Direct enzymatic assay for %HbA1c in human whole blood samples

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Abstract

Objectives: Development and validation of a direct enzymatic HbA1c assay that utilizes a single channel on chemistry auto-analyzers without the need to run separate glycated hemoglobin and total hemoglobin assays.

Design and methods: An enzyme based single channel assay was developed to measure %HbA1c in human whole blood samples. The performance characteristics of the Diazyme Direct Enzymatic HbA1c Assay were evaluated on the Hitachi 917 auto-analyzer using whole blood samples, appropriate controls and a reference lot of manufactured reagents. Accuracy studies were completed by comparing the Direct Enzymatic Assay to existing HPLC and immunoassay methods. Interference testing was performed to determine the effect of total hemoglobin, glycated serum proteins, chemical substances and hemoglobin variants in patient samples.

Results: The Direct Enzymatic HbA1c Assay showed within run precision and total precision results of ≤2% CV for both normal and abnormal level samples. Method comparison studies showed that there was a good correlation between the Direct Enzymatic HbA1c and the HPLC ($R^2=0.98$) or the immunoassay ($R^2=0.97$) methods. The assay measured within the range of 4–16% HbA1c and showed excellent performance with variant hemoglobin in samples.

Conclusions: Diazyme Direct Enzymatic HbA1c Assay is accurate and precise when compared to currently marketed medical devices. The assay is designed to report %HbA1c values directly without need for a separate measurement of total hemoglobin and is not adversely affected by interferences from common hemoglobin variants in samples. It is a cost effective, user-friendly method and is adaptable to most general chemistry analyzers.

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Keywords: HbA1c; Glycated hemoglobin; Enzymatic HbA1c; Human whole blood; Diabetes

Introduction

Hemoglobin A1c (HbA1c) is an important test recommended by the American Diabetes Association (ADA) and other diabetes organizations worldwide, for management of patients with diabetes mellitus [1]. The United Kingdom Prospective Diabetes Study (UKPDS) and Diabetes Control and Complications Trials (DCCT) have showed the direct relationship between HbA1c and the risk for complications due to hyperglycemia [2,3]. HbA1c specifically refers to glucose-modified hemoglobin A (HbA) at N-terminal valine residues of the beta chains of human hemoglobin. The glycohemoglobin is produced by the non-enzymatic addition of glucose residues to amino groups in hemoglobin. There is a linear relationship between the levels of %HbA1c and the mean blood glucose concentrations [4]. Since red blood cells have an average life span of 2–3 months in the blood circulation, %HbA1c becomes a better indicator of patient glycemic control in the preceding 2–3 months.

Recently, the International Federation of Clinical Chemistry and Laboratory medicine (IFCC) developed a new reference method that specifically measures the concentration of only one molecular species of glycated A1c [5,6]. The results by the new reference method have also been compared with the results obtained by current methodologies [7], and the relation between the assays can be expressed by simple regression equations (master equations). Despite recent advances in the standardization of HbA1c testing [8], several issues remain that prevent

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effective field utilization of commercially available clinical testing methods of HbA1c [9]. First, when determining % of HbA1c using the most common method, the immunnoassay, two separate tests are required, a test for glycated hemoglobin (GHb) and a test for total hemoglobin (THb). The final %HbA1c value is expressed as a ratio of the specific GHb in relation to the THb found in the whole blood sample. This type of dual channel testing is usually achieved by running both assays in parallel on chemistry analyzers and utilizing a calculation program to present final data in %HbA1c. Assay imprecision, in theory, is exacerbated in such dual channel testing methodologies since errors from both assays find representation in the final HbA1c values reported. Secondly, samples from patients with hemoglobinopathies may contribute to incorrect HbA1c value reporting. Methodologies that are based on the structural aspects of hemoglobin encounter this problem with varying degrees [9]. An enzymatic assay for HbA1c determination has previously been published [10]. This published enzymatic HbA1c test also requires dual channel tests for both GHb and THb; however the enzymatic assay is reported to have less interference in samples with variant hemoglobin [11]. The effect of interferences of a whole blood sample on the reporting of %HbA1c is important as an expert group in the field of diabetes mellitus suggested that laboratories consider the interferences of an assay when selecting an HbA1c assay method [12].

We developed an improved enzymatic HbA1c assay, Diazyme Direct Enzymatic HbA1c Assay, which uses a single channel test and reports %HbA1c values directly, without the need for a separate THb test. Oxidizing agents in the lysis buffer react with the sample to eliminate low molecular weight and high molecular weight signal interfering substances. After lysis, whole blood samples are subjected to extensive proteolytic digestion with Bacillus sp proteases. This process releases amino acids, including glycated valines, from the hemoglobin beta chains. The glycated valines serve as substrates for a specific recombinant fructosyl valine oxidase (FVO) enzyme, produced in Escherichia coli. The recombinant FVO specifically cleaves N-terminal valines and produces hydrogen peroxide in the presence of selective agents. This, in turn, is measured using a horseradish peroxidase (POD) catalyzed reaction and a suitable chromagen, capable of measuring at a wavelength sufficiently removed from the natural absorbance of hemoglobin. The signal produced in the reaction is used to directly report the %HbA1c of the sample using a suitable linear calibration curve expressed in %HbA1c.

Materials and methods

Patient samples

Fresh whole blood samples collected with EDTA anticoagulant were obtained from a certified commercial source (ProMedDx LLC, Norton, MA). Each sample was provided with a Tosoh G7 HPLC %HbA1c value. From this set, samples were randomly chosen for the precision and interference studies. For the method comparison study, an effort was made to ensure that the samples chosen encompassed the broad measuring range of the assay. For the hemoglobin variant analysis, whole blood samples were obtained from the Diabetes Diagnostic Laboratory (DDL, University of Missouri, Columbia, MO). These samples were tested by DDL using a UMC Primus assay to determine the %HbA1c and the UMC Tosoh HPLC to determine the genetic variant. Whole blood samples containing variants HbS, HbC and HbE were obtained [9].

The Direct Enzymatic HbA1c Assay uses whole blood directly and hence no centrifugation steps were involved in separating blood cells from plasma. Prior to testing, whole blood samples were mixed by gentle inversion to re-suspend settled erythrocytes.

Controls

The controls used for each of the validation studies were obtained from a certified commercial source (Aalto Scientific LTD, Carlsbad, CA). The stabilized freeze-dried whole blood material was reconstituted with sterile distilled water prior to use.

Test reagents

Freshly prepared lots of Diazyme Direct Enzymatic HbA1c assay reagents were used in the testing. Unless otherwise indicated, the analytical grade chemicals were from Sigma Chemical Company, St. Louis, MO. Lysis buffer was composed of 100 mM CHES (2-(Cyclohexylamino)ethanesulfonic acid); pH 8.7, 1% Triton X-100, 0.45% SDS (Sodium dodecyl sulfate) and 0.5 mM oxidizing agents. Reagent 1AB mixture was composed of 5 mM MES (4-Morpholinoethanesulfonic acid); pH 7.0, 4 kU/mL purified Bacillus sp. proteases (Toyobo, Osaka, Japan) and 1 mM oxidizing agents. Reagent 2 was composed of 15 mM Tris pH 8.0, >10 U/mL recombinant fructosyl valine oxidase enzyme (Diazyme), 90 U/mL peroxidase (Toyobo, Osaka, Japan) and 0.8 mM of chromagen DA-64 (Wako chemicals, USA). The HbA1c calibrators used for the assay were prepared from stabilized freeze-dried human whole blood (Aalto Scientific LTD, Carlsbad CA). Prior to usage, the Direct HbA1c calibrator vials were reconstituted with 0.5 mL sterile distilled water and allowed to equilibrate at room temperature for 30 min. Reconstituted calibrators were found to be stable for 14 days when capped tightly and stored at 2 to 8 °C. Calibrators were assigned a single value for %HbA1c based upon extensive replicate analysis using Diazyme enzymatic assay reagents and HbA1c control materials from at least two sources and a representative number of patient blood samples with known HbA1c values assigned by a currently marketed device. The Diazyme Direct Enzymatic HbA1c Assay requires weekly calibration on a standard clinical chemistry analyzer.

Enzymatic assay

The Diazyme Direct Enzymatic HbA1c Assay method is designed so that the signal production for GHb is balanced by a
redox status that is dependent on the amount of THb in the whole blood sample. The first step of the assay allows the sample to react with oxidants found in the lysis buffer and in the R1AB. The oxidants quench small molecule interfering substances such as ascorbic acid and large molecule reducing interfering substances such as hemoglobin. Increasing hemoglobin in the sample results in a change in the redox status of the reaction system, which counterbalances the signal generated after the addition of R2. By using a suitable calibration curve, the %HbA1c values of samples can be obtained directly from a single reading without the need of any post-analytical calculations.

Hemolysis of the sample was performed by mixing 20 μL of whole blood with 250 μL of lysis buffer and incubating for at least 10 min at room temperature. The calibrators and controls were treated the same as human whole blood samples. The lysis step is achieved off-line or on-board, if the test instrumentation allows pre-dilution and incubation with lysis buffer. The Direct Enzymatic HbA1c Assay consists of two reagents (R1AB, and R2). The R1A and R1B components are supplied in separate bottles for longer stability of the reagents. For instruments capable of handling three reagent bottles (i.e. the Hitachi 917), the assay application can be directly used with the supplied reagent bottles. For analyzers capable of handling only two reagents, Diazyme Direct Enzymatic HbA1c reagents R1A and R1B are designed to be premixed, in a specific ratio, before use on the instrument. The lysed human whole blood samples are subjected to extensive proteolytic digestion upon addition of the R1AB mixture. The proteolytic digestion releases amino acids (including glycated valines from the hemoglobin beta chains), which then serve as substrates for the fructosyl valine oxidase (FVO) enzyme, contained in Reagent 2, which specifically serves as substrates for the fructosyl valine oxidase (FVO) enzyme, contained in Reagent 2, which specifically cleaves N-terminal valines, resulting in production of hydrogen peroxide (H₂O₂). The amount of H₂O₂ produced by FVO is measured by a Trinder reaction using a unique long wavelength chromagen (>700 nm) to avoid absorption interferences from hemoglobin. For studies reported in this paper, all Enzymatic HbA1c tests were performed with a Hitachi 917 chemistry analyzer.

**Immuoassay method**

The Roche Tina-quant II assay method was used to determine HbA1c values in samples per the manufacturer’s instructions on the Hitachi 917 auto-analyzer. For method comparison studies, the same set of samples was analyzed by both methods. The Roche Tina-quant immunoassay required the following calculation to convert the GHb and THb results into %HbA1c (NGSP/DCCD): %HbA1c=(91.5 *[GHb/THb])+ 2.15 [13].

**HPLC method**

The Tosoh G7 HPLC method (Tosoh Medics Inc, San Francisco, CA) was used to determine HbA1c values in fresh human whole blood samples per the manufacturer’s instructions by ProMedDx, Inc or its affiliates. The samples were analyzed per manufacturer’s instructions and shipped frozen for method comparison studies. For the variant samples, the boronate affinity-based Primus HPLC system values, believed to be accurate for variant samples [14], were obtained from DDL.

**Interferences and limitations**

The interference effects of common substances found in patient blood were tested using chemicals from Sigma Chemical Company, St. Louis, MO, unless otherwise indicated. The substances tested were: ascorbic acid, bilirubin, bilirubin conjugated (Frontier Scientific, Logan, UT), glucose, uric acid, urea (Fisher Scientific, Chino, CA), triglycerides (Indofine Chemicals, Hillsborough, NJ) and glycated serum proteins. The substances were tested in various concentrations in the lysis buffer to determine the effect on the %HbA1c reported. Each substance was tested until a 10% deviation from the true value of the specimen was reached, or such that the tolerance limit for the interfering substance is greater than the concentration in normal human blood [15].

**Statistical analysis**

Statistical analysis was done with Excel 2003 (Microsoft, Richmond, WA) unless otherwise indicated. The Bland-Altman analysis and T-tests were completed with the MedCalc software (Broekstraat 52, 9030 Mariakerke, Belgium). The method comparison studies between the enzymatic, immuno-logic and HPLC methods followed the CLSI EP5A protocol for Method Comparison and Bias Estimation Using Patient Samples [16]. No calculations were required to obtain the %HbA1c of samples for the Diazyme Direct Enzymatic Assay. Precision of the Direct Enzymatic HbA1c Assay was calculated using the following formula: $S_t = \sqrt{\frac{(1 + 2)^2}{4}}$, where $I$ = number of days, $S_t = \sqrt{\frac{(1 + 2)^2}{4}}$, and $A = \frac{3}{21}$, $B$ = Standard deviation of “Daily Means” [17].

**Results**

**Sensitivity and specificity**

To study the chemical sensitivity or detection limit for this assay, the lowest concentration of the analyte that can be detected with a stated reasonable uncertainty, we used a modified HbA1c whole blood test sample (whole blood sample diluted with buffer containing human serum and redox agents).

<table>
<thead>
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<th>HPLC assay</th>
<th>Immunoassay</th>
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<td>*p-value &lt;0.05</td>
<td>**p-value &lt;0.0001</td>
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Replicate analysis yielded a lower detection limit of 3.90% with a confidence interval of 99.7%, which was calculated by adding the value equal to three times the standard deviation (3SD) to the mean (mean=3.78%, SD =0.039) [18]. This limit of detection is comparable to the HPLC method [4.21%] [19]. The chemical sensitivity for %HbA1c of the Tina-quant II immunoassay is not available as the assay measures the GHb and THb analytes separately, with detection limits of 0.2 g/dL and 0.3 g/dL respectively [13]. The Direct Enzymatic HbA1c Assay reagents are comprised of redox balanced components and, therefore, a mixed reagent blank or water blank cannot be used in this assay. For instruments that require a blank reaction, a blank solution was artificially prepared with balanced redox agents and replicate analysis indicated the blank limit to be at 1.94% HbA1c, well below the effective measuring range of the assay (4–16% HbA1c).

We tested Direct Enzymatic HbA1c Assay tolerance limits for interfering chemical substances and found no adverse affect on assay performance in the presence of 12 mg/dL Ascorbic acid, 15 mg/dL total Bilirubin, 13 mg/dL conjugated Bilirubin, 4000 mg/dL Glucose, 30 mg/dL Uric acid, 80 mg/dL Urea, and 4000 mg/dL Triglycerides in the samples. Stable glycated hemoglobin serves as a substrate for enzymatic reaction used in the Diazyme Direct Enzymatic HbA1c Assay.

The clinical sensitivity and specificity were calculated against well established reference methods. The Tina-quant II immunoassay has a reference range of 4.8–5.9% HbA1c for metabolically healthy patients [13], which is consistent with the American Diabetes Association’s recommended normal range of less than 6% HbA1c based on data from clinical trials [1]. Using this range the sensitivity and specificity of the Direct Enzymatic HbA1c Assay were found to be comparable to those of the immunoassay and Tosoh HPLC based on the method comparison data shown in Fig. 3A and Table 1.

**Influence of hemoglobin**

To assess total hemoglobin tolerance, paired samples with similar %HbA1c value, but with different total hemoglobin concentrations were tested using the Direct Enzymatic HbA1c Assay reagents. In this experiment, the reported values obtained were within 0.5% HbA1c deviation from the target HbA1c values in the total hemoglobin range from 9 g/dL to 21 g/dL. To further test the total hemoglobin tolerance range, a series of lysates were prepared from two whole blood samples with similar %HbA1c and different total hemoglobin concentrations. Different volumes of whole blood were spiked into a constant volume of lysis buffer. The hemolysates thus prepared contained the same %HbA1c, but had different total hemoglobin content ranging from 5–42 g/dL. The physiological range of hemoglobin for healthy patients ranges from 10–20 g/dL, thus the assay covers the necessary range for healthy patients [15]. The results showed that in the range of 9–21 g/dL THb, the sample HbA1c was measured with less than 0.5% HbA1c deviation from the target value (Fig. 1).

**Influence of other glycated serum proteins**

Interference due to presence of Glycated serum proteins (GSP) in sample was tested and the results confirmed that non-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Primus HPLC</th>
<th>Direct Enzymatic HbA1c</th>
<th>Immunoassay</th>
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<tr>
<td>HbC</td>
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<td>5.5%</td>
<td>6.4%</td>
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<tr>
<td>HbE</td>
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<td>7.5%</td>
<td>8.3%</td>
</tr>
<tr>
<td>HbE</td>
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<td>6.6%</td>
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<td>HbS</td>
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<td>11.3%</td>
<td>9.5%</td>
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<tr>
<td>HbS</td>
<td>7.3%</td>
<td>7.5%</td>
<td>6.3%</td>
</tr>
<tr>
<td>HbS</td>
<td>14.7%</td>
<td>15.1%</td>
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Table 3

<table>
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<th>Abnormal sample ID 10989897</th>
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<td>A. Within run precision</td>
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<tr>
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<tr>
<td>Within run SD</td>
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<tr>
<td>Within run CV%</td>
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<td>0.7%</td>
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<tr>
<td>B. Total Precision</td>
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<tr>
<td>Total mean</td>
<td>5.7</td>
<td>10.3</td>
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<tr>
<td>Total SD</td>
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<td>0.18</td>
</tr>
<tr>
<td>Total precision CV%</td>
<td>1.8%</td>
<td>1.8%</td>
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</table>
specific glycated proteins do not adversely affect the assay accuracy. This experiment was performed using lyophilized GSP (Sigma Chemical Co., St Louis, MO), reconstituted in lysis buffer, as the interfering substance. Varying concentrations of GSP, 0.1 to 1.0 mM, in lysis buffer, were tested by spiking in both normal and abnormal samples and testing for recovery. The results obtained showed that in the range of 0.1–1.0 mM GSP, the sample %HbA1c recovery deviation was within 0.3% HbA1c from the target value (Fig. 2). It should be noted that the reference level of GSP in adults serum is <0.3 mM [20,21] and is well below tolerance limits of the Diazyme Direct Enzymatic HbA1c Assay.

Influence of variant hemoglobin in samples

It is known that some HbA1c testing methods may be plagued by inconsistent or incorrect performance due to interfering factors in the sample, such as the presence of variant hemoglobin [9]. The effects of hemoglobin variants on the assay were examined using samples identified by DDL to

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**Figure 3**

A method comparison study was done with a set of whole blood samples tested in parallel with the Diazyme Direct Enzymatic HbA1c Assay and currently marketed Tosoh G7 HPLC method (Tosoh Medics Inc, San Francisco, CA). A correlation plot is shown for the Direct Enzymatic HbA1c Assay method compared with the Tosoh G7 HPLC in panel A and the related Bland-Altman analysis plot is shown in panel B. The Bland-Altman analysis was done with the MedCalc software (Broekstraat 52, 9030 Mariakerke, Belgium).
be variants of hemoglobin S, C and E by analysis of HPLC chromatogram peaks. In a method comparison study using 73 samples with the presence of hemoglobin variants S, C and E, the results obtained by the Direct Enzymatic HbA1c Assay showed good correlation to the results obtained by Primus HPLC method ($R^2=0.963$, $y=1.0x+0.001$). Several samples, including some variant samples that had produced large bias errors during method comparison testing, are shown in Table 2. For these variant samples, the Direct Enzymatic HbA1c Assay reported values in agreement with the Primus HPLC, and did not agree with a commercially available immunoassay method (Pointe Scientific, Canton, MI). It is important to note, however, that these results were obtained with a single lot of reagents and calibration materials. Two additional samples (HbA) were identified that reported high values with the Tosoh G7 method (bias of >3% HbA1c when compared to the Primus HPLC method and enzymatic HbA1c assay). These samples are being further studied to determine root causes of the discrepant reporting by assay methods. In a separate experiment, the effect of variant hemoglobin F (HbF) on the assay was tested, and the results shown that at low levels of HbF (<10%), the patient %HbA1c levels were not affected when using the Direct Enzymatic HbA1c Assay method (data not shown).

**Assay precision**

Two patient samples, one each in the normal and abnormal ranges, were used to determine the assay imprecision (Table 3A and B) by estimating within run and total standard deviations and by calculation of coefficient of variation (% CV), according to the CLSI (formerly NCCLS) EP5A protocol [17]. The Direct Enzymatic HbA1c Assay showed within run precision and total precision results within the current NGSP requirement of ≤4% [22]. For the normal level patient whole blood sample (ID 11004462, mean value 5.7% HbA1c), the within run precision was 1.0% CV ($S_{wr}=0.06$), between run precision was 0.99% ($S_{br}=0.06$) and total precision was 1.8% CV ($S_T=0.10$). For the abnormal level patient whole blood sample (ID 10989897, mean value 10.3% HbA1c), the within run precision was 0.7% CV ($S_{wr}=0.07$), between run precision was 0.84% ($S_{br}=0.09$) and total precision was 1.8% CV ($S_T=0.10$).

**Assay linearity**

To demonstrate the linearity of the Diazyme Enzymatic HbA1c Assay, samples containing different HbA1c levels were prepared in appropriate ratios and tested with a reference lot of Diazyme Enzymatic HbA1c Assay reagents. The linearity study tests were done on the Hitachi 917 auto-analyzer instrument. The six samples used to obtain linearity data were prepared by dilution of two samples, Linearity Level 1 and Level 2 with known HbA1c content of 4.0% and 21.90% respectively. The dilution was done using the proportion method to create a dilution series with predictive %HbA1c values [23]. Results with the six-level linearity set indicated that the assay was linear in the 4–16% HbA1c range (data not shown).

**Stability of Direct Enzymatic HbA1c Assay kit**

The assay reagents were stable for at least three weeks when stored on-board the Hitachi 917 system. For this study, a reference lot of the Direct Enzymatic HbA1c Assay reagents were loaded on the Hitachi 917 instrument and samples were tested on day 0. The reagents were stored on board and tested on subsequent testing days.

To establish shelf life of Direct HbA1c reagents, a specific lot of the Diazyme Direct Enzymatic HbA1c Assay kit reagents were used in an accelerated study. In the study, two levels of HbA1c samples with normal %HbA1c and abnormal %HbA1c values were used as test samples. The first baseline testing was done on Day 0. At different time intervals, Direct Enzymatic HbA1c Assay kit reagents were removed from 37 °C storage and tests performed to determine the deviation from control %HbA1c values obtained on Day 0. The data obtained from accelerated studies at 37 °C indicated that the reagents were stable, without loss in assay performance, up to an equivalent of at least 12 months when stored at 2–8 °C.

**Method comparison**

A method comparison study was done with a set of whole blood samples tested in parallel with the Diazyme Direct Enzymatic HbA1c Assay, the Tosoh G7 HPLC method (Tosoh Medics Inc, San Francisco, CA) and the Roche Tina-quant II immunoassay method (Roche Diagnostics, USA). The Direct Enzymatic HbA1c Assay method showed good correlation when compared with both the Tosoh G7 method ($R^2=0.98$, $y=0.97x+0.11$, $n=66$) and the Roche Tina-quant II method ($R^2=0.97$, $y=1.10x-0.421$, $n=66$) [16]. A correlation plot is shown for the Direct Enzymatic HbA1c method compared with the Tosoh G7 HPLC in Fig. 3A and the related Bland-Altman analysis plot is shown in Fig. 3B demonstrating small bias between the methods. Paired T-tests were completed and showed that there is no significant difference between the Direct Enzymatic HbA1c and the HPLC ($P<0.025$) and immunoassay methods ($P<0.001$). In addition, a one-way ANOVA was completed and also showed no significant difference between the Enzymatic HbA1c and the HPLC ($P<0.001$) and immunoassay methods ($P<0.001$) (Table 1).

**Discussion**

We present a validated Enzymatic HbA1c assay that is designed to report %HbA1c values directly without measuring total hemoglobin content in the sample. This novel assay uses several techniques that render the assay functional. The enzymatic assay relies on the specific cleavage of the hemoglobin in a whole blood sample matrix followed by contacting the A1c residues with the fructosyl valine oxidase enzyme. During the development phase of the assay, we found that only certain types of proteases such as those found in the Bacillus species were
found to be effective in generating complete proteolysis in the short period of time allowed by chemistry auto-analyzers. Several types of fructosyl amino oxidase enzymes have been reported that are potentially useful for determining glycated hemoglobin [24,25]. The fructosyl valine oxidase (FVO) enzyme used in the assay presented here was isolated from Aspergillus species, and has a much higher specific activity using glycated valines as a substrate than other glycated amino acids. The recombinant FVO used in this assay is genetically engineered so that the enzyme is stable under physiological buffer conditions and pairs well with the selected proteases in order to provide a suitable reaction mixture in which to assess the analyte concentration.

The performance characteristics of the new Direct Enzymatic HbA1c Assay were found to be accurate and precise and also present certain advantages over several existing HbA1c methods. As detailed in the Results section, the assay is specific and is not adversely affected by the presence of non-specific glycated serum proteins and has a wide tolerance for total hemoglobin in the samples. Method comparison studies also showed that the assay reports values directly in %HbA1c and is accurate over a linear range of %HbA1c in human whole blood samples.

The immunoassays currently on the market utilize some of the structural aspects of hemoglobin while reporting the total HbA1c in the sample. Consequently, many of the immunoassays are affected by hemoglobin variants in the whole blood sample [26]. However, the Direct Enzymatic HbA1c Assay was designed so that the structure of the hemoglobin does not interfere with the assay. Hemoglobin variants S, C and E were tested and found not to interfere with the assay. The Direct Enzymatic HbA1c Assay showed within run precision and total precision results of ≤2% CV for both normal and abnormal level samples. The low precision can be attributed to the fact that the assay is in a single channel format. Unlike other tests on the market, error is only introduced from one channel on an auto-analyzer rather than from both the GHb and THb channels of the assay. It is recommended that the precision of HbA1c assays be less than 4% [27]. The Direct Enzymatic HbA1c Assay correlated well with the Roche Tina-quant II immunoassay methods and a currently marketed HPLC assay.

Conclusion

Diazyme Direct Enzymatic HbA1c Assay uses a single channel on a clinical chemistry auto-analyzer to measure %HbA1c without the need for a separate measurement of total hemoglobin. The assay was compared to currently marketed medical devices and is accurate and precise. The data reported also indicate that the Diazyme Direct HbA1c Assay is not adversely affected by interferences from common hemoglobin variants in samples. Due to the cost effectiveness of the assay and the format clinicians may save both time and money by using this user-friendly method. The single channel assay requires both less reagents than a dual channel test and less time to obtain results. The Diazyme Direct Enzymatic Assay meets the recommendations by expert groups in the field of diabetes mellitus for HbA1c testing [12].

Acknowledgments

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References

[18] Armbruster DA, Tillman MD, Hubbs LM. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the


