Reverse complementary oligonucleotides of the anchor and sensor probes were used as positive controls (see Table 1).

Representative melting curves for the CYP2C9*2 and CYP2C19*2 alleles are depicted in Fig. 1. The differences in the T_m,s among individual genotypes were sufficient to permit reliable discrimination of single alleles (dT, +5–7 °C). There was no difference between the theoretically predicted and sequenced PCR products (data not shown). We thus show corroborative and conclusive evidence for accurate DNA amplification of individual alleles.

In our cohort of unrelated individuals from Southern Germany, the allelic frequencies were 0.125 for the CYP2C9*2 and 0.083 for the CYP2C9*3 allele (n = 24), and 0.158, 0.003, and 0.000 for the CYP2C19*2, *3, and *4 alleles, respectively (n = 165).

We report a new genotyping assay for identification of the CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, and CYP2C19*4 alleles, which contribute substantially to the genetic variability in the pharmacokinetics of drugs and other xenobiotics that are metabolized by CYP2C9 and CYP2C19 in Caucasians (4, 5). Other molecular biology methods may be used to detect genetic polymorphisms, including direct sequencing (6), restriction fragment length polymorphism analysis (7), and single-strand conformation polymorphism analysis (8), but these routine methods are laborious and cumbersome, which is a major drawback for their routine use in clinical practice. Others have developed a fluorescence-based assay that can be performed with the TaqMan System, but not with the LightCycler technology (9, 10).

FRET provides a powerful tool for the rapid, inexpensive, and reliable determination of certain genetic polymorphisms, as recently shown by us for the molecular diagnosis of the Gilbert syndrome (11).

Recently, the clinical significance of cytochrome P450 genotyping before drug treatment has been shown for patients treated with the antiepileptic drug phenytoin: patients carrying at least one variant CYP2C9 allele required dose adjustment approximately two-thirds of standard doses to achieve a therapeutic serum concentrations (12).

In conclusion, our assay can be used in routine clinical practice to provide guidance on dose adjustments for drugs that are metabolized by CYP2C9 and CYP2C19.

**References**


**Monitoring Analytical Quality in Routine Glycohemoglobin Measurements, Paola Luraschi,1 Simona Brambilla,2 Roberta Mozzì,1 Gianpaulo Cattozzo,2 and Carlo Franzini1**

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Maintenance of hemoglobin (Hb)A1c (a specific glycohemoglobin) at <7% has been recommended for effective
treatment of both type 1 and type 2 diabetes (1–5). Because a higher frequency of HbA1c testing may lead to superior glycemic control (6), it can be anticipated that an increased number of HbA1c assays will be performed and increased attention will be given to analytical standardization (7,8). HbA1c assays based on different methods are available (1), but most laboratories in Italy (e.g., 164 of 201 participants in a quality assessment scheme during the year 2001) use HPLC. Several HPLC instruments for measuring HbA1c have been evaluated recently (9–12).

We report our experience to demonstrate how use of routine internal quality control and participation in external quality assessment schemes (EQAS), with occasional method comparison exercises, may provide information that laboratory performance is being kept within acceptable limits.

Leftover EDTA-blood samples, stored at 4 °C and assayed within 3 days, were used in this study for method comparison exercises. Internal quality-control (QC) samples were prepared as follows (13,14): samples with either low or high HbA1c were hemolyzed by shaking one volume of saline-washed erythrocytes with one volume of water and one volume of carbon tetrachloride and were pooled to yield “low” and “high” QC pools (mean HbA1c, 5.07% and 12.7%, respectively). The pools were stored in 0.5-mL aliquots at −80 °C. Lyophilized QC materials from Bio-Rad (Lypocheck Diabetes Control, Levels 1 and 2; mean HbA1c, 5.14% and 9.84%, respectively) were also used. Both the frozen and the reconstituted lyophilized QC materials, properly identified, were introduced in the analyzer after being diluted 1:201 (5 μL of QC material plus 1.0 mL of diluent). Because of the total hemoglobin concentrations in these samples, their total areas in the chromatograms were well within the set limits of the instrument and were comparable to the areas of the fresh samples, which are automatically diluted by the instrument. We participated in a professional EQAS, run essentially as described previously (15), as a joint activity among national scientific associations in the fields of clinical chemistry and diabetes. The scheme is based on the use of lyophilized materials at two concentrations, four pairs per year. Such materials are prepared by the European Reference Laboratory for Glycohemoglobin as well.

HbA1c was measured with a Bio-Rad Variant II system (VaII/a), an ion-exchange-based HPLC instrument, calibrated by the manufacturer to be traceable to the DCCT standard. The system had been in routine use for 5 months, performing ~80 measurements per day, and was calibrated and run as recommended by the manufacturer. Results were expressed as a fraction of the total hemoglobin, in “% Units”. Over 9 months, our VaII/a had five major extraordinary maintenance calls: QC values obtained on such occasions, which were outside the ±3 SD interval, were not included in the statistical evaluations.

Short-term instrumental drift was checked by assaying the high and low pools (frozen) at the start and end of each daily series during a period of 71 days. Mean differences (last – first) ± SD were as follows: 0.04 ± 0.18% Units and 0.04 ± 0.31% Units (not significantly different from 0). On the basis of the two sets of 71 pairs of results, the within-series imprecision (CV) at two HbA1c values was 2.5% (mean HbA1c value, 5.35% Units) and 1.6% (mean HbA1c value, 13.5% Units).

The four QC materials (two frozen hemolysates and two Lypochecks) were assayed daily over a 9-month period. Regression/correlation analysis of daily values against measurement number in the sequence showed a significant slope (~0.006) only with one frozen pool. Overall imprecision values (as CV) over the whole 9-month period were 9.3% and 9.0% for the frozen pools (249 measurements each) and 2.9% and 2.8% for the lyophilized materials (167 measurements each). Monthly means were calculated over the period and regressed against the month’s number. Statistically significant slope values were observed with the two frozen hemolysates (~0.062 and -0.143 for the low and high pool, respectively) and with the Level 2 lyophilized control (~0.048), this last corresponding to a decay of <0.5% per month. Monthly (9 months) overall CVs were 1.7–3.9% for the lyophilized materials and 1.7–7.2% for the frozen hemolysates; no significant trend of CV values with time was apparent. It is difficult to determine whether these results are attributable to instability and/or inhomogeneity of the materials or to a downward trend in the analytical system, although the different behaviors observed with the different materials favor the first hypothesis.

Three HPLC systems widely used in Italy, as shown by the number of laboratories using each instrument in the EQA survey, were used in method comparison studies, including another Bio-Rad Variant II instrument (VaII/b; 19 users in the EQAS); a Menarini HA 8140 instrument calibrated by the manufacturer to be traceable to the DCCT standard (MeHA; 56 users), and a Hitachi LC 9100 instrument, not calibrated to be traceable to the DCCT standard (HiLC; 11 users). Different sets of 48–194 fresh patient samples were assayed in the split-sample mode, in 4–10 discrete batches, each including 10–20 samples. Results from each alternative instrument were compared with those obtained by the VaII/a by means of Deming regression/correlation analysis, as shown in Fig. 1. In spite of good correlation (r = 0.983), the HiLC system showed significant negative bias: the two components of error [constant (intercept = −0.68% Units) and proportional (slope = 0.83)] combined to give high negative bias (~1.7 to ~2.1% Units) at critical HbA1c concentrations (6.0, 7.0, and 8.0% Units). The alternative VaII/b system in use gave results fairly comparable to the VaII/a system. The MeHA system (r = 0.988) gave a significant intercept (0.68% Units) and slope (0.90), but the two components of systematic error compensated each other, giving a small bias (from ~0.1 to +0.1% Units) at critical HbA1c values (6.0, 7.0, and 8.0% Units). The intermethod differences (in % Units) were also calculated for each blood sample. The distributions of such differences, as the mean ± SD, were...
as follows: HiLA minus VaII/a, −1.98 ± 0.50% Units; VaII/b minus VaII/a, 0.07 ± 0.41% Units; MeHA minus VaII/a, −0.03 ± 0.38% Units.

The results from the HiLA system were mathematically corrected on the basis of the ratio (median system value)/(DCCT assigned value) in the EQAS to simulate recalibration to the DCCT standardization. The correction improved the agreement with the VaII/a systems, with a negative intercept (−0.82% Units) partially corrected by the slope (1.088); however, bias was still evident at critical HbA1c values (from −0.3 to −0.1% Units), and the inter-method (HiLA minus VaII/a) mean difference (± SD) was −0.25 ± 0.44% Units.

The quality specifications for effective HbA1c measurement have been addressed recently, considering three different approaches: the opinion of patients (16), the biological variation (17), and the opinion of experts (18).

In spite of a few discrepant results and opinions (15, 17), the HbA1c concentration in blood appears to be rather stable and is characterized by low within-subject biological variation (19). To avoid adding a significant portion of additional variation to such a low biological variation, the analytical variation must be kept low. Indeed, for certification as a level II or level I laboratory, according to the criteria issued by the National Glycohemoglobin Standardization Program (18), imprecision ±5% or ±3% is required. Imprecision observed in this study is at the borderline of acceptability (19); therefore, deterioration of precision cannot be tolerated, and careful maintenance of instruments and monitoring by internal quality control are essential in this respect.

Accuracy of measurement is of particular concern in the case of HbA1c. It has been suggested that patient results are better compared with fixed agreed values than with population-based (method-specific) reference values (so-called normal values) (4, 5, 18, 20). For a proper comparison of patient results, the analytical methods need to be calibrated to produce DCCT-compatible results (18, 21), as recommended by the National Glycohemoglobin Standardization Program (18), so that physicians can apply the DCCT outcome data to patient care and relate their patients’ HbA1c to risk for complications. From the results of six exercises in the EQAS, we recorded a mean bias of −0.02% Units (interval, −0.2 to +0.1% Units) vs the instruments’ median and a mean bias of −0.33% Units (interval, −0.2 to −0.5% Units) vs the DCCT values assigned. This may reflect some difficulty in either assignment of values to the EQAS material or manufacturer calibration. The participants using the MeHA system recorded a mean bias vs the DCCT value of −0.35% Units.

Fig. 1. Method comparison results.

In all three panels the solid line is the regression line and the dashed line is the equivalence line (y = x). Statistical analysis was by Deming regression. (A), Hitachi LC-9100 vs Bio-Rad Variant II/a (n = 194; r = 0.983; S_yx = 0.32% Units; y-intercept, −0.68 ± 0.09% Units; slope, 0.83 ± 0.01). (B), Bio-Rad Variant II/b vs Bio-Rad Variant II/a (n = 47; r = 0.992; S_yx = 0.33% Units; y-intercept, 0.22 ± 0.16% Units; slope, 0.98 ± 0.02). (C), Menarini HA 8140 vs Variant II/a (n = 133; r = 0.988; S_yx = 0.030% Units; y-intercept, 0.68 ± 0.09% Units; slope, 0.90 ± 0.01).
and those using the HiLC instrument showed a mean bias vs the DCCT value of ~1.26% Units. The two values for the mean bias shown in the EQAS by the VaI/a system (~0.33% Units vs the DCCT assigned values and ~0.02% Units vs the instrument’s median values) combined with a mean CV of 3% gave theoretical 95% confidence intervals for a single measurement of approximately ± 0.7% Units and ± 0.2% Units, respectively. Such values for the total error may be regarded as acceptable and fairly good performance for a routine laboratory (18), leading to the conclusion that coupling of a laboratory’s alertness with the manufacturer’s cooperation may produce clinically valid results in routine work. We point out that our performance evaluation results did not stem from an instrument evaluation exercise, but arose mainly from routine work over a 9-month period, supplemented with occasional method comparison exercises. One instrument included in these comparisons gave markedly different results, as it did in the EQAS. Single-point, mathematically simulated recalibration substantially improved the performance, confirming the possibility of improving accuracy through calibration. Two-point calibration is likely to permit even better improvement when significant slope and intercept values are shown by comparison.

We conclude that clinically useful analytical quality can be achieved in the measurement of HbA1c by the use of commercial dedicated HPLC systems and monitoring their performance by means of QC programs with appropriate materials. Occasional method comparison studies may increase the operator’s confidence on performance; appropriate calibration may improve poor performances.

Simonetta Granata (Ospedale Niguarda, Milan, Italy) and Ferruccio Ceriotti (Ospedale S. Raffaele, Milan, Italy) performed the measurements with the alternative systems. Andrea Mosca (Università degli Studi di Milano, Milan, Italy) contributed many suggestions and assistance.

References

Newly Identified Apolipoprotein AV Gene Predisposes to High Plasma Triglycerides in Familial Combined Hyperlipidemia

Familial combined hyperlipidemia (FCHL) is the commonest form of hereditary hyperlipidemia (1, 2). Its primary defect is increased secretion of hepatic triglyceride (TG)-rich apolipoprotein B (apoB)-containing particles (VLDL) (3) and impaired clearance of postprandial lipoproteins (4), which increases the number of circulating TG-rich lipoproteins. FCHL is present in up to 20% of survivors of myocardial infarction, and it is considered a significant genetic risk factor for developing cardiovascular disease (1, 5). The underlying genetic defect is unknown, although the disease has been linked to chromosomes 1 (6) and 11 (7). With regard to the latter, linkage...