Measurement of Urea in Human Serum by Isotope Dilution Mass Spectrometry: A Reference Procedure

Anja Kessler and Lothar Siekmann*

Background: A reference measurement procedure is needed to demonstrate the traceability of results of urea measurements in human serum. We developed a measurement procedure using the principle of isotope dilution gas chromatography/mass spectrometry.

Methods: \[^{13}\text{C},^{15}\text{N}_2\] Urea as internal standard was added to a serum sample and equilibrated with endogenous nonlabeled urea. For the preparation of calibrators, the same amount of labeled urea was mixed with known amounts of nonlabeled urea. The serum samples were treated with ethanol to remove proteins by precipitation. The labeled and nonlabeled urea of the samples was converted into a trimethylsilyl derivative of 2-hydroxypyrimidine. The gas chromatography/mass spectrometry system was adjusted to monitor m/z 153 and 168 for the nonlabeled urea derivative and m/z 156 and 171 for the isotopically labeled analogs. The results of the determination were calculated from peak ratios by a hyperbolic calculation function based on the theory of isotope dilution analysis.

Results: The procedure was applied to control samples and patient samples and evaluated with respect to its trueness and precision. The standard uncertainty of the results was 0.47–1.72%.

Conclusions: This reference measurement procedure allows values to be assigned to controls and calibrators that are traceable to the primary urea reference material of NIST and, therefore, to the Système International unit “mole” with a low degree of uncertainty. This procedure provides a tool for the highly accurate determination of urea in control materials as well as in patient sera.

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The determination of serum urea in the clinical chemical routine is based on different reactions, e.g., the diacetyl reaction (1), the Berthelot reaction (2), and some enzymatic reactions. To demonstrate traceability of the results of these methods, control samples for internal and external quality control must be available with urea concentration values assigned by a procedure of higher metrological order, a reference measurement procedure. At present, the only analytical principle that appears to be suitable for establishing such a reference procedure is isotope dilution mass spectrometry (ID-MS).1

Björkhem et al. (3) first reported an isotope dilution-gas chromatography/mass spectrometry (ID-GC/MS) procedure using \[^{15}\text{N}_2\] urea as the labeled analog and derivatization of urea to a dimethylated 5,5-diallylbarbituric acid. Welch et al. (4) applied \(^{18}\text{O}\)-labeled urea and analyzed the mixture of labeled and natural urea after formation of 6-methyl-2,4-bis[(trimethylsilyl)oxy]pyrimidine by ID-GC/MS. In other investigations, ID-MS methods were developed for studying urea kinetics and urea metabolism (5–9).

Previously described derivatives (4–6) have been tested, and the trimethylsilyl ether derivative of 2-hydroxypyrimidine as described by Wolthers et al. (5) has been shown to be the most suitable derivative for GC/MS analysis; it can also be prepared easily.

For ID-MS measurements, it is of great advantage to use an isotopically labeled internal standard that has a mass difference of at least two or, preferably, three mass units. The use of \[^{13}\text{C},^{15}\text{N}_2\] urea as an internal standard showing a three-mass unit difference to nonlabeled urea ensures that only minimal contributions from naturally occurring isotopes can be expected when the labeled substance is measured.

1 Nonstandard abbreviations: ID-MS, isotope dilution mass spectrometry; ID-GC/MS, isotope dilution-gas chromatography/mass spectrometry; SRM, standard reference material; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; and GUM, Guide to the Expression of Uncertainty in Measurement.

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The reference procedure described by Welch et al. (4) is designed for measuring urea in high-quality matrix reference materials, e.g., standard reference material (SRM) 909 of NIST, with a very low degree of uncertainty. It was the objective of the present investigation to develop a measurement procedure for assigning traceable urea concentration values not only to calibrators for routine testing and control materials for internal and external quality control but also to selected panels of patient sera, which can be used for method comparison studies and validation of routine test kits. For this purpose, it is desirable to have a measurement procedure available that on the one hand is more easily performed and on the other hand still fulfills the requirements of a reference procedure in terms of an adequately low degree of uncertainty for its results.

Materials and Methods
CHEMICALS
Urea SRM 912a (purity 99.9% ± 0.1%) is certified by NIST. [13C,15N2]Urea (90 atom%; 15N, 95 atom%) was obtained from ICN Biomedicals. The reagents used for derivatization were malondialdehyde bis(dimethylacetal) from Sigma, hydrochloric acid (250 g/L) from Merck, and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) from Macherey-Nagel. Methanol (SupraSolv) and ethanol (p.a.) from Merck were used as solvents.

Sera from patients of the University Hospital Bonn, Germany, were stored at −20 °C before use. The aggregate of patient samples used represented the routine situation of the clinical chemical laboratory of the hospital. Fifteen percent of the collected samples were from patients attending the outpatient clinic; the samples of inpatients were from all departments of the hospital (internal medicine, urology, surgery, gynecology, and others).

Lyophilized control sera were reconstituted with distilled water, as prescribed by the manufacturer, using calibrated volumetric equipment.

INSTRUMENTS AND SETTINGS
ID-GC/MS. The instrument used was a Fisons MD 800 combined gas chromatograph quadrupole mass spectrometer system (Fisons), equipped with a septum-less injection system (Jade; Analyt).

GC conditions. The carrier gas was helium at an inlet pressure of 50 kPa. The split ratio of the inlet splitter was set to a value of 1:50. A fused-silica capillary column coated with OV-1 (30 m × 0.32 mm i.d.; 0.25 μm film thickness; Mega) was used. The injector temperature was 300 °C. Before injection of the sample, the column oven was kept isothermal at 70 °C for at least 1 min. This temperature was maintained for 1 min after injection and then increased to 150 °C at a programmed rate of 10 °C/min. At the end of the program, the column was heated to 240 °C for 1 min.

During the first 2 min of GC, vacuum (rotary vacuum pump) was applied to a T-connection in the transfer line between the end of the column and the mass spectrometer, using a computer-controlled valve, to prevent reagents from entering the ion source. The transfer line was an uncoated fused silica capillary column (0.6 m × 0.15 mm i.d.).

MS conditions. The ionization energy was 70 eV, and the temperature of the ion source was set to 250 °C. For the calibration of the mass scale of the instrument, the exact positions of the peak maxima of the ions at m/z 153 and 156 (M+15) and at m/z 168 and 171 (M+) were determined in a separate chromatographic run of a calibrator mixture of labeled and nonlabeled urea derivatives before a series of quantitative analyses. For example, for calibrating the mass scale at m/z 153, 10 different selected ion recordings were monitored simultaneously in the mass range m/z 153.00–153.45 (e.g., m/z 153.00, 153.05, 153.10, 153.15, 153.20, 153.25, 153.30, 153.35, 153.40, and 153.45). The mass for which the highest intensity was obtained was selected for the ID-GC/MS measurements.

For selected-ion recording of calibrators and samples, each mass was scanned for 0.03 s in a mass range of ± 0.05 mass units surrounding the peak maxima determined in the mass calibration experiment; a complete recording cycle of the four preselected masses including settling times took 0.16 s.

GLASSWARE
A volumetric flask used for the preparation of the urea calibrator solution was calibrated by adjusting the flask to the calibration mark with water at room temperature and weighing the contents. For the sampling of serum as well as for dispensing the nonlabeled calibrator solution, a mechanized pipetter, MicrolabM (Hamilton), equipped with a 2.5-mL syringe was calibrated by weighing corresponding amounts of water at room temperature. Before every weighing procedure, the balance was calibrated using officially calibrated test weights. For pipetting the [13C,15N2]urea calibrator solution, a 50-μL syringe (SGE) equipped with a repeating adapter was adjusted to 23 μL.

The density of air and water at the temperature during calibration were considered in calculations, and the exact volumes were calculated and reported as standard volumes at 20 °C. When the volumetric equipment was used at different temperatures, the volumes were recalculated. The impression of the volumetric steps using the syringe, the pipetter, and the flask was determined by weighing respective samples of water 10 times.

PROCEDURES AND MEASUREMENTS
Weighing procedures. The urea certified reference material and the water samples for calibrating the pipetter and the syringe were weighed with a microbalance (model MCI; Sartorius). The weighing procedures for calibrating the volumetric flask were carried out with an electronic balance (model 1474; Sartorius). Before every weighing,
the balances were calibrated with test weights (class E2) certified by the German National Metrology Institute (Physikalisch-Technische Bundesanstalt, Braunschweig, Germany).

Preparation of the calibrator solutions of nonlabeled and \(^{13}\text{C,15}\text{N}_2\)urea for ID-GC/MS measurement. The nonlabeled urea calibrator solutions were prepared by dissolving a specified amount of certified SRM 912a between 29.9 and 30.3 mg (e.g., 30.112 mg) in 100 mL of methanol or ethanol in the calibrated volumetric flask at 20 °C. The weight was multiplied by the factor 0.999 according to the purity certified for the urea reference material. For the calculation of the resulting urea concentration, the calibrated volume of the volumetric flask at the relevant temperature was taken into account.

The \(^{13}\text{C,15}\text{N}_2\)urea calibrator solution was prepared by dissolving \(~48~\text{mg}\) of the labeled compound in 15 mL of 900 mL/L water-100 mL/L methanol. The solution was kept in a refrigerator at 4 °C; at this temperature, it was stable for at least 3 months. The material was equilibrated to room temperature before use.

Preparation of the calibrators for the ID-GC/MS measurement. Three different calibrators were prepared by mixing 23 \(\mu\text{L}\) of the labeled urea solution containing \(~60~\mu\text{g}\) of \(^{13}\text{C,15}\text{N}_2\)urea with 150, 200, and 250 \(\mu\text{L}\) of the nonlabeled urea calibrator solution, giving isotope ratios of \(~0.75, 1.00, \text{and} 1.25\). These mixtures were diluted with 3 mL of ethanol, and 100-\(\mu\text{L}\) aliquots were transferred into tapered autosampler test tubes (volume, 0.7 mL). Each of the three calibrators was prepared in quadruplicate. In addition, one calibrator containing only nonlabeled urea and a calibrator containing only labeled urea were prepared.

Preparation of the serum samples for the ID-GC/MS measurement. The approximate concentration of urea in the serum samples to be investigated was first estimated by the use of a routine method (10). Aliquots of the serum containing \(~60~\mu\text{g}\) urea were then transferred with the calibrated pipetters into glass tubes (volume, 10 mL). The samples were diluted with distilled water to a final volume of 0.7 mL, and 23 \(\mu\text{L}\) of the labeled urea solution was added. After the sample equilibrated for 30 min at room temperature, 3 mL of ethanol was added and the precipitation of the proteins was completed by intensive shaking with a vortex-type mixer for 5 min. The samples were centrifuged (2800g) for 10 min at 10 °C for phase separation. Aliquots (100 \(\mu\text{L}\)) of the supernatant were transferred into tapered autosampler test tubes (volume, 0.7 mL).

Derivative formation. The ethanolic solutions of the samples and calibrators were mixed with 20 \(\mu\text{L}\) of malondialdehyde bis(dimethylacetal) solution (0.3 mol/L) and 40 \(\mu\text{L}\) of hydrochloric acid (250 g/L). The reaction (leading to 2-hydroxypyrimidine) was complete at room temperature after 1 h. The samples and calibrators were evaporated in a vacuum centrifuge at a pressure of 400 Pa overnight to dryness at room temperature. The dry residues were reacted 1 h with 20 \(\mu\text{L}\) of MSTFA at 60 °C.

A 0.4-\(\mu\text{L}\) aliquot of the reaction mixture was injected into the gas chromatograph-mass spectrometer. For continuous control of the stability of the system, serum samples and calibrators were analyzed alternately.

CALIBRATION AND CALCULATIONS
The areas and heights of the peaks obtained after selected-ion recording were determined by the Masslab Software provided by the manufacturer of the GC/MS instrument. The subsequent calculation of the urea concentration was based on a hyperbolic calibration function obtained from the substance concentrations and the isotope ratios of the three different calibrator mixtures (11). For calculations, a computer spread sheet was used.

The combined standard uncertainty of measurement was calculated from the random (type A) and systematic (type B) components of uncertainty according to the Guide to the Expression of Uncertainty in Measurement [GUM; Ref. (12)]. The random component of uncertainty was given by the experimental standard deviations of the results. One of the systematic components of uncertainty involves the purity of the certified reference material as stated by NIST (0.1%). Additional systematic components included the uncertainties of the certified values of the calibrated test weights (Physikalisch-Technische Bundesanstalt) that were used for balance calibration when weighing the primary reference material and calibrating the volumetric equipment.

Results
To demonstrate the applicability of this reference measurement procedure, the urea concentrations of serum samples from 53 patients and 15 lyophilized control sera were determined. The lowest urea concentrations were 5.005 mmol/L (control serum) and 1.834 mmol/L (patient serum). The highest urea concentrations were 31.93 mmol/L for a control serum and 61.24 mmol/L for a patient serum.

For quantification, the GC peak area ratios measured at \(\text{m/z}~153\) for the nonlabeled derivative \(\text{O-}\text{trimethylsilyl-2-hydroxypyrimidine}\) and \(\text{m/z}~156\) for the analogous \(^{13}\text{C,15}\text{N}_2\)-labeled derivative in calibrators and samples were used. Another independent calculation of the results was based on the ratios of the peak areas of the masses \(\text{m/z}~168\) and 171. The four selected-ion monitoring signals were recorded in the same gas chromatographic run (Fig. 1).

TRUENESS
The results of the two independent detection procedures calculated from the peak ratios (\(\text{m/z}~153/156\)) and (\(\text{m/z}~168/171\)) were compared using the Passing-Bablok regression procedure (Fig. 2) (13). The following relationships were obtained for the comparison of the results of patient sera (Fig. 2A):
The trueness of the method was also examined by analyzing the control sera SRM 909a-1 and SRM 909a-2. NIST has certified these sera for the concentration of urea. We analyzed them on three different occasions, each time in duplicate. For SRM 909a-1, the urea target concentration and uncertainty, as certified by NIST, was 5.535 ± 0.071 mmol/L; the urea concentration and uncertainty measured by ID-GC/MS was 5.563 ± 0.15 mmol/L. For SRM 909a-2, the urea target concentration and uncertainty, as certified by NIST, was 19.47 ± 0.25 mmol/L; the urea concentration and uncertainty measured by ID-GC/MS was 19.50 ± 0.67 mmol/L. For these analyses, the uncertainty was calculated according to GUM (12), with coverage factor $k = 2$.

IMPRECISION

The total imprecision of measurement is a characteristic parameter for the quality of a reference method. Both imprecision observed under repeatability conditions (within series) and imprecision observed under reproducibility conditions (series to series) contribute to the overall imprecision.

The within-series imprecision was determined by analyzing a control serum (761.02) 10 times in the same analytical series. The mean value was 6.940 mmol/L, the SD was 0.046 mmol/L, and the CV was 0.67%.

Another measure for the within-series imprecision is the SD obtained from duplicate measurements of patient and control sera (15). For patient sera, the mean value of all results was 20.4 mmol/L, and the SD was 0.15 mmol/L, which corresponded to a mean relative SD of 0.76%. The mean concentration of the control sera was 15.0 mmol/L, and the SD was 0.11 mmol/L (mean relative SD, 0.77%).

There are two components that contribute to the within-series standard deviation: (a) the standard deviation of sampling the serum and internal standard and (b) the standard deviation of the isotope ratio measurements using the GC/MS system.

Each volume was calibrated by weighing water samples eight times. The CVs were 0.09 – 0.40% for the volumes of the sera (100 – 800 μL) and 0.03% for calibrator solutions (150 – 250 μL). The volume of the syringe for sampling the labeled standard was 23.454 μL, and its imprecision was 0.038 μL (CV = 0.16%).

To evaluate the imprecision of the GC/MS system, a mixture of the labeled and nonlabeled derivative was injected and measured 10 times. The mean value of the isotope ratios ($m/z$ 153 and 156) was 1.0078, the SD was 0.0020, and the CV was 0.19%.

The control sera were analyzed as duplicates in at least three different series. For this investigation, the total imprecision was 0.47 – 1.72% (relative standard deviation). The series-to-series standard deviation (SDs) was calculated from the total standard deviation (SDT), and the within-series standard deviation (SDW) was calculated using the equation, $SD_S = \sqrt{SD_T^2 - SD_W^2}/2$, according to the analysis of variance. This ranges from 0.13% to 1.21% (relative standard deviation).

UNCERTAINTY OF MEASUREMENT

The combined standard uncertainty consists of random (type A) and systematic (type B) components of uncertainty. The total imprecision of the results expressed as overall standard deviation reflects the combined effects of the different random components, such as the imprecision of the volumetric steps and the weighing procedures and the imprecision of the isotope ratio measurements of the GC/MS system. The relative standard deviations deter-
mined by repetitive measurements of different series are the measure for the random components of uncertainty; in our study, they were 0.47–1.72%.

According to GUM (12), the systematic components of uncertainty may also be expressed as standard deviations or “type B” standard uncertainties. These are not determined by multiple measurements but are based on the information on certificates issued by manufacturers of equipment or accredited calibration laboratories.

For one control serum (761.06), as a typical example, all contributing factors of the systematic component, their nominal and corrected values, and their uncertainties are listed in Table 1. The systematic component of uncertainty was calculated to be 0.084% by the formula $u_{cB} = \sqrt{\sum u_B^2}$. The random component of uncertainty for this serum is 0.63%. The combined standard uncertainty is calculated to be 0.63%. Using a coverage factor $k = 2$, this yields an expanded uncertainty of 1.26%. The standard uncertainties for the control materials investigated here were 0.47–1.72%, which correspond to expanded uncertainties of 0.94–3.44% (coverage factor $k = 2$).

**Limit of Detection**

Because no serum with a zero concentration of urea is available, a blank signal cannot be measured. In this case, the detection limit can be estimated by measuring the signal-to-noise ratio or by extrapolating a precision profile (16) to zero concentration of urea. It was assumed that a peak of a GC/MS chromatogram with a signal-to-noise ratio of 3:1 is clearly detectable. The chromatogram of the serum with the lowest concentration (1.834 mmol/L) detected in this investigation showed a signal-to-noise ratio of ~1000:1 (for peaks of $m/z$ 153 and 156); therefore, the lower limit of detection was calculated to be 0.0055 mmol/L. When a precision profile was extrapolated from low sample concentrations to zero concentration, a lower limit of detection of 0.006 mmol/L was estimated, which was very similar to the result calculated from the signal-to-noise ratio.

**Limits of Quantification**

The limits of quantification may be defined as upper and lower concentration values for which the imprecision of measurement does not exceed a certain predefined relative standard deviation. For the reference procedure performed here, the standard deviations for control and patient sample measurements were <1.8% over the whole range of urea concentrations (1.834–61.24 mmol/L).

**Discussion**

The concentration of urea in serum is one of the most frequently determined clinical chemical indices for monitoring renal function. At present, a variety of methods based on different analytical principles are in use in routine laboratories. Most of the methods are performed automatically; usually the precision of the results is very good. This can be checked easily by multiple measurements of the same serum sample. However, the evaluation of traceability, in particular of trueness of these results, still remains a problem.

For external quality control for several clinical chemical quantities (e.g., creatinine, total glycerol, cholesterol, uric acid, and glucose), control sera whose concentrations have been determined by a metrologically superior method (a reference measurement procedure) are available. The reference procedure values, which are directly traceable to the Système International unit “mole”, form an independent basis for the evaluation of the results of

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Fig. 2. Comparison of the urea concentrations calculated from the peak ratios ($m/z$ 153/156) and ($m/z$ 168/171) for 53 patient sera (A) and 15 control sera (B).

The regression was calculated according to the Passing-Bablok procedure.
the participants in external quality-control surveys (17) provided the matrix of the control materials behaves similar to natural human patient sera.

In the present investigation, a reference measurement procedure for the determination of urea in serum based on the principle of ID-MS is described. The combination of GC/MS and ID is a reference method principle that is internationally accepted to yield high specificity and trueness. This has been described previously (18). Not just for its high specificity has the ID-MS technique been defined by the Consultative Committee on Amount of Substance as a “primary method of measurement” (19).

The use of a primary standard material of high and well-known purity is a necessary prerequisite for establishing a reference measurement procedure. The urea reference material from NIST (SRM 912), which was used during this investigation, fulfills this requirement; the certified purity of this material is 99.9% ± 0.1%.

Because an unpredictable portion of the analyte can be lost in the preinstrumental part of analytical procedure or even during GC, it is necessary to control these losses by using an internal standard. An isotopically labeled variant of the analyte is the ideal internal standard because it can be expected to exhibit the same physico-chemical properties as the nonlabeled substance during extraction, derivative formation, and GC. It seemed to be advantageous to make use of a compound, such as \([^{13}C,^{15}N_2]\)urea, that differs from the nonlabeled urea by at least three mass units.

For the evaluation of the procedure, both patient sera and control sera were analyzed. The method was evaluated with regard to trueness, precision, detection limits, and limits of quantification according to requirements described in a standard document of the International Organization for Standardization (17).

For checking the trueness of the reference procedure, human control sera certified by NIST were analyzed. These certified urea concentrations had been determined previously by an ID-GC/MS-method, but a different urea derivative was prepared and a differently labeled internal standard, \([^{18}O]\)urea, was used (4). A comparison (see “Trueness” in Results) showed a very good agreement of the results. The values obtained with the present procedure are within the limits of uncertainty of the NIST certificate; they, therefore, confirm the trueness of the procedure described here within the stated limits of uncertainty. Furthermore, the results of the two independent detection procedures for the peak ratios (m/z 153/156) and (m/z 168/171) were compared in a plot (Fig. 2). All data points were situated very close to the identity line, which demonstrates perfect agreement between the two sets of results. This can be demonstrated for the results of the patient sera as well as for the results of the control sera. Outliers, which would become visible if there was a lack of specificity for the detection of the one or the other pair of masses, were not observed.

Several components (e.g., vial-to-vial variation, volumetric steps, and instability of isotope ratio measurements using the GC/MS system) contribute to the total imprecision of the procedure. This imprecision was in the

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Table 1. Uncertainty of a reference procedure value assigned to a typical serum control material.

<table>
<thead>
<tr>
<th>Component</th>
<th>Uncertainty, mmol/L</th>
<th>Relative uncertainty, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Random components of uncertainty (uA) of a urea concentration value (31.93 mmol/L) in a control serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-series imprecision (SDw and CVw)</td>
<td>0.091</td>
<td>0.29</td>
</tr>
<tr>
<td>Series-to-series imprecision (SDs and CVs)</td>
<td>0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Total imprecision (SDT and CVT)</td>
<td>0.20</td>
<td>0.63</td>
</tr>
<tr>
<td>B. Systematic components of uncertainty (uB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purity of reference material</td>
<td>99.9%</td>
<td>99.9%</td>
</tr>
<tr>
<td>Weight of reference material</td>
<td>30.271 mg</td>
<td>30.241 mg</td>
</tr>
<tr>
<td>Volume of flask</td>
<td>100 mL</td>
<td>99.946 mL</td>
</tr>
<tr>
<td>Volume to reconstitute the freeze-dried serum</td>
<td>5 mL</td>
<td>4.998 mL</td>
</tr>
<tr>
<td>Volume of calibrator</td>
<td>200 µL</td>
<td>200.037 µL</td>
</tr>
<tr>
<td>Volume of sample</td>
<td>100 µL</td>
<td>99.161 µL</td>
</tr>
<tr>
<td>C. Combined uncertainty (uC) of a urea concentration value (31.93 mmol/L) assigned to a control serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random component uA</td>
<td>0.20</td>
<td>0.63</td>
</tr>
<tr>
<td>Systematic component uB</td>
<td>0.025</td>
<td>0.084</td>
</tr>
<tr>
<td>Combined standard uncertainty uC</td>
<td>±0.20</td>
<td>0.63</td>
</tr>
<tr>
<td>Expanded uncertainty U</td>
<td>±0.40</td>
<td>1.26</td>
</tr>
</tbody>
</table>

a Symmetric, rectangular a priori probability distribution assumed (e.g., uB = 0.1%/√3 = 0.058%).

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range 0.47–1.72%. In relation to the large biologically variation (within- and between-subject), such precision may be regarded as quite satisfactory.

The lower limit of detection (0.0055 mmol/L) was by a factor of 150 lower than any realistic urea concentration in a human serum. With respect to the limits of quantification, which are more relevant than the detection limits, several recommendations have been published (16). For the present procedure, it was most useful to set a fixed maximum CV (2.5%). It can be expected that even patient and control samples with higher or lower urea concentrations than measured here fall within the limits of quantification.

A value is traceable when it can be related to stated references, usually national or international measurement standards, through an unbroken chain of comparisons, all having stated uncertainties of measurement (20). According to this, the result of each measurement procedure consists of a value of concentration and its uncertainty of measurement. The uncertainty of the result is calculated according to GUM (12). To achieve a low degree of uncertainty, it is desirable to reduce all of its components as much as possible. By using calibrated direct displacement pipetting devices (Hamilton dispenser or syringe with repeating adapter) instead of grade A pipettes for all volumetric steps (sampling the water for reconstitution, the serum samples to be analyzed, and the calibrator solutions), we minimized the random component of uncertainty. The most important contribution to the random uncertainty probably originates from the instrumental imprecision of isotope ratio measurements. It is difficult to reduce this imprecision beyond certain limits (typically 0.19%). In comparison with the random components ($u_{r} = 0.47–1.72\%$), the uncertainty of the systematic components ($u_{s} = 0.084\%$) does not significantly contribute to the overall combined standard uncertainty ($u_{c} = 0.47–1.72\%$). The systematic components were minimized in this reference procedure by using a primary reference material (NIST urea SRM 912) that has a certified purity and a low uncertainty of purity. Furthermore, the use of test weights with certified low uncertainties for calibration of all relevant volumetric equipment and for weighing the primary reference material makes the measurement procedure described here traceable to national and international standards.

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References