

Evaluation of high performance liquid chromatography for routine estimation of haemoglobins A₂ and F

G B Tan, T C Aw, R A Dunstan, S H Lee

Abstract

Aims—To compare high performance liquid chromatography (HPLC) with conventional methods for the estimation of blood haemoglobin A₂ (HbA₂) and haemoglobin F (HbF) concentrations in routine thalassaemia screening.

Methods—An HPLC system (the VARIANT Hemoglobin Testing System) was tested for precision and reproducibility in the measurement of HbA₂ and HbF, and reference ranges were obtained for a local healthy adult population. HPLC was compared with column anion exchange chromatography for HbA₂ measurement, and radial immunodiffusion, or alkaline denaturation for HbF measurement. The reliability of HbA₂ measurement by HPLC for the detection of β thalassaemia and HbE was assessed in 200 consecutive samples for routine thalassaemia screening.

Results—HPLC was rapid, technically easy, and gave good precision and reproducibility. In all comparisons linear regression analysis showed good correlation between HbA₂ or HbF concentrations determined by HPLC and by the respective conventional methods. All β thalassaemia or haemoglobin E carriers presumptively identified by conventional methods in 200 consecutive samples were detected by HbA₂ measurement using HPLC, without any false positive or false negative results.

Conclusions—The measurement of HbA₂ and HbF by HPLC is rapid, reproducible, and precise. It is as reliable as column chromatography for the measurement of HbA₂ and radial immunodiffusion or alkaline denaturation for the measurement of HbF. HPLC may be an appropriate method for rapid screening in population surveys for β thalassaemia and HbE carriers.

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Useful variables in the routine laboratory diagnosis of thalassaemia include the haemoglobin concentration, erythrocyte indices and morphology, haemoglobin electrophoresis, haemoglobin A₂ (HbA₂) and haemoglobin F (HbF) estimation, and the detection of erythrocyte haemoglobin H (HbH) inclusions.¹⁻⁴ These data may need to be supplemented with clinical information such as the age and

family history of the patient, and tests to exclude iron deficiency.^{1,2} A practical means of diagnosis of β thalassaemia is by detection of an increased concentration of HbA₂.⁴ Indeed, successful prevention programmes for β thalassaemia in Greece and Italy have relied on screening by erythrocyte indices and HbA₂ concentrations.⁵ Haemoglobin E (HbE), which co-migrates with HbA₂ in conventional electrophoretic or chromatographic methods,¹⁻³ is another haemoglobin disorder with a high incidence in many parts of South East Asia.^{6,7} An accurate and simple method for the measurement of HbA₂ and HbF concentrations could be a useful tool in screening programmes for β thalassaemia and HbE.

Although cellulose acetate electrophoresis is the best routine method for separating abnormal haemoglobins,³ chromatographic procedures are also useful for confirmation and quantitation.⁸ Low pressure macro-column chromatography, using a weak cation exchange material, such as cm-cellulose or an anion exchange material, such as DEAE-cellulose is most commonly used.⁹ This procedure, however, is labour intensive and time consuming. Microchromatographic techniques decrease the chromatographic time but sacrifice resolution.

High performance liquid chromatography (HPLC) is a sensitive and precise method for detecting abnormal haemoglobins.¹⁰ It has advantages over conventional chromatographic techniques in terms of its speed and reliability. In recent years it has been applied to separate and quantitate various haemoglobin fractions.¹¹⁻¹⁴

Methods

As normal adult blood concentrations of HbA₂ and HbF may vary with methodology, reference ranges of HbA₂ and HbF were obtained for the VARIANT Hemoglobin Testing System (Bio Rad Laboratories, Hercules, California, USA) in a population of 104 healthy adults. The VARIANT was compared against a column ion exchange method of HbA₂ measurement in 200 consecutive samples for routine thalassaemia screening, and against radial immunodiffusion (RID) or alkaline denaturation^{15,16} for HbF measurement in 147 consecutive samples for routine thalassaemic screening.

Erythrocyte indices were determined using the Technicon H*1 cell counter (Bayer Diagnostics, Hants, United Kingdom). Haemoglobin electrophoresis in cellulose

Division of
Haematology,
Department of
Laboratory Medicine,
National University
Hospital, Singapore
0511

G B Tan
S H Lee

School of Biomedical
Sciences, Curtin
University of
Technology, Perth,
Western Australia
R A Dunstan

Division of
Biochemistry,
Department of
Laboratory Medicine,
National University
Hospital, Singapore
T C Aw

Correspondence to:
S H Lee

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acetate at pH 8.4 or citrate agar at pH 6.0 was performed using standard methods.³ HbH inclusions were stained by brilliant cresyl blue as described.³

All reagents were provided by the manufacturers, and equipment and reagents were used according to the manufacturers' instructions. The VARIANT is a fully automated cation exchange HPLC system which uses buffers and conditions specifically designed to separate and quantitate HbA₂ and HbF. The analyser makes use of two dual piston pumps and a pre-programmed gradient to allow two different sodium phosphate buffers of increasing ionic strength to pass through a column containing spherical cation exchange resin during a 6.5 minute program. Haemolysed samples are kept at a constant 12°C (SEM 2°C) in the autosampler chamber until they are automatically injected into the

analysis stream. The optical density of the eluate is determined by spectrophotometry incorporated in the equipment which reads at two wavelengths, 415 nm and 690 nm. Specific haemoglobin variants consistently elute within certain retention time frames. A built-in microprocessor and integrator analyses test data to produce a retention profile with percentage HbA₂ and HbF values. Each subfraction is presumptively identified based on predefined retention times. The instrument was calibrated with a haemoglobin A₂/F haemolysate supplied by the manufacturer. Samples were run together with two levels of A₂/F controls, also supplied by the manufacturer, in each run.

HbA₂ was estimated using an anion exchange method, the β-thal Quik column (Helena Laboratories, Beaumont, Texas, USA). HbF quantitation was carried out by alkaline denaturation using Betke's method^{15,16} or by RID, using the HbF Quiplate procedure (Helena Laboratories, Beaumont, Texas, USA). All commercial methods were carried out according to manufacturers' protocols.

Regression analysis was carried out by the method of least squares fit.

Results

REFERENCE RANGES FOR HbA₂ AND HbF CONCENTRATIONS BY HPLC

Blood samples from healthy blood donors were used to establish the reference range. Subjects with abnormal erythrocyte indices were excluded. The remaining 104 adults (26 women and 78 men aged between 18 to 52 years) had haemoglobin concentrations within the normal range (130–170 g/l for men and 115–155 g/l for women). The mean blood HbA₂ concentration of these 104 adults was 2.4%, with values ranging from 1.4 to 2.9, and a standard deviation (SD) of 0.26%. The 95% confidence limits were 1.9 to 3.0 (fig 1). The concentrations of blood HbF ranged from 0.0 to 2.1%, with a mean (SD) of 0.2 (0.29)%. The 95% confidence limits were 0 to 0.8% (fig 2).

PRECISION STUDIES OF HbA₂ AND HbF MEASUREMENT BY HPLC

Precision studies were performed using two blood samples with normal or raised values of HbA₂ or HbF. Each sample was analysed in 10 replicates on three different days. Results (table 1) show low inter- and intra-assay variability for the measurement of normal or raised HbA₂ concentrations, and for the measurement of raised HbF concentrations. An increase in interassay variability was apparent in the measurement of normal HbF concentrations.

THALASSAEMIA SCREENING

Two hundred consecutive subjects for routine thalassaemia screening were evaluated at the National University Hospital by erythrocyte indices and morphology, haemoglobin electrophoresis in cellulose acetate (pH 8.4),

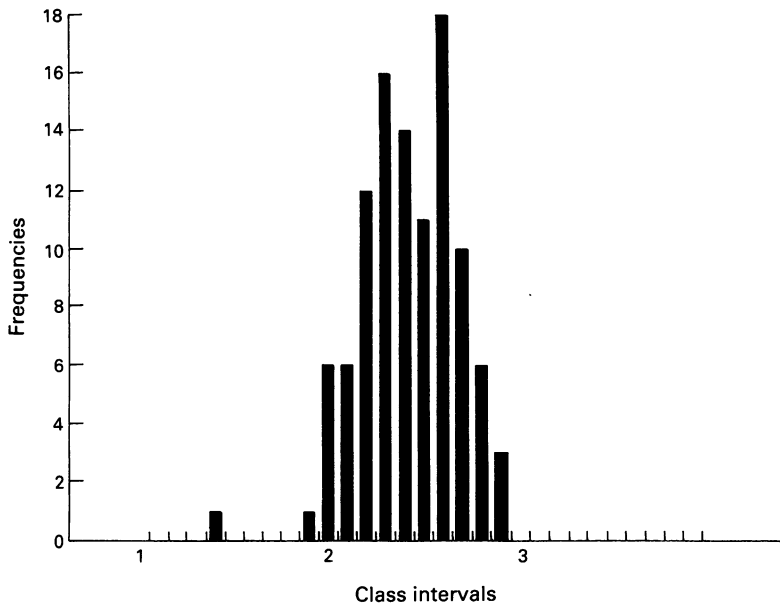


Figure 1 Frequency histogram of the HbA₂ concentration in 104 healthy adults.

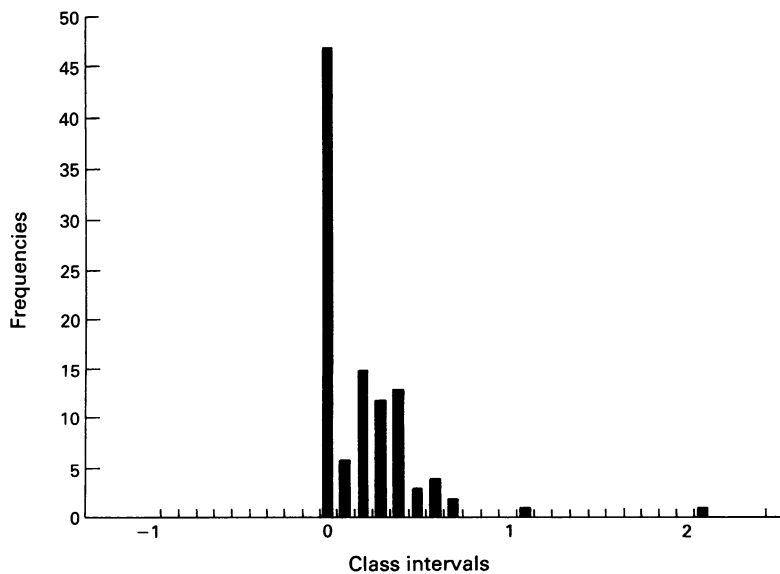


Figure 2 Frequency histogram of the HbF concentration in 104 healthy adults.

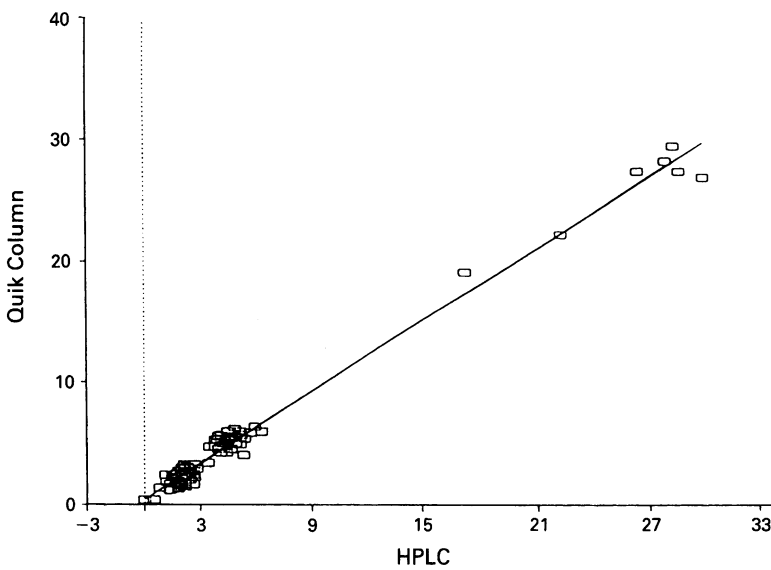
Table 1 Precision of HbA₂ and HbF measurement by HPLC

	N	HbA ₂			HbF		
		Mean %	SD	CV	Mean %	SD	CV
Sample 1							
Day 1	10	2.73	0.082	3.0	0.43	0.048	11.2
Day 2	10	2.66	0.052	1.9	0.41	0.032	7.7
Day 3	10	2.67	0.067	2.5	0.43	0.067	15.7
Sample 2							
Day 1	10	4.07	0.082	2.0	6.93	0.150	2.2
Day 2	10	3.92	0.092	2.3	6.81	0.088	1.3
Day 3	10	3.78	0.079	2.1	6.71	0.145	2.2

Table 2 Cases detected among 200 consecutive subjects screened for thalassaemia

	β -thalassaemia trait	α -thalassaemia trait	HbH disease	HbE trait
N	39	25	3	7
Hb* (g/l)	106 (68-154)	108 (74-140)	93 (84-111)	111 (80-153)
MCV* (fl)	64.7 (59.2-77.9)	68.5 (58.1-78.7)	71.0 (57.2-80.1)	71.9 (63.3-75.8)
MCH* (pg)	19.9 (17.5-21.7)	21.1 (16.5-23.4)	17.7 (16.6-18.3)	23.4 (19.5-26.4)
MCHC* (g/l)	309 (270-328)	310 (271-362)	256 (235-290)	324 (308-342)
% HbA ₂ *	4.6 (3.8-6.3)	2.0 (1.4-2.6)	1.2 (1.1-1.4)	25.8 (17.3-29.9)
% HbF*	0.9 (0.0-4.9)	0.4 (0.0-2.2)	0.5 (0.4-0.7)	0.6 (0.0-2.4)

* = mean (range).

HbA₂ and HbF concentrations were determined by HPLC. All subjects tabulated are aged 1 year old and over. Two infants with α -thalassaemia trait were excluded from this table.Figure 3 Linear regression plot of HbA₂ concentrations measured by the β -thal Quik Column method against HbA₂ concentrations measured by HPLC. Correlation coefficient $r = 0.99$; $n = 200$.

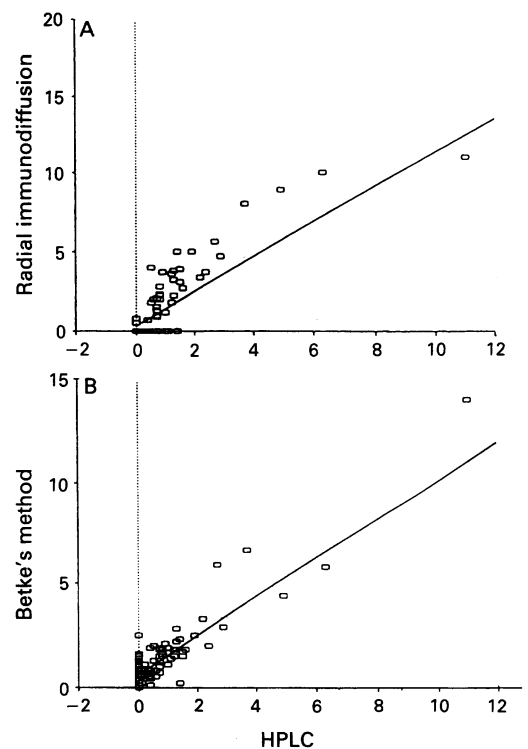
erythrocyte HbH inclusions, and HbA₂ and HbF concentrations. Subjects came from the routine inpatient and outpatient hospital population, including antenatal clinics. In most subjects with microcytic and hypochromic indices iron deficiency was excluded by serum iron or ferritin estimation. Using this analysis, 39 had β thalassaemia trait, 27 α thalassaemia trait, and three haemoglobin H disease (table 2). Of the subjects found to have β thalassaemia trait, HbA₂ concentrations were raised in all cases, while HbF values were raised ($\geq 0.8\%$) in 18 out of 39 (46%) cases. Presumptive diagnoses of α thalassaemia trait or HbH disease were all confirmed by finding erythrocyte HbH

inclusion in very scanty or in large numbers, respectively. The three subjects with HbH disease had low HbA₂ values (range 1.1-1.4%). Investigations to exclude α thalassaemia 2 carriers in whom HbH inclusions may be undetectable,⁴ were not carried out in this study.

Seven samples with apparently high HbA₂ concentrations of more than 7% (range 17%-30%) were further screened by electrophoresis in citrate agar at pH 6.0. None of these samples showed any additional bands in citrate agar electrophoresis at pH 8.0. These results, together with other variables including erythrocyte indices, morphology, and HbF concentrations (table 2), were consistent with heterozygous HbE in all seven samples. No other abnormal haemoglobin variants were detected in this study.

COMPARISON OF HPLC WITH OTHER METHODS

Quantitation of HbA₂ by HPLC was compared with the β -thal HbA₂ Quik Column, an anion-exchange column system. The comparison was performed on all the 200 samples. The linear regression equation of HbA₂ values, measured by anion exchange, against HbA₂ values, measured by HPLC, was $y = 0.337 + 0.984x$, with a correlation coefficient of 0.99 (fig 3). HbA₂ results determined by HPLC gave excellent agreement with those obtained using the β -thal Quik Column. Both methods identified the same individuals with β -thalassaemia trait or HbE among the 200 cases screened, without any false negative or false positive results. In the

Figure 4 (A) Linear regression plot of HbF concentrations measured by RID against HbF concentrations measured by HPLC. Correlation coefficient $r = 0.90$; $n = 147$. (B) Linear regression plot of HbF concentrations measured by Betke's method against HbF concentrations measured by HPLC. Correlation coefficient $r = 0.93$; $n = 147$.

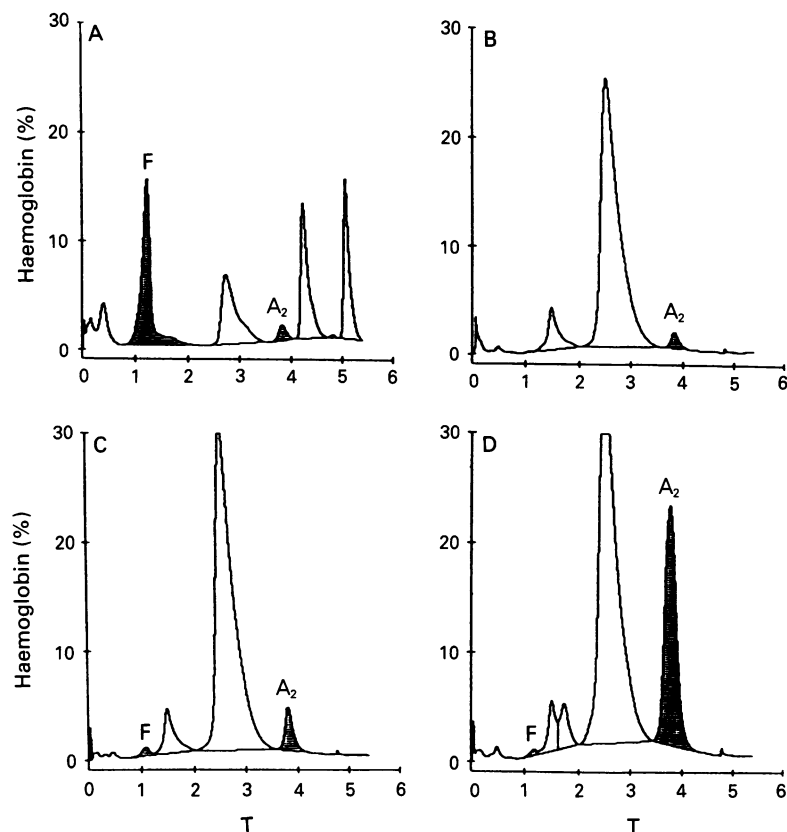


Figure 5 Graphical output of the VARIANT Hemoglobin Testing System. *T* = retention time in minutes. HbF and HbA₂ appear as shaded peaks. (A) Control haemoglobin samples. Successive eluted peaks are haemoglobins F, A, A₂, S and C. HbF = 27.8%, HbA₂ = 2.4%. (B) Normal subject; HbF = 0%, HbA₂ = 2.2%. (C) β -thalassaemia trait; HbF = 0.9%, HbA₂ = 4.9%. (D) Haemoglobin E trait; HbF = 0.3%, apparent HbA₂ concentration = 23.6%.

124 samples from patients in whom thalassaemia or HbE was not detected, the mean (range) HbA₂ concentration was 2.1% (0.5–3.4) by HPLC, and 2.3% (0.3–3.3) using the β -thal Quik column.

HbF, measured by Betke's method or RID, was compared against HbF quantitation by HPLC in a total of 147 samples. The linear regression equation for HbF values measured by RID against HbF values measured by HPLC was $y = 0.155 + 1.397x$ (fig 4A), with a correlation coefficient of 0.90. The linear regression equation for HbF values measured by Betke's method against HbF values measured by HPLC was $y = 0.510 + 1.114x$ (fig 4B), with a correlation coefficient of 0.93. HbF concentrations were determined by all the three methods (HPLC, RID, and Betke's method) in 94 samples from patients in whom thalassaemia or HbE was not detected. In these 94 samples, the mean (range) HbF concentration was 0.36% (0.0–11.0) by HPLC, compared with 0.48% (0.0–11.0) by RID, and 0.85% (0.0–14.0) by Betke's method.

DETECTION OF ABNORMAL HAEMOGLOBINS

The VARIANT was capable of differentiating several different haemoglobins that were detected during routine screening (figs 5A–5D). It did not, however, consistently distinguish HbH, which elutes before the start of the integration program (at 1 minute), in three cases of HbH disease (not shown).

HbH was not detected in any of the retention profiles from 27 subjects with α -thalassaemia trait, which all showed scanty erythrocyte HbH inclusions by staining.

Although the VARIANT could separate HbC from HbA₂ (fig 5A), it could not distinguish between HbE and HbA₂ (fig 5D).

Small companion peaks probably due to glycosylated and degraded haemoglobins (Bio-rad Laboratories; unpublished data) were found to elute between the HbF and HbA₂ peaks in most of the patient samples tested (figs 5B–5D).

Discussion

Currently, anion exchange column chromatography or radial immunodiffusion and alkaline denaturation are established methods for the determination of HbA₂ or HbF, respectively.^{2,3} The VARIANT Haemoglobin Testing system introduces cation-exchange HPLC for the estimation of HbA₂ and HbF for routine thalassaemia screening. Linear regression analysis of HbA₂ values measured by HPLC showed excellent correlation with values obtained by column anion exchange chromatography. For HbF, HPLC measurements also showed good correlation with values obtained using RID or Betke's method, although a better correlation was observed with the latter technique. The reference ranges obtained for HbA₂ and HbF in this study agree with those of several previous investigators using HPLC.^{14,17,18}

An increase in the HbA₂ in the range of 4–6% is specific for β -thalassaemia trait after the third month of life,^{1,4} and high HbA₂ concentrations are a result of β -thalassaemia in almost all instances.¹⁹ Some individuals with β thalassaemia trait, however, have normal indices,^{1,4,19} so that in population surveys when the HbA₂ is measured only in cases with a mean corpuscular volume below a certain cutoff value (≤ 80 fl), such individuals may not be detected. Conversely, HbA₂ concentrations may be normal in the rare "silent" β -thalassaemia trait with a normal HbA₂,¹ so that an increased HbA₂ cannot be used as the sole discriminant for β -thalassaemia.

A low HbA₂ concentration may be seen in HbH disease,¹ as illustrated by the three cases of HbH disease in our study.

Both electrophoretic or conventional chromatographic methods are unable to differentiate HbA₂ from haemoglobin variants such HbE or HbC, that contain similar charge differences.¹⁹ HPLC, however, has been reported to be capable of resolving HbC,^{11–14} O-Arab,¹¹ and Agenogi¹⁴ from HbA₂. The VARIANT resolved haemoglobins A, A₂, F, S, and was able to differentiate HbC from HbA₂. It could not distinguish, however, HbE from HbA₂ (see below) or consistently resolve HbH. A simple and accurate method for HbH and Hb Barts estimation would be highly effective for α -thalassaemia screening in South-East Asian populations.⁴

Of the haemoglobin variants which comigrate with HbA₂, it is HbE principally which

interferes with HbA₂ estimation in South-East Asian populations. Its clinical importance resides in the fact that while heterozygous or homozygous HbE do not cause clinically important anaemia, compound heterozygotes for HbE and β -thalassaemia ($\beta^E/\beta^{\text{thal}}$) may express a phenotype indistinguishable from homozygous β -thalassaemia.^{1,2,7} In practice, HbE carriers may be readily distinguished from β -thalassaemia trait by the apparent concentration of HbA₂. The highest concentration of HbA₂ in a large population of β -thalassaemia carriers of diverse ethnic backgrounds is reported to be 6.8%, but HbA₂ concentrations in some 5' β -gene deletion thalassaemias may range from 7–12%.¹⁹ HbE concentrations in heterozygotes are typically in excess of 20%.^{2,7} Therefore, HbA₂ concentrations in excess of perhaps 10% should suggest coelution of an abnormal haemoglobin such as HbE. In our study all samples with apparently high HbA₂ concentrations (17% to 30%) were consistent with heterozygous HbE by other variables. Additional family or molecular studies are sometimes necessary to confirm $\beta^E/\beta^{\text{thal}}$ or $\beta^E/\alpha^{\text{thal}}$ compound heterozygosity.^{1,2} In this study all cases of β thalassaemia trait or presumptive heterozygous HbE could be readily identified using the HbA₂ concentration determined by HPLC as the sole discriminant.

The HbF concentration was increased ($\geq 0.8\%$) in 46% of the cases of β -thalassaemia trait in this study, in agreement with previous findings.^{1,4} Although the HbF concentration is not a useful discriminant for β -thalassaemia trait, an increased HbF concentration may be useful for the detection of homozygous β -thalassaemia variants, $\delta\beta$ -thalassaemia, hereditary persistence of fetal haemoglobin or $\beta^E/\beta^{\text{thal}}$.^{1,2,4,19}

Important features of the VARIANT Hemoglobin Testing system are that it requires only 5 μ l of whole blood, and that the procedure is fully automated, and provides a retention profile in only 6.5 minutes. These features are considerable advantages in population screening programmes for thalassaemia and other haemoglobin disorders. We estimate the cost per test to be comparable with that of existing methods for laboratories with large work volumes.

Our results show that HPLC is a reliable and technically easy method which can give accurate quantitation of haemoglobin fractions HbA₂ and HbF. The saving of labour and rapid turnaround time are added advantages. Our study supports the notion that the efficiency of HPLC may be exploited in screening on a large scale for β -thalassaemia and HbE in South-East Asian populations.

The HbA₂ and HbF concentrations obtained, however should be interpreted together with other variables such as erythrocyte indices, and iron studies, or family studies in some individuals. Furthermore, because of the large number of known haemoglobin variants (almost 400)² screening data would need to be supplemented in individual cases by methods such as isoelectric focusing, haemoglobin chain separation studies, or haemoglobin gene analysis.

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