Clin Chem Lab Med 1998; 36(11):859–865 © 1998 by Walter de Gruyter · Berlin · New York

Quantitative Automated Particle-Enhanced Immunonephelometric Assay for the Routinary Measurement of Human Cystatin C

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Human cystatin C is a low molecular mass protein of 13359 Dalton recently proposed as a new very sensitive marker of changes in glomerular filtration rate. Serum cystatin C concentration correlates negatively with glomerular filtration rate as well as or better than creatinine. We evaluated a recently introduced automated nephelometric immunoassay for cystatin C in serum or EDTA-plasma samples on the Behring Nephelometer System. The assay consists of incubating the 100-fold diluted sample for 6 minutes with latex particles covalently coated with anti-human cystatin C antibodies, and then quantifying the change of light-scatter produced. Method reproducibility is satisfactory, the intra- and inter-assay coefficients of variation ranging from 1.58 % to 3.77 % and from 5.6 % to 11.47 % respectively. Rheumatoid factor (≤ 1116 IU/ml), bilirubin (≤ 418 µmmol/l), triglycerides (10.47 mmol/l), and haemoglobin (12 g/l) do not significantly interfere in the assay. No significant difference was found in cystatin C concentration between serum and EDTA-plasma samples. Cystatin C is stable in serum samples stored under different conditions up to one month. This method correlates well (mean difference = –0.536 ± 0.307 mg/l) with another commercially available particle-enhanced turbidimetric immunoassay. Cystatin C offers better clinical sensitivity than creatinine for discriminating patients with normal renal function and those with mild-to-moderate reduction in renal function. This method is suitable for routine cystatin C measurement, including emergencies.

Key words: Cystatin C; Glomerular filtration rate; Latex immunoassay; Nephelometry; Renal function.

Introduction

The kidney plays a major role in the metabolism of low molecular weight plasma proteins. Most of these proteins are freely filtered through the glomerulus and almost completely reabsorbed and catabolized in the proximal tubular cells (1). Human cystatin C, alias γ-trace protein, is a basic low molecular mass protein (M_r = 13359) with two disulphide bridges, a positive charge at physiological pH, and an isoelectric point of 9.3. The sieving coefficient for cystatin C is close to one, the molecule being an ellipsoid with axes of about 30 and 45 Ångström (Å) (2). Containing one non-glycosylated polypeptide chain with 120 amino acid residues, cystatin C belongs to a recently defined superfamily of proteins, called the cystatins superfamily (3), since all its members are cysteine proteinase inhibitors (4–6). The human cystatin C gene has been cloned, sequenced and located to chromosome 20p11.2 (7, 8), and seems to be of the so-called housekeeping types, which are constantly expressed in most human tissues (9–11). Cystatin C is steadily produced by all human nucleated cells; however, its production is not influenced by inflammation and thus it cannot be considered an acute phase protein (12). Also malignancy does not significantly affect cystatin C production (13). The serum cystatin C concentration seems to be not significantly influenced by gender and age beyond the first year of life (14), while during the first year of life its serum levels inversely correlate with the logarithm of age (15). In the neonate, serum cystatin C levels are significantly higher than those in the adult (16); however, cystatin C seems to derive only from the neonate himself, because no correlation has been demonstrated between maternal and neonatal serum levels (17). Because of its low molecular mass, cystatin C easily crosses the glomerular filter and is then reabsorbed and catabolized by the proximal tubular cells (18, 19). These findings confirm that this protein cannot return into the blood stream after its filtration through the glomerulus and that serum cystatin C considerably increases in patients with renal failure, as other low molecular mass plasma proteins (20, 21). Although the diagnostic potential role of cystatin C has been described in the past (22), the utilized analytical methods, such as enzyme immunoassay (EIA) (23), radioimmunoassay (RIA) (24), fluoroimmunoassay (25), and simple radial immunodiffusion (RID) were not well standardized and were time consuming. Consequently, the test has been not widely introduced in clinical practice, especially because of the impracticability of these methods in emergency situations. Recently, new immunological methods based on turbidimetric or nephelometric techniques, such as the particle-enhanced turbidimetric immunoassay (PETIA), have been proposed for a simple, accurate, and rapid estimation of cystatin C in the clinical laboratory (26). Thus, cystatin C has been clinically reevaluated as a new endogenous serum marker for the early assessment of changes in glomerular filtration rate (GFR) (27, 28), especially in conditions, such as the monitoring of renal transplant patients (29). By using new standardized methods, it has been demonstrated that very small reductions in GFR

cause significant increases in serum cystatin C levels (30).

We describe the performance of a fully automated and rapid particle-enhanced nephelometric immunoassay (PENIA) on the Behring Nephelometer System for determining serum levels of cystatin C. We compared results with those obtained from a commercially available analytical PETIA (26), using serum samples from patients with normal to markedly reduced GFR in order to investigate also the clinical significance of serum cystatin C levels.

Patients and Methods

Analytical methods

N-Latex nephelometric cystatin C immunoassay is based on rabbit monospecific anti-human cystatin C antiserum, covalently coated with 80-nm diameter chloromethylstyrene particles (31). Additional reagents utilized to prevent non-specific interactions and to enhance stability of the reaction mixture are supplement A and supplement B; they are both available in liquid form (Dade Behring Diagnostics, Milan, Italy). The assay is performed with a six-point cystatin C calibration curve, generated from multiple dilutions of a human cystatin C calibrator (Dade Behring Diagnostics, Milan, Italy). The calibrator consists of lyophilized purified cystatin C obtained from human urine. The technique utilizes a fixed-time method with an incubation time of six minutes. Samples are automatically prediluted 1:100 in two stages with a phosphate buffered saline (N-Diluent, Dade Behring Diagnostics, Milan, Italy) before being analyzed. The antigen-antibody complexes scatter an incident beam of light, originating from an infrared high-performance light-emitting diode (840 nm), and changes in the signal are converted into concentrations (mg/l). The intensity of the signal is proportional to the cystatin C sample concentration. In this study, we used a Behring Nephelometer Analyzer (BNA) for the evaluation of the cystatin C nephelometric assay (Dade Behring Diagnostics, Milan, Italy). The evaluation was performed by using a single reagent lot (N. 167801) as well as a single calibrator lot (N. 085201). The proposed method has been compared with the PETIA technique (Dako, Milan, Italy) by using a commercially available kit (Cat. K0071, Lot.: 0.57[101]). The PETIA method consists of polystyrene particles of uniform size (38 nm in diameter), chemically coupled with rabbit antibody against human cystatin C (26). The reaction between sample cystatin C and these immunoparticles results in the formation of agglutinates. Absorbance due to the formation of agglutinates is measured at 340 nm. Sample values are determined by interpolation on a calibration curve, obtained by using an appropriate cystatin C calibrator consisting of lyophilized recombinant human cystatin C (Dako, Milan, Italy). In this study, the assay was performed on an automated single-unit centrifugal analyser (Cobas Fara II, Roche Diagnostic Division, Milan, Italy). Creatinine (serum and urine) was measured by the Vitros enzymatic assay (Vitros 950 System Chemistry, Ortho Clinical Diagnostics, Milan, Italy). The method is based on the enzymatic reaction of creatinine with creatinine iminohydrolase (creatinine deaminase) to form ammonia and *N*-methylhydantoin. The ammonia formed is quantified by reaction with bromphenol blue (32).

Patients and samples

To analyze the relationship between GFR and serum cystatin C, we selected 135 patients (72 males, 63 females) aged from 21 to 88 years with normal to reduced renal function. GFR was estimated by calculating endogenous creatinine clearance (CrCl). To calculate creatinine clearance (ml/min), we used the classical formula: CrCl = UxV/P, where U is urinary creatinine concentration, V is the urinary volume, expressed as litre, and P is plasma creatinine concentration. All values were normalized for body surface area referred to 1.73 m^2. All samples were analyzed for serum cystatin C and creatinine; in addition, an accurate 24-h urine sample was obtained for each patient to assess urinary creatinine and to measure accurately the urinary volume. Serum samples were collected early in the morning, with the patients fasting. On the basis of creatinine clearance values, patients were divided into three groups:

– 52 patients with normal renal function (24 males, 28 females, aged from 21 to 79 years), defined by a creatinine clearance above 80 ml/min/1.73 m^2. This group was used to establish preliminary reference range. Thus, subjects were selected on the basis of an accurate history, using as major exclusion criteria: a) the presence of renal diseases or of other disease predisposing for renal complications; b) serum urea concentration above 7.5 mmol/l; c) the administration of any drug in the six months before the study.
– 49 patients with mild-to-moderate decrease in glomerular filtration (29 males, 20 females, aged from 21 to 66 years), defined by a creatinine clearance ranging from 40 to 80 ml/min/1.73 m^2.
– 34 patients with moderate-to-severe decrease in glomerular filtration (19 males, 15 females, aged from 31 to 88 years), defined by a creatinine clearance below 40 ml/min/1.73 m^2. In the last two groups of patients the major exclusion criteria were: a) concomitant cardiac diseases (e.g. myocardial infarction, etc.); b) concomitant muscle diseases; c) malignancies; d) renal transplantation.

Blood samples were collected from a peripheral vein directly into minicaps without application of an anticoagulant. Samples were centrifuged within 1 hour from the collection, and analyzed within 4 hours. Plasma samples were also collected in 30 subjects, by using 3 ml vacuum tube contained 0.07 ml 7.5% EDTA K_3 anticoagulant (Vacutainer™, Becton Dickinson, Milan, Italy).

Assay validation

The intra-assay reproducibility was assessed using 20 replicate analyses of four serum pools with cystatin C concentration 0.91, 1.05, 2.33, and 7.26 mg/l. The inter-assay reproducibility was assessed by duplicate measurement at the serum pools over 10 working days. All runs utilized the same calibration curve. Linearity was analyzed by using 5 fresh serum samples with high levels of cystatin C (from 2.0 to 6.8 mg/l). Samples were serially diluted with isotonic phosphate buffer, in order to cover the whole measuring range of the assay. The undiluted samples and the dilution series were analyzed within 4 hours. To assess the effect of anticoagulants and clotting, 30 serum and EDTA-treated plasma samples were collected from healthy subjects and from patients with decreased creatinine clearance. All samples were analyzed within 4 hours in the same run, utilizing the same calibration curve. The effects of incomplete clotting and storage of serum samples were evaluated by collecting 20 samples with cystatin C concentration ranging from 0.64 to 5.73 mg/l. All samples were allowed to clot for 1 hour, separated, and analyzed within 8 hours after their collection. Subsequently, they were divided into aliquots, stored under different conditions, and

were analyzed again as follows: after 2 days at room temperature, after 1 week at +2 °C – +8 °C, and after 1 month frozen at –25 °C. The frozen aliquots were subjected to further 3 freeze-thaw cycles, with each additional freezing period lasting at least one day. After each cycle, cystatin C was determined. All sample containers were well closed in order to avoid evaporation or freeze-drying effects. Potential interferences with the N-latex cystatin C immunoassay were investigated using 5 serum samples containing different amounts of the potentially interfering substances mixed in fixed ratios with other serum samples containing elevated cystatin C levels but no interfering factors. The study included samples with high levels of rheumatoid factor, bilirubin, triglycerides, haemoglobin. A total of 117 fresh patient samples, covering the whole measuring range of N-latex cystatin C immunoassay, were selected in order to compare the nephelometric method with the PETIA technique (Dako, Milano, Italy). The comparison was performed in one day, utilizing the same calibration curve. Samples containing potentially interfering factors, such as bilirubin, haemoglobin, triglycerides, and rheumatoid factor were excluded.

Statistical analysis

The statistical analysis was made using StatView SE+Graphics™ statistical software (Abacus Concepts Inc., Berkeley, CA, USA) on a Macintosh PowerBook 5300cs computer (Apple Computer Inc., Cupertino, CA, USA). Data were evaluated with standard parametric tests. Comparisons between creatinine clearance and the serum concentration of cystatin C were performed by using the reciprocal of the measured concentrations. This procedure results in a linearization of the curvilinear relationship between renal filtration and the serum marker concentration, and greatly reduces the skewness of the frequency distribution for cystatin C data. Differences in means between groups were analyzed by one-way analysis of variance (ANOVA) followed by the Scheffé multiple comparison test; $p<0.05$ was considered statistically significant. Method comparison was made by simple linear regression analysis and by using the approach of Bland and Altman to assess the agreement between methods (33). The reference interval for cystatin C was calculated in the group of healthy adults, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) guidelines (34), after checking for normal distribution by the Kolgomorov-Smirnov test. To determine differences in cystatin C and in creatinine values between males and females, we used the Student's t-test, and a p value less than 0.05 was considered statistically significant.

Results

Range and linearity

The basic measuring range covers cystatin C concentrations from 0.20 up to about 6.8 mg/l. When sample dilution was twentyfold concentrations as low as 0.17 mg/l could be detected. Samples with high cystatin C concentration can automatically be re-run by the analyzer by a further sample dilution up to 1:400, and 1:2000. We found that the standard curve of the nephelometric assay was linear from 0.7 to 6.8 mg/l (Fig. 1). Within the assay's measuring range, the deviations of measured from theoretical values ranged from 0.56 to 3.54 %.

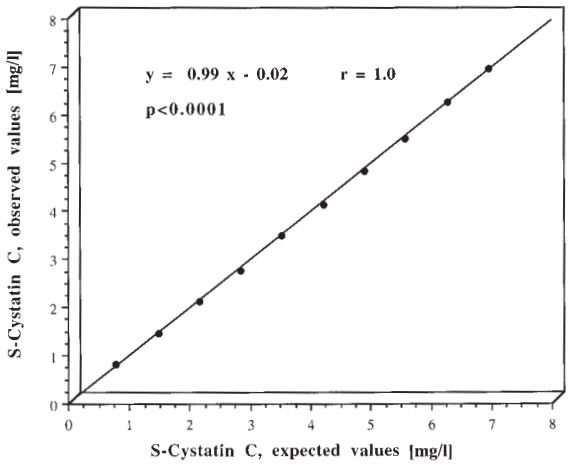

Fig. 1 Linearity of latex cystatin C immunonephelometric assay.

Imprecision

The within-run imprecision expressed as coefficient of variation (CV, %) ranged from 1.58 % to 3.77 %, with mean value of 2.31 %, while the between-run imprecision ranged from 5.67 % to 11.47 % with a mean value of 7.92 % (Tab. 1).

Tab. 1 Reproducibility of latex cystatin C immunonephelometric assay.

Samples (serum pools)	Mean (mg/l)	SD (mg/l)	CV (%)
1	0.91	0.02	2.20
2	1.05	0.02	1.71
3	2.33	0.04	1.58
4	7.26	0.27	3.77

Within-run imprecision (n = 20)

Samples (serum pools)	Mean (mg/l)	SD (mg/l)	CV (%)
1	0.89	0.05	5.67
2	1.03	0.06	6.30
3	2.24	0.18	8.25
4	6.63	0.76	11.47

Between-run imprecision * (n = 10)
*The same calibration curve stored for 10 days

Comparison between serum and plasma

Comparison between cystatin C values in EDTA plasma and serum specimens simultaneously drawn from 30 subjects, showed that cystatin C mean concentration ± standard deviation (SD) was 1.12 ± 0.67 mg/l, and 1.14 ± 0.70 mg/l respectively. ANOVA demonstrated no significant difference between values found from EDTA plasma samples and those from serum samples. By using simple linear regression, we found that EDTA plasma and serum values significantly correlated each other (Fig. 2).

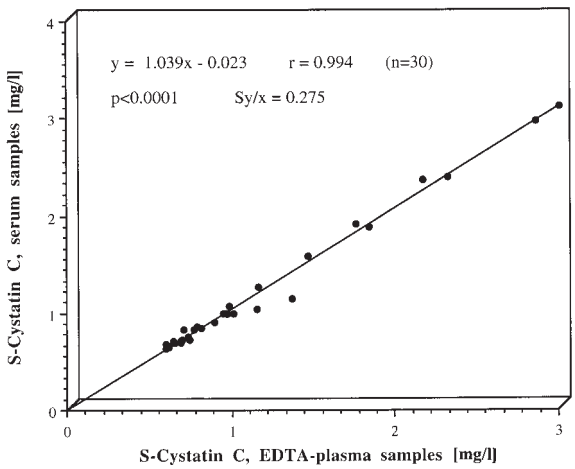

Fig. 2 Comparison between measurement of cystatin C in serum and 7.5 % EDTA plasma samples.

Analyte stability

Storage of serum samples at room temperature for 48 hours, refrigerated at 4 °C up to 1 week, and frozen 1 month at –25 °C, did not affect cystatin C significantly. After 48 hours at room temperature, we found a mean increase in values of 8.9 %, ranging from 3 to 15 %. After 1 week at +2 °C – +8 °C, we found a mean increase in values of 5.19 % (range: 0–12.6 %), and after 1 month at –25 °C we found a mean decrease in values of 2 %, ranging from 0 to 8.2 %. ANOVA demonstrated no significant difference between values found in specimens stored at different temperatures over these time periods.

Tab. 2 Analytical interferences on latex cystatin C nephelometric immunoassay.

Cystatin C (mg/l)				
	Added	Observed	Expected	Recovery (%)
Rheumatoid factor (IU/l)	0	6.6	–	–
	372	5.29	5.06	104
	744	3.52	3.47	101
	1116	1.94	1.88	104
Bilirubin (µmol/l)	0	3.11	–	–
	139.2	2.79	2.74	102
	278.4	2.35	2.37	99
	417.6	2.00	2.00	100
Triglycerides (mmol/l)	0	5.47	–	–
	3.49	4.03	4.18	97
	6.98	2.83	2.88	98
	10.47	1.54	1.58	98
Haemoglobin (g/l)	0	9	–	–
	4	6.25	6.3	99
	8	3.56	3.6	99
	12	0.91	0.9	101

Analytical interferences

Addition of rheumatoid factor (final concentration up to 1116 IU/l), bilirubin (final concentration up to 417.6 µmol/l), triglycerides (final concentrations up to 10.47 mmol/l), and haemoglobin (final concentration up to 12 g/l) to serum samples with high cystatin C concentrations did not interfere with the nephelometric assay (Tab. 2).

Correlation with PETIA method

Cystatin C was simultaneously determined by both methods in serum samples from a group of 117 subjects (64 males and 53 females, aged from 21 to 88 years) with normal to reduced renal function. Specimens were collected and frozen at –25 °C up to analysis, performed within 20 days from their collection. Simple linear regression between values measured by the two methods showed a good correlation (Fig. 3). The bias plot graph, in according to Bland and Altman method (33), demonstrated that the mean difference between the two methods was –0.536 mg/l, and that less than 5 % of values were out of the limits of agreement, which were –1.15 and 0.078 (Fig. 4).

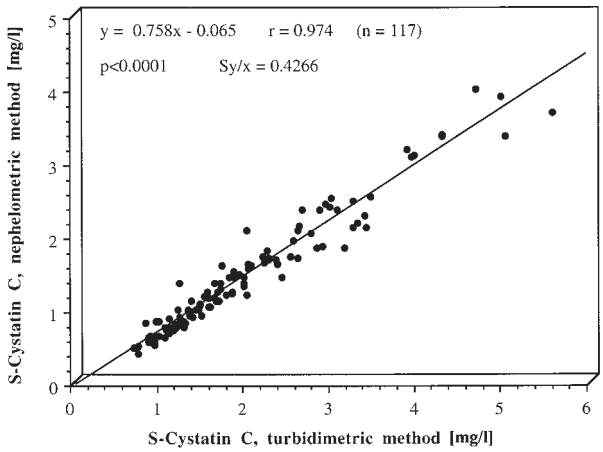

Fig. 3 Method comparison between the latex cystatin C nephelometric assay and cystatin C PETIA assay.

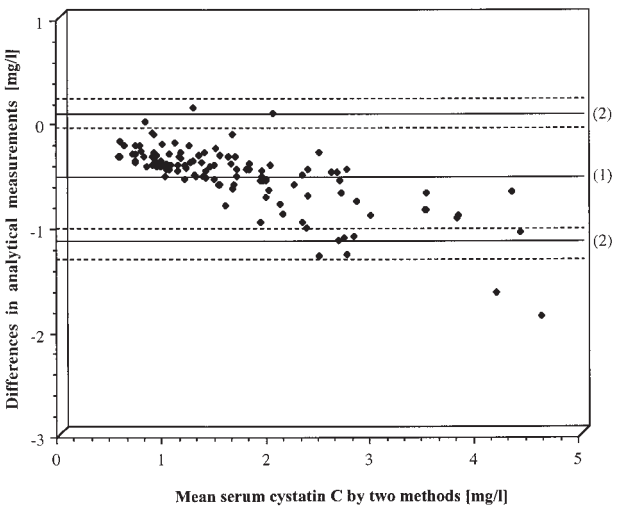

Fig. 4 Bias plot of 117 samples measured by latex nephelometric immunoassay vs. particle-enhanced turbidimetric assay (PETIA) with the mean difference (1) and limits of agreement (2) with 95 % confidence limits (dotted lines).

Reference values and clinical applications

In the group of healthy adults with creatinine clearance more than 80 ml/min/1.73 m^2, we found that cystatin C values were normally distributed, with a mean value of 0.80 mg/l and a median value of 0.80 mg/l (Fig. 5). The

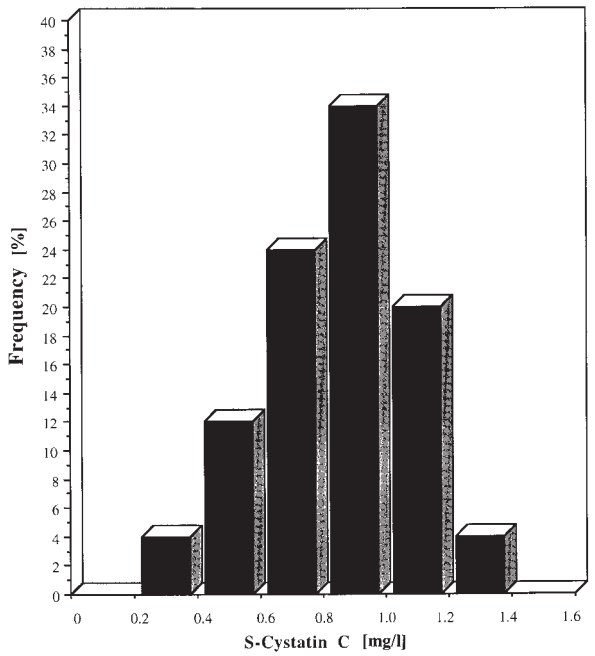

Fig. 5 Frequency of distribution of serum cystatin C values in a group of healthy adults (n=52).

upper value was found to be 1.33 mg/l, while the lower value was 0.36 mg/l. We noticed that serum levels of cystatin C were 11.8 % higher in males than in females, and the difference between values in males and females was greater (22.7 %) for serum creatinine levels. By using the Student's t-test, we found no significant differences between cystatin C values in males (mean value 0.85 mg/l) and females (mean value 0.75 mg/l), while a significant difference (p=0.001) was found between creatinine values in males (mean value 84.0 µmol/l) and females (mean value 65.3 µmol/l). Additionally, no significant effect of age was found on serum cystatin C concentration. As cystatin C is normally distributed, we computed a Gaussian-based reference interval (mean ± 1.96 standard deviation) following the recommendations of Solberg (34). Thus, cystatin C reference intervals in healthy adults (men and women) were 0.37 – 1.22 mg/l. In this study, the relationship between the reciprocal values of serum cystatin C and creatinine clearance varies with renal filtration: by using simple linear regression, we found a significant correlation between the two variables in patients with creatinine clearance less than 80 ml/min/1.73 m^2, while no correlation was found in subjects with creatinine clearance more than 80 ml/min/1.73 m^2 (Fig. 6). Serum cystatin C and serum creatinine values correlated in patients with creatinine clearance less than 80 ml/min/1.73 m^2 (cystatin C (mg/l) = 0.007 creatinine (µmol/l) + 0.664, r=0.76); however, this correlation significantly decreased in patients with creatinine clearance more than 80 ml/min/1.73 m^2 (cystatin C (mg/l) = 0.002 creatinine (µmol/l) + 0.732, r=0.39). Finally, by comparing (ANOVA) serum cystatin C and creatinine values among patient groups, we found that cystatin C levels significantly differ between patients with normal renal filtration and patients with mild-to-moderate decrease in glomerular filtration (p<0.001). Also serum creatinine levels differ between the above mentioned groups, but less clearly (p<0.05) (Fig. 7).

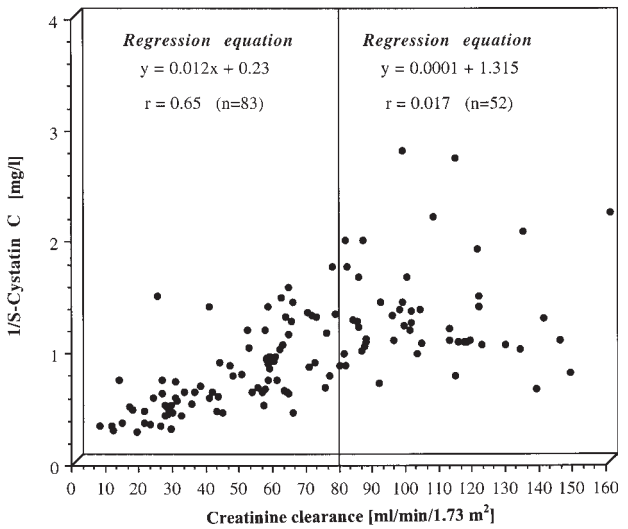

Fig. 6 Relationship between creatinine clearance and reciprocal values of serum cystatin C in patients with creatinine clearance less than 80 ml/min (left) and in patients with creatinine clearance more than 80 ml/min (right).

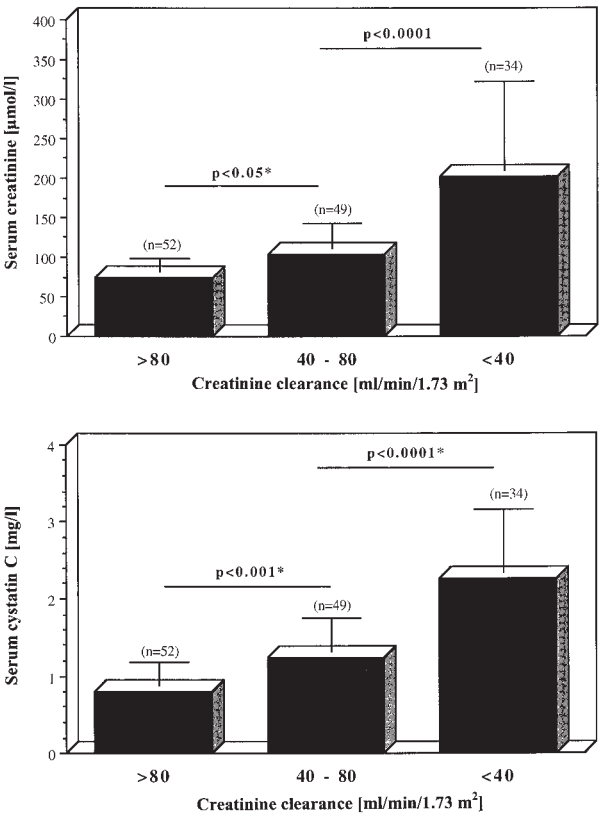

Fig. 7 Mean values and standard deviation (SD) of serum creatinine (top) and serum cystatin C (bottom) in three groups of patients with different ranges of creatinine clearance.
* Test comparison between groups: ANOVA followed by Sheffé multiple comparison test.

Discussion

Because the diagnostic value of cystatin C determinations in several biological fluids has recently received increasing interest, especially as a serum endogenous marker for the assessment of early changes in GFR, the development of accurate, precise, and rapid automated analytical methods is necessary in order to introduce the test in clinical practice and make it competitive. N-latex cystatin C by Dade Behring Diagnostics is a novel particle-enhanced reagent for the immunonephelometric assay of cystatin C in human serum samples. Although the method described here was evaluated employing a BNA, it could easily be performed by using the Behring Nephelometer II (BN II), with the advantage of stabilized temperature in the reaction cups (+37 °C). Assay results are available within 6 minutes, making the method suitable for use in emergency. This method demonstrates a good analytical performance, with satisfactory reproducibility. The relatively high between-run imprecision (from 5.67 to 11.47 %) might derive from the utilization of the same calibration curve for ten days. In fact, in the course of the reproducibility studies (within- and between-run) all other variables (serum pools, reagents, etc.) were kept rigorously unchanged. Analytical sensitivity of the N-latex-cystatin C method is 0.17 mg/l, comparable to that of the PETIA method (0.15 mg/l) even if significantly lower than that reported for previous methods based upon enzyme-labeled or radioactive reagents (23, 24). However, the measuring range comprises the entire span of serum concentrations of cystatin C seen in healthy subjects and in most of patients with impaired renal function. Linearity of this test extends up to 6.8 mg/l without prozone phenomena. Cystatin C demonstrates a good stability in serum, showing no significant differences between values measured in fresh samples (within 8 hours from their collection) and those in samples stored under various conditions (p=n.s.). However, the change in values demonstrates two different trends with time. In contrast to Finney *et al.* (35), the recovery in samples stored up to 1 week under different conditions was found to be in all cases ≥100 %, ranging from 103 to 115 % in samples stored 48 hours at room temperature and from 100 to 113 % in those stored 1 week at +2 – +8 °C. On the other hand, the recovery in samples frozen 1 month at –25 °C was found to be in all cases ≤100 %, ranging from 92 to 100 %. These findings seem to suggest that freezing at –25 °C may be considered the best condition to store serum samples for measuring cystatin C. By using this method, it is reasonable to assume that serum samples and plasma-EDTA samples may be equally used to measure cystatin C concentration. Interferences resulting from the presence of rheumatoid factor, bilirubin, triglycerides, and haemoglobin were found to be negligible under routine conditions. The comparison with the PETIA method shows that N-latex cystatin C nephelometric assay has a high degree of correlation in 117 patient samples. The bias plot produced five outliers, showing that two of them were found when the average serum cystatin by the two methods was more than 4.0 mg/l, and other two when the average was between 2.5 and 4 mg/l. Many factors may contribute to these discrepancies: the nature and the size of latex-particles, the different source of cystatin C calibrator material, and, finally, the antibodies. However, the most relevant differences between methods are found in a range of high concentration of cystatin C.

Results obtained in the group of healthy adults suggest that male/female differences are significantly greater for creatinine than those for cystatin C. Additionally, in the group of healthy adults with creatinine clearance >80 ml/min/1.73 m^2 we found no influence of age on serum cystatin C levels. The relationship between creatinine clearance and serum cystatin C (expressed as reciprocal value) is greatly influenced by the selection of the population studied. Evaluations that include subjects with normal renal function produce weaker correlations, whereas clinical studies that include patients with moderate-to-severe reduction in renal function tend to give much stronger relationships because of polarization of the data. Results shown in Figure 6 seem to confirm that the relationship between serum cystatin C and creatinine clearance varies in parallel or in agreement with renal filtration: in patients with creatinine clearance <80 ml/min/1.73 m^2 the correlation coefficient (r) is 0.65, while no correlation has been demonstrated in subjects with creatinine clearance >80 ml/min/1.73 m^2 (r=0.011). Creatinine and cystatin C correlated well with each other in patients with creatinine clearance less than 80 ml/min/1.73 m^2; this correlation significantly decreased in patients with creatinine clearance more than 80 ml/min/1.73 m^2. Therefore, creatinine and cystatin C may be considered two independent serum markers for the assessment of changes in GFR. Cystatin C seems to be a promising serum marker of changes in GFR: results presented in Figure 7 strongly indicates that cystatin C mean values significantly differ between patients with normal renal function and patients with mild-to-moderate decrease in renal function (p<0.0001). Creatinine mean values significantly differ between the above mentioned groups, but less strongly (p<0.01). Thus, since creatinine is a relatively specific but not sensitive marker of changes in GFR, cystatin C might be useful to assess early changes in GFR.

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Received 22 June 1998; accepted 28 September 1998

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