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A quantitative method for the analysis of glycated and glutathionylated hemoglobin by matrix-assisted laser desorption ionization-time of flight mass spectrometry

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Abstract

The quantization of glycated isoforms of hemoglobin has been increasingly used in clinical practice in recent years. Glycated hemoglobin is currently considered the most important measurement for long-term control of the glycemic state and it has become a reference tool for the management of diabetes. Glutathionylated hemoglobin is an increasingly clinically relevant covalent adduct of glutathione with β chain of the globin and its concentration has been correlated with oxidative stress. We have developed an innovative technique based on linear mode matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry for quantitative analysis of hemoglobin species. This method was applied to the quantification of glycated and glutathionylated hemoglobin. A rigorous comparison was pursued to evaluate the analytical performances in quantifying glycated hemoglobin in comparison to an established high-performance liquid chromatography method. Our results indicated a complete equivalence between the two methods. The same analysis enabled the quantitative determination of the glutathionylated hemoglobin fraction. This isoform was investigated in an adult Italian population (184 individuals, 101 males and 83 females), indicating a bimodal distribution of this species. In fact 65.22% of screened individuals had glutathionylated hemoglobin levels lower than 0.50% while 34.78% had glutathionylated hemoglobin levels higher than 0.50%. A semiautomatic robotic procedure was developed for fast analysis of a large number of samples. This is the first report of a quantitative application of linear MALDI-TOF mass spectrometry for the determination of glutathionylated hemoglobin in blood samples. This method allows fast screening of this hemoglobin isoform, therefore opening the route to explore its specificity and sensitivity as a molecular biomarker.

Keywords: Hemoglobin; Glutathione; HbA_{1C}; MALDI-TOF; Mass spectrometry; Proteomics

The analysis of hemoglobin chains has been of particular interest in recent years since the observation of specific posttranslationally modified isoforms could be correlated with a particular disease. The evaluation of

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glycohemoglobin levels (HbA_{1C})[1] in diabetic patients is a valuable technique for monitoring glucose levels throughout a large time window [1]. Recently a new species of β hemoglobin adduct, glutathionylated hemoglobin, has been introduced in clinical practice to evaluate oxidative stress disorders and it has been proposed as a biochemical marker connected to the onset of cardiovascular disease [2,3]. The value used to quantify the glycohemoglobin is HbA_{1C}, which is a general term referring to the products of glucose, binding slowly and nonenzymatically to hemoglobin by a ketoamine bond [4]. These products include the condensation of glucose with N-terminal valines of β chains [5] and the glycation of N-terminal valine(s) of α chain(s) [6]. Moreover, ε amino groups of the globin lysine residues might also undergo glycation [6]; experimental evidence indicates that in vitro the sites of modification can vary, depending on the physico-chemical conditions [6,7]. However in vivo, glycation occurs exclusively on a single site at the time and no species with multiple glycoside moieties have been reported, even at high percentage of HbA_{1C}. Detailed analysis, performed with enzymatic digestions of glycated proteins coupled with liquid chromatographic separation and mass spectrometric detection, uniquely identified these primary glycation sites on α and β chains involving N-terminal residues [8]. Several procedures and numerous commercial instruments, based mainly on chromatographic separation methods, are currently available for the determination of HbA_{1C} in blood samples. Measurement of this parameter every 6 months is strongly recommended by the American Diabetes Association (ADA) in all diabetic patients, even under glycemic control regime [9]. Therapeutic protocols should reduce the percentage of HbA_{1C} lower than 7.00%, and such treatments undergo reevaluation if this level gets higher than 8.00% (ADA). Measurement of glycated hemoglobin (HbA_{1C}) adduct is a key parameter to predict the risk of developing diabetic complications.

Quantification of glutathionylated hemoglobin is an interesting parameter to verify in vivo balance between antioxidant- and oxidant-producing systems [10]. This ratio determines the ''oxidative stress,'' which has been associated with some diseases such as cardiovascular [2], neurodegenerative [11], and diabetes and atherosclerosis [1]. Glutathione (γ-L-glutamyl-L-cystein-glycine) is a tripeptide that represents the major sulfhydryl compound in the intracellular compartment present at millimolar concentration in the blood cells and at micromolar concentration in the plasma. This molecule plays a pivotal role in the protection of cells and tissue from oxidative damage, by shifting redox equilibrium between reduced (GSH) and oxidized (GSSG) glutathione forms. The balance between these two states is regulated by a NADPH-dependent glutathione reductase. In the reduced form, GSH inhibits injury of free radical by eliminating reactive groups (e.g., reactive oxygen species) and protects proteins from oxidative damage. The redox couple (GSH-GSSG) has been used for directly monitoring oxidative stress [12,13]. The oxidized glutathione can be present in two forms as homo- and as heterodisulfide dimer with other thiol compounds, such as proteins. This latter form is a reversible protein posttranslational modification, which might well influence and modulate protein functions by an oxidative stress response mechanism [14]. The mixed disulfide protein–glutathione adducts represent a direct picture of an imbalance effect in the oxidant/antioxidant system. In fact, the use of glutathionylated hemoglobin has been proposed as a clinical marker and its increased levels have been associated with diabetes, hyperlipidemia, and uremia [10,12,15].

Electrospray ionization (ESI) triple–quadrupole mass spectrometry has been introduced as a primary reference method for accurate quantitative measurements of hemoglobin species, such as glycated hemoglobin, single nucleotide polymorphism, and glutathionylated hemoglobin [16–19]. Unfortunately, this technique has relatively low tolerance for salts and sample impurities. Moreover, instrumentation costs and laborious analysis procedures strongly limit the possibility to employ this technique on medium-large sample numbers. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a promising technique for clinical applications given its high sensitivity and resolution, even in the presence of limited amount of salts, buffers, and other contaminants [20,21]. Moreover, time and cost of analysis for each sample are particularly low, given the ion source setup, which eventually might handle thousands of samples in a single acquisition run. These qualities have made MALDI-TOF MS an instrument of choice for the investigation of large numbers of clinically relevant molecules in serum, blood, urine, tissue extracts, and whole cells [22–24]. MALDI-TOF MS analyses of blood samples have been already employed to accurately measure molecular average mass of hemoglobin α and β chains [25,26] to potentially identify allelic variants and/or posttranslationally modified species, such as the glycated chains. Unfortunately, the resolution observed in such experiments has limited the capability to quantify all the different species. As a matter of fact none of these studies has reported a rigorous quantitative comparison of obtained data versus an

[1] *Abbreviations used:* HbA_{1C}, glycohemoglobin; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; GSH, reduced glutathione; GSSG, oxidized glutathione; TFA, trifluoroacetic acid; DTT, dithiothreitol; GHb_M, glycated β hemoglobin quantified by MALDI-TOF MS; GSS-Hb_M, glutathionylated β hemoglobin quantified by MALDI-TOF MS; ESI, electrospray ionization; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; SA, sinapinic acid; MTP, multi titer plate.

established method, such as HPLC ion exchange coupled with spectrophotometric detection assay.

Our work has addressed the development and improvement of a quantitative linear MALDI-TOF mass spectrometric technique to analyze blood samples. In the following we describe an investigation into the potential use of MALDI-TOF MS for quantifying glycated and glutathionylated hemoglobin as a percentage of the modified β-chain forms (glycated and glutathionylated) with respect to the β-chain free form (named GHb_M and $GSS\text{-}Hb_M$, respectively). These values have been compared with HbA_{1C} obtained by an HPLC method, which has an analytical imprecision within the International Federation of Clinical Chemistry (IFCC) recommendation limits. An improved procedure has been developed to implement a robotic format for handling of 750 samples/day, minimizing the operator manual work.

Materials and methods

Materials

Dithiothreitol (DTT), horse heart cytocrome *c*, and myoglobin were obtained from Sigma–Aldrich (Milan, Italy). Sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid; SA), solvents for sample preparation and spotting on 384 Multi Titer Plate (MTP) target, and trifluoroacetic acid (TFA) were purchased from Fluka (Milan, Italy).

Preparation of blood samples

For glycated and glutathionylated hemoglobin analysis, blood specimens were taken from 101 (49 males and 52 females) and 184 (101 males and 83 females, age >20 years) adult patients, respectively, attending the Policlinico Tor Vergata (Rome, Italy). Informed consent was obtained for all blood samples used in this investigation.

Blood samples were collected in EDTA-containing tubes. Whole blood (1 mL) was washed three times with 8 mL of saline solution and centrifuged for 10 min at 15,000 rpm at room temperature. Hemolysate was prepared by mixing sedimented cells (about 500 μL) with 1.5 mL of water.

Analyses of glycated hemoglobin were performed on hemolyzed samples that were fresh or stored at −80 °C for no longer than 1 month, while analyses of the glutathionylated hemoglobin were performed both on fresh and on singly frozen and thawed blood samples.

Glycated hemoglobin by routine analysis

Routine hemoglobin analyses were carried out by an HPLC ion exchange chromatography system (Hi-AUTO A1c A. Menarini Diagnostics—Arkray KDK) equipped with a photometric detection unit operating at 415–500 nm, where the range of values for HbA_{1C} of a healthy subject was 4.30–5.90%. This instrument is certified by A. Menarini Diagnostics European Reference Lab ESRL 7 according to NGSP following the methods and reagents as having documented traceability to the Diabetes Control and Complications Trial (DCCT) Reference Method.

Matrix and sample preparation for MALDI-TOF MS

Samples were prepared with a sandwich layer method. First, a matrix layer was prepared by applying a droplet (0.5 μL) of 30 g/L of saturated solution of SA matrix dissolved in 100% ethanol to the surface of a smooth stainless steel sample plate and allowing it to dry. A fine crystalline layer was formed within a few seconds. A droplet (0.5 μL) of 30 g/L of saturated solution of sinapinic acid matrix, dissolved in acetonitrile and 0.1% trifluoroacetic acid (1:1), and analyte solution (1:100 dilution), mixed with equal volumes (5 μL), was placed on the surface of the crystalline seed layer and dried in air at room temperature. The stainless steel sample plate was then inserted into the MALDI instrument.

Linear MALDI-TOF MS analysis and calculation of glycated or glutathionylated hemoglobin

All MALDI analyses were performed with a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. Ion source and flight tube were kept at a pressure of about 4×10^{-7} mbar by turbo molecular pumps. All spectra were acquired in linear mode at 20, 18, and 9.40 kV for ion sources 1 and 2 and lens, respectively. Laser power was fixed to 25% with a pulsing rate of 10 Hz to allow about 1000 ion counts for each single acquisition series. A single acquisition run was the sum of at least 30 series with 990 total added shots. Mass spectra were analyzed using Bruker software Xtof. All spectra were processed by smoothing and baseline subtraction using a polynomial function. The tentative interference of such a smoothing data transformation routine was evaluated by calculating variation percentage (2.37%) for glycated hemoglobin in a range between 5.6 and 12.7% for transformed and untransformed spectra (15 samples). $GSS\text{-}Hb_M$ was affected by the smoothing routine by a variation percentage of 3.03% (15 samples). Spectra were first calibrated using an external calibration by horse heart cytocrome *c* $[MH]^+$ 12,361.19 and Myoglobin $[MH]^+$ 16,952.61. Internal calibration was performed on the screened samples by α and β non-modified singly charged signals produced by nonallelic variant hemoglobin chain [25,26]. Glycated and glutathionylated β hemoglobin quantified by MALDI-TOF

(GHb_M and GSS-Hb_M, respectively) were calculated as percentages of the modified form, glycated or glutathionylated, with respect to the free form of β-chain hemoglobin, according to the following equations:

$$GHb_M = \frac{\text{Intensity } \beta\text{\_Glycated Hb}_{m/z\,16031}}{\text{Intensity } \beta\text{\_free Hb}_{m/z\,15868}} \quad (1)$$

and

$$\text{GSS-Hb}_M = \frac{\text{Intensity } \beta\text{\_Glutathionylated Hb}_{m/z\,16174}}{\text{Intensity } \beta\text{\_free Hb}_{m/z\,15868}}. \quad (2)$$

Automatic spotting and acquisition

Automatic sample preparation, spotting on 384 MTP target, and acquisition of spectra for patients were also performed. Samples (hemolysate or blood) were prepared (see above) using a Biomek2000 (Beckman Coulter) instrument. After dilution and mixing with equal volume of 30 g/L of saturated solution of sinapinic acid, a droplet (1.5 μL) of matrix–analyte mixture was deposited on matrix seed layer (preformed with 0.5 μL of saturated solution of sinapinic acid dissolved in 100% ethanol on the 384 MTP target). To avoid evaporation, each solution was prepared in a plate sealed by an aluminum foil lid (Beckman Coulter) and then perforated by a multipipette by the robot. Samples were then left to dry completely at atmospheric pressure at a temperature of 23–25 °C. Crystallized samples so prepared on a 384 MTP target were ready for mass spectrometric analysis. Acquisition was developed by AutoXecute routine method on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics) using SpreadSheet generator from Bruker Daltonics. Conditions were similar to the manual mode setup. However, some specific parameters were developed: (a) laser range was 25–60%, (b) peak resolution for both nonmodified hemoglobin forms to be added had to be higher than 500 (FWHM), and (c) measurement of the sample surface area was accomplished at each MALDI target position by the spiral large algorithm provided by Bruker Daltonics to optimize maximal surface sampling. Mass spectra were processed, smoothed, and baseline subtracted and GHb_M and GSS-Hb_M calculated according to Eqs. (1) and (2).

Statistical method

Data consisted of percentages of glycated or glutathionylated species with respect to free hemoglobin β chain. Moreover, to compare and assess the agreement between HPLC HbA_{1C} and GHb_M MALDI-TOF techniques for glycated hemoglobin, differences between results of two methods were plotted against averages of two methods, using the Bland and Altman plot by MedCalc software. A distribution plot was performed to analyze GSS-Hb_M levels in the population.

Results

MALDI-TOF MS and analytical performance of glycated hemoglobin

We initially investigated the suitability of MALDI-TOF MS for the detection and analysis of a hemoglobin sample. The corresponding positive ion mass spectrum is shown in Fig. 1. We can observe two major peaks at 15,127 and 15,868 *m/z*, corresponding to the singly protonated α and β chains of hemoglobin, respectively. Peaks at mass values 15,289 and 16,031 *m/z* can be assigned to the singly charged species, belonging to the condensation of a glucose moiety on the α and β chains of hemoglobin, respectively. Other visible peaks at higher masses can be assigned to heme adducts for each hemoglobin chain (15,740 and 16,483 *m/z*, α and β chains, respectively) and to sinapinic acid matrix adducts with α and β hemoglobin (15,334 and 16,077 *m/z*, respectively). The value of glycated hemoglobin (GHb_M) is calculated as percentage of intensity ratio of β-glycated hemoglobin (16,031 *m/z*) over β hemoglobin (15,868 *m/z*). Recorded mass spectra are analyzed by calculating the percentages of glycated hemoglobin species and measuring their relative peak intensities. In the sample reported in Fig. 1, the GHb_M ratio is 7.10%.

To evaluate the analytical performance of this method, we investigated the reproducibility in intra- and interday series. For this purpose, 10 samples at different ratios (low, medium, and high) of GHb_M were analyzed and the coefficients of variation (CV%) for intra- and interday assays determined (Table 1). The average imprecision (CV%) values of the linear MALDI-TOF MS method were 2.62% for intraday and 5.52% for interday analyses in all investigated GHb_M ranges. Since protein adducts with ionization matrix might slowly take place after deposition of the sample, we have investigated variations of GHb_M values at different acquisition times after spotting. In Fig. 2 is reported the average value of GHb_M in a time course experiment (seven different times) performed on the same sample spotted three times. GHb_M value does not significantly change over the investigated time window. Samples acquired within 3 h after spotting show imprecision of 2.20%. This time window is well suited to the sample preparation and acquisition procedure here described. Longer analysis times might lead to higher imprecision values due to the formation of SA adducts. In fact we observed interday imprecision of about 10% when the presence of the matrix adduct significantly altered the GHb_M value (GHb_M 7.13%, CV% 10.70%; GHb_M 5.83%, CV% 9.60%).

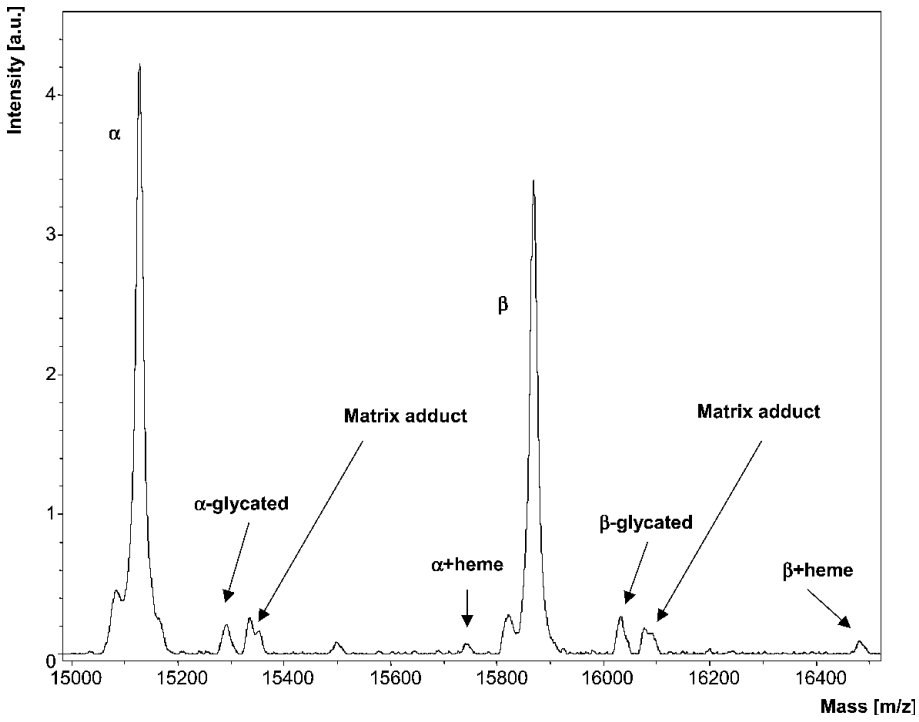

Fig. 1. MALDI-TOF spectrum of a hemoglobin sample. Spectrum annotation is according to data obtained from other authors [25].

Table 1
Coefficient of variation (CV%) for intraday and interday assays for GHb_M performed in triplicate experiment

Sample	Mean value (%GHb_M)	Intraday (%CV)	Interday (%CV)
1	3.69	3.23	3.86
2	5.47	3.39	5.52
3	6.60	2.70	6.53
4	7.00	2.85	7.05
5	10.51	2.48	5.86
6	10.70	2.36	5.98
7	5.50	1.58	5.93
8	7.00	2.80	4.50
9	11.40	2.82	4.60
10	8.50	2.00	5.38
Mean CV (%)		2.62	5.52

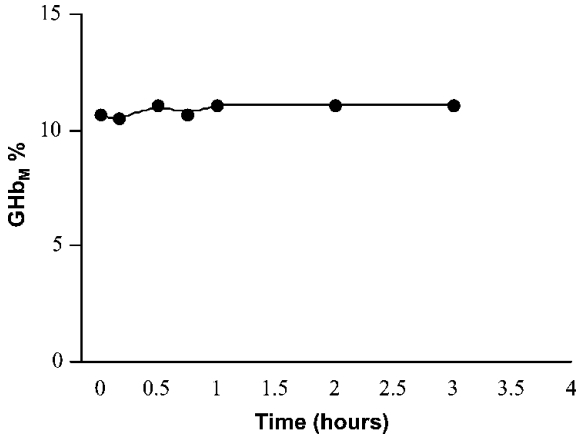

Fig. 2. Time course acquisition of a hemoglobin sample acquired in triplicate with a standard deviation ±3% for the seven experiments.

Automated procedure

To investigate a larger population by this method, we have developed an automatic procedure to prepare, crystallize, and analyze glycated and glutathionylated hemoglobin species. In this setup we have used a Biomek2000 (Beckman Coulter) instrument coupled to an automatic acquisition method, developed by AutoXecute suite on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics). This latter procedure has been optimized to enhance linear response on the TOF analyzer. In particular, the constraint of a minimal resolution (FWHM) of 500 is set as a specific parameter to evaluate spectra goodness before data averaging.

This method has been developed to work directly with whole blood, without the need to prepare hemolysate for each sample. A dilution of whole blood directly in deionized water is operated by the robotic system, thus inducing erythrocyte breakage. This step decreases time of acquisition and the actual cost for preparation. Sinapinic acid matrix solution is kept in an aluminum-foil-sealed vessel to avoid evaporation of organic solvent during preparation of the layer. This lid is perforated by a multipipette tool and mixed with a preformed blood solution; aspiration and dispensation are set to avoid the problems determined from the blood samples. After spotting on the target, all mass spectra were processed

and GHb_M and GSS-Hb_M calculated. From these data, we have estimated a coefficient of variation for the GHb_M of about 2.70%, comparable with the imprecision estimated for the manual procedure.

Glycated hemoglobin determination by MALDI-TOF MS vs HPLC

An important issue has been to compare data obtained with a linear MALDI-TOF MS method and with a validated routine assay, such as A. Menarini-Arkray Hi-AUTO A1c. This investigation initially used 10 replicate experiments in an interday setup with 10 samples spanning a wide range of HbA1c concentrations. Results show an average absolute percentage difference of 1.98% throughout the explored range (Table 2). This investigation was then extended to a higher number of samples (101 patients, 49 males and 52 females) using an automatic acquisition procedure in an intraday setup. Direct comparison of linear MALDI-TOF MS results versus the HPLC data has shown a good agreement, with a correlation coefficient of $r^2 = 0.92$ ($p < 0.0001$) and an angular coefficient of 1.07 ± 0.04. To better highlight possible differences between HPLC and MALDI-TOF MS data, we have employed a Bland–Altman graph (Fig. 3) which is indicative of a high correlation between the two assays. In fact, only 4 samples were outside ± 1.96 standard deviation borders, which lie on percentages of difference of +1.94% and −1.40%.

MALDI-TOF MS and analytical performance of glutathionylated hemoglobin

In linear MALDI-TOF MS of hemoglobin sample, it was possible to observe a peak at 16,174 *m/z* (Fig. 4). This signal has been reported to correspond to the conjugation of β hemoglobin Cys^{93} with glutathione in the singly protonated form [12]. In fact, this reversible

Table 2
Independent measurements of glycated hemoglobin by MALDI-TOF MS and by HPLC performed in triplicate experiment

Sample	HPLC value (%)	MALDI-TOF value (%)	Percentage of difference (%)
1	5.40 ± 0.09 SD	5.50 ± 0.36 SD	1.85
2	5.70 ± 0.12 SD	5.83 ± 0.17 SD	2.23
4	6.20 ± 0.07 SD	6.60 ± 0.31 SD	6.06
5	6.45 ± 0.10 SD	6.55 ± 0.36 SD	1.55
6	7.10 ± 0.14 SD	7.13 ± 0.13 SD	0.42
7	10.71 ± 0.17 SD	10.51 ± 0.20 SD	−1.90
8	10.90 ± 0.23 SD	10.70 ± 0.31 SD	−1.87
9	5.50 ± 0.15 SD	5.38 ± 0.32 SD	−2.23
10	7.00 ± 0.04 SD	6.92 ± 0.31 SD	−1.15
11	11.40 ± 0.13 SD	11.12 ± 0.51 SD	−2.52
Absolute mean			1.98

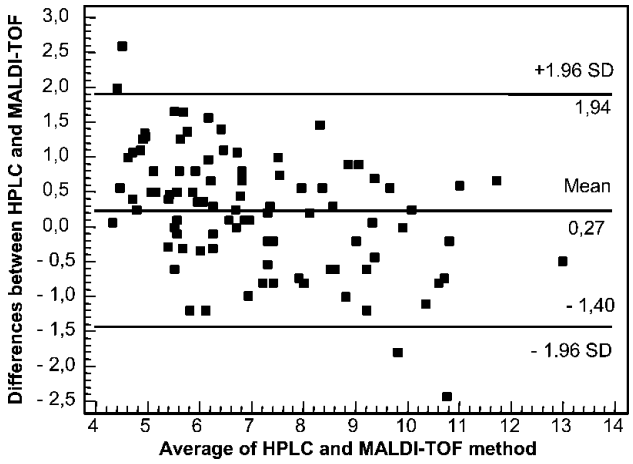

Fig. 3. Comparison between HPLC and MALDI-TOF data using a Bland–Altman plot.

chemical bond has been confirmed by preincubating the sample with a reducing agent, 100 mM dithiothreitol (DTT). After 5 min at room temperature peak intensity is reduced by 50% (Fig. 5B) with respect to that of the nonreduced form (Fig. 5A) and after 1 h at 37 °C glutathione adduct was absent (Fig. 5C). Quantitative analysis, integrating all peaks before and after addition of DTT, revealed a significant modification only of the peak referred to GSS-Hb_M in the investigated mass range (data not shown).

To evaluate the analytical performance of this method, we have investigated the reproducibility of analyzing blood samples of GSS-Hb_M to evaluate the coefficient of variation (CV%) for intra- and interday assays (Table 3). The average imprecision (CV%) values of the linear MALDI-TOF MS method were 4.62% for intraday and 7.55% for interday analyses in all investigated GSS-Hb_M ranges.

These values were obtained from each sample after a single thawing process. To establish whether the GSS-Hb_M value varies with time and storage condition, we have calculated the imprecision values for a sample affected by multiple cycles of freezing and thawing (CV% = 15.05%) and for samples stored on ice after 1, 2.5, or 4 h (CV% = 18.24%) or at room temperature (CV% = 21.84%) after a single thawing process. Comparing these values with those obtained from a single sample after having been thawed once (CV% = 8.83%), we can observe that the GSS-Hb_M value changed significantly with the storing conditions. Single samples were aliquoted and stored at −20 °C before use and each aliquot was used within a short time after thawing.

Given the increasing interest in quantization of GSS-Hb_M, we have evaluated the possibility to analyze this hemoglobin adduct on a population of 184 subjects (101 males and 83 females). GSS-Hb_M levels are estimated as percentage of glutathionylated hemoglobin β with respect to total hemoglobin β, see Eq. (2). Fig. 6

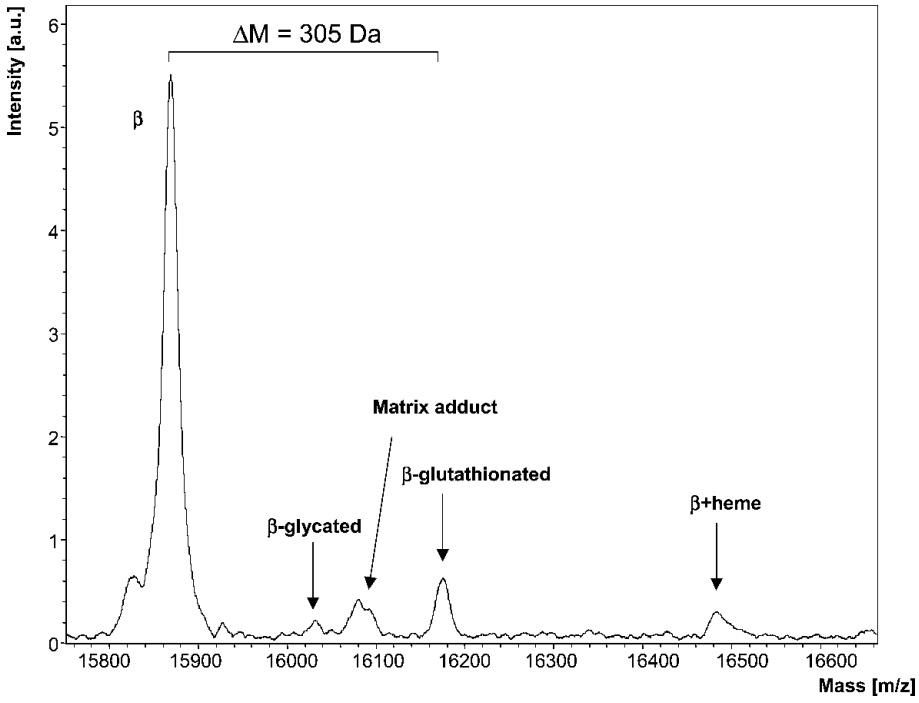

Fig. 4. MALDI-TOF spectrum of β hemoglobin sample.

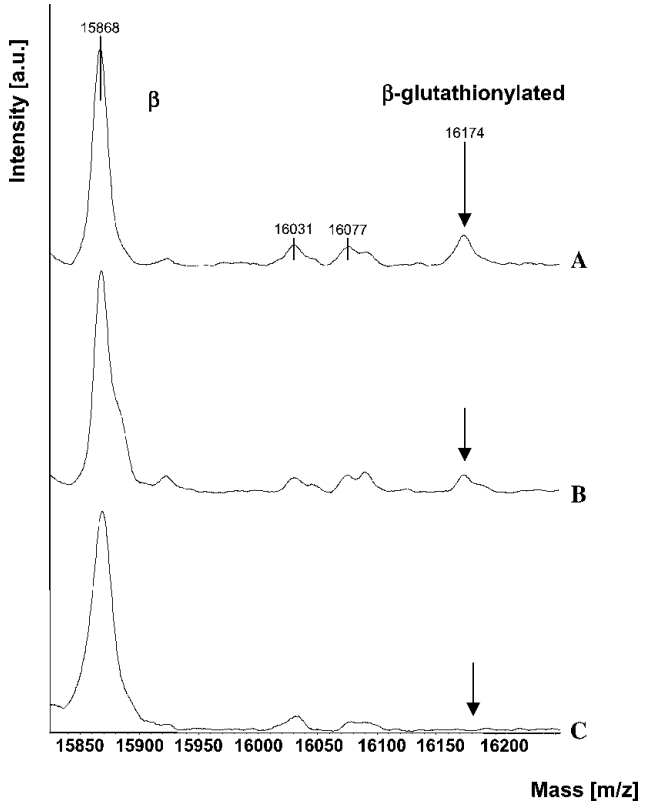

Fig. 5. MALDI-TOF spectrum of a hemoglobin sample with: (A) normal, (B) 100 mM DTT 5 min at room temperature, and (C) 100 mM DTT 1 h at 37 °C.

Table 3
Coefficient of variation (CV%) for intraday and interday assays for GSS-Hb_M performed in triplicate experiment

Sample	Mean value (%GSS-Hb_M)	Intraday (%CV)	Interday (%CV)
1	8.10	3.89	5.51
2	7.80	3.98	8.83
3	4.10	4.90	8.63
4	6.30	5.70	7.22
Mean CV (%)		4.62	7.55

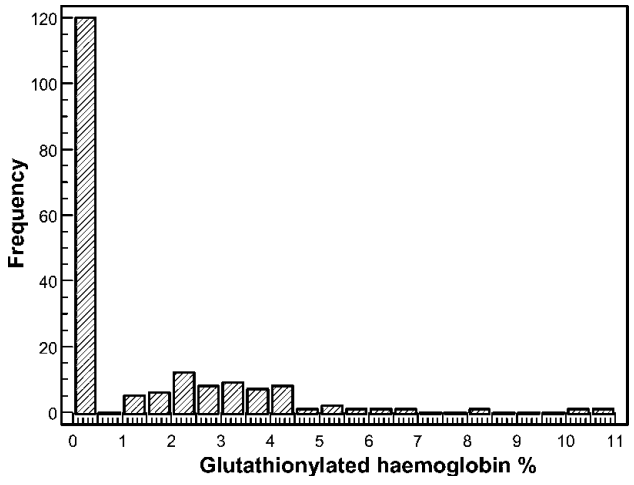

Fig. 6. Distribution plot of 184 samples of a population (101 males and 83 females) of glutathionylated hemoglobin.

is a distribution plot of calculated GSS-Hb_M percentages over the investigated population. Each data point is the average of two independent measurements. Several subjects showed GSS-Hb_M levels lower than our arbitrary threshold value, which has been evaluated at 0.50%. Samples belonging to this group are indicated

at the arbitrary value of 0.50% in the distribution plot. For these reasons, the population is equally distributed between two groups: group A with 34.80% of the population with GSS-Hb_M > 0.50% and group B with 65.20% of the population with GSS-Hb_M < 0.50%. Group A has a median value of 3.00% and 10th and 90th percentiles of 1.60 and 5.20%, respectively.

Discussion

We have described an innovative technique to detect and quantify hemoglobin posttranslational modification species as glycated and glutathionylated hemoglobin. We have developed a linear MALDI-TOF mass spectrometric technique, which is able to work both from hemolysed and from whole-blood samples, with good analytical imprecision. In fact, the described semirobotic procedure might represent a first development of an innovative cost-effective method for routine glycated hemoglobin analysis. Moreover, the applied method is simple and fast and, in our opinion, possesses numerous advantages over other methods.

The application of MALDI-TOF MS in the investigation of hemoglobin variants and glycated species already has been proposed [25]. Nevertheless a stringent comparison of the glycated hemoglobin quantified by this method and by a validated method has not yet been explored. Initial investigations have been pursued by Lapolla et al. [26] using different sample preparation and matrix, highlighting a correlation between the HbA1c values and the combined percentages of glycated and glycooxidized hemoglobin species. We have developed and evaluated the quantification performances of a linear MALDI-TOF MS method which enables higher protein mass spectra resolution [25,26] using sinapinic acid matrix. Initially we have improved preparation and crystallization of samples, which has returned a strong optimization of sensitivity and peak resolution [25,26]. This resolution allowed us to calculate a mass discrimination of approximately 15–20 Da in the working mass window, therefore, much smaller than the mass difference between glycated (+162 Da) and glutathionylated (+305 Da) species. In fact this resolution is comparable with that obtained by deconvolution of the multiple charges distribution in liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) [13,17].

MALDI-TOF MS has been often reported as a poor quantitative technique, given the fast sampling properties of the time of flight analyzer and the problems connected with heterogeneity of sample crystallization [27]. To overcome these problems we have first optimized our crystallization conditions to obtain a single homogenous layer of sinapinic acid and hemolysate. Secondly, the mass spectrometric data collection has been specifically adjusted to sample the largest crystal area while keeping a maximum number of 1000 ion counts on the detector for each acquisition series. This specific setup has shown good quantitation properties in our experiments and the evaluated analytical performances have been particularly promising. Average coefficients of variation were 2.62% for the intraday and 5.52% for the interday series (Table 1), thus demonstrating a high reproducibility in quantifying glycated β chain. Critical evaluation of the acquired data must be undertaken especially concerning the presence of interfering matrix adducts. We have performed a time course investigation on the sample (Fig. 2), which indicates an optimal acquisition time frame within 3–5 h after sample crystallization. Therefore, possible increments of GHb_M value due to matrix adducts can be excluded when operating in this time window.

Analysis of GHb_M has been performed on hemolyzed fresh samples or samples stored at −80 °C for no longer than 1 month, since we have experimentally determined that this parameter is not altered with storage (data not shown).

Interassay variations were extensively evaluated over a wide range of glycated hemoglobin percentages in both a manual and a semiautomatic setup. The interday manual design gives an average absolute percentage of 1.98% (Table 2). To fully validate and compare the observed results with those using a technique of choice in clinical routine we have studied a much larger number of potential diabetic subjects, 101 patients (49 males and 52 females). Samples were analyzed and their GHb_M values were compared with those using an established HPLC method such as A. Menarini-Arkray Hi-AUTO HbA_{1C}. Comparison of mass spectrometric results versus data obtained by the HPLC method, by a Bland–Altman plot, indicates equivalence of glycated hemoglobin results over the range encountered in routine analysis (Fig. 3). These values are well inside IFCC recommendation limits for the optimal interlaboratory dispersion (CV% < 2.50%).

A signal at *m*/*z* 16,174 was clearly visible in spectra of a few blood samples (Fig. 4). Such a signal has been previously assigned to the disulfide-conjugated form of β chain hemoglobin with the tripeptide glutathione [12,13]. This observation was further confirmed by adding DTT (Figs. 5A–C) and checking the reversibility of these adducts. Average coefficients of variation for GSS-Hb_M were 4.62 and 7.55% for intra and interday series, respectively (Table 3).

Analysis of GSS-Hb_M has been performed on fresh blood samples, since we have observed that GSS-Hb_M value is altered with replicate freezing and thawing of samples before analysis. Given the lack of a well-defined international standard assay for quantization of glutathionylated hemoglobin, we did not pursue a comparative analysis. However, a strict comparison of this method with respect to other published techniques, such as

LC/ESI-MS [12,13] and UV-VIS HPLC [28], would be of interest for our future work.

A larger study on a representative population of 184 subjects (101 males and 83 females) using the same experimental setup employed for acquisition of glutathionylated hemoglobin was then undertaken. The distribution plot (Fig. 6) of GSS-Hb_M does not have a normal distribution. The bimodal profile is mostly due to the large number of subjects showing a GSS-Hb_M level lower than 0.50%. This value represents a threshold value of detection of the presented method and it has been arbitrarily assigned to all samples with undetectable GSS-Hb_M levels. The reason for such a bimodal distribution in the total population ($n = 184$) is not yet clear, and further investigations will be necessary to screen a larger population and investigate the possible clinical and molecular correlations. In this light the presented study might strongly enhance screening and validation of GSS-Hb_M as a molecular marker, by providing a swift and reliable tool for its quantization. Moreover, data acquired with this technique might be directly correlated to GHb_M levels and to the possible presence of hemoglobin polymorphism or drug adducts [29].

We have developed a semiautomatic robotic method that enables preparation, crystallization, and analysis of hemoglobin species. Polymorphism variants and glycated and glutathionylated hemoglobin could all be detected using the very same analytical procedure. A Biomek2000 (Beckman Coulter) robotic preparing unit was coupled to an automatic acquisition method on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics). Overall analysis times are not yet comparable with those of commercial fully automatic instrumentation such as A. Menarini-Akray Hi-AUTO HbA_{1C}, roughly one sample every 4 min, or with even higher-performance instruments such as the immunochemistry-based systems. However, mass spectrometric analysis on the MALDI-TOF MS instrument was about 3 min/sample with the presented setup. Moreover, this acquisition time could be easily reduced down to 30 s/sample by simply reducing the number of acquisition series without altering the methods analytical performance (data not shown). It is relevant to mention that a few hardware optimizations might significantly reduce acquisition times.

This study presents a development in the potential use of MALDI-TOF MS in medicine for quantification of hemoglobin species, in particular of glycated hemoglobin. Large population studies on glutathionylated hemoglobin could be easily pursued by this method, given the simple preparation setup and the fast analysis time. A number of new issues have been identified for future investigations and discussions with regard to the extension of quantitative applications of MALDI-TOF MS in other clinically relevant molecules.

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