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Rapid determination of human globin chains using reversed-phase high-performance liquid chromatography

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) of human globin chains is an important tool for detecting thalassemias and hemoglobin variants. The challenges of this method that limit its clinical application are a long analytical time and complex sample preparation. The aim of this study was to establish a simple, rapid and high-resolution RP-HPLC method for the separation of globin chains in human blood. Red blood cells from newborns and adults were diluted in deionized water and injected directly onto a micro-jupiter C18 reversed-phase column (250 mm × 4.6 mm) with UV detection at 280 nm. Under the conditions of varying pH or the HPLC gradient, the globin chains (pre-β, β, δ, α, $^{G}\gamma$ and $^{A}\gamma$) were denatured and separated from the heme groups in 12 min with a retention time coefficient of variation (CV) ranging from 0.11 to 1.29% and a peak area CV between 0.32% and 4.86%. Significant differences ($P < 0.05$) among three groups (normal, Hb H and β thalassemia) were found in the area ratio of α/pre-β+β applying the rapid elution procedure, while $P \geq 0.05$ was obtained between the normal and α thalassemia silent/trait group. Based on the ANOVA results, receiver operating characteristic (ROC) curve analysis of the δ/β and α/pre-β+β area ratios showed a sensitivity of 100.0%, and a specificity of 100.0% for indicating β thalassemia carriers, and a sensitivity of 96.6% and a specificity of 89.6% for the prediction of hemoglobin H (Hb H) disease. The proposed cut-off was 0.026 of δ/β for β thalassemia carriers and 0.626 of α/pre-β+β for Hb H disease. In addition, abnormal hemoglobin hemoglobin E (Hb E) and Hb Westmead (Hb WS) were successfully identified using this RP-HPLC method. Our experience in developing this RP-HPLC method for the rapid separation of human globin chains could be of use for similar work.

1. Introduction

Hemoglobin (Hb), the oxygen-carrying moiety of erythrocytes, is a polypeptide tetramer of two α-like chains (ζ or α) and two β-like chains (ε, $^{G}\gamma$, $^{A}\gamma$, δ or β) surrounding a heme molecule. During embryonic development, the ζ, α, ε or γ chains contribute to embryonic Hbs such as Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Gower 2 ($\alpha_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$). The α and γ chains contribute to Hb F ($\alpha_2\gamma_2$) during fetal development [1]. In normal adults, the major Hb type is Hb A, which consists of two α and two β chains, while the minor Hb fractions are Hb A2 ($\alpha_2\delta_2$) (<3.5%) and Hb F (<2.0%). In normal newborns, Hb F fraction is approximately 70% while that of Hb A is approximately 30%.

Thalassemias are inherited disorders of hemoglobin synthesis that are found at high frequencies in countries, which are historically afflicted with endemic malaria, including Southern China. The geographical correlation of the disease distribution with the historical endemicity of malaria suggests that thalassemias have risen in frequency through natural selection by malaria because of genes conferring genetic resistance to malaria in humans [2]. The two main types of thalassemia are α and β [3]. Point mutations or deletions cause low or no expression of the globin gene leading to imbalance between the α-like and β-like chains, which is the etiology of thalassemias. The ultimate method for the diagnosis of different types of thalassemias is DNA analysis based on polymerase chain reaction (PCR) [4]. Thalassemia caused by deletion mutations is usually detected by gap-PCR while reverse dot blot (RDB) is useful for point mutations [5,6]. Many other strategies such

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as denaturing HPLC, multiplex ligation-dependent probe amplification, and high-resolution melting have been applied [7–9]. However, a direct DNA approach without a precise biochemical hematological indication can be time-consuming, expensive and subject to false negative results or misinterpretations [10]. The complex relationship between genotype and phenotype make the diagnosis difficult. Thus, a combination of different tests is required for accurate diagnosis.

In fact, phenotype studies still occupy a key position in the diagnosis of hemoglobin disorders despite the development of a number of molecular biology techniques [11]. Several markers such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and Hb A2 are recommended as complementary tests. Globin chain analysis is always helpful for detecting thalassemias and abnormal Hbs. Proper identification of an unknown Hb variant is important to determine if it is causing a clinical abnormality or is simply a polymorphism. Reversed phase high-performance liquid chromatography (RP-HPLC) could be useful for this approach [12–14]. Globin chain analysis is also useful for monitoring gene therapy and other related hematologic studies [15–19].

To date, techniques such as cellulose acetate electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis and capillary zone electrophoresis have been used for globin chain detection [20]. However, these methods have long analysis times or use extracted denatured globin chains. RP-HPLC, a generally applied tool for the separation, purification and quantification of proteins from biological materials, has greatly influenced and stimulated research in the analysis of human globin chains [21]. This methodology has a number of advantages: it is fast and accurate, uses minute amounts of material, has good resolution and reproducibility, and most importantly, can be completely automated. A variety of RP-HPLC gradient programs with long analytical times have been used to analyze human globin chains. This method reveals differences in hydrophobicity, improving the separation of Hb, which is difficult to achieve with electrophoretic systems (e.g. urea-triton electrophoresis and isoelectrofocusing) [22]. The separation is usually based on the application of an increasingly hydrophobic environment to the chromatographic column, which is filled with an interacting lipophilic stationary phase. Practically, the condition of the dissociated heme group could be well created at a pH below 3 using a constant concentration of trifluoroacetic acid and the order of elution, therefore, primarily depends on the hydrophobicity of the individual chains [21].

Rapid separation of human globin chains by RP-HPLC has been reported, but the technique still required time-consuming globin precipitate extraction and the resolution was limited [23]. The analytical time and sample pre-treatment of this method were the two main problems for its clinical application. Therefore, a simple, fast and high-resolution method is required. Furthermore, a challenge to analysis of human globin chains is the selection of initial separation conditions and subsequent optimization of the appropriate experimental parameters for a column without a ready gradient program.

In this paper we describe a rapid chromatographic procedure for human globin chain separation without globin chain extraction or even cell washing. Such a similar protocol for simple sample preparation has been described in the previous assay of globin chains using the capillary electrophoresis technique [24]. Our RP-HPLC protocol could be used to determine α, β, $^{G}\gamma$, and $^{A}\gamma$ chains both from newborn and adult blood samples although there are different levels for each of these Hb components in the two group samples. In addition to this, heme groups and globin chains (pre-β, β, α, δ, $^{G}\gamma$ and $^{A}\gamma$) gave excellent separation.

2. Materials and methods

2.1. Chemicals and reagents

Trifluoroacetic acid (TFA) was from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and methanol were from Fisher Scientific (Fair Lawn, NJ, USA). NaCl (purity ≥ 99.5%), NaOH (purity ≥ 96.0%), acetone (purity ≥ 99.5%), and HCl (36–38%) were from Jinhuada Chemical Reagent Co., Ltd. (Guangzhou, China). Water for HPLC was treated with a Milli-Q A10 purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation and chromatography

Although any conventional HPLC machine could be used, we used a Shimadzu LC-20AT chromatographic system (Shimadzu, Kyoto, Japan) with a CBM-20A system controller, a LC-20AT binary pump, a CTO-20A column oven, a SPD-20A UV–vis detector, a SIL-20A auto-injector and an LC-SOLUTION work station. Chromatographic separation was with a Jupiter C18 HPLC column (4.6 mm × 250 mm, 5 μm, 300 Å, Phenomenex, Torrance, CA, USA) and a SecurityGuard C18 column (4.0 mm × 30 mm, 5 μm, 300 Å, Phenomenex, Torrance, CA, USA), that is shielded ahead of Jupiter C18 HPLC column for filtering some small particles. Elution was obtained at 40 °C, a temperature chosen for lower pressure, better resolution and higher reproducibility than could be obtained at uncontrolled room temperature. Solvent A was a mixture of acetonitrile–methanol, at 90:10 (v/v). Solvent B was 0.5% TFA in deionized water at pH 2.6 adjusted by a few drops of 10 N NaOH. A flow rate of 2.0 ml/min was applied during a linear gradient of acetonitrile–methanol. The UV detection wavelength was 280 nm as previously reported [20], and the injection volume was 10 μl.

2.3. Study subjects

A total of 301 samples with normal (n = 106), α thalassemia silent/trait (n = 60, including 2 ones with Hb WS), Hb H disease (n = 59), β thalassemia carriers (n = 46, including 2 ones with Hb E and 3 co-inheritence of α thalassemia ones with Hb WS) and β thalassemia intermediate/major (n = 30, including 11 ones with Hb E and 2 Hb WS) were collected using EDTA as anticoagulant in this study. Full blood counts and a hemoglobin test of direct DNA analysis was used as described previously [3]. The nondeletional forms of α thalassemias and β thalassemia mutations were identified by RDB assay while the deletional forms of α thalassemia were determined by gap-PCR [3].

2.4. Three types of sample preparation

2.4.1. Simple pre-treatment of blood samples

Samples of 50 μl of venous blood collected in anticoagulant were taken from healthy adults, newborns and thalassemia patients from a clinical laboratory and diluted in 1 ml deionized water for elution. Samples were centrifuged at 3000 r/min for 5 min to remove cells debris [25]. An equal volume of plasma was prepared in the same way for a blank control.

2.4.2. Preparation of hemoglobin solution

For the preparation of a hemoglobin solution, we adopted the procedure previously described by Masala and Manca et al. with slight adjustment [21]. Blood samples were diluted with about 5 volumes of saline (0.9% NaCl), and washed three times by centrifugation (3000 r/min for 5 min) to remove plasma. Lysates were prepared by mixing a volume of the washed red cells with an equal volume of deionized water and 0.4 volumes CCl_4, with occasional stirring. The mixture was centrifuged at 3000 r/min for 10 min to

remove cell debris, and the clear hemoglobin solution, floating over the stromata, was collected using a pipette.

2.4.3. Extraction of globin precipitate

Hemoglobin solution prepared as above was added dropwise to eight volumes of acidified acetone cooled to −20 °C. Heme-depleted globin chains were obtained by centrifugation at 3000 r/min for 10 min, washed three times with about 1 ml acid acetone, and dried under vacuum. The globin powder was dissolved in 1 ml buffer (solvent B with a low pH making the powder easier to be dissolved) [20], filtered using a 0.45 μm Millipore filter, and injected onto the column.

2.5. HPLC gradient design for human globin chain separation

By summing the change in the law of hydrophobicity (increase between 25% and 60% acetonitrile) according to previous similar works [12–14,21,23], the gradient was designed with a high percentage of solvent B at the beginning and ended with a lower percentage during several minutes within the determined scope of hydrophobicity. Our goal was obtaining a procedure for satisfactory separation of human globin chains. Relative quantification was carried out by measuring the percentage of the peak area of the heme and globin chains. The gradient program could be modified according to the machine, the geometry of the column, and the separation to be achieved. Resolution was adjusted by changing the gradient slope and run time.

2.6. Statistical analysis

Using an analysis of variance (ANOVA) test, we compared normal groups with patient groups for area ratio of α/pre-β+β. The ROC curve [26], which was used to validate the sensitivity and specificity for a binary test as a function of the discrimination threshold (cut-off value), was plotted using SPSS 17.0 (IBM, Armonk, NY, USA) to select the best indicator and to determine suitable cut-off values for the prediction of thalassemia patients. The area under the curve (AUC) quantifies the ability of the test to discriminate between normal individuals and thalassemia traits [26]. In order to clearly demonstrate the actually two γ-globin peaks and α peak, bivariate correlation analysis to determine the association between Hb F levels and the ratio of $^{G}\gamma + {}^{A}\gamma/\alpha$ in the samples from β thalassemia intermediate/major group were conducted by using the spearman correlation coefficient For all tests, statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Method development and optimization

For the separation of γ subunits, an Hb solution from a normal newborn (high Hb F) was used for method optimization. We set the elution protocol starting with 51% solvent B, continued for 1 min, and then changed from 51 to 50% for 1 min, followed by the gradient elusion of solvent B from 50 to 40% (with a corresponding increase in solvent A from 49 to 60%) for 10 min, and re-equilibration (51% solvent B) was required for the next run.

The prepared Hb solution was injected, and the gradient started and developed. As shown in Fig. 1a, heme groups and four globin chains (β, α, $^{G}\gamma$, and $^{A}\gamma$) were separated clearly. The elution pattern showed the appearance of the most hydrophilic heme groups at 5.0 min, followed in 7.8 min by the β peak, 11.4 min by the α chain, 10.7 min by the $^{G}\gamma$ chain and 12.0 min by the $^{A}\gamma$ chain. As the total γ chains contributed to Hb F, 30 samples with different Hb F levels from β thalassemia intermediate/major group were analyzed to verify the actually two γ-globin peaks and α-globin peak.

Table 1
Reproducibility in sets of five assays on the same sample from normal adults.

Globin chains/ heme group	Retention time (min)		Peak area (%)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Heme	5.03 ± 0.04	0.73	49.37 ± 0.16	0.32
Pre-β	6.97 ± 0.09	1.29	1.50 ± 0.02	1.63
β	7.70 ± 0.07	0.92	25.93 ± 0.17	0.64
δ	8.59 ± 0.06	0.68	0.40 ± 0.02	4.86
α	11.37 ± 0.01	0.11	18.99 ± 0.21	1.11

An obvious positive correlation was obtained between the ratio of $^{G}\gamma + {}^{A}\gamma/\alpha$ (α as a reference) and Hb F levels ($r_s = 0.887$, $P < 0.01$). In our assay, this correlation could not be observed assuming the actually α chain was eluted before $^{G}\gamma$ chain like in other previous similar works [21,23]) ($r_s = -0.787$, $P < 0.01$) or at 12.0 min ($r_s = 0.267$, $P > 0.05$), and therefore the peak at a 11.4 min retention time was the actually α chain. Our results of the elution order of $^{G}\gamma$ and $^{A}\gamma$ peaks is similar to the other previous works [21,23], $^{G}\gamma$ had a faster retention time than $^{A}\gamma$ in the present method, thus indicating the corresponding to the separation mechanism of due to less hydrophobic for glycine than alanine under an increasingly hydrophobic condition.

When the Hb solution of a normal adult was injected, the heme group peak was present at 5.0 min, followed by the pre-β peak at 7.4 min and the very close β-chain peak at 8.0 min, then the δ-chain at 9.0 min, and the α-chain at 11.7 min (Fig. 1b). We injected the same sample five times, obtaining a reproducibility of retention time CV ranging from 0.11 to 1.29%, and the peak area CV was between 0.32% and 4.86% (Table 1). The CVs were all below 5% thus indicating the good reproducibility of our established method. Fig. 1c shows a chromatogram after heme-depleted globin powder was injected. The α and β chains were separated without the heme peak, but the peak shape was poor under these conditions. Degradation of the globin chains caused by acidified acetone might have been a possible reason. Fig. 1d and e shows the plasma injection and simple preparation of a sample without cell washing. The plasma eluted before 3 min without an observable peak at the retention time of the globin chains or heme groups. The elution pattern of the sample with simple pre-treatment was similar to the prepared Hb solution. As shown in Fig. 1d and e, the corresponding peaks show that the plasma protein fractions had no influence on the separation of globin chains.

Supplementary Fig. 1a shows the effect of flow rate on the retention time of the major hemoglobin components in adults. The high flow rate reduced the retention time, but caused higher pressure and decreased the resolution of detection. To maintain the resolution, save solvent and protect the column, a 2.0 ml/min flow rate was used. Supplementary Fig. 1b and c shows the influence of pH and gradient slope on the retention time. A lower pH mainly enabled a faster retention time for heme, while a shorter gradient time achieved a faster elution time for globins. As shown in Supplementary Fig. 2, wavelength was an important factor for the absorption area. The area ratio of α/β between 210 and 220 nm was about one, which was specific for the peptide bond, and gave markedly better detection limits compared to other wavelengths. However, the slope of the baseline increased. This problem was not seen at 280 nm, which corresponds to the aromatic amino acids tryptophan and tyrosine and gave a higher absorption for the β chain than for the α chain. Therefore, the wavelength was set at 280 nm for quantitative analysis of human globin chains.

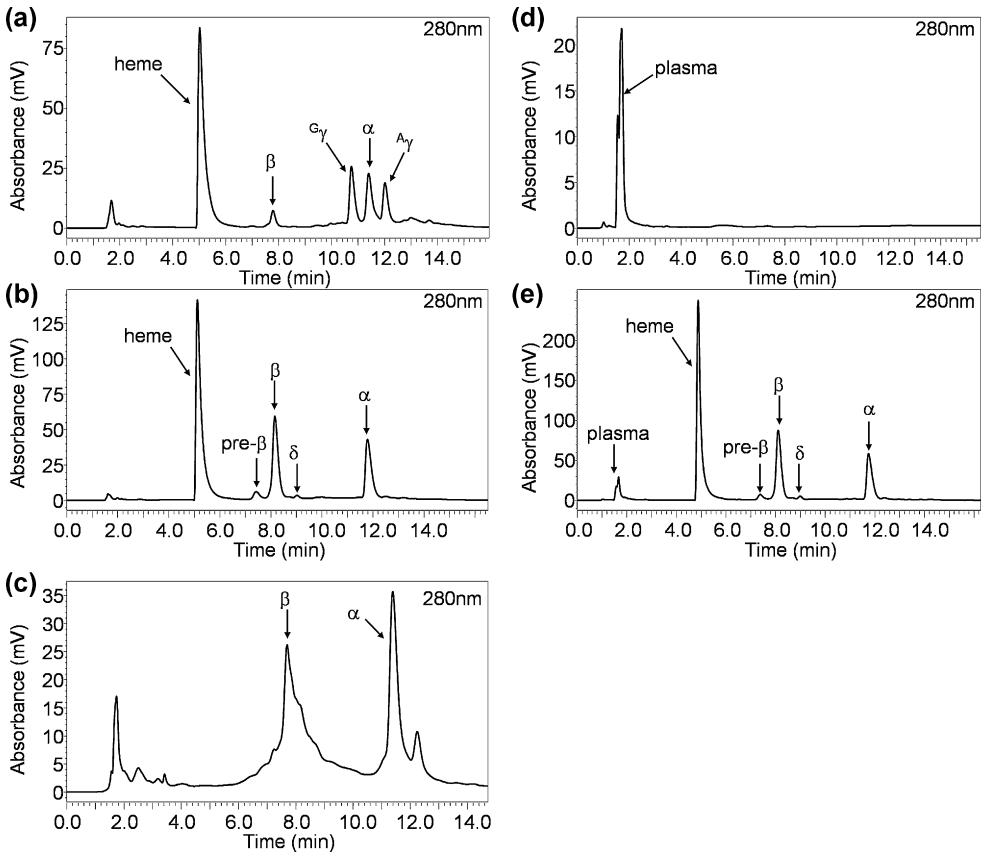

Fig. 1. Representative chromatograms from normal newborns and adults. (a) Hb solution prepared from a normal newborn with cell washing; (b) Hb solution prepared from a normal adult with cell washing; (c) globin powder extracted from the same normal adult; (d) blank plasma; (e) venous blood with simple pre-treatment from the same normal adult. Pre-β, pre-β chain; β, β chain; δ, δ chain; α, α chain; $^{G}\gamma$, $^{G}\gamma$ chain; $^{A}\gamma$, $^{A}\gamma$ chain.

3.2. Application study

3.2.1. Multiple comparisons

As shown in Fig. 2, significant differences ($P < 0.05$) between groups (normal, Hb H and β thalassemia) were found in the area ratio of α/pre-β+β applying the rapid elution procedure, while $P \geq 0.05$ was obtained between the normal and α thalassemia silent/trait group.

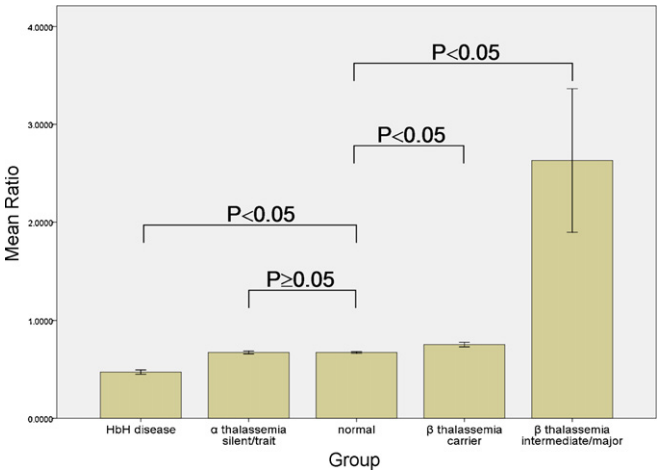

Fig. 2. Multiple comparisons of α/pre-β+β ratio between normal and patient groups. Cohort of 301 subjects analyzed using ANOVA. Error bars represent 95% confidence interval. Column indicates mean ratio.

3.2.2. ROC curve analysis

Based on the results of multiple comparisons, data sets from 106 normal persons and 46 β thalassemia carriers applying the established procedure were used to construct ROC curves. The AUC were 1.000 for δ/β, 1.000 for δ/pre-β+β, 0.999 for δ, 0.980 for α/β, 0.879 for α/pre-β+β and 0.605 for α/pre-β+β+δ. As shown in Fig. 3a and Table 2, the δ/β ratio was selected as the best indicator for the prediction of β thalassemia carriers. Based on the ROC curve analysis, the optimal cut-off value for indicating β thalassemia carriers using the ratio of δ/β was 0.026 with a sensitivity of 100.0% and a specificity of 100.0%. The same analysis was employed in plotting a curve for the ratio of α/β and α/pre-β+β for the prediction of Hb H based on ANOVA results. The ROC curve area was 0.981 for α/β and 0.958 for α/pre-β+β. The ratio of α/pre-β+β was a better marker for Hb H disease, as shown in Fig. 3b and Table 3, and the proposed cut-off value was 0.626 with a sensitivity of 96.6% and specificity of 89.6% for Hb H disease.

3.2.3. Characterization of hemoglobin variants

Among the 301 subjects, each of 7 Hb WS and of 13 Hb E were well identified by the respective specific peaks using this RP-HPLC method. As shown in Fig. 4a and b, the abnormal α^{WS} chains were observed at 13.6 min while the β^{E} chains eluted at 7.3 min.

3.3. Discussion

Here we present a rapid method for the separation of human globin chains with simple sample preparation. Hemoglobin was released from red blood cells because of osmotic pressure. The presence of 0.5% TFA in solvent B created a pH below 3.0, and was

Table 2
Results of ROC curve analysis of β thalassemia carriers from simple prepared samples.

Ratio	AUC	Cut-off	Sensitivity (%)	Specificity (%)
δ/β	1.000	0.026	100.0	100
δ/pre-β+β	1.000	0.022	100.0	99.1
δ	0.999	0.573	97.8	100
α/β	0.980	0.804	93.5	98.1
α/pre-β+β	0.879	0.719	69.6	91.6
α/pre-β+β+δ	0.605	–	–	–

AUC, area under the ROC curve.

Table 3
Results of ROC curve analysis for Hb H disease from simple prepared samples.

Ratio	AUC	Cut-off	Sensitivity (%)	Specificity (%)
α/pre-β+β	0.981	0.626	96.6	91.5
α/β	0.958	0.657	89.8	94.3

AUC, area under the ROC curve.

required as the ion-pairing agent. Under these conditions, heme groups were removed from tetramers, and the constituent globins dissociated. Pre-β, β, δ, α, ^{G}γ, and ^{A}γ were clearly separated based on different hydrophobicities, but unlike the previous report [21], in which α chain was eluted before ^{G}γ, in our procedure, α chain was recorded between ^{G}γ and ^{A}γ, and the actually α chain was verified by bivariate correlations analysis between Hb F levels and the ratio of ^{G}γ+^{A}γ/α. Small peaks observed before 2 min with the prepared Hb solution (Fig. 1b) may be carbonic anhydrase and superoxide dismutase, that should exist. Our RP-HPLC profiles were similar with results observed from other similar reports [21,23] and we may need to do further work on identifying the actually peaks of them in chromatogram if necessary. In fact, they have little influence on the analysis of results because of the significantly lower absorption compared with Hb components. Of note, small quantities of globins would be easily denatured under low pH conditions (using solvent B as a buffer with 0.5% TFA), resulting in a poor chromatogram map and a lower detection sensitivity. However, this

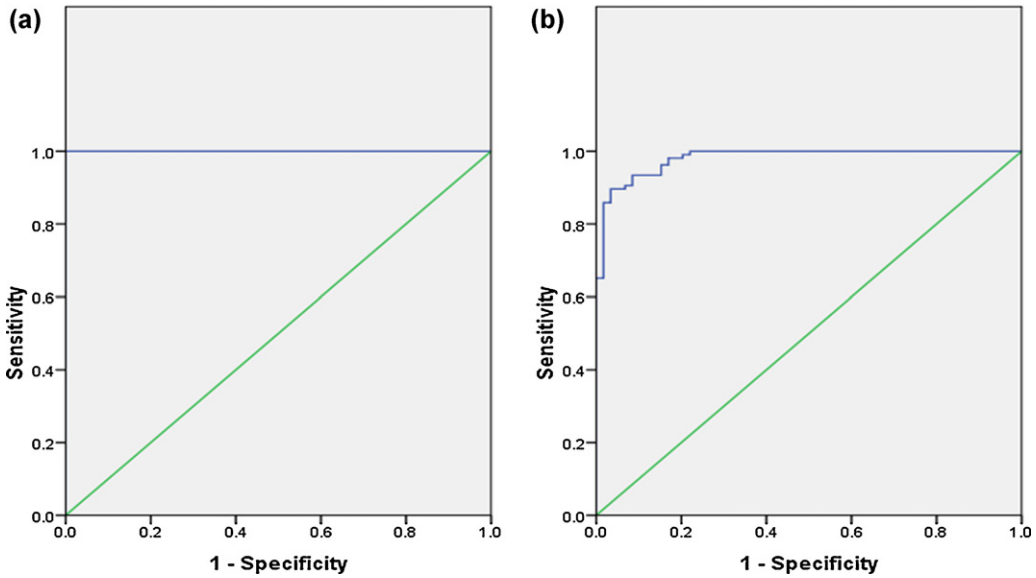

Fig. 3. Results from β thalassemia carriers (a) and Hb H disease (b) based on ROC curve analysis of the HPLC assay.

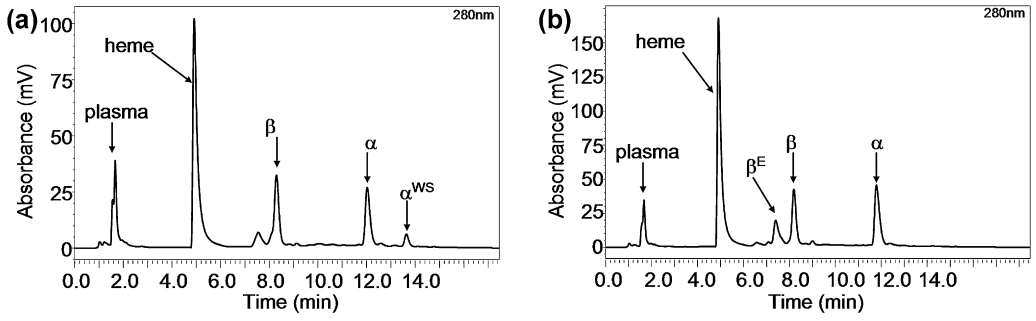

Fig. 4. Characterization of abnormal globin chains. (a) $\alpha^{WS}\alpha/\alpha\alpha$; (b) β^{E}/β. β, β chain; α, α chain; α^{WS}, α^{WS} chain; β^{E}, β^{E} chain.

was not a problem when deionized water was used as a buffer. The pre-β peak observed before the normal β-peak in this procedure was a posttranslationally modified protein, presumably containing a β-globin glutathione adduct as its major component of β chains [27].

Because of the down-regulation of β chain or relative elevation of δ chain, the area ratio of δ/β was selected as the best indicator for β thalassemia carriers. It also had a high sensitivity and specificity in the procedure presented here for β thalassemia intermediate/major in our study. To evaluate this marker, a larger number of samples are required for a validation study. β thalassemia heterozygote with normal Hb A2 may be detected by this marker, thus a comparison between this marker and Hb A2 for β thalassemia would be interesting. ANOVA tests for the area ratio of α/pre-β+β showed no significant difference between the normal and α thalassemia silent/trait, possibly because of the compensatory over-expression of the α-globin gene or the degradation of excess globin chains. As a result, the sensitivity of this direct method was lower than globin as described previously [4]. Therefore, this method cannot be used as the sole diagnostic tool for α thalassemia silent/trait because of the considerable overlap of α/pre-β+β ratio ranges. Despite these limitations, the ratio of α/pre-β+β had a good sensitivity and specificity for Hb H disease prediction in this simple and rapid method. In fact, for monitoring gene therapy for β thalassemia major or Hb H disease, the sensitivity of this established procedure would be sufficient.

For abnormal hemoglobin, Hb WS is difficult to detect using the Bio-Rad Variant II Beta-Thal short program (Bio-Rad, Hercules, CA, USA), but was easy in the method described here. Furthermore, the retention time on the Bio-Rad VARIANT II makes discriminating between Hb E and HB A2 difficult, but the β^E and δ globin chains, representing Hb E and HB A2, were clearly separated in the RP-HPLC method, demonstrating its high resolution. As a matter of fact, Hb WS and Hb E occur frequently in the Chinese population. Because of the degradation of abnormal chains or the limited resolution of this rapid chromatographic program, Hb CS and Hb QS, also common in China, were undetected [28].

If globins from adult lysates without detectable amounts of Hb F were studied, a gradient that saves time (less than 8 min for the separation of heme, pre-β, β, δ, and α in this study) and solvents could be designed by changing the gradient program slope for a shorter time or by increasing the flow rate. However, the presence of a variant chain may escape observation in these conditions. To obtain additional variants or more hydrophobic chains (i.e. ζ) based on the gradient described here, the run time and hydrophobicity might need to be extended.

The design of the gradient program might depend on the protein column. Using a new RP column without a gradient program for globin chain separation and establishing new conditions is time consuming. In summary, in separating human globin chains by RP-HPLC, we found that hydrophobicity between 25% and 60% acetonitrile is effective for various protein columns. The scope of hydrophobicity described here has reference value for similar work.

Discrimination between the two types of γ chains in this procedure could be of interest for diagnosis of the hereditary persistence of fetal hemoglobin or δβ thalassemia, or for suggesting a haplotype in cases of sickle cell anemia [11]. This method might also be useful for the separation of globin chains in non-human blood [29], which is important for studying gene therapy in animal experiment.

4. Conclusions

A simple, rapid and high resolution RP-HPLC procedure was developed for the simultaneous separation of six types of globin chains and heme groups. This method had good resolution and simple sample pretreatment and could be an addition to methods for prediction of both thalassemias and Hb variants. The strategy for the development of methods for human globin chain separation by RP-HPLC described here could be of use in similar work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.041.

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