Serum Cystatin C, Determined by a Rapid, Automated Particle-Enhanced Turbidimetric Method, Is a Better Marker than Serum Creatinine for Glomerular Filtration Rate

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We describe a fully automated particle-enhanced turbidimetric assay for cystatin C in undiluted serum and EDTA-plasma. The throughput is 90 samples per hour and urgent samples can be analyzed in 7 min. The assay range (0.4–14.1 mg/L) covers the concentration range in health and disease. The within- and between-run imprecision is 0.9% and 2.2%, respectively. Analytical recovery of additions of recombinant cystatin C averaged 98%. Rheumatoid factors (≤323 000 IU/L), bilirubin (≤150 μmol/L), hemoglobin (≤1.2 g/L), and triglycerides (≤8.5 mmol/L) do not interfere in the assay. In view of the superior (by ROC analysis) diagnostic accuracy of serum concentrations of cystatin C for reduced glomerular filtration rate (GFR) in comparison with creatinine, cystatin C seems an attractive alternative to creatinine for estimation of GFR.

Indexing Terms: ROC curve/kidney function

Cystatin C is a nonglycosylated 13-kDa basic protein of the cystatin super-family of cysteine proteinase inhibitors (1–4). Produced by all investigated nucleated cells, its production rate is unaltered in inflammatory conditions (4–8). Determination of the structure of the cystatin C gene and its promoter has shown that the gene is of the house-keeping type, which is compatible with a stable production rate of cystatin C by most cell types (7). The low molecular mass of cystatin C, in combination with its stable production rate, strongly suggests that the major determinant of cystatin C concentrations in blood plasma is the glomerular filtration rate (GFR).6 Three investigations, employing enzyme-amplified single radial immunodiffusion to quantify cystatin C, supported this hypothesis and demonstrated that the serum concentration of cystatin C is at least as good an indicator for GFR as that of creatinine (5, 6, 9). A recent investigation with enzyme-linked immunoassay for determining cystatin C also supports this hypothesis (10). However, the reported methods for quantification of cystatin C are tedious and do not easily allow automation. They are, therefore, far from ideal for routine clinical use and do not allow analysis of urgent samples.

Here, we describe the development of a rapid, automated method for determining serum and plasma concentrations of cystatin C, based upon particle-enhanced turbidimetry (PET). We tested the method with samples from a group of patients with normal to markedly reduced GFR to investigate whether the cystatin C measurement was a better marker than serum creatinine for GFR.

Materials and Methods

Materials

A Cobas Fara instrument (F. Hoffmann-La Roche, Basel, Switzerland), an automated single-unit centrifugal analyzer, was used for the development of the present PET assay of cystatin C. The light source is a high-intensity xenon flash used with a holographically inscribed grating monochromator. The change in absorbance at 340 nm was measured.

Cystatin C immunoparticles. Carboxylate-modified latex particles, 38 nm in diameter, were obtained from Duke Scientific Corp., Palo Alto, CA. Rabbit antibodies against human cystatin C were from Dako, Glostrup, Denmark (code no. A 451). We covalently attached the antibodies to the uniform microparticles by a carbodiimide reaction (11, 12). The immunoparticles were used at a final concentration of ~4 g/L. The number of IgG molecules on each particle was ~32.

Cystatin C-free normal human serum. To produce a suitable matrix for developing a quantitative assay for cystatin C, we diluted 50 mL of a serum pool from 10 healthy volunteers with an equal volume of 0.05 mol/L Tris buffer, pH 7.4, containing 0.5 mol/L sodium chloride. To this solution was then added 20 mL of CNBr-Sepharose with 10 mg of the IgG fraction of polyclonal rabbit antiserum against human cystatin C covalently coupled per milliliter. The mixture was gently rocked at room temperature for 2 h and the CNBr-Sepharose removed by filtration on a glass filter funnel. The resulting solution was concentrated to 50 mL by pressure ultrafiltration with a membrane having a nominal retention limit of 10 kDa. Enzyme-amplified single radial immunodiffusion (9) was used to corroborate the complete removal of cystatin C from the serum pool.

Calibrator. Lyophilized recombinant human cystatin C produced and isolated as described earlier (13, 14) was used to prepare the calibrator. Agarose and sodium do-
decyl sulfate–polyacrylamide gel electrophoresis (15, 16) failed to demonstrate any contamination in the lyophilized cystatin C preparation. Amino acid analysis of 1.00 mg of the cystatin C preparation dissolved in 1.00 mL of 0.15 mol/L NH₄HCO₃ and absorbance measurements [absorbitivity at 280 nm, 0.91 L · g⁻¹·cm⁻¹ (17)] gave concordant results, demonstrating the absence of significant amounts of water in the lyophilized cystatin C preparation. The cystatin C calibrator used in the present method was made by dissolving this preparation of lyophilized cystatin C in cystatin C-free normal human serum to a concentration of 17.6 mg/L and allowing the mixture to incubate at 4°C overnight. A calibration curve covering the range 0.4 to 14.1 mg/L was prepared by dilution with 0.15 mol/L NaCl. The calibrators could be stored at 4°C for at least 7 days without changes in the dose–response curves produced.

Reaction buffer. Phosphate buffer, 0.1 mol/L, pH 7.2, containing 20 g/L polyethylene glycol 6000 and 2 g/L Tween 20, was used as the reaction buffer. Polyethylene glycol 6000 (code no. 807491) and Tween 20 (code no. 822184) were obtained from Merck Schuchardt, Munich, Germany.

Interference tests. To obtain samples having various bilirubin concentrations, we dissolved 11.8 mg of bilirubin (code no. 24520; Merck, Darmstadt, Germany) in 6.73 mL of dimethyl sulfoxide (code no. 802912; Merck Schuchardt) to give a stock solution of 3000 μmol/L. The stock solution was diluted further in dimethyl sulfoxide to give final concentrations of bilirubin in donor samples in the range 0–300 μmol/L (serum: bilirubin solution = 9:1 by vol).

To obtain samples with various hemoglobin concentrations, we prepared as earlier described (18) a hemolysate containing 115 g/L hemoglobin. This hemoglobin stock solution was diluted further in 0.15 mol/L NaCl to give final concentrations of hemoglobin in donor samples in the range 0–2.00 g/L (serum:hemoglobin solution = 4:1 by vol).

Serum samples with increased triglyceride (TG) concentrations, as determined by the Kodak Ektachem 700 XR-C system (Eastman Kodak, Rochester, NY), were used to investigate possible interference from hyperlipemia in the cystatin C assay.

Patients’ Samples

Serum and EDTA-treated plasma samples from healthy subjects and from patients with decreased GFR or increased concentrations of rheumatoid factor or TG were investigated. The rheumatoid factor values were estimated by an ELISA method (19). To analyze the relationships between GFR, cystatin C, and creatinine concentrations, we used serum samples from 27 male and 24 female patients (ages 8–81 years) with various renal conditions. Their GFR, determined as described below, ranged from 7 to 141 mL/min per 1.73 m² body surface (reference range 80–120 mL/min per 1.73 m²). The procedures involving patients and healthy subjects were in accordance with the Helsinki Declaration of 1975.

Final Assay Procedure

The following protocol was found to be optimal for routine assay of cystatin C in serum and plasma and was used in subsequent experiments. The assay is performed at 37°C with a seven-point cystatin C calibration curve covering the range 0.4–14.1 mg/L (produced with solutions of isolated recombinant cystatin C). The assay steps are: 20 μL of sample (or calibrator) is pipetted into a cuvette followed by 10 μL of distilled water for washing and 230 μL of reaction buffer. The rotor is spun for 120 s, after which a sample blank recording is made at 340 nm. Thereafter, 21 μL of a cystatin C immunoparticle suspension is added, followed by 20 μL of distilled water for washing. The increase in absorbance at 340 nm produced by the agglutination reaction is then measured after 240 s to give an endpoint value. After endpoint values are corrected for sample blanks and reagent blank, the results are calculated in the Cobas Fara instrument by logit-log function analysis. The throughput of the procedure is 90 samples per hour, and urgent samples can be analyzed in 7 min.

Other Procedures

Determination of GFR. GFR was determined by measuring the plasma clearance of iohexol (20), a radiocontrast agent used as a reliable marker for GFR (21). Clearance was calculated from iohexol concentrations in four plasma samples drawn 3–4 h after injection of the marker. The method has a total variation (CV) of 11%, most of which is accounted for by biological variation (20). In patients with markedly reduced renal function (serum creatinine >200 μmol/L), the sampling period was extended to as much as 48 h to ensure correct determination of the slope of the elimination curve.

Serum creatinine concentrations were determined by the Kodak Ektachem 700 XR-C system, with use of the enzyme creatine amidinohydrolase.

Statistical analysis. The data were evaluated with standard parametric tests. Comparisons between GFR and the serum concentrations of either creatinine or cystatin C were performed by using the reciprocals of the measured concentrations, the serum level of a substance being inversely related to its clearance. This procedure results in a linearization of the curvilinear relationships between the GFR and the serum marker concentrations and greatly reduces the skewness of the frequency distribution for creatinine and cystatin C data.

Evaluation of diagnostic accuracy. To estimate the diagnostic accuracy of the serum concentrations of cystatin C and creatinine in predicting reduced GFR (i.e., GFR <80 mL/min per 1.73 m²), we constructed receiver-operating characteristic (ROC) plots (22). This procedure facilitates comparisons of the diagnostic sensitivity and specificity of the two variables (their ability to correctly classify subjects into groups with normal or reduced GFR) over a broad range of hypothetical cutoff limits. The resulting ROC plot areas were compared by nonparametric procedures (22).
Results

Analytical Variables

Calibration curve and linearity. Fig. 1 shows a calibration curve covering the analytical range 0.4–14.1 mg/L. Virtually identical curves were obtained when the cystatin C calibrator (17.6 mg/L) cystatin C dissolved in cystatin C-free serum) was diluted in 40 g/L bovine serum albumin in distilled water, in 0.15 mol/L NaCl, or in cystatin C-free serum. The detection limit of the assay, defined as the concentration corresponding to the mean of 10 determinations of the zero calibrator signal + 3 SD, was 0.15 mg/L. The linearity of the method was analyzed by diluting five serum samples in 0.15 mol/L NaCl to produce cystatin C values 75%, 50%, 25%, and 10% of those in the undiluted samples (i.e., covering the cystatin C concentration range 1.19–10.7 mg/L). The results obtained for the diluted samples (y) did not differ significantly from those expected (x). Regression analysis produced the equation: \( y = 0.982x + 0.117 \text{ mg/L} (r^2 = 0.9998, n = 25) \).

Imprecision. To determine within-run, between-run, and total imprecision, we analyzed five serum pools with cystatin C values between 0.84 and 4.24 mg/L. The imprecision was calculated by using a one-way analysis of variance (ANOVA). Data from five replicate analyses assayed in six different runs (days) were compiled (Table 1). The within-run imprecision averaged 0.9% and never exceeded 2%, the between-run imprecision averaged 2.2%, and the total imprecision averaged 2.4% and was always below 3.2%.

Analytical recovery. Supplemented samples were prepared by adding different amounts of a recombinant cystatin C solution (64.4 mg/L) to three normal donor samples of known concentration. The percentage recovery was calculated as the percentage ratio between the measured and added concentrations of cystatin C. The average analytical recovery of cystatin C was 98% (Table 2).

Interference tests. To assess whether hyperlipemia interferes in the assay, we analyzed patients' samples of known increased TG concentrations after supplementing the samples with the cystatin C calibrator (17.6 mg/L) and by dilution experiments. Testing for linearity, we observed no interference for samples with TG contents as great as 9.4 mmol/L. The measured analytical recovery of cystatin C was quantitative in samples with TG as great as 8.5 mmol/L.

Samples from 19 patients with increased rheumatoid factor concentrations (21 000–323 000 IU/L) were analyzed for cystatin C. No significant correlation between the concentrations of rheumatoid factor and of cystatin C could be observed, indicating that rheumatoid factor does not interfere in the assay.

Interference from bilirubin was investigated by assaying six samples of normal blood donor serum or EDTA-plasma containing added bilirubin. Bilirubin concentrations <150 μmol/L did not interfere, but at 150–300 μmol/L bilirubin the cystatin C concentrations in samples were slightly higher than expected. The increase was <10%, however.

Interference from hemoglobin was investigated in six normal donors' serum samples or EDTA-plasma samples with added hemoglobin. Hemoglobin concentrations ≤1.2 g/L did not interfere in the assay.

Table 1. Analytical Imprecision of the cystatin C assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>Within-run</th>
<th>Between-run</th>
<th>Total</th>
<th>CV, %</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.84</td>
<td>0.015</td>
<td>0.019</td>
<td>0.024</td>
<td>2.8</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>0.97</td>
<td>0.008</td>
<td>0.030</td>
<td>0.031</td>
<td>3.2</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>1.41</td>
<td>0.015</td>
<td>0.041</td>
<td>0.044</td>
<td>3.1</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>3.36</td>
<td>0.016</td>
<td>0.044</td>
<td>0.047</td>
<td>1.4</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>4.24</td>
<td>0.018</td>
<td>0.051</td>
<td>0.054</td>
<td>1.3</td>
<td>26</td>
</tr>
</tbody>
</table>

* Five serum pools were analyzed as many as five times on each of six separate days.

Table 2. Analytical recovery of cystatin C, tested by addition of recombinant cystatin C to three serum samples.

<table>
<thead>
<tr>
<th>Added Concentration (mg/L)</th>
<th>Measured Concentration (mg/L)</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1 (0.67 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91</td>
<td>2.68</td>
<td>105</td>
</tr>
<tr>
<td>2.86</td>
<td>3.61</td>
<td>103</td>
</tr>
<tr>
<td>5.82</td>
<td>6.43</td>
<td>101</td>
</tr>
<tr>
<td>8.59</td>
<td>9.01</td>
<td>97</td>
</tr>
<tr>
<td>12.88</td>
<td>13.22</td>
<td>97</td>
</tr>
<tr>
<td>Sample 2 (0.56 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91</td>
<td>2.49</td>
<td>101</td>
</tr>
<tr>
<td>2.86</td>
<td>3.26</td>
<td>94</td>
</tr>
<tr>
<td>5.72</td>
<td>6.03</td>
<td>96</td>
</tr>
<tr>
<td>8.59</td>
<td>8.77</td>
<td>96</td>
</tr>
<tr>
<td>12.88</td>
<td>13.14</td>
<td>98</td>
</tr>
<tr>
<td>Sample 3 (0.90 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91</td>
<td>2.62</td>
<td>101</td>
</tr>
<tr>
<td>2.86</td>
<td>3.62</td>
<td>95</td>
</tr>
<tr>
<td>5.72</td>
<td>6.52</td>
<td>98</td>
</tr>
<tr>
<td>8.59</td>
<td>8.95</td>
<td>94</td>
</tr>
<tr>
<td>12.88</td>
<td>13.23</td>
<td>96</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td></td>
<td>98 ± 3</td>
</tr>
</tbody>
</table>

Fig. 1. Calibration curve for determination of cystatin C. Lyophilized recombinant cystatin C was dissolved in cystatin C-free serum and diluted in 0.15 mol/L NaCl to give concentrations of 0.4–14.1 mg/L.
Sample type and stability. Measurements of cystatin C in serum and EDTA-plasma did not differ significantly. In 10 serum and plasma samples stored at 4°C (range 0.68–0.81 mg/L) cystatin C concentrations were stable for at least 5 days. The concentrations obtained at 5 days did not differ significantly from those obtained on the day of sampling, the mean value on day 5 being 102% (SD 4%) of that on the sampling day. Finally, three cycles of freezing and thawing of 33 samples did not significantly change the cystatin C concentrations determined. The average value after three cycles was 102% (SD 6%) of that before freezing.

Serum Cystatin C and Creatinine as Markers for GFR

When data from all 51 patients were included in the calculations, both the serum creatinine and the cystatin C concentrations were significantly related to GFR (Table 3, Fig. 2). The correlation between the reciprocal cystatin C concentration and GFR (r = 0.87) was significantly stronger than that between the reciprocal creatinine concentration and GFR (r = 0.71). Fig. 2 makes evident that these significant relationships were largely accounted for by data from patients with reduced renal function. However, although there was no significant relationship between the reciprocal creatinine concentration and GFR in subjects with normal GFR, the correlation between the reciprocal cystatin C concentration and GFR extended over the entire GFR range and remained significant also in subjects with normal renal function (Table 3).

The cystatin C concentrations of the limited number of subjects (27) with normal GFR (>80 mL/min per 1.73 m²) ranged from 0.61 to 1.21 mg/L and may be used to define a preliminary reference interval. No sex-related difference was observed.

Table 3. Correlation between GFR and creatinine and cystatin C concentrations in plasma of 51 patients.

<table>
<thead>
<tr>
<th>GFR</th>
<th>All</th>
<th>Normal</th>
<th>Reduced*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>51</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>1/cystatin C vs GFR</td>
<td>0.87b</td>
<td>0.39c</td>
<td>0.91</td>
</tr>
<tr>
<td>1/creatinine vs GFR</td>
<td>0.71</td>
<td>0.17d</td>
<td>0.83</td>
</tr>
<tr>
<td>1/cystatin C vs 1/creatinine</td>
<td>0.73</td>
<td>0.19d</td>
<td>0.79</td>
</tr>
</tbody>
</table>

* lmerol clearance <80 mL/min per 1.73 m².
  b Significantly (P <0.05) better than the corresponding value for 1/creatinine vs GFR.
  c P = 0.03.
  d Not significant.

Diagnostic Accuracy of Serum Cystatin C and Creatinine

ROC plots describe the alterations in diagnostic sensitivity and specificity that occur when the (hypothetical) cutoff limit is gradually increased. As demonstrated in Fig. 3, the cutoff limit for cystatin C can be increased to correspond to a sensitivity of almost 70% while maintaining 100% specificity. Increasing the cystatin C cutoff limit so as to attain 100% sensitivity moderately reduces the specificity (to ~75%). In contrast, the specificity of the serum concentration of creatinine begins to decrease at a cutoff limit corresponding to a sensitivity of <50%. A sensitivity of 100% for serum creatinine would require a cutoff limit yielding a specificity of close to zero. The areas under the curves differed significantly (P <0.001), demonstrating that the diagnostic accuracy of the serum concentration of cystatin C is superior to that of creatinine.

Discussion

Three earlier studies, using enzyme-amplified single radial immunodiffusion to determine the concentration
of cystatin C, indicated that this value might be as good
a marker for GFR as the concentration of creatinine (5,
6, 9). However, radial immunodiffusion procedures are
usually much slower and less precise than the available
procedures for determining creatinine. Therefore it is
important to develop automated, rapid, and precise
methods for quantifying cystatin C in serum and plasma
to explore the diagnostic potential of cystatin C in clinical
practice. The present cystatin C assay by PET ful-
fulls all these criteria. The PET technology significantly
increases the sensitivity of turbidimetric assays and allows
determination of protein concentrations at least
10- to 100-fold below what can be determined by con-
ventional nonenhanced assays. The detection limit of
the present PET assay of cystatin C is 0.15 mg/L, well
below the lowest serum concentrations reported for
healthy individuals (5, 6, 9, 10, 23–25). The overall assay
performance also compares favorably with conventional
immunoassays with regard to simplicity of assay pro-
dure, sample handling time, rapidity, and precision.

Although the method described here was developed
employing a Cobas Fara instrument, it could easily be
adapted for a Cobas Mira instrument, which makes it
even more practical for analysis of single urgent sam-
ple. Thus, the present method, in either Cobas version,
is considerably more convenient than the previously
described methods for quantifying cystatin C, which
rely on manual procedures and require analysis periods
of 2–24 h (5, 6, 9, 10, 23–27). The sensitivity of the
present method is lower than that reported for previous
methods based upon enzyme-labeled or radioactive re-
agents, but its assay range comprises (without sample
dilution) the entire span of serum concentrations of
cystatin C seen in healthy subjects and in the vast majority
of sick individuals (5, 6, 9, 10, 23–25).

The mean cystatin C concentration determined in
subjects with normal GFR by the present PET assay was
0.86 (range 0.61–1.21) mg/L, which is compatible with
the concentration ranges for healthy persons given by
most earlier reports on concentration of human cystatin
C (5, 6, 9, 23–25).

Comparisons of the relations between cystatin C and
creatinine concentrations and GFR are greatly influ-
enced by the selection of the population studied. Evalu-
ations that include mainly subjects with normal renal
function generally produce weaker correlations,
whereas clinical studies that include patients with
markedly reduced renal function tend to give much
stronger relationships because of polarization of the
data. Our study group represents a typical clinical ma-
terial and is therefore likely to produce clinically rele-
vant information.

Our results (Table 3, Figs. 2 and 3) demonstrate that
the serum concentration of cystatin C is a better marker
for GFR than that of creatinine. The overall correlation
between cystatin C and GFR was significantly stronger
than that between creatinine and GFR. Moreover, the
cystatin C concentration was significantly correlated to
GFR for subjects with normal renal function as well as
for patients with reduced GFR. This was not the case for
creatinine. Earlier comparisons of the serum concentra-
tions of cystatin C and creatinine as markers for GFR,
in which enzyme-amplified single radial immunodiffu-
sion was used to determine cystatin C, failed to clearly
demonstrate the superiority of cystatin C as a marker
for GFR (5, 6). However, the immunodiffusion-based
method is much less precise than the PET assay de-
scribed here and therefore does not fully exploit the
advantages of cystatin C concentrations over creatinine
concentrations for diagnostic purposes.

Theoretically, cystatin C should have several advan-
tages as a marker for the GFR. Because the production
rate of creatinine is determined mainly by muscular
mass, the rate is quite variable. In addition, the elimi-
nation pathways for creatinine are complex and include,
besides glomerular filtration, tubular secretion and
elimination via the intestine. Cystatin C, on the other
hand, is a direct gene product, produced at a constant rate
by virtually all body tissues, and is probably eliminated
from blood almost exclusively by glomerular filtration
(4–8).

Consequently, it is not surprising that the diagnostic
accuracy of serum cystatin C was superior to that of
creatinine in discriminating between subjects with nor-
mal renal function and those with reduced GFR (Fig. 3).
For example, accurate detection of renal dysfunction in
90% of our subjects with reduced GFR (90% diagnostic
sensitivity) would require cutoff limits of 65 mmol/L for
creatinine and 0.90 mg/L for cystatin C. Such limits for
creatinine would result in falsely positive results in 67%
of the subjects with normal renal function. The corre-
sponding false-positive rate for cystatin C is 20%. Sen-
sitivity and specificity were greater for serum cystatin
C than for serum creatinine over the entire range of pos-
sible cutoff limits. Thus, both when the object is to ex-
clude with certainty individuals with normal GFR (specificity)
and when it is important to identify individ-
uals with GFR impairment (sensitivity), serum cystatin
C is a more efficient diagnostic tool than serum creati-
inine. A recent report on the serum concentration of
cystatin C as determined by a sandwich enzyme immu-
noassay supports the notion that the cystatin C concen-
tration has a greater diagnostic sensitivity for demon-
strating reduced kidney function than does the concen-
tration of creatinine (10).

In conclusion, serum cystatin C seems to be a prom-
ising marker for GFR, and PET assay for cystatin C
we describe appears suitable for both urgent and nonur-
gent routine quantification of cystatin C in serum. How-
ever, further studies, including the establishment of
reliable age- and sex-related reference intervals, are
needed before the usefulness of serum concentrations
of cystatin C as a GFR marker can be definitely deter-
ned. Such studies are under way.

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