ORIGINAL ARTICLE

Silent haemoglobin variants and determination of HbA_{1c} with the HPLC Bio-Rad Variant II

T Lahousen, R E Roller, R W Lipp, W J Schnedl

See end of article for authors' affiliations

Correspondence to: Dr W J Schnedl, Karl-Franzens University, Department of Internal Medicine, Auenbruggerplatz 15, A-8036 Graz, Austria; wolfgang.schnedl@kfunigraz.ac.at

Accepted for publication 14 May 2002

J Clin Pathol 2002;**55**:699–703

Aims: To evaluate the determination of HbA_{1c} with an automated high performance liquid chromatography (HPLC) method in patients with clinically silent haemoglobin variants.
Methods: HbA_{1c} values were determined with the ion exchange HPL Bio-Rad Variant II using the high resolution β thalassaemia programme in patients with silent haemoglobin variants, namely: Hb Graz, Hb Sherwood Forest, Hb O Padova, and Hb D.
Results: All of these haemoglobin variants caused additional peaks in the chromatograms. No clinically useful HbA_{1c} results were produced for patients with Hb Graz and Hb Sherwood Forest, the results for the patient with Hb D were too low, but the results for patients with Hb O Padova were acceptable.
Conclusions: The development of this automated HPLC method modification with high resolution mode aids the identification of interference caused by the described clinically silent haemoglobin variants in HbA_{1c} determination.

In patients with diabetes glycated haemoglobin, measured as HbA_{1c}, is used for evaluating long term control of the disease. Glycated haemoglobin is the result of irreversible non-enzymatic glycation at one or both N-terminal valines of the haemoglobin β chain. The extent of glycation and the relative involvement of the α and β chains of haemoglobin remain unclear.[1 2] HbA_{1c} is expressed as the percentage of total haemoglobin, although the value of the total is (when all glycated sites are included) approximately 50% higher than HbA_{1c} alone. Depending on the determination method used the concentration of HbA_{1c} is approximately 4–6% in healthy patients without diabetes. Glycated haemoglobin most accurately reflects the previous two to three months of glycaemic control. In clinical practice, the measurement of HbA_{1c} is required every three months to determine whether the patient's metabolic control has remained continuously within the target range.[3]

> "All possible and all known interference caused by haemoglobin variants needs to be evaluated for each single HbA_{1c} determination method"

Despite advances in the standardisation of methods for glycohaemoglobin determination an increasing number of haemoglobinopathies have been reported to cause false HbA_{1c} results.[4] Although several methods based on different principles (high performance liquid chromatography (HPLC), immunoagglutination, boronate affinity assays, and electrophoresis) have been developed,[5] the designated DCCT comparison method is a cation exchange HPLC (Diamat; Bio-Rad, Richmond, California, USA).[6] The measurement of glycated haemoglobin is interfered with by non-glucose adducts, such as carbamylation in uraemia,[7] aspirin, penicillin, and metabolites occurring in alcoholism, which may attach to the haemoglobin.[8 9] Samples with variant haemoglobins or those that contain compounds that are known to interfere with the measurement of HbA_{1c} are specifically excluded from certification testing, and there are no specific guidelines or requirements for comparability of samples containing haemoglobin variants.[10] Nevertheless, all possible and all known interference caused by haemoglobin variants needs to be evaluated for each single HbA_{1c} determination method.[11]

We describe the interference caused by Hb Graz, Hb Sherwood Forest, Hb O Padova, and Hb D in the determination of glycated haemoglobin with the automated HPLC Bio-Rad Variant II using the high resolution β thalassaemia programme.

MATERIALS AND METHODS

The blood samples were collected in EDTA anticoagulation bottles and sent cooled at 4°C, by fast mail to the Bio-Rad Laboratories in Munich, Germany. Determinations of HbA_{1c} were performed within three days. The fully automated HPLC Variant II (Bio-Rad Laboratories, Munich, Germany) was used with the high resolution β thalassaemia programme (figs 1–3). If interference by haemoglobin variants is assumed, the Variant II dual kit allows fast switching between the routinely used 3.5 minute haemoglobin A_{1c} programme and the extended 6.5 minute β thalassaemia programme without changing reagents or cartridges. As a second HPLC method the Hi-Auto A_{1c} HA-8140 (Menarini, Florence, Italy) was used. The immunoagglutination method used was the DCA 2000 (Bayer, Vienna, Austria), which uses a specific antibody against the first six amino acid residues of the glycated N-terminal of haemoglobin. Here we describe HbA_{1c} determinations in two patients with type 2 diabetes and the β chain variant Hb Graz ($\alpha_2\beta_2$2(NA2) His → Leu), a patient without diabetes but with β chain variant Hb Sherwood Forest ($\alpha_2\beta_2$104(G6) Arg → Thr), two patients (one without diabetes and one with type 1 diabetes) with α-chain variant Hb O Padova ($\beta_2\alpha_2$30(B11) Glu → Lys), and a patient with type 2 diabetes with β chain variant HbD ($\alpha_2\beta_2$121(GH4) Glu → Gln).[12] Amino acid analysis and DNA sequence analysis were performed as described previously and the routine haematological data of all patients were within the normal range.[13 14] Fasting blood glucose was determined with a hexokinase/glucose-6-phosphate dehydrogenase colorimetric method (Gluco-Quant; Roche, Vienna, Austria) and fructosamine was determined with a colorimetric test using nitroblue tetrazolium in alkaline solution (Unimate FRA; Roche). All determinations were analysed blindly and the procedures were in accordance with the Declaration of Helsinki and the local ethics committee recommendations.

Abbreviations: HbA_{1c}, glycated haemoglobin; Hb, haemoglobin; HPLC, high performance liquid chromatography

Peak name	Calibrated area %	Area %	Retention time (min)	Peak area
Unknown	–	0.1	0.110	3196
Unknown	–	1.5	0.162	44 603
A1a	–	9.0	0.244	271 472
Unknown	–	44.3	0.628	1 341 993
P3	–	3.5	1.515	106 236
Ao	–	39.6	1.743	1 197 649
A2	2.9	–	2.869	61 478
			Total area:	3 026 626

F concentration = %
A1c concentration = %
A2 concentration = 2.9%

Analysis comments:

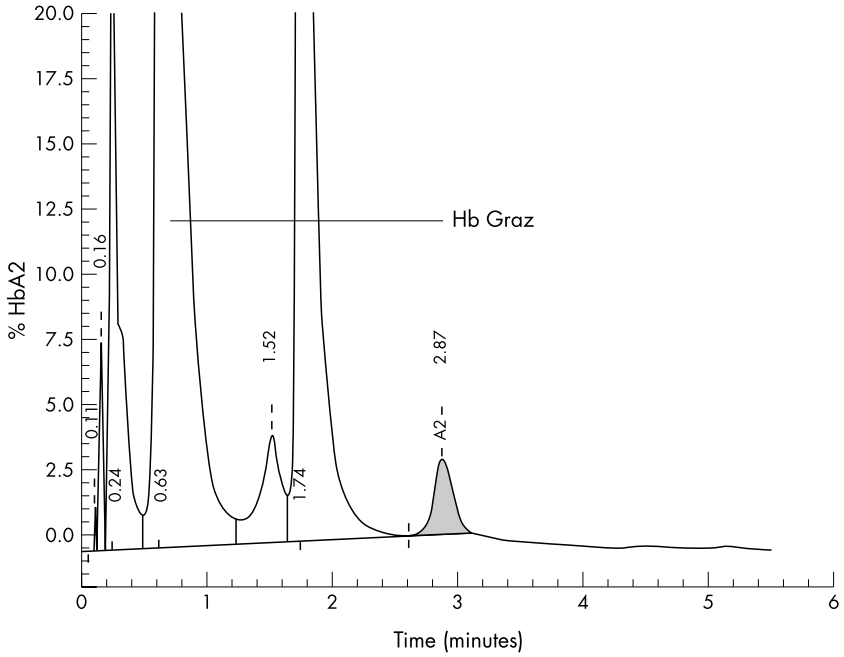

Figure 1 Chromatogram from the ion exchange high performance liquid chromatography Bio-Rad Variant II using the high resolution β thalassaemia programme in a patient with diabetes and the Hb Graz variant.

Table 1 HbA_{1c} results in patients with haemoglobin (Hb) variants

Haemoglobin variant	HbA_{1c} Variant II	HbA_{1c} HA-8140	HbA_{1c} DCA 2000	Fructosamine	Fasting blood glucose
Non-diabetic reference range	4.7–6%	4.5–5.7%	4.5–5.7%	<285 µmol/l	<6.1 mmol/l
Hb Graz 1	No result	Abnormal sep	5.9	466	11.8
Hb Graz 2	No result	Abnormal sep	5.2	334	10.6
Hb Sherwood Forest	No result	Abnormal sep	4.6	238	5.5
Hb D	4.8	9.1 (variant Hb)	5.7	302	6.5
Hb O Padova 1	4.9	6.8 (variant Hb)	4.5	251	6.0
Hb O Padova 2	9.5	10 (variant Hb)	8.8	338	5.2

Variant II, HPLC variant II (Bio-Rad); HA-8140, HPLC high-auto A_{1c} HA-8140 (Menarini); DCA 2000, immunoagglutination DCA 2000 (Bayer); Abnormal sep, abnormal separation; variant Hb, variant haemoglobin.
HPLC, high performance liquid chromatography.

RESULTS

In the two patients with type 2 diabetes and Hb Graz the chromatogram from the Bio-Rad Variant II using the high resolution β thalassaemia programme showed that Hb Graz migrates with fetal haemoglobin and labile HbA_{1c} (LA1c), overlapping HbA_{1c}, as shown in the analysis data (fig 1). No result was given for HbA_{1c} determination in both patients with Hb Graz (fig 1). The determination of HbA_{1c} with the HPLC Menarini described the results as "abnormal separation" and the immunoagglutination method DCA 2000 showed values

Peak name	Calibrated area %	Area %	Retention time (min)	Peak area
Unknown	–	0.1	0.109	4033
Unknown	–	1.2	0.156	47 479
A1a	–	3.9	0.230	150 588
F	1.7	–	0.390	84 844
LA1c	–	45.3	0.623	1 770 488
P3	–	2.6	1.492	101 615
Ao	–	42.7	1.714	1 665 609
A2	2.8	–	2.836	75 652
Unknown	–	0.1	3.256	4049

F concentration = 1.7% Total area: 3 904 358*
A1c concentration = %
A2 concentration = 2.8%

Analysis comments:

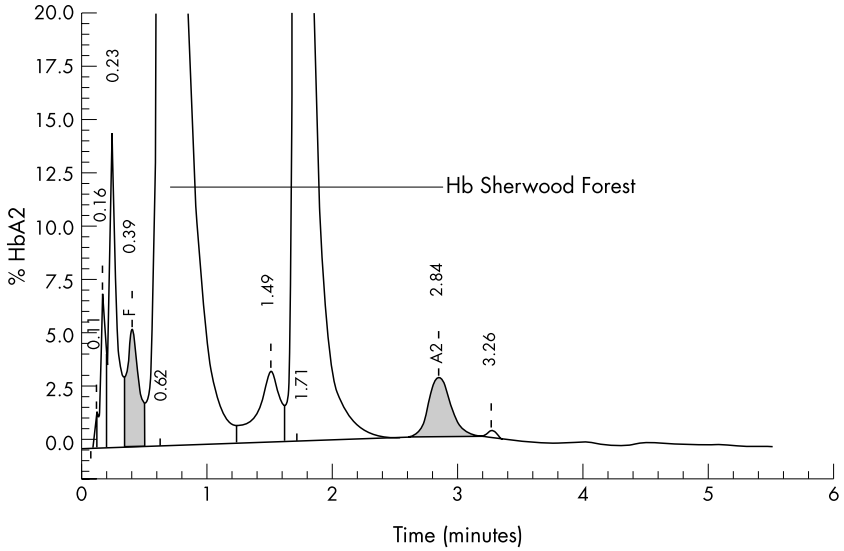

Figure 2 Chromatogram from the ion exchange high performance liquid chromatography Bio-Rad Variant II using the high resolution β thalassaemia programme in a patient without diabetes but with the Hb Sherwood Forest variant.

within the non-diabetic reference range for both patients with diabetes (table 1).[12]

In the patient without diabetes with the Hb Sherwood Forest variant the chromatogram from the Bio-Rad Variant II using the high resolution β thalassaemia programme demonstrated an additional peak with HbA_{1c}. No HbA_{1c} result was given in the analysis data (fig 2). The result of HbA_{1c} determination with the HPLC Menarini was "abnormal separation" and the immunoagglutination method DCA 2000 showed a value within the non-diabetic reference range for this patient without diabetes (table 1).[12]

In the two patients (one without diabetes and one with type 1 diabetes) who had the Hb O Padova variant the chromatogram from the Bio-Rad Variant II using the high resolution β thalassaemia programme demonstrated an additional late (at four minutes) migrating peak (fig 3). In the patient without diabetes the result for HbA_{1c} was within the non-diabetic reference range and compared well with values of fasting blood glucose and fructosamine (table 1). In the patient with type 1 diabetes, the result was above the non-diabetic reference range, indicating (in agreement with the fructosamine result) that blood glucose regulation was unsatisfactory. The determinations of HbA_{1c} with the HPLC Menarini gave results in both patients described as "variant haemoglobin"[12] and the immunoagglutination method DCA 2000 showed values within the non-diabetic reference range for the patient without diabetes and a diabetic value for the patient with type 1 diabetes (table 1).[12]

In the patient with type 2 diabetes and Hb D the chromatogram from the Bio-Rad Variant II using the high resolution β thalassaemia programme showed several additional "unknown" peaks, as published previously.[15] The result for HbA_{1c} was within the low non-diabetic reference range. Compared with the values of fructosamine and fasting blood glucose (table 1), which were in the diabetic range, the HbA_{1c} result seemed too low. The determination of HbA_{1c} with the HPLC Menarini gave the results as "variant haemoglobin"[16] and the immunoagglutination method DCA 2000 showed values in the upper non-diabetic reference range for this patient (table 1).[12]

DISCUSSION

Most mutations in the globin genes of haemoglobin are a single base pair change in the DNA code, resulting in an amino acid substitution. More than 700 haemoglobin variants are known and about half of these variants are clinically silent like the ones investigated in our study.[17] Methods to determine HbA_{1c} include cation exchange HPLC, boronate affinity,

Peak name	Calibrated area %	Area %	Retention time (min)	Peak area
A1a	–	0.4	0.165	12 105
A1b	–	1.1	0.257	35 579
LA1c	–	1.0	0.668	34 073
A1c	9.5*	–	0.805	184 523
P3	–	3.6	1.480	118 796
A0	–	64.6	1.687	2 143 380
A2	1.9	–	2.840	48 494
C	–	0.6	4.246	20 186

Total area:

*** Values outside of expected ranges**

HbF concentration = %
HbA1c concentration = 9.5%*
HbA2 concentration = 1.9%

Analysis comments:

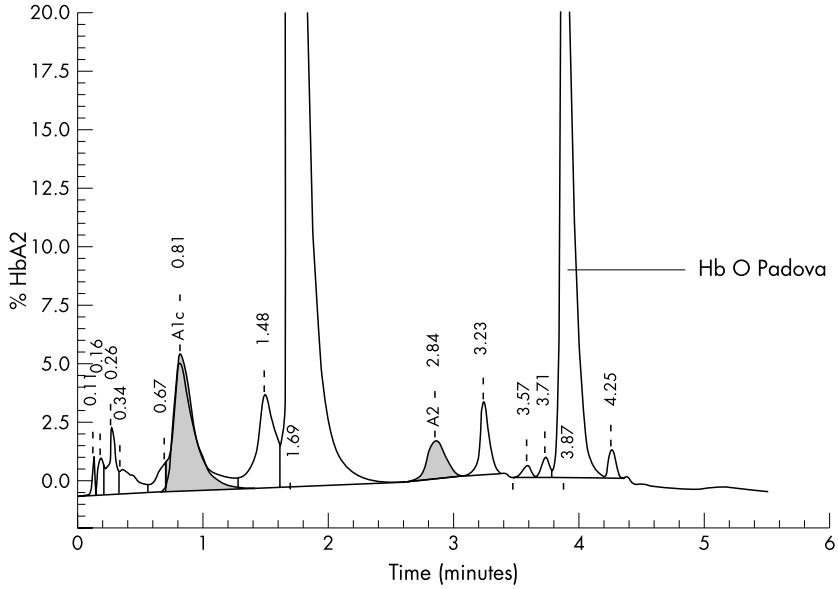

Figure 3 Chromatogram from the ion exchange high performance liquid chromatography Bio-Rad Variant II using the high resolution β thalassaemia programme in a patient with type 1 diabetes and the Hb O Padova variant.

electrophoresis, and immunoassays. The first clinically useful cation exchange chromatographic method for HbA_{1c} determination was published in 1978.[18] Haemoglobin A_{1c} was originally a term for an ion exchange chromatographic peak and is now defined as irreversibly glycated haemoglobin molecules at one or both N-terminal valines of the β chains. Here, we describe the interference of Hb Graz, Hb Sherwood Forest, Hb O Padova, and Hb D in the determination of HbA_{1c} with an extended automated HPLC method modification, namely the Bio-Rad Variant II high resolution β thalassaemia 6.5 minute programme, which was developed to measure HbA_2 in the diagnosis of β thalassaemia trait.

HPLC methods usually indicate the presence of a haemoglobin variant, but they lack the resolution necessary to differentiate between them. They may demonstrate additional peaks in the chromatograms and these may be combined with clinically low or high results (figs 1–3).[4] The Diamat HPLC method was once used for the screening of certain haemoglobin variants,[19] and the Hi-Auto A_{1c} HA-8140 HPLC method has separation conditions that seem to detect haemoglobin variants and describe the chromatogram as "abnormal" or "variant" haemoglobin.[20] The HLC-723 GHb V A1c2.2 HPLC (Tosoh, San Francisco, California, USA) has an enhanced resolution using the 3.0 minute instead of the 2.2 minute protocol, which allows the detection of haemoglobin variants.[21] Chromatograms from the Variant HPLC using the β thalassaemia short programme helped to establish the diagnosis of certain haemoglobinopathies.[22] However, most HPLC systems are not able to resolve additional peaks in their chromatograms and this leads to the overestimation and underestimation of HbA_{1c} results.[4 12]

The HPLC Variant II is described as giving an acceptable analytical performance and the results compared well with an HPLC method (Variant; Bio-Rad) certified by the National Glycohaemoglobin Standardisation Programme.[15] We found that the determination of HbA_{1c} values in patients with early migrating haemoglobin variants, such as Hb Graz and Hb Sherwood Forest, was not possible. The HbA_{1c} value for the late migrating Hb O Padova variant was acceptable, but the HbA_{1c} results for the Hb D variant appeared to be too low compared with the blood glucose and fructosamine results (table 1). Using the high resolution β thalassaemia programme of the Variant II in patients with silent haemoglobin variants showed that HPLC

Take home messages

- The development of this automated high performance liquid chromatography method modification with high resolution mode aids the identification of interference caused by clinically silent haemoglobin variants in glycated haemoglobin (HbA_{1c}) determination
- Such interference should be investigated in all newly developed and/or modified HbA_{1c} assays
- Affinity chromatography may provide a more accurate measure of glycaemic control in samples with haemoglobin variants

method modifications with high resolution modes aid identification of interference caused by haemoglobin variants.

> "The determination of HbA_{1c} values in patients with early migrating haemoglobin variants, such as Hb Graz and Hb Sherwood Forest, was not possible"

However, different commercially available methods measure different fractions of glycated haemoglobin, causing different HbA_{1c} results depending on the method used.[11 12] The degree of interference of haemoglobin variants may vary with each method and even with each method modification. Several haemoglobin variants are known to interfere with measurement of HbA_{1c} by HPLC,[4] and an increasing number of interfering haemoglobin variants have been reported.[23] Only a few haemoglobin variants are known to affect HbA_{1c} results in immunoassays.[4 5] Boronate affinity methods measure glycohaemoglobin regardless of the glycation site. If an erroneous result is caused by haemoglobin mutations affinity chromatography may provide a more accurate measure of glycaemic control in samples with haemoglobin variants.[12] New methods are being developed, such as electrospray mass spectrometry[24] and a method based on quenching of the fluorescence of an eosin-boronic acid solution.[25] Preliminary results with these methods on samples containing haemoglobin variants (Hb S and Hb C) demonstrated no apparent bias against the comparison method used.[24 25]

We conclude that this modification of the HPLC method enables interference with HbA_{1c} determination as a result of silent haemoglobin variants to be recognised more easily. However, these results emphasise the need for additional investigations into the interference caused by haemoglobin variants in all newly developed and/or modified HbA_{1c} assays.

ACKNOWLEDGEMENT

Thanks to Bio-Rad Laboratories (Munich, Germany) for performing the HbA_{1c} determinations with the HPLC Variant II high resolution β thalassaemia programme.

Authors' affiliations
T Lahousen, R E Roller, R W Lipp, W J Schnedl, Department of Internal Medicine, Karl-Franzens University School of Medicine, Auenbruggerplatz 15, A-8036 Graz, Austria

REFERENCES

1 **Kilpatrick ES**. Glycated haemoglobin in the year 2000. *J Clin Pathol* 2000;**53**:335–9.
2 **John WG**. Glycated haemoglobin analysis. *Ann Clin Biochem* 1997;**34**:17–31.
3 Tests of glycemia in diabetes (position statement). *Diabetes Care* 2001;**24**(suppl 1):S80–2.
4 **Schnedl WJ**, Korninger RE, Liebminger A, *et al*. Hemoglobin variants and determination of glycated hemoglobin (HbA_{1c}). *Diabetes Metab Res Rev* 2001;**17**:94–8.
5 **Bry L**, Chen PC, Sacks DB. Effects of hemoglobin variants and chemically modified derivates on assays for glycohemoglobin [review]. *Clin Chem* 2001;**47**:153–63.
6 **Little RR**, Rohlfing CL, Wiedmeyer H-M, *et al*. The National Glycohemoglobin Standardization Program: a five-year progress report. *Clin Chem* 2001;**47**:1985–92.
7 **Flückiger R**, Harmon W, Meier W, *et al*. Hemoglobin carbamylation in uremia. *N Engl J Med* 1981;**304**:823–7.
8 **Nathan DM**, Francis TB, Palmer JL. Effect of aspirin on determination of glycosylated hemoglobin. *Clin Chem* 1983;**29**:486–69.
9 **Karsegard J**, Wicky J, Mensi N, *et al*. Spurious glycohemoglobin values associated with hydroxyurea treatment. *Diabetes Care* 1997;**20**:1211–12.
10 **Stott A**, Casson IF, Higgins GJ. Glycated haemoglobin assays. Approaches to standardization of results. *Diabet Med* 2001;**18**:274–9.
11 **Little RR**. Recent progress in glycohemoglobin (HbA_{1c}) testing [editorial]. *Diabetes Care* 2000;**23**:265–6.
12 **Schnedl WJ**, Krause R, Halwachs-Baumann G, *et al*. Evaluation of HbA_{1c} determination methods in patients with hemoglobinopathies. *Diabetes Care* 2000;**23**:339–44.
13 **Schnedl WJ**, Reisinger EC, Katzensteiner S, *et al*. Haemoglobin O Padova and falsely low haemoglobin A1c in a patient with type I diabetes. *J Clin Pathol* 1997;**50**:434–5.
14 **Liu JS**, Molchanova TP, Gu LH, *et al*. Hb Graz or $\alpha_2\beta_2$2(NA2)His→Leu; a new β chain variant observed in four families from southern Austria. *Hemoglobin* 1992;**16**:493–501.
15 **Higgins TN**, Blakney GB, Dayton J. Analytical evaluation of the Bio-Rad Variant II automated HbA(1c) analyzer. *Clin Biochem* 2001;**34**:361–5.
16 **Schnedl WJ**, Lipp RW, Trinker M, *et al*. Hemoglobin D [β 121(GH4)Glu→Gln] causing falsely low and high HbA_{1c} values in high performance liquid chromatography [letter]. *Clin Chem* 1998;**44**:1999–2000.
17 Alphabetical hemoglobin variant list [editorial]. *Hemoglobin* 1996;**20**:313–35.
18 **Abraham EC**, Huff TA, Cope ND, *et al*. Determination of glycosylated hemoglobins (HbA_1) with a new microcolumn procedure: suitability of the technique for assessing the clinical management of diabetes mellitus. *Diabetes* 1978;**27**:931–7.
19 **Delahunty T**. Convenient screening for hemoglobin variants by using the Diamat HPLC system. *Clin Chem* 1990;**36**:903–5.
20 **John WG**, Braconnier F, Miedema K, *et al*. Evaluation of the Menarini-Arkay HA 8140 hemoglobin A1c analyzer. *Clin Chem* 1997;**43**:968–75.
21 **Gibb I**, Parnham A, Fonfrede M, *et al*. Multicenter evaluation of Tosoh glycohemoglobin analyser. *Clin Chem* 1999;**45**:1833–41.
22 **Riou J**, Godart C, Hurtel D, *et al*. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. *Clin Chem* 1997;**43**:34–9.
23 **Moriwaki Y**, Yamamoto T, Shibutani Y, *et al*. Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, detected during the analysis of glycated haemoglobin (HbA_{1c}) by high performance liquid chromatography. *J Clin Pathol* 2000;**53**:854–7.
24 **Nakanishi T**, Miyazaki A, Iguchi H, *et al*. The effect of hemoglobin variants on routine HbA1c measurements: an assessment by a mass spectrometric method. *Clin Chem* 2000;**46**:1689–92.
25 **Blincko S**, Anzetse J, Edwards R. Measurement of glycated haemoglobin in whole blood by a novel fluorescence quenching assay. *Ann Clin Biochem* 2000;**37**:492–7.