The intermethod variabilities of control materials and patient blood samples for the measurement of glycohemoglobin were compared. Sets of 50 blood samples and 15 control materials were analyzed by HPLC and affinity and immunochemical methods. For each pair of methods, the distances of the materials from the regression line of patient blood results (expressed as normalized residuals) were calculated. Only two of 15 controls had normalized residuals exceeding 3 standard deviations from the regression line. Total hemoglobin (Hb) content, Hb derivatives, and cellulose acetate electrophoresis demonstrated that only a minority of controls could be considered similar to patients' blood samples. We selected Menarini’s and our home-prepared controls to simulate calibration of the different techniques by these materials. Intermethod calibration succeeded mostly in harmonizing results obtained by HPLC methods. On the contrary, calibration of the immunochemical techniques (Boehringer and Roche) did not improve intermethod agreement to a clinically useful level.

In the last 10 years >20 papers on the commutability of reference materials, i.e., the ability that such materials possess to show interassay properties comparable with those of human samples, have been published. The majority deal with the measurement of enzyme activities [1, 2], serum proteins [3], and cholesterol [4]. The lack of commutability of control materials has been considered a major problem when transferring the accuracy between methods [5] and the fact that half of the papers that have been published after 1995 support the idea that awareness of lack of commutability is an emerging problem in clinical chemistry.

Glycohemoglobin (glyHb) standardization is another subject that has been intensively debated over the last few years, especially after the evidence that the measurement of glyHb in blood is the best tool for the long-term monitoring of glycometabolic control in diabetics [6]. To this purpose an IFCC working group on glyHb standardization is actually working to develop and validate a reference metrological system for Hb A1c determination, including primary and secondary reference materials, a reference method, and adjusted reference and target values [7–9].

However, up to now no study has been performed on the commutability of control materials for glyHb determination. Furthermore, the latest proposed secondary reference material for glyHb has been tested for stability, matrix effect, and homogeneity, not for commutability [10]. Because control and reference materials are essential in assessing analytical methods and for data alignment, we decided to check the commutability of a set of commercially available control materials among the most widely used techniques for whole-blood glyHb determination.

Our goal was to find a control material with good commutability that could be used as calibrator for the diverse assay methods actually used to measure glyHb in blood, and to try to ascertain which kind of harmonization of results obtained by different methods can be achieved by calibrating all the methods with the same material.

Materials and Methods

Specimens

Human blood samples (n = 250) covering a wide range of glyHb values and collected in the presence of EDTA were taken from the routine laboratory. The following commercially available control materials were also analyzed for
their glyHb content: Abbott Control L, M, and H (Abbott Diagnostics; Abb L, Abb M, and Abb H, codes 1A86–10, lot no. 13207M100); DCA 2000 normal and abnormal controls (Bayer Diagnostics; Bay N, code 5072, lot no. 0068; Bay A, code 5073, lot no. 0056); GlycoHb control I and II (Menarini diagnostics; Gly I, code U8312, lot no. 6031; Gly II, code U8312, lot no. 6031); Lyphochek (Bio-Rad Labs.; Lyp I, lot no. 33531; Lyp II, lot no. 33532); Precinorm and Precipath (Boehringer Mannheim; Pre N, code 1488422, lot no. 187994; Pre P, code 1488449, lot no. 187995); and Roche HbA1c control N and P (Roche Diagnostics; Roc N, code 0755672, lot no. T1937; Roc P, code 0755680, lot no. T1937). We also analyzed two home-prepared lyophilized materials (Sam 1, Sam 3) tested for homogeneity and stability; one has already been used in an Italian interlaboratory exercise [11]. All the control materials, with the exception of the Abbott, were available in the solid state as lyophilized hemolysates. They were reconstituted with distilled water, or with the appropriate reconstitution fluid (Bayer, Roche) according to the manufacturer’s instructions.

For each method comparison the glyHb concentration was measured simultaneously in 50 samples and in 15 control materials, in five separate studies. In fact, for logistic reasons, 50 blood specimens were analyzed simultaneously by the Bio-Rad Variant, the Kontron s400, and by the Menarini H8140 methods. Different batches of 50 samples each were then analyzed separately by the other methods (Abbott IMx, Boehringer Tina-quant, Hitachi L-9100, Kontron s400, Menarini H8140, Roche Unimate A1c, Tosoh A1c 2.2). Particular attention was dedicated to sample selection, to study homogeneous specimen groups. As a matter of fact, the 50 sample batches were selected on five different occasions from the routine laboratory, by collecting samples from healthy and diabetic subjects to cover approximately the same Hb A1c range. The Hb A2 ranges (Bio-Rad Variant values) analyzed in the five studies were: 5.1–14.5%, 5.2–14.3%, 4.4–12.6%, 4.4–12.8%, and 4.7–13.0%.

**Analytical methods**

The following methods for glyHb measurement in blood were used according to the manufacturers’ instructions. **Affinity chromatography methods.** Abbott IMx test (Abbott Diagnostics).

**HPLC methods.** Bio-Rad Variant (Bio-Rad Labs.); Hitachi system L-9100 (Merck Bracco); Kontron system 400 (Kontron Instruments); Menarini H8140 (Menarini diagnostics); and Tosoh system A1c 2.2 (Eurogenetics Tosoh).

**Immunochemical methods.** Boehringer hemoglobin A1c Tina-quant test (Boehringer Mannheim); Roche Unimate A1c (Roche Diagnostics).

Hb electrophoresis was performed on cellulose acetate followed by staining with Ponceau Red and counterstaining with tetramethyl benzidine, as described [12]. Total Hb content, spectral analysis of Hb derivatives, and methemoglobin (MetHb) concentration were measured by standard laboratory techniques [13].

**Statistical analysis**

Method imprecision was evaluated by means of the test of differences of duplicates on 10 normal and 10 diabetic samples. In this test, the mean is equal to the sum of all results divided by their number, whereas $SD = (\Sigma d^2 / 2n)^{1/2}$, where $\Sigma d^2$ is the sum of the squares of the differences among the duplicates, and n is the number of duplicates.

Methods comparison was elaborated with the standardized principal component analysis [14]. In all the comparisons the results obtained by the Bio-Rad Variant method were plotted on the x-axis and the regression equation was calculated from the measurements on blood samples. The results obtained on the control materials were reported as normalized residuals, i.e., as $(y - y') / S_{y|x}$, where $y$ is the results obtained by the y-method (Abbott IMx, Boehringer Tina-quant, Hitachi L-9100, Kontron s400, Menarini H8140, Roche Unimate A1c, Tosoh A1c 2.2), $y'$ the values calculated from the equations of the regression line, and $S_{y|x}$ the residuals calculated on blood samples.

Data correction after calibration was achieved by constructing, for each method, a calibration curve with the assigned Hb A1c value on the x-axis (i.e., the Bio-Rad Variant value obtained on each control), and the reported y-method value on the ordinate. The original data obtained on blood samples with the y-method were then recalculated with the calibration curve and plotted in terms of bias units with respect to the measurements performed with the x-method.

**Results**

The control materials were first analyzed with regard to their composition. Table 1 reports data on total Hb, MetHb, and Hb derivatives content. As can be seen, most of the materials possess a total Hb content similar to that of whole blood, with the tendency of being in the normal-low or anemic range. Their MetHb concentrations were found generally higher than in normal whole blood (usually MetHb is <1%), from moderate (Gly I and II, Sam) up to high (Bay and Roc materials). It was not possible to determine MetHb concentration in the Abbott materials because these controls were found mainly in the cyanMetHb form.

The composition of the controls was then investigated by cellulose acetate electrophoresis. Fig. 1 reports typical patterns obtained after fixation and staining. The main bands visible under these conditions were those of Hb A, Hb A2, and carbonic anhydrase, but in most of the materials this pattern was altered because of various reasons. In the case of the Abbott L, M, and H materials (Fig. 1, lanes 2–4), the Hb A band was more diffuse than in normal controls, with a cue prominent at the cathodic...
end (arrow). This pattern was common in the Bayer (lanes 6 and 7) and Roche materials (lanes 26 and 27), which also shared a heavier background developed during migration. The Bio-Rad controls (lanes 10 and 11) showed grossly diffuse Hb bands, with a lateral width almost double with respect to that of normal samples. On the contrary, band width was markedly reduced in the Menarini controls (lanes 14 and 15). Finally, a totally unusual migration pattern was observed in the Boehringer materials (lanes 30 and 31) where no Hb A, Hb A₂, and carbonic anhydrase bands could be detected. The electrophoretic pattern was normal for the Cal and Sam controls (lanes 18–20 and 22–24, respectively).

The regression analyses of results obtained by different methods for glyHb determination are reported in Table 2. The regression and correlation analysis calculated from the data obtained on blood samples (Table 2, upper part) proved that method–instrument pairs gave quite often different results. The intercept values were always significantly different from 0 and slope values ranged from 0.964 to 1.053. In the case of comparisons among HPLC methods (the four most right columns in Table 2) the correlation coefficients ($r$) were constantly higher and sample residuals ($S_yu_x$) constantly smaller than those of the affinity chromatography (Abbott) and immunochromatographic methods (Boehringer, Roche). The imprecision of the methods (intraassay CV) was as follows: Abbott IMx, 2.7%; Bio-Rad Variant, 0.6%; Boehringer Tina-quant, 1.4%; Kontron s400, 1.2%; Menarini H8140, 1.1%; Merck-Hitachi L-9100, 0.9%; Roche Unimate A1c, 0.9%.

In the lower part of Table 2 the results obtained on control materials, measured by the different techniques in the same runs with patient samples, are presented in terms of normalized residuals. The results from the Abbott controls were not reported, since these materials analyzed by methods other than Abbott IMx gave very low Hb A₁c values, always below the physiopathological interval. Boehringer controls were excluded because of their very low total Hb content. In two cases (Lyp II, Abbott IMx; Roc P, Menarini H8140) normalized residuals exceeding the $±3$ interval were found when comparing the control materials to patient samples.

Among the different control materials that we tested, by taking into account the results obtained from Hb analysis (content of total Hb and derivatives, cellulose

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**Table 1. Some properties of the control materials for glyHb analysis.**

<table>
<thead>
<tr>
<th>Control materials</th>
<th>Hb total, g/L</th>
<th>MetHb, % of total Hb</th>
<th>Hb A₁c, % of total Hb</th>
<th>Main Hb derivatives present in the materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abb L</td>
<td>138</td>
<td>ND</td>
<td>4.6</td>
<td>CyanometHb</td>
</tr>
<tr>
<td>Abb M</td>
<td>126</td>
<td>ND</td>
<td>7.7</td>
<td>CyanometHb</td>
</tr>
<tr>
<td>Abb H</td>
<td>126</td>
<td>ND</td>
<td>12.7</td>
<td>MetHb</td>
</tr>
<tr>
<td>Bay N</td>
<td>156</td>
<td>15.9</td>
<td>5.2</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Bay A</td>
<td>124</td>
<td>16.0</td>
<td>12.0</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Gly I</td>
<td>84</td>
<td>5.3</td>
<td>3.8–5.8*</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Gly II</td>
<td>76</td>
<td>5.1</td>
<td>9.9–11.9*</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Lyp 1</td>
<td>135</td>
<td>6.8</td>
<td>5.9</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Lyp 2</td>
<td>108</td>
<td>2.7</td>
<td>9.7</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Pre N</td>
<td>2.0</td>
<td>100</td>
<td>4.1</td>
<td>MetHb</td>
</tr>
<tr>
<td>Pre P</td>
<td>2.0</td>
<td>100</td>
<td>9.7</td>
<td>MetHb</td>
</tr>
<tr>
<td>Roc N</td>
<td>120</td>
<td>20.0</td>
<td>5.3</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Roc P</td>
<td>116</td>
<td>22.9</td>
<td>11.5</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Sam 1</td>
<td>86</td>
<td>4.0</td>
<td>5.9</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Sam 3</td>
<td>154</td>
<td>0.5</td>
<td>11.4</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>Normal blood</td>
<td>117–173c</td>
<td>0.78 ± 0.37d</td>
<td>4.5–5.7e</td>
<td></td>
</tr>
<tr>
<td>reference changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Codes as explained under Materials and Methods.

**Fig. 1.** Electrophoretic Hb migration patterns obtained by alignment of eight cellulose acetate plates (four lanes per plate).

The reproduction of the last plate (lanes 29–31) was compressed to keep the abnormal Hb bands (arrows) in frame. The anodic compartment was at the top of each plate. c, normal blood sample; Abb, Abbott control materials; Bay, Bayer; Lyp, Bio-Rad Labs.; Gly, Menarini; Cal, Sam, home-prepared controls; Roc, Roche materials; Pre, Boehringer; CA, carbonic anhydrase.
Table 2. Methods comparison with Bio-Rad Variant system: regression parameters calculated from the analyses on blood samples (top) and normalized residuals relative to control materials (bottom).

<table>
<thead>
<tr>
<th>Regression parameters</th>
<th>Methods</th>
<th>Abbott IMx</th>
<th>Boehringer Tina-quant</th>
<th>Roche Unimate</th>
<th>Menarini H8140</th>
<th>Merck-Hitachi L-9100</th>
<th>Tosoh Alc 2.2</th>
<th>Kontron s400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope ± SE</td>
<td></td>
<td>1.027 ± 0.056</td>
<td>1.031 ± 0.038</td>
<td>1.089 ± 0.038</td>
<td>1.053 ± 0.017</td>
<td>1.010 ± 0.027</td>
<td>1.012 ± 0.021</td>
<td>0.964 ± 0.016</td>
</tr>
<tr>
<td>Intercept ± SE</td>
<td></td>
<td>0.24 ± 0.43</td>
<td>-0.04 ± 0.30</td>
<td>0.38 ± 0.30</td>
<td>-1.44 ± 0.14</td>
<td>-2.10 ± 0.22</td>
<td>-0.93 ± 0.17</td>
<td>-1.72 ± 0.13</td>
</tr>
<tr>
<td>r</td>
<td></td>
<td>0.924</td>
<td>0.966</td>
<td>0.969</td>
<td>0.993</td>
<td>0.981</td>
<td>0.990</td>
<td>0.993</td>
</tr>
<tr>
<td>$S_{y</td>
<td>x}$</td>
<td></td>
<td>0.76</td>
<td>0.51</td>
<td>0.52</td>
<td>0.23</td>
<td>0.36</td>
<td>0.27</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>49</td>
<td>48</td>
<td>49</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Control materials

Bay N 0.43 0.02 -0.63 0.14 0.33 0.69 -0.58
Bay A -0.42 2.61 1.49 -2.07 -0.77 -0.45 -1.42
Gly I -0.20 -1.64 -2.11 0.03 0.81 1.34 0.88
Gly II -1.01 -2.38 -0.79 -0.12 0.54 2.41 0.69
LyP I -1.28 -1.76 -1.89 -0.61 -0.16 -0.15 -0.05
LyP II -3.29 -2.45 -1.85 0.56 -1.39 -0.89 -0.48
Roc N 0.11 -0.51 -1.79 -0.54 0.21 -0.40 -1.28
Roc P 0.85 2.38 0.77 -3.07 -0.64 -0.08 -1.18
Sam 1 -0.79 -1.33 -2.03 0.13 0.34 0.15 -0.76
Sam 3 -0.34 -1.76 -0.90 0.16 0.10 0.72 0.10

The various methods currently available to measure glyHb in blood are known to be well correlated, but frequently biased among each other [15–17]. To this regard, our results were quite consistent with those from others, except that in most cases the Bio-Rad Diamat HPLC was used as the x-method in method comparisons trials. However, glyHb results obtained by Bio-Rad Diamat and Bio-Rad Variant HPLC were very similar and closely correlated [18, 11], so our comparison among Bio-Rad Variant and other methods can be compared with other studies in which the Diamat system was used (16). In the case of Abbott IMx, the standardized Hb A1c Abbott data were correlated to Bio-Rad Variant results similarly to that found previously [19]. However, sample residuals found in this study were slightly higher than those reported by Wilson et al. ($S_{y|x}$ 0.76% vs 0.57%, respectively). Also for the immunoturbidimetric Roche method, we found results well correlated to the Bio-Rad HPLC, with sample residuals slightly higher than those reported by Gey et al. ($S_{y|x}$ 0.52% vs 0.39%, respectively; [20]). A somewhat better agreement between the standardized immunoturbidimetric Boehringer method and Bio-Rad Variant HPLC was found, as already reported [21] with sample residuals from both immunoturbidimetric assays and Bio-Rad Variant, very similar in this and the previous study (0.51% and 0.52%). However, also taking into account the electrophoresis pattern and the ability of minimizing the normalized residuals, we then selected Menarini’s and our home-prepared controls as optimal materials to be used as calibrators. Therefore, to examine the potential utility of these control materials in standardizing glyHb results obtained by different techniques, the original data relative to the differences between pairs of methods on patient samples were recalculated after calibration of the dependent method with pairs of materials. The cumulative distributions of such differences (in the original units of Hb A1c, % of total Hb) are presented in Fig. 2. For the purpose of clarity, in all plots of Fig. 2 we reported the distribution centiles 5th, 50th, and 95th as dotted lines, and the no-bias value with a vertical continuous line.

As can be seen, the distribution plots of the original data (Fig. 2A) were variously biased against Bio-Rad Variant HPLC, in agreement with the intercept values relative to method comparisons reported in Table 2. Globally, glyHb results obtained by the different methods ranged from $-2.12\%$ Hb A1c units from the Bio-Rad Variant result (low limit of the Roche immunochemical method) up to $+3.1\%$ units (upper limit of the Merck-Hitachi HPLC).

After recalculation, patient results calibrated with Menarini controls (Fig. 2B) were found to have a greater overlapping. Particularly, Menarini and Kontron results were almost superimposable to the Bio-Rad Variant data, and Table 3 reports such deviations in Hb A1c units. Calibration with Menarini controls was not so effective in reducing the bias of the other two HPLC methods (Tosoh and Merck-Hitachi). Moreover, calibration with the homemade controls Sam 1 and Sam 3 (Fig. 2C) produced a more marked reduction in bias between methods, since only 13 of 200 Hb A1c results (a total of n = 7 from Merck-Hitachi, n = 3 from Tosoh, n = 2 from Kontron, and n = 1 from Menarini) were $\pm 0.5\%$ (Hb A1c units) off the value measured by the Bio-Rad Variant method. Calibration with such materials was also effective in reducing the mean bias between Abbott and Bio-Rad Variant results, although they had minor success in standardizing Roche Unimate and Boehringer Tina-quant data.

**Discussion**

The various methods currently available to measure glyHb in blood are known to be well correlated, but frequently biased among each other [15–17]. To this regard, our results were quite consistent with those from others, except that in most cases the Bio-Rad Diamat HPLC was used as the x-method in method comparisons trials. However, glyHb results obtained by Bio-Rad Diamat and Bio-Rad Variant HPLC were very similar and closely correlated [18, 11], so our comparison among Bio-Rad Variant and other methods can be compared with other studies in which the Diamat system was used (16). In the case of Abbott IMx, the standardized Hb A1c Abbott data were correlated to Bio-Rad Variant results similarly to that found previously [19]. However, sample residuals found in this study were slightly higher than those reported by Wilson et al. ($S_{y|x}$ 0.76% vs 0.57%, respectively). Also for the immunoturbidimetric Roche method, we found results well correlated to the Bio-Rad HPLC, with sample residuals slightly higher than those reported by Gey et al. ($S_{y|x}$ 0.52% vs 0.39%, respectively; [20]). A somewhat better agreement between the standardized immunoturbidimetric Boehringer method and Bio-Rad Variant HPLC was found, as already reported [21] with sample residuals from both immunoturbidimetric assays and Bio-Rad Variant, very similar in this and the previous study (0.51% and 0.52%). However, also taking into account the electrophoresis pattern and the ability of minimizing the normalized residuals, we then selected Menarini’s and our home-prepared controls as optimal materials to be used as calibrators. Therefore, to examine the potential utility of these control materials in standardizing glyHb results obtained by different techniques, the original data relative to the differences between pairs of methods on patient samples were recalculated after calibration of the dependent method with pairs of materials. The cumulative distributions of such differences (in the original units of Hb A1c, % of total Hb) are presented in Fig. 2. For the purpose of clarity, in all plots of Fig. 2 we reported the distribution centiles 5th, 50th, and 95th as dotted lines, and the no-bias value with a vertical continuous line.

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account differences in sample residuals among the various comparisons, it is noteworthy that three methods (Roche, Menarini, and Kontron) had slopes significantly different ($P \# 0.024$) from a value of 1.0. This is another confirmation of the need for glyHb standardization.

To reduce bias among methods, different approaches to glyHb standardization have been launched, these being essentially the one promoted first by the National Glycohemoglobin Standardization Program (NGSP) and, later on, that diffused by the International Glycohemoglobin Standardization Program (IGSP). It is actually impossible to anticipate if the NGSP and the IGSP will converge to the same reference and target values and which of the two Standardization Programs will involve the greater number of manufacturers. As a time provision, in our personal experience the NGSP trial required approximately 6 months before getting the certification (www.missouri.edu/~diabetes/ngsp/) and we estimate that the IGSP may take probably the same amount of time. It is becoming common opinion that the practical benefit return in terms of glyHb standardization will not come about before a couple of years from now [22].

While awaiting for international glyHb standardization at the manufacturers’ level, in some countries national standardization activities have been started with commercially available [23] or home-prepared control materials ([10, 11, 24]). With regard to the commercial controls, some manufacturers report limitations in the intended use of their controls, and some do not. In some cases (Sweden, Germany) serious difficulties to get good control materials were reported, especially because of considerable matrix effects found for some methods (J.O. Jeppson and R. Kruse, personal communications). For these reasons, we decided to perform a homogeneous and comprehensive commutability study on most of the currently available control materials, by comparing the results with those previously obtained in an Italian standardization study with home-prepared controls ([11]). For technical reasons it has not been possible to include in our study all the techniques and materials used for glyHb analysis. For instance, we regret not to have analyzed methods such as the Bayer DCA 2000 and the Primus affinity HPLC, both very popular in some countries. However, since new methods and materials are continuously coming to the light, the DCA 2000 and the Primus methods, together with other methods and materials, could be the object of future investigations on calibration and commutability.

Therefore, the intrinsic characteristics of various hemolysates proposed as controls for glyHb determinations were investigated. Since commutability depends not only on the methods tested, but also on the nature and source

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**Table 3. Intermethod differences of Hb A1c measurements on blood (Bio-Rad Variant was assumed as x-method).**

<table>
<thead>
<tr>
<th>y-method</th>
<th>Bias (x-method) - (y-method), A1c, % of total Hb (mean ± SD)</th>
<th>Raw data</th>
<th>Gly corrected</th>
<th>Sam corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbot IMx</td>
<td>-0.44 ± 0.75</td>
<td>-0.35 ± 0.83</td>
<td>-0.08 ± 0.76</td>
<td></td>
</tr>
<tr>
<td>Boehringer Tina-quant</td>
<td>-0.18 ± 0.50</td>
<td>-0.97 ± 0.53</td>
<td>-0.61 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>Roche Unimate HbA1c</td>
<td>-1.03 ± 0.53</td>
<td>-0.75 ± 0.50</td>
<td>-0.61 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>Menarini H8140</td>
<td>1.03 ± 0.24</td>
<td>-0.01 ± 0.22</td>
<td>0.03 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Merck-Hitachi L-9100</td>
<td>2.03 ± 0.36</td>
<td>0.26 ± 0.36</td>
<td>0.10 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Tosoh Alc 2.2</td>
<td>0.83 ± 0.27</td>
<td>0.44 ± 0.28</td>
<td>0.09 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Kontron s400</td>
<td>2.00 ± 0.23</td>
<td>-0.05 ± 0.24</td>
<td>-0.11 ± 0.23</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2. Cumulative distributions of the differences between various methods (y-methods) and Bio-Rad Variant (x-method) with regard to the Hb A1c measurement in blood samples.**

The units plotted on the x-axis are Hb A1c concentrations, expressed as a percentage of total Hb. (A) Original data; (B) data corrected after calibration with Menarini controls; (C) data corrected after calibration with home-prepared control materials. 

- [ ]: Abbott IMx; [ ]: Boehringer Tina-quant; [ ]: Roche Unimate HbA1c; [ ]: Menarini H8140; [ ]: Merck-Hitachi L-9100; [ ]: Tosoh Alc 2.2; [ ]: Kontron s400. Dashed lines are drawn at centiles 5, 50, and 95; a continuous vertical line is drawn at 0 bias.
of the materials, the matrices in which they are dissolved, and the addition of preservative agents [25], we started our work by carefully looking at the Hb pattern in the chosen materials. Particularly, we decided to investigate total Hb concentration, the Hb derivatives pattern, and Hb electrophoresis. The analysis of Hb derivatives was considered important because most of the actual glyHb methods are based on spectrophotometric measurements in the Soret region (400–430 nm). In this spectral region the absorptivities of Hb derivatives are very high and even small changes in the wavelength may cause large changes in the glyHb value if an abnormal concentration of Hb derivatives or nonnative derivatives is present in the materials [26].

With regard to total Hb concentration, most of the materials were found similar to patient samples, with the exception of the Boehringer controls (Precinorm N and Precipath P), which were very dilute. These materials were therefore excluded from the intermethod comparisons, also because of the high proportion of MetHb and because of the totally unusual pattern obtained from cellulose acetate electrophoresis. To this regard, Boehringer declares that Precinorm controls contain a mixture of human and sheep blood, but we were unable to find any trace of human Hb A, even on our high-resolution HPLC system (data not shown here). It is unclear if the other controls, which are all of human origin, do really originate from healthy subjects and from diabetic patients. Only in the case of the Bayer materials is the origin from diabetic patients stated relative to the Bayer A (abnormal) control.

The composition of other materials was more similar to native fresh blood, with the exception of Bayer and Roche controls, which were found to contain >10% MetHb. Abbott controls too were considered nonnative from this point of view, since they were all in the cyanMetHb form. The electrophoresis patterns confirmed the results of the spectral analysis, and also showed unusual findings with regard to the Bio-Rad controls, possibly because of the high content of preservatives added by the manufacturer to protect Hb during the lyophilization process. Despite such findings, Abbott, Bayer, and Roche controls were also tested together with the remaining controls (Menarini and ours) for commutability by the linear regression analysis of methods comparison.

The commutability criterion of the linear regression approach was adopted from other studies ([3, 4]), where the limits of ±3 normalized residuals were chosen as cutoff values for noncommutability. From this point of view, most of the materials listed in Table 2 were considered commutable, with the exception of two. For one (Bio-Rad Lyp II, in the comparison between Bio-Rad Variant and Abbott IMx), the finding of noncommutability was in agreement with the manifested matrix effect found in Sweden when using Bio-Rad freeze-dried controls in national surveys including laboratories performing the Abbott IMx method.

By taking together the results from Hb pattern analysis and intermethod comparisons, we concluded that only Menarini and our materials were best ranked to be used as calibrators. An a posteriori correction of the original data indeed showed that bias between methods was drastically reduced after correction, especially for all the HPLC methods. For some methods (Boehringer and Roche) the cumulative distributions were still too wide for clinical use (i.e., >1 Hb A1c % unit) and harmonization of results obtained by these methods was not successful. Harmonization of Abbott’s results is achievable, especially by the use of our home-prepared materials, but only with regard to the mean bias obtained after calibration.

A possible explanation for the poor success obtained by calibrating Abbott, Boehringer, and Roche methods by Menarini’s and our materials used as calibrators could be the higher imprecision of these techniques with respect to the HPLC methods. As a matter of fact, sample residuals were higher in these method comparisons with respect to the comparisons among HPLC techniques only. With regard to the results obtained with the correction of the data from the Boehringer immunochemical method, the quasiparadox result (i.e., correction with calibration increased the absolute bias value) could be partially explained by greater imprecision of this method with respect to HPLC. In such a case the study would have to be repeated with a greater number of replicates, either for patient or control specimens.

The inability of Menarini’s and our materials to harmonize data obtained by the immunochemical techniques needs further investigation. An interlaboratory trial actually under development in Italy, with >160 laboratories and several techniques, will compare the ability of Menarini’s and of our home-prepared controls in reducing the intermethod variability for several different techniques, including the Boehringer, Roche, and Bayer immunochemical methods. In such a study the measurements on the different materials are being performed in triplicate, so that the impact of method-dependent imprecision will be minimized. As a matter of fact, we have already demonstrated that, by using our home-prepared controls (previous lots), we were able to reduce intermethod variability also for Abbott IMx and Boehringer Tina-quant procedures ([11]).

In conclusion, in this study about methods and control materials for glyHb analysis, we proved that relevant differences still exist among different methods and that commercial materials are very different among each other with respect to Hb composition and commutability properties. Noncommutability appears to be rather frequent, and only our specially prepared human controls and Menarini’s were commutable for the majority of method pairs. Differential sources, derivatization of Hbs, and lyophilization processes can explain the absence of commutability. Since human source materials, which most closely mimic patients’ samples, may be used as inter-
method calibrators only for those pairs of methods for which commutability has been proven, we strongly suggest that, also in the case of glyHb measurement, the commutability of calibrators and controls should be tested before the materials are used in routine methods. We also propose that manufacturers clearly state that their own materials are intended for users of their products only, unless commutability studies have proven their commutability with other methods. Moreover, we think that the criterion of not exceeding ±3 normalized residuals from the regression line of patient samples could be acceptable for control materials. However, a more stringent criterion should be used for the acceptance of calibrators, and our data seem to indicate that ±1 normalized residuals is a more appropriate criterion in such cases. Finally, it is essential that the IGSP proceeds, since the intermethod calibration of some techniques (i.e., the immunochemical methods) by common commercially available calibrators may not succeed in harmonizing the glyHb results to a clinically useful degree.

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