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ATR-FTIR detection of secondary structural stability of hemoglobin in hemolysate samples stored at freezing temperature

Htet Htet Htin Khine[1], Siriporn Proungvitaya[1], Patcharaporn Tippayawat[1,2], Chirapond Chonanant[3], Molin Wongwattanakul[1,2*]

[1] Center for Research and Development of Medical Diagnosis Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[2] Center for Innovation and Standard for Medical Technology and Physical Therapy, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[3] Department of Medical Technology, Faculty of Allied Health Sciences, Burapha University, Chonburi, Thailand.

KEYWORDS
ATR-FTIR;
HbA1c;
Hemoglobin structure;
Structural stability.

ABSTRACT

As for the importance of HbA1c for the diagnosis and monitoring of diabetes patients, many research studies evaluate HbA1c concentration upon storage. However, there are limited studies concerning the structural compositions during storage. The objective of this study was to examine the stability of secondary structure of hemoglobin in hemolysate samples stored for four months using the attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectrometer. The leftover EDTA samples with known HbA1c values were separated into normal and diabetes groups, prepared in hemolysate form, and stored at -20°C. To evaluate the secondary structure of hemoglobin, FTIR spectra were collected for four months using the Agilent 4500 portable FTIR spectrometer (Agilent Technologies, CA). Qualitative and quantitative comparisons of amide I bands were performed in Spectragryph software and Origin software. The difference between the relative intensity ratios of amide A/B and amide I/II were not significant (p-value > 0.05). In qualitative comparison, the position, pattern, and signal intensity of the second derivative spectra remained identical up to four months. In quantitative comparison graph, alpha helix and beta sheet compositions did not show increasing or decreasing trend. This study demonstrated that the structural firmness of hemoglobin in samples remains unchanged after four months of storage at -20°C.

***Corresponding author:** Molin Wongwattanakul, PhD. Faculty of Associated Medical Sciences Khon Kaen University, Khon Kaen, Thailand. Email address: moliwo@kku.ac.th

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease indicated by consistently high blood glucose, caused by either inadequate secretion of insulin from the pancreas or insulin resistance of the body cells. Statistics indicate a total of 4.2 million deaths by diabetes[1]. Glycosylated hemoglobin (HbA1c) has proven to be a keystone for glycemic control and DM diagnosis because it indicates the risk of diabetic complication development[2]. The American Diabetes Association (ADA) recommends that diabetic patients should maintain their HbA1c level below 7%[3]. The UK Prospective Diabetes Study (UKPDS) publication stated that every 1% reduction of HbA1c levels can lower the risk of diabetes complications along with the usefulness of HbA1c, the reliability of HbA1c results from clinical laboratories has become pivotal[2].

In the literature, many studies have used HbA1c analyzers to evaluate HbA1c concentrations upon storage. Bergmann and Sypniewska et al[4] evaluated the effect of a single freeze/thaw cycle on HbA1c concentrations measured by the HPLC method for 2-12 weeks. Farshad et al[5] studied the effect of different sample storage conditions on HbA1c concentrations using Cobas Integra 400 assays. Vijayachandrika et al[6] validated the stability of glycated hemoglobin measurements in blood samples stored at -20 °C for up to one month. Liotta et al[7] showed the reliability of HbA1c using the IE-HPLC method in frozen blood samples stored for 1.5 years. However, there is still a lack of structural detection in HbA1c samples during storage.

Fourier transform infrared (FTIR) spectroscopy is an analytical technique that provides information about the structure and composition of molecules by obtaining an infrared spectrum of absorption or emission. In FTIR spectroscopy, high-resolution spectral data are obtained over a wide spectral range, 4000 - 400 cm^{-1}. The attenuated total reflectance (ATR) technique uses a crystal with a high refractive index to analyze solid or thin film samples by simplified measurement. Diamond, Zinc selenide (ZnS), and Germanium crystals are commonly used for ATR. ATR-FTIR has many advantages such as rapid, non-destructive samples and requiring a small amount of sample for measurements. All forms of materials-- solid, liquid, and gas--can be identified[8]. ATR-FTIR spectroscopy can provide structural information on biomolecules like proteins, nucleic acid, lipids, and carbohydrates in their specific spectral regions[9,10]. Previous studies used ATR-FTIR to establish structural changes in hemoglobin caused by increased glucose concentration in diabetes samples, temperature and pH change[11], irradiation[12], and magnetic field[13]. However, there are limited studies in the literature regarding the structure of Hb upon storage. This study aimed to illustrate the secondary structural information of hemoglobin in HbA1c samples during four months of storage at freezing temperature.

Materials and methods

Sample collection and preparation

The leftover EDTA samples with known HbA1c results were obtained from the clinical laboratory of Srinagarind Hospital, Khon Kaen, Thailand. Samples were divided into normal and abnormal groups at 6.5% of HbA1c level as a cut-off. This study was approved by Khon Kaen University ethics committee for Human research (HE651015). The red blood cells were separately pooled for normal and abnormal groups. The hemolysate was performed by adding an equal volume of distilled water. Next, cell debris were filtrated using Whatman No.1 filter paper (Whatman, England). The pure hemolysates were aliquoted into 1.5 ml polystyrene capped tubes. After pooling, the HbA1c levels of normal and abnormal HbA1c control were measured in the pure hemolysates by Bio Rad D-10 TM analyzer (Bio Rad Laboratories, CA) based on the ion-exchange HPLC method. Then, the aliquots were immediately stored at -20°C for further measurements over four months.

Ethical Approval

This study was approved by the Khon Kaen University ethics committee for human research (HE651015).

FTIR Spectrum Acquisition

The Agilent 4500 portable FTIR spectrometer (Agilent Technologies, CA) was used to collect spectra. The ATR crystal was cleaned with deionized water and methanol before and after sample measurement. For measurement, 3 µl of sample was pipetted and placed on ATR crystal surface, spreading gently over the area. A low speed of hairdryer was applied to make a dry film. Then, spectrum was collected in spectral range 4000 - 650 cm^{-1}, with 64 scans and a spectral resolution of 4 cm^{-1}. Five replicate measurements were performed for each vial and averaged in Spectragryph software V1.2. The average spectra of each month were normalized to remove variations caused by unequal initial quantity of sample. Relative intensity ratios of amide A/B and amide I/II were compared monthly with the initial month using paired t-test (*p*-value = 0.05). The second derivative spectra of amide I band were produced for qualitative comparison of hemoglobin secondary structures using Spectragryph software V1.2.

For quantitative comparison, the baseline correction of collected raw spectra was performed and a fit was obtained in spectral range 1700 - 1600 cm^{-1} by using Gaussian curve fitting analysis in Origin software. First, the collected spectra of each month were averaged and smoothed with Savitsky-Golay smoothing at 17 smoothing points to avoid interference. Then, peaks within the spectral part 1600-1700 cm^{-1} were normalized. Second, derivative spectra were produced to detect the exact position of secondary structures of hemoglobin. After that, the spectra were put in Origin software, the baseline was corrected and Gaussian curve fitting analysis of the amide I band was performed for peak deconvolution.

Statistical analysis

Using SPSS software 28.0, the mean difference of intensity ratio between the first month and the other months was compared using a paired sample t-test. H_0 (null hypothesis): the mean difference between two groups is equal to zero. H_a (alternate hypothesis): the mean difference between the two groups is different from zero. If *p*-value < 0.05, reject the null hypothesis; if *p*-value > 0.05, accept the null hypothesis.

Results

The infrared spectra of normal and abnormal HbA1c control

The average raw spectral patterns of HbA1c normal level (Figure 1A) and HbA1c abnormal level (Figure 1B) were collected for four months. The spectra exhibited the expected absorption peaks associated to human blood[9]. As shown in figure 1, IR peaks could be described briefly as: 3290 cm^{-1} (amide A band), 3060 cm^{-1} (amide B band), 2956 cm^{-1} (asymmetric vibration of CH_3 stretching of proteins and lipids), 2872 cm^{-1} (symmetric vibration of CH_3 stretching of proteins and lipids), 1645 cm^{-1} and 1534 cm^{-1} (vibration of C=O and N-H stretching of amide I and amide II bands), 1448 cm^{-1} (bending vibration of CH_2 and CH_3 of phospholipid, fatty acids and glycerides), 1390 cm^{-1} (symmetric vibration of COO- of lipids and proteins), 1299 cm^{-1} (amide III band), 1242 cm^{-1} (asymmetric vibration of phospholipids), 1164 cm^{-1} (asymmetric vibration of COOC- of carbohydrates and proteins), 1102 cm^{-1} (symmetric vibration of phospholipids), 929 cm^{-1} (phosphodiester stretching bands region), 891 cm^{-1} (vibration of N-H of thymine).

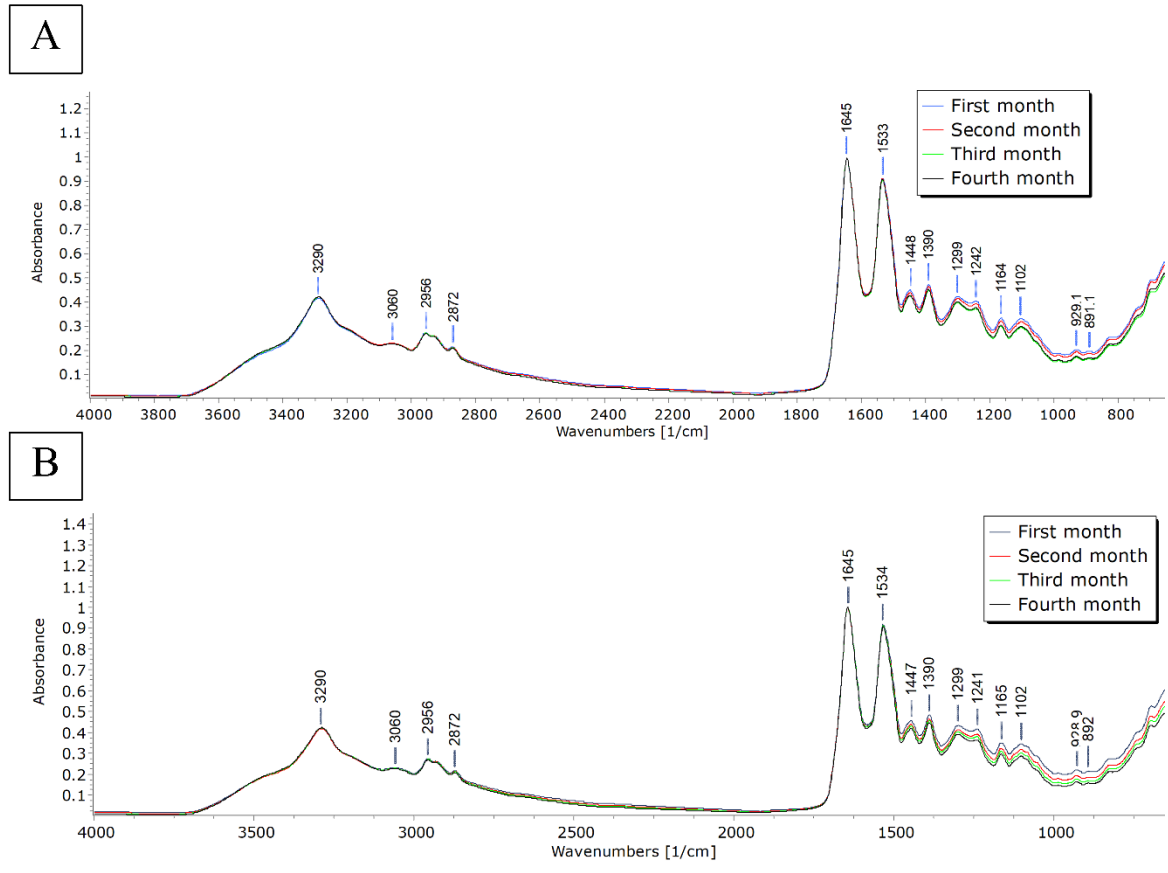

Figure 1 Average raw infrared spectra of hemolysate samples stored at 1st, 2nd, 3rd and 4th month.
(A) Normal HbA1c level
(B) Abnormal HbA1c level

Comparison of the relative intensity ratio of protein bands

FTIR spectroscopy has been proven to be a non-destructive tool for detecting secondary structural changes in proteins. The relative intensity ratio of FTIR spectra at different peaks and wavenumbers could provide information for qualitative changes in specific functional groups. The changes in protein composition were monitored using the relative intensity ratios of amide A/B and amide I/II. The values of I_{3290}/I_{3061} and I_{1650}/I_{1542} were calculated based on the averaged intensity of amide A, amide B, amide I and amide II bands. Then, the relative intensity ratios for each month were analyzed using SPSS software and paired t-tests were performed (p-value = 0.05). As shown in table 1, the difference was not statistically significant (p-value > 0.05) for both ratios, leading to the conclusion that protein functional groups did not change during storage months.

Table 1 Relative intensity ratio of amide bands in normal and abnormal groups

	Months	Normal group		Abnormal group	
		Intensity ratio	*p*-value	Intensity ratio	*p*-value
Amide A/B (I_{3290}/I_{3061})	First month	1.83±0.01		1.82±0.018	
	Second month	1.85±0.013	0.077	1.83±0.017	0.272
	Third month	1.85±0.007	0.049	1.85±0.026	0.230
	Fourth month	1.87±0.022	0.054	1.85±0.02	0.103
Amide I/II (I_{1650}/I_{1542})	First month	1.13±0.006		1.14±0.003	
	Second month	1.14±0.005	0.122	1.13±0.003	0.289
	Third month	1.14±0.003	0.295	1.14±0.007	0.314
	Fourth month	1.14±0.008	0.240	1.14±0.006	0.786

Note: The data are presented by mean ± SD.

Qualitative analysis of amide I band

The second derivative spectra of amide I bands of each month were produced to detect the qualitative composition of secondary structures of hemoglobin. Protein secondary structures such as alpha helices, beta sheets, and beta turns were observed in second derivative spectra at 1650 cm^{-1}, 1685 cm^{-1} and 1627 cm^{-1} respectively. A comparison of the position, pattern, and signal intensity of the second derivative spectra of the first month with those of 2nd, 3rd, and 4th months as shown in figure 2 and figure 3, revealed identical spectra in both groups. According to qualitative comparison, we may suggest that the secondary structure of hemoglobin remained unchanged in both groups after four months of storage at -20°C.

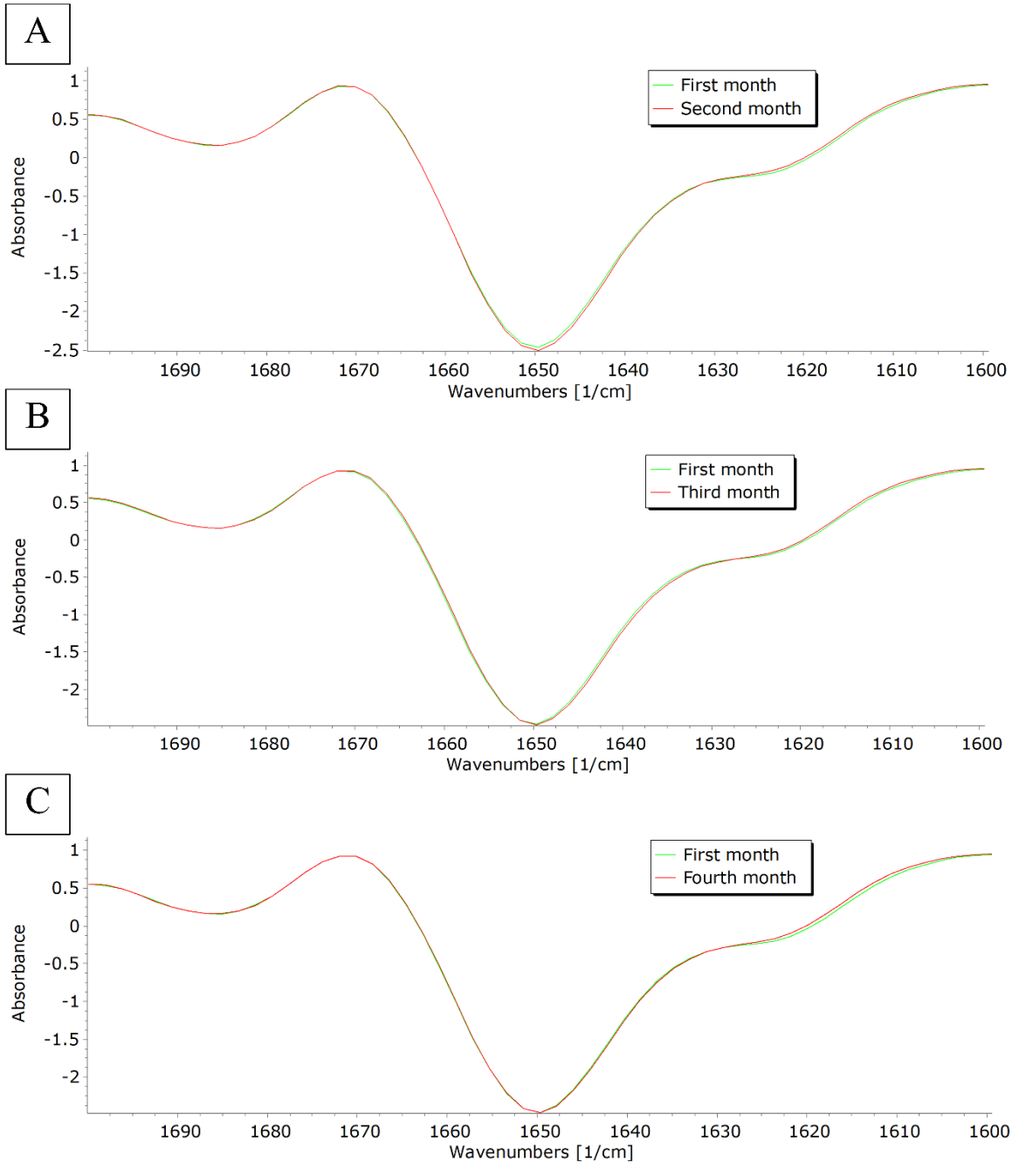

Figure 2 Second derivative spectra of amide I region of normal HbA1c level.
(A) Spectrum of the first month and the second month
(B) Spectrum of the first month and the third month
(C) Spectrum of the first month and the fourth month

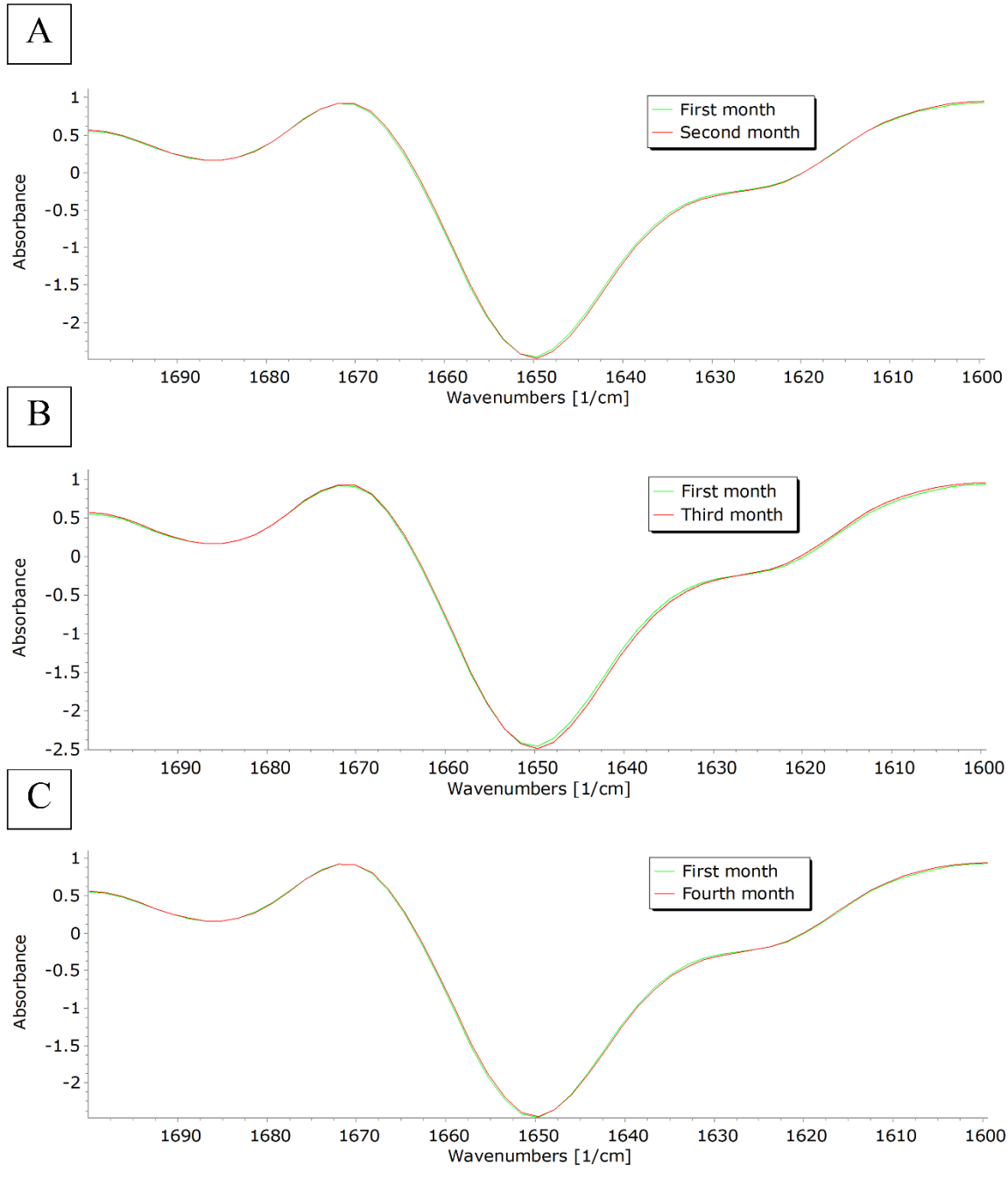

Figure 3 Second derivative spectra of amide I region of abnormal HbA1c level.
(A) Spectrum of the first month and second month
(B) Spectrum of the first month and third month
(C) Spectrum of the first month and fourth month

Quantitative comparison of amide I band

For quantitative determination of secondary structures of hemoglobin, Gaussian curve fitting analysis was performed on the amide I region using Origin software. The percentage compositions of alpha helices and beta sheets were demonstrated in figure 4. There was no change in alpha helix and beta sheet compositions in both groups. According to this finding, the secondary structure of hemoglobin remained unchanged in both groups after four months of storage.

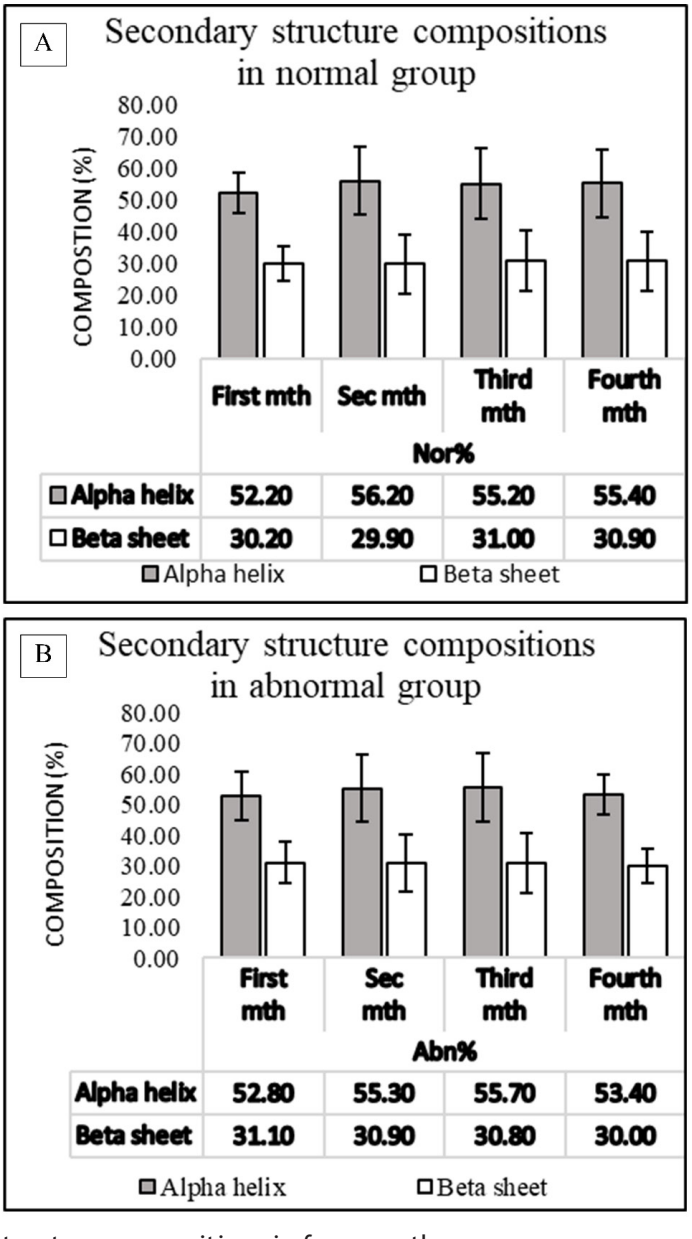

Figure 4 Secondary structure compositions in four months.
(A) Alpha helix and beta sheet compositions of normal group
(B) Alpha helix and beta sheet compositions of abnormal group

Discussion

Hemoglobin molecule is the major protein in red blood cells, and secondary structures include alpha helices, beta sheets, and other elements such as beta turns and unordered structures. In the FTIR spectrum, amide I (1600 - 1700 cm^{-1}) and amide II (1500 - 1600 cm^{-1}) bands, which exhibit the highest absorption peaks in the entire spectrum, mainly reflect protein secondary structures[14]. Andleeb et al[15] used the spectral region of 1600 – 1700 cm^{-1} to detect hemoglobin secondary structure at higher levels of hemoglobin A1c in type 2 diabetes. Their findings indicated a decrease in the intensity ratio of amide I/II in patients with HbA1c > 9% due to a change in Hb structure[15]. Ye et al[16] examined the spectra between 1600 - 1700 cm^{-1} to access the impact of HbA1c levels on the hemoglobin structure in type 2 diabetes patients. They reported that the variations of protein structures were detected by comparing the relative intensity ratio of different peaks between group H (healthy controls), group A (patients with HbA1c < 7%), and group B (patients with HbA1c > 9%). The ratio of amide I/II was slightly lower in group A (p-value > 0.05) and significantly lower in group B (p-value < 0.05) than in group H. They concluded that the Hb structure of group B may have changed[16]. Therefore, this study used the spectra between 1600 - 1700 cm^{-1} region to detect the secondary structure of hemoglobin in HbA1c samples.

Previous studies have reported the structural changes in Hb due to temperature, pH, irradiation, glucose concentration, and magnetic field changes. Increasing temperature leads to the weakening of hydrogen bonds[17]. Calabro and Magazu[13] demonstrated the unfolding of hemoglobin after exposure to a low-frequency electromagnetic field[13]. In the study of Ye et al[16], the secondary structures of hemoglobin changed as the HbA1c level exceeded 9.0%[16]. In the study of Saeed et al[12], the secondary protein structures in rat erythrocytes upon neutron irradiation were detected[12]. In the present study, we studied the secondary structure of hemoglobin at four months of storage at -20 °C. The relative intensity ratio of amide bands reflected the change in the composition of protein portions in samples. In our study, the relative intensity ratios of amide A/B and amide I/II of 2nd, 3rd and 4th months were compared with those of the initial month. There was no statistically significant difference (p-value > 0.05) between the relative intensity ratio of amide bands in both groups, suggesting that protein composition remained the same as the initial month after four months of storage.

Protein secondary structures include alpha helix, beta sheet, and beta turns. The second derivative spectra of the amide I region can indicate the exact position of these structures[18]. In Ye et al[16] study, the second derivative spectra of 1600-1700 cm^{-1} region were compared among three groups. Obvious differences were observed in the number, position, signal intensity, and pattern of the underlying components among the three groups. Andleeb et al[15] also observed that the qualitative comparison of the second derivative spectra clearly differed in pattern, number, and intensity among the three groups. In our study, the qualitative comparison in pattern, number, and intensity of second derivative spectra of both groups did not show any difference after four months of storage. We suggest that the hemoglobin structure did not change in both groups after four months of storage at -20°C. However, a limitation of ATR-FTIR technique is the interference from water vapor; therefore, lyophilized hemolysate may reduce the effects of water vapor. Moreover, the stability of hemoglobin in various temperatures and freeze-thawed conditions should be further investigated. This information can be applied to monitor the stability of hemoglobin as control material in further studies.

Conclusion

In conclusion, our study illustrated the secondary structural composition of hemoglobin in HbA1c samples over four months of storage using ATR-FTIR spectroscopy, along with the relative intensity ratio and peak deconvolution technique. According to the relative intensity ratio of amide bands and secondary structural compositions in both groups, there was no evidence of protein structures unfolding. This study concludes that the structural integrity of hemoglobin in HbA1c samples remains unchanged after four months of storage at -20°C.

Take home messages

The ATR-FTIR spectra showed the stability of the secondary structure of hemoglobin, which was stored at -20°C for four months.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Author contributions

Htet Htet Htin Khine: Conceptualization, Design of the experiments, Samples collection, Research methodology, Formal analysis, Writing – Original draft.

Siriporn Proungvitaya: Conceptualization, Design of the experiments, Research methodology validation, Writing – Review & Editing.

Patcharaporn Tippayawat: Conceptualization, Design of the experiments, Research methodology validation, Writing – Review & Editing.

Chirapond Chonanant: Conceptualization, Design of the experiments, Research methodology validation, Writing – Review & Editing.

Molin Wongwattanakul: Conceptualization, Design of the experiments, Research methodology validation, Formal analysis, Resources, Project administration and funding acquisition, Writing – Review & Editing.

Data availability

Data available on request due to privacy/ ethical restrictions

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