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ORIGINAL ARTICLE

Novel method for screening for the presence of haemoglobin S in blood

T. N. Higgins,* B. Ridley* and G. Clarke† *Chemistry Department, and †Transfusion Medicine Department, Dynacare Kasper Medical Laboratories, Edmonton, Alberta, Canada

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SUMMARY. A high-performance liquid chromatography (HPLC) method designed for the measurement of haemoglobin (Hb)A_{1c} in blood was investigated for use as a screening method for the presence of HbS in blood. In the Bio-Rad VARIANT II HbA_{1c} method, HbS was found to have a specific retention time and percentage Hb value that enabled the detection of HbS in blood. Other Hb variants did not have the same combination of retention time and percentage Hb as HbS. The HPLC method was superior to the HbS solubility test in ease of performance and readability. Also, the proposed method showed less interference than the solubility test and could be used with samples from all age groups. The proposed method takes 3 min per sample to perform and is thus suitable for large-scale screening.

Key words: HbS screening, HPLC.

Transfusion medicine regulatory agencies in Canada mandate policies for screening for the presence of haemoglobin S (HbS) in blood products to be given to certain classes of patients. In particular, the Canadian Standards Association (2004) Guidelines for Blood and Blood components state that in the case of massive transfusion (including exchange transfusion), all units of blood to be transfused to infants under 4 months of age be screened and found negative for the presence of HbS. The American Association of Blood Banks in the 2003 Guidelines for Blood and Blood Components mandates that a policy regarding the indications for the transfusion of red cells known to lack HbS be in place.

In order to meet these requirements, it is possible to screen for the presence of HbS in blood using the S solubility test performed on lysed cells. Electrophoresis at alkaline pH may also be used for screening for the presence of HbS.

Recent papers report (Joutovsky *et al.*, 2004; Higgins & Ridley, 2005) that identification of several Hb variants could be made by high-performance liquid chromatography (HPLC) on the Bio-Rad VARIANT II HbA_{1c} and β-thalassaemia methods using retention time and percentage Hb data. We wished to investigate whether there were specific retention time and percentage Hb characteristics of HbS that would allow for the identification of HbS as distinct from other Hb variants. This would allow for the use of HPLC as a screening test for the presence of HbS in blood.

METHODS AND MATERIALS

Blood samples were collected in tubes containing potassium ethylenediaminatetraacetic acid (BD Preanalytical solutions, Oakville, Ontario, Canada). The HbA_{1c} program on the Bio-Rad VARIANT II analyser (Bio-Rad Laboratories, Montreal, Quebec, Canada) (as opposed to the Bio-Rad β-thalassaemia program) was used as directed by the manufacturer. In brief, blood samples are mixed on the system before a portion of the sample is introduced into the analyser, mixed with haemolysing reagent and injected into a specific analytical cartridge. A buffer of increasing ionic strength forces the blood sample through the cartridge to a flow cell where the absorbance of separated Hb fractions is measured at a primary wavelength of 415 nm and a background-reducing secondary wavelength of 690 nm. The identification of Hbs in the chromatogram is based on

Correspondence: Trefor Higgins, Co-Director of Clinical Chemistry, Dynacare Kasper Medical Laboratories, Edmonton, Alberta, Canada T5J 5E2.
Tel.: +1 780 451 3702; fax: +1 780 452 8488;
e-mail: trefor.higgins@dkml.com

preset retention times and windows (that are based on retention times) that are intended to help in the interpretation of normal and abnormal chromatograms. The VARIANT II designates retention times of 1·89–1·99 min as an EDS window and retention times of 1·99–2·09 min as a C window. Hbs E, D and S are normally found in the EDS window, and HbC is normally found in the C window. If an abnormality, usually in the form of a split peak, in the chromatogram was noted in these windows, the retention time, identity of the variant after confirmation and the percentage of variant on the HbA_{1c} method were tabulated in an Excel file. The presumptive identities of all Hb variants found in the HbA_{1c} program were established using the Bio-Rad VARIANT II β-thalassaemia program and Hb electrophoresis at alkaline and acid pH with the Sebia Hydrasys (Somagen, Edmonton, Alberta, Canada) electrophoretic system. These methods were performed as directed by the manufacturer except that visualization of the Hb electrophoresis gel at acid pH was performed using Acid Violet stain rather than the recommended Amido Black stain. A card file containing the patient name, date of birth, sex, Alberta Health Care number and the identity of the Hb variant was kept on all individuals in whom a Hb variant was detected.

The mean (1 SD) of retention time and percentage Hb variant present was calculated for all Hb variants detected.

RESULTS

Figure 1 shows chromatograms from patients with normal Hb (Fig. 1A), HbS trait (Fig. 1B), homozygous HbS (Fig. 1C) and sickle cell (SC) disease (Fig. 1D). The chromatogram from a patient with normal Hb shows the presence of HbA at a retention time of 1·77 min (1 SD, 0·007). The chromatogram from a patient with HbS trait shows an HbA peak at 1·77 min and a peak in the EDS window with a mean retention time of 1·965 min (1 SD, 0·006). A single peak in the EDS window with a retention time of 1·96–1·97 min is seen in the chromatogram of patients who are homozygous for HbS. No HbA_{1c} peak is found on these patients, and the *y*-axis of the chromatogram is in volts rather than percentage Hb. The chromatogram from patients with SC disease have an absurdly high HbA_{1c} value (however, it is obviously not HbA_{1c}) with one peak in the EDS window at 1·96/197 min retention time and another in the C window with a retention time of 2·01/2·02 min.

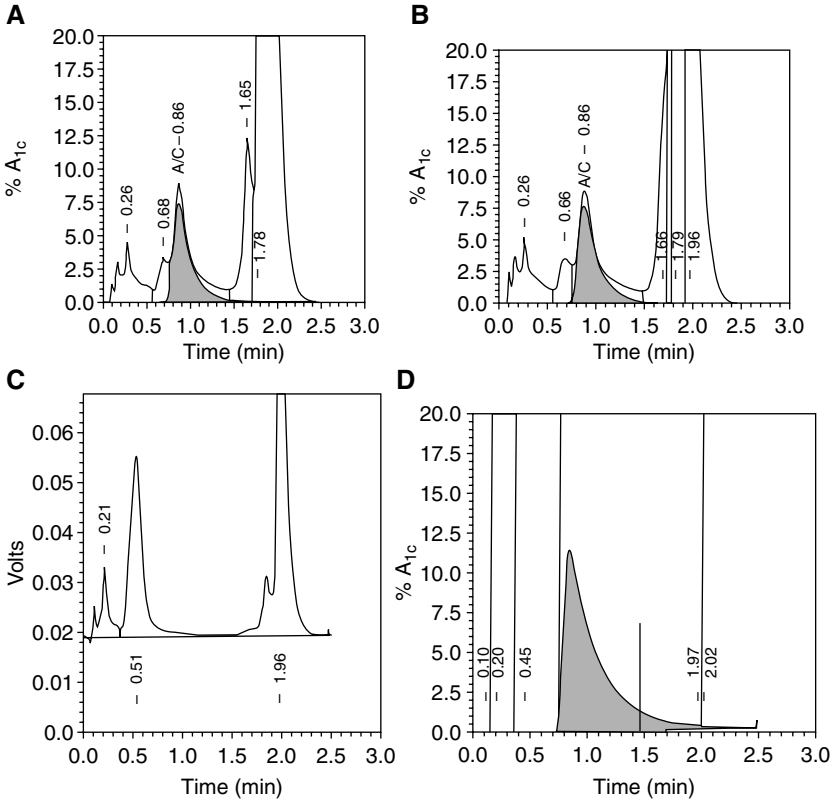

Fig. 1. Chromatograms from patients with normal haemoglobin (Hb) (A), HbS trait (B), homozygous HbS (C) and sickle cell disease (D).

Table 1. Haemoglobin (Hb) variants detected on the Bio-Rad VARIANT II HbA_{1c} method with the retention time and percentage Hb

Hb variant	*n*	Mean retention time (min) (1 SD)	Mean % of total Hb (1 SD)
HbE	50	1.904 (0.008)	34.37 (3.1)
HbD	56	1.945 (0.01)	42.81 (2.71)
HbS	148	1.965 (0.006)	40.99 (3.21)
HbC	43	2.018 (0.02)	37.07 (4.1)
HbOsu Christianburg	3	1.90	46.5
HbD Iran	3	1.88	44.7
Hb Manitoba	1	1.97	25.8
Hb Montgomery	3	1.97	25.1
HbQ Thailand	1	1.97	28.8
Hb Guangzhou Hangzhou	1	1.96	29.5
HbG Honolulu	1	1.96	53.2
Hb Presbyterian	1	2.00	47.8
HbO Arab	2	1.99	41.3
HbQ India	2	1.99	24.6
Hb Spanish Town	1	1.92	22.9

Table 1 lists the identity of Hb variants detected within the EDS and C windows over a 19-month period, together with the retention time and percentage Hb found. The data were acquired using five different lot numbers of reagents on three different Bio-Rad VARIANT II analysers, and the percentage Hb and retention time do not appear to be affected by the age of the column or buffer. Samples from patients homozygous for HbS, HbE, HbD and HbC showed a major peak at 1·96, 1·90, 1·94 and 2·02 min, respectively. Other Hb variants elute outside the EDS and C windows, but the retention times of these variants are so different from HbS that they are not presented in the table.

DISCUSSION

The HbA_{1c} method on the Bio-Rad VARIANT II analyser can discriminate normal Hb from the major Hb variants resulting in changes in the chromatogram which are readily observed by the analyst. The retention time for HbS shows very little variance [95% confidence interval (CI) limits of 1·964–1·966] and is sufficiently removed from the common Hb variants of E, D, C and O Arab to allow for positive identification of HbS. The amount of HbS present in the setting of SC trait is also characteristic at 40·99% (95% CI limits of 40·47–41·52%). The presence of HbS/α-thalassaemia may diminish the proportion of HbS present, and in this circumstance, the retention time is of paramount importance in determining the presence of HbS.

Hb Memphis gives a positive result in the S solubility test but may be identified in the described method by a difference in percentage Hb.

The commonly used methods for distinguishing the presence of HbS include HbS solubility testing and Hb electrophoresis. These methods have significant disadvantages in the context of blood donor screening.

The HbS solubility test is based on the deoxygenating of HbS with sodium metabisulfite (Daland & Castle, 1948) or sodium hydrosulfite (Loh, 1971) or the differential solubility of HbS in 2·24 M phosphate buffer (Matusik *et al*., 1971; Del Guidice *et al*., 1979). In all of these methods, blood samples with HbS show increased turbidity that is observed by reading a ruled card through the treated sample. The commercial versions of some of these tests use reagents that have a short reagent dating once reconstituted and are therefore expensive for occasional use. To achieve optimal performance, it is important to include a centrifugation step in the analysis to increase reliability and sensitivity. Furthermore, the visualization and interpretation of the test are very subjective and fraught with interpretative error, especially with only occasional use. Other Hb variants, such as HbC Harlem, HbC Ziguinchor, Hb Memphis, HbS Travis can give a positive S solubility test. False-positive results may be obtained with the presence of Heinz bodies and high concentrations of monoclonal proteins as well as in samples with high concentrations of cold agglutinins unless the red cells are washed with saline at 37 °C and the suspension is kept at 37 °C for some time before

analysis. False-negative results may be obtained on patients who are anaemic (Hb $<8{\cdot}0$ g dL^{-1}), and when the haematocrit is less than 15%, the amount of sample tested should be doubled prior to analysis in order to obtain correct results. False negatives may also occur in neonates less than 3 months of age, and recommendations (Bain, 2001) have been made not to perform the S solubility test in patients under 6 months of age due to the low concentration of HbS in this age group.

The reagents used in some HbS solubility tests are listed as harmful because they are strong reducing agents and may cause mucus and skin damage while others are made from a flammable chemical. Incubation times required for the S solubility test range from 2 to 20 min with some methods recommending that no readings be done before a minimum incubation of 6 min. All steps in the procedure are performed manually although a semiautomated method has been described (Wilson *et al*., 1974).

Electrophoresis at alkaline pH may be used for screening for the presence of HbS, but the procedure is slow, requires about 2 h for an analysis and considerable technical expertise to perform and is limited by the number of tests that the electrophoresis gel can analyse simultaneously. HbD Punjab, HbD Iran and HbG family give false-positive results.

Transfusion medicine regulatory bodies mandate the performance of HbS screening of donor blood prior to high volume or exchange transfusion for neonatal patients and for transfusion to patients with known SC disease. The frequently used SC solubility tests have demonstrated false positives and negative results due to a number of interferences and variable visual interpretations of the test. In contrast, the HPLC method described here does not require a centrifugation step for optimal performnce, which is not affected by low Hb concentration or the presence of Heinz bodies or cold agglutinins in the sample. Furthermore, the difference in appearance in the chromatogram between patients with normal Hb and patients with HbS trait is readily noted. In addition, the HPLC method allows for discrimination between HbS trait, homozygous HbS and SC disease, which the solubility method cannot do. In addition, the described HPLC method may be completed in less time than the solubility test.

Many laboratories have access to HPLC as a rapid, automated method for the quantification of HbA_{1c} within their facility. Use of this well-established technique to screen donor units for HbS provides a rapid (approximately 3 min/test) means of testing these donor units for HbS without the problems that may be encountered when the S solubility test or Hb electrophoresis is used. The HPLC method avoids the subjective interpretation associated with the visual endpoint detection used in standard commercial SC solubility testing. Performance of SC testing within the blood bank environment may also require additional resources for the kit and associated reagents as well as external quality assurance/proficiency testing. In contrast, use of HPLC when it is available within the laboratory allows rapid determination of the presence of HbS in donor blood and is an efficient means of conforming to transfusion medicine standards while utilizing existing test resources within the laboratory.

Although we have shown that, in Alberta (a multicultural and multiracial society), the most clinically significant and common Hb variants may be distinguished from HbS, it is possible that there is an Hb variant that has retention time and Hb percentage similar to those of HbS.

If a peak is found on a sample with a retention time of 1·96/1·97 min and a percentage Hb of 37·8–44·2, then a report is issued, 'presumptive HbS, confirmation to follow'. If a peak is found that does not meet the criteria for HbS, the following report is issued: 'Hb variant noted. Confirmation to follow'. In either case, the particular donor unit of red blood cells would not be used.

In laboratories that already have access to a Bio-Rad VARIANT II analyser, the reagent costs of the proposed HPLC method compare very favourably with that of commercial SC reagents (HPLC € 0·9–1·3; commercial SC reagents € 1·6–3·3). However, the purchase cost of the VARIANT II analyser € 18 000–30 000 may make it prohibitive to use in laboratories performing only a small number of HbS screening.

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