ANALYTICAL SCIENCES MARCH 2010, VOL. 26

Sequential Injection-Cation Exchange Micro-column System for Hemoglobin Typing to Differentiate HbE Carriers

Supaporn KRADTAP HARTWELL,*† Worathip SRIPAORAYA,* Somchai LAPANANTNOPPAKHUN,* Torpong SANGUANSERMSRI, and Kate GRUDPAN***

**Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
***Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

A weak cation exchange micro-column was incorporated into a sequential injection (SI) system to perform automatic hemoglobin (Hb) typing as an alternative way to measure HbE. Separation of HbF, HbA and HbA_2/HbE was performed using phosphate buffer solutions in the pH range of 6 – 7 to create pH gradient mobile phase. The resultant chromatogram showed relative amounts of HbE to other types of hemoglobins in more quantitative detail than the conventional techniques such as dichlorophenol indophenol precipitation and micro-column anion exchange. The system is more economical than a commercially available ion-exchange HPLC analyzer for hemoglobin testing, though analysis time per run is longer due to the aspiration operation of the syringe pump of the SI system. It has been demonstrated that the system can differentiate negative (normal) from positive (HbE carriers) subjects.

(Received September 28, 2009; Accepted October 29, 2009; Published March 10, 2010)

Introduction

HbE is one of the abnormal hemoglobin variants found abundantly in Southeast Asia.[1,2] Carriers of HbE or people with homozygous HbEE normally have no serious health problems. However, when HbE coexists with β-thalassemia gene, severe symptoms may result.[3-6] In many areas around the world, there are populations of β-thalassemia carriers and HbE carriers. Neither group by itself has any indication of a health problem and therefore, most people who are carriers do not know it. With the lack of knowledge about thalassemia and hemoglobinopathies, along with the migration and marriages among peoples from different regions, the combination of 2 carrier genes (*i.e.* HbE and β-thalassemia gene) is highly possible. Thus, it becomes important for the countries at risk to screen for HbE carriers as much as they do for other hemoglobinopathies.

The screening techniques should be low cost to make them affordable for the entire population. Although, the main objective of a screening technique is to cut down on the number of samples that are unnecessarily tested using higher cost extensive diagnosis techniques, it is important that the screening techniques provide sufficiently accurate and reliable results. Two main screening techniques for HbE commonly used in hospitals in Thailand are dichlorophenol indophenol precipitation (DCIP test) and ion exchange chromatography.[7,8] Chromatography is the more specific technique. A simple version of anion exchange chromatography, where diethyl aminoethyl (DEAE) anion exchanger was packed into a 10-ml syringe barrel that acts as a column, has been introduced. It has been used as part of a routine screening process at the Thalassemia Laboratory at Maharaj Nakorn Chiang Mai Hospital, Thailand.[9-11] This technique pays attention to the presence of HbE which is eluted first (with HbA_2) from an anion exchange column. Therefore, the volume of mobile phase and the analysis time can be limited just for adequately eluting HbE. Other Hbs (*i.e.* HbA and HbF) will not be eluted with this limited volume of mobile phase. Eluates from positive samples will show a reddish color due to the presence of a high amount of HbE, while negative samples have clear colorless eluates (due to a low amount of HbA_2) after the same elution time and with the use of the same volume of mobile phase. Although this result is sufficient to predict the existence of HbE, the detailed chromatogram can be useful for further studies or diagnosis. Ion exchange HPLC has been reported for most thalassemia studies[12-15] due to its ability to reveal various hemoglobin variants in one run. However, the high costs of instrumentation and of the mobile phase are drawbacks that make HPLC unsuitable for use as a screening process, especially where one's budget is very limited.

Our group incorporated the micro-anion exchange column with the first generation flow injection system.[16] The system successfully performed hemoglobin typing with a chromatogram by using much lower amounts of sample and mobile phase, as compared to the conventional simple version anion exchange micro-column chromatography. However, the operational process including buffer gradient control was still only semi-automatic. Therefore, this work attempts to develop a micro-chromatographic system based on a sequential injection technique to automate the mobile phase gradient. At the same time, the cation exchange resin is tested for use as an alternative to an anion exchange resin. Cation exchange has been used in the commercial HPLC analyzer for hemoglobin testing (BIO-RAD). Therefore, this can be a closer comparison of the

† To whom correspondence should be addressed.
E-mail: kradtas@yahoo.com

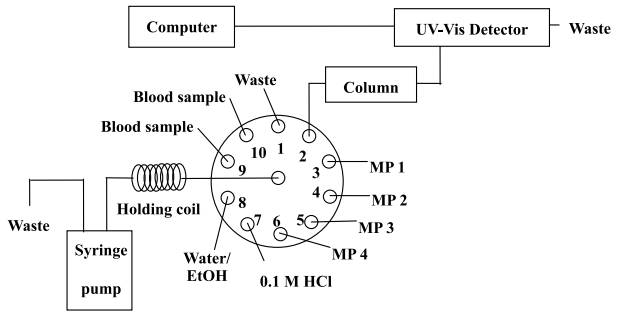

Fig. 1 Schematic diagram of the SI-CatEx system (MP is mobile phase).

proposed system with the commercial HPLC analyzer. The sequential injection-cation exchange chromatographic system (SI-CatEx) is evaluated with packed red cells of negative (normal) and positive (HbE carrier) subjects.

Experimental

Apparatus

The SI system was composed of a 5-ml syringe pump (Carvo), a 10-ports selection valve (Valco), and a spectrophotometer (UVIS-200 detector, LINEAR Instruments) with a flow through cell of 3 mm path length and 1.2 µl internal volume (SST Cell). All tubings for delivery of reagents were PTFE of 0.03″ i.d. (UpChurch). The holding coil has the volume of 1000 µl. Figure 1 shows the diagram of the system with positions of buffer solutions, sample, and column. The analytical signals were recorded with a personal computer through Labview™ 7 Express and the peak area was integrated with eDAQ chart software (PowerChrom 280, Australia).

Reagents

A stock NaCl solution (1.5 M) was prepared and was diluted with Milli-Q water to obtain working solutions of 0.3 and 0.15 M. Phosphate buffer solutions at a concentration of 0.05 M and in a pH range of 6.0 – 7.0 were prepared by adjusting the pH of the appropriate volume of 0.5 M KH_2PO_4 with 2 M H_3PO_4 or 2 M NaOH. All the reagents were purchased from Sigma.

Cation exchange micro-column

A micro-column was made by drilling a straight channel through a piece of acrylic (1 cm × 1 cm × 4 cm) with threads made on both ends to fit the normal nuts (1/16″, UpChurch) used in SI assemblies. The size of the channels available for packing of stationary phase is 2.5 cm in length and 0.3 cm i.d. with an estimated volume of 177 µl.

Cation exchange resin used here is carboxy [COO^-] functional groups of sizes 40 – 90 µm suspended at 70% resin in 20% EtOH and 150 mM NaCl (Fractogel® EMD COO^- (M)). The total volume of resin suspension used was approximately 270 µl per column. This volume was obtained from a calculation based on the volume of column available for packing, percent gel suspension, and the manufacturer's suggestion for the desired compression of 25 – 30%.[17]

The resin was washed prior to packing into the column. Resin was first centrifuged at 1800 rpm for 10 min and then the supernatant was removed. Distilled water was added at the volume of 10 times the volume of resin and was removed after centrifugation. This step was repeated 3 times. After that, 0.3 M NaCl was added at the volume of 1:1 ratio to the volume of resin. Using a micro-pipette, this suspension was transferred into the micro-column, which has a thin piece of cotton and a nut placed at one end. After resin was packed into the micro-column while solution was flowing out, 0.15 M NaCl was added to condition the resin. Finally, another thin piece of cotton and a nut were placed at the other end of the column.

Blood samples

All blood samples were obtained from the Thalassemia Laboratories in Chiang Mai University Hospital. Blood samples were hemolysated as follows. A portion (200 µl) of packed red cells was mixed with 200 µl of 0.05 M phosphate buffer. The mixture was shaken by using a vortex machine for 10 min to break the cells. Then, the mixture was centrifuged at 2000 rpm for 10 min to remove supernatant. The sediment was reconstituted with 0.05 M phosphate buffer at the ratio of 1:100 (v/v) (sediment:buffer). It appears as a red clear liquid when observed with one's bare eyes. The micro-centrifuge tube containing 1000 µl of the reconstituted sediment was connected to port No. 9 or 10 of the selection valve (Fig. 1).

It should be noted that the HPLC operation at the Chiang Mai University Hospital employs a blood sample at 1:200 dilution ratio to avoid the problem of column clogging which can occur when using a too concentrated sample. The 1:200 dilution ratio can also be used with the proposed SI-CatEx system, however, since our column can more easily be changed and is of lower cost as compared to the HPLC column, we prefer to use red cells sample at 1:100 dilution ratio in order to reduce any error that may occur with too much dilution.

Results and Discussion

Detection wavelength

According to previous works, two wavelengths at 415 and 450 nm were reported for the detection of Hb.[8,16] Therefore, both wavelengths were tried. It was found that the absorption spectra obtained at both wavelengths had minimal noise and the detection wavelength at 415 nm offered a higher analytical signal (both peak height and peak area) at the same separation condition (*i.e.* mobile phase pH gradient 5.0 – 7.0 with the same volumes of sample and mobile phase).

pH gradient range and concentration of phosphate buffer solution

Concentration of the mobile phase relates directly to its ionic strength, which also affects the degree of the ion exchange process. Therefore, both pH gradient and concentration of the mobile phase are important parameters for effective separation of different Hbs.

pH gradient in the range of 5 – 9 and concentrations of phosphate buffers (0.03 – 0.06 M) were studied. Here, we are interested in comparing the relative amounts of HbE in negative and positive subjects. Therefore, a preliminary study was carried out by passing the eluates that had been separated by the conventional anion exchange micro-column at the hospital into the proposed SI-CatEx system. As previously mentioned, the conventional anion exchange micro-column technique uses a limited volume of mobile phase, enough to just elute out HbA_2 or HbA_2/HbE. Other major hemoglobins (*i.e.* HbA and HbF) cannot be eluted with this limited volume of mobile phase. Therefore, eluate from a negative blood sample should contain only a small amount of HbA_2, while eluate from a positive blood sample should contain a high amount of HbA_2/HbE.

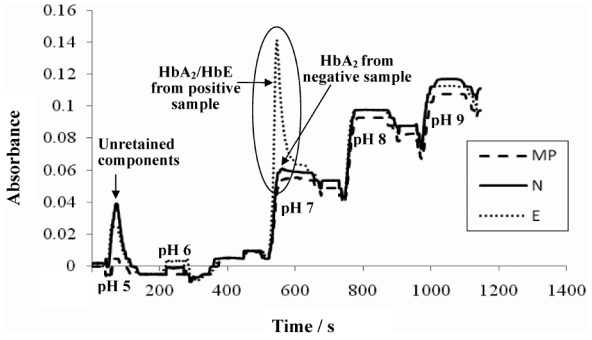

Fig. 2 SI-CatEx elution of the eluate portions, obtained from the conventional anion exchange micro-column, containing HbA_2 (from negative sample, N) and HbA_2/HbE (from positive sample, E). HbA_2 and HbA_2/HbE could be eluted with phosphate buffer of pH 7.0 (MP is mobile phase).

After introducing these eluates into the SI-CatEx system and eluting them with phosphate buffers of different pHs (pH 5, 6, 7, 8 and 9), we found that non-retained components were eluted out with mobile phase buffer pH 5; after that, HbA_2 from a negative sample and HbA_2/HbE from a positive sample were eluted out at pH 7.0 (Fig. 2).

According to the chromatogram obtained from separation of hemoglobins using a cation exchange HPLC analyzer, HbA_2/HbE were eluted last, which is in the reverse order as compared to using an anion exchange chromatographic technique. This suggested that only the pH range 5 – 7 should be studied further in more detail. Finally, we found that the pH gradient should be created by varying the pH of the phosphate buffer from 6.0 (500 µl) to 6.1 (1500 µl), 6.7 (5000 µl) and 7.0 (3000 µl), using the same flow rate of 1.5 ml/min, for the best time savings with good resolution.

Various concentrations of phosphate buffer mobile phase (0.03, 0.04, 0.05 and 0.06 M) were tested. The concentration of phosphate buffer at 0.05 M leads to separation of hemoglobins at approximately the same relative ratio as the theory suggests: HbF 1%, HbA 96% and HbA_2 3%.[18] Lower concentrations could be used to separate hemoglobins but the relative amounts of each type of hemoglobin do not follow the theory. This is probably because of the co-elution of HbA and HbA_2/HbE peaks.

Flow rate

The flow rate of the mobile phase affects the separation of hemoglobins. When the flow rate was too high, hemoglobins did not have time to separate, causing co-elution. Too low of a flow rate causes peak tailing and low sample through put. The flow rates of 0.5, 1.0 and 1.5 ml/min were tested. It was found that 1.5 ml/min (set as 25 µl/s in the SI software) yields good resolution with a reasonable separation time. A higher flow rate was not used because it may damage the packed bed column and cause leaking.

Operational steps

Operational steps were automatically controlled by a computer system. The details of the operational sequences are shown in Table 1. Steps 7 – 8, 11 – 12, and 15 – 16 are for getting rid of the mobile phase used in the earlier steps. In this way, the total separation time was shorter than in the case of having too long of a pH gradient between each mobile phase, as occurs when operating without those steps. The total volume of buffer mobile phases was 13 ml with a 50-µl sample (1:100 dilution ratio). After each run, the column was flushed with 0.1 M HCl (2000 µl at 1.5 ml/min), followed by 0.01 M NaCl (3000 µl at 1.5 ml/min), in order to re-equilibrate the column. To change the sample (at ports 9 and 10), one removes the previous sample tube and replaces it with a waste container. Water (2 ml from port 8) was flushed out to port 9 or 10 to remove the residual sample, followed by 10% ethanol (1 ml) and again water (2 ml) to completely clean the sample line. Total washing volume was 5 ml for the sample line length of 5 cm (0.03″ i.d.) at the flow rate of 1 ml/min.

Table 1 Summarization of the operational steps for the SI-CatEx system

Step	Port (see Fig. 1)	Action	Flow rate/ µl s^{-1}	Volume/ µl
1	No. 8 Milli-Q water	Aspirate	90	5000
2	No. 2 Column	Dispense	25	5000
3	No. 9 or 10 Blood sample	Aspirate	25	50
4	No. 2 Column	Dispense	25	50
5	No. 3 Mobile phase (MP1)	Aspirate	90	500
6	No. 2 Column	Dispense	25	500
7	No. 4 Mobile phase (MP2)	Aspirate	90	1000
8	Pump waste	Dispense	90	1000
9	No. 4 Mobile phase (MP2)	Aspirate	90	1500
10	No. 2 Column	Dispense	25	1500
11	No. 5 Mobile phase (MP3)	Aspirate	90	1000
12	Pump waste	Dispense	90	1000
13	No. 5 Mobile phase (MP3)	Aspirate	90	5000
14	No. 20 Column	Dispense	25	5000
15	No. 6 Mobile phase (MP4)	Aspirate	90	1000
16	Pump waste	Dispense	90	1000
17	No. 6 Mobile phase (MP4)	Aspirate	90	3000
18	No. 2 Column	Dispense	25	3000

MP1, Phosphate buffer 0.05 M, pH 6.0; MP2, phosphate buffer 0.05 M, pH 6.1; MP3, phosphate buffer 0.05 M, pH 6.7; MP4, phosphate buffer 0.05 M, pH 7.0.

Hb typing profile

The chromatogram obtained from the SI-CatEx system is represented in Fig. 3(a). The order of the hemoglobins eluted out of the SI-CatEX system is HbF, HbA and then HbA_2/HbE, the same as that obtained from the commercial cation exchange HPLC analyzer. This order of elution could also be concluded based on the relative absorbance values which relate to the amount of each type of hemoglobin in a normal human adult and are also based on theory that the order should be reversed as compared to when the anion exchange column was used.[16,18] The chromatogram that started recording from step 4 (see Table 1) took approximately 9.5 min per run. Retention times of HbF, HbA and HbA_2/HbE were 112, 244, and 485 s, respectively. An example of the chromatogram showing integrated peak areas for use in calculation of percentage of each type of hemoglobin is shown in Fig. 3(b). The points for integration of each peak were selected from baseline to baseline by considering detailed absorbance values collected by the computer software. HbF is normally present at a very low percentage in adults, therefore, the scale must be expanded to ease the selection of the points for integration.

System evaluation

Precision. Negative blood sample was used for this study because it was expected that the integration of the small HbA_2 peak area would yield lower precision as compared to the integration of the larger HbA_2/HbE peak areas of a positive

sample. Therefore, a negative blood sample should better estimate the real precision of the system. The same negative blood sample was run consecutively 10 times. The relative standard deviation (RSD) of the peak area of HbA_2 was found to be 6.5%.

Memory effect. Since the column was packed simply by using a piece of cotton at each end of the column to retain the resin, it is important to ensure that blood sample residue does not become absorbed in the cotton and cause a memory effect. We have proven this point by running one more cycle of buffer gradient through the column after the separation and finding that there were no more peaks shown. Then, the same blood sample was introduced for two repeated separations. The peak height and the peak area were compared to those from the first separation and we found that they were not significantly different. These results indicated that there was no memory effect.

Reusability. The same blood sample was separated repeatedly using the same column. It was found that the same column could be reused with no significant change of peak shape and area for 24 times. Beyond that, the peak shape and the baseline became distorted and accurate integration of peak area was impossible.

SI-CatEx system for screening for HbE carriers. Figure 4 shows relative amounts of HbF, HbA and HbA_2/HbE found in negative and positive subjects. These values were calculated from relative peak areas by setting the total peak area as 100%. Although the cut off value may not be precisely identified due to the small number of subjects, the trend of the results shows that the relative amounts of HbA_2/HbE found in positive subjects (4.3 – 22.6%, mean = 10.9%) are significantly higher than those found in negative subjects (2.0 – 3.8%, mean = 2.9%) at 95% confidence when tested with *t*-test.

It was found that the column could be reused 24 times, therefore, when dealing with a higher number of samples, it is necessary to change or re-pack the column. The precision of different columns was tested by separating 5 of the same normal samples (randomly selected) that were separated in the previous column and comparing the chromatograms. There was no significant difference between peak areas of HbA_2/HbE from chromatograms of the same sample obtained from different columns (RSDs were 0 – 5% from 5 samples).

Table 2 summarizes the advantages and disadvantages of the SI-CatEx as compared to the cation exchange HPLC analyzer for hemoglobin typing. The main benefit of the SI-CatEx system over the commercial HPLC analyzer lies in the lower cost of instrumentation, while sample and mobile phase volumes are comparable. It is also possible to construct a portable SI-CatEx system by replacing the commercial UV-Vis detector with a low cost compact LED lab-made colorimeter.

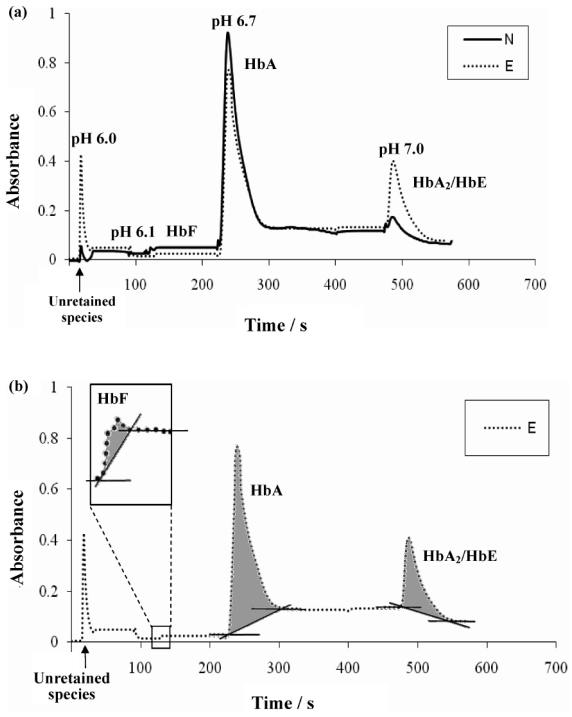

Fig. 3 (a) Chromatogram of hemoglobin typing of negative (normal) and positive (HbE carrier) samples obtained from the SI-CatEx system using a phosphate buffer with pH gradient of 6.0 – 7.0. (b) Example of chromatogram showing peak area integration used for calculation of percentage of each type of hemoglobin. Note: To be able to integrate the peak area for HbF, one must expand the scale.

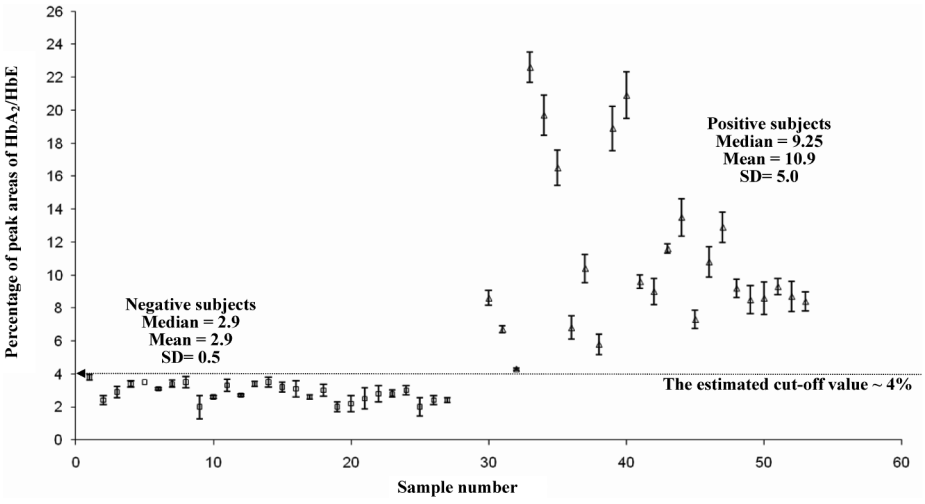

Fig. 4 Summarization of the amounts of HbA_2/HbE found in negative (normal) and positive (HbE carrier) blood samples calculated from the relative peak area of the chromatograms obtained from the SI-CatEx system.

Table 2 Comparison of some benefits (SI-CatEx *vs.* HPLC) for hemoglobin testing

Parameter	SI-CatEx	HPLC	Comparison of SI-CatEx with HPLC
Instrumentation cost[a]	10000 USD	100000 USD	10 times cheaper
Analysis cost/sample[a,b]	0.30 USD	5 USD	15 times cheaper
Sample volume	50 µl (1:100 or 1:200 dilution)	20 µl (1:200 dilution)	2.5 times higher but still in µl level
Separation time/sample	9.5 min	6.5 – 8 min	Slower
Equilibration and washing time	3.5 min column equilibration and 5 min washing of sample lines between each sample	1 h equilibration daily before use and no washing between samples due to continuous flow of buffer	More washing between samples

a. Prices are based on quotations in Thailand. b. Calculated based on prices of column, mobile phase and reusability.

The disadvantage of the SI-CatEx system is the lower sample throughput because of the limitations in the pumping operation of the SI system. Therefore, the SI-CatEx system is suitable for analysis where having a small number of samples may make the operation of HPLC less cost effective.

Conclusions

HbE is eluted out from a cation exchange column later than HbF and HbA_2 while it is eluted out first from an anion exchange column. Therefore, if one intended to just consider the presence of HbE without quantitative information relative to other hemoglobins, anion exchange chromatography should be used. However, if one is interested in a full chromatogram, either cation or anion technique can be chosen as available. Here, we would like to investigate the possibility of using a lower cost sequential injection system to couple with a micro-cation exchange column as an alternative to the higher cost commercial HPLC analyzer for hemoglobin typing. The pH gradient could be controlled automatically by the sequential injection system, but the system involves aspiration and dispensation of solution by a syringe pump; this delays the analysis time. Total separation time of the sequential injection-cation exchange chromatographic system (SI-CatEx) is therefore longer than HPLC (9.5 *vs.* 6.5 min). Additional operation times of the two systems are different. HPLC requires long equilibration time of 1 h once daily before use with no washing step between samples. SI-CatEx requires shorter equilibration time and washing the sample line between each sample. However, this drawback is compensated with some other benefits. The SI-CatEx is more cost effective for low to medium numbers of samples. The SI-CatEx exhibits lower cost than HPLC in terms of instrumentation and cost of mobile phase. The system is a better quantitative system of HbE as compared to the conventional techniques such as DCIP, though the details of separation (*i.e.* separation of HbA and HbA1c) are not as good as with HPLC. However, further studies on the ingredients and conditions of the mobile phase may lead to overcoming this limitation in the future.

Acknowledgements

The authors thank financial support from the Commission on Higher Education (CHE, through Research Group Grant). Additional supports from the Thailand Research Fund and the Center for Innovation in Chemistry (PERCH-CIC) are also acknowledged. Partial support from the Graduate School, Chiang Mai University, Chiang Mai, Thailand (to W. S.) is also appreciated.

References

1. P. Winichagoon, S. Svasti, P. Winichagoon, C. Chitchumroonchokechai, and S. Fucharoen, *Haematologica*, **2007**, *92*, 702.
2. D. Bachir and F. Galacteros, *Hemoglobin E. Orphanet*, **2004**, http://www.orpha.net/data/patho/GB/uk-HbE.pdf.
3. North Dakota Department of Health, Newborn Screening Fact Sheet (Hemoglobin E Disease), **2008**, http://www.ndhealth.gov/familyhealth.
4. I. Panigrahi, S. Agarwal, T. Gupta, P. Singhal, and M. Pradhan, *Ind. Pedtr*., **2005**, *42*, 357.
5. P. A. Wahidiyat, D. Gatot, T. Tjitrasari, H. P. Ringoringo, N. S. Marzuki, R. A. Taufani, I. Setianingsih, and A. Harahap, *Paediatrica Indonesiana*, **2006**, *46*, 82.
6. L. Chapple, A. Harris, L. Phelan, and J. Bain, *J. Clin. Pathol*., **2008**, *59*, 74.
7. P. Jaiwang, I. Meemungthum, and V. Wiwanitkit, *J. Chula. Med*., **2007**, *51*, 103.
8. S. Fucharoen, P. Winichagoon, R. Wisedpanichkij, B. Sae-Ngow, R. Sripanich, W. Oncoung, W. Muangsapaya, J. Chowthaworn, S. Kanokpongsakdi, A. Bunyaratvej, A. Piankijagum, and C. Dewaele, *Clin. Chem*., **1998**, *44*, 740.
9. T. Chamrasratanakorn, T. Sanguansermsri, and A. Punyakeaw, *Bull. Chiang Mai Assoc. Med. Sci*., **1998**, *31*, 32.
10. T. Sanguansermsri, P. Tanpaiboon, S. Wongmaeta, S. Chomchuen, and T. Chamrasratanakorn, *Thai J. Hematol. Trans. Med*., **2000**, *10*, 17.
11. S. Sirichotiyakul, F. Tongprasert, and T. Tongsong, *Int. J. Gynecol. Obstet*., **2004**, *86*, 390.
12. F. P. L. van de Dijs, G. A. van der Berg, J. G. Schermer, F. D. Muskiet, H. Landman, and F. A. J. Muskiet, *Clin. Chem*., **1992**, *38*, 1864.
13. G. B. Tan, T. C. Aw, R. A. Dunstan, and S. H. Lee, *J. Clin. Pathol*., **1993**, *46*, 852.
14. R. Galanello, S. Barella, D. Gasperini, L. Perseu, E. Paglietti, C. Sollaino, L. Paderi, M. G. Pirroni, L. Maccioni, and A. Mosca, *J. Automatic Chem*., **1995**, *17*, 73.
15. J. Riou, C. Godart, D. Hurtrel, M. Mathis, C. Bimet, J. Bardakdjian-Michau, C. Préhu, H. Wajcman, and F. Galactéros, *Clin. Chem*., **1997**, *43*, 34.
16. B. Srisawang, P. Kongtawelert, S. K. Hartwell, J. Jakmunee, and K. Grudpan, *Talanta*, **2003**, *60*, 1163.
17. Chromatography Technical Center, EMD Chemicals Inc., Packing Procedure for Fractogel EMD Resins, http://www.emdchemicals.com.
18. T. Watanabe, K. Kato, D. Yamada, S. Midorikawa, W. Sato, M. Shiga, Y. Otsuka, M. Miura, K. Harano, and T. Harano, *Clin. Chem*., **1998**, *44*, 1562.