**Are Cystatin C and $\beta_2$-Microglobulin Better Markers than Serum Creatinine for Prediction of a Normal Glomerular Filtration Rate in Pediatric Subjects?** *Guido Filler,*[1]* *Iris Witt,*[1] *Friedrich Priem,*[2] *Jochen H.H. Ehrich,*[1] *and Klaus Jung*[3] ([1]Dept. of Paediatr. Nephrol., [2]Clin. Biochem., and [3]Urol., Charité Hosp., Humboldt Univ., Schumannstr. 20–21, D-10098 Berlin, Germany; *author for correspondence: fax +49 30 2802 8844, e-mail ehrich@rz.charite.hu-berlin.de)

Serum creatinine is the marker most widely used to predict glomerular filtration rate (GFR). In childhood, there is age and muscle mass dependency of serum creatinine, and assessing a normal GFR accurately even with the use of body length/creatinine ratios remains difficult *[1, 2]*. Recently, cystatin C (Cys-C), a 13-kDa protein, was found to correlate closely with GFR in adults *[3–5]*. $\beta_2$-Microglobulin ($\beta$2-MG) serum concentrations also correlate with GFR, and have been shown to be age independent in infants *[6]*. Here, we report reference limits of Cys-C and $\beta$2-MG in children between ages 0.8 and 18 years and demonstrate age independency of these parameters as an essential advantage compared with the conventionally used serum creatinine values.

We studied serum Cys-C and $\beta$2-MG in 216 urological pediatric patients (age range 0.8 to 18 years, mean 11.0 ± 6.2 years) with normal GFR, using leftover serum after routine serum chemistry measurements, including serum creatinine. The study is in accordance with the ethical standards of the Helsinki declaration of 1975 (revised in 1983), and parents' (in the case of 18-year-olds: patients) oral consent was obtained in each case. $^{51}$Cr–EDTA clearance studies with a modified method of Chantler and Barratt *[7]* served as a gold standard for GFR assessment; values between 90 and 150 mL/min per 1.73 m$^2$ were defined as normal GFR. Cys-C was measured with the particle-enhanced turbidimetric assay (Dako, Glostrup, Denmark) in the Hitachi 717 analyzer, $\beta$2-MG was measured with the microparticle enzyme immunoassay (Abbott, Wiesbaden, Germany), and serum creatinine was determined enzymatically (PAP; Boehringer Mannheim, Mannheim, Germany).

We calculated correlation coefficients according to Spearman and regression coefficients between age and serum concentrations of creatinine, Cys-C, and $\beta$2-MG, and a strong correlation between creatinine concentration and age could be demonstrated (Fig. 1). Cys-C and $\beta$2-MG showed slopes of the regression lines not significantly different from zero and there was no correlation between their concentrations and age. Thus, age-independent reference values for both low-molecular-mass proteins can be considered.

We calculated the central 95% reference intervals according to the IFCC guidelines by using the nonparametric method *[8]*. The upper 97.5 percentiles amounted to 1.38 mg/L for Cys-C and 2.27 mg/L for $\beta$2-MG. Likewise, the lower 2.5 percentiles amounted to 0.18 mg/L for Cys-C and 0.361 mg/L for $\beta$2-MG. Those data correspond to values found in adults *[4, 5]*. In subjects with normal GFR (>80 mL/min per 1.73 m$^2$), a preliminary reference

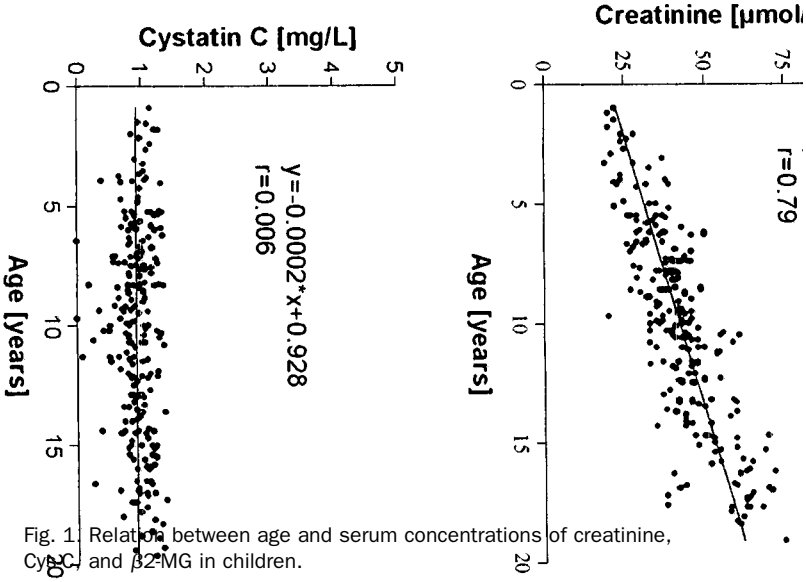


Fig. 1. Relation between age and serum concentrations of creatinine, Cys-C, and $\beta$2-MG in children.

The slope for serum creatinine was significantly different from zero ($P < 0.0001$). By contrast, Cys-C vs age and $\beta$2-MG vs age showed slopes not significantly different from zero.

interval between 0.61 and 1.21 mg/L was defined *[4]*. Newman et al. *[5]* used 1.25 mg/L as the upper reference value. However, all these references values were established in a very limited number of subjects (n = 27) *[4]*. Using an ELISA method, we previously found higher values *[3]*. These differences are very likely explained by

the different calibrator material used in the different studies. In the present study, the same calibrator material (recombinant Cys-C) was used as in the studies of Kyhse-Anderson et al. *[4]* and Newman et al. *[5]*, whereas we calibrated the ELISA method with a urinary protein calibrator of Cys-C (Behring, Marburg, Germany). To date, there are no published Cys-C reference limits for children. Our β2-MG reference values match well with the published reference values *[9]* for healthy children (95th percentile 2.20 mg/L).

Measurement of serum creatinine can be performed by various methods. The results can be influenced by numerous factors, and each method will produce different normal values for adults and children *[10]*. In addition to GFR, muscle mass and physical activity also influence the creatinine concentration *[10]*, and thus impairment of GFR may easily be overlooked in young children. Here, Cys-C or β2-MG may serve as a useful alternative. Our data clearly demonstrate age independency for serum concentrations of both Cys-C and β2-MG. Cys-C concentrations >1.4 mg/L and β2-MG concentrations >2.3 mg/L suggest an impaired GFR irrespective of age; hence, measurement of Cys-C or β2-MG concentrations may be advantageous compared with measurements of serum creatinine concentration for the detection of an impaired GFR.

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**Interference of Methylene Blue with CO-Oximetry of Hemoglobin Derivatives**, *Hervé Gourlain,*[1]* *Françoise Buneaux,*[1] *Stephen W. Borron,*[2] *Bernard Gouget,*[3] *and Pierre Levillain*[1] ([1] Lab. de Toxicol., and [2] Réanimation Toxicol., Hôpital Fernand Widal, 200 rue du Fg-St-Denis, 75475 Paris Cedex 10, France; [3] C.N.E.H., 9 rue Antoine Chantin, 75014 Paris, France; *author for correspondence: fax +33 1 40 05 48 78)

Methylene blue (MB) is frequently used as an antidote in treating methemoglobinemia *[1]* because it facilitates the reducing activity of the NADPH-dependent methemoglobin reductase system in erythrocytes *[2]*. However, MB absorbs strongly between 550 and 700 nm (Fig. 1), the same spectrophotometric region as that of the various hemoglobin derivatives: oxyhemoglobin ($O_2$Hb), deoxyhemoglobin (HHb), methemoglobin (MetHb), and carboxyhemoglobin (COHb). To evaluate the potential magnitude and direction of errors linked to the presence of MB for the results for total hemoglobin (tHb) and its derivatives, we evaluated six CO-Oximeters. The wavelengths used by each instrument for these determinations are as follows: IL 482 (Instrumentation Laboratory, Lexington, MA), 535, 585.2, 594.5, and 626.6 nm; CCD 270 (Chiron Diagnostics, Medfield, MA), 557, 577, 597, 605, 624, 635, and 650 nm; CCD 835 (Chiron; wavelengths not communicated); OSM3 (Radiometer, Copenhagen, Denmark), 535, 560, 577, 622, 636, and 670 nm; ABL 520 (Radiometer; same wavelengths as OSM3); AVL 912 (AVL Scientific Corp., Roswell, GA), 530, 536, 542, 548, 554, 560, 566, 572, 578, 584, 590, 604, 612, 622, 630, 640, and 648 nm.

Blood was collected from five healthy volunteers with informed consent. Because the study involved only blood sampling, Institutional Ethics Committee Review was not required in France. The five samples were combined to obtain 120 mL of pooled blood, which were then separated into three 40-mL fractions:

–Fraction N, which had no enrichment in CO or MetHb.

–Fraction CO, which was enriched in CO by tonometry with use of an IL 237 tonometer (Instrumentation Laboratory) and CO in nitrogen, 10 mL/L (Société Cosma, Igny 91430, France). Because of the time required to analyze a large number of samples, the tonometry was carried out separately on four 10-mL specimens just before analysis.

–Fraction Met, which was treated with 4 mg of hydroquinone (Prolabo, Paris, France) to obtain samples enriched in MetHb. Hydroquinone, a known inducer of MetHb, was selected for its lack of absorbance in the spectral range of hemoglobin. Again, this enrichment step was carried out on four 10-mL specimens just before analysis.

Each fraction was separated into aliquots. Four aliquots were adulterated with MB by dilution with a stock 10 g/L solution (Pharmacie Centrale des Hôpitaux de Paris, Paris, France) to obtain final concentrations of 0.1, 0.25, 0.50, and 1 g/L. We then added 1.0 mL of one of these solutions to 9.0 mL of blood to obtain a blood MB concentration of 10, 25, 50, or 100 mg/L. These MB concentrations were chosen to correspond to the plasma concentrations clinically anticipated when MB is slowly injected intravenously as 5–25 mL of a 10 g/L solution (the 100 mg/L concentration is rarely attained). Four control aliquots were prepared for each fraction as well, the MB being replaced with NaCl, 9 g/L. Each adulterated sample was compared with its own control, and measurement was performed immediately after treatment of the blood with either MB or NaCl. Measurements were performed in triplicate with all six CO-Oximeters.

The tHb concentrations of controls measured by CO-Oximetry were ~146 g/L. The COHb and MetHb percent-

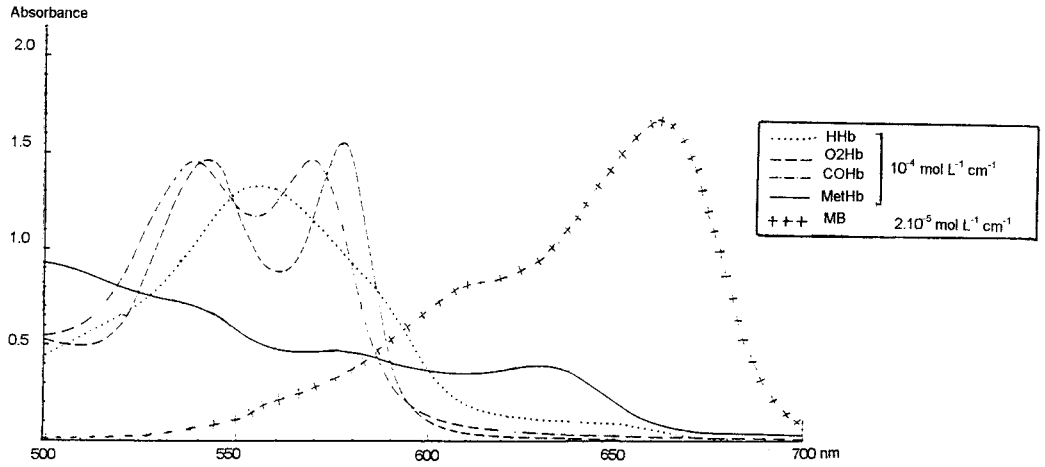


Fig. 1. Spectra of hemoglobin derivatives and methylene blue.
The absorbance of MB in physiological (isotonic) saline solution peaks at 663 nm.

ages of control specimens obtained from CO-Oximeters were respectively 1.05–1.55% and 0.50–0.55% for the N samples, 23.1–36.6% and 0.1% for the CO samples, and 0.9–1.2% and 4.6–14.5% for the Met samples.

Using uncorrected data, we calculated the difference, negative or positive, between the mean of three values of the adulterated specimen and the mean for the corresponding control. The results (Table 1) are the absolute differences in values, as reported by the instruments. The values for tHb are reported in g/L, the values for hemoglobin derivatives in % of tHb. The relative errors for the derivatives are thus much greater than the absolute differences in reported percentage. For example, comparing the IL 482 results for an untreated N control (i.e., physiological COHb and MetHb; no MB) with those for a sample to which MB (100 mg/L) had been added shows that the percentage of MetHb increased from 0.5% to 42.9%, an absolute difference of +42.4% MetHb. Given that the known concentration of MetHb in these specimens is ~0.5%, this would mean a relative error of 8500%!

Large negative differences are observed as well. For instance, in samples with small percentages of COHb or MetHb, negative readings for samples adulterated with MB result in negative tHb percentages. In the CCD 270 results for the untreated (N) control and the specimen adulterated with MB (100 mg/L), the reported MetHb decreased from 0.5% to −8.5%—giving an absolute difference of −9%, but a relative error of 1800%. Furthermore, one sample adulterated with 100 mg/L MB gave a result >100% (CCD 270 $O_2$Hb result for specimen Met) with a difference of +26.5%. Several of the AVL 912 data points are missing, because the software for that analyzer eliminates most of the unreasonable results.

In interpreting the clinical significance of these findings, the relative errors are of greater importance, with each laboratory determining what constitutes an acceptable deviation. If, for example, relative errors of 20% for MetHb, HHb, and COHb; 3% for $O_2$Hb; and 2 g/L for tHb are considered "acceptable," only a handful of the values reported in Table 1 would be retained. The "acceptable" relative errors are indicated in bold type in Table 1. Thus, we consider the majority of the values in this study clinically unacceptable.

The presence of MB in the samples perturbs most of the measurements (Table 1). The results obtained for unadulterated samples or for samples enriched with CO or MetHb vary greatly not only from one instrument to another, but also in direction. A precise interpretation of the errors is difficult. Indeed, one must take into account several types of problems of analysis, i.e., spectral order and software.

Regarding spectral order, analysis of a mixture of the four major derivatives of hemoglobin presupposes a spectral measurement of four wavelengths followed by a mathematical treatment of the signals. To correct for any eventual interference, one must make these measurements at other wavelengths and integrate into the system of calculation the absorption data specific to the interfering substance. MB absorbs strongly at 600–700 nm and more weakly at 550–600 nm (Fig. 1). Thus, its presence mainly affects the determination of MetHb near 620 nm and, more weakly, determinations of the other hemoglobin derivatives near that wavelength. This interference may be observed with the IL 482, which works uniquely at four wavelengths: MB absorbance at 626 nm simulates the presence of MetHb, resulting in a large increase for this derivative and consequently for tHb. This positive error for MetHb results in overcorrection for the other derivatives in the calculation system, so the values reported for them are too small.

The importance of software-related errors can be appreciated by comparing the results reported by the OSM3 and ABL 520, which are identical instruments; in the absence of an alternative explanation from the manufacturer, we believe the differences (error) in reported results may be attributed to differences in the software, which calculates the contributions of the various hemoglobin species. All CO-Oximeters tested other than the IL 482 measure at several wavelengths in the 630–670-nm region, which might be used to correct for MB. Generally,

**Table 1. Differences (absolute errors) between MB-adulterated blood specimens and controls.**

| | | | | | % of tHb | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | tHb, g/L | | | | MetHb | | | | $O_2Hb$ | | | | HHb | | | | COHb | | | |
| Specimens[a] | 10[b] | 25 | 50 | 100 | 10 | 25 | 50 | 100 | 10 | 25 | 50 | 100 | 10 | 25 | 50 | 100 | 10 | 25 | 50 | 100 |
| IL 482 | | | | | | | | | | | | | | | | | | | | |
| N | 2 | 6 | 12 | 30 | 8.2 | 14.1 | 23.5 | 42.4 | −3.2 | −5.2 | −9.9 | −16.7 | −2 | −4.3 | −5 | −9.3 | −2.4 | −4.8 | −8.8 | −17.4 |
| CO | **1**[c] | 7 | 12 | 28 | 6.4 | 13.8 | 24.7 | 46 | −1.7 | −1.7 | −6.8 | −14.5 | −2.3 | −5.3 | −5.2 | −8.1 | **−2.4** | −6.8 | −12.7 | −23.4 |
| Met | 3 | 9 | 13 | 28 | 3.4 | 9.9 | 16.3 | 34 | −1.2 | −4.3 | −3.5 | −14.5 | −0.9 | −2.1 | −3.8 | −3.1 | −1.2 | −4.2 | −8.3 | −15.8 |
| OSM3 | | | | | | | | | | | | | | | | | | | | |
| N | 6 | −11 | −14 | −16 | 2.0 | −0.2 | −2.2 | −0.9 | 2.8 | 2.7 | −2.1 | −1.7 | ND | ND | ND | ND | **−0.9** | −2.8 | −6 | −13.3 |
| CO | −3 | −16 | −15 | −22 | −0.5 | −3.4 | −4.3 | −0.3 | 2.3 | 5 | 0.4 | −0.1 | ND | ND | ND | ND | **0.1** | **0.2** | **−4.1** | −14.8 |
| Met | −5 | −10 | −20 | −30 | −3.6 | −5.8 | −12.8 | −18.4 | −0.4 | −1 | −1 | −6.2 | ND | ND | ND | ND | **−0.5** | −1.8 | −6.4 | −19.2 |
| ABL 520 | | | | | | | | | | | | | | | | | | | | |
| N | 6 | −14 | −20 | −31 | 1.9 | −1.3 | −5.7 | −8.9 | 1.4 | 6.6 | 6.8 | 17.3 | −2.7 | −3.5 | 3.7 | 8.8 | **−0.8** | −1.8 | −5 | −16.3 |
| CO | −3 | −11 | −17 | −30 | **−0.1** | **−0.1** | **−0.1** | −0.1 | 1.4 | 3.4 | 5.8 | 14 | −1 | −1.8 | −1.3 | −0.9 | **−0.3** | **−1.6** | **−4.4** | −13 |
| Met | −2 | −14 | −28 | −45 | −3.1 | −6.3 | −16 | −34 | 3.1 | 6.1 | 21.1 | 46.8 | 0.5 | 1.2 | −0.8 | 1.8 | **−0.5** | −1.4 | −4.4 | −15.4 |
| CCD 270 | | | | | | | | | | | | | | | | | | | | |
| N | −5 | −11 | −23 | −37 | 2.1 | 0.1 | −4.5 | −9 | 0.3 | 4.1 | 4 | 18.5 | −5.2 | −10.7 | −8.5 | −12.1 | 2.8 | 6.4 | 8.6 | 2.5 |
| CO | −4 | −15 | −24 | −38 | **0** | −2.7 | −6.4 | −8 | 1 | 1.8 | 9.9 | 21.4 | −3.6 | −9.3 | −19.7 | −27.8 | **2.6** | 10.3 | 16.3 | 14.4 |
| Met | −4 | −8 | −20 | −37 | **−0.4** | −3.8 | −7.3 | −17.4 | 2.7 | 1.6 | 9.6 | 26.5 | −1.4 | −3.2 | −12.7 | −22.6 | 2.1 | 5.4 | 10.3 | 14.2 |
| CCD 835 | | | | | | | | | | | | | | | | | | | | |
| N | −9 | −13 | −17 | −29 | **0.2** | **−0.2** | **−0.3** | **−0.3** | 3 | 5.8 | 2.9 | 9.1 | −2.9 | −3.5 | −0.6 | −6.1 | **0.1** | −2.1 | −2.6 | −2.8 |
| CO | −7 | −12 | −19 | −30 | −0.9 | −4.1 | −7.7 | −5.1 | 2.8 | 4.2 | 12.2 | 18.6 | −2.2 | −0.8 | −2.4 | 0.5 | **0.3** | **−0.8** | **−1.8** | −14 |
| Met | −7 | −8 | −27 | −32 | −3.6 | −3.2 | −13.4 | −12 | 6.1 | 5.6 | 17.6 | 15.7 | −2.3 | −0.2 | −2.5 | −1.8 | **−0.1** | −2.2 | −1.7 | −2 |
| AVL 912 | | | | | | | | | | | | | | | | | | | | |
| N | −3 | −7 | – | – | 2.3 | 0.4 | – | – | 3.1 | 7.3 | – | – | −7 | −11 | – | – | 1.6 | 3 | – | – |
| CO | – | – | – | −24 | – | – | – | 0.8 | – | – | – | 0.5 | – | – | – | −2.6 | – | – | – | 0.3 |
| Met | −3 | −6 | −14 | −2.3 | −2.4 | −4.4 | −6.6 | −10.8 | 3.5 | 8.3 | 3 | 8 | −4.8 | −7 | −1 | 1.2 | 1.7 | 2.9 | 4.4 | 0.7 |

[a] N, normal blood specimens not enriched with CO or MetHb (tHb: 13.1–14.6 g/L, COHb: 1.05–1.55%, MetHb: 0.50–0.55%); CO, blood specimens enriched in carbon monoxide (tHb: 120–133 g/L, COHb: 23.1–36.6%, MetHb: 0.1%); Met, blood specimens enriched in methemoglobin (tHb: 125–141 g/L, COHb: 0.9–1.2%, MetHb: 4.6–14.5%). ND, not done due to technical difficulties; –, result not provided by instrument. For each group of specimens (N, CO, Met) a control (no added MB) and four MB-adulterated specimens (10, 25, 50, 100 mg/L) were evaluated. Values shown represent the differences between control and adulterated specimens.

[b] Concentration of added MB.

[c] Data in bold type indicate "acceptable" relative error, i.e., <20% for MetHb, HHb, and COHb; <3% for $O_2Hb$; and <2 g/L for tHb.

however, these wavelengths are used to correct for turbidity, and no instrument takes into account the absorption of MB in their calculation program. The corrections are therefore poorly adapted to the presence of MB and may produce errors of potentially significant magnitude (Table 1). The AVL 912 system of calculation eliminates the most perturbed results, explaining the great number of missing values in Table 1.

The effect of MB on measured tHb concentrations deserves further comment. The IL 482 apparently detects MB as an increase in the peak at 626 nm, registering it as an increase in MetHb and, consequently, in tHb. The other instruments, which measure near the maximum for MB absorption (650–700 nm), apparently recognize this peak outside the maximum range of hemoglobin as "nonhemoglobin" and correct the tHb downward.

We conclude, therefore, that these six CO-Oximeters should not be used to determine the concentration of hemoglobin derivatives in blood samples containing MB. This is particularly important in cases of methemoglobinemia, because the samples with high proportions of MetHb give the most affected results (Table 1). Furthermore, pending further refinements, CO-Oximetry cannot be safely used clinically to evaluate the efficacy of treatment of methemoglobinemia with MB.

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**Determination of MEGX by HPLC with Fluorescence Detection**, *Maria Andreeva,* Paul Dieter Niedmann, Ekkehard Schütz, Eberhard Wieland, Victor William Armstrong, and Michael Oellerich* (Abt. Klin. Chem., Zentrum Innere Med., Georg-August-Universität Göttingen, Robert Koch Str. 40, D-37075 Göttingen, Germany; *author for correspondence: fax 49/551/398551)

The hepatic conversion of lidocaine to its metabolite monoethylglycinexylidide (MEGX) is catalyzed by the cytochrome P450 enzyme CYP3A4 in humans. This metabolic capacity of the liver led to the development of the MEGX test, in which serum MEGX concentrations are determined, as a relatively simple real-time test of liver function *[1]*. A large body of evidence currently documents the clinical utility of this test *[2]*, demonstrating that MEGX concentrations <20 μg/L reflect a compromised hepatic function. Furthermore, several studies have shown that test results <10 μg/L are associated with an extremely high risk for pretransplant nonsurvival *[3, 4]*. The assessment of serum MEGX concentrations <5 μg/L might be particularly relevant in pediatric patients with end-stage liver disease *[4]*.

The automated fluorescence polarization immunoassay (FPIA; Abbott Diagnostics, N. Chicago, IL) of MEGX in serum is almost universally available and widely used. It is sensitive (detection limit: 3 μg/L) and easy to perform. However, the test is subject to interferences, most importantly from high bilirubin concentrations *[5, 6]*. One approach to deal with this interference was proposed by Zoppi and Fumagalli, who precipitated the protein-bound bilirubin with the precipitation reagent from the Digoxin II assay supplied by Abbott *[6]*. Given the lack of sensitive HPLC methods capable of covering the low MEGX values usually observed in patients with severe hyperbilirubinemia, this modification of the FPIA procedure has been neither compared with more-definitive methods, nor validated with patients' samples. In addition, lidocaine concentrations >10 mg/L cross-react in the FPIA *[1]*, which can present problems in experiments with cell cultures or microsomal preparations and in assaying samples from patients whose blood still contains lidocaine from the test dose. Furthermore, OH-MEGX, which is formed from lidocaine in rats, also cross-reacts in the FPIA, thus precluding investigations with this species *[7]*. To achieve analytical sensitivity comparable with that of the FPIA but with a superior specificity, many different HPLC protocols involving liquid–liquid or solid-phase extraction have been used *[8]*. Using UV or electrochemical detection, however, investigators have not been able to accurately measure MEGX concentrations <10 μg/L *[8]*.

We here describe a reliable and simple isocratic reversed-phase HPLC procedure for determining MEGX in serum. Based on the combination of HPLC separation and fluorescence detection—after derivatization of MEGX with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F; Aldrich Chemical Co., Steinheim, Germany)—this method demonstrates superior specificity and sensitivity, the lower limit of the working range being 2.5 μg/L.

For sample preparation we used a modification of the extraction procedure of O'Neal et al. *[9]*. Briefly, a 500-μL aliquot of specimen and 250 μL of acetonitrile containing the internal standard monopropylglycinexylidide (MPGX), 200 μg/L, were mixed for 15 s in a 5-mL polypropylene tube. MPGX was synthesized according to a two reaction-step method, with use of 2,6-dimethylaniline, chloroacetylchloride, and propylamine *[10]*. Saturated borate buffer (500 μL, 0.75 mol/L, pH 9.5) was added to the tube and mixed by vortex-mixing for 15 s. Dichloromethane (2 mL) was added, and the sample was centrifuged for 10 min at 4000*g* after 1 min of vortex-mixing. The supernatant was carefully aspirated and discarded. The lower organic layer was transferred into a clean polypropylene tube and evaporated in a vacuum centrifuge at 38 °C. The residue of the extraction was redissolved in 100 μL of a solution of saturated sodium tetraborate/methanol (2/1 by vol). We then added 10 μL of NBD-F solution [10 g/L NBD-F in ethanol/acetonitrile (3:1 by vol)], vortex-mixed for 3 s, and heated to 60 °C for 10 min. The reaction was stopped by adding 10 μL of HCl (5 mol/L) and cooling in ice water. Any precipitates formed were removed by centrifugation (5 min, 10 000*g*), and 50 μL of the clear supernatant was used for chromatography.

The HPLC system consisted of a chromatographic pump, an automated injector, a spectrofluorometric detector, a system controller linked to a PC (Shimadzu System LC-10A; Shimadzu, Kyoto, Japan), and a 250 mm × 4.6 mm (i.d.) reversed-phase column packed with Ultrasphere ODS (5-μm particle size; Beckman Instruments, Fullerton, CA). The column was maintained at 47 °C to improve separation. The mobile phase (flow rate 1.5 mL/min) was prepared by mixing 370 mL of acetonitrile, 40 mL of tetrahydrofuran, and 590 mL of potassium dihydrogen phosphate buffer (50 mmol/L, pH 5.0) and then degassing. Fluorescence was detected at 340 nm excitation and 520 nm emission. For calculation, we used the internal standard mode, with peak-height ratios. One-point calibration was routinely performed in each run by assaying an in-house-prepared MEGX calibrator (MEGX added to drug-free serum to a final concentration of 50 μg/L). Quality control was assessed by assaying commercially available serum specimens from Abbott, either undiluted (25 and 125 μg/L) or diluted with drug-free serum to 5 μg/L.

A chromatogram of a blank serum sample and two chromatograms of serum samples from patients collected 15 min after a bolus intravenous injection of lidocaine, 1 mg/kg of body wt., are shown in Fig. 1. Retention times of MEGX and MPGX were 8.2 and 12.3 min, respectively. Both substances were rapidly eluted as symmetrical peaks and were fully separated at the baseline (Fig. 1). The majority of the front peaks and the changing pattern were caused by impurities in the fluorescence reagent.

Because lidocaine cannot be derivatized, no interference from it is observed in this assay. 3-OH-MEGX was well separated from MEGX because of a considerably shorter retention time. The presence of endogenous inter-

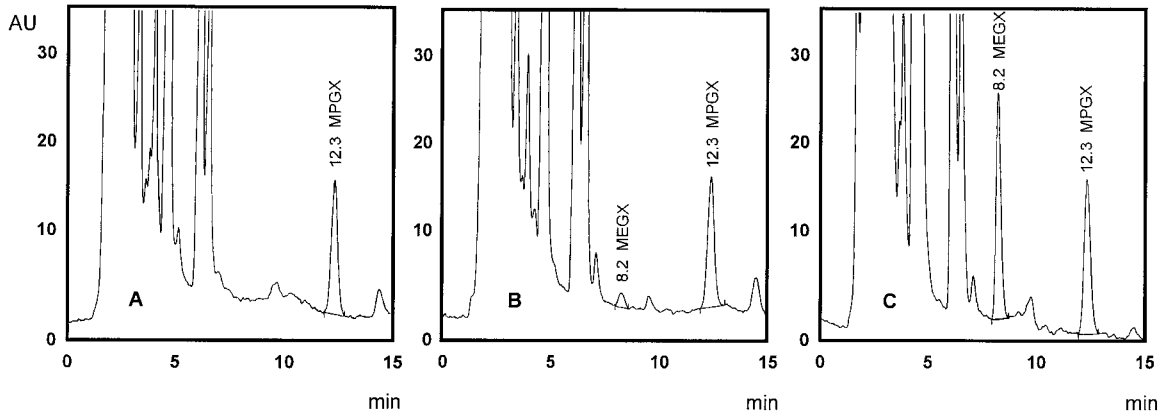


Fig. 1. Representative chromatograms of: (*left*) blank serum sample, collected before the application of lidocaine; and serum samples of patients with MEGX concentrations of 3.5 μg/L (*middle*) and 74 μg/L (*right*), collected 15 min after the application of lidocaine.
The internal standard MPGX was added to all samples.

ferences was evaluated by separate analysis of 92 MEGX-free patients' specimens. Interference by commonly administered drugs (acetaminophen, *N*-acetylprocainamide, amikacin, amitriptyline, amoxicilline, amphotericin B, bupivacaine, caffeine, carbamazepine, cephazolin, chloramphenicol, cimetidine, clemastine, clonazepam, cyclosporin A, desipramine, diazepam, digoxin, disopyramide, dopamine, ethosuximide, famotidine, gentamicin, imipramine, lamotrigin, lidocaine, mexiletine, mycophenolate mofetil, netilmicin, phenobarbital, phenytoin, physostigmine, prilocaine, primidone, procainamide, quinidine, rapamycin, salicylate, tacrolimus, theophylline, tobramicin, valproic acid, vancomycin) was evaluated by analyzing (*a*) patients' specimens received for routine therapeutic drug monitoring (TDM), (*b*) human TDM quality-control sera (Chiron Diagnostics, Fernwald, Germany), or (*c*) drug-containing methanol calibrators. None of the drugs interfered: They either did not react with NBD-F, were insufficiently retained by the column, or were not detected during a chromatographic run of 20 min.

The detection limit (signal-to-noise ratio = 3) was 1.7 μg/L. The assay was linear over the working range, between 2.5 and 250 μg/L ($r$ >0.999). Performance characteristics were tested at several concentrations of MEGX added to drug-free serum (Table 1). Within-run imprecision was adequate (CV 11.5%) at the lower limit of the working range (2.5 μg/L), better at higher concentrations. [In contrast, the FPIA CV was 15.6% at 4 μg/L for MEGX (n = 20).] The between-run CVs were ≤12.7%. The deviation from target values (Abbott controls: 25, 50, 100, and 200 μg/L) was −4.8%, 2.6%, 4.2%, and 5.1%, respectively (n = 5). Analytical recovery was calculated from the ratio of the peak heights for the MEGX-supplemented serum samples and for sodium tetraborate/methanol (2/1 by vol) solutions that contained the same amounts of MEGX, were derivatized, and were directly injected onto the column. The extraction efficiency was >88% for MEGX and 90.6% for the internal standard MPGX (Table 1).

We compared the present HPLC-fluorometric method and the TDx FPIA (Abbott) by assaying with both methods 96 serum samples from 51 patients routinely undergoing the MEGX test; samples that could not be directly analyzed with the FPIA because of high bilirubin concentrations (TDx reading: MX BKG) were pretreated with the Digoxin II precipitation reagent according to Zoppi and Fumagalli *[6]*. According to regression analysis by Passing and Bablock *[11]*, the HPLC ($x$) method correlated well with the FPIA ($y$) for analysis of the 96 patients' samples, of which 21% had MEGX values <10 μg/L: $y = 0.974x + 1.308$ ($r = 0.987$).

The purpose of the present study was to develop a highly sensitive and specific method for MEGX determination that would cover the complete clinically relevant decision range, including very low MEGX concentrations. Accordingly, we developed a derivatization procedure that substantially improved the sensitivity and specificity

**Table 1. Imprecision of the HPLC method and recovery of MEGX added to serum.**

| MEGX concn, μg/L[a] | Imprecision: CV, %[b] | | Recovery, %[c] |
|---|---|---|---|
| | Within-run | Between-run | |
| 2.5 | 11.5 | 12.7 | 92.4 |
| 5.0 | 8.1 | 8.7 | 94.6 |
| 25.0 | 5.5 | 5.9 | 100.2 |
| 125.0 | 2.6 | 3.8 | 88.1 |
| 250.0 | 2.4 | 3.9 | 89.0 |

[a] Added to drug-free serum.
[b] n = 12 each.
[c] n = 3 at each concentration.

of MEGX determination, by using NBD-F, which is highly reactive with primary as well as secondary amino groups *[12]* (the latter being found in MEGX). The reaction is simple, short (10 min), and does not require special conditions or equipment. The reaction product is stable for at least 2 days at 4 °C in the dark, allowing repeat analyses within this period. The combination of a simple liquid–liquid extraction procedure with isocratic chromatography makes the method easy to perform in almost every laboratory. The internal standard MPGX can be readily synthesized, but as an alternative one can use the commercially available butanilicaine citrate (Hoechst AG, Frankfurt, Germany; data not shown). Use of the latter compound, however, increases the run time because of its later elution (~18 min) from the column. If required, an even lower detection limit can be achieved by increasing the sample volume to 1 mL.

We have now had experience with >800 chromatographic runs in one $C_{18}$ column without any deterioration of the separation performance. The technician time required to process 30 samples is ~1.5 h, and each chromatographic run takes 15 min. This seems to be adequate even in laboratories with a high workload, a same-day turnaround being sufficient for most clinical indications. We conclude that this HPLC method is an attractive and cost-effective alternative to FPIA.

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**PCR-Based Test for Two Cystic Fibrosis Mutations (A455E, 711+1 G→T) Common Among French Canadians,** *Paula M. Strasberg,[1,2]* Danielle Noreau,[1] Leslie McGlynn-Steele,[1] Galina Koultchitski,[1] and Peter N. Ray[1,3]* ([1] Dept. of Paediatr. Lab. Med., Div. of Genetics, Rm. 3420, Hospital for Sick Children, 555 University Ave., Toronto, ON M5G 1X8; [2] Dept. of Clin. Biochem., Faculty of Med., Univ. of Toronto, Toronto, ON M5G 1L5; and [3] Dept. of Mol. and Med. Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada; *corresponding author: fax 416-813-7732, e-mail pstras@sickkids.on.ca)

Since identification in 1989 of the gene responsible for cystic fibrosis (CF), the CF transmembrane conductance regulator (CFTR) *[1–3]*, >600 mutations have been identified *[4]*. Within the North American Caucasian population, 10–12 mutations account for >80% of CF chromosomes *[5]*, this number varying somewhat according to ethnic origin. Within a diverse French Canadian population, three mutations, 621+1G→T (intron 4), A455E (exon 9), and 711+1 G→T (intron 5), are much more prevalent than in other ethnic groups, representing as much as 23% of the mutant chromosomes in some subpopulations *[5–7]*. The A455E mutation also represents 3% of all Dutch CF mutations *[5]*. In contrast, overall, these three mutations represent 0.7%, 0.1%, and 0.1%, respectively, of CF chromosomes *[5]*. DNA diagnostic laboratories often include these mutations in their analyses when searching for mutations within these specific ethnic groups.

The 621+1G→T CF mutation has been shown to produce a new *Hin*dII restriction site, which can be detected after gel electrophoresis of *Hin*dII-digested PCR products *[6]*. Detection of the A455E and 711+1 G→T mutations, however, has thus far generally been by the allele-specific oligonucleotide method *[6–9]*, single-strand conformation polymorphism, or denaturing-gradient gel electrophoresis *[10]*. Allele-specific oligonucleotide assay, either normal or reverse dot-blot format, is a multistep, cumbersome technology, the commercially available forms of which do not assay the normal allele. As an alternative, we have developed a rapid and simple PCR restriction enzyme detection procedure, utilizing unique primers for the amplification of the A455E and 711+1 G→T regions of the CFTR gene on chromosome 7.

The PCR primers used to detect the A455E mutation are: forward, -5′-AGATAGAAAGAGGACAGTTG**C**T-3′ (nucleotides 1472–1494), and reverse, -5′-GAACAAAAGAACTACCTTGCCT-3′ (nucleotides 1539–1517). In the upstream primer, the C used in place of T is indicated here in underlined type. The C-to-A transversion produced by A455E at nucleotide 1496, 3-bp downstream of the forward primer, creates a new *Gsu*I restriction site (5′-CTGG**A**G$(N)_{16}$↕-3′, the underlined A denoting the site of mutation) between nucleotides 1513 and 1514, in addition to the one ordinarily present between nucleotides 1531 and 1532, 7-bp from the 5′ end of the reverse primer (see Fig. 1).

For 711+1 G→T, the sequences of the forward and reverse primers are respectively as follows: 5′-GCTGT-

CAAGCCGTGTTCTAG-3′ (nucleotides 630–649) and 5′-GCCTAAAAGATTAAATCAATAGGTAC<u>T</u>TA-3′ (nucleotides 741–713). The underlined T in the reverse primer marks replacement of the normal A. The G-to-T transversion produced by the 711+1 G→T mutation, 1-bp 5′ to the reverse primer, creates a new *Mse*I restriction site (T ↕ TAT) (Fig. 1).

"Hot-start" PCR was performed with a Perkin-Elmer Cetus (Mississauga, ON) Model 9600 thermal cycler after a 7-min denaturation at 94 °C. The PCR used 300 ng of genomic DNA and 50 pmol of each primer in a final volume of 50 μL; the buffer consisted of 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 5 mL/L Triton X, and 2.5 U of AmpliTaq (Perkin-Elmer Cetus). Amplification cycles (n = 30) of 94 °C for 30 s, 57 °C (A455E) or 60 °C (711+1, G→T) for 30 s, and 72 °C for 1 min were followed by a 10-min elongation step at 72 °C.

After the PCR, 15 μL of PCR product was digested with *Gsu*I (MBI Fermentas, Hamilton, ON) or *Mse*I (New England Biolabs, Burlington, ON) as directed for 2 h at 37 °C. Products were analyzed by electrophoresis on 12% polyacrylamide gel (PAGE). Aliquots of DNA isolated from carriers of the A455E and 711+1G→T mutations were a generous gift of Julian Zielenski, Department of Genetics, The Hospital for Sick Children, Toronto. DNA samples carrying no CF mutation were obtained from our laboratory collection.

The amplified PCR product obtained from nonmutant DNA with the A455E primers was 68 bp, and was cleaved to 61 and 7 bp by *Gsu*I. In the presence of an A455E mutation, an extra restriction site was created, yielding restriction fragments of 42, 19, and 7 bp (Fig. 1). Amplification of nonmutant DNA with the 711+1 G→T primers produced a fragment of 112 bp, cleaved by *Mse*I to 99 and 13 bp. The new restriction site produced in the presence of a 711+1 G→T mutation resulted in fragments of 83, 16, and 13 bp (Fig. 1). Heterozygote carriers of these mutations have a combination of normal and mutant patterns. Homozygous forms of these mutations are rarely found.

In conclusion, this design and use of "engineered" primers represent a rapid and efficient procedure for the analysis of the A455E and 711+1G→T CF mutations.

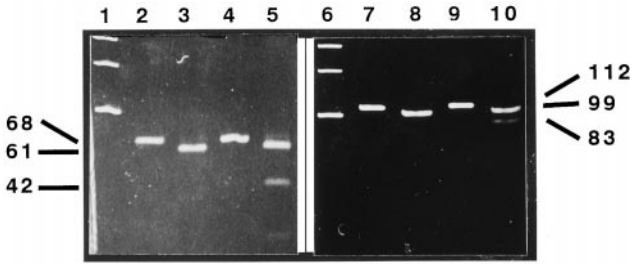


Fig. 1. PCR and restriction digestion analysis of the A455E (*left panel*) and 711+1 G→T (*right panel*) CF mutations.

*Lanes 1 and 6*: 100-bp ladder (BRL, Burlington, ON); *lanes 2 and 3*, uncut and *Gsu*I-cut control DNA; *lanes 4 and 5*, uncut and *Gsu*I-cut heterozygote DNA; *lanes 7 and 8*, uncut and *Mse*I-cut control DNA; *lanes 9 and 10*, uncut and *Mse*I-cut heterozygote DNA.

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**Effect of High Room Temperature on Urinary Iodine Assay**, *Nguyen Tri Dung* (Dept. of Biochem., Hosp. of Endocrinol., Hanoi, Vietnam) *and Maurice L. Wellby** (Dept. of Clin. Chem., Queen Elizabeth Hosp., Adelaide, S. Australia 5011; *author for correspondence: fax 618 8222 6032, e-mail wholdback@tqehsmtp.tqeh.sa.gov.au)

Assay of urinary iodine, along with other measurements, is essential for assessing the iodine status of populations in iodine-deficient areas and for monitoring the effectiveness of control programs. The International Council for Control of Iodine Deficiency Disorders has published a number of urinary iodine assays that are applicable to specific situations *[1]*. One commonly used method, their "Method A," is based on an initial mild chloric acid digestion to remove interfering substances, after which the iodine is quantified by its catalytic effect on reducing yellow ceric ion to colorless cerous ion in the presence of arsenious acid (Sandell–Kolthoff reaction). This is usually done manually in a colorimeter at 405 nm, the details having been described by Dunn et al. *[2]*. In brief, arsenious acid solution is added to each digested urine, blanks, and calibrators. After 15 min, ceric ammonium sulfate is added to each tube and all tubes are left at room temperature for exactly 20 min, at which time the remaining absorbance at 405 nm is measured.

In establishing this method in Hanoi, Vietnam, during the summer months, we consistently obtained very low absorbance readings for all tubes, including the blanks, and so made attempts to remove the supposed iodine

**Table 1. Effect of temperature on absorbances at 405 nm for potassium iodate standards.**

| I concn., μg/L | Temperature, °C | | | |
|---|---|---|---|---|
| | 33–35 | 28 | 25 | 22 |
| 0 | 0.236 | 0.817 | 0.984 | 1.107 |
| | 0.245 | | | 1.085 |
| 20 | 0.214 | 0.670 | 0.737 | 0.983 |
| | 0.204 | | | 0.944 |
| 50 | 0.160 | 0.492 | 0.566 | 0.814 |
| | 0.148 | | | 0.773 |
| 100 | 0.108 | 0.270 | 0.341 | 0.567 |
| | 0.104 | | | 0.556 |
| 150 | 0.078 | 0.156 | 0.195 | 0.405 |
| | 0.071 | | | 0.386 |

contamination. The derived calibration curves were too flat to be useful. We then realized that the low absorbance readings, determined with a Jenway Model 6100 monochromator-type spectrophotometer (Felsted-Dunmow, Essex, UK), actually reflected rapid catalytic conversion of ceric to cerous ions—the speed of the reaction probably being due to the high ambient room temperatures (measured at 33–35 °C). This conjecture was confirmed by performing the catalytic process at various lower temperatures in a water bath cooled with ice (Table 1).

Iodine deficiency occurs in many of the hotter equatorial regions, some of which are served by laboratories that cannot afford efficient air conditioners. Where laboratorians encounter this problem of rapid catalysis of ceric to cerous ions, they may resolve it by using a cooled water bath or by installing air conditioning if funds are available.

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**Clinical Evaluation of the Cell-Dyn® 1700CS Blood Counter,** *Sandra Petani, Elizabeta Topić,* Graciela Turčić, and Mathias Däschner* (Clin. Inst. of Chem. Hematol. Lab., School of Med., Sestre milosrdnice University Hosp., Zagreb, Croatia; *address for correspondence: Clin. Inst. of Chem., Sestre milosrdnice University Hosp., Vinogradska 29, 10.000 Zagreb, Croatia; Fax + 385 1 57 27 30)

New generations of hematology analyzers produce fast and reliable data on blood count, while at the same time offering screening information on differential blood count. The aim of this study was to evaluate a Cell-Dyn® (CD) 1700CS (Abbott Diagnostics, Abbott Park, IL) hematology analyzer that determines 18 parameters, including three-part differential blood count.

A single unit instrument consists of a sample analyzer, data module section, and printer as a separate component. The analyzer contains the hardware to aspirate, dilute, and analyze each whole-blood sample from open and closed collection tubes or prediluted samples. Aspiration volume in open mode is 30 μL, in closed mode 450 μL, and in predilute mode 40 μL. The data module section includes a computer controlling all operations and storing a total of 5000 numeric and graphic data results, color video display monitor, and keyboard. The keyboard allows entry of sample identification number, patient name, sex, date of birth, physician, collection date and time, and comments. In addition, limits and reference ranges can be printed with each patient's results.

Counting of blood cells is based on the volumetric impedance method, directly measuring white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), platelets, mean corpuscular volume (MCV), and mean platelet volume, and automatically calculating hematocrit (HCT), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), RBC distribution width, plateletcrit, and platelet distribution width. The instrument differentiates the subpopulations of lymphocytes, granulocytes, and the mid-cell fraction (eosinophils, basophils, monocytes, and precursors of WBCs) by electronic sizing. Specially formulated reagents cause the WBC membrane to shrink around the nucleus while keeping the cell intact, allowing separation of white cells according to their volume. Lymphocytes fall within the small-cell region, neutrophils within the large-cell region, and the remaining cells into the mid-size cell region. The three-part differential screen is provided with a region-flagging criteria (R-flags) system based on computer check of the three different cell populations' peaks and valleys histogram. Suspect flags are generated after the instrument has evaluated the three-part differential indicating the possible distribution or morphological abnormalities. CD 1700CS utilizes five different alerts: R0, R1, R2, R3, and R4, plus a multiple alert designated RM (Fig. 1). "R" is an abbreviation for the region of the histogram, and the associated number indicates the portion of the histogram that is abnormal. "M" is displayed when multiple regions are abnormal. White cells falling beyond the anticipated normal range will trigger one of the alerts.

The instrument is calibrated for directly measured parameters with the Cell-Dyn calibrator according to the manufacturer's guidelines and does not require frequent recalibration when it is operated and maintained according to the manual recommendations. The system offers several quality-control (QC) options to monitor and validate instrument performance. Also, QC programs are designed to provide continual monitoring and confirmation of the instrument calibration.

Results of analytical evaluation showed that within-run precision (n = 30) for all parameters either measured or calculated was satisfactory and ranged from 0.63% (MCV) to 4.7% (mid-cell fraction) in open mode, and from 0.59% (MCV) to 4.93% (mid-cell fraction) in closed mode. Between-run CVs tested on three levels, low (n = 3 × 20),

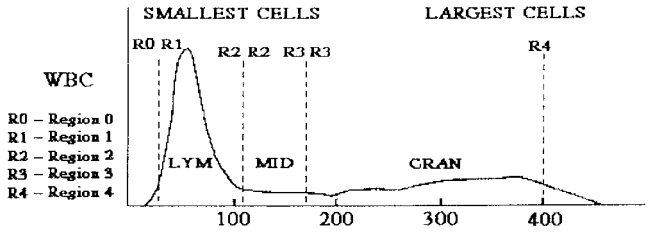


Fig. 1. A CD 1700CS leukocyte histogram with suspect population flags for three-part differential blood count.
LYM, lymphocytes; MID, mid-cell fraction; GRAN, granulocytes

normal (n = 3 × 20), and high (n = 3 × 20) generally were <3%; higher values were observed only for platelet count determination in the abnormal low range for closed tube sampling (CV of 5.10% with an average platelet count of 63 × $10^9$/L) and for mid-cell percentage for closed tube sampling (CV of 4.40–6.98%). The CVs for closed tube sampling were observed to be slightly higher than those for open tube sampling. Satisfactory CVs of between-day precision tested (n = 3 × 20) on three-level control material were obtained for most parameters, except for leukocyte count in the abnormal low range (CVs of 3.92% and 4.01% with an average leukocyte count of 2.1 × $10^9$/L), platelet count in the abnormal low range (CV of 5.73%), and mid-cell percentages in both open and closed tube sampling (CV of 6.00–6.20%).

Results of blood count comparison obtained by simultaneous analysis of 156 patient samples on CD 1700CS and MEK-8118K (Nihon Kohden, Tokyo, Japan) hematologic analyzers showed a satisfactory correlation for all the parameters tested, ranging from 0.967 for platelets to 0.994 for WBC, except for MCHC ($r$ = 0.389 in closed and $r$ = 0.344 in open mode). A low correlation coefficient for MCHC was also found by other authors *[1–3]*, indicating a serious problem in MCHC measurement assuming that the differences probably reflected variations in cell sizing techniques used in various instruments, which would be of minor clinical significance. The comparison of open and closed mode of tube sampling was excellent for all the parameters tested, except for MCHC, showing a slightly higher CV ($r$ = 0.570) than those between the two instruments, but still not acceptable. Carryover of WBC, RBC, HGB, HCT, and platelets for open and closed tube sampling, as assessed by the method of Broughton *[4]*, was <1%.

A sample stability study showed that all RBC parameters were stable for 12 h *[5]*. After this period, a slight increase in MCV (4.5%/48 h), and a fall of RBC (5.5%/48 h) and HCT (constantly for 48 h) were observed. A gradual loss of platelets starting after 6 h was 4.7% at 12 h and 8.5% at 48 h. WBC count was constant for 24 h; after 48 h, an increase by 3.7% was observed. Neutrophil and lymphocyte percentages remained stable for 8 h. After this period, a pronounced decrease in neutrophils and an increase in lymphocyte percentages were observed *[6, 7]*. The mid-cell fraction was less stable; after 6 h, an increase by 19.6%, and, after 48 h, an increase by 96.6% was observed. Samples with leukocytosis or leukopenia showed a similar time stability as those with leukocyte count within the normal range.

Linearity determined by serial dilutions of three separate samples selected to cover the clinically important ranges indicated the counter to be linear for HGB concentration for up to 210 g/L, leukocyte count for up to 35 × $10^9$/L, and platelet count for up to 900 × $10^9$/L.

On testing the instrument reliability of leukocyte differentiation by the CD 1700CS, leukocyte three-part differential was compared with manual differentials as a reference method differentiating 400 cells from 269 patient samples *[7, 8]*. The linear regression analysis of lymphocytes, mid-cells, and granulocytes, presented in Fig. 2, showed satisfactory correlation coefficients for granulocytes ($r$ = 0.924) and lymphocytes ($r$ = 0.939), whereas the correlation coefficient for mid-cell fraction was notably lower ($r$ = 0.435). A good relation between two technologists was observed, with correlation coeficient for granulocytes and lymphocytes of $r$ = 0.983 and $r$ = 0.985, respectively, but lower for the mid-cell fraction ($r$ = 0.677), indicating the presence of technologist-to-technologist variability for eosinophil, basophil, and monocyte subpopulations calculated from the manual differential as mid-cell fraction. According to our experience, one of the problems of intertechnologist variance for monocytes could be attributed to an unequal distribution of monocytes in the blood smear and to a low number (percentage) of monocytes in the blood smear *[9, 10]*.

Sensitivity and specificity of the three-part differentials produced by the analyzer were assessed by analyzing the results of manual differentiation and instrument differentiation *[11–13]*. Each of 269 samples was classified as truly negative (TN), denoting a differential blood count within the reference range by both methods; truly positive (TP), denoting a differential blood count signalized by the analyzer as pathologic, which was confirmed by manual differentiation; falsely positive (FP), denoting a result

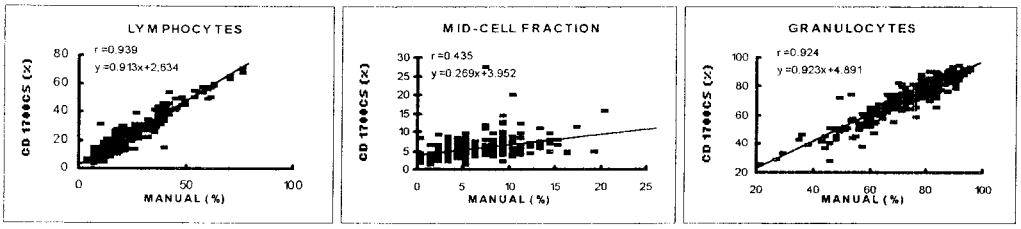


Fig. 2. Linear regression analysis of results of differential blood count on CD 1700CS against manual 200-WBC differential results for lymphocytes, mid-cell fraction, and granulocytes.

signalized by the analyzer as pathologic, whereas on manual differentiation it showed no deviations from a sample of a healthy subject; and falsely negative (FN), denoting a differential blood count result that the analyzer failed to signalize as pathologic, whereas on manual differentiation the sample was found to be pathologic. Of 269 samples tested, 175 (65.1%) samples yielded TP results, 70 (26.0%) samples yielded TN results, 9 (3.3%) samples yielded FP results, and 13 (5.6%) samples yielded FN results. The causes of FN results (n = 13) obtained by the instrument were a failure in signalizing increased granulocytes in three cases, increased granulocytes with left shift in two cases, increased monocytes in four cases, increased eosinophils in two cases, decreased lymphocytes in two cases, and increased lymphocytes in two cases. Analysis of FP results showed that the analyzer falsely signalized increased mid-cell fraction in four cases, increased granulocytes in three cases, decreased granulocytes in one case, and decreased lymphocytes in one case.

Suspect population flags for three-part differential were also evaluated by comparing the instrument flagging with manual differential results *[14]*. Each sample was analyzed in relation to suspect flags (RO, R1, R2, R3, R4, RM) as indicators of possible distribution and (or) morphological leukocyte abnormalities. Suspect flags were found in 113 (42%) of the total number of samples (n = 269) tested for differential comparability. Of 113 samples flagged, 90 (79.6%) samples showed TP flags. Comparison with the microscopic method confirmed the presence of a distribution and (or) morphologic abnormality in these cases. Of 113 samples flagged, only 8 (7.1%) samples showed FP flags. In these cases, a differential blood count obtained by the analyzer produced suspect population flags, whereas on manual differentiation it showed no deviation from a sample of a healthy subject. Furthermore, 15 (13.3%) samples confirmed by microscopic examination as morphologically abnormal were not flagged by the instrument, so these samples showed FN results related to the instrument flagging system. Among leukocyte flags, the "lymphocyte area anomaly" was most common. Lymphocyte R2 or RM alerts were found in 31 cases, indicating the presence of blasts, variant lymphocytes, lymphopenia, and lymphocytosis. Lymphocyte RO or RM alerts indicate interference to the left of the lymphocyte peak. These alerts were found in 4 cases and referred to the presence of nucleated red cells. Lymphocyte R1 or RM alerts were found in 18 cases and indicated lymphocytosis or lymphopenia. Mid-cell R2 or RM alerts are prompted by interference between the lymphocytes and mononuclear areas secondary to eosinophilia, monocytosis, or blast cells. These alerts were found in 7 cases. Mid-cell R3 or RM alert was found in only one case and indicated the presence of blast cells. Granulocyte R3 or RM alert was found in 22 cases. These alerts were caused by immature granulocytes, band cells, granulocytosis, and neutropenia. Granulocyte R4 or RM alert was found in 13 cases and indicated granulocytosis or neutropenia. Two different flags were found in 17 cases, indicating the presence of distribution and (or) morphological abnormalities for two different leukocyte subpopulations. Reliability of the suspect flags generated by the instrument as detectors of leukocyte morphological abnormalities suggested an 85.4% reliability of the flags for immature granulocytes or left shift, 57% for nucleated RBC and atypical lymphocytes, and 80% for blast cells.

Differential leukocyte count maintains diagnostic significance in two fields: (*a*) measurement of the relative proportions of normal leukocyte populations, with important implications, for instance, in the diagnosis and monitoring of infectious diseases and in monitoring of cytotoxic therapies; and (*b*) search for and definition of abnormal cells in the peripheral blood. It is related to the diagnosis of hematologic disorders and requires good test sensitivity for identification of abnormal cells *[15]*. When the analyzer is used in hospital laboratories that give simultaneous access to healthy subjects and those with widely varying pathologies, it is important to know the percentage of samples submitted to microscopic control. The advantages of automated differential count over manual differentiation using 100 or 200 cells are greater analytical precision, because the differential leukocyte count obtained by the analyzer is performed by counting thousands of cells instead of hundreds and is accompanied by excellent accuracy, at least for neutrophils and lymphocytes, and reduction in costs and turnaround time due to high throughput *[15]*. The simultaneous production of leukocyte histograms and suspect flags is also an aid to the interpretation of differential blood counts produced by the instrument. Nevertheless, the usefulness of the differential blood count obtained by the analyzer is limited in cases of acute infective processes, hematologic diseases, and allergic states accompanied by changes in hematologic parameters. In these cases, only microscopic differentiation can produce the finding of differential blood count with certainty. This evaluation showed that the CD 1700CS analyzer is reliable in separation of normal and pathologic differential blood counts.

In conclusion, the Cell-Dyn 1700CS automated hematology analyzer is a suitable and reliable blood counter for complete blood count determination in daily routine. The three-part differential blood count with simultaneous production of leukocyte histograms and interpretative suspect flags can be used for screening and separation of normal and pathologic differential blood counts, thereby considerably reducing daily manual differentiation, in case of normal samples free of distribution and morphological alterations in leukocyte populations. This is a substantially time- and material-saving method as compared with the manual method, at the same time allowing the laboratory personnel to thoroughly examine and follow the samples indicated or found to be pathologic. The analyzer should not be expected to completely replace manual determination and interpretation of differential blood count. Therefore, microscopic analysis of the differential blood count and morphological knowledge will continue to be very important in hematology.

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**Analytical Performance of the Sanofi Access® Cardiac Troponin-I Procedure,** *Diego Lozano,* Pino Carreño, Inmaculada Moreno, and Manuel Méndez* (Servicio de Bioquim., Hosp. La Paz, Paseo de la Castellana 261, 28046 Madrid, Spain; *present address and address for correspondence: c/Ferrocarril del Puerto 16 5-C, 29002 Malaga, Spain)

Troponin-I (TnI), a regulatory protein of the thin filaments of striated muscle, is part of the troponin complex (I, T, C) involved in the calcium-sensitive switch that regulates the interaction of actin and myosin in these tissues *[1]*. TnI exists in three isoforms: one for slow-twitch, one for fast-twitch, and one for cardiac muscle. Cardiac TnI (cTnI) is differentiated from the skeletal muscle isoform by a significant difference in amino acid sequence *[2]*. cTnI is not expressed in human fetal skeletal muscle or in adult human skeletal muscle in response to any pathological stimuli but is specific to cardiac muscle. Consequently, an increasing interest has been paid to this protein as a marker of myocardial injury *[3, 4]*.

Several different immunoassays have been reported for quantification of cTnI in serum. They use two cTnI-specific monoclonal antibodies, do not show cross-reactivity with skeletal muscle TnI or other cardiac proteins, and are not subject to nonspecific binding effects from the sample matrix *[5, 6]*. The Access® cTnI is an automated procedure performed with the Access Immunoassay Analyzer (Sanofi Diagnostics Pasteur, Madrid, Spain), a random and continuous-access instrument that performs various immunoenzymatic assays. Paramagnetic particles coated with monoclonal antibodies against the analyte constitute the solid phase, and chemiluminescence is used to detect antigen–antibody binding. The protocol is initiated by adding the sample and anti-cTnI antibody labeled with alkaline phosphatase to a reaction vessel containing the paramagnetic particles in a buffered solution. After incubation, the unbound material is separated and removed by applying a magnetic field and washing. Finally, a chemiluminescent substrate is added to the reaction vessel. The signal is directly proportional to the amount of the antigen, which is determined by comparison with the stored calibration curve. The test takes ~15 min. The Access cTnI procedure resembles the previous manual assay designed by Pasteur *[6]* only through use of the same monoclonal antibodies.

To evaluate the analytical characteristics of the Access cTnI assay, we followed the protocol proposed by the Sociedad Española de Química Clínica (SEQC) *[7]*. Access reagents, calibrators, and controls were provided by the manufacturer. The control materials and calibrators were prepared and stored according to the manufacturer's instructions. Imprecision was calculated by assaying in triplicate, in 2 runs per day for 20 days, cTnI controls at three concentrations covering the analytical range. The linearity study was carried out by diluting a specimen pool of high concentration with various volumes of the S0 control (zero cTnI concentration) and analyzing the diluted solutions in duplicate. The detection limit of the method was calculated by determining 5 times the S0 control and a sample with low cTnI concentration (0.1 $\mu$g/L calibrator) and establishing a calibration curve from these two points. The detection limit was taken as 2 SD above the mean measured for the S0 control. We repeated this process on 3 days in a 1-month period. To determine the amount of carryover, we analyzed in quadruplicate the sequence AABBB, where A is a specimen pool with high cTnI concentration (40 $\mu$g/L) and B is a specimen pool with low concentration (0.1 $\mu$g/L). The percentage of carryover was calculated with the formula proposed by Broughton *[8]*:

$$\text{carryover, \%} = 100 \times (B1 - B3)/(A2 - B3)$$

The imprecision of the method (CV) ranged from 4.8% to 7.8% (Table 1)—values comparable with the published data for the automated analyzers Stratus® (Dade) *[9]* and Opus Plus® (Behring Diagnostics) *[10]* and better than those of the manual procedures (CVs between 2.1% and

**Table 1. Imprecision of the Access cTnI assay.**

| Within-run | | Between runs | |
|---|---|---|---|
| Mean cTnI, μg/L | CV, % | Mean cTnI, μg/L | CV, % |
| 0.183 | 5.68 | 0.209 | 7.75 |
| 6.180 | 4.78 | 5.920 | 7.87 |
| 22.420 | 5.10 | 26.810 | 6.32 |

26.7% *[5, 6]*). The detection limit was determined as 0.037 μg/L, lower than the reported minimum detectable concentrations for other assays: Stratus assay, 0.5 μg/L; Bodor et al. *[5]*, 1.9 μg/L; Larue et al. *[6]*, 0.2 μg/L. The manufacturer's recommended cutoff for myocardial injury (cTnI 0.1 μg/L) is also lower than the decision points recommended for the Stratus analyzer—0.6 μg/L *[11]*, 0.8 μg/L *[12]*, and 1.5 μg/L *[13, 14]*—and the Opus Plus analyzer—2 μg/L *[15, 16]*. The method showed excellent linearity over the range of concentrations studied (1–50 μg/L), yielding a linear regression equation of $y_{measured} = 0.95x_{expected} - 0.68$ ($r = 0.999$), and the percentage difference between the expected and measured data was 86–94%. The amount of sample-related carryover was insignificant, i.e., 0.008%.

We conclude that the Sanofi Pasteur Access assay of cTnI is a linear and precise method.

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