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Performance Characteristics of a Cystatin C Immunoassay with Avian Antibodies

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Abstract

Background: A new particle-enhanced turbidimetric immunoassay (PETIA) with avian antibodies for measuring serum/plasma cystatin C has been developed. The performance characteristics of the assay are described.

Methods: Measurements were performed on a Roche Modular P and on an Abbott Architect ci8200 using Gentian cystatin C immunoassay.

Results: Measuring range was 0.3–8.0 mg/L. Reference range was 0.57–1.09 mg/L. Total analysis time was 10 minutes. Linearity was absolute over the whole assay range. Recovery of samples and controls was within 98.6–109.4%. Total imprecision CV, measured over 20 days with two lots, was ≤4.2%. Comparison with a particle enhanced nephelometric cystatin C immunoassay (PENIA) by linear regression resulted in a slope within 0.97–1.02 and intercept within ±0.05 mg/L. Interference studies with drugs, anticoagulants, intralipid (≤11g/L), triglycerides (≤14 g/L) and bilirubin (≤420 mg/L) showed no significant interference. Due to the use of avian antibodies, no interference with rheumatoid factor was observed. No carry-over was detected. Lower detection limit and lower quantification limit (CV ≤6%) were both below 0.33 mg/L, which is less than the lowest standard. Sample stability was up to one month at 2–8 °C. Stability of the reagents at 2–8 °C was estimated to be 24 months. Stability of the reagents in use was minimum 9 weeks.

Conclusions: Gentian cystatin C PETIA is shown to have excellent performance characteristics. Correlation with existing PENIA shows no significant difference between methods . Interference results are improved due to avian antibodies and a broader span of the calibration curve. Avian antibodies are also known to have better immune response than mammalian antibodies towards mammalian antigens.

Introduction

Glomerular filtration rate (GFR) is defined as the volume of plasma that can be completely cleared of a particular substance by the kidneys in a unit of time. Changes in GFR may be due to:

– High age
– High bloodpressure
– Acute and chronic renal insufficiency
– Tumor in renal and urine system
– Inflammatory conditions in the renal/urine system
– Proteinuria (Proteins in urine)

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It is therefore important to have an efficient and reliable method for determining GFR. Serum/plasma creatinine has become the most common marker for GFR as it holds several desirable properties for a GFR marker. It is freely filtered by the glomerulus and not reabsorbed by the proximal tubules. The concentration of SCr in the bloodstream depends on variables as muscle mass, age, diet and gender. With increased plasma concentrations, there is increased tubular secretion, leading to an overestimation of GFR in patients with moderate and severe decreases in GFR. SCr is also insensitive for detecting small decreases in GFR. Even a 50% reduction of GFR is not infrequently associated with a normal serum creatinine concentration [1].

Cystatin C

Cystatin C was proposed as a new marker for GFR by Anders Grubb in 1981 [2]. Cystatin C is a non-glycosylated protein that belongs to the cystatin superfamily of proteins. The protein is 13kDa and is almost ellipsoidal (30 and 45 Å). It has a pI around 9 [3]. Due to the small size and basic pI, it is freely filtered by the glomerulus. It is reabsorbed by tubular epithelial cells and 99% is catabolized. The serum level correlates with the pathophysiological states influencing GFR, which is also compatible with a stable secretion of cystatin C from most human tissues [4]. The cystatin C concentration in serum/plasma is uninfluenced by age, gender and muscle mass, and some authors state cystatin C to be a better GFR marker than SCr, especially in the creatinine-blind range [5,6]. The relationship between cystatin C and GFR has been estimated through several studies [7,8]. It is also suggested that cystatin C may be a better GFR tool for special patient groups [9–11].

Methods and materials

The following reagents were used:

Cystatin C Immunoparticles, Code.No. 1014
Cystatin C Reaction Buffer, Code.No. 1007
Cystatin C Calibrator, Code.No. 1012
Cystatin C Low Control, Code.No. 1020
Cystatin C High Control, Code.No. 1021

All reagents are produced by Gentian AS, Moss, Norway.
Saline solutions from Roche Diagnostics/Abbott Laboratories were used as diluters.
Cystatin C controls were made by spiking human serum with human cystatin C.
The following turbidimetric instruments were used:

- Modular P (Roche Diagnostics) situated at Rikshospitalet University Hospital, Oslo, Norway.
- Architect ci8200 (Abbott Laboratories) situated at Uppsala University Hospital, Uppsala, Sweden.

For method comparison, the nephelometric cystatin C method from Dade Behring was performed using a BN ProSpec situated at Uppsala University Hospital, Uppsala, Sweden. Measuring range for the Gentian cystatin C immunoassay is 0.3–8.0 mg/L. For Modular P, a serum/plasma sample of 3 μl and 230 μl of assay buffer is mixed. The assay buffer is used to enhance the reaction and to prevent unspecific interactions. After 16.5 cycles (each cycle is 17.9 seconds), 40 μl immunoparticles and 20 μl water are added. After 18 cycles the first absorbance measurement is made, and after 34 cycles the last measurement is made. The difference between these two absorbance measurements are converted to mg/L in accordance with the regression fit of the calibration curve. In total the analysis time is 10 min and 50 seconds, in which the agglutination reaction lasts for 5 minutes and 25 seconds. Primary/secondary wavelength is 546/700 nm.

For Architect ci8200 a sample of 3 μl and 220 μl of assay buffer is mixed. After 16 photometric points (the time between each point is 18 seconds), 45 μl immunoparticles are added. After 18 photometric points, the first absorbance measurement is made, and after 31 and 33 photometric points the last measurements are made. In total an analysis time of 9 min and 36 seconds, in which the agglutination reaction lasts for 4 minutes and 48 seconds. The mean of these two last measurements is used in the conversion of absorbance difference to mg/L. Primary/secondary wavelength is 548/700 nm.

Avian antibodies

This Gentian cystatin C immunoassay uses avian antibodies. Antibodies derived from avians have several advantages over mammalian antibodies due to the phylogenetic distance between Mammalia and Aves. Avian antibodies do not react with human rheumatoid factor and do not activate the human complement system. This reduces false positive reactions that may occur between anti-mammalian IgG antibodies and the mammalian antibodies [12–14].

Principle of the test

The Gentian cystatin C assay is a particle enhanced turbidimetric immunoassay (PETIA). The immunoparticles are made from activated polystyrene microspheres to which anti-human cystatin C produced from chicken eggs is covalently attached. When immunoparticles and cystatin C react, there is a formation of agglutinates which changes the absorbance signal, depending on the amount of cystatin C present. From a calibrator of known human cystatin C concentration, a 6 point calibration curve is created by diluting the calibrator according to a predefined dilution regime. From this interpolation curve the cystatin C content in a patient sample can be quantified.

Statistical analysis

Statistical Analysis were performed with *Kaleidagraph* (Synergy Software) and *Analyse-it* (Analyse-It Software, Ltd., UK) for Microsoft Excel. All regression analysis are performed with 95 % confidence interval.

Antigen excess

The prozone/critical hook effect will cause very high concentrations of cystatin C to produce signals that are similar to the signals generated by moderate concentrations of cystatin C. The critical hook concentration of the Gentian cystatin C immunoassay was determined by spiking a serum sample up to 50 mg/L and performing a dilution series on Modular P. By plotting the measured cystatin C concentration (measured in duplicates) vs theoretical cystatin C concentration the critical hook concentration was determined, defined as concentration corresponding to the absorbance 10 % above the highest calibrator. The calibrator used had a cystatin C concentration of 7.90 mg/L, and when reading from the antigen excess curve, no antigen excess effect was found below 50 mg/L cystatin C. In this study a theoretical concentration of 50 mg/L cystatin C was reported as 9 mg/L cystatin C. In this context it useful to mention that cystatin C concentrations above 8 mg/L rarely has been reported.

Interference studies

Interference studies were performed according to the NCCLS protocol EP7-A, Vol.22, No.27 [15]. This protocol tests interference according to a two-sided hypothesis test with 95% Confidence Interval. To determine the cut-off value for accepting or rejecting the hypothesis of no interference, a clinically relevant difference is set to 7.5% of the original cystatin C concentration for samples below 1 mg/L and 5% for samples above 1 mg/L. Two cystatin C levels were used in the experiments. Rheumatoid factor interference, drug interference and anticoagulant interference were only studies on Modular P.

Bilirubin

Serum samples were spiked with bilirubin. No interference was observed below 800 mg/L bilirubin on Modular P. On Architect ci8200 no interference was observed below 420 mg/L bilirubin.

Intralipid

Serum samples were spiked with intralipid. No interference was observed below 16 g/L intralipid on Modular P. On Architect ci8200 no interference was observed below 11 g/L.

Haemoglobin

Serum samples were spiked with haemoglobin. Interference was observed at 10 g/L haemoglobin. A dose response study of the low sample showed that haemoglobin interference g/L is not present below 7 g/L on Modular P. On Architect ci8200 no interference was observed below 8 g/L.

Table 1. Drug interference: Drugs tested according to recommendations in [16]

Drug	Test Concentration (mg/L)
Acetaminophen	200
Acetylcysteine	150
Acetylsalicylic acid	1000
Ampicillin	1000
Ascorbic Acid	300
Calsium Dobesilate	200
Cefoxitin	2500
Cyclosporine	5
Doxycycline	50
Heparin	5000 U
Ibuprofen	500
Levodopa	20
Methyldopa	200
Metronidazole	200
Phenylbutazone	400
Rifampicin	60
Theophylline	100

Triglycerides

The recommended method for detecting triclycerides (TG) interference is to use an ultracentrifugated control pool and compare the control pool and test pool as described in [15]. No interference below 12.5 mmol/L=14 g/L TG was observed on Modular P. On Architect ci8200 no interference was observed below 16 g/L.

Drug interference

The drugs in table 1 were investigated at the given concentrations in [16], and at two cystatin C levels. No significant interference was observed.

Anticoagulant interference

The anticoagulants Heparin (1000 U/ml), Disodium EDTA (2 g/L), Sodium Citrate (38 g/L), Sodium Fluoride (10g/L),Potassium Oxalate (2 g/L) and a 50/50 mixture of Sodium Fluoride and Potassium Oxalate (2 g/L / 2 g/L) were investigated at the given concentrations in *Burtis CA, et al, fourth edt* [17], and at two cystatin C levels. No significant interference was observed.

Rheumatoid factor (RF)

Concerning rheumatoid factor interference, it is known that avian IgG has no immunological cross reactivity with mammalian IgG, and can be used to avoid interference due to RF [13]. As the the Gentian cystatin C antibodies are from chicken egg origin, RF interference is not present in the Gentian cystatin C immunoassay.

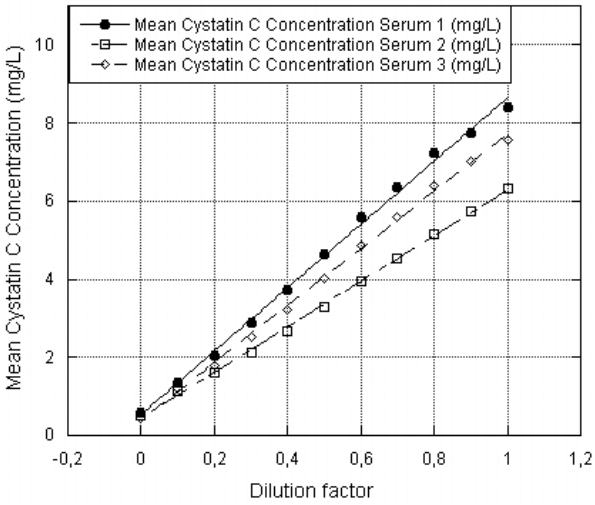

Figure 1. Linearity Modular P: First order regression analysis where measured mean cystatin C concentration of the three sera is plotted vs dilution factor.

Linearity

Modular P

The linearity experiment followed the NCCLS protocol EP6-A, Vol.23, No.16 [18]. This protocol describes a statistical approach to evaluate linearity. The main purpose is to show that, when diluting a high cystatin C serum sample with a very low cystatin C serum, the regression that fits the data best is a first order linear regression, and not higher order polynomial. If the data fits a higher order regression analysis (determined by a Student's t-test on the higher order coefficients), the difference between the higher order regression and the first order regression should not exceed 6%. 11 dilutions of three different spiked sera were measured in tripli-

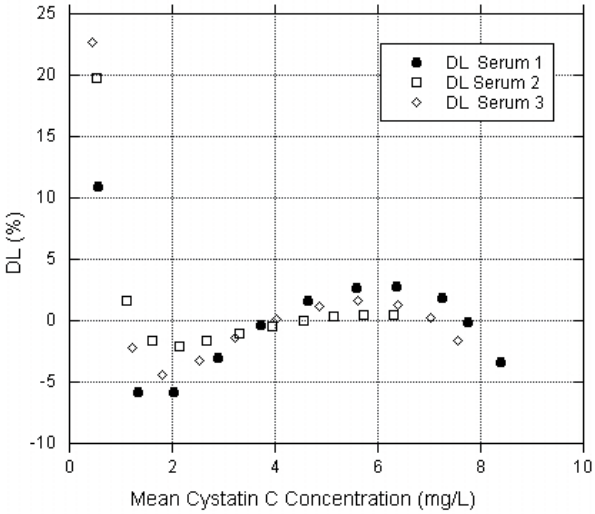

Figure 2. Linearity Modular P: Difference plot (in %) between calculated cystatin C concentration (mg/L) in first order and third order regressions vs measured mean cystatin C concentration in serum 1, 2 and 3 (mg/L).

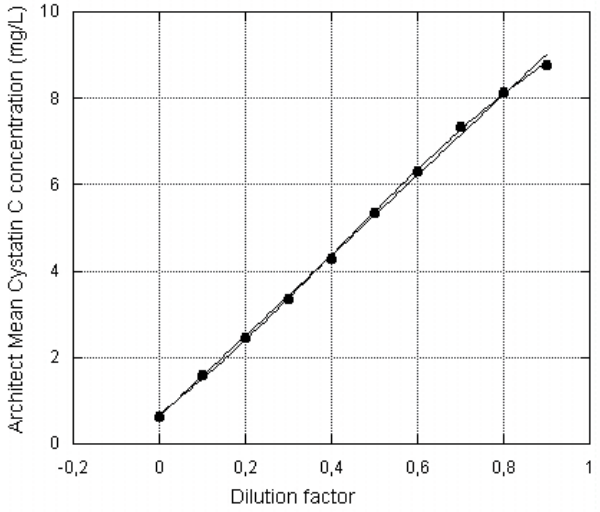

Figure 3. Linearity Architect ci8200: First and third order regression analysis where measured mean cystatin C concentration is plotted vs dilution factor.

cate. The three sera also fitted a higher order regression, but the difference between the linear regression and the third order regression was within the specifications. Figure 1 shows the first order regression analysis and figure 2 shows a difference plot (in %) between calculated cystatin C concentration (mg/L) in first order and third order regressions vs measured mean cystatin C concentration in serum 1, 2 and 3 (mg/L).

Architect ci8200

The experiment was performed as in section 4.1, but with only one serum. The dilution series also fitted a higher order regression, but the difference between the linear regression and the third order regression was within the specifications. See figures 3 and 4.

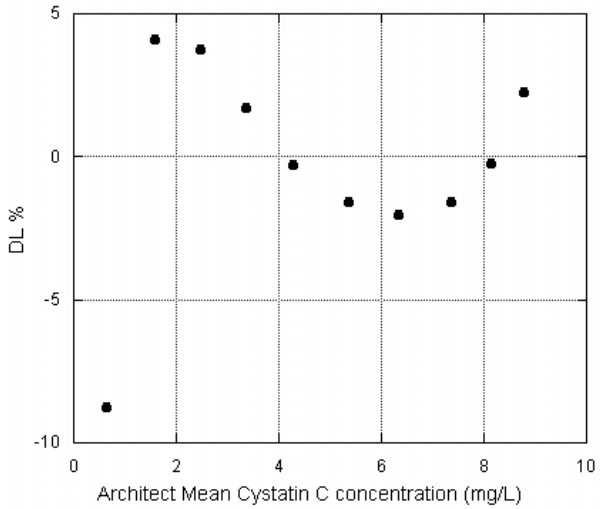

Figure 4. Linearity Architect ci8200: Difference plot (in %) between calculated cystatin C concentration (mg/L) in first order and third order regressions vs measured mean cystatin C concentration (mg/L).

Lower detection/Quantification limit

To obtain the LDL and LQL on Modular P, the NCCLS protocol EP17-A, Vol.24, No.34 [20] was followed as far as possible. LQL was defined as the lowest dilution of a serum sample that gives a CV of ≤6% (≥20 replicates). With this criteria, the LQL on Modular P was estimated to be:

LQL=0.28 mg/L

The LDL was found by first determining the so called Lower Limit of Blank (LoB). With a series of dilutions in the range up to 4xLoB and the pooled Standard Deviation of these dilutions, the LDL on Modular P was estimated to be:

LDL=0.03 mg/L

On Architect ci8200 only the LQL was determined:

LQL=0.33 mg/L

Measures of reproducibility

Modular P

The precision experiment on Modular P was performed according to the recommendations in the NCCLS protocol EP-5A, Vol.19, No.2 [19]. Two different lots of all reagents were used. 3 serum samples and 2 controls were measured in duplicate with each lot for 20 days with at least 2 hours between each measurement. A re-calibration was performed on both lots each day, and the sequence of the samples and lots were changed every day to minimize systematic effects. The results are shown in table 2. In total 80 measurements on each sample/control were collected.

Table 2. Precision data from Modular P with 80 observations on each sample

Sample ID	Mean (mg/L)	Within Run CV (%)	Between Day CV (%)	Between Run CV (%)	Total Precision CV (%)
103	0.66	2.23	1.92	2.25	3.70
111	3.96	1.17	1.54	0.84	2.11
150	1.54	1.70	2.24	2.51	3.77
Control L	0.88	1.81	1.25	3.54	4.17
Control H	2.42	1.68	1.45	1.33	2.58

Architect ci8200

The precision experiment on Architect ci8200 was performed as in section 6.1, but over 5 days. Four samples with mean concentrations of 5.17 mg/L(CV=4.18%),3.38 mg/L(CV=3.75%),1.35 mg/L(CV=2.67%),0.69 mg/L(CV=2.81%) and two controls with mean concentrations of 0.88 mg/L(CV=3.72%) and 3.58 mg/L(CV=1.41%) were measured and the CVs were all below 4.18%.

An additional precision experiment at Uppsala University Hospital on Architect ci8200 has shown an imprecision below 2%.

Recovery

Modular P

Three serum samples with known cystatin C concentration were spiked with a serum of very high and known cystatin C concentration. The expected cystatin C concentration was calculated, and the recovery between expected and measured cystatin C concentration is shown in table 3.

Architect ci8200

The experiment was performed as in section 7.1, but with only one serum sample. The recovery results of serum samples between 1.64 mg/L and 5.64 mg/L were between 104.7% and 108.3%.

Table 3. Recovery results on Modular P

Expected cystatin C concentration (mg/L)	Measured mean cystatin C concentration (mg/L)	Recovery (%)
SERUM 1		
2.21	2.18	98.6
3.21	3.19	99.4
4.21	4.43	105.1
5.21	5.69	109.1
6.21	6.80	109.4
SERUM 2		
1.55	1.67	107.7
2.55	2.59	101.6
3.55	3.52	99.0
4.55	4.70	103.3
5.55	5.91	106.4
SERUM 3		
2.40	2.39	99.4
3.40	3.43	100.7
4.40	4.63	105.1
5.40	5.85	108.3
6.40	6.86	107.1

Table 4. Lotvariation results. Three different lots used to measure the same 35 serum samples

Comparison	Intercept (mg/L)	Slope
Lot 2 vs lot 1	0,00	1,00
Lot 3 vs lot 1	0,08	1,03
Lot 3 vs lot 2	0,10	1,02

Lot variation

The same set of serum samples (≥35) was measured on three different Gentian reagent lots on Modular P, where all reagents were changed. The goal was to obtain a slope of 1 ± 0,05 and an intercept of 0±0.15 mg/L with a passing bablock regression analysis in order to consider lot variations acceptable, see table 4.

These results fulfill the predefined criteria for lot variation.

Method comparison

Modular P

In this experiment the Gentian cystatin C immunoassay on Modular P was compared with the Dade-Behring cystatin C N Latex method on BN ProSpec. This was done by measuring the same 80 (of which 5 samples were outliers) serum samples in duplicates on both methods (on one calibration and in two runs on Modular P

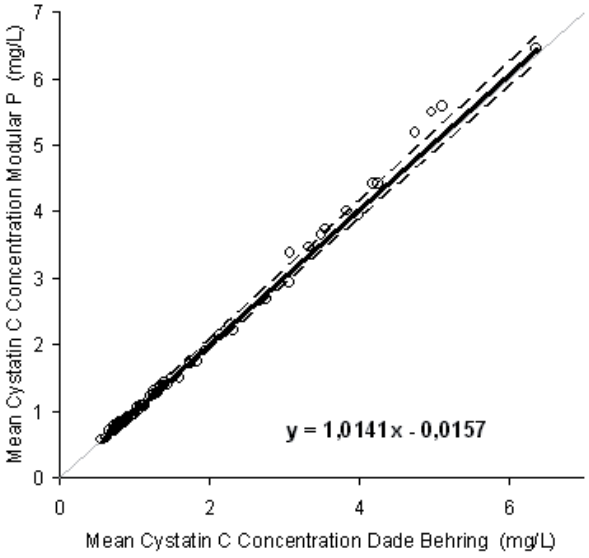

Figure 5. Method Comparison study between Gentian cystatin C immunoassay on Modular P and Dade-Behring cystatin C N Latex Immunoassay on BN ProSpec. Passing bablock regression analysis (N=75). Gentian cystatin C method plotted vs Dade Behring cystatin C method.

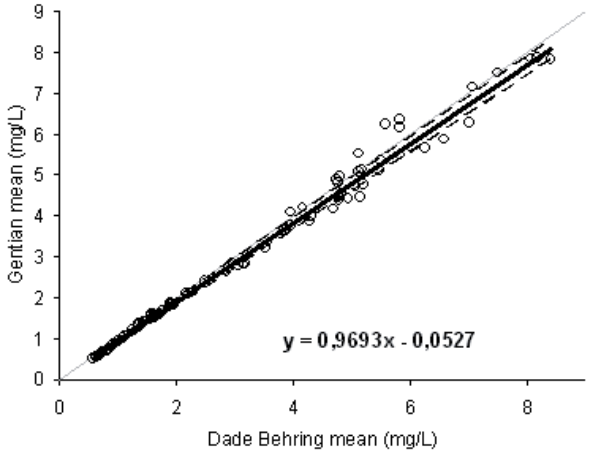

Figure 6. Method comparison study between Gentian cystatin C immunoassay on Architect ci8200 and Dade-Behring cystatin C N Latex Immunoassay on BN ProSpec. Passing bablock analysis (N=82).

and on two runs and two days on BN ProSpec) and comparing them with bias analysis, linear regression analysis and passing bablock analysis, see figure 5. In addition, the difference from Dade-Behring's upper confidence limit, also called medical decision point, was determined. The experimental design and data analysis mostly followed the recommendations in the NCCLS protocol EP9-A2,Vol.22, No.19 [21].

The Dade-Behring cystatin C immunoassay has a medical decision point of 0.95 mg/L. Using a linear regression analysis, we find that a sample measuring 0.95 mg/L with the Dade Behring method will measure between 0.92–0.95 mg/L with the Gentian cystatin C immunoassay on Modular P.

From the passing bablock analysis we see that a slope of 1.01 (95% confidence interval of 0.99 to 1.04) and an intercept of –0.016 mg/L (95% confidence interval of –0.045 to 0.007) is obtained.

Architect ci8200

The Gentian cystatin C immunoassay on Architect ci8200 was compared with Dade-Behring Cysatin C method on BN ProSpec at Uppsala University Hospital in Uppsala, Sweden. This was done by measuring the same 86 (of which 4 samples were outliers) serum samples in duplicates on both methods (on one calibration and in one run on Architect ci8200 and BN ProSpec) and comparing them with bias analysis, linear regression analysis and passing bablock regression analysis, see figure 6. In addition, the difference from Dade-Behring's upper confidence limit, also called medical decision point, was determined.

Using a linear regression analysis, a sample measuring 0.95 mg/L with the Dade Behring method will measure between 0.81–0.95 mg/L with the Gentian cystatin C immunoassay on Architect ci8200.

From the passing bablock analysis, a slope of 0.97 (95% confidence interval of 0.95 to 1.04) and an intercept of –0.05 mg/L (95% confidence interval of –0.09 to –0.008) is obtained.

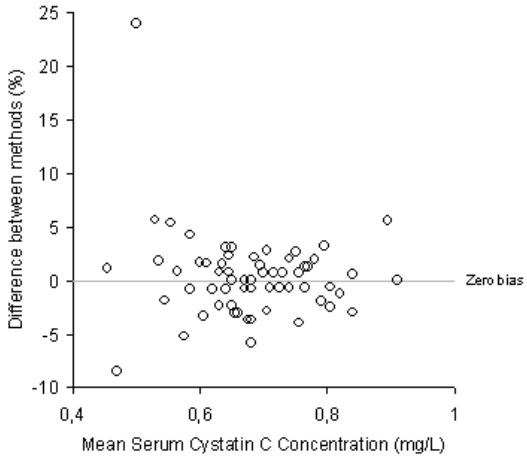

Figure 7. Serum and plasma samples from same self-declared healthy individual measured on Modular P. Bias plot where % difference between serum and plasma cystatin C concentration is plotted vs mean serum cystatin C concentration (mg/L)(N=73).

Serum and plasma comparison

This study was performed by measuring a set of serum and plasma samples from 73 self-declared healthy individuals. This study was only performed on Modular P.

The results support that serum and plasma are equivalent as sample material for Gentian cystatin C immunoassay on Modular P, see figures 7 and 8.

The corresponding 95% confidence intervals are:

Slope 0.913 to 1.037
Intercept –0.024 to 0.061 mg/L

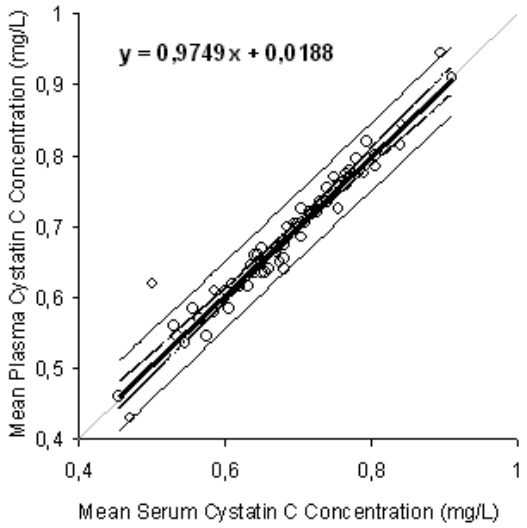

Figure 8. Serum and plasma samples from same self-declared healthy individual measured on Modular P. Linear regression analysis where mean plasma cystatin C concentration is plotted vs mean serum cystatin C concentration (mg/L)(N=73).

This shows that the slope is not significantly different from 1 and the intercept is not significantly different from 0. This supports the idea that serum and plasma are equal sample material.

Instrument variation

Modular P

As with the lot variation study in section 8, a set of serum samples (≥35) were measured on three different instruments, Modular P, Hitachi 911 (an in-house instrument) and Hitachi 917 (at Ullevål University Hospital, Oslo) . The goal is to obtain a slope of 1±0,075 and an intercept of 0±0,15 mg/L with a passing bablock regression analysis. The results shows that instrument variation is within the predefined acceptance limits.

Architect ci8200

The instrument variability of the Gentian cystatin C immunoassay between Architect ci8200 and Modular P was investigated in a separate experiment. This was done by measuring more than 70 serum samples on both instruments and comparing them with bias analysis, and passing bablock regression analysis. The regression analysis showed that the goals were achieved. In the bias plots no samples differed by more than 6%. The regression analysis showed a very small but significant intercept of 0.019 mg/L of and a slope of 0.999 (not significantly different from 1), see figure 9.

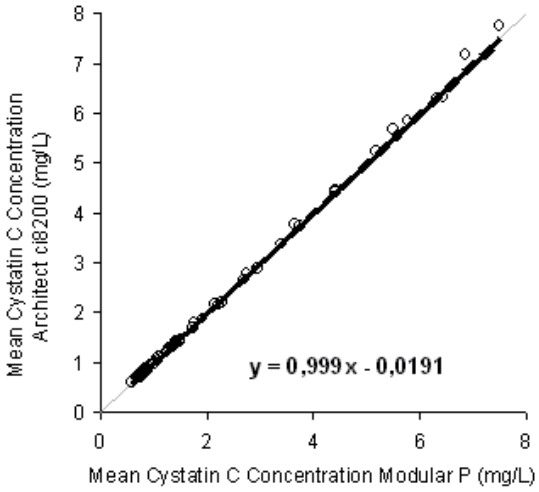

Figure 9. Instrumentvariation study between Gentian cystatin C immunoassay on Architect ci8200 and Modular P. Passing bablock analysis (N=74).

Sample carry over

Sample carry over of the Gentian cystatin C immunoassay on Modular P and Architect ci8200 was studied by following the experimental design in the NCCLS protocol EP10-A2, Vol 22, No 29 [22]. This protocol has a recommended sample sequence for detecting sample carry over effects. The sequence was run for five days and showed that carry over effects were not significant. The mean sample carry over was 0.10% on Modular P and 0.37% on Architect ci8200.

Stability studies

Accelerated stability study and room temperature stability

This study was performed by stressing the Gentian cystatin C reagents at 27°C, 37°C and 45°C, and measuring a series of serum samples until prefixed criteria were not fulfilled. Assuming that the Arrhenius equation is valid [23], the calculated stability at 4°C was 24 months. The reagents were stable for at least 14 days in room temperature.

Sample stability

Five serum samples were stored at room temperature and at 2–8°C. Baseline values were collected, and the samples were measured at predefined intervals, and recovery calculated. With recovery claims of ±10%, a stability of 15 days at room temperature was observed, and a stability of 36 days was observed at 2–8°C. However, serum coagulation and bacterial growth were observed before recovery claims failed.

During one week five serum samples were repeatedly frozen and thawed every day. This did not affect the cystatin C concentration in the samples.

Stability of calibration curve and in use stability

Nine weeks calibration curve stability and nine weeks in-use stability was observed.

Reference interval

By measuring 138 serum samples from self-declared healthy individuals, a non-parametric 95% reference interval was estimated:

0.57–1.09 mg/L

However, the data was normally distributed according to the Kolmogorov-Smirnov test for normality after removal of an outlier, and a parametric method for determination of the reference interval may also be used

Mean± 2SD estimates the reference interval:

0.51–1.09 mg/L

Calculations are made according to the NCCLS protocol C28-A, Vol. 15, No. 4 [24].

Sex distribution:

Males: 42%
Females: 58%

Age distribution:

Age	Number of persons
Below 20 years:	1
20–29	31
30–39:	36
40–49	19
50–59	31
60–69	11
70–79	8
80–89	1
TOTAL	138

Discussion

Since 1981, Cystatin C has been widely discussed as a marker for GFR. This study shows that the new cystatin C PETIA (Gentian AS) introduced is comparable in function to both cystatin C PETIA (Dako Cytomation) and PENIA (Dade Behring). This cystatin C PETIA has a strong correlation to the cystatin C PENIA (Dade Behring)(y=0.16+1.01x). The corresponding correlation between existing cystatin C PETIA (Dako Cytomation) and PENIA (Dade Behring) is reported as [25](y=0.15+0.76x). The new cystatin C PETIA (Gentian AS) offers a great advantage by using chicken egg antibodies that eliminates interference with, for example, rheumatoid factor. The new cystatin C PETIA (Gentian AS) has a larger delta absorbance of the calibration curve since absorbance per mg/L of the calibrator is higher than previously published turbidimetric products. This improves precision of the measurements. Moreover, it reduces the interference with lipids since the signal to noise ratio is improved. The new cystatin C PETIA (Gentian AS) is therefore a robust and rapid assay with excellent performance characteristics, enabling routine testing on clinical chemistry platforms.

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