Stability and Reproducibility of a Single-Sample Urinary C-Peptide/Creatinine Ratio and Its Correlation with 24-h Urinary C-Peptide

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INTRODUCTION: C-peptide measurement in blood or 24-h urine samples provides useful information regarding endogenous insulin secretion, but problems related to the rapid degradation of C-peptide in blood and difficulty of 24-h urine collection have limited widespread routine clinical use of this test. We assessed the feasibility of measuring urinary C-peptide (UCP) with correction for creatinine concentration in single urine samples.

METHODS: We analyzed UCP using a routine electrochemiluminescence immunoassay in samples from 21 healthy volunteers. We investigated the stability of UCP with different preservatives and storage conditions and compared the reproducibility of urinary C-peptide/creatinine ratio (UCPCR) in first- and second-void fasting urines, then assessed correlations with 24-h collections.

RESULTS: UCPCR was unchanged at room temperature for 24 h and at 4°C for 72 h even in the absence of preservative. UCPCR collected in boric acid was stable at room temperature for 72 h. UCPCR remained stable after 7 freeze-thaw cycles but decreased with freezer storage time and dropped to 82%–84% of baseline by 90 days at −20°C. Second-void fasting UCPCRs were lower than first-void (median 0.78 vs 1.31, P = 0.0003) and showed less variation (CV 33% vs 52%), as second-void UCPCRs were not influenced by evening food-related insulin secretion. Second-void fasting UCPCR was highly correlated with 24-h UCP (r = 0.8, P = 0.00006).

CONCLUSIONS: Second-void fasting UCPCR is a reproducible measure that correlates well with 24-h UCP in normal samples. The 3-day stability of UCPCR at room temperature greatly increases its potential clinical utility.

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C-peptide is secreted by the pancreatic β cells in equimolar quantities with insulin, after enzymatic cleavage of proinsulin into insulin. C-peptide measurement is used to assess endogenous insulin production in both diabetic and nondiabetic individuals, even if treated with insulin (1, 2). Widespread use of C-peptide measurement in blood is limited by practical considerations: C-peptide concentration rapidly falls as a result of proteases, requiring samples to be rapidly spun and frozen after collection (3, 4). Thus it is difficult to use in routine clinical practice.

Measuring C-peptide excretion in urine is an alternative approach to the assessment of insulin secretion. C-peptide metabolism occurs largely in the kidneys, in contrast to insulin, which is metabolized and extracted in the liver. The total quantity of C-peptide excreted in the urine per day represents 5%–10% of pancreatic secretion, compared with only 0.1% of secreted insulin (5). Previous studies suggest that 24-h urinary C-peptide (UCP)3 measurements accurately assess β cell–secretory capacity and correlate with fasting and stimulated serum insulin and C-peptide (5–8). However, 24-h urine collections are cumbersome, often incomplete, and impractical in a busy clinic (9). Serum insulin and C-peptide are correlated with single and 4-h poststimulated UCP in both nondiabetic (10) and insulin-treated diabetic (11) patients.

Correcting UCP for urinary creatinine adjusts for differences in urine dilution, enabling spot samples to be taken at different times of the day rather than in a 24-h collection, an approach already used for routine microalbuminuria screening (12). This urinary C-pep-
tide/creatinine ratio (UCPCR) has been shown to correlate with serum C-peptide and could be used to assess whether patients are likely to be insulin dependent (11). These studies were more than 20 years ago, however, when C-peptide assays used polyclonal antibodies with radioisotope antibody labels that were labor intensive and commonly required incubations of 48–72 h (13). Now, routine immunoassays use sensitive nonisotopic labels and sophisticated automation, which means analysis takes only minutes to generate results instead of days (3). If UCP is stable on storage, there would be potential for UCPCR analysis in routine clinical practice.

Our study was undertaken as a preliminary step to assess the feasibility of measuring UCP corrected for creatinine concentration in a single urine sample. We assessed the stability of C-peptide in urine and identified its potential for routine clinical use.

Materials and Methods

Materials
We undertook C-peptide analysis on a routine analyzer (Roche Diagnostics E170) at the Biochemistry department at the Royal Devon and Exeter National Health Service Foundation Trust, Exeter, UK. The assay, a direct electrochemiluminescence immunoassay for human serum, plasma, or urine, is a 2-site immunoassay employing monoclonal antibodies against human C-peptide, calibrated to WHO International Reference Reagent (IRR) for C-peptide of human insulin for immunoassay (IRR code 84/510) (14).

We purchased sodium azide and sodium carbonate from Sigma and boric acid–containing urine containers from Sterilin.

Study Participants
We studied 21 healthy nondiabetic volunteers, median age 36 years (range 24–54 years), with body mass index 25.0 kg/m² (range 19.6–36.9); 40% were men.

Analytical Characteristics of C-Peptide Assay
We assessed the precision of the C-peptide assay using random urine samples from healthy volunteers (n = 4) and Elecsys PreciControl multianalyte quality control material (n = 2) (Roche Diagnostics). Imprecision data are presented as mean C-peptide (nmol/L) and CV.

Stability of UCPCR

Stability study. Two urine samples were stored at −20 °C for up to 3 months to test longitudinal stability of C-peptide in urine. Two additional urine samples were exposed to 7 freeze-thaw cycles over 1 week to assess the impact on C-peptide concentration.

Preservative study. Single random urine samples were taken from 20 healthy individuals and separated into 36 aliquots: 9 containing no preservative, 9 with sodium azide (0.85 g/L), 9 with sodium carbonate (8.5 g/L), and 9 with boric acid (13.3 g/L). One sample from each preservative was frozen immediately at −20 °C. Half the remaining samples were then stored at room temperature (21 °C) and half in the refrigerator at 4 °C. One sample from each preservative at each temperature was then frozen at −20 °C after 12, 24, 48, and 72 h.

We assessed differences between baseline and 72-h UCPCR measurements using the Wilcoxon test.

Clinical Sampling

First- vs second-void samples. Seventeen healthy individuals provided their first and second fasting void urine samples of the day over a period of 5 successive days. (The first fasting void is the first urine passed in the morning while the patient is fasting; the second fasting void is the subsequent urine passed while remaining fasting.)

We analyzed urinary C-peptide and creatinine and calculated a CV for the UCPCR of the first- and second-void samples.

24-h vs second-void fasting sample. Seventeen healthy individuals provided a second-void overnight fasting urine sample. These same volunteers then collected the rest of their urine over 24 h beginning from the next void. The volume of urine over the 24-h collection was recorded. We analyzed urinary C-peptide and creatinine and assessed the correlation between 24-h UCP and second-void fasting UCPCR using the Pearson correlation coefficient.

Results

Analytical Characteristics of C-Peptide Assay

Intraassay and interassay CVs showed good precision (<3.3% and <4.5%, respectively) (see Supplemental Table 1, which accompanies the online version of this article at www.clinchem.org/content/vol55/issue11).

Stability of UCPCR

Freezer storage. UCPCR decreased with storage time and dropped to 82%–84% of baseline by 90 days (online Supplemental Table 2). UCPCR remained stable after 7 freeze-thaw cycles (online Supplemental Table 3).

Storage and preservatives. When stored at 4 °C, UCPCR remained stable for up to 72 h regardless of preservative added (Fig. 1A) (mean 103% of baseline at 72 h in all
Fig. 1. Storage of UCPCR with different preservatives at room temperature and 4 °C.
Samples were taken from 20 nondiabetic volunteers. The samples were added to 4 different preservatives stored in the refrigerator at 4 °C (A) and at room temperature (B), each for 12, 24, 48, and 72 h before freezing. Data are presented as mean percentage of baseline at each time point, with error bars representing the 95% CIs. Black straight line, no preservative; dashed line, sodium azide; gray line, sodium carbonate; dotted line, boric acid.
preservatives). No samples dropped to below 90% of baseline by 72 h.

Fig. 1B shows the mean (95% CI) percentage baseline of UCPCR in samples stored with the 3 preservatives and the absence of preservative at room temperature over 72 h. At room temperature, urinary C-peptide remained stable for 24 h in all samples [mean 102% (95% CI 101%–103%) of baseline at 24 h in all samples]. By 72 h, however, C-peptide concentrations in the samples stored with no preservative decreased significantly (mean 69% of baseline, \( P < 0.001 \)), and those on sodium azide decreased to a mean of 92% baseline (\( P = 0.053 \)). Eleven of the samples stored with no preservative and 4 of the samples stored on sodium azide had UCPCR levels that were <90% at 72 h. In contrast, the UCPCR in samples stored on boric acid and on sodium carbonate remained stable for up to 72 h (mean 103% and 102% of baseline, respectively). None of the samples stored on these preservatives dropped to <90% of baseline.

**CLINICAL SAMPLING**

**First vs second void.** Second-void fasting UCPCR was lower than first-void fasting UCPCR (median 0.78 vs 1.31, \( P = 0.0003 \)). UCPCR in second-void fasting samples showed less variability and was more reproducible over a 5-day period than first-void fasting samples (CV 33% vs 52%, respectively).

**24-h UCP vs second-void fasting UCPCR.** Second-void fasting UCPCR was highly correlated with 24-h urinary C-peptide (\( r = 0.8, P = 0.00006 \); Fig. 2).

**Discussion**

We have shown that UCPCR measured in a second-void fasting urine sample is stable, reproducible, and strongly correlated with 24-h urinary C-peptide excretion.

The stability of UCPCR is critical for its clinical utility. We found UCPCR to be stable without preservative for 3 days when stored at 4 °C and for 24 h at room temperature. In contrast, blood C-peptide degrades unless rapidly separated and stored frozen (15, 16). UCPCR was stable for 72 h at room temperature in boric acid and sodium carbonate. Boric acid is routinely used as a bacteriostatic agent in urine samples to be assessed for pathological microbial infection (17, 18). The stability of UCPCR in the widely available borate urine containers will mean that physicians working far from the analyzing laboratory can measure UCPCR in their patients. Samples could be sent by mail for analysis without any special storage conditions, a situation impossible with serum C-peptide.

Storage conditions influence urinary C-peptide; in our study, UCPCR decreased by 18% when stored at −20 °C for 90 days, in keeping with previous work on serum C-peptide stability at −20 °C (16). This decline will have little consequence for routine practice when samples are analyzed rapidly after being taken, but is an important consideration for research samples stored long term. In an ongoing study to assess the long-term stability of UCPCR at −80 °C, no significant decrease was seen after 4 months of storage (data not shown). Seven freeze-thaw cycles over 5 days did not reduce UCPCR.

As a measure of baseline C-peptide production, second-void fasting UCPCR displayed considerably less variation than first-void fasting UCPCR. This probably reflects that first-void fasting UCPCR levels are influenced by food-related insulin secretion from the previous evening. Whereas the CV for the second-void fasting UCPCR was lower than that of the first void (33% vs 52%) this figure still indicates a sizeable day-to-day intraindividual variation. This may represent, in part, the normal biological variation found in insulin secretion in the healthy population, as well as imprecision in the assays. Future studies comparing UCPCR with fasting serum concentrations of C-peptide will address these issues.

Twenty-four-hour urinary C-peptide collections are a good measure of endogenous β cell reserve, correlating well with serum insulin and C-peptide concentrations in diabetic subjects (6, 7). We have shown that second-void fasting UCPCR is strongly correlated with 24-h urinary C-peptide excretion (explaining 64% of the variation), suggesting that UCPCR should be investigated further as a convenient single-sample test of β-cell function.
Our study has limitations. Although UCPCR as a test for β-cell function will be used principally in diabetic patients, our study was limited to healthy volunteers, and future work will therefore concentrate on diabetic subjects. No comparisons with blood measures of serum C-peptide were made; again, further work in a diabetic population will be needed.

The results of this study suggest that UCPCR could be a useful test to assess β-cell status. The test has potential applications in assessing requirement for insulin therapy in type 2 diabetic patients, predicting response to specific oral hypoglycemic agents and mapping β-cell status in the honeymoon period in newly diagnosed type 1 diabetic patients. Future applications may include a role in the assessment of novel immunological modifiers in type 1 diabetes and of success of islet and β-cell transplantation.

In conclusion, our study has demonstrated that UCPCR is a stable and reproducible measure that correlates well with 24-h UCP in nondiabetic subjects. We suggest second-void fasting UCPCR as the optimum approach for the assessment of baseline endogenous C-peptide production using a spot urine test. The stability of UCPCR up to 3 days at room temperature greatly increases its potential clinical utility.

**References**