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Evaluation of MALDI-TOF MS for the measurement of glycated hemoglobin

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ABSTRACT

Background: Glycated hemoglobin (Hemoglobin A_{1c}, HbA_{1c}) plays a key role in monitoring long-term blood glucose levels in diabetics mellitus. Therefore, it is of great importance to ensure test quality of HbA_{1c} methods.
Objectives: We aimed to evaluate analytical performances of a matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) system for the measurement of HbA_{1c}.
Methods: We assessed the analytical performances of the method including imprecision, accuracy, and linearity. In addition, comparison with Variant II Turbo 2.0 and Capillarys3 TERA, correlation between glycation rate of α and β globin as well as the influence of most frequent analytical interferences in HbA_{1c} assays were also investigated.
Results: As measurement of imprecision, within-run CVs and total CVs were lower than 1.6% and 2.4%, respectively. Discrepancy of test results (< 0.2%) of IFCC value-assigned external quality control samples indicated a good accuracy of the method. The linearity was excellent with a correlation coefficient of 0.999. The QuanTOF results were well correlated with those obtained by Variant II Turbo 2.0 and Capillarys3 TERA. Good correlation between glycation rates of α and β globin were found. QuanTOF was not prone to common interferences including bilirubin, triglyceride, labile A_{1c}, and carbamylated hemoglobin. However, unacceptable positive bias was observed when the amount of HbF were greater than approximately 8.0% or in the presence of HbS.
Conclusions: QuanTOF perform well for the determination of HbA_{1c} and meet quality criteria requested for clinical use.

1. Introduction

Glycated hemoglobin (Hemoglobin A_{1c}, HbA_{1c}), a modified hemoglobin with a covalent bond connection of glucose to the N-terminal valine of the β chain, is widely used as an important marker for monitoring long term glycemic control performance in diabetics, as well as screening and diagnosis of diabetes [1,2]. The routine methods currently used for HbA_{1c} quantification are based on different principles such as cation exchange high-pressure liquid chromatography, affinity chromatography (HPLC), capillary electrophoresis (CE), or immunological reaction [3]. Each technology has its specific characteristics and performance. MALDI-TOF MS was previously employed to precisely measure molecular mass of α and β globin chains for identification of potential hemoglobin variants, and also modified species such as the glycated globin [4,5].

As compared with liquid chromatography-mass spectrometry (LC-MS), MALDI-TOF MS offers important advantages such as lower instrumentation costs, easier sample preparation, and simpler analysis procedures. With the development of modern MALDI-TOF MS, it becomes more suitable for routine quantitative measurements of HbA_{1c}. Although the protocol for HbA_{1c} quantification based on MALDI-TOF MS had been described [4,5], to our knowledge, MALDI-TOF MS is not yet used in the laboratory routine to quantify HbA_{1c}. Moreover, systematic evaluation of MALDI-TOF MS for HbA_{1c} determination has never been reported.

In this study, we assessed the analytical performances of a MALDI-TOF MS system for HbA_{1c} assay with respect to imprecision, accuracy, linearity, comparison with Variant II and Capillarys3 TERA, correlation of glycation rates between α and β globin and the influence of the most frequent analytical interferences in HbA_{1c} assays.

2. Materials and methods

The QuanTOF (Intelligene Biosystems, China), a newly developed

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MALDI-TOF MS system, was evaluated for HbA_{1c} measurement in this study. Residual patient samples collected in EDTA tubes were obtained from Laboratory Medicine of Peking University Shenzhen Hospital. This study was approved by the Institutional Medical and Ethics Committee of Peking University Shenzhen Hospital.

2.1. Sample preparation and mass spectrometry analysis

Sample preparation was performed following the manufacturer's instruction: whole blood samples collected in EDTA tubes were diluted 1:200 in DI water and mixed 1:9 with 10 mg/mL sinapinic acid (Sigma-Aldrich, US). Two point five μL of this mixture was then spotted onto a stainless steel MALDI target plate (6 × 16 sample array). Simultaneously, a series of standard samples with differing glycation level assigned by National Glycohemoglobin Standardization Program (NGSP) was prepared in the same manner to establish the standard curve on each target.

All spectra were acquired on QuanTOF with the following settings: source voltage 19 kV, laser frequency 5 kHz, laser energy 8 μJ, scanning speed 2 mm/s, mass range 5000–30,000 *m/z*, 10-rows scan per spot. Mass spectrometric analysis speed by QuanTOF was about 15 s per sample spot with the above settings. Mass spectra were processed by QuanGHb software developed by Intelligene Biosystems for QuanTOF instrument.

2.2. HbA_{1c} standard curve

The standard curve was constructed in similar manner as described by Hattan and collaborators [5]. Commercially available Lyphochek hemoglobin A_{1c} standards was purchased from Bio-Rad (Hercules, CA, US). These six blood-based HbA_{1c} reference standards vary systematically in their % HbA_{1c} values traceable to NGSP. Mass spectrometric peak areas from the βHb and glycated-βHb were used to calculate the ratio of [glycated βHb/(βHb + glycated βHb)]. Linear regression analysis was performed between computed ratio and HbA_{1c} level of each standard by the software and subsequently used as the standard curve for quantification analysis.

2.3. Evaluation protocol

2.3.1. Imprecision

Three samples with low (5.5%, 37 mmol/mol), medium (7.2%, 55 mmol/mol), and high (11.9%, 107 mmol/mol) HbA_{1c} levels were used to evaluate precision of the method. For intra-assay imprecision, samples were run in 20 technical replications on a target plate. Total imprecision was determined according to the Clinical and Laboratory Standards Institute (CLSI) EP05-A2 guidelines. The three samples with different HbA_{1c} levels were tested in two runs per day on twenty consecutive days. Results were expressed in both NGSP (%) and IFCC units (mmol/mol).

2.3.2. Accuracy

Accuracy was evaluated by analyzing 5 external quality control samples (NO. 201,911, 201,912, 201,913, 201,914, 201,915) from National Center for Clinical Laboratories in China, which were assigned by IFCC Network Laboratories for HbA_{1c} with IFCC reference method. Biases between HbA_{1c} results obtained with QuanTOF and target values were calculated.

2.3.3. Linearity study

Linearity was evaluated according to the CLSI EP06-A guidelines by proportionally mixing two samples with a high (17.5%, 168 mmol/mol) and a low (4.5%, 26 mmol/mol) HbA_{1c} value. Each mixed sample was assayed in duplicate and regression analysis was performed with obtained mean values versus expected values. The expected HbA_{1c} values were calculated according the following formula: Expected value = $(HbA_{1cL} \times V_L \times Hb_L + HbA_{1cH} \times V_H \times Hb_H)/(V_L \times Hb_L + V_H \times Hb_H)$. $V_{L/H}$: volume of the sample with low/high HbA_{1c} value; $Hb_{L/H}$: Hb concentration of the sample with low/high HbA_{1c} value. Hb concentration was measured by a Sysmex XN9000 hematology analyzer (Sysmex Co., Japan).

2.3.4. Method comparison

A total of 55 samples, HbA_{1c} range from 4.0% (20 mmol/mol) to 17.5% (168 mmol/mol), were analyzed on QuanTOF, a CE method analysis system (Capillarys3 TERA, Sebia, France), and a cation exchange HPLC system (Variant II Turbo 2.0, Bio-rad, US). HbA_{1c} results obtained with the QuanTOF were compared with those obtained with Capillarys3 TERA and Variant II respectively. Data were further analyzed using linear regression and a Bland-Altman plot.

2.3.5. Correlation of glycation rate of α globin and β globin

Glycation rate of α globin and β globin, defined as the proportion of the glycated form in its total globin, can be calculated by glycated α(β) Hb/[α(β)Hb + glycated α(β)Hb]). Glycation rates of α globin and β globin can be obtained because MALDI-TOF MS can measure intact α and β globin chains, as well as their glycated forms. The correlation of glycation rates between α globin and β globin were investigated by analyzing 56 samples with HbA_{1c} values range from 4.3% (23 mmol/mol) to 13.8% (127 mmol/mol) using QuanTOF.

2.4. Assessment of interfering substances

2.4.1. Labile A_{1c}

Erythrocytes of three samples, containing a normal (4.8%; 29 mmol/mol), medium (6.6%; 49 mmol/mol), and high (10.0%; 86 mmol/mol) HbA_{1c} level, were incubated with glucose solutions (56 mmol/L) at 37 °C for 1.5 h. HbA_{1c} and labile A_{1c} were measured every 30 min. Labile A_{1c} was quantitated using Variant II analyzer. After analysis, the bias with different labile A_{1c} concentrations was calculated.

2.4.2. Carbamylated hemoglobin

The interference of carbamylated hemoglobin (cHb) on HbA_{1c} measurements was evaluated by the same three samples mentioned above. Erythrocytes were incubated with potassium cyanate (1 mmol/L) at 37 °C for 3 h. HbA_{1c} and cHb were measured every 1 h. cHb values were determined using Variant II analyzer. HbA_{1c} values of samples with different concentration of cHb were compared with baseline HbA_{1c} values.

2.4.3. Bilirubin and triglyceride

Influence of triglyceride and bilirubin was assessed with two samples with normal (5.6%, 38 mmol/mol) and high (8.7%, 72 mmol/mol) HbA_{1c} levels. Erythrocytes of the two samples were mixed with various dilutions of triglyceride and bilirubin plasma to achieve final concentrations of 22.8 mmol/L triglyceride and 304.0 μmol/L bilirubin, respectively.

2.4.4. HbF interference

The influence of HbF on HbA_{1c} quantification was assessed by mixing umbilical cord blood with three samples containing a normal (5.6%, 38 mmol/mol), medium (6.7%, 50 mmol/mol), and high (9.2%, 77 mmol/mol) HbA_{1c} level. HbF levels achieved were ranged from 0.8% to 14.2% determined with Capillarys3 TERA.

2.4.5. Common hemoglobin variants

Interference of hemoglobin variants was investigated by analyzing samples containing HbAS (n = 2), HbAD (n = 5), HbAC (n = 3), and HbAE (n = 10). All heterozygous hemoglobin variants were confirmed by Sanger sequencing. The variants were measured by QuanTOF, as well as a boronate affinity HPLC system (Ultra^2, Trinity Biotech,

Table 1
Performance characteristics of the QuanTOF for HbA_{1c} measurement.

Parameter	Results NGSP units, %			Results IFCC units, mmol/mol		
Precision (EP5)	Low	Medium	High	Low	Medium	High
HbA_{1c} values	5.50	7.20	11.90	37	55	107
Within-run CV	1.63	1.14	0.90	2.79	1.68	1.14
Total CV	2.40	1.37	1.41	4.00	2.00	1.78
Trueness (EP9)	Measured values	Target values	Bias	Measured values	Target values	Bias
201,911	5.46	5.67	−0.21	36	38	−2
201,912	9.82	9.73	0.09	84	83	1
201,913	4.95	4.89	0.06	31	30	1
201,914	7.10	6.87	0.23	54	52	2
201,915	7.98	7.90	0.08	64	63	1
Linearity	Measured values	Expected values	Bias	Measured values	Expected values	Bias
Low	4.51	4.51	0.00	26	26	0
mixture 1	6.12	6.20	−0.08	43	44	−1
mixture 2	8.05	8.18	−0.13	65	66	−1
mixture 3	10.45	10.61	−0.16	91	92	−1
mixture 4	13.49	13.47	0.02	124	124	0
High	17.47	17.47	0.00	168	168	0

Interferences	Bias, %	Bias, mmol/mol
Bilirubin (≤304.0 μmol/L)	≤0.1	≤1
Triglycerides (≤22.8 mmol/L)	≤0.1	≤1
cHb (≤8.7%)	≤0.2	≤2
Labile A_{1c} (≤12.2%)	≤0.2	≤2
HbF(≤8.0%)	≤0.2	≤2
HbF(> 8.0%)	> 0.2	> 2
HbAS: globin separation	S globin separated with β globin	
Trueness HbA_{1c} (n = 2)	0.5, 0.4	5, 4
HbAC: globin separation	C globin unseparated with β globin	
Trueness HbA_{1c} (n = 3)	≤0.2	≤2
HbAD: globin separation	D globin unseparated with β globin	
Trueness HbA_{1c} (n = 5)	≤0.2	≤2
HbAE: globin separation	E globin unseparated with β globin	
Trueness HbA_{1c} (n = 10)	≤0.2	≤2

Ireland) used as a comparative method because results from boronate affinity HPLC are not thought to be affected by the presence of hemoglobin variants [6]. Subsequently, the HbA_{1c} values obtained with QuanTOF were compared with those obtained with Ultra^2.

2.5. Statistical analysis

All statistical and graphical analyses in the above evaluation protocols were performed with SPSS 19.0 (IBM, USA).

3. Results

3.1. Imprecision and accuracy

For QuanTOF imprecision study, within-run CVs are 1.6%, 1.1% and 0.9% (NGSP units) for low, medium, and high HbA_{1c} level samples, respectively. Accordingly, total CVs are 2.4%, 1.4% and 1.4% (Table 1). The CVs in IFCC units are higher those in NGSP units. For QuanTOF accuracy study, the absolute difference between measured HbA_{1c} value and respective IFCC target value is less or equal to 0.2% (2 mmol/mol) for all five control samples (Table 1). These results have shown that the QuanTOF demonstrates excellent performance on precision and accuracy.

3.2. Linearity

The linearity of the method proved to be prominent within the range of HbA_{1c} values from 4.5% (26 mmol/mol) to 17.5% (168 mmol/mol). The equation of the linear regression was Y (measured HbA_{1c}, %) = 0.995 × X (expected HbA_{1c} values, %) + 0.106, with a correlation coefficient of 0.999 (Table 1).

3.3. Correlation study

HbA_{1c} values obtained with QuanTOF were well correlated with respective results from Variant II and Capillarys3 TERA ($P < .001$ for both analyzers). The linear regression analysis results were: Y (QuanTOF HbA_{1c}, %) = 0.898 × X (Variant II Turbo 2.0 HbA_{1c}, %) + 0.472, with a correlation coefficient of 0.996 (Fig. 1A); or Y (QuanTOF HbA_{1c}, %) = 0.951 × X (Capillarys3 TERA HbA_{1c}, %) + 0.258, with a correlation coefficient of 0.997 (Fig. 1C). The Bland-Altman plot showed mean differences of −0.384% (QuanTOF vs Variant II Turbo 2.0) and − 0.146% (QuanTOF vs Capillarys3 TERA), respectively with the standard deviations (SD) of 0.414 and 0.260 (Fig. 1B and D).

3.4. Glycation rates correlation between α globin and β globin

After analysis of α globin glycation rates and β globin glycation rates obtain from 56 normal samples, we found the glycation rate of α globin was well correlated with that of β globin ($P < .001$). And linear regression analysis resulted in the following equations: Y (Glycation rate of β globin, %) = 1.655 × X (Glycation rate of α globin, %)-0.229, with a correlation coefficient of 0.996 (Fig. 1E).

3.5. Effects of analytical interferences

3.5.1. Labile A_{1c}

No modification of hemoglobin was detected on the mass spectrum over the range of m/z 15,000–16,000. As compared with the baseline HbA_{1c} values, all HbA_{1c} level biases of glucose treated samples with various duration were within 0.2% (3 mmol/mol) at proportion of labile A_{1c} up to 12.2% (Table 1, Supplementary Table 1).

3.5.2. Carbamylated hemoglobin

After treatment with potassium cyanate, cHb-α globin (m/z 15,168.6) peak, as demonstrated earlier [7], were observed in mass spectrum (Fig. 2B). All HbA_{1c} values were within 0.2% (2 mmol/mol) of the baseline HbA_{1c} values with proportion of cHb up to 8.7% (Table 1, Supplementary Table 2).

3.5.3. Bilirubin and triglyceride

Measured HbA_{1c} values were within 0.1% (1 mmol/mol) ranges of the baseline HbA_{1c} values with concentrations of bilirubin and triglyceride up to 304.0 μmol/L and 22.8 mmol/L, respectively (Table 1).

3.5.4. HbF interference

When HbF was present, mass spectrum showed the peaks of γ globin chain in Fig. 2C (m/z = 15,997.4). HbA_{1c} results were not modified by HbF significantly when its percentage was lower than 8.0%. However, the bias of HbA_{1c} values exceeded 0.2% (2 mmol/mol) when greater than approximately 8.0% HbF was present. Additionally, the bias increased with the increase of the percentage of HbF (Table 1, Supplementary Table 3).

3.5.5. Common hemoglobin variants

Of the four most common Hb variants (i.e. HbS, HbD, HbC, and HbE), only S globin (m/z = 15,838.7) can be detected by QuanTOF (Fig. 2D). Nevertheless, the bias of measured HbA_{1c} results with QuanTOF exceeded 5.0% (NGSP criteria, < ± 5%) in comparison with Ultra^2. Although HbD, HbC, and HbE were not detected, acceptable biases compared with Ultra^2 were observed (Table 1).

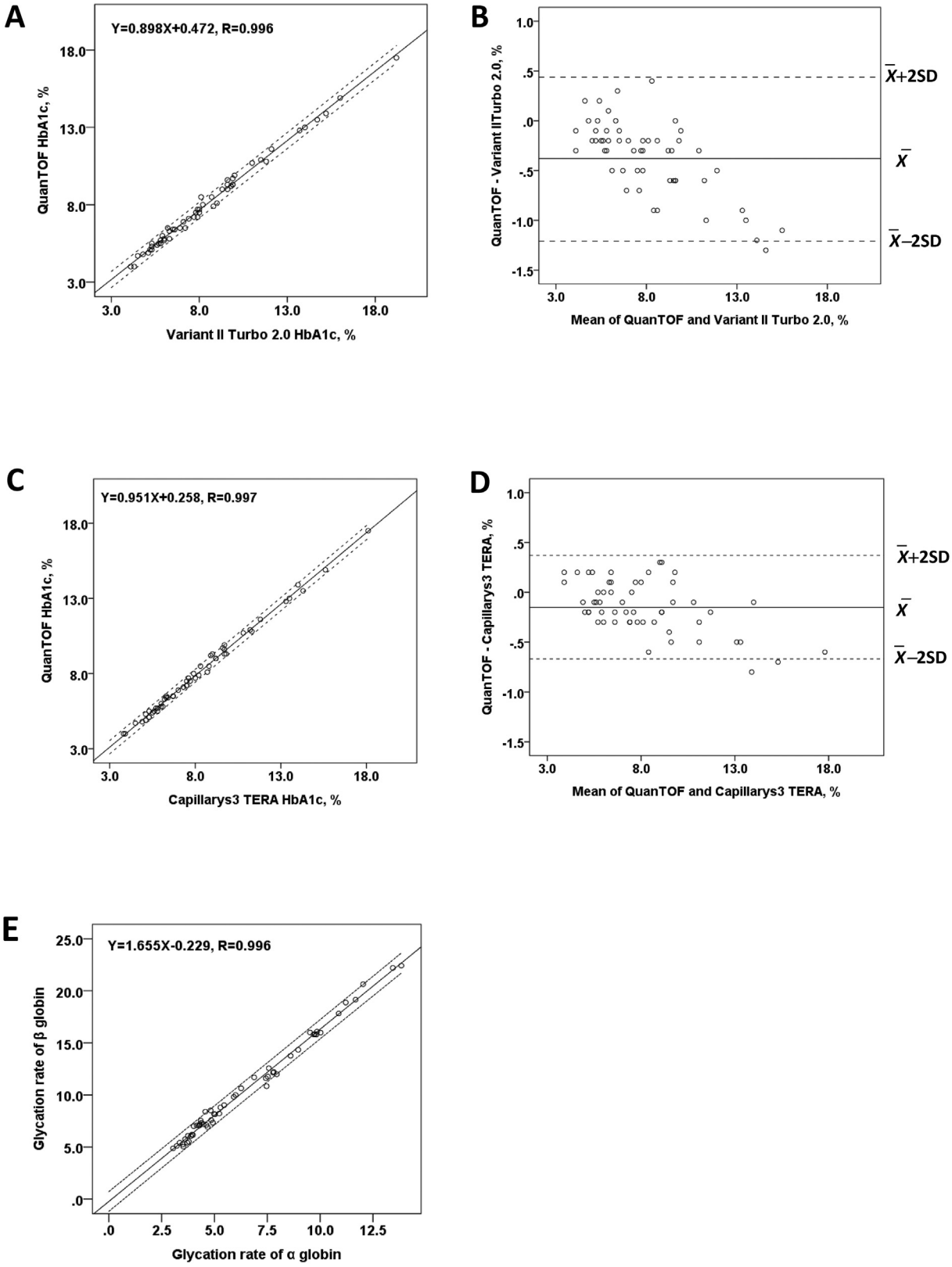
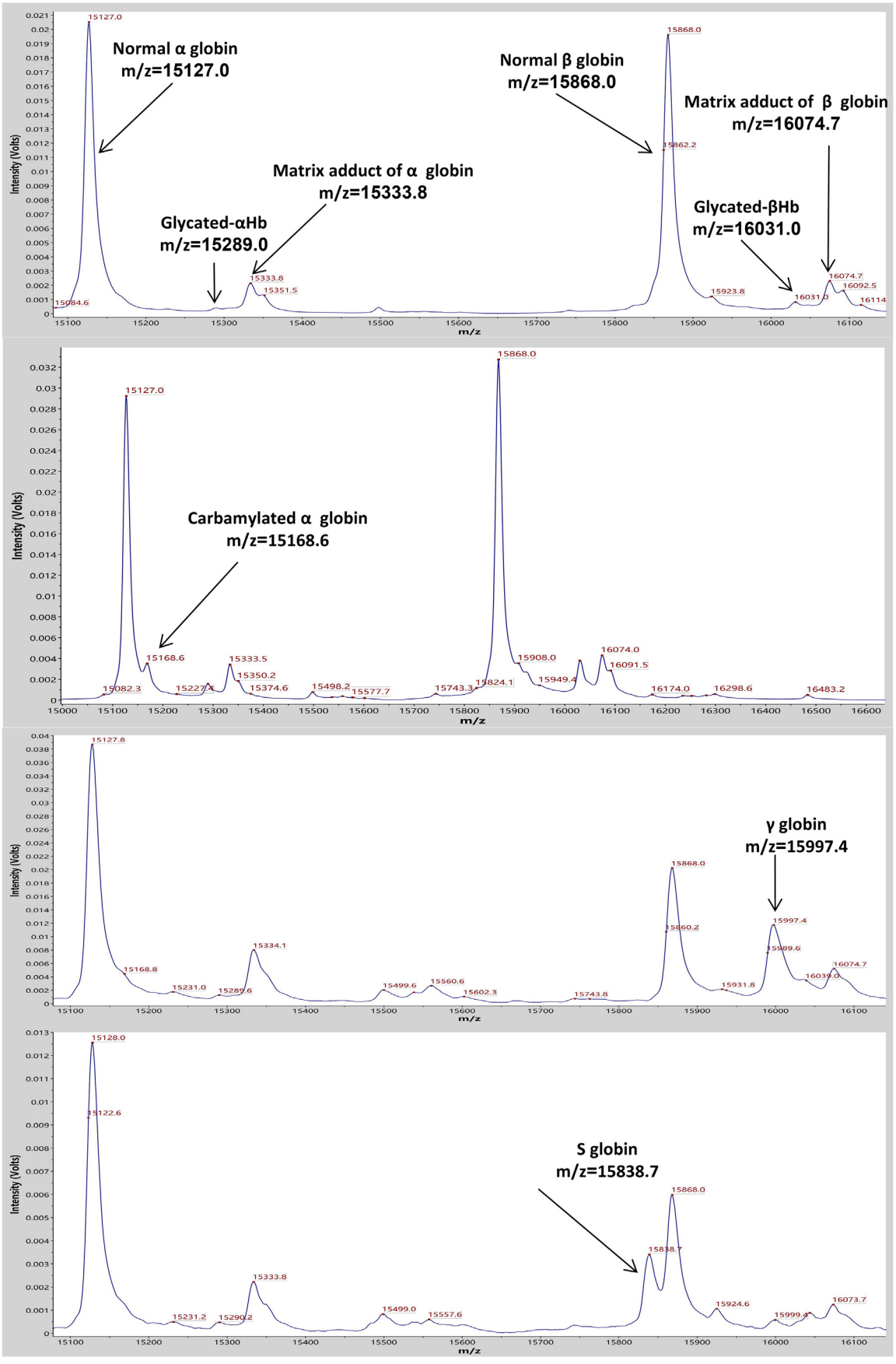


Fig. 1. Method comparison with linear regression analysis and Bland-Altman plots. HbA_{1c} results obtained with the QuanTOF were compared with those obtained with Variant II Turbo 2.0 (A and B) and Capillarys3 TERA (C and D) respectively. The solid line shows the estimations of mean differences $\bar{X}$, and the dashed lines indicate the $\bar{X} \pm 2SD$. Fig. 1E shows linear regression analysis of glycation rates correlation between α globin and β globin.



(caption on next page)

Fig. 2. MALDI-TOF spectrum of normal and variant hemoglobin. Fig. 2A shows a mass spectrum of a normal sample with the known masses for MH + 1 of α globin (*m/z* = 15,127.0) and β globin subunits (m/z = 15,868.0), as well as the corresponding glycated β globin (m/z = 16,031.0) and glycated α globin (m/z = 15,289.0). Other known peaks with high masses are sinapinic acid matrix adducts [m/z = 15,334 (α globin), m/z = 16,077 (β globin) m/z] [Reference NO. 4]. Fig. 2B shows a mass spectrum of a normal sample treated with potassium cyanate; cHb-α globin (m/z 15,168.6) peak were observed in mass spectrum. Mass spectrum showed the peaks of γ globin (m/z = 15,997.4) in the presence of HbF (C). Fig. 2D shows detection of S variant globin (m/z = 15,838.7) by QuanTOF.

4. Discussion

HbA_{1c} plays a key role in assessing long term glycemic control quality in diabetes mellitus. It is crucial to measure HbA_{1c} with an analyzer as well as related quantification method to provide reliable results. HbA_{1c} is defined as glucose attachment to the N-terminal valine residue of the β globin. The greatly enhanced reproducibility of MALDI-TOF MS makes it well suited for the relative quantitation of glycated β globin vs. non-modified β globin - glucose modification results in a 162 Da increase in β globin mass which can be readily distinguished.

Glucose may also modify other amino acid residues such as lysine residues present on β globin [8]. Since MALDI-TOF MS cannot distinguish these glycated species with same molecular weight, HbA_{1c} results obtained with QuanTOF contain various glycated species. Nevertheless, this study showed good performances of QuanTOF in routine HbA_{1c} analytical test. Reproducibility study showed total CVs is well within the recommended precision [9]. In addition, the results presented in this study are in good agreement with target values of external control samples as well as those obtained from Variant II and Capillarys3 TERA.

The study indicated that HbA_{1c} quantification by MALDI-TOF MS was not prone to common interferences such as triglyceride, bilirubin, labile A_{1c}, and cHb. Labile A_{1c} forms at early stage of hemoglobin glycation process; its concentration varies with change of blood glucose level. Carbamylation hemoglobin, detected by QuanTOF in this study, results from the modification of urea-derived isocyanic acid (-$OCNH_2$-, molecular weight 42) and mostly increases in patients with renal failure [10]. Molecular weight of cHb-α globin (*m/z* = 15,168.6) observed in mass spectrum consist with the alteration of cHb modification on α globin (m/z = 15,127.0). Recent research shows Labile A_{1c} and cHb are still interfere with HbA_{1c} measurement by HPLC [11]. QuanTOF Data demonstrate that MALDI-TOF MS is superior to HPLC in this matter.

HbF, consist of 2α chain and 2γ chain, remain a very common and important interfering substance in HbA_{1c} determination. It was documented that significant bias was induced by HbF when its proportion increased up to 20% or higher [12]. QuanTOF can detect the presence of γ globin. However, when HbF percentages exceeded approximately 8.0%, unacceptable positive bias was found, suggesting that high level of HbF interfere with HbA_{1c} measurement.

HbA_{1c} determination in the presence of hemoglobin variants is a crucial issue [13]. Hemoglobin variants could interfere with HbA_{1c} measurement due to the effects of the hemoglobin variants on the specific method used or on the alteration of erythrocyte lifespan [14,15]. To date, there are > 1300 hemoglobin variants identified [16]. The four most common hemoglobin variants, known as HbS, HbC, HbD, and HbE, result from a single amino acid substitution in β globin chain of valine to glutamic, lysine to glutamic, glutamine to glutamic and lysine to glutamic, respectively [16]. Accordingly, the respective mass difference is ~30 Da (S globin), ~1 Da (C globin), ~1 Da (D globin), and ~1 Da (E globin). Consequently, QuanTOF could only distinguish the presence of S globin among the four most common Hb variants due to mass resolution limitation of a linear time-of-flight analyzer. However, unacceptable positive biases were observed as compared with an affinity HPLC method, which was routinely used as a comparative method in previous studies [6]. This may due to limitation of resolution resulting in overlapping mass peaks arising from molecular components of normal and variant β globins, their glycated forms and matrix adducts, giving rise to interference with HbA_{1c} quantification.

In case of HbD, HbC, and HbE, although mass spectra cannot discern the globin variants from normal one due to merged mass peaks, HbA_{1c} quantification results do not seem to affected. Acceptable bias suggest that these variants probably have no interfering effects on HbA_{1c} quantification by QuanTOF, mainly because mass peaks of respective forms (glycated and non-glycated) of variant and normal globins were merged due to 1 Da mass difference between normal and the variant globins. Since a small amount of samples with hemoglobin variants were used in this part of evaluation study, more data are needed to further investigate the interference of Hb variants on HbA_{1c} measurement by QuanTOF. Although MALDI-TOF MS may not be able to distinguish variant globin when their mass differences are too small, many hemoglobin variants can be easily detected [7].

Correlation of glycation rate of α globin and β globin in this study confirmed the previous finding [5,17]. Similar to β globin subunit, α globin subunit can also be glycated on its N-terminal valine residue and other amino acid residues [8]. Our study showed an excellent correlation between glycation rates of α globin and β globin. Furthermore, glycation rate of β globin are much higher than that of α globin. The correlation can be used as an additional information to validate the HbA_{1c} values; any discrepancy between the glycation rate of α globin and β globin them may indicate an error in the HbA_{1c} assay. Another potential use of the correlation is to validate and calculate HbA_{1c} values in the presence of variant hemoglobin. For example, glycation rate of α globin can be used to calculate HbA_{1c} values in the presence of variant β globin and vice versa. To calculate HbA_{1c} values based on the intensity ratio of glycated α globin, a reliable correlation standard curve should be established firstly.

In conclusion, the QuanTOF HbA_{1c} assay evaluated in this study showed good analytical performances in precision, accuracy, linearity, and good correlation with other popular methods. What's more, it was not prone to the most frequent analytical interferences in HbA_{1c} assay. Comparing with classical methods such as CE and HPLC, MALDI-TOF MS have costs and time advantages due to low costs of matrix consuming and fast scanning speed. Moreover, MALDI-TOF MS provides more information regarding glycation rate of α globin, of which it may offer more potential applications for HbA_{1c} assay of hemoglobin variants.

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Appendix A. Supplementary data

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