Clinical Biochemistry 49 (2016) 1288–1291

Contents lists available at ScienceDirect

Clinical Biochemistry

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

Q Sepharose micro-column chromatography: A simple screening method for identifying beta thalassemia traits and hemoglobin E carriers

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ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form 3 June 2016
Accepted 4 July 2016
Available online 15 July 2016

Keywords:
Q Sepharose
Micro-column chromatography
Hemoglobin E carrier
Beta thalassemia carrier

ABSTRACT

Objectives: For beta thalassemia control program in pregnancy, mass screening of the carrier state by determination of the hemoglobin (Hb) A_2 and Hb E proportions and mutation analysis is a preferred method for making prenatal diagnoses. Q Sepharose micro-column chromatography, developed for the determination of Hb A_2 and Hb E for screening purposes, was compared with high performance liquid chromatography (HPLC) to ascertain its relative accuracy and reliability.

Design and methods: Results using Q Sepharose micro-column chromatography in 350 blood specimens, including 50 samples genetically proven to be beta thalassemia heterozygotes, were compared to HPLC for validation. An additional study was conducted to test a clinical application on a large-scale survey for beta thalassemia in 1581 pregnant women and their spouses.

Results: The mean (±SD) Hb A_2 proportions in the normal and genetically proven beta thalassemia heterozygotes were 2.70 ± 0.40% and 6.30 ± 1.23%, respectively, as determined by Q-Sepharose micro-column chromatography, and 2.65 ± 0.31% and 5.37 ± 0.96%, respectively, as determined by HPLC. The mean Hb E proportions in the Hb E heterozygotes were 23.25 ± 4.13% and 24.72 ± 3.5% as determined by Q Sepharose micro-column chromatography and HPLC, respectively. In the large-scale survey for beta thalassemia, 23 at risk couples were detected. Seven affected fetuses were identified by prenatal diagnosis.

Conclusions: Q Sepharose micro-column chromatography was found to be reliable, reproducible and well-suited for large-scale surveys. Additionally, by being reusable and convenient, this simple and economical chromatography method may be an alternative means to screen for beta thalassemia and Hb E carriers in the mass population.

1. Introduction

Beta thalassemia major and hemoglobin (Hb) E-beta thalassemia disease are leading genetic problems in Southeast Asian countries. Mass screening of the carrier state by determination of the Hb A_2 and Hb E proportions and mutation analysis is a preferred method for making prenatal diagnoses (PND). Normal adults have an Hb A_2 between 2.5% and 3.5% [1]. When the proportion of Hb A_2 exceeds this value, phenotypic diagnosis of beta thalassemia carrier can be made. Hb E, a common Hb variant caused by a beta globin gene mutation (HBB:c.79G>A), is also highly prevalent in the population. The heterozygous Hb E is prevalent in 25–30% of individuals [2]. To date, many automated Hb analyses are available for mass screening of the population, including high performance liquid chromatography (HPLC), low pressure liquid chromatography and capillary electrophoresis. However, these standard investigations are costly for screening large populations. Actually, screening of beta thalassemia and Hb E carriers by standard Hb analyses in thalassemia prevention programs seems immoderate since detection of the carrier state by determination of the Hb A_2 and Hb E proportions can be done without quantitation of other types of Hb. Specific determination of the Hb A_2 and Hb E proportion by micro-column chromatography may be a suitable choice for screening purposes. In contrast with Hb A_2 and Hb E measurements by standard chromatography methods, micro-column chromatography is economically well-suited for large-scale surveys in thalassemia prevention programs. Diethylaminoethyl (DEAE) Sephadex is a commonly used anion exchanger for packing micro-columns for trapping Hb in alkaline pH. When the mobile phase is adjusted to a more acidic pH

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http://dx.doi.org/10.1016/j.clinbiochem.2016.07.003
0009-9120/

by the addition of a small amount of HCl, Hb A_2 and Hb E will be replaced by the Cl ion and will be eluted before other Hb types for easy measurement. Q Sepharose is a strong anion exchanger that uses quaternary amines as a ligand [3]. This anion exchanger has been introduced in recent years and has been used in various clinical applications [4–6]. Q Sepharose chromatography procedures have been shown to remove many negatively charged biological impurities, including viruses, during the purification process of biological products. This is also because the high salt concentration can disrupt the binding of most negatively charged particles to the Q Sepharose resin [6], reusability by intra-column regeneration is another advantage. By not requiring a strict pH adjustment as DEAE Sephadex micro-column chromatography and being reusable, Q Sepharose micro-column chromatography may be a suitable choice for Hb A_2 and Hb E determinations in population surveys. The objective of this study is to compare Q Sepharose micro-column chromatography with HPLC for the detection of beta thalassemia and Hb E carriers for screening purposes.

2. Materials and methods

Three hundred pregnant women, singly or together with their spouses if present, were consecutively recruited from the antenatal care (ANC) clinic at Naresuan University Hospital between December 2012 and September 2013. Their blood specimens were labeled as the general population group. Fifty other blood samples, which were phenotypically diagnosed as beta thalassemia heterozygotes by HPLC analysis, were also consecutively collected from the thalassemia screening program at Naresuan University Hospital during the same period. These beta thalassemia carriers were further genetically documented via an allele-specific polymerase chain reaction (PCR) and were labeled as the beta thalassemia carrier group. Quantitation of Hb A_2 or Hb E was performed on both general population and beta thalassemia carrier group by using Q Sepharose micro-column chromatography in parallel with HPLC. Since the micro-column chromatography technique may be limited by inability to quantitate Hb A_2 in the presence of slow moving Hb such as Hb S, Hb D or Hb G [7–9], any abnormal Hb, except Hb E, identified by HPLC had to be excluded from the study. The study was approved by the institutional ethical committee. Written informed consent was obtained from all of the blood donors before entering the study.

Before commencing this study, a pilot study on reusability of Q Sepharose column was conducted by the same investigators. Samples with normal, beta thalassemia heterozygote, and Hb E heterozygote, 8 specimens each, were collected to quantitate Hb A_2 or Hb E by using Q Sepharose micro-column chromatography. The Q Sepharose column was reused 5 times on each specimen. HPLC was performed in parallel on the first and the last test of micro-column chromatography.

Q Sepharose (0.5 ml) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was packed into the tip of a well-designed disposable syringe with a 10 ml reservoir (Fig. 1). Before starting the procedure, Q Sepharose micro-columns were equilibrated with 10 ml of working buffer (0.05 M Tris-HCl-KCN, pH 8.5). Hemolysate was prepared by mixing 20 μl of whole blood with 2 ml of the working buffer. One milliliter of hemolysate was applied to the column and was fixed in the column with another 2 ml of the working buffer. Hb A_2 or Hb E was then eluted from the column with 4 ml of eluting buffer (0.05 M Tris-HCl-KCN and 12 mM NaCl, pH 8.5). The absorbance of the eluate was measured at 415 nm with a spectrophotometer (SPECORD 30™, Analytik Jena AG, Thuringia, Germany) and was labeled as optical density-1 (OD1). OD2 was made by mixing 1 ml of the previously prepared hemolysate with 3 ml of working buffer and measured as a reference. The proportion of Hb A_2 or Hb E was calculated using the following formula:

$$\text{Hb } A_2/\text{Hb E } \% = \frac{(\text{OD1x100})}{\text{OD2}}$$

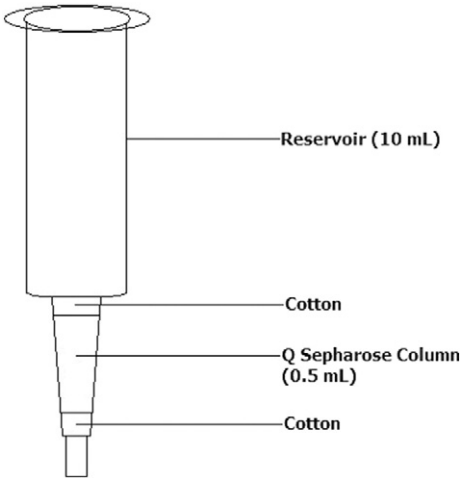

Fig. 1. Q Sepharose column.

Q Sepharose micro-column chromatography was performed by the same technologist throughout the study. The test was conducted within 7 days of drawing the blood. One Q Sepharose column was reused for 3 Hb A_2 or Hb E quantitation tests. Intra-column regeneration was performed by washing with 2 ml of high concentration NaCl, as a washing buffer (0.05 M Tris-HCl-KCN and 1 M NaCl pH 8.5), before restarting equilibration with 10 ml of working buffer for the next testing. Quantitation of Hb A_2 and Hb E by HPLC (VARIANT™) was performed in parallel using the β-thalassemia Short Program (Bio-Rad Laboratories, California, USA). Because of its well-accepted high accuracy and our center's experience, HPLC, a cation-exchange automated chromatography was the method selected in this study as the gold standard for determining the Hb A_2 and Hb E values.

A precision study was conducted using blood specimens collected from normal, beta thalassemia and Hb E carrier group, one sample each, to be examined by micro-column chromatography and HPLC with ten repetitive tests for each method on days 0, 7, and 14 after blood collection.

The mean values of Hb A_2 and Hb E proportion and standard deviation (SD) were calculated for Q Sepharose micro-column chromatography and HPLC method in each population group. The Pearson correlation coefficient (r^2) of Hb A_2 and Hb E values between micro-column chromatography and HPLC method was also calculated. The sensitivity, specificity, and positive and negative predictive values of Q Sepharose micro-column chromatography for screening of beta thalassemia heterozygote were analyzed, using the cut-off Hb A_2 value of 4.0%. For the reusability test of Q Sepharose column in the pilot study, values of Hb A_2 or Hb E proportion from each episode of column reusing were compared using repeated ANOVA. Differences of Hb A_2 or Hb E values between micro-column chromatography and HPLC were analyzed using paired *t*-test. All data analyses were performed using the Statistical Package for the Social Sciences software.

To test a clinical application of Q Sepharose micro-column chromatography on a large-scale survey in a thalassemia prevention program, we conducted an additional clinical study. One thousand five hundred and eighty one pregnant women and their spouses (834 pregnant women) were consecutively recruited from the ANC clinic at Phayao Hospital between January 2015 and January 2016. Using the cut-off Hb A_2 value of 4.0%, Q Sepharose micro-column chromatography was performed in all specimens, screening for couples at risk for beta thalassemia. Detection of beta thalassemia mutations was conducted in all samples with beta thalassemia phenotype using a real-time PCR with high-resolution DNA melting analysis and direct DNA sequencing.

Table 1
Hemoglobin A_2 and hemoglobin E proportions in general population samples (n = 297) and genetically proven beta thalassemia heterozygotes (n = 50) from each method.

	Minimum (%)	Maximum (%)	Hb A_2 (%)[a]
Normal (n = 230)			
Q Sepharose	2.00	3.50	2.70 ± 0.40
HPLC	1.20	3.90	2.65 ± 0.31
Beta thalassemia heterozygote (n = 50)			
Q Sepharose	4.10	8.50	6.30 ± 1.23
HPLC	4.20	8.80	5.37 ± 0.96
	Minimum (%)	Maximum (%)	Hb E (%)[a]
Hb E heterozygote (n = 55)			
Q Sepharose	16.4	31.4	23.25 ± 4.13
HPLC	16.0	33.7	24.72 ± 3.50

Abbreviations: Hb, hemoglobin; Q Sepharose, Q Sepharose micro-column chromatography; HPLC, high performance liquid chromatography.
[a] Values demonstrate as mean ± SD.

3. Results

Of the 300 blood samples in the general population group, HPLC determined that there were 230 normal, 55 heterozygous Hb E, 9 heterozygous beta thalassemia, 3 homozygous Hb E, and 3 other abnormal Hb specimens. Three specimens with abnormal Hb other than Hb E were excluded from the study. The mean (±SD) Hb A_2 proportions in the normal specimens were 2.70 ± 0.40% and 2.65 ± 0.31% as determined by Q Sepharose micro-column chromatography and HPLC, respectively (Table 1). Excluding the homozygous Hb E specimens, the mean Hb E values determined by Q Sepharose micro-column chromatography and HPLC were 23.25 ± 4.13% and 24.72 ± 3.50%, respectively (Table 1). There was a significant correlation of the proportions of Hb A_2 and Hb E as measured between Q Sepharose micro-column chromatography and HPLC ($r^2 = 0.987$, $p < 0.01$) (Fig. 2). Using a cut-off Hb A_2 value of 4.0%, after excluding the Hb E samples, the diagnostic accuracy of the Q Sepharose micro-column chromatography for screening beta thalassemia carriers was 100% for sensitivity, specificity, and positive and negative predictive values compared to HPLC, which is the gold standard. Data on the micro-column chromatography screening accuracy was demonstrated with a 2 × 2 table shown in Table 2.

In the beta thalassemia carrier group, the mean Hb A_2 proportions in the fifty genetically proven beta thalassemia heterozygotes were 6.30 ± 1.23% and 5.37 ± 0.96% as determined by Q Sepharose micro-column chromatography and HPLC, respectively (Table 1).

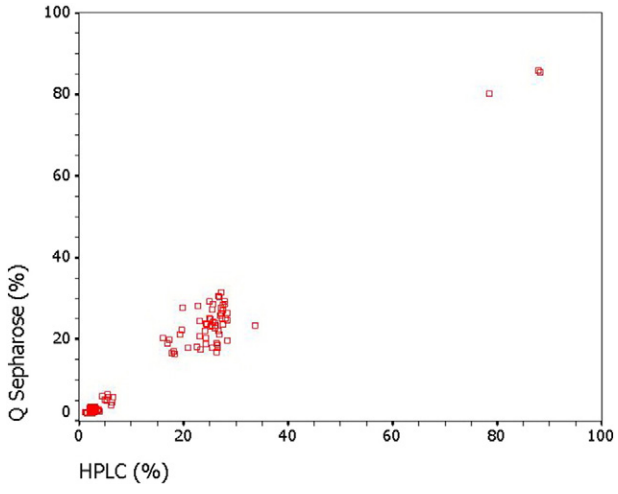

Fig. 2. Scatterogram showing correlation of hemoglobin A_2 and E values between Q Sepharose micro-column chromatography and high performance liquid chromatography. Abbreviations: Q Sepharose, Q Sepharose micro-column chromatography; HPLC, high performance liquid chromatography

Table 2
Sensitivity and specificity of Q Sepharose microcolumn chromatography for screening of beta thalassemia heterozygote using high performance liquid chromatography as gold standard (samples with hemoglobin E were excluded.) (n = 239).

Disease status		
Test result	Beta thalassemia heterozygote	Normal
Positive	9	0
Negative	0	230

A precision study with the normal, beta thalassemia and Hb E carrier specimens via both micro-column chromatography and HPLC is shown in Table 3. Results on reusability of the Q Sepharose column in the pilot study are shown in Table 4. There were no differences of Hb A_2 or Hb E values between each episode of column reusing in Q Sepharose micro-column chromatography ($p > 0.05$). The three excluded blood samples with abnormal Hb from HPLC were investigated by using DNA techniques. These samples included an Hb Tak heterozygote (HBB:c.441_442insAC), an Hb Q Thailand heterozygote (HBA1:c.223G>C) and Hb H-Constant Spring (CS) compound heterozygote [Alpha thalassemia-1 heterozygote (NG_000006.1: g.26264_45564del19301)/Hb CS heterozygote (HBA2:c.427T>C)]. Data of these Hb analyses from HPLC is shown in Table 5. The Hb A_2 proportions determined by Q Sepharose micro-column chromatography and HPLC for the abnormal Hbs are also shown in Table 5.

For the large-scale survey of beta thalassemia using Q Sepharose micro-column chromatography, among 1581 cases, there were 271 (17.14%) Hb E heterozygotes, 63 (3.98%) beta thalassemia heterozygotes, 22 (1.39%) Hb E homozygotes, and 9 (0.56%) Hb E – beta compound heterozygotes. Excluding the Hb E alleles, all 72 beta globin mutations were detected by using DNA technique. Twenty-three at risk couples were identified; 20 at risk for Hb E – beta compound heterozygote and 3 at risk for beta thalassemia homozygote. Amniocentesis was performed for PND in all at risk couples. Seven affected fetuses were found; 6 Hb E – beta compound heterozygotes [3 Hb E – CD 41/42 (HBB:c.126_129delCTTT), 2 Hb E – CD 17 (HBB:c.52A>T), and 1 Hb E – CD 35 (HBB:c.108C>A)], and 1 beta thalassemia homozygote [CD 17 (HBB:c.52A>T)].

4. Discussion

Our results indicate that Q Sepharose micro-column chromatography is accurate for quantitating Hb A_2 and Hb E compared with HPLC with acceptable precision. Both methods gave almost the same range

Table 3
Precision study with normal subject, beta thalassemia and hemoglobin E heterozygote by Q Sepharose micro-column chromatography and high performance liquid chromatography with ten repetitive tests for each method.

Day tested	N	Hb A_2/Hb E (%)[a]			
		Q Sepharose	CV (%)	HPLC	CV (%)
Normal					
Day 0	10	2.14 ± 0.12	5.60	2.40 ± 0.00	0.00
Day 7	10	2.10 ± 0.11	5.23	2.42 ± 0.08	3.30
Day 14	10	2.17 ± 0.12	5.52	2.33 ± 0.11	4.72
Beta thalassemia heterozygote					
Day 0	10	8.05 ± 0.27	3.35	8.50 ± 0.05	0.58
Day 7	10	7.84 ± 0.23	2.93	8.57 ± 0.24	2.80
Day 14	10	7.71 ± 0.33	4.28	7.93 ± 0.17	2.14
Hb E heterozygote					
Day 0	10	26.49 ± 1.06	4.00	26.14 ± 0.05	0.19
Day 7	10	24.84 ± 1.10	4.42	26.34 ± 0.56	2.12
Day 14	10	21.64 ± 1.52	7.02	25.66 ± 0.15	0.58

Abbreviations: Hb, hemoglobin; Q Sepharose, Q Sepharose micro-column chromatography; HPLC, high performance liquid chromatography; CV, coefficient of variance.
[a] Values demonstrate as mean ± SD.

Table 4
Reusability study of Q Sepharose column with normal subject, beta thalassemia and hemoglobin E heterozygote with five episodes of column reusing compared with high performance liquid chromatography.

Reused episode number		1	2	3	4	5	
Normal	N			Hb A_2 (%)*			
Q Sepharose	8	2.48 ± 0.24	2.48 ± 0.22	2.39 ± 0.16	2.33 ± 0.18	2.28 ± 0.21	p** = 0.157
HPLC	8	2.50 ± 0.36	–	–	–	2.49 ± 0.36	
				p*** = 0.084			
Beta thalassemia heterozygote	N			Hb A_2 (%)*			
Q Sepharose	8	4.59 ± 0.45	4.48 ± 0.36	4.29 ± 0.29	4.44 ± 0.48	4.44 ± 0.32	p** = 0.097
HPLC	8	4.74 ± 0.33	–	–	–	4.63 ± 0.33	
				p*** = 0.214			
Hb E heterozygote	N			Hb E (%)*			
Q Sepharose	8	25.84 ± 3.83	24.80 ± 3.89	23.69 ± 3.43	23.63 ± 3.12	24.61 ± 1.80	p** = 0.181
HPLC	8	24.96 ± 2.08	–	–	–	24.05 ± 1.54	
				p*** = 0.074			

Abbreviations: Hb, hemoglobin; Q Sepharose, Q Sepharose micro-column chromatography; HPLC, high performance liquid chromatography.
* Values demonstrate as mean ± SD.
** p-Value comparing between Q Sepharose micro-column chromatography and high-performance liquid chromatography using paired *t*-test.
*** p-Value comparing between each episode of column reusing of Q Sepharose micro-column chromatography using repeated ANOVA.

of Hb A_2 values in both the normal and beta thalassemia heterozygote specimens without overlapping values. Using a cut-off Hb A_2 value of 4.0%, Q Sepharose micro-column chromatography could completely discriminate the beta thalassemia carriers from the normal population. There were also no demonstrated false negative cases of beta thalassemia carriers. However, the technique gave higher Hb A_2 values that were compatible with being a beta thalassemia carrier in some cases for Hb Q Thailand heterozygote and Hb H- CS compound heterozygote. As mentioned in the literature, the presence of slow moving Hb such as Hb S, Hb D or Hb G, and in our finding with Hb Q Thailand and Hb H-CS, could disturb with quantitation of Hb A_2 by micro-column chromatography. Thus, with this false positive limitation, all of the blood samples with beta thalassemia carrier results must be confirmed by molecular mutation analysis prior to thalassemia counseling. However, this disadvantage seems negligible since mutation analysis has to be routinely performed in all results indicating beta thalassemia carrier for genetic counseling before conducting PND.

Our pilot study had indicated that reusing the Q Sepharose micro-column 5 times was acceptable. However, to be more confident, we chose to reuse the column 3 times in this study. Further study on reusability of the Q Sepharose column may be needed for economic reasons.

In the large-scale survey of beta thalassemia in couples having a child, Q Sepharose micro-column chromatography could identify at risk couples before PND is considered. No false positive results were found in this survey.

The Q Sepharose micro-column chromatography screening strategy may be more economical and suitable for beta thalassemia prevention in many Southeast Asian countries. In Thailand, the osmotic fragility test or value of mean corpuscular volume from the complete blood count and the dichlorophenol indophenol precipitation test for Hb E are routinely used for screening for beta thalassemia and Hb E carriers. However, Q Sepharose micro-column chromatography is able to replace all of these screening tests. One lot of blood specimens, which on average includes 20 samples, takes at most 2.5 h to elute Hb A_2 or Hb E. Q Sepharose micro-column chromatography also has the additional advantages of convenience without strict pH adjustment during Hb A_2 or Hb E elution. Moreover, with its intra-column regeneration property, the Q Sepharose micro-column can be reused after washing with high salt concentrations and a working buffer.

Table 5
Hemoglobin proportions from high performance liquid chromatography of the three excluded blood samples compared with hemoglobin A_2 values from Q Sepharose micro-column chromatography.

	Abnormal Hb (%)	Hb A (%)	Hb A_2 (%)	Hb A_2 from Q Sepharose (%)
Hb Tak	39.2 (D window)	50.3	2.3	2.9
Hb Q Thailand	26.4 (S window)	62.7	2.7	4.5
Hb H-Constant Spring	2.2 (C window), undetermined amount of Hb H	82.4	0.8	5.1

Abbreviations: Hb, hemoglobin; Q Sepharose, Q Sepharose micro-column chromatography.

5. Conclusions

Q Sepharose micro-column chromatography is a reliable and well-suited method for large-scale surveys. Furthermore, with its reusable properties and convenience, this simple chromatography method may be an alternative means for Hb A_2 and Hb E determinations in population screenings. The method might facilitate the identification of a couple at risk of having a fetus with beta thalassemia disease, which would result in an early PND.

Disclosure statement

All authors have no relevant conflicts of interest to disclose.

Acknowledgements

This work was supported by a research grant from the Faculty of Medicine (fiscal year 2012), Naresuan University (R2556C020).

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