Measurement of Urinary Cystatin C with a Particle-Enhanced Turbidimetric Immunoassay on Architect Ci8200

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Background: Cystatin C is a low-molecular-weight protein that is freely filtered by the glomerulus and catabolized after reabsorption by the proximal tubular cells in healthy subjects. Urinary cystatin C is a potential biomarker for tubular damage including acute kidney injury (AKI) in the acute phase when patients are submitted to the intensive care unit. **Methods:** The aim of this study was to perform a method validation of urinary analysis of cystatin C by particle-enhanced turbidimetric immunoassay (PETIA) on a high-throughput chemical analyzer. Total assay time was 10 min. The antigen excess, linearity, lower limit of quantification (LoQ), recovery, assay precision, stability, and interference caused by hemoglobin were evaluated. **Results:** The LoQ was calculated to 0.020 mg/l with a coefficient of variation (CV) $\leq 10\%$. No

hook effect was observed and the assay was linear over the studied interval less than 0.020–0.950 mg/l with a regression of $R^2 = 0.9994$. The assay had a recovery between 93–100% and the assay precision had a total CV of less than 3.5%. Cystatin C was stable for 3 days in room temperature and 14 days in $+4^\circ\text{C}$. The assay did not show any major interference with hemoglobin at a hemoglobin concentration of 10 g/L. The reference interval for urine cystatin C was less than 0.166 mg/l. **Conclusion:** The urinary cystatin C PETIA showed good precision and performance characteristics including short turnaround times that are necessary qualifications for a biomarker at a routine laboratory. J. Clin. Lab. Anal. 26:358–364, 2012. © 2012 Wiley Periodicals, Inc.

Key words: avian antibodies; cystatin C; method validation; particle-enhanced turbidimetric immunoassay; urine

INTRODUCTION

A kidney holds approximately one million nephrons with each nephron consisting of capillary tufts or glomerulus, proximal tubule, loop of Henle, distal tubule, and the collecting ducts. Proteins lower than 50 kDa are filterable in the glomerulus, this means low-molecular-weight proteins, monosaccharides, polypeptides, amino acids, and ions freely pass through to the proximal tubules. This liquid is known as the primary urine. Reabsorption of primary urine occurs in the proximal tubules where polypeptides and amino acids are reabsorbed completely.

Acute kidney injury (AKI) is a common and serious complication highly associated with morbidity and high mortality among hospitalized patients (1, 2) and is frequently occurring in hospitalized patients with an incidence of 25% in the intensive care unit (3). AKI causes an accumulation of toxins and other nitrogenous waste

products, which under normal conditions are excreted in the urine. AKI is commonly characterized as a sudden decrease in renal function and glomerular filtration rate, resulting in major disturbance of the kidneys homeostasis and apoptosis of renal tubular cells (4, 5). AKI is seen in patients with prerenal azotemia, patients undergoing cardiothoracic surgery or cardiopulmonary bypass but the most important cause is sepsis (1, 6, 7). Five percent of intensive care unit patients with AKI are unfortunately

Grant sponsor: Uppsala Hospital Research Fund; grant sponsor: Norwegian Research Council.

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Received 29 February 2012; Accepted 6 June 2012

DOI 10.1002/jcla.21531

Published online in Wiley Online Library (wileyonlinelibrary.com).

forced to undergo renal replacement therapy and data collected over the last 20 years indicate an increase of renal replacement therapy incidence (8).

Risk, injury, failure, loss, and end-stage renal disease (RIFLE) and acute kidney injury network (AKIN) are two recently standardized systems of criteria for the clinical classification of AKI. But these systems are not ideal for the diagnosis of AKI as the criterion is based on elevations of the current gold standard for AKI serum creatinine (9). Serum creatinine and other traditional markers for detection of renal failure are considered unspecific and unreliable because of its delayed response to acute renal injury. The need for novel biomarkers in the diagnosis of AKI is widely appreciated by intensive care units (2, 10). AKI is a condition usually treated at the intensive care units. It is a condition associated with considerably higher mortality and costs than for acute myocardial infarctions. The demand for STAT tests for AKI is thus at least as high as for myocardial infarctions.

Cystatin C is an endogenous nonglycosylated low-molecular-weight protein with a molecular mass of 13 kDa and a total of 120 amino acids in its polypeptide chain. Cystatin C belongs to the type II cystatin gene superfamily and is a strong inhibitor of cysteine proteases that causes proteolysis and tissue damage such as papain-like, legumain-like cysteine protease, ficin, and cathepsins to name a few. Cystatin C is produced at a constant rate by all nucleated cells and secreted extracellularly shortly after synthesis and is present in all body fluids and tissues (11–13). The 7.3 kb gene encoding for cystatin C is localized on chromosome 20 and considered as a housekeeping-type of gene, which may explain the stable protein production throughout the body. After its secretion, cystatin C is filtered freely through the glomerulus due to its positive charge at physiological pI and low molecular weight (14). After filtration, the low-molecular-weight protein is reabsorbed by proximal tubular cells and almost completely catabolized and degraded in healthy individuals. The remaining cystatin C protein that is not catabolized is eliminated with the urine in very low concentrations ranging 0.03–0.3 mg/l in healthy individuals (15).

Today, cystatin C is used primarily as a biomarker to estimate glomerular filtration rate and kidney function measured in serum or plasma (16). The assessment of cystatin C in other body fluids is a new and exciting field. Studies have shown cystatin C concentrations to be 0.80–2.5 mg/l in plasma, 8–14 mg/l in cerebrospinal fluid, and 0.03–0.3 mg/l in urine (15). Urinary cystatin C has recently been proposed as a biomarker for renal failure, in particular diagnosing the acute stage of kidney failure, such as AKI and acute tubular necrosis (1, 17). Urinary cystatin C has also been suggested as a

marker in the assessment of glomerular filtration rate, which sounds appealing because of its noninvasive requirement (18), but there are authors who disprove the use of urinary cystatin C as a glomerular filtration rate marker (19,20). Thus, cystatin C in urine has mainly been used in research as a good indicator of renal disorders affecting the tubular cells, which is believed to be the general cause of AKI (1). For tenofovir-treated patients with AIDS, urinary cystatin C has proven useful when monitoring the renal safety in follow-up examinations (21). It is also usable in patients suffering from hemorrhagic fever with renal implications and patients with type II diabetes with general nephropathy (22, 23). These findings may indicate urinary cystatin C as a broad marker for tubular damage in patients with varied tubular kidney complications. The absence of circadian variations simplifies the interpretation of test results and allows intensive care unit personnel a fast and reliable estimation of renal damage (24).

The aim of this study was to evaluate the assay performance of a urinary analysis of cystatin C with particle enhanced turbidimetric immunoassay (PETIA) on a fully automated high-throughput turbidimetric analyzer for routine diagnostic.

MATERIALS AND METHODS

Samples

Samples were collected from the routine at the Department of Clinical Chemistry and Pharmacology, Uppsala University Hospital. Human urine samples with varying concentrations of cystatin C were found by searching urine samples with elevated albumin concentrations in the laboratory computer system. The patient identities that the samples were always removed before work commenced. Urine samples were centrifuged at $2,400 \times g$ for 5 min. The supernatant was transferred to a new tube and the pellet was discarded. To achieve samples with a low cystatin C concentration, samples were either diluted with saline (0.9% NaCl) or pooled with urine samples containing low concentrations of cystatin C. The study was approved by the local ethical board at Uppsala University.

Urinary Cystatin C assay

Assay buffer (REF 1101), immunoparticles (REF 1101), calibrator (REF 1012) with a concentration of 7.8 mg/l and purified delipidated human serum cystatin C with a concentration of 35 mg/l were all provided by Gentian AS (Moss, Norway) (16). BioRAD Seronorm level 1 (diluted 1:6) and BioRAD Immunoassay Liquicheck (Hercules, CA) were used as controls. Before

analyzing, controls and calibrator were mixed slowly for 3–5 min.

The cystatin C method is based on a PETIA technique. Immunoparticles consist of purified avian antibodies (IgY) directed to human cystatin C that are covalently attached on uniform polystyrene particles. The cystatin C reagent solution is ready to use and is preserved with 15 mmol/l sodium azide and antibiotics. The assay buffer contains of a 3-(N-morpholino)-propanesulfonic acid buffered saline, preserved with sodium azide. Urinary cystatin C measurements were performed on Abbott Architect ci8200 analyzer (Abbott Park, IL). Ten microliters of sample and 180 μ L assay buffer were mixed in a cuvette and incubated for approximately 5 min. Forty-five microliters of immunoparticles were added at point 16 (each photometric point represents 18 s) and incubated for another 5 min. Total assay time was 10 min. The immune complexes formed by agglutination were measured at 444 nm and the absorbance was proportional to the concentration of cystatin C expressed in mg/l. The calibration was set at six points and run automatically in duplicates covering the range 0.042–1.050 mg/l. Test results over the highest calibrator point were simply displayed as greater than 1.050 mg/l and were automatically rerun on the instrument with a 1:8 dilution, using saline solution contained in the instrument. Further instrument settings were as follows: Absorbance blank at point 19, main absorbance measure at point 31–33, and spline calibration method. Test results were reported in mg/l with 3 decimals.

Assay Precision

The assay precision was evaluated by three patient samples with different levels of cystatin C: 0.13 mg/l, 0.57 mg/l, and 0.90 mg/l. The samples were divided into ten equal portions with a total volume of 700 μ L and frozen at -20°C . Before each run, the assay was calibrated and controls were analyzed. Each sample was thawed in a $+37^{\circ}\text{C}$ water bath, vortex mixed, and measured in duplicate twice a day with at least 2 h between each run. For each sample within run coefficient of variation (CV), between-run CV, between-day CV, and total CV was calculated.

Lower Limit of Quantification (LoQ)

LoQ study was performed to determine the lowest actual amount of cystatin C in urine that could be reliably detected with a $\text{CV} \leq 10\%$. A sample pool with cystatin C concentration of 0.50 mg/l was determined by measuring in duplicate. Sample dilutions were made (1:5, 1:10, 1:15, and 1:20) with saline. The diluted samples were run in five replicates, four times with 1 h between

each run. Within each run controls were measured in singles.

Analyte Stability

Five urine samples were divided into seven portions in which, three portions were stored at room temperature and four portions were stored in $+4^{\circ}\text{C}$. All samples were measured initially, at day 3 and 7 and one additional sample that had been stored in $+4^{\circ}\text{C}$ was measured at day 14. The assay was calibrated before each run. Controls and samples were measured in duplicates. The percentage change in cystatin C concentration was calculated, using Excel 2007.

Antigen Excess

The antigen excess was determined to identify the critical hook concentration. A dilution series of spiked purified serum cystatin C with a concentration of 35 mg/l was prepared by diluting the stock solution from 35 mg/l to 0.436 mg/l. The sample absorbance values were plotted against the calculated theoretical sample concentrations.

Assay Recovery

Purified serum cystatin C (35 mg/l) was diluted with saline to a concentration of 5 mg/l and was used as stock solution, and 2.5%, 5%, and 10% dilutions of the stock solution were made and analyzed in triplicates to determine the concentration. A pooled urine sample at 0.156 mg/l was determined in triplicate. The urine sample was spiked with the stock solution matching the theoretical calculated concentrations at 0.156, 0.300, 0.444, 0.588, 0.732, 0.877, and 1.021. Samples were measured in duplicates and the difference between calculated and measured values was expressed in percent. A recovery $\pm 10\%$ was considered acceptable.

Hemoglobin Interference

Erythrocytes were centrifuged and washed with cold isotonic saline four times. The erythrocytes were lysed by adding three volumes of distilled water and then frozen at -20°C to disrupt the erythrocyte membranes. After thawing, the cellular fragments were removed by centrifugation at $2,000 \times g$ for 10 min. The hemoglobin concentration of the lysate was 57 g/l. Two cystatin C samples with concentrations of 1.0 mg/l and 0.1 mg/l, respectively were measured ten times. The CV for these ten replicates was used to determine the number of replicates further in the study. The samples were mixed with hemolysate to obtain a volume of 5 ml with a final hemoglobin concentration of 10 g/l. An additional 5 ml of the two samples were mixed preparing a control by adding the same volume saline as volume hemolysate. Samples were measured

in four replicates and CV and percentage changes were calculated.

Assay Linearity

A pooled sample A, with a high cystatin C concentration (0.953 mg/l) and a pooled sample B, with almost no detectable concentration of cystatin C were pooled and determined by analyzing in triplicate. A dilution series was made by adding different portions of the high sample and the low sample (A10+ B0, A9+B1, A8+B2 ... A0+B10). Linearity was evaluated by plotting the measured values of sample pools run in duplicates, against the theoretical values.

Reference Material

Urine samples were collected as spot urine from 140 apparently healthy volunteers (98 females and 42 males, median age: 36 years, range 21–74). The volunteers were recruited among the laboratory staff and they all stated that they were healthy. The samples were centrifugated at +4°C for 10 min at $2,000 \times g$ and stored frozen at –70°C until the time of testing. Prior to analyzing, the samples were centrifuged at $2,000 \times g$ for 10 min at room temperature.

Statistical Calculations

Statistical analysis and figures were performed utilizing Excel 2000 (Microsoft Corporation, Seattle, WA) and Statistica 7.1 (StatSoft, Tulsa, OK). Calculations of reference intervals were performed by bootstrap estimation using RefVal 4.0 (Department of Clinical Chemistry, Rikshospitalet, Oslo, Norway). RefVal fulfils the recommendations of the International Federation of Clinical Chemistry on the statistical treatment of reference values. The statistical program used in this study performs log transformations of the values to achieve normal distribution. The distribution is checked against normal distribution to verify that the transformed material is normally distributed. When normal distribution of the material is achieved, the program then randomly selects subsets from this population and calculates reference intervals. This calculation is performed 500 times and this forms the basis for the calculation of the reference intervals.

RESULTS

Assay Precision

The assay precision was determined by calculating CV for three cystatin C samples. The obtained CV values did not exceed 3.5% at any level (Table 1).

Lower Limit of Quantification (LoQ) and Limit of Detection (LoD)

The LoQ was determined by measuring urine sample dilutions made with saline in four runs, using five replicates in each run. All results showed a CV less than 10%, which did not exceed the limit and was considered acceptable (Table 2). LoD was determined by measuring a blank sample ten times. The LoD was defined as mean +3SD and was calculated to be 0.004 mg/l.

Hemoglobin Interference

The samples and controls were measured in four replicates and single, respectively. The low cystatin C sample without hemolysate had a concentration mean of 0.078 mg/l and the high sample had a mean of 0.722 mg/l. The low sample with hemolysate had a loss in cystatin C concentration by 8.0% (CV 17.1%). The high sample increased in cystatin C concentration with 12.3% (CV 0.31%).

Analyte Stability

Storing samples at +4°C resulted in a loss of cystatin C concentration after 14 days ranging between –0.6 and –11.0% (Fig. 1A and B). The samples stored in room temperature gave a varied result with both increasing and decreasing concentrations of cystatin C after 7 days. For four samples, the result ranged from –11.1 to +9.0%. One sample (Δ) decreased drastically after 7 days and had a 51.0% loss of cystatin C concentration from 0.204 to 0.100 mg/l (Fig. 1B).

Antigen Excess

There was no hook effect observed at any concentration ranging from 0.436 to 35 mg/l with samples measured in duplicates run on the instrument (Fig. 2). Test result

TABLE 1. Assay Precision for the Urine Cystatin C Assay. The Mean Value and CV for Three Urine Samples are Presented

Mean concentration (mg/l)	0.129	0.568	0.898
Within-run CV (%)	1.64	0.71	0.35
Between-day CV (%)	1.27	1.89	1.76
Between-run CV (%)	2.11	2.49	1.98
Total CV (%)	2.96	3.20	2.67

TABLE 2. The Mean Concentration of Cystatin C and Total CV for Urine Sample Dilutions Made With Saline

Mean concentration (mg/l)	0.100	0.045	0.028	0.020
Total CV (%)	1.97	3.91	6.28	8.76

Fig. 1. The cystatin C stability of three (A) and two (B) urine samples over a period of 7 days in room temperature and 14 days in +4°C. Samples were measured initially, also at days 3, 7, and 14. The open shapes ($\diamond$, $\square$, $\triangle$) represent samples stored at room temperature and the filled shapes ($\blacktriangle$, $\blacklozenge$, $\blacksquare$) represent samples stored at +4°C.

Fig. 2. Increasing theoretical concentrations of cystatin C plotted against the observed delta absorbance values ($\blacksquare$). The calibration standard curve ($\blacktriangle$) is also plotted and the dashed line represents the 6th standard value + 10% which is the cut-off value for antigen excess.

Fig. 3. The linearity of urinary cystatin C assay with expected (x-axis) values plotted against observed (y-axis) values measured with PETIA.

that exceeded the highest calibration point generated a result of greater than 1.050 mg/l. The absorbance values were retrieved from the instrument software for each measurement.

Assay Recovery

The recovery ranged from 93 to 100% for the seven prepared dilutions, which covered the calibrator range, with a $CV \leq 0.5\%$ at all levels. The 2.5%, 5%, and 10% stock solution dilutions with saline gave a mean value of 5.27 mg/l when multiplying the dilution factor for each measurement.

Assay Linearity

The assay showed good linearity and the assay linearity equation was $y = 1.0125x - 0.0065$ with a regression of $R^2 = 0.9994$ over the studied interval greater than 0.001 mg/l to 0.950 mg/l (Fig. 3).

Reference Interval

Fifteen of the values were below the LoQ (0.020 mg/l). Thus, the lower limit of the reference interval could not be calculated. Reference interval (0.975 fractions) calculated estimates for the population ($n = 140$) with RefVal less than 0.166 mg/l, and 90%-confidence interval for the 97.5 percentile was 0.119–0.213 mg/l.

DISCUSSION

Presently, cystatin C is an established biomarker for glomerular filtration rate estimating measurements in plasma or serum to detect glomerulus kidney abnormalities (25). In the past few years, measurements of cystatin C in urine have attracted attention as a potential biomarker for AKI or acute tubular necrosis and also other

pathological changes of the kidney tubules (1, 26). Thus, the aim of this study was to validate an assay with a potential future capacity of detecting tubular damage with good precision and performance on a high-throughput chemical analyzer that may be useful in the acute phases of patients who are admitted to the intensive care unit with renal implications. All three levels of cystatin C showed low imprecision with an acceptable CV of less than 3.5%, which complies with results reported by Herget-Rosenthal with coworkers and Conti with coworkers. In these studies, the used method for measuring cystatin C was particle-enhanced nephelometric immunoassay (PENIA) (11, 27). Earlier publications have shown that differences between PETIA and PENIA often are very small (28). Accordingly, the LoQ study confirmed the assay precision study but it showed a slightly lower CV at similar concentrations of cystatin C. This phenomenon may be explained because of the many substantial factors affecting the precision study as the between-day variance, between-run variance, the calibrator batch-to-batch differences, and handling of samples with different starting points, etc. The LoQ result allows precise measurements at cystatin C concentrations of 0.02 mg/l. In healthy subjects, the concentration of cystatin C is low (0.03–0.3 mg/l) and is within the studied concentration interval chosen for the LoQ study. The stability of cystatin C in urine is acceptable after 3 days and 14 days in room temperature and +4°C, respectively. This allows urine samples to be shipped from health centers and nearby health clinics to the laboratory by mail at room temperature, and after analysis store samples in +4°C in case a reanalysis is required. There are also daily transports to the laboratory from nearby health clinics and test results are usually reported at the same day after sample arrival at the laboratory. The stability of cystatin C in urine is sufficient for the laboratory when the assay is eventually included in the routine. One sample stored at room temperature showed a pronounced decrease of cystatin C after 7 days (Fig. 1B). This observation caused us to limit the stability at room temperature to 3 days which is more than sufficient for our clinical use. We have no clear explanation regarding the decrease in this sample but a possibility could be degradation by proteolytic enzymes in the urine (11). The same sample stored at +4°C did not show similar tendency. In a study by Conti with coworkers, the collection of urine samples was performed with addition of a cocktail containing antiprotease and antimicrobial agent. They reported a decrease in cystatin C values without this cocktail (11). Nevertheless, we have shown that cystatin C in urine is stable for 3 days in room temperature without addition of antiproteases (Fig. 1A and B).

There was no hook effect observed for the assay up to a cystatin C concentration of 35 mg/l. In healthy indi-

viduals, the concentration of cystatin C in urine is low as stated before but in patients suffering from severe renal tubular disorders, the concentration can lead to a 200-fold increase (29). The assay will not report incorrect test result up to 35 mg/l because none of the samples fell below the dashed line (Fig. 2), which represents the cut-off value of antigen excess. The cut-off absorbance value for antigen excess was defined as the absorbance value of the highest standard point +10%. Samples with absorbance values higher than the highest standard point were automatically rerun with a 1:8 dilution on the instrument and based on the assay recovery, test results can be accepted. Hemoglobin did not interfere at a clinical significance. Testing the cystatin C–hemoglobin interference at such a high concentration as 10 g/L hemoglobin should give a satisfactory margin for analyzing urine samples from patients with hematuria. The assay was linear over the calibration interval with good correlation and interception.

In this study, particles coated with IgY antibodies were used to measure cystatin C in urine. IgY, or chicken antibodies, is the major serum immunoglobulin in chickens and other avian species. IgY is transported to the egg from the hen in a similar way as mammalian IgG crosses the placenta barrier. IgY is found in high concentrations in egg yolk and the recovery of antibody is far greater after purification from egg yolk than after antibody purification from mammalian sources. IgY does not interact with rheumatoid arthritis factors, complement, human antimurine antibodies or bacterial Fc-receptors due to its phylogenetic difference (30). Studies have, however, shown that IgY is more suitable for turbidimetric measurements and gives a better analytical performance (28, 31).

In summary, the urinary cystatin C turbidimetric immunoassay is a rapid and easy method for measurements with short turnaround time.

ACKNOWLEDGMENTS

This study was financially supported by the Uppsala Hospital Research Fund and the Norwegian Research Council.

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