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On-line microheterogeneity analysis and rapid phenotyping of haptoglobin by capillary electrophoresis using sodium dodecyl sulfate as additive

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ABSTRACT

To improve the detection sensitivity and determine phenotypes of haptoglobin (Hp), a prefilling technique was developed and tested in capillary electrophoresis (CE) with UV–vis absorbance detection. Adding 0.01% sodium dodecyl sulfate (SDS) to the protein sample and 0.1% SDS to the prefilling buffer solution, on-line stacking and microheterogeneity separation of Hp were achieved. In addition, the influences of pH, buffer concentration, sample and prefilling buffer SDS concentration upon resolution were examined. Under optimized conditions, Hp-microheterogeneity was well resolved and two phenotypes of Hp (Hp 1-1 and Hp 2-2) were differentiated. This method was applied to the analysis of sera from normal individuals and β-Thalassemia patients. After the depletion of albumin (HSA) and immunoglobulin G (IgG), this method allowed to determine two phenotypes in different individuals and to detect the decrease of Hp in β-Thalassemia patients. Featuring high efficiency, speed and simplicity, the proposed method shows great potential for use in clinical diagnosis and proteome research.

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1. Introduction

Human serum haptoglobin (Hp), synthesized in the liver, is an acute phase plasma protein that specifically binds hemoglobin (Hb). This protein is closely associated with a number of diseases, e.g., hepatitis, evolution and prognosis of liver transplantation, atherosclerosis, hemolytic anemia, AIDS, etc. [1–6]. Based on the existence of two different alleles (Hp1, Hp2), hp is characterized by a genetic polymorphism, resulting in three major phenotypes of human serum haptoglobin: Hp 1-1 (98 kDa), Hp 2-1 (86–300 kDa) and Hp 2-2 (170–900 kDa) [1,7]. Due to its specific binding to Hb, current studies of Hp are mostly based upon the stable Hb–Hp complex [8]. According to the different molecular masses and varying Hb-binding capacity for the three Hp phenotypes, several methods of Hp phenotyping have been established. Historically, the determination of Hp phenotypes was based on electrophoretic separation in starch or agarose gels, though it has been outdated due to low sensitivity and poor selectivity. In this regard, the use of polyacrylamide gel electrophoresis (PAGE) with chemiluminescence (CL) detection (PAGE-CL) has become one of the most important methods for Hp phenotyping [7,9–12]. Applying the Western-blotting technique with enhanced CL imaging detection or using a direct CL imaging method with Hb supplemented to serum samples in polyacrylamide gels, Hp phenotypes have been separated [7,11]. In order to eliminate the interference of Hb, the use of ammonium persulfate to enhance the CL signals has also been well documented [12]. Additionally, high pressure gel permeation chromatography and mass spectrometry have also been used for Hp phenotyping [13–15]. However, Hp phenotyping based on PAGE shows disadvantages of time consuming procedures and poor capacities of quantitative analysis.

Recently, capillary electrophoresis (CE) has been developed as a popular and widely used method for protein subunit analysis because of its high resolution, high sensitivity and high speed. For example, with the addition of 1, 4-diaminobutane to the background electrolyte, characterization of human serum albumin (HSA) heterogeneity can be accomplished and HSA from different vendors can be differentiated by CE [16]. Using detergents such as Tween 20, CE has been used to separate the A, B and C variants of β-lactoglobulin (β-Lg) and detect β-Lg phenotypes of a large sample of dairy cattle [17] as well as polymorphism of caprine milk caseins [18]. In addition, high-density lipoprotein (HDL) and two distinct phenotypes of low-density lipoprotein (LDL) can be effectively separated by microchip CE using sodium dodecyl sulfate (SDS) as an additive [19]. Several protocols with or without sample pretreatment, and introducing different additives have been

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described for the detection of protein microheterogeneity using CE [20–22]. All previous studies imply that types differentiating and microheterogeneity analysis of proteins are undoubtedly possible under suitable conditions.

Based on the difference in charge-to-mass ratios of their Hb–Hp complex, three major Hp phenotypes have been resolved by dynamic double coating CE [23]. However, this study actually investigated the Hb–Hp complex limiting applicability of this technique due to a poor detection limit of Hp and complicated procedure of capillary pretreatment. Other recent research paid their attention to the relationship between Hp polymorphisms and the protein patterns [24]. The primary goal of this work was to develop a fast technique that would allow microheterogeneity analysis and direct determination of Hp phenotypes by SDS-CE without Hb as the probe. Hereby, we developed a prefilling technique with SDS in the buffer solution resembling the analysis of small solutes by micellar electrokinetic capillary chromatography [25–27]. After conditions optimization, phenotypes of Hp 1-1 and Hp 2-2 can be differentiated by the microheterogeneity analysis. The results suggest that this method offers great applications for the analysis of Hp in human serum samples and for Hp depletion monitoring in β-Thalassemia patients.

2. Experimental

2.1. Apparatus

A 4-tray PrinCE capillary electrophoresis apparatus (PrinCE Technologies, Emmen, The Netherlands) equipped with a UV–vis detector (Lambda 1010, Bischoff Chromatography, Leonberg, Germany) at 214 nm was used for all measurements. Fused-silica capillary columns of 75 μm I.D. × 375 μm O.D. (Yongnian Photoconduction Fibre, Hebei, China) were used in the study, with a total length of 65.4 cm and an effective length of 48.9 cm. All electropherograms were obtained at average ambient laboratory temperatures of 23 ± 1 °C.

2.2. Reagents and solutions

Haptoglobins (Hp 1-1, 98 kDa and Hp 2-2, 170–900 kDa) were purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) and sodium dodecyl sulfate (SDS) were obtained from Sino-American Biotechnology Co. (Beijing, China). Sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), phosphoric acid (H_3PO_4) and N, N-dimethylformamide (DMF) were purchased from Beijing Chemical Plant (Beijing, China). All chemicals for the preparation of buffer solutions were of analytical grade. All solutions were prepared using ultrapure water (Millipore, Milford, MA, USA) and passed through a 0.45 μm filter prior to use.

Hp stock solutions of 1 mg/mL stored at −20 °C were prepared by adding precisely weighed standard to ultrapure water, and the working standard was made by dilution to corresponding multiples. Stock solutions of 100 mM Tris and 100 mM NaH_2PO_4 were employed for preparing buffer solutions of desired concentrations and pH values. The concentration of stock solution of SDS was 10% (w/v), which was greater than its critical micelle concentration (CMC, 0.23% or 8.1 mM in pure water). Then it was diluted by buffer solution or added to the sample with a final concentration far below its CMC.

2.3. Procedures

The fresh capillaries were consecutively treated with 1 M NaOH (60 min), then ultrapure water (30 min) by 1500 mbar pressure for silanol activation. Prior to analysis, 0.2 M NaOH, ultrapure water were used to rinse the capillaries, each for 5 min. Then, the prefilling technique was actualized through a 5 min rinse of Tris–phosphate buffer solution containing SDS with different concentrations before sample injection. Samples were hydrodynamically injected at 50 mbar pressure for 12 s (Hp standards) or 6 s (serum samples). Once a high voltage (18.0 kV) was applied after the sample injection, buffer solution without SDS in the anodic reservoir entered the capillary under the electroosmotic flow (EOF). Capillaries were washed with 0.2 M NaOH at 1.0 kV and 100 mbar for 20 min to refresh the capillary wall between every 10 runs.

2.4. Analysis of human serum samples

Fresh blood samples from healthy people were obtained from the affiliated hospital of the Beijing Normal University. After careful settlement for 1 h, the supernatant was extracted as serum with a mini-sample collector and then centrifuged at 2500 rpm (three times 10 min). Then the serum samples were treated with the Aurum Serum Protein Mini Kit (Biorad, USA) to remove both HSA and immunoglobulin (IgG). Next, the serum samples were stored at −20 °C and prepared for electrophoresis analysis after the addition of SDS. Serum samples of β-Thalassemia patients purchased from Guangzhou Children's Hospital (Guangzhou, China) were treated as the healthy sera.

3. Results and discussion

3.1. On-line concentration of protein

Due to the low sensitivity of Hp detection by UV, additives such as SDS have been chosen for protein stacking. Compared to direct electrophoresis analysis without any additive, the migration time and peak shape of Hp showed obvious differences while applying a prefilling technique by rinsing the capillary with buffer solution containing SDS before sample injection. On-line concentration and extended migration times have been demonstrated for both Hp 1-1 and Hp 2-2 as shown in Fig. 1, which demonstrates that an improvement in sensitivity of at least 10-fold can be obtained, without increasing analysis time dramatically. In Fig. 1A, broad peaks are produced due to the poor UV absorbance of Hp and adsorption on the capillary inner surface, while in Fig. 1B stacking of protein by SDS is clearly observed due to the formation of a protein–SDS complex (as only SDS is transparent at the wavelength of 214 nm, data not shown), as well as repulsion of protein particles from the negatively charged wall. As only a slight decrease of EOF was achieved, with the EOF mobility values of 6.892×10^{-4} and 6.817×10^{-4} $cm^2 V^{-1} s^{-1}$ (DMF being chosen as neutral EOF marker, with the concentration of 0.1%, v/v) in the absence and presence of SDS, respectively, the prolongation for the migration time must be attributed to lower apparent electrophoretic velocities of the protein–SDS complexes.

3.2. Microheterogeneity analysis of Hp

The electropherograms shown in Fig. 1, for samples of Hp without additive, indicate that no obvious difference between Hp 1-1 and Hp 2-2 was observed, thus the addition of SDS to the standard samples was investigated. It is interesting to note that, with the addition of proper concentrations of SDS in the sample, there were two peaks for each protein being detected (Fig. 2). Hp consists of two different polypeptide chains, the α-chain and the β-chain [1], which are linked by disulfide linkages. Both Hp 1-1 and Hp 2-2 contain the same β-chain (40 kDa) which is heavier than the α-chain. Identified by differences of molecular weight, α-chains are mainly divided into two variants: α^1-chain expressed in Hp 1-1 and α^2-chain present in Hp 2-2, with molecular weight of 8.9 and 16 kDa,

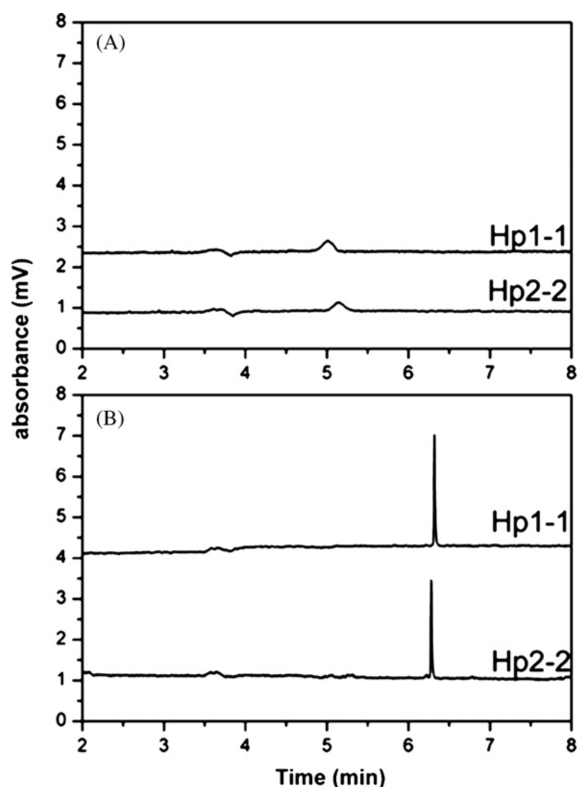

Fig. 1. Effects of the absence (A) and presence (B) of 0.1% SDS in the prefilling buffer solution upon two phenotypes of Hp by CE with UV detection at 214 nm. Protein samples with a concentration of 200 μg/mL were hydrodynamically injected at 50 mbar for 12 s. Allied conditions: 65.4 cm (48.9 cm to detector) × 75 μm I.D. fused-silica capillary, 16 mM Tris–phosphate (1:1) buffer solution at pH 9.9, +18 kV applied voltage, 23 ± 1 °C.

respectively. Peaks with close migration times might represent the β-chain of each protein, while the other two peaks indicate that α^2-chain and α^1-chain exist in Hp 2-2 and Hp 1-1, respectively. To explore the impact of SDS added to the Hp sample, we examined the concentrations ranging from 0.0033 to 0.013% and found that two peaks appeared when 0.0066–0.01% SDS were present, attending by better peak profiles and different migration times between two phenotypes (Fig. 2A and B). Comparing the electropherograms, the addition of 0.01% SDS to the protein sample was the most appropriate condition providing the highest resolution separating both peaks. It is also should be noticed that increasing the negative charge of protein–SDS complex – as this results from additional binding of SDS – causes a delay in peak detection, corresponding to an increase in mobility to the anode (inlet), but only a slight change of the EOF.

3.3. Optimization of separation conditions

Fig. 3 illustrates the effect of the pH of running buffer solution on resolution of microheterogeneities of proteins. To obtain Fig. 3, pH values ranging from 9.6 to 10.8 were examined. Buffer pH is an important factor affecting the characteristics of inner surface of the capillary wall, corresponding to the influence of the zeta potential and EOF and thus determining migration times and separation of the analytes. In the present study, with pH values varying from 9.6 to 10.8, there is a high EOF by which the separation is being determined. From Fig. 3, it can be observed that the resolution of both peaks of each Hp improved with the pH increasing up to 9.9, then decreasing as the pH continues to increase. Considering selectivity, pH obviously affected Hp 1-1 but with little impact, only, on the separation of microheterogeneity of Hp 2-2. In order to obtain good resolution and, simultaneously, satisfying selectivity, we chose 9.9 as the final pH value for the further experiments.

Experiments with running buffer concentrations (Tris) from 12 to 28 mM were performed with increasing resolution of both proteins upon increasing the buffer concentration. These effects are mainly attributed to the reduction of the EOF resulting from an increased ionic strength with higher buffer concentration. As the resolution did not improve further when the buffer concentration was above 16 mM, a final running buffer concentration of 16 mM was selected in further experiments to minimize Joule heating and to avoid a bad peak profile.

One of the difficulties of protein analysis in CE concerns the strong adsorption of proteins on the capillary wall, leading to peak broadening or even to disappearance of peaks. Several methods have been established to suppress wall adsorption and then to increase the detection sensitivity [28]. In this experiment, prefilling with buffer solutions containing SDS was demonstrated to be an effective method for this purpose as described in Section 3.1. On the other hand, the concentration of SDS in the prefilling buffer solution played an important role in improving resolution and selectivity. In the prefilling buffer solution, SDS concentrations lower than 0.1% brought on poor resolution efficiency, whereas bad peak profiles

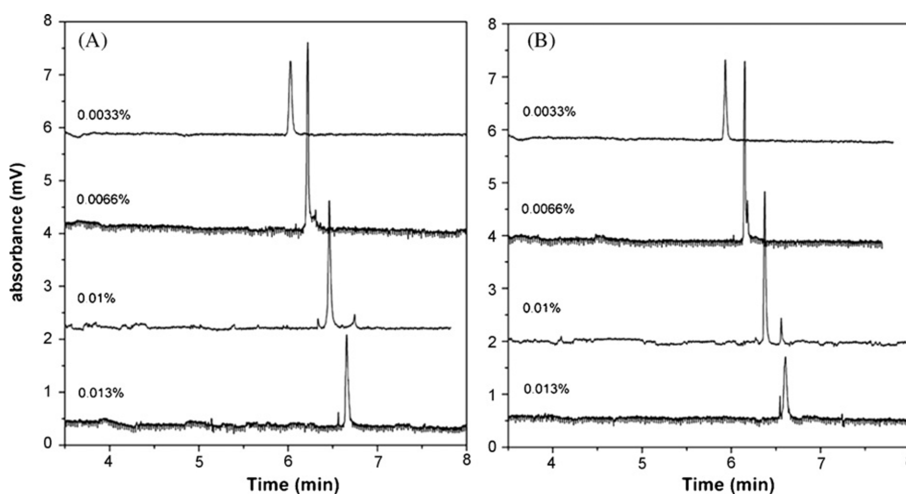

Fig. 2. Comparison of microheterogeneity analysis of two Hp phenotypes (A, Hp 1-1 and B, Hp 2-2) under the same conditions at 18 kV using SDS in the protein samples with concentrations of 0.0033, 0.0066, 0.01, and 0.013%, respectively, as shown in the electropherograms. The value of pH was 10.0 and the other conditions were the same as provided in Fig. 1.

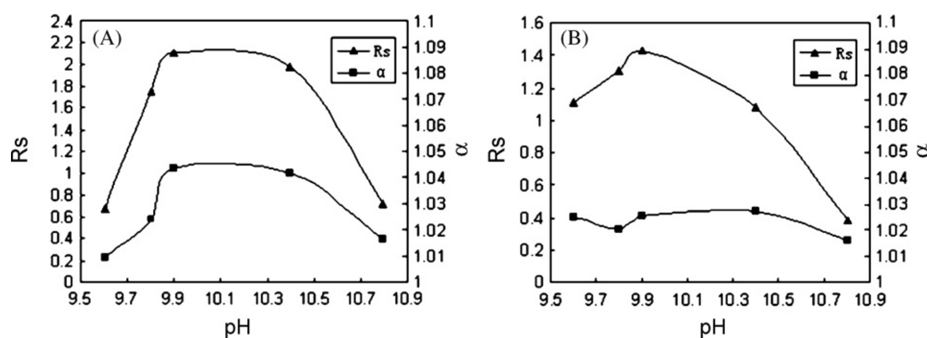

Fig. 3. Effect of buffer solution pH on the separation of microheterogeneity of proteins (Rs = resolution; α = selectivity factor). Protein samples containing 0.01% SDS are (A) Hp 1-1 and (B) Hp 2-2. Other conditions as provided in Fig. 1.

were obtained with higher SDS concentrations (>0.1%, yet lower than its CMC). In further experiments, 0.1% SDS prefilling buffer was used as it provided sufficient resolving power and peak profiles for the separation of the Hp microheterogeneities.

3.4. Phenotyping of Hp

After condition optimization, CE allowed the identification of both Hp phenotypes. Fig. 4 indicates the results that microheterogeneities of two phenotypes have been absolutely baseline-resolved, due to the introduction of the most optimized condition. Likewise, from the electropherogram it can be observed that two phenotypes have been differentiated by the fact that peaks of Hp 2-2 migrate faster than those of Hp 1-1, with migration times of 6.11, 6.28 min and 6.23, 6.49 min for the two peaks of each protein, respectively; also, more effective and symmetric peak patterns were obtained. Considering the discussion in Section 3.2, peaks with close migration times of 6.28 and 6.23 min might denote the β-chain, while the peak with migration time of 6.11 min corresponds with the α^2-chain and 6.49 min with the α^1-chain.

3.5. Possible mechanisms for interaction of SDS and Hp

Serving as a pseudostationary phase, SDS can interact with the proteins which provide additional interaction sites after denaturization. Since the concentration of SDS in the prefilling solution is far below its CMC, one reason causing on-line stacking in Section 3.1 is that negatively charged SDS monomers will bind with protein molecules, in addition to denaturalizing and unfolding the protein [29,30]; furthermore, with more negative charges, such protein–SDS complexes show increases in their migration mobility toward the anodic end, which leads to a sequence of apparent mobilities as below:

$$\mu_{(\text{protein})\text{ap}} > \mu_{(\text{complex})\text{ap}} > \mu_{(\text{SDS})\text{ap}} \quad (1)$$

where $\mu_{(\text{protein})\text{ap}}$ is the apparent mobility of native protein, $\mu_{(\text{complex})\text{ap}}$ is the apparent mobility of protein–SDS complex, and $\mu_{(\text{SDS})\text{ap}}$ is the apparent mobility of SDS; protein–SDS complexes with lower velocities are therefore stacked before they migrate through the detection window. This mechanism is likely to be at the basis of the observation that a wide protein sample zone is shortened after the denaturation.

Former studies have shown that adding SDS to the sample was effective to increase resolving power for separation of the protein microheterogeneity [21,22]. We have documented in Section 3.2 that microheterogeneity of Hp could be separated by the addition of SDS to the sample besides to the prefilling solution. Here, we assume that proteins in the samples are denaturized and thus disulfide linkages between the chains are broken, resulting in formation of α- and β-chains, which are then combined with SDS monomers to migrate in the capillary. As documented by the native PAGE [12], Hp 1-1 migrates faster than Hp 2-2, which can be interpreted that an effective mobility of Hp 1-1 higher than Hp 2-2 has been obtained during the electrophoresis in the gel. In the current system, polypeptides interacting with SDS bear negative charges and exhibit an effective mobility to the anode as well as a reversed EOF to the cathode. Because the electroosmotic mobility (takes the main role of the separation) is the main determinant of the separation, the apparent mobility of polypeptides has a direction to the cathode as well. Due to the difference among the apparent mobilities, α^2-chain, β-chain and α^1-chain pass sequentially through the detection window.

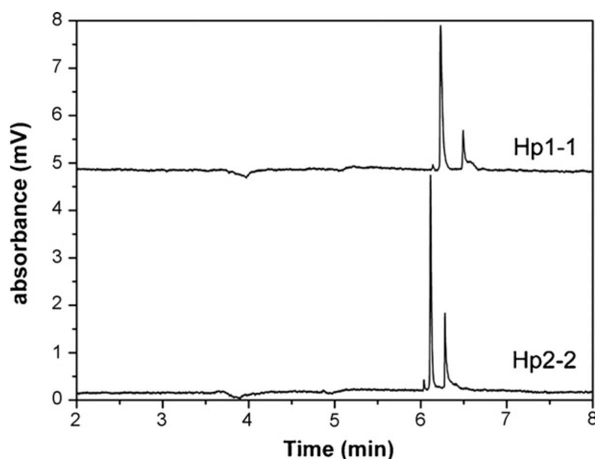

Fig. 4. Phenotyping of Hp. Protein samples with a concentration of 200 μg/mL contained 0.01% SDS; other conditions as those provided in Fig. 1.

3.6. CE-UV performance

There was only a short and flat-topped peak observed at the concentration of 200 μg/mL by direct injection, showing a poor detection limit (Fig. 1). This is likely to be attributable to the low sensitivity of UV detection for the whole protein molecule. For this method, a series of standard solutions with concentrations ranging from 62.5 to 500 μg/mL were analyzed to determine the signal linearity for each protein tested. Run-to-run repeatability data were obtained by investigating a protein sample at a concentration of 200 μg/mL. The results of regression analysis on the calibration curves, detection limits and repeatability are presented in Table 1. Under the optimal conditions, the detection limits for Hp 1-1 and Hp 2-2, defined as the S/N to be 3, were 40.0 and 31.3 μg/mL, respectively. The percent relative standard deviations (RSD) for peak areas were less than 2.5%, and for migration times less than 0.6% (expressed by the first peak for each protein), which is typical for CE.

Table 1
Detection limits, linearity and repeatability[a].

Analyte	Equation for calibration curves	Linear range (μg/mL)	R^2	Detection limit (μg/mL) (S/N = 3)	Concentration level[b] (μg/mL)	Peak area RSD (%), n = 3	Migration time RSD (%), n = 3
Hp 1-1	$y = 0.0105x + 0.358$	40.0–500	0.9945	40.0	200	2.5	0.1
Hp 2-2	$y = 0.011x + 0.0142$	31.3–500	0.9996	31.3	200	1.8	0.6

[a] Separation conditions: pH 9.9, separation voltage +18 kV, buffer solution of 16 mM Tris–phosphate (prefilling solutions containing 0.1% SDS), hydrodynamic injection time: 12 s.
[b] Sample composition of 200 μg/mL Hp with 0.01% SDS.

3.7. Applications

Serum samples from healthy subjects have been analyzed by SDS-CE to determine the Hp phenotypes. As the most abundant human serum proteins are HSA and IgG, which display a high UV absorbance and thus interfere with the detection of other proteins, the Aurum Serum Protein Mini Kit was used to remove these two proteins from the human serum. HSA and IgG depleted sera were then injected into the capillary. Electropherograms of Hp 1-1 and Hp 2-2 individual serum samples are shown in Fig. 5 and peaks of Hp were identified by the addition of standards. We note that Hp 1-1 and Hp 2-2 individuals could be differentiated by the different migration times of Hp, though the peak of β-chain was not absolutely resolved from another composition in the serum. It also should be noted that Hp in the serum migrated much slower than the standard samples. As we know, there are still other proteins, i.e., transferrin, fibrinogen, C3 complement and lipoprotein in serum, which might also form complexes with SDS [19,31]; besides, serum samples diluted by commercial binding buffer take a higher ionic strength after injection to the capillary, resulting in slower apparent mobilities for the proteins. Thus the delay of migration time is likely to be attributable to other protein–SDS complexes and different compositions between standards and clinical samples. It has been reported that reference values of Hp concentration differ between Hp types, with reference range of Hp 2-2 in serum (0.38–1.50 g/L) being lower than that of Hp 1-1 (0.57–2.27 g/L) [1]. This can explain the lower peak heights of Hp 2-2.

Table 2
Recovery of Hp from human serum samples[a].

Analyte	Human serum samples		
	Added (μg/mL)	Recovered (μg/mL)	Recovery ± RSD (%) (n = 3)
Hp 1-1	62.5	57.6 ± 1.5	92.1 ± 2.2
	125.0	115.7 ± 3.0	92.6 ± 2.3
	250.0	233.9 ± 7.7	93.6 ± 3.0
Hp 2-2	125.0	114.8 ± 4.7	91.8 ± 3.8
	200.0	188.1 ± 10.6	94.1 ± 4.9
	250.0	239.9 ± 14.9	96.0 ± 5.7

[a] Separation conditions: pH 9.9, separation voltage +18 kV, buffer solution of 16 mM Tris–phosphate (prefilling solutions containing 0.1% SDS), hydrodynamic injection time: 12 s.

For human serum samples, calibration curves were established by changing the dilution ratio of real samples. The UV signal linearity was observed between 56.8–180.0 and 48.0–166.5 μg/mL for Hp 1-1 and Hp 2-2, respectively, with detection limits of 56.8 and 48.0 μg/mL for each protein. RSD of Hp in the serum samples for peak areas were less than 5.84%, and for migration times less than 0.91% at 100 μg/mL (n = 3). To validate this method, we have carried out a recovery study: 20 times diluted human serum samples with most of HSA and IgG depleted were spiked with series of Hp stan-

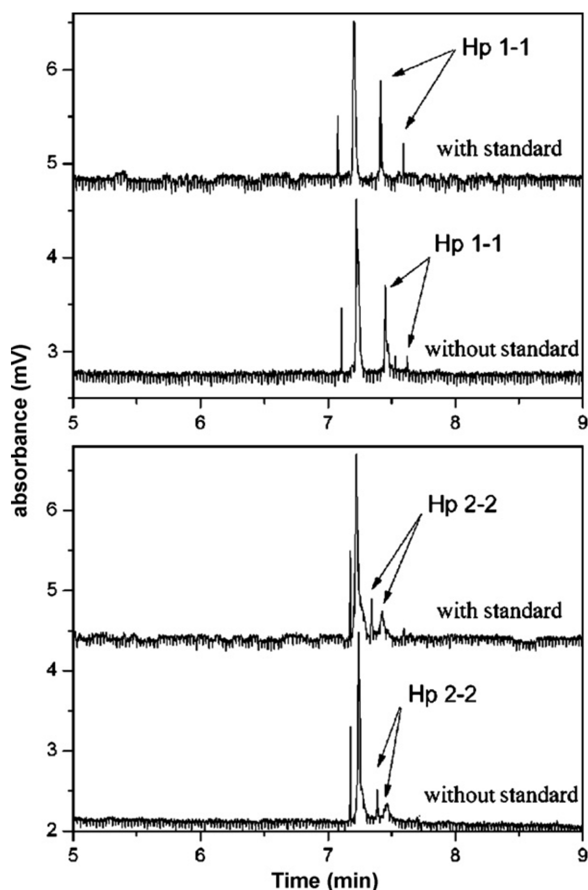

Fig. 5. Electropherograms of healthy subject's sera with and without the addition of standard (as shown in the electropherograms). Samples were diluted 16 times, with an injection time of 6 s. Other conditions as provided in Fig. 4.

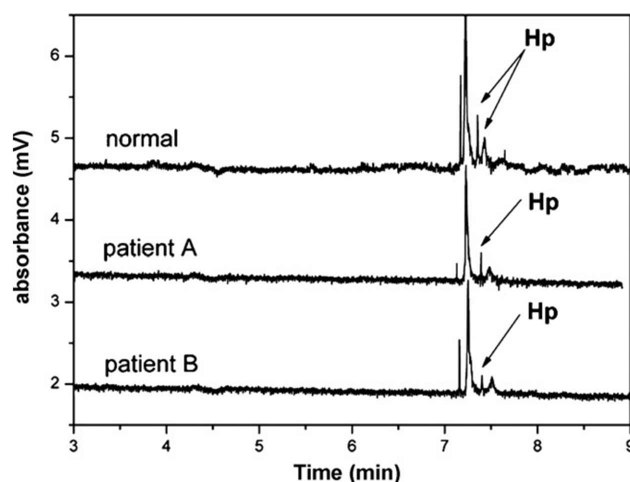

Fig. 6. Comparison of healthy subject's and β-Thalassemia patients' serum samples (A, β-Thalassemia major patient and B, β-Thalassemia intermedia patient) which were diluted 16 times, with an injection time of 6 s. Other conditions as those provided in Fig. 4.

dards, to reach a final concentration from 62.5 to 250 μg/mL for Hp. The recoveries for different concentration levels were higher than 90%, as shown in Table 2.

As an acute phase protein which is consumed following hemolysis, Hp usually shows lower concentrations in patients with hemolysis. β-Thalassemia is a kind of hemolytic anemia, due to an abnormal Hb [5,32]. Fig. 6 lists electropherograms of β-Thalassemia patients' sera compared with a healthy individual. There is an obvious decrease of Hp concentration in β-Thalassemia, which is even more pronounced in a β-Thalassemia major than in a β-Thalassemia intermedia, in line with previous data [5,32]. Therefore this approach can be used to assess the severity of hemolysis in Thalassemia or other hemolytic anemias.

4. Conclusions

A direct, rapid, simple and efficient method for Hp phenotype (Hp 1-1 and Hp 2-2) detection has been developed using CE-UV. We demonstrated that applying a prefilling technique with buffer solution containing 0.1% SDS allowed protein stacking and resolution improvement. Addition of 0.01% SDS to Hp standards was found useful for resolving the microheterogeneity of Hp and for detecting Hp phenotypes (Hp 1-1 and Hp 2-2). With the proposed method, clinical serum samples of healthy individuals and Thalassemia patients have been analyzed. The different Hp phenotypes (Hp 1-1 and Hp 2-2) could be differentiated. Furthermore, the described assay has proven to be useful for detecting Hp decreases in β-Thalassemia patient serum. Apart from these applications, this method offers potential perspectives for the analysis of other proteins in clinical samples.

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