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Sensitive Automated ELISA for Measurement of Vitamin D-Binding Protein (Gc) in Human Urine, Anna Lis Lauridsen,1,* Michael Aarhus,2 Anna Lisa Christensen,3 Bente Jespersen,2 Kim Brixen,4 and Ebba Nexø1 (1) Department of Clinical Biochemistry, Norrebrogade, Aarhus University Hospital, Aarhus, Denmark; Departments of 2 Nephrology and 3 Endocrinology, Odense University Hospital, Odense, Denmark; * address correspond ince to this au-thor at: Department of Clinical Biochemistry, Aarhus University Hospital, Norrebrogade 44, DK-8000 Aarhus C, Denmark; fax 45-8949-3060, e-mail all@dandinet.dk

We report a sensitive automated ELISA for measurement of group-specific component (Gc; also known as Gc globulin and vitamin D-binding protein) that detects lower concentrations than any other published method. This new ELISA enables measurement of Gc in human urine.

Gc is a 50- to 58-kDa multifunctional plasma protein synthesized mainly by hepatocytes and usually present in plasma in concentrations between 4 and 6 μmol/L. The functions of Gc are diverse. Gc is an important player in the actin-scavenger system, which prevents the harmful consequences of actin in the blood stream during tissue injury. It binds actin monomers with high affinity, and Gc–actin complexes are readily cleared from the circulation (1). Gc also has functions in the immune system, acting as a co-chemotactic factor together with complement C5a (2) and, after deglycosylation, as a very potent macrophage-activating factor (Gc-MAF) also capable of activating osteoclasts (3, 4). The name “vitamin D-binding protein” is derived from its ability to bind and transport vitamin D metabolites. Usually, <0.1% of 25-hydroxyvitamin D (25OHD) and <1% of 1,25-dihydroxyvitamin D [1,25(OH)2D] circulate in their free forms (5). Gc is probably also important for the renal activation of 25OHD to 1,25(OH)2D. Mice lacking the multifunctional receptor megalin lose Gc–25OHD complexes in the urine and are deficient in 1,25(OH)2D (6). Thus, there is evidence that, generally, Gc and Gc–25OHD complexes are filtered in the glomerulus and reabsorbed by megalin-mediated endocytosis into the proximal tubular cells, where vitamin D activation takes place. This theory postulates that, under healthy conditions, only trace amounts of Gc should be excreted in the urine, whereas urinary loss of Gc is expected to increase with decreases in the capacity for reabsorption in the proximal tubules. To evaluate the fate of Gc in humans with various kidney diseases, there is a need for an assay sensitive enough to measure the minute amounts of Gc excreted in urine. This prompted us to develop a sensitive ELISA.

We used the principles described by Engbaek (7) to optimize the ELISA for Gc. The assay is based on an immobilized polyclonal antibody that captures the Gc, which subsequently binds a biotinylated monoclonal detection antibody that reacts with peroxidase–avidin–tetracytosine-labeled benzidine, producing a color reaction.

As capture antibody, we added to each well 8 μg of rabbit anti-human Gc globulin (DakoCytomation Denmark A/S) in 100 μL of 50 mmol/L sodium carbonate (pH 9.6). We incubated the ELISA plates (F96-Maxisorp Nunc immunoplates; Nunc A/S) at 4 °C for 20 h before emptying the wells and adding 200 μL of 1 mol/L ethanolamine (pH 8–9). After another 20 h at 4 °C, the plates were stored at −20 °C. We biotinylated the monoclonal mouse anti-human Gc globulin (AntibodyShop; SSI) after overnight dialysis of 200 μL against 0.1 mol/L sodium bicarbonate (pH 8.3) by mixing gently for 4 h in the dark at room temperature with 10 μL of 4.4 mmol/L biotin–amidocaproate–N-hydroxysuccinimide ester (Sigma). Subsequently, we added 10 μL of 100 mmol/L lysine monohydrochloride (Fluka), waited for 15 min, and added 10 μL of rabbit γ-globulin (50 g/L, Calbiochem) and bovine γ-globulin (100 g/L, Sigma) in 10 mmol/L sodium phosphate (pH 7.4). We then dialyzed the mixture for 48 h against 10 mmol/L sodium phosphate (pH 7.4) and for 24 h after addition of 1 g/L sodium azide (Merck).
The biotinylated antibody was stored at \(-20^\circ\text{C}\) and before use was diluted 1:10 000 in the assay buffer, 100 mmol/L sodium phosphate (pH 8.0) containing 1 g/L bovine albumin (Sigma). We used an 8-point calibration curve with purified human Gc-globulin, mixed type (Calbiochem), diluted in assay buffer (0, 1.25, 2.5, 5, 10, 20, 40, and 80 pmol/L) and obtained the calibration curve by cubic regression.

For the ELISA, we used an automated analyzer (BEP-2000; Dade Behring) at 37°C. Between each step, we incubated the plates for 30 min and washed the wells three times with a washing buffer consisting of 10 mmol/L sodium phosphate (pH 7.4), 145 mmol/L sodium chloride, and 1 g/L Tween 20 (Merck). On the BEP-2000, controls and samples were diluted 1:10 in assay buffer, and 100-μL duplicates of the calibrators and diluted controls and samples were added. The biotinylated detection antibody (100 μL) was then added, followed by 100 μL of peroxidase–avidin (DakoCytomation Denmark A/S) diluted 1:2000 in 10 mmol/L sodium phosphate (pH 7.4) containing 400 mmol/L sodium chloride and 0.2 g/L lysozyme (Sigma). The color reaction was started by addition of 10 μL of TMB-One ready-to-use substrate (Kem-Entec Diagnostics A/S) and stopped after 9 min by addition of 100 μL of 1 mol/L phosphoric acid to each well. Color development was measured photometrically at 620 nm.

We checked for linearity by measuring 5 dilutions of each of 10 urine samples. The linear regression lines between measured and expected Gc concentrations all had intercepts not differing from 0, and 7 of 10 slopes did not differ from 1, whereas 3 slopes differed slightly (95% confidence intervals, 0.94–0.99, 1.02–1.36, and 1.01–1.10). Imprecision was estimated at 4 concentrations by 4 measurements on each plate and 2 plates a day for 6 days. At urinary Gc concentrations of 14, 184, 198, and 521 pmol/L, the within-plate, within-day, between-day, and total imprecisions calculated as recommended by Krouwer and Rabinowitz (8) were 1.8%–3.6%, 2.0%–4.1%, 1.4%–4.2%, and 2.6%–5.8%, respectively. Recovery was 95%–108% for 221 and 426 pmol/L added to five different urines and each measured four times. The detection limit, defined as the concentration corresponding to a signal 3 SD above the mean for the calibrator free of Gc, was 2.5 pmol/L, but in practice we used 10 pmol/L. The effect of storage for 3 and 8 months at \(-20^\circ\text{C}\) and \(-80^\circ\text{C}\) until measurement. We recommend either measurement of Gc in fresh urine or storage of urine samples at \(-80^\circ\text{C}\) until measurement.

To examine the urinary loss of Gc as a function of kidney function, we enrolled 99 patients with various kidney diseases [dialysis (n = 0), glomerulonephritis (35),

Table 1. Urinary and plasma concentrations of Gc, albumin, and creatinine in 10 healthy controls (28–66 years of age) and in 99 patients with various kidney diseases (20–88 years of age).

<table>
<thead>
<tr>
<th></th>
<th>Median (range)</th>
<th>Correlation* with urinary Gc in patients</th>
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<tbody>
<tr>
<td><strong>Healthy controls</strong></td>
<td></td>
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<tr>
<td>Urinary Gc, nmol/24 h</td>
<td>0.62 (0.03–1.49)</td>
<td>27 (0.03–897)</td>
</tr>
<tr>
<td>Gc in second-void morning urine, nmol/L</td>
<td>0.70 (0.07–1.11)</td>
<td>13 (0.04–375)</td>
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<tr>
<td>Gc/Creatinine in second-void morning urine, nmol/mmol</td>
<td>0.05 (0.01–0.09)</td>
<td>2.8 (0.01–62)</td>
</tr>
<tr>
<td>Urinary albumin, mg/24 h</td>
<td>17 (10–27)</td>
<td>734 (14–8204)</td>
</tr>
<tr>
<td>Urinary creatinine, mmol/24 h</td>
<td>13 (10–22)</td>
<td>11 (4–20)</td>
</tr>
<tr>
<td>Plasma Gc, μmol/L</td>
<td>3.9–6.4*</td>
<td>4.8 (3.4–7.7)</td>
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<tr>
<td>Plasma albumin, g/L</td>
<td>36–51*</td>
<td>41 (22–47)</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/L</td>
<td>44–134*</td>
<td>224 (59–712)</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>72–138*</td>
<td>37 (6–186)</td>
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*Spearman nonparametric correlation coefficients between 24-h urinary Gc and the other variables.

**p**<0.001; **p** < 0.01; **p** < 0.05.

*95% central reference interval.

![Fig. 1. Effect of freezer storage.](image)
diabetic nephropathy (17), polycystic kidney disease (9), and other or nonspecified kidney disease (38) and 12 healthy individuals (of those, 2 were excluded because of urinary albumin >30 mg/24-h) in a cross-sectional study approved by the local ethics committee (No. 20020055). Plasma and urine samples (24-h urine and second-void morning urine) were stored immediately at −80 °C and, without thawing, moved to a −20 °C freezer before measurement. We measured plasma Gc by our immunonephelometric method on a Behring Nephelometer 2 (Dade Behring) (9) and albumin and creatinine on an Integra 700 (Roche). We used Kruskal–Wallis and Mann–Whitney tests for comparisons between groups and the Spearman nonparametric method to look for correlations. We performed the calculations with SPSS 10.0.5 and set 0.05 as the significance level.

In the 99 patients, the median 24-h excretions of Gc and albumin did not differ significantly between patients in the various kidney disease groups but were significantly (P < 0.001) higher in patients than in healthy controls (Table 1). Measurement of Gc in second-void morning urine gave a good estimate of the 24-h urinary excretion, equivalent to that given by the Gc/creatinine ratio, as seen from the high correlations in Table 1. Urinary Gc did not correlate with plasma Gc, but correlated with markers of kidney disease, particularly with urinary albumin excretion (Table 1). Linear regression with Gc as the dependent and albumin as the independent variable (after transformation to obtain approximate gaussian distributions of residuals) showed a highly significant relationship in urine [r = 0.86; P < 0.001; mean (95% confidence interval) slope, 1.2 (1.04–1.3); mean (95% confidence interval) intercept, −4.6 (−5.5 to −3.7)], but not in plasma (P = 0.23). The median urinary Gc/albumin ratio was significantly lower in patients with glomerulonephritis than in patients with polycystic, other, or nonspecified kidney diseases. The correlation between creatinine clearance and the Gc/albumin ratio in 24-h urine was high (r = −0.54; P < 0.001) as was the correlation in second-void morning urine (r = −0.59; P < 0.001). Longitudinal studies, however, are needed to test the utility of the urinary Gc/albumin ratio as a marker of kidney function.

In the kidneys, both Gc and albumin are believed to be filtered in the glomerulus and subsequently reabsorbed by megalin-cubilin–mediated endocytosis in the proximal tubule (10). Because of this shared fate, the high correlation between urinary Gc and urinary albumin was expected. However, contrary to the negative correlation between plasma and urinary albumin, we found no significant correlation between plasma and urinary Gc. The missing decrease in plasma Gc concentration is in agreement with findings in megalin-knockout mice (6) and in some studies of patients with kidney diseases (11, 12), but not in others (13, 14).

In conclusion, we have developed a sensitive automated ELISA capable of measuring very low Gc concentrations with low imprecision (functional detection limit, 0.01 nmol/L). In 99 patients, the urinary loss of Gc increased with increasing severity of kidney disease, but had no relationship with plasma Gc concentration.

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References