urinary hormone values in US women

	Uncorrected Urine Concentrations		Creatinine Corrected Urine Concentrations		Specific Gravity Corrected Urine Concentrations	
	E1C vs. E2	PDG vs. P4	E1C vs. E2	PDG vs. P4	E1C vs. E2	PDG vs. P4
Lag urine 1 day before serum	0.47	0.79	0.45	0.85	0.51	0.83
No lag	0.72	0.91	0.45	0.85	0.76	0.95
Lag urine 1 day after serum	0.91	0.95	0.91	0.98	0.93	0.98
Lag urine 2 days after serum	0.84	0.91	0.80	0.92	0.84	0.93
Lag urine 3 days after serum	0.61	0.57	0.61	0.53	0.61	0.55

E1C indicates urinary estrone conjugates; E2, serum estradiol; PDG, urinary pregnanediol glucuronide; P4, serum progesterone. For E1C vs. E2, N = 34 mean paired urine and serum cycle days from 30 US women; for PDG vs. P4, N = 17 mean paired urine and serum cycle days from 30 US women.

Table 2

Weighted correlations between serum and urinary hormone values in US women

Urine/Serum	Uncorrected Urine Concentration	Specific Gravity Corrected Urine Concentration	Creatinine Corrected Urine Concentration
E1C vs. E2	0.92	0.93	0.94
PDG vs. P4	0.92	0.98	0.97

E1C indicates urinary estrone conjugates; E2, serum estradiol; PDG, urinary pregnanediol glucuronide; P4, serum progesterone. Correlations incorporate a one day lag of urine behind serum. For E1C vs. E2, N = 34 mean paired urine and serum cycle days from 30 US women; for PDG vs. P4, N = 17 mean paired urine and serum cycle days from 30 US women.

Table 3

Mean differences (95% CI) between correlations of serum with urinary hormone values corrected by creatinine, or specific gravity, and without correction from US specimens.

	Creatinine Corrected vs. Uncorrected	Specific Gravity Corrected vs. Uncorrected	Specific Gravity Corrected vs Creatinine Corrected
E1C vs.E2	0.013 (-0.032, 0.079)	0.019 (-0.007, 0.058)	0.006 (-0.024, 0.043)
PDG vs. P4	0.063 (0.011, 0.197)*	0.056 (0.017, 0.179)*	-0.007 (-0.036, 0.015)

E1C indicates urinary estrone conjugates; E2, serum estradiol; PDG, urinary pregnanediol glucuronide; P4, serum progesterone. Correlations incorporate a one day lag of urine behind serum. For E1C vs. E2, N = 34 mean paired urine and serum cycle days from 30 US women; for PDG vs. P4, N = 17 mean paired urine and serum cycle days from 30 US women.

Figure Legends

Figure 1. Distribution of CR values from one menstrual cycle for 30 US (*triangles*) and 13 Bangladeshi (*circles*) participants. Each line shows all CR measurements for one participant from one menstrual cycle with CR concentration plotted on the x-axis.

Figure 2. Uncorrected (*closed squares*), SG corrected (*open circles*), and CR corrected (*open triangles*) PDG results for one Bangladeshi participant.

Figure 3. Correlations in Bangladeshi specimens between all CR corrected and uncorrected PDG values (*panel A, N = 105*) which includes one outlier not shown (uncorrected PDG value 1304.47 nmol/L, CR corrected value 13044666.7 nmol/nmol); between CR corrected and uncorrected PDG, excluding specimens with CR values <0.1 nmol/L (*panel B, N = 66*), and between SG corrected and uncorrected PDG for specimens with CR values <0.1 nmol/L (*panel C, N = 39*).

Figure 4. Distribution of SG values from one menstrual cycle for 30 US (*triangles*) and 13 Bangladeshi (*circles*) participants. Each line shows all SG measurements for one participant from one menstrual cycle with SG plotted on the x-axis.

Subject







