Talanta 115 (2013) 323–328

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Impact of glutathione-HbA_{1c} on HbA_{1c} measurement in diabetes diagnosis via array isoelectric focusing, liquid chromatography, mass spectrometry and ELISA

Si Li [a,1], Chen-Gang Guo [a,1], Lu Chen [b,1], Xiao-Yang Yin [a], Yi-Xin Wu [c], Liu-Yin Fan [a], Hui-Zhi Fan [b,*], Cheng-Xi Cao [a,**]

[a] Laboratory of Bio-separation and Analytical Biochemistry, State Key Laboratory of Microbial Metabolism, and School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] Department of Chemistry, Research Center of Proteomics, Fudan University, Shanghai 200433, China
[c] Ruijin Hospital, Medical School, Shanghai Jiao Tong University, Shanghai 200025, China

ARTICLE INFO

Article history:
Received 10 March 2013
Received in revised form
16 May 2013
Accepted 19 May 2013
Available online 23 May 2013

Keywords:
HbA_{1c}
HbA_3
Liquid chromatography
Isoelectric focusing
ELISA
Mass spectrometry

ABSTRACT

Hemoglobin A_{1c} (HbA_{1c}) has been proven to be a key biomarker for diabetes screening, and glutathiolation of HbA_{1c} (viz., GSS-HbA_{1c}) has been identified. However, the impact of GSS-HbA_{1c} on the measurement of HbA_{1c} for diabetes screening has not been quantitatively assessed yet. To address the issue, the micropreparative capillary isoelectric focusing (cIEF) developed in our previous work was used for the high resolution separation and purification of hemoglobin (Hb) species. The main fractions of HbA_0, HbA_3 and HbA_{1c} extracted from the developed cIEF were identified by validated Mono S method. The proposed GSS-HbA_{1c} fractions in the cIEF were pooled and identified by electrospray ionization mass spectrometry (ESI-MS). The HbA_{1c} enzyme-linked immunosorbent assay (ELISA) kit was employed for further quantitative analysis of GSS-HbA_{1c}. A total of 34 blood samples with HbA_{1c} levels from 4.2% to 13.4% were assessed via the above comprehensive strategy of IEF-HPLC-MS-ELISA. It was demonstrated that the HbA_{1c} levels detected by cation exchange LC were considerably influenced by the glutathiolation of Hb and the range of detected GSS-HbA_{1c} values was between 0.23% and 0.74%. The results and developed cIEF methods have considerable significances for investigation of diabetes and clinical diagnosis.

1. Introduction

The prevention and treatment of diabetes have already become serious world-wide public health issues. The global prevalence of diabetes in 2000 was 171 million adults, and the number would increase to 366 million in 2030 [1]. The overall prevalence of diabetes and pre-diabetes in China increased to 92 million and 148 million adults in 2010 respectively, as a result of the rapid change in lifestyle [2]. Hemoglobin A_{1c} (HbA_{1c}) was the major glycated Hb (GHb) species, which was formed via non-enzymatic reaction in vivo between glucose and β-chains of HbA in human erythrocytes [3,4]. The HbA_{1c} level was positively correlated to the average blood glucose concentration over the last four weeks to three months. Thus, the measurement of HbA_{1c} had been used for screening and assessment of long-term serum glucose regulation of diabetes [5,6].

Glutathiolated HbA, viz., HbA_3, has gradually become a potential biomarker for clinical diagnosis. As early as 1957, Beutler et al. showed that GSH played a crucial role in protecting oxyhemoglobin against oxidative breakdown by measuring the reduced glutathione (GSH) content in drug-sensitive red blood cells [7], and Mills revealed that GSH served as the most effective and dominant hydrogen donor in anti-oxidization [8,9]. Allen and Jandl in 1960–61 unveiled the reaction mechanism between GSH molecule and sulfhydryl group of Hb in vivo [10,11]. Srivastava and Beutler further reported that GSH could attach to the thiol group of cysteine residue at the position of β-93 of Hb [12]. In 1980s, Beuzard's group [13–15] systematically unveiled the detailed physicochemical properties of HbA_3. Up to now, HbA_3 has greatest potential to be a key pathogenic factor in several diseases [16].

Glutathiolated HbA_{1c} (GSS-HbA_{1c}), which may also be described as glycated HbA_3, has recently stepped into the spotlight as one of the disturbing factors in HbA_{1c} measurement. In theory, glucose molecule mainly combined with the N-terminal valine of

* Corresponding author. Tel./fax: +86 21 3420 5820.
** Corresponding author.
E-mail addresses: hzfan@fudan.edu.cn (H.-Z. Fan), cxcao@sjtu.edu.cn (C.-X. Cao).
[1] The first three authors have equal contribution to the work.

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.05.040

β-chains of Hb, while GSH might attach to the residues of Cys-β93, Asp-β94 and His-β146 [15,17], which indicated GSH and glucose could simultaneously attach to the same hemoglobin molecule. Al-Abed et al. demonstrated the existence of glycated HbA_3 or GSS-HbA_{1c} in diabetic patients via liquid chromatography-mass spectrometry (LC-MS) [18]. However, the published work was primarily focused on the elevated level of HbA_3 in diabetic patients. The quantitative analysis of GSS-HbA_{1c} and its impact on HbA_{1c} determination have not been pursued with further research [19].

The cation exchange-based HPLC (e.g. Bio-Rex 70) was formally accepted as the designated method in National Glycohemoglobin Standardization Program (NGSP) programme [20]. Because of the distinct charge difference between GSS-HbA_{1c} and HbA_{1c}, it could be easily expected that the glutathiolation of HbA_{1c} should lead to an underestimated HbA_{1c} value in the cation exchange-based HPLC. This hypothesis has been demonstrated by the facts that HbA_{1c} values were found unacceptable in some of blood samples with reinforced glutathiolation of Hb [21,22]. Unfortunately, there was a limited amount of quantitative information regarding the proportion of glutathiolated HbA_{1c}.

To address the issues mentioned above, the main purposes herein are therefore to (i) separate and identify Hb species, especially GSS-HbA_{1c}, from diabetic blood sample; (ii) quantitatively display the variation of HbA_{1c} value in glutathiolated blood sample; (iii) determine GSS-HbA_{1c} level, viz., the hidden part of HbA_{1c} by glutathiolation, in diabetes blood; and (iv) reveal the impact of GSS-HbA_{1c} on HbA_{1c} quantification in diabetes diagnosis. In order to achieve the four targets, a specific capillary isoelectric focusing (cIEF, 600 μm i.d., 1200 μm and 20 mm length) had to be developed for the micropreparative separation and purification of Hb fractions from blood samples. In addition, the validated HPLC, MS and ELISA methods were used for the relevant investigations given below.

2. Experimental

2.1. Chemicals

Unless stated otherwise, all chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent (Shanghai, China). Acrylamide (ultra-pure grade, $>99.9\%$) was purchased from Aladdin Reagent (Shanghai, China). Lithium Chloride (99%) was obtained from Alfa Aesar Chemicals (Tianjin, China). Disodium Malonate was purchased from TCI Co., Ltd. (Tokyo, Japan). N, N′-methylen- bis-acrylamid and N, N, N′, N′-tetramethyl-ethylenediamine (TEMED) were from Sigma-Aldrich (St. Louis, MO, USA). Bio-Lyte pH 6–8 carrier ampholyte was purchased from Bio-Rad (Hercules, CA, USA). The specific fused silica capillary (600 μm i.d. and 1200 μm o.d.) was from Ruipu Co., Ltd. (Hebei, China). The Human HbA_{1c} ELISA Kit and micro-plate reader ST-360 was from Kehua (Shanghai, China).

2.2. Preparation of samples

Adult blood samples were taken from healthy volunteers and patients ($n=34$) with diabetes mellitus attending the Ruijin Hospital (Shanghai Jiao Tong University School of Medicine) for health evaluation. The HbA_{1c} concentrations of the samples have a range of values from 4.2% to 13.4%, which initially assessed by the commercial VARIANT II system (Bio-Rad, CA, USA) in accordance with the introduction of equipment. The collection of blood samples and HPLC analyses were complied with the operation standards and the ethical direction of the hospital.

Hemolysates was prepared from whole blood samples (within 2 days of collection). Briefly, the blood sample was centrifuged at 2000*g* for 10 min to remove plasma. Erythrocytes were washed three times with isotonic saline. For 1 ml of washed sample, 1 ml of ultrapure water was added to lyse erythrocytes and 0.4 ml of carbon tetrachloride was used to remove membrane fragments. The hemolysates were centrifuged (ca. 6000*g*) for another 10 min, the clear supernatant was extracted and saturated with carbon monoxide, so as to prevent auto-oxidation of hemoglobin. Except trace quantity of HbF, all subjects have no other Hb variants.

The experiment of glutathiolation on hemoglobin was carried out according to previous reports [22]. The Mono S method was conducted on HPLC system (Thermo Fisher Scientific, USA) with Mono S 5/50 GL column (GE Healthcare life science, Buckinghamshire, UK), according to the published work by Jeppsson et al. [23]. In brief, HbA_{1c} was separated from other hemoglobin species by a complex LiCl gradient. The original buffer included 0.01 M sodium malonate and 0.2 g sodium azide per liter (pH=5.7). The elution buffer was original buffer plus 0.3 M of LiCl per liter. The gradient profile was started with 0.2% elution buffer, then increased to 40% in 5.5 min, followed 50% in 2 min, and reached the point of 100% in 5 min and lasted for 2 min. The elution time was set in 20 min, including 4 min of regeneration. The separated fractions were detected in the absorbance at 415 nm.

2.3. cIEF procedure

A miniaturized capillary array device was used to carry out the preparative separation of hemoglobins [24]. Briefly, the loading volume of Hb sample was approximately 0.2 μl in each capillary. The capillary (600 μm i.d., 1200 μm o.d. and 20 mm length) was pretreated with the cross-linked polyacrylamide (CPA) coating method as the separation channel [25]. A mixture of 5%(v/v) 3-(trimethoxysilyl)-propylmethacrylate and 60% (v/v) acetone were prepared as silane coupling solution. The CPA gel (5% T, 4% C) contained 2% pH 6–8 ampholytes (bio-lyte). Two electrolyte trays were respectively added into 20 mM NaOH and 20 mM sulfuric acid to achieve stable pH gradient [26]. A power supply (DYY-4C, Beijing Liu-yi Scientific Instrument Factory, Beijing, China) was set at 20 V/cm for 2 min, 40 V/cm for 2 min, and 60 V/cm for 2 min, and then set at constant voltage of 400 V/cm until the complete of IEF run. The integrated system of stereomicroscope SZX7 (Olympus, Japan) and EXCCD (Touptek, China) was used to record the focusing images of Hb species in cIEF.

2.4. Mass spectrometry

The Hb samples were dried in a vacuum centrifuge (Eppendorf, New Brunswick) and re-dissolved with equal volumes of water and acetonitrile, which was used as the working solvent. Then 2 mL/L formic acid was added to acidize the protein samples. Samples were introduced to the glass-tip of LTQ-ORBITRAP Mass Spectrometer (Thermo Electron Corporation) via infusion with 20 μL volume prepared above. The spray voltage was set at 1.6 kV and source temperature was at 200 °C. Data were acquired over a mass range of 2000–32,000 m/z and analyzed with Thermo Xcalibur Qual Browser.

2.5. HbA_{1c} ELISA assay

The HbA_{1c} ELISA was an immunoassay that the monoclonal antibody (Mab) was used to specifically recognize the first eight amino acid residues and the binding glucose at the N-terminal of β-chain of HbA_{1c} [27]. Briefly the hemoglobins were oxidized with the treatment-reagent for 10 min at room temperature. Then the micro-plate was washed three times. The Mab-enzyme (horseradish peroxidase (HRP)) conjugate was added to each micro-well (incubate for 60 min at 37 °C). Next, the HRP in the conjugate would react with the substrate tetramethylbenzidine (TMB) to

generate blue color (incubate for 15 min at 37 °C in the dark). Finally, stop reagent was added into each well and the color turned to yellow. The optical density (OD) of solution was measured at 450 nm using a microplate reader in 15 min. A standard curve of OD versus HbA_{1c} concentration was generated. Finally, the HbA_{1c} content in sample was determined by comparing the OD values to the standard curve. Each data point of the samples was the average of three times parallel tests.

3. Results and discussion

3.1. Analysis of glutathiolation on HbA_{1c} by mono S HPLC

To clearly illustrate the impact of glutathiolation on HbA_{1c} measurement, the HbA_{1c} levels of fresh blood samples and glutathiolated samples were compared via Mono S HPLC method at first. The result of the sample with 5.2% HbA_{1c} value were displayed in Fig. 1 (not all results shown herein). Fig. 1A showed the routine analysis of the presented sample for HbA_{1c} quantification. The chromatogram of the same but glutathiolated sample was shown in Fig. 1B. The main Hb species were eluted successively as follows: HbA_{1a+b}, HbA_{1c}, HbA_3 and HbA_0. The comparative results between Fig. 1A and B clearly demonstrated that the amount of HbA_3 was highly elevated in the glutathiolated sample as expected, in accordance with the previous work [22].

An important point worth noting was that HbA_{1c} value of the presented sample was considerably affected. To quantitatively demonstrate the alteration of HbA_{1c} value, we calculated the corresponding HbA_{1c} values from normal individuals and diabetic patients. The decrement of HbA_{1c} values in four representative

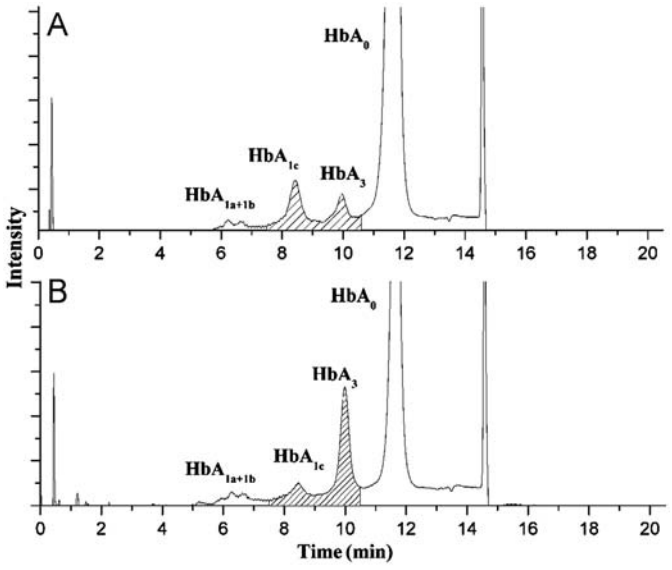

Fig. 1. Chromatograms of the fresh sample (A) and the glutathiolated sample (B) obtained by the Mono S. The main hemoglobin fractions were HbA_{1a+1b}, HbA_{1c}, HbA_3 and HbA_0, . Conditions: chromatography with the Mono S HR 5/50 column. Samples were separated by a complex LiCl-sodium malonate gradient in 20 min, including 4 min regeneration. The elution buffer contained 0.2 g/L sodium azide (pH 5.7). Flow rate was 2 mL/min, the sample size injected was 0.2 µl. The separated peaks were detected in 415 nm.

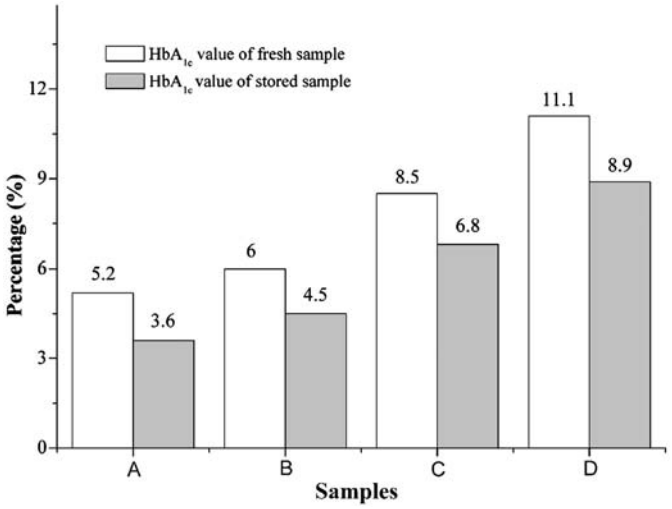

Fig. 2. Comparison of HbA_{1c} values between fresh (White bar) and glutathiolated (gray bar) samples.

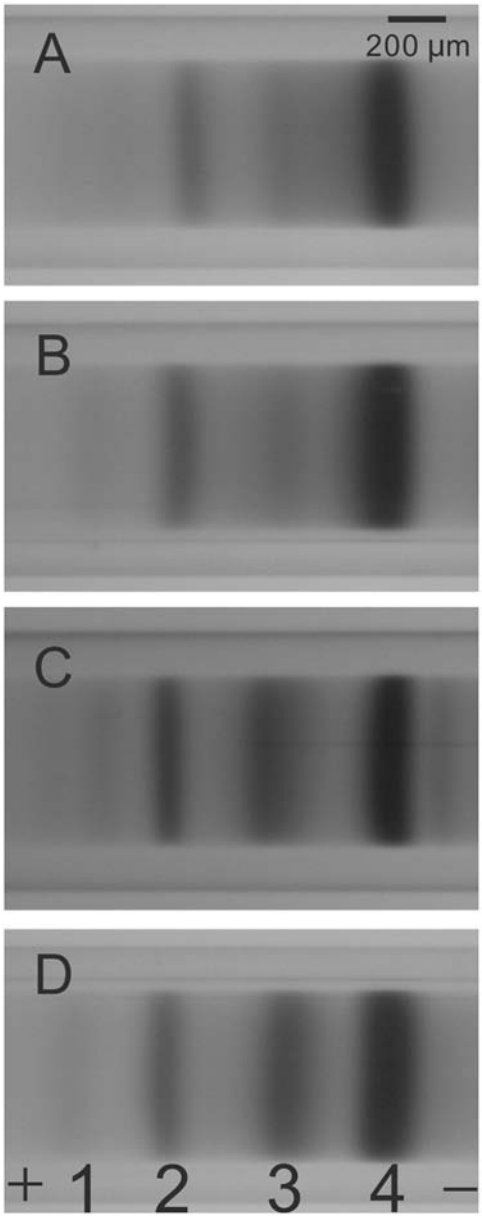

Fig. 3. High-resolution IEF images of the four glutathiolated samples. The HbA_{1c} value was: (A) 4.5% (B) 10% (C) 11.1% and (D) 12.6%. The numbers designate the following fractions: (1) GSS-HbA_{1c}, (2) HbA_3, (3) HbA_{1c} and (4) HbA_0. Experimental conditions: 600 µm i.d., 1200 µm o.d. and 20 mm length capillary, cross-linked polyacrylamide coating for inner wall of capillary, 2% pH 6–8 Bio-lyte, 20 mM NaOH used as the catholyte, 20 mM sulfuric acid used as the anolyte.

samples was shown in Fig. 2. In Panel C, for instance, the HbA_{1c} value showed a decrease from 8.5% at the onset to 6.8% after glutathiolated. This compelling result apparently demonstrated that glutathiolation reacted not only on HbA_0 but also HbA_{1c}, resulting in the formation of GSH and HbA_{1c} adduct as kind of triple-binding complex. In light of the fact that glutathiolation in erythrocytes maintains a considerable level even under normal physiological condition, it is not at all surprising that the detected HbA_{1c} values by cation exchange HPLC should be systematically underestimated due to the charge difference between GSS-HbA_{1c} and HbA_{1c}. It also offered one possible interpretation that why the HPLC affinity method performs better than cation exchange method on HbA_{1c} quantification [28,29]. Thus it was essential to quantitatively assess its impact on HbA_{1c} measurement in blood samples.

3.2. Separation and identification of Hb fractions

The separation and collection of GSS-HbA_{1c} was conducted by using the specific cIEF (600 μm i.d., 1200 μm o.d. 20 mm length) method [24]. The cIEF images of four glutathiolated samples were shown in Fig. 3. The most distinctively focused band 2, 3 and 4 were extracted from gel and confirmed as HbA_3, HbA_{1c} and HbA_0, respectively, by the Mono S HPLC method. The results were in agreement with that of the previous work [30,31].

To locate the focused position of GSS-HbA_{1c} in cIEF, there was a hypothesis that based on the electric point (p*I*) difference of different Hb species. It was known that the glycation in erythrocytes lead to the separation of HbA_{1c} from other Hb species, which was focused at anode side of HbA_0 in pH gradient. The GSS-HbA_{1c}, a sort of GSH, glucose and HbA complex, could be also regarded as the glycation product of HbA_3. Similarly, the position of glycated HbA_3 would be focused, if any, at the anode side of HbA_3 exactly in the same way. It could be observed in Fig. 3 that a visible fraction was appeared at the anode side of HbA_3 (band 1). Compared with the sample with 4.5% HbA_{1c} value (the IEF images of samples with low HbA_{1c} values were not all shown herein), the focused band 1 in the samples with 10%, 11,1% and 12.6% HbA_{1c} level (Fig. 3B–D) were more distinct.

The identification of the proposed GSS-HbA_{1c} was carried out via ESI-MS. The CPA gel including GSS-HbA_{1c} and HbA_3 (used as internal reference for mass calibration) was extracted from micro-column. In the electro-elution process, the sample was dissolved in phosphate buffered saline (PBS). To reduce the signal interference, the desalination of Hb sample was conducted via the pre-equilibrated C-18 column before MS analysis. The MS results shown in Fig. 4 indicated the accurate molecular weight of beta chain from whole blood sample and the pooled GSS-HbA_{1c} sample. In Panel A, the mass peaks at 15856 (normal β-chain), 16018 (singly glycated β-chain, 15856 plus 162) and 16164 (glutathiolated β-chain, 15856 plus 307 and H^+) were observed as control group. In contrast, the spectrum obtained from GSS-HbA_{1c} in Fig. 4B presented two elevated mass peaks (arrow marked), the larger one of which indicative of the β-chain from HbA_3 (16164), and the second one at 16325 was the corresponding peak for the β-chain of GSS-HbA_{1c}, consistent with the theoretical mass (15856 plus 307 and 162). The results shown above verified our assumption about the location of GSS-HbA_{1c} in pH gradient. In addition, the absence of mass peak at 16018 in Fig. 4B testified that HbA_{1c} fraction (band 3 in cIEF) was excluded from the separated GSS-HbA_{1c}, eliminating the possibility of false positive result in the following ELISA assessment. Taken together, the results of ESI-MS offered the direct evidence for the confirmation of the proposed GSS-HbA_{1c} complex.

3.3. Quantitative analysis of GSS-HbA_{1c} by ELISA

By presenting nine of the whole samples, Fig. 5 shows the standard curve of optical density value versus glycation degree of sample. The HbA_{1c} values of the nine samples were between 4.5%

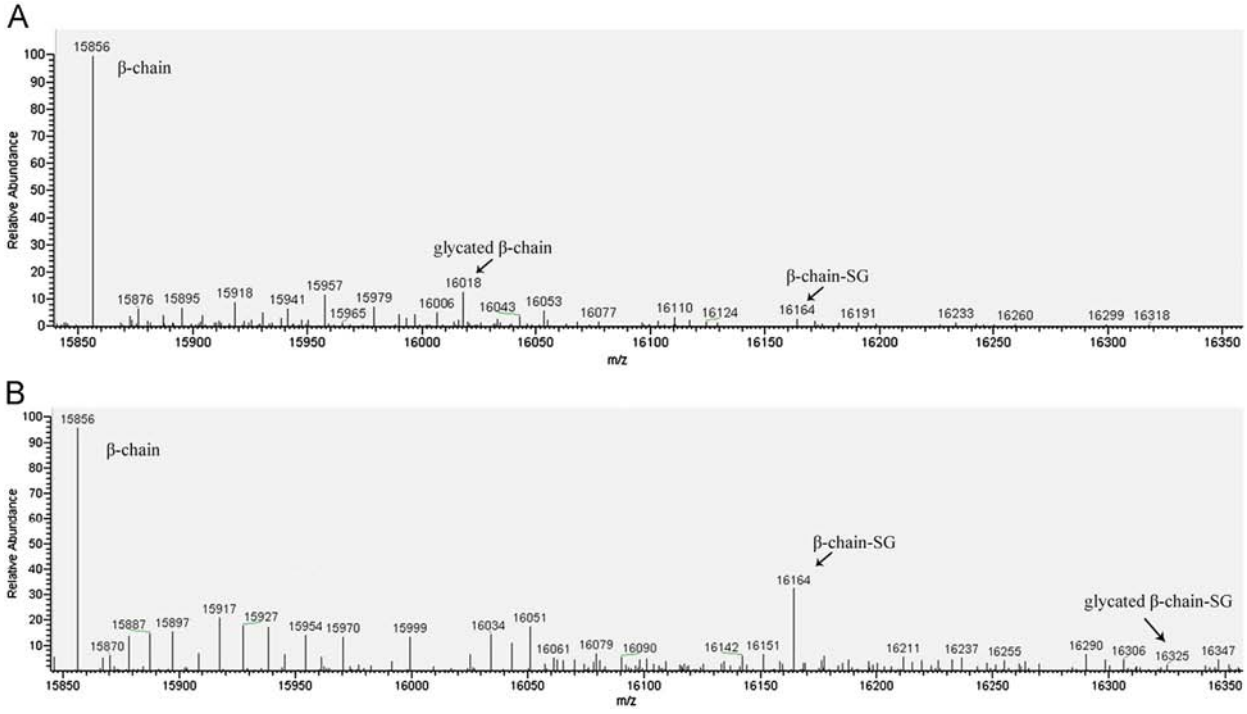

Fig. 4. ESI spectra of hemoglobin species from whole blood sample (A) and extracted GSS-HbA_{1c} sample (B). Conditions: samples were mixed with 2 mL/L formic acid to aid ionization and introduced to the glass-tip via infusion with 20 μL volume prepared above. The spray voltage was set at 1.6 kV and the heated capillary at 200 °C. Acquired mass range: 2000–32000 m/z. Data were analyzed with Thermo Xcalibur Qual Browser.

and 12.6% (Fig. 5A–I). The linearity of HbA_{1c} value to sample volume gradient was generated, with a range from 0.5 μl to 5 μl. The data points of triangle mark in blue represented the glycation degree of HbA_3 purified from cIEF, viz. the ratio of GSS-HbA_{1c} to HbA_3. The tested sample was collected from a total of 10 μl whole blood sample.

To readjust the ultimate HbA_{1c} value in whole blood sample, the ratio of GSS-HbA_{1c} to HbA_3 should be determined first. By comparing the glycation degree between purified HbA_3 and HbA in the same volume, we found that the glycation degree of HbA_3 (point of triangle mark in blue) was identical to that of HbA. The result in Fig. 5E, for instance, showed that the relative difference (RD) between the two values was 2.70%. The RD values of the presented samples were ranged from 1.13% to 5.21%. The statistical analysis of confidence level shows the paired *t*-test value is 2.02, less than the critical level of $t_{95\%}$ (2.31, $n=9$), indicating that there was no significant difference between the glycation degree of HbA_3 and total HbA. The detailed data were shown in Table 1. For the 34 tested samples, no significant difference was observed. Therefore, the glycation degree of HbA_3, viz. the ratio of GSS-HbA_{1c} -to- HbA_3, was assessed in agreement with the glycation degree of HbA.

As we discussed above, HbA_{1c} value of 6.5% was evaluated as the critical line for diagnosis of diabetes. The notion that the underestimated HbA_{1c} value was minor and/or negligible for the diagnosis of diabetes might be an inexact supposition. The HbA_{1c} value of patient who was diagnosed with prediabetes ($5.7 \le HbA_{1c} \le 6.4\%$), with high possibility, might equal/over the critical line of 6.5%. Fig. 6 shows the adjusted HbA_{1c} levels of 34 subject samples via the means of adding GSS-HbA_{1c} value. The underestimated HbA_{1c} values of whole fresh samples were calculated, ranging from 0.23%–0.74%. Remarkably, it should be mentioned in Fig. 6 that

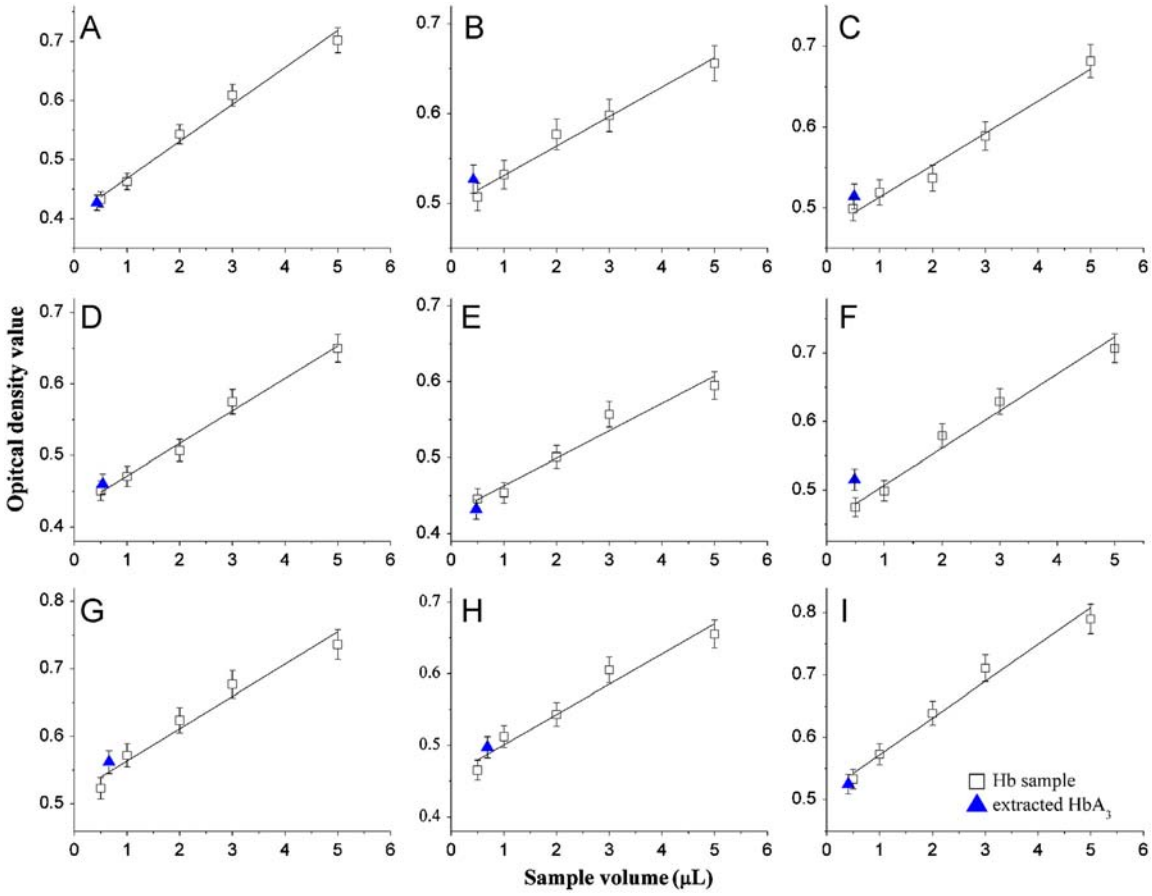

Fig. 5. Correlation between glycation degree of HbA_3 and total Hb. The HbA_{1c} values of the given blood samples were initially analyzed as follows: (A) 4.5%, (B) 5.2%, (C) 6.5%, (D) 7%, (E) 8.5%, (F) 9%, (G) 11.1%, (H) 11.6% and (I) 12.6%. The purified HbA_3 fractions were extracted from the special cIEF from a total of 10 μl loading volume of Hb sample. The corresponding HbA_3 values were calculated at a range from 4.2% to 6.3%. Error bars represent standard deviation of plots data (SD < 3%). The relative differences of glycation degree between HbA_3 and total Hb in the presented samples were shown in Table 1.

Table 1
The detailed data values of the presented samples in Fig. 5.

Sample No.[a]	A	B	C	D	E	F	G	H	I
HbA_{1c} value (%)	4.5	5.2	6.5	7.0	8.5	9.0	11.1	11.6	12.6
HbA_3 value (%)	4.2	4.7	5.8	5.0	5.3	5.4	6.1	6.3	5.1
OD value of glutathiolated Hb	0.43	0.51	0.49	0.45	0.44	0.48	0.55	0.48	0.53
OD value of equal-volume HbA sample	0.42	0.53	0.51	0.46	0.43	0.52	0.56	0.49	0.52
RD (%)	1.40	2.93	4.05	2.01	2.70	5.21	2.18	2.05	1.13
Adjusted HbA_{1c} value (%)	4.7	5.4	6.9	7.4	9.0	9.5	11.8	12.3	13.2

[a] The numbers herein correspond with the samples A–I in Fig. 5. Not all samples were shown herein.

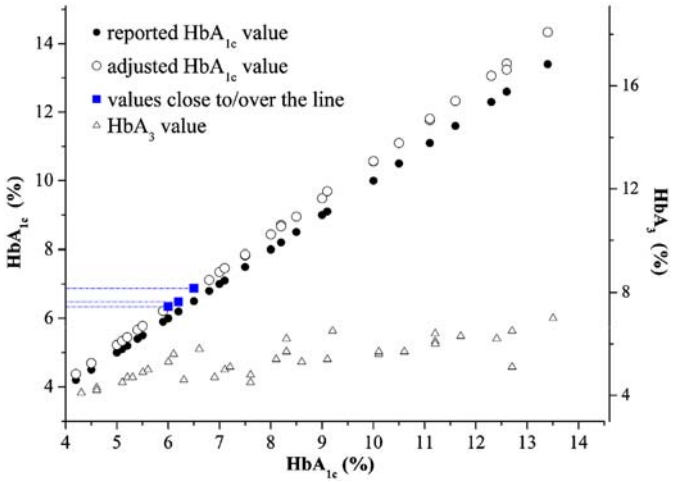

Fig. 6. Relationship between original and adjusted HbA_{1c} values. Square spots represent the most affected samples. The HbA_{1c} levels of the presented samples were at a range between 4.2% and 13.4%. The HbA_3 values were ranged from 4.1% to 7%. The detailed data of nine samples were shown in Table 1.

the HbA_{1c} values of three samples of No. 10 (6.1% adjusted to 6.4%), 11 (6.3% adjusted to 6.5%) and 12 (6.5% adjusted to 6.9%) were substantially equal to or over the key value of 6.5%, which increases the risk for false assessment of the actual HbA_{1c} value.

4. Conclusion

We performed the efficient separation of GSS-HbA_{1c} from other Hb species via the specific cIEF method, and the focused location of GSS-HbA_{1c} in pH gradient was validated for the first time. The impact of the proposed complex on HbA_{1c} measurement was quantitatively evaluated via the comprehensive strategy. Currently, the one-step collection of GSS-HbA_{1c} via cation exchange HPLC used for diabetes screening has not been reported, in that case, the cIEF method developed in our lab could be a useful approach to directly collect the fraction of GSS-HbA_{1c}. In order to enhance the detection accuracy of HbA_{1c} value in diabetes screening, we cautiously hold the thought that the further investigations on the GSS-HbA_{1c} adduct deserve more attention.

Acknowledgments

The authors are grateful for the funding provided by the National Natural Science Foundation of China (No. 21035004), the National Key Program of Scientific Instrument of China (No. 2011YQ030139) and the Shanghai Jiao Tong University (No. YG2010ZD209).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.040.

References

[1] World Health organization. Diabetes programme. Country and regional data on diabetes. ⟨http://www.who.int/diabetes/facts/world_figures/en/⟩ (accessed 2012).
[2] W. Yang, J. Lu, J. Weng, W. Jia, L. Ji, J. Xiao, Z. Shan, J. He, New Engl. J. Med. 362 (2010) 1090–1101.
[3] R.J. Koenig, A. Cerami, Proc. Nat. Acad. Sci. 72 (1975) 3687–3691.
[4] H.F. Bunn, D.N. Haney, S. Kamin, K.H. Gabbay, P.M. Gallop, J. Clin. Invest. 57 (1976) 1652–1659.
[5] R.J. Koenig, C.M. Peterson, R.L. Jones, C. Saudek, M. Lehrman, A. Cerami, New Engl. J. Med. 295 (1976) 417–420.
[6] American Diabetes Association, Diabetes Care 33 (2010), S4–10.
[7] E. Beutler, M. Robson, E. Buttenwieser, J. Clin. Invest. 36 (1957) 617–628.
[8] G.C. Mills, J. Biol. Chem. 229 (1957) 189–197.
[9] G.C. Mills, J. Biol. Chem. 234 (1959) 502–506.
[10] J.H. Jandl, L.K. Engle, D.W. Allen, J. Clin. Invest. 39 (1960) 1818–1836.
[11] D.W. Allen, J.H. Jandl, J. Clin. Invest. 40 (1961) 454–475.
[12] S.K. Srivastava, E. Beutler, Biochem. J 119 (1970) 353–357.
[13] M.C. Garel, C. Domenget, J. Caburi-Martin, C. Prehu, F. Galacteros, Y. Beuzard, J. Biol. Chem. 261 (1986) 14704–14709.
[14] C.T. Craescu, C. Poyart, C. Schaeffer, M.C. Garel, J. Kister, Y. Beuzard, J. Biol. Chem. 261 (1986) 14710–14716.
[15] S.J. Wodak, J.L.D. Coen, S.J. Edelstein, H. Demarne, Y. Beuzard, J. Biol. Chem. 261 (1986) 14717–14724.
[16] J.E. Muscat, W. Kleinman, S. Colosimo, A. Muir, P. Lazarus, J. Park Jr., J.P. Richie, Free Radic. Biol. Med. 36 (2004) 464–470.
[17] B.J. Mills, C.A. Lang, Biochem. Pharmacol. 52 (1996) 401–406.
[18] Y. Al-Abed, S. VanPatten, H. Li, J.A. Lawson, G.A. FitzGerald, K.R. Manogue, R. Bucala, Mol. Med. 7 (2001) 619–623.
[19] A. Pastore, A.F. Mozzi, G. Tozzi, L.M. Gaeta, G. Federici, E. Bertini, A.L. Russo, L. Mannucci, F. Piemonte, Anal. Biochem. 312 (2003) 85–90.
[20] J.-O. Jeppsson, U. Kobold, J. Barr, A. Finke, W. Hoelzel, T. Hoshino, K. Miedema, A. Mosca, P. Mauri, R. Paroni, L. Thienpont, M. Umemoto, C. Weykamp, Clin. Chem. Lab Med. 40 (2002) 78–89.
[21] R.R. Little, J.D. England, H.M. Wiedmeyer, D.E. Goldstein, Clin. Chem. 29 (1983) 1113–1115.
[22] C.L. Rohlfing, S. Hanson, A.L. Tennill, R.R. Little, Diabetes Technol. Ther. 14 (2012) 271–275.
[23] J.-O. Jeppsson, P. Jerntorp, G. Sundkvist, H. Englund, V. Nylund, Clin. Chem. 32 (1986) 1867–1872.
[24] S. Li, J.-Y. Dong, C.-G. Guo, Y.-X. Wu, W. Zhang, L.-Y. Fan, C.-X. Cao, W.-B. Zhang, Talanta (2013), http://dx.doi.org/10.1016/j.talanta.2013.05.041, in press.
[25] L. Gao, S. Liu, Anal. Chem. 76 (2004) 7179–7186.
[26] C.-X. Cao, L.-Y. Fan, W. Zhang, Analyst 133 (2008) 1139–1157.
[27] W.G. John, M.R. Gray, D.L. Bates, J. Beacham, Clin. Chem. 39 (1993) 663–666.
[28] L. Bry, P.C. Chen, D.B. Sacks, Clin. Chem. 47 (2001) 153–163.
[29] M. Campbell, J.S. Henthorn, S.C. Davies, Clin. Chem. 45 (1999) 969–975.
[30] H.B. Mortensen, C. Christophersen, Biochim. Biophys. Acta. 707 (1982) 154–163.
[31] M. Conti, C. Gelfi, A.B. Bosisio, P.G. Righetti, Electrophoresis 17 (1996) 1590–1596.