Certification of Creatinine in a Human Serum Reference Material by GC-MS and LC-MS

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Background: To meet recommendations given by the Laboratory Working Group of the National Kidney Disease Education Program for improving serum creatinine measurements, NIST developed standard reference material (SRM) 967 Creatinine in Frozen Human Serum. SRM 967 is intended for use by laboratories and in vitro diagnostic equipment manufacturers for the calibration and evaluation of routine clinical methods.

Methods: The SRM was produced from 2 serum pools with different creatinine concentrations. The concentrations were certified using a higher-order isotope-dilution GC-MS method and an isotope-dilution LC-MS method. The LC-MS method is a potential higher-order reference measurement procedure.

Results: The GC-MS mean (CV) concentrations were 67.0 (0.9%) μmol/L for serum pool 1 and 346.1 (0.45%) μmol/L for serum pool 2. The LC-MS results were 66.1 (0.2%) μmol/L and 346.3 (0.2%) μmol/L, respectively. For serum pool 1, there was a 1.4% difference between the mean GC-MS and LC-MS measurements, and a 0.10% difference for serum pool 2. The results from the 2 methods were combined to give the certified concentrations and expanded uncertainties.

Conclusions: The certified concentration (expanded uncertainty) of SRM 967 was 66.5 (1.8) μmol/L for serum pool 1 (a value close to the diagnostically important concentration of 88.4 μmol/L) and 346.2 (7.4) μmol/L for serum pool 2 (a concentration corresponding to that expected in a patient with chronic kidney disease).

The concentration of creatinine in serum is a diagnostic marker for chronic kidney disease (CKD). An estimated 20 million Americans have kidney disease (1), and the number is rising, primarily owing to the increasing incidence of diabetes and high blood pressure. Between 1988 and 2003, the number of patients on dialysis nearly tripled (2). Early detection of CKD, followed by drug treatments, can prevent or postpone kidney failure. The simplest and most widespread method of detecting kidney disease is through measurement of blood creatinine concentrations. Recognizing that more accurate blood creatinine measurements will lead to better diagnosis of early stage kidney disease, the Laboratory Working Group of the National Kidney Disease Education Program (NKDEP) outlined a series of recommendations, including the development of a reference material (3).

The NKDEP recommended that a serum reference material with a creatinine concentration of 88.4 μmol/L (1.00 mg/dL) be developed (3). This value corresponds to a glomerular filtration rate (GFR) of approximately 60 mL·min⁻¹·1.73 m². CKD is defined as a GFR of <60 mL·min⁻¹·1.73 m² for ≥3 months (3). The GFR describes the ability of the kidneys to filter waste products from the blood and is estimated based on the patient’s serum creatinine concentration, age, sex, and race. Therefore, 88.4 μmol/L is near the critical concentration that would determine a positive or negative diagnosis. Also, compared with higher creatinine concentrations, errors associated with the calibration or measurement precision of creatinine at this relatively low concentration will have a greater impact on the error of the estimated GFR (3).

To meet the recommendations set by the NKDEP, NIST has developed standard reference material (SRM) 967, Creatinine in Frozen Human Serum (4). This SRM consists of 2 serum pools with target creatinine concentra-

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3 Nonstandard abbreviations: CKD, chronic kidney disease; NKDEP, Laboratory Working Group of the National Kidney Disease Education Program; GFR, glomerular filtration rate; SRM, standard reference material; JCTLM, Joint Committee on Traceability in Laboratory Medicine.
tions of approximately 88.4 μmol/L (the diagnostically critical concentration) and 354 μmol/L (4 mg/dL, a concentration corresponding to that in a patient with CKD). The creatinine concentrations were certified using 2 independent methods. The isotope-dilution gas chromatography–mass spectrometry (GC-MS) method (5) is considered to be a higher-order reference measurement procedure by the NCCLS (6) and the Joint Committee on Traceability in Laboratory Medicine (JCTLM) (7). The isotope-dilution LC-MS method is similar to a procedure developed at the Laboratory of the Government Chemist that was approved by the JCTLM as a higher-order reference measurement procedure (7, 8). The LC-MS method has fewer sample preparation steps and is a higher-throughput method than the GC-MS method. The commutability of SRM 967 was then verified in a study organized by the NKDEP.

SRM 967 has a frozen serum matrix. Frozen serum more closely matches the native state of clinical samples than a lyophilized matrix. Reference materials supplied by other metrology institutes are either lyophilized (9) or do not have the target creatinine concentrations described above (10).

Materials and Methods
We used 2 methods, based on GC-MS and LC-MS, to confirm the accuracy of the quantification; both used isotope dilution. The methods were independent; i.e., they used different procedures to measure the same analyte. The sample preparation (ion exchange chromatography vs protein precipitation), chromatography (gas chromatography vs liquid chromatography), ionization (electron impact vs electrospray), internal standards (creatinine-13C2 vs creatinine-d3), and quantification protocol (Bracketing vs linear regression) (11, 12) differed between the methods.

Preparation of Human Serum
The pools of human serum used for SRM 967 were prepared by Solomon Park Research Laboratories. Blood was collected from healthy, postmenopausal, adult women following CLSI guidelines (13). The resulting serum master pool of approximately 3 L was split into 2 pools. Pool 1 was not enriched with additional creatinine. Pool 2 was enriched with an appropriate amount of reagent-grade creatinine to bring the concentration up to approximately 354 μmol/L. Both pools were passed through filters with a 0.2-μm pore size. No preservatives were added. One-milliliter aliquots of the pools were placed in 3-mL amber glass vials and capped with Teflon stoppers and aluminum seals. The vials were frozen at −80 °C until analysis.

Preparation of the Calibration Standards
Certification of the creatinine concentrations in SRM 967 was performed at NIST. We made calibration solutions that contained known unlabeled: labeled creatinine mass ratios and internal standard solutions that contained known masses of labeled creatinine. The internal standard solutions were added to the samples at the beginning of the sample preparation; the mass of the added labeled creatinine was approximately equal to the mass of unlabeled creatinine in the sample. To achieve this 1:1 ratio, we performed a preliminary quantification in which a wider range of mass ratios was used. Once the approximate creatinine concentration was measured, the quantity of internal standard necessary for a 1:1 ratio was calculated. After sample processing, we ran the calibration standards and samples in the same set on the mass spectrometer. The unlabeled labeled creatinine peak area ratios in the samples were converted to mass ratios using data from the calibration standard runs and either a bracketing method or linear regression method, as described below. The mass ratios were then solved for the mass of the unlabeled creatinine, and the concentration of unlabeled creatinine in each sample was calculated.

For each analytical method, we gravimetrically prepared an independent stock solution of labeled creatinine to make the calibration standards and sample internal standard solutions. We weighed approximately 1.25 mg labeled creatinine (either creatinine-13C2 or creatinine-d3) into a 50-mL volumetric flask, added 50 mL water, and calculated the concentration of the solution (approximately 0.025 mg/g). This solution was split into 3 sets, each set containing a different mass of labeled creatinine. One set was used as internal standards for the samples from serum pool 1, the 2nd set as internal standards for the samples from serum pool 2, and the 3rd set to prepare the calibration standards. The internal standard aliquots were stored at −20 °C.

The accuracy of the quantification was limited by the accuracy of the mass of unlabeled creatinine in the calibration standards. To test for bias in the GC-MS calibration standards, 2 independent solutions of unlabeled creatinine were gravimetrically prepared as follows. We weighed approximately 20 mg solid creatinine SRM 914a (14) into a volumetric flask and added 20 mL water. We transferred 2.5 mL of the solution to a 100-mL volumetric flask and added water to a final volume of 100 mL. We then calculated the concentration (approximately 0.025 mg/g) of the stock solution.

We made calibration standards from both unlabeled stock solutions such that the unlabeled:labeled creatinine mass ratios of the 2 sets of calibration standards were offset from each other by 0.1 units and ranged from 0.8 to 1.2. To test for bias, we ran the calibration standards as a single set. The linearity of the resulting calibration curve

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4 Certain commercial instruments and materials are identified in this report to adequately specify the experimental procedures. Such identification does not imply endorsement by the National Institute of Standards and Technology, nor does it imply that the instruments and materials identified are the best available for the purpose.
confirmed that the 2 independently prepared calibration standard sets were not biased. The calibration standards for the GC-MS method were derivatized as described below and reconstituted in hexane to the same concentration as the GC-MS samples, approximately 10 mg/L, and stored at −20 °C until analysis. The calibration standards for the LC-MS method were made in a similar manner, except we prepared an independent solution of unlabeled creatinine for each of 3 sample sets. We diluted the LC-MS calibration standards with 10 mmol/L ammonium acetate to the same concentration as the LC-MS samples, approximately 1.6 mg/L, and stored them at −20 °C until analysis.

QUALITY CONTROL SAMPLES
SRM 909b Human Serum, which had been previously certified for creatinine concentrations (15, 16), was analyzed along with SRM 967 to confirm the accuracy of the analysis. SRM 909b consists of lyophilized serum (2 pools), and each vial was reconstituted with 10.00 mL water and allowed to equilibrate for 1.5 h before being prepared along with SRM 967.

QUANTIFICATION BY GC-MS
Two aliquots from each SRM 967 vial and 1 aliquot from each SRM 909b vial were measured. The aliquots were added gravimetrically to the creatinine-13C2 internal standard solutions and equilibrated overnight at 5 °C. Ion-exchange chromatography was necessary to separate creatinine from creatinine before derivatization, because these 2 compounds will form the same derivative. Amberlite IRC-50 ion-exchange resin (Chemical Dynamics) was washed in water and soaked in 1.0 mol/L HCl for 3.5 h with occasional agitation. The resin was then rinsed with water and stored in excess 0.1 mol/L HCl until use. The resin was slurry packed into 20 cm by 10-mm columns with occasional agitation. The resin was then rinsed with 150 mL water and soaked in 1.0 mol/L HCl for 3.5 h.

We eluted the creatinine with 75 mL of 1.0 mol/L ammonium acetate, followed by 75 mL water; this fraction was discarded. The resin was then rinsed with water and allowed to equilibrate for 1.5 h before being prepared along with SRM 967.

We performed the measurements by use of an Agilent 5972 GC-MS. The injection volume was 1 μL. The GC column was 30-m long, with a 0.25-mm internal diameter, and a 0.25-μm thick DB-5ms stationary phase (Agilent). The GC oven temperature program was 130 °C for 2 min, 12 °C/min to 250 °C, 250 °C for 0.5 min. The mass spectrometer was operated in the electron impact ionization mode with selected ion monitoring of the [M-73]+ ions at m/z 150 and 152 for the unlabeled and labeled forms, respectively.

We ran 3 separately prepared sets of samples on the GC-MS. Each set consisted of 10 samples: 4 samples of SRM 967 pool 1, 4 samples of SRM 967 pool 2, 1 sample of SRM 909b pool 1, and 1 sample of SRM 909b pool 2. Calibration was by bracketing, i.e., each sample was measured in duplicate, in between duplicate measurements of the 2 calibration standards with unlabeled: labeled peak area ratios just below and above that of the sample. We calculated the mass ratios by linear interpolation between the bracketing standards for each sample. We then repeated the measurements on a 2nd day, with the order of the standards reversed. The results of the 2-day measurements were averaged to arrive at the mass ratios from which the creatinine concentrations in the samples were calculated.

QUANTIFICATION BY LC-MS
Two aliquots from each SRM 967 vial and 1 aliquot from each SRM 909b vial were measured. Aliquots of SRM 967 and SRM 909b were added gravimetrically to the creatinine-d3 internal standard solutions and equilibrated overnight at 5 °C. The proteins were precipitated by adding 3 volumes of ice-cold ethanol to each cold tube and vortex-mixing. After standing for 5 min, the samples were centrifuged at 900g for 20 min. The supernatant, containing the creatinine, was removed and concentrated to dryness using a N2 stream. Each sample was reconstituted in 1 mL water and filtered through a polyvinylidene fluoride syringe filter (13-mm diameter, 0.2-μm pore size).

We diluted the samples to a concentration of approximately 1.6 mg/L with 10 mmol/L ammonium acetate and stored them at −20 °C until analysis.

We performed the measurements by use of an Agilent 1100 series LC-MS. The injection volume was 4 μL, corresponding to approximately 6 ng creatinine. The liquid chromatography column was a 15-cm-long, 2.0 mm internal diameter, 3 μm particle diameter, LUNA C18 (2) (Phenomenex). The gradient mobile phase program was 10 mmol/L ammonium acetate for 7 min, ramped to 20% 10 mmol/L ammonium acetate and 80% acetonitrile by 7.1 min, and held for 13 min. The flow rate was 0.2 mL/min. The column temperature was 23 °C. The mass spectrometer was operated using positive mode electrospray ionization and selective ion monitoring of the (M+H)+ ions at m/z 114 and 117 for creatinine and creatinine-d3, respectively. The ionization source parameters were drying gas temperature 350 °C, N2 gas flow 12...
L/min, nebulizer pressure 170 kPa (25 psi), capillary 1500 V, and fragmentor 120 V.

We ran 3 separately prepared sets of samples on the LC-MS. Each set consisted of 14 samples: 6 samples of SRM 967 pool 1, 6 samples of SRM 967 pool 2, 1 sample of SRM 909b pool 1, and 1 sample of SRM 909b pool 2. Each set was run as follows: the 5 calibration standards were run 1st; followed by the samples; then the samples were measured again in the reverse order; and last, the 5 calibration standards were run in reverse order. We calculated a composite linear regression, using a slope-intercept model, from the peak areas of the calibration standards. We used the linear calibration to calculate the mass ratios using the average of the duplicate sample peak area measurements, which we then used to calculate the creatinine concentration in the samples.

Results

Table 1 lists the measured concentrations of creatinine in SRM 967. Examples of selected ion chromatograms from each serum pool are shown in Fig. 1. The concentration data were corrected for the purity (estimated uncertainty) of the reference standard SRM 914a: 99.7% (0.3%). The concentration unit conversion from mg/g to μmol/L was performed using the densities of SRM 967. The pool 1 material had a density of 1.0231 g/mL; the pool 2 material had a density of 1.0226 g/mL.

Among 12 GC-MS measurements, the largest was 69.2 μmol/L. This point was identified as an outlier by Grubb and Dixon tests and excluded from the statistical analysis and calculation of the certified concentrations. The GC-MS mean concentrations (SD) for SRM 967 were 67.0 (0.6) μmol/L for pool 1 and 346.1 (1.6) μmol/L for pool 2. The LC-MS results were 66.1 (0.2) μmol/L for pool 1 and 346.3 (0.8) μmol/L for pool 2. For pool 1, there was a 1.4% difference between the mean GC-MS and LC-MS measurements, and a 0.10% difference for pool 2. There was no evidence of inhomogeneity within or among the vials, or of a concentration trend corresponding to the vial filling order. The results of the control measurements are listed in Table 2 and were within 1.0% of the certified values for SRM 909b.

Discussion

The measurement of creatinine in the SRM 967 pool 1 samples by GC-MS was slightly more variable and biased toward higher values than the LC-MS method. This effect was obscured at higher creatinine concentrations and led

<table>
<thead>
<tr>
<th>Table 1. Quantification of creatinine in SRM 967 by GC-MS and LC-MS.</th>
<th>GC-MS method</th>
<th>LC-MS method</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRM vial</td>
<td>Pool 1, μmol/L</td>
<td>SRM vial</td>
</tr>
<tr>
<td>Set 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>69.2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>66.6</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>67.7</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>66.9</td>
<td>4</td>
</tr>
<tr>
<td>Set 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>68.1</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>67.2</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>67.1</td>
<td>8</td>
</tr>
<tr>
<td>Set 3</td>
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<td></td>
</tr>
<tr>
<td>9</td>
<td>66.4</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>66.6</td>
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<td>10</td>
<td>66.4</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>66.4</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>67.0</td>
<td>346.1</td>
</tr>
<tr>
<td>SD</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>CV</td>
<td>0.9%</td>
<td>0.45%</td>
</tr>
</tbody>
</table>

* Two aliquots from each SRM vial were measured. The serum pool 1 data point 69.2 μmol/L from the GC-MS method was excluded from the statistical analysis.
to the difference between the average results obtained by
the 2 methods to be greater for the pool 1 samples (1.4%)
than the pool 2 samples (0.10%). The difference between
the pool 1 measurements was considered small enough
such that the results could be combined in the same way
as the pool 2 measurements, as described below.

CALCULATION OF CERTIFIED CONCENTRATIONS AND
EXPANDED UNCERTAINTIES

The results from the 2 methods were combined using a
Bayesian approach (18–20). This approach, intended for
certifying data from a small number of analytical meth-
ods, assumes that both the means and the variances of the
methods could be different. The means were combined by
Eq. 1, where  is the combined mean and  and  are the
means from the 2 methods. The combined mean is the
certified concentration.

\[
\hat{c} = \frac{c_1 + c_2}{2}
\]

Table 2. Quality control measurements of SRM 909b from
the GC-MS and LC-MS sample sets.

<table>
<thead>
<tr>
<th>Method</th>
<th>Pool 1 ((\mu\text{mol/L}))</th>
<th>Pool 2 ((\mu\text{mol/L}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>55.6</td>
<td>467.0</td>
</tr>
<tr>
<td>2</td>
<td>55.8</td>
<td>468.0</td>
</tr>
<tr>
<td>3</td>
<td>55.4</td>
<td>466.7</td>
</tr>
<tr>
<td>Mean</td>
<td>55.6</td>
<td>467.2</td>
</tr>
<tr>
<td>SD</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>LC-MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>56.1</td>
<td>465.6</td>
</tr>
<tr>
<td>2</td>
<td>55.5</td>
<td>465.9</td>
</tr>
<tr>
<td>3</td>
<td>56.1</td>
<td>467.5</td>
</tr>
<tr>
<td>Mean</td>
<td>55.9</td>
<td>466.3</td>
</tr>
<tr>
<td>SD</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Certified SRM value</td>
<td>56.18</td>
<td>467.4</td>
</tr>
<tr>
<td>Expanded uncertainty</td>
<td>0.55</td>
<td>5.3</td>
</tr>
<tr>
<td>Difference, GC-MS, %</td>
<td>−1.0</td>
<td>−0.44</td>
</tr>
<tr>
<td>Difference, LC-MS, %</td>
<td>−0.50</td>
<td>−0.23</td>
</tr>
</tbody>
</table>

The variances of the combined means, ², were calculated
by Eq. 2, where ² and ² are the variances of the
measurements of the 2 methods.

\[
\hat{\sigma}^2 = \frac{\sigma_1^2 + \sigma_2^2 + (c_1 - c_2)^2}{4}
\]

The expanded uncertainties of the measurements were
calculated following NIST guidelines (21). A combined
standard uncertainty for each serum pool was calculated
from type A and type B uncertainties. Type A uncertain-
ties are calculated using statistical methods; type B uncer-
tainties are estimated based on judgment (nonstatistical
methods). The combined variances, ², were the type A
uncertainties. The corresponding type B uncertainties
were assigned a value of 1% of the combined mean,  for
each serum pool, to account for undetected interferences
and the uncertainty in the purity of the reference standard
SRM 914a (other sources of type B uncertainty were
considered negligible). The type A and B uncertainties
were combined using Eq. 3 to give combined standard
uncertainties, , for each serum pool.

\[
u_c = \sqrt{\hat{\sigma}^2 + (0.01c)^2}
\]

Table 3. Calculation of the expanded uncertainty for
SRM 967.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pool 1 ((\mu\text{mol/L}))</th>
<th>Pool 2 ((\mu\text{mol/L}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined mean,</td>
<td>66.53</td>
<td>346.20</td>
</tr>
<tr>
<td>Combined variance, ²</td>
<td>0.38</td>
<td>1.60</td>
</tr>
<tr>
<td>Combined standard uncertainty,</td>
<td>0.91</td>
<td>3.69</td>
</tr>
<tr>
<td>Expanded uncertainty,</td>
<td>1.82</td>
<td>7.37</td>
</tr>
</tbody>
</table>
to encompass a large fraction (approximately 95%) of the measurements obtained by subsequent analyses. Table 3 lists the values at each step of the calculation. The certified, SI traceable, concentrations (expanded uncertainty) for SRM 967 are 66.5 (1.8) μmol/L for serum pool 1 and 346.2 (7.4) μmol/L for serum pool 2, or 0.753 (0.021) mg/dL for serum pool 1 and 3.916 (0.083) mg/dL for serum pool 2.

COMMUTABILITY VALIDATION

A commutability validation study was organized by the NKDEP. Commutability refers to the ability of the SRM to give similar results to real patient samples when analyzed by different analytical methods. The experimental design followed a protocol recommended by the CLSI (22). Briefly, creatinine was measured in SRM 967 and individual patient serum samples using routine laboratory methods and the NIST LC-MS method described above. SRM 967 was found to be commutable with 15 methods from 7 in vitro diagnostic equipment manufacturers (23).

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References