Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 791 (2003) 73–83
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of haemoglobin A_{1c} by liquid chromatography using a new cation-exchange column

Maria Beatriz de la Calle Guntiñas*, René Wissiack, Guy Bordin, Adela Rosa Rodríguez

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, 2440 Geel, Belgium

Received 30 October 2002; received in revised form 5 February 2003; accepted 24 February 2003

Abstract

The use of a new cation-exchange column, ProPac SCX-10, for the determination of haemoglobin A_{1c} (HbA_{1c}) by high-performance liquid chromatography is described. After optimization of the analytical method for the separation of the various isoforms of haemoglobin with the ProPac SCX-10 column, the method was applied to the determination of HbA_{1c} in blood from 59 volunteers. Three of the 59 had previously been diagnosed as diabetics. Interference studies for carbamylation, acetylation and pre-HbA_{1c} were carried out via ''in-vitro'' experiments. No interference due to carbamylation was observed at the urea values normally found in uremic patients undergoing dialysis. No interference from pre-HbA_{1c} was detected either. The method is able to separate haemoglobin A ($\alpha_2\beta_2$), haemoglobin S (haemoglobin from sickle cell anaemia patients) and haemoglobin A_2 ($\alpha_2\delta_2$) without interference. The method of Hampel was applied to detect outliers. A value of $3.29\pm0.44\%$ (2σ) for HbA_{1c} was obtained in the analysis of 56 blood samples from non-diabetics. This average value is lower than that reported by most of the methods currently used in routine analyses.

Keywords: Diabetes; Haemoglobin A_{1c}

1. Introduction

The global prevalence of diabetes mellitus is predicted to rise from 135 million patients in 1995 to 300 million by 2025 [1]. The Diabetes Control and Complications Trial Research Group (DCCT) [2] reported that careful control of diabetic patients is required to avoid long-term complications, including retinopathy, nephropathy and neuropathy. Also, a slight decrease in the prevalence of elevated LDL-cholesterol was detected in a DCCT intensive therapy group. The glycated haemoglobin A_{1c} (HbA_{1c}) concentration is an indicator of average blood glucose concentration over 3 months and has been suggested as a diagnostic or screening tool for diabetes [3–5].

The concentration of this isoform is known to predict cardiovascular risk in diabetic patients [6,7]. Khaw et al. [8] studied men in the ''Norfolk Cohort of the European Prospective Investigation into Cancer and Nutrition'' to determine the effect of glycated haemoglobin in the whole male population without diabetes and at concentrations below those used to diagnose diabetes. The study led to the

*Corresponding author. Fax: +32-14-584-273.
E-mail address: beatriz.de-la-calle@irmm.jrc.be (M. Beatriz de la Calle Guntiñas).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00202-2

conclusion that the glycated haemoglobin concentration seems to explain most of the excess mortality risk due to diabetes in men and to be a continuous risk factor throughout the whole population studied, also in non-diabetics.

An important chemical effect of hyperglycemia is the posttranslational modification of proteins by glucose. Some authors suggest that the spontaneous reaction between the aldehyde group with primary amino groups of proteins and DNA is a pathogenic mechanism that is responsible for some non-desirable long-term effects of diabetes [9]. However, it remains unclear if glycation products are responsible for some of the diabetes complications. According to Gould et al. [10], glycated Hb per se is an unlikely factor in the pathophysiology of diabetic complications.

Several methods are currently applied to determine glycated haemoglobin, which, however, measure different glycated components. Methods based on affinity chromatography determine total glycated haemoglobin [11–13]. Immunoassay methods on the basis of structural differences measure only HbA_{1c} [13]. Cation-exchange chromatography and electrophoretic methods separate HbA_{1c} from other haemoglobin isoforms on the basis of molecular charge. Interferences due to the labile intermediate pre-HbA_{1c} (Schiff base) [14], to carbamylated haemoglobin in uremic patients and to reaction with salicylic acid [11,15] in alcoholics and patients treated with high doses of aspirin have been reported using ion-exchange chromatography. Despite this, cation-exchange chromatography remains the most widely applied method for the determination of glycated haemoglobin and it has been chosen by the DCCT as the reference method using Bio-Rex 70 as resin. It has been reported that this method is only 60% specific [16]. A more specific method is based on the use of a Mono S column [17], although an increase of 1% in the level of HbA_{1c} is also reported in uremic patients due to carbamylation. Cation-exchange chromatography can also provide non-accurate results when a haemoglobinopathy is present [11], although some ion-exchange columns can be used to detect certain haemoglobinopathies [4,18]. Measurement of haemoglobin A_2 (HbA_2) using cation-exchange HPLC is complicated in individuals with haemoglobin S (HbS) because HbA_2 is falsely increased by the presence of HbS adducts [19].

Very recently, the International Federation of Clinical Chemistry (IFCC) approved a reference method for the measurement of HbA_{1c} in human blood [20].

Working in an institute mainly dedicated to the production of reference materials and measurements, our group is frequently faced with participation in inter-laboratory comparisons and with the need of making a large number of measurements (for example, in stability and homogeneity studies). Analytical methods characterized by high precision, accuracy, robustness and specificity are thus required.

In 1998 a new type of cation-exchange column packing was introduced [21] involving a hydrophilic-coated, pellicular polymeric support with grafted tentacular surface chemistry. According to the authors, this type of column is highly suited for the resolution of closely related protein variants and has extremely high selectivity. On the basis of this information, the ProPac SCX-10 column was selected to develop a method to analyze HbA_{1c} in human blood. The method seems to be free from the interference from pre-HbA_{1c} and carbamylated haemoglobin at the urea levels that can be found in uremic patients undergoing dialysis. Moreover, HbS does not interfere in the determination of HbA_{1c} or HbA_2. The throughput of the method is one sample per hour, which makes it suitable for our purpose, although it would present some logistical problems as a ‘‘field’’ method.

2. Experimental

2.1. Instrument

A Waters (Milford, MA, USA) high-performance liquid chromatography (HPLC) system was used consisting of a four-channel vacuum degasser, a Model 626 quaternary gradient pump, a Model 717 autosampler equipped with a heater/cooler device, which has temperature control ranging between 4 and 40 °C, and a 996 diode-array detector (DAD). Haemoglobin was measured at 416 nm. The whole system was controlled with a Millenium^{32} Workstation. A Mistral column oven (Holland Spark Instrumenten, Emmen, Netherlands) was used which allows temperature regulation in the range 5–90 °C. Pump tubing and connections between the different

subparts of the instrument were made of PEEK. Glass total recovery vials (Waters) (8×40 mm) were used for the autosampler.

2.2. Column and reagents

Separation by cation exchange of haemoglobin isoforms was achieved by injecting 20 μL of diluted hemolyzed blood sample onto a ProPac SCX-10 analytical column (4×250 mm) (Dionex, Sunnyvale, CA, USA). A ProPac SCX-10G guard column (4×50 mm) (Dionex) was used to protect the analytical column. The temperature of the column during the separation was 22 °C.

The eluents used for the chromatographic separation were: A, 0.01 mol/L NaH_2PO_4–Na_2HPO_4 (pH 6.0); and B, 0.01 mol/L NaH_2PO_4–Na_2HPO_4 (pH 6.0)+0.5 mol/L NaCl (Table 1). NaN_3 was added to both solutions up to a concentration of 0.01 mol/L to avoid met-Hb, which is generated in vitro and is frequently present in commercialized standards, disturbing the separation of Hb isoforms [22]. NaN_3 is also used to avoid bacterial growth. All the reagents used were of the highest available purity and were purchased from Sigma (Munich, Germany). Deionized water from a Milli-Q Plus system (>18.3 MΩ) (Millipore, Billerica, MA, USA) was used.

2.3. Sample preparation

Three milliliters of blood were collected in a tube containing EDTA. Twenty microliters of the whole blood were diluted up to 1 mL with deionized water.

Table 1
Gradient applied for the separation of haemoglobin isoforms using the ProPac SCX-10 cation-exchange column

Time (min)	Flow (mL/min)	A (%)	B (%)
	1.00	99.0	1.0
20.00	1.00	88.0	12.0
30.00	1.00	75.0	25.0
40.00	1.00	60.0	40.0
45.00	1.00	60.0	40.0
46.00	1.00	99.0	1.0
50.00	1.00	99.0	1.0

A, 0.01 mol/L NaH_2PO_4–Na_2HPO_4 (pH 6.0)+0.01 mol/L NaN_3; B, 0.01 mol/L NaH_2PO_4–Na_2HPO_4 (pH 6.0)+0.5 mol/L NaCl+0.01 mol/L NaN_3.

The hemolysate was filtered through a 0.45 μm cellulose acetate filter (Alltech, Deerfield, IL, USA) and directly collected on a total-recovery glass vial to be placed on the autosampler of the chromatographic equipment. In the autosampler the samples were kept at 7 °C. Twenty microliters of the filtered sample were injected on top of the cation-exchange column and the signal at 416 nm was recorded. Whole blood samples were stored at 4 °C and analyzed within 1 week to avoid degradation problems. In analyses carried out 7–10 days after the sampling, a slight decrease in the percentage of HbA_{1c} (%HbA_{1c}) was obtained when compared with the results obtained for the same sample analyzed immediately after sampling. No systematic study was made on storage conditions, although it would be interesting to analyze samples which had been stored at room temperature. In some intercomparison studies the temperature during shipping of samples cannot be controlled.

Studies with human blood samples were approved by the European Commission responsible committee.

2.4. Study of interferences

The following tests were carried out in order to evaluate the performance of the method in the presence of some potential interferents of cation-exchange-based methods.

2.4.1. Pre-HbA_{1c} interference

Following the procedure applied in the IFCC method to eliminate the pre-HbA_{1c} fraction [20], 1.5 mL of blood were washed twice with 10 mL of saline solution (9 g/L NaCl) and centrifuged for 10 min at 3000 *g*. The sedimented cells were incubated in 10 mL of saline solution at 37 °C for 4 h to eliminate pre-HbA_{1c}. After 4 h the hemolysate was prepared by mixing the cells with 1.5 mL of water. Cell debris were eliminated by centrifugation at 3000 *g* for 20 min. The supernatant was diluted 25 times and the sample was filtered through a 0.45 μm cellulose acetate membrane and injected onto the chromatographic system. This procedure was applied to two blood samples corresponding to a non-diabetic (serum glucose 5.4 mmol/L) and to a diabetic patient (serum glucose 9.7 mmol/L). The results obtained for HbA_{1c} after incubation of the samples were compared with the values obtained for the same

samples after applying the normal sample pre-treatment procedure, without elimination of pre-HbA_{1c}.

2.4.2. Interference due to carbamylated haemoglobin

One milliliter of blood from a non-diabetic donor was diluted five times with pure water and urea was added to a final concentration of approximately 36 mmol/L, which falls within the concentration range which can be expected in uremic patients [23]. The sample was then incubated for 24 h at 37 °C following the procedure described by Brunnekreeft and Eidhof [15]. A second aliquot of the same sample was treated in an identical way but without the addition of urea, and a third aliquot was treated following the normal sample treatment. The results obtained for the three aliquots were compared.

2.4.3. Interference due to acetylated haemoglobin

To carry out this study, 12 mL of a solution of 4 mmol/L salicylic acid were added to 0.5 mL of two blood samples, one from a non-diabetic and one from a diabetic patient. The samples were kept in a water bath at 37 °C for 7 h, following the procedure applied by Nathan et al. [24]. Filtration and injection onto the chromatographic column were carried out as described in the general procedure for sample pre-treatment.

A second set of experiments was carried out in which the only parameter changed was the concentration of salicylic acid solution, which was diluted five times.

2.5. Studies with HbS and HbA_2

Standard solutions of HbA_2 and HbS, both obtained from Sigma, were prepared in order to evaluate the performance of the method when applied to HbS and to establish whether interferences in the determination of HbA_{1c} can take place in the presence of HbS. It was also the purpose of this study to evaluate whether HbS can interfere in the determination of HbA_2.

The standard solution of HbS was prepared by weighing 1 mg of haemoglobin and dissolving it in 1 mL of water. HbA_2 was delivered in vials containing 5 mg of protein, which made it practically impossible to make aliquots for weighing. For this reason, 5 mL of water were added directly to the vial, also obtaining in this way a final concentration of approximately 1 mg/mL.

2.6. Statistical evaluation of the method

Fifty-nine blood samples were obtained from volunteers from the institute during annual medical controls. The samples were tested for HIV, hepatitis B and hepatitis C and analyzed within 48 h of sampling. All samples were stored at 4 °C for a maximum of 1 week. After integration of the chromatograms following the procedure indicated hereafter for the determination of HbA_{1c}, the outlier test of Hampel was applied [25].

3. Results

Fig. 1 shows a typical chromatogram obtained applying the described method. The area between the two arrows corresponds to the total haemoglobin content. The HbA_{1c} peak percentage of the total area directly provides the percentage of HbA_{1c} with regards to the total content of haemoglobin.

The mean $\%HbA_{1c}$ value obtained for 56 blood samples using the present procedure was $3.29 \pm 0.44\%$ (2σ) (Fig. 2). This value is in quite good agreement with that of $3.33 \pm 0.48\%$ (2σ) reported by Jeppsson et al. [20] for a population of 120 non-diabetics using the IFCC reference method. It is important to stress that the mean value obtained for the $\%HbA_{1c}$ for non-diabetics with the ProPac SCX-10 column is lower than the 5% or even 6% reported when using some routine methods.

Three blood samples out of the batch of 59 analysed were considered as outliers when the Hampel test was applied (Fig. 2). The Medical Service of the European Commission confirmed that those three samples, with $\%HbA_{1c}$ values of 4.15 ± 0.06, 4.41 ± 0.02 and 4.32 ± 0.05, respectively, were the only ones among the 59 belonging to diabetic patients (Table 2). The Medical Service made its report on the basis of the levels of glucose in serum (the reference value for the VITROS 950, Ortho Clinical Diagnostics, system used is 3.3–6.1 mmol/L) and considering the clinical history of the

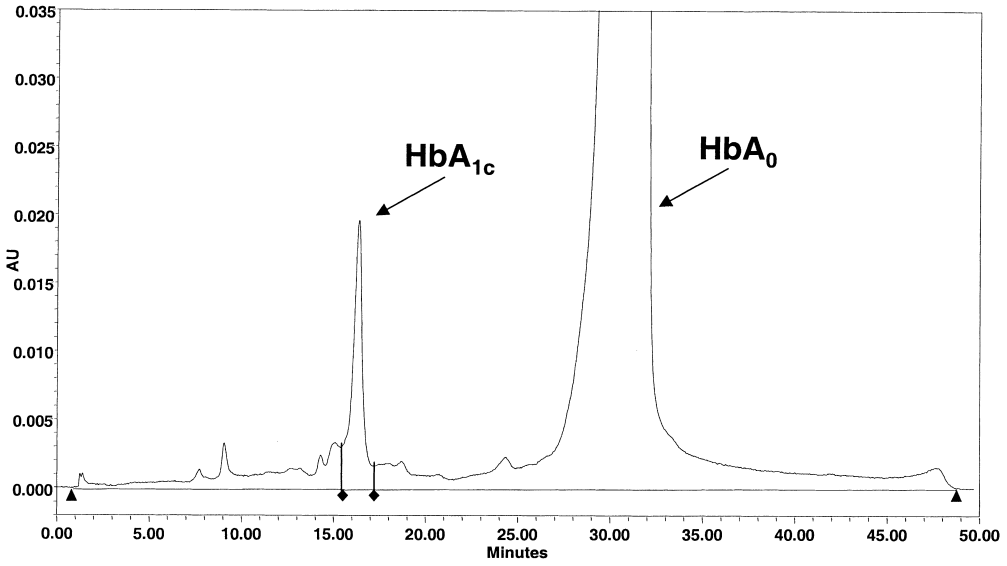

Fig. 1. Chromatogram obtained after injection of 20 μL of blood from a non-diabetic onto the ProPac SCX-10 cation-exchange column.

patient (diabetes diagnosed in previous controls) [26]. Only for the patient with a higher level of glucose in serum did the Medical Service determine %HbA_{1c} using an immunoassay (Table 2).

3.1. Precision and robustness studies

To estimate the precision of the method, blood samples from a non-diabetic and from a diabetic

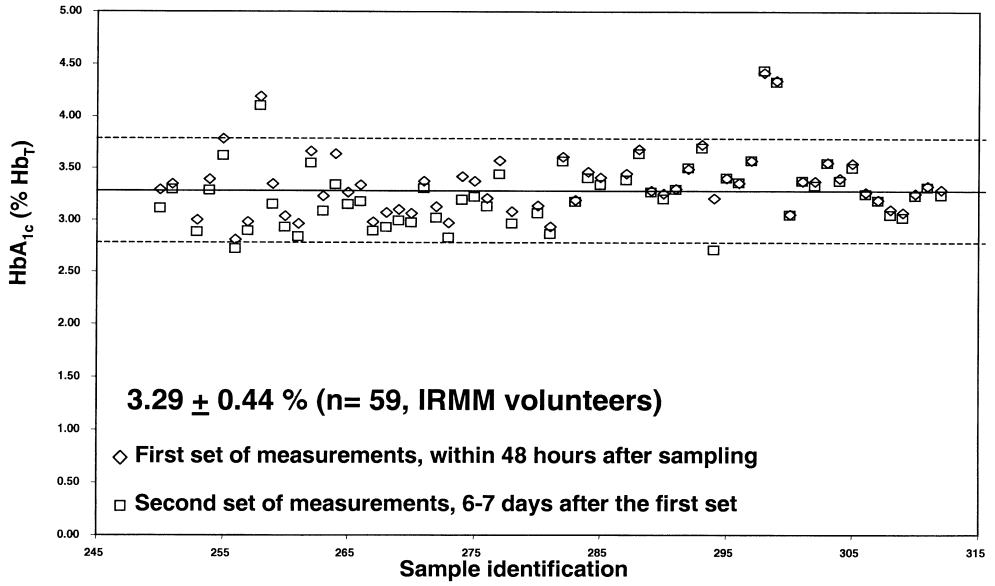

Fig. 2. Percent HbA_{1c} values obtained for 59 blood samples after separating the haemoglobin isoforms on a ProPac SCX-10 column.

Table 2
Values of glucose in serum and urine and %HbA_{1c} as determined by immunoassay and by cation-exchange chromatography (ProPac SCX-10 column) in samples identified as belonging to diabetic patients

Sample	Glucose in serum[a] (mmol/L)	%HbA_{1c}	
		ProPac SCX-10	Immunoassay[b]
1	6.3	4.15±0.06	ND[c]
2	7.2	4.41±0.02	6.9
3	9.7	4.32±0.05	ND

[a] The concentration of glucose in serum was determined with a VITROS 950 system (Ortho Clinical Diagnostics). The reference value with this system is 3.3–6.1 mmol/L.

[b] %HbA_{1c} by immunoassay was determined with a Bayer DCA 2000 system. The reference value with this system is 5–7%.

[c] ND, not determined.

patient were analyzed. In both cases the within-day precision, calculated by injecting the same sample three times, was <1.5%; the between-day precision, calculated by injecting the same sample once on each of three different days (within a week), was <2%. The results obtained for the precision evaluation are summarized in Table 3. The NCCLS EP5-T recommendation of two replicates per specimen per run and two runs per day for 20 days was not followed because, as already mentioned in Sample preparation, the samples were only stable for 1 week.

Other analytical characteristics of the method such as linear range or detection limit are not reported due to the lack of a certified reference material or even a commercially available standard of pure HbA_{1c}.

As a complementary study to check the robustness of the method, three sets of experiments were carried out.

(1) One sample obtained from a non-diabetic donor was injected 23 times within 1 day, obtaining a %HbA_{1c} value of 3.36±0.04 (C.V. 1.19%).

(2) Blood from the same non-diabetic donor was taken three times within half a year with sampling intervals of 3 months. The values obtained for HbA_{1c} were: 3.36±0.04 (n=23), 3.29±0.04 (n=9) and 3.31±0.04 (n=2), respectively. The first important information that can be deduced from this study is that, independent of the number of replicates, a coefficient of variation of approximately 1% was obtained. Secondly, a constant %HbA_{1c} value was obtained (considering the standard deviation of the method) for the same donor within a period of half a year, which indicates that the method is not affected by non-controlled parameters related to the analytical method itself or to the biological status of the donor.

(3) A set of 37 blood samples (including one sample from a diabetic patient) were analyzed independently, on two different days, with an interval of 7 days, one replicate per sample and per day, by an untrained and a trained operator, respectively. The mean %HbA_{1c} values for the 36 non-diabetics were 3.27±0.24 and 3.17±0.28, respectively. For the diabetic patient the values obtained were 4.10 and 4.19 %HbA_{1c}. In the latter case, no standard deviation is given because only one sample was analyzed and only one injection was made on each of the 2 days.

Table 3
Precision evaluation

Sample	Within-day (n=3)		Between-day (n=3)	
	%HbA_{1c} (mean±SD)	C.V. (%)	%HbA_{1c} (mean±SD)	C.V. (%)
Non-diabetic	3.26±0.05	1.38	3.28±0.06	1.83
Diabetic	4.14±0.02	0.37	4.14±0.05	1.11

3.2. Study of interferences

The results obtained for the interference studies are illustrated in Fig. 3. As can be seen in Fig. 3a, the labile fraction pre-HbA_{1c} is sufficiently resolved from the HbA_{1c} fraction so as not to interfere in its determination. After incubating the blood samples, the peak indicated in Fig. 3a as corresponding to pre-HbA_{1c} disappeared, showing the efficiency of the method used to eliminate pre-HbA_{1c}. The results, summarized in Table 4, confirm that, after incubation to eliminate pre-HbA_{1c}, no significant change in %HbA_{1c} occurred in blood from a non-diabetic or from a diabetic patient.

With respect to the interference due to carbamylated haemoglobin, no increase was observed in %HbA_{1c} (considering the standard deviation of the method) for urea concentrations of approximately 36 mmol/L (Table 4). In Fig. 3b the appearance of an extra peak with a retention time of around 23 min can be observed. Such a peak was detected both for the aliquot of blood that was incubated but to which no urea was added, as well as for the aliquot

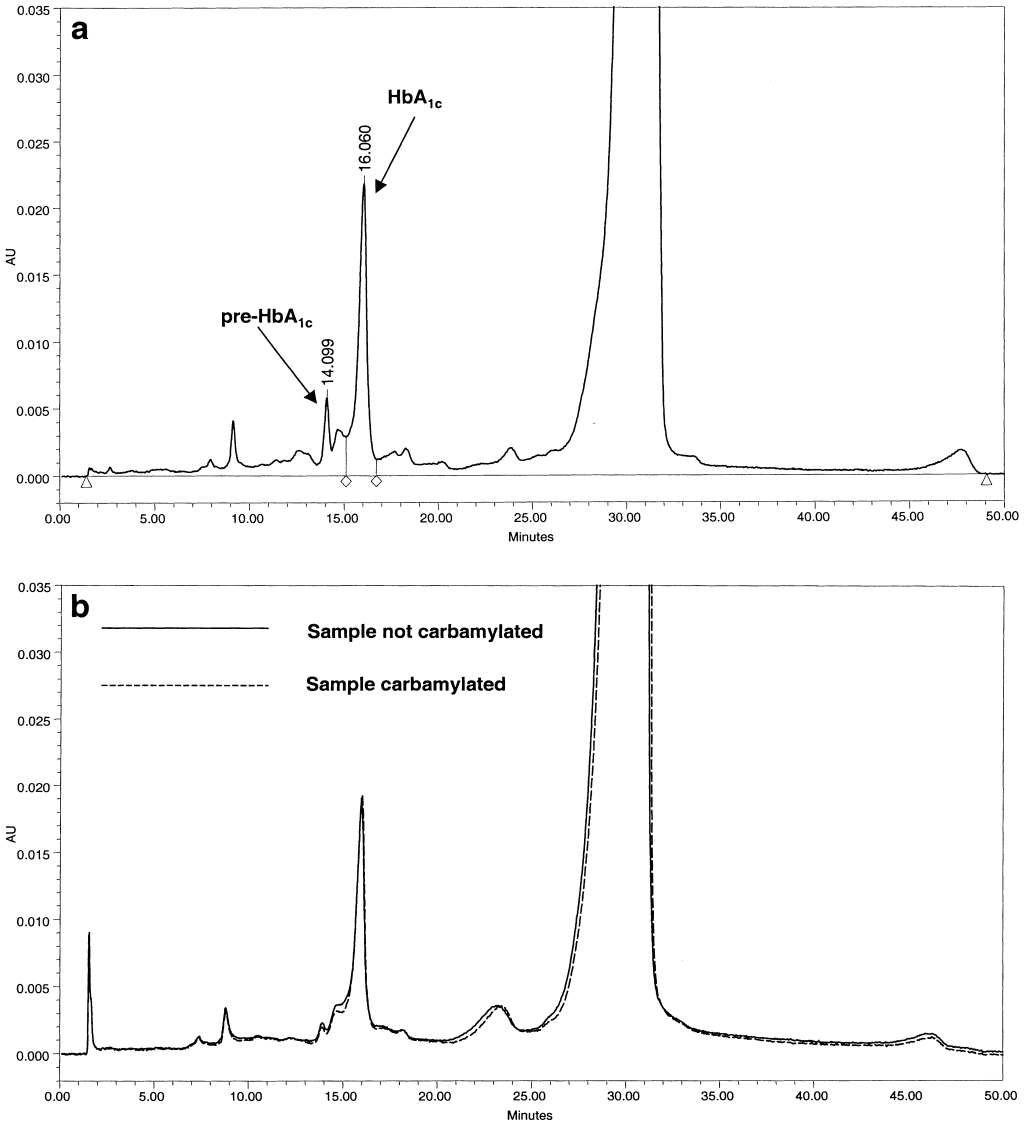

Fig. 3. Chromatograms obtained in the presence and absence of possible interferences: (a) pre-HbA_{1c}, (b) carbamylated haemoglobin and (c) acetylated haemoglobin.

incubated in 36 mmol/L urea. Thus, this unknown peak is very likely due to the incubation itself and not to a compound product of the reaction between haemoglobin and urea.

Incubating aliquots of blood from a non-diabetic and from a diabetic donor in a solution of 4 mmol/L salicylic acid produces an increase from 3.01 to 4.36% and from 4.39 to 5.17%, respectively, in the value of HbA_{1c} (Table 4). An extra peak appeared with a retention time of between 20 and 21 min after incubation with salicylic acid, which can be used as an indicator of interference due to acetylated haemoglobin (Fig. 3c). Similar results were obtained by Brunneckreeft and Eidhof using an ion-exchange Protein-Pak SP-8HR column [15]. Reducing the concentration of salicylic acid to 0.8 mmol/L, acetylated haemoglobin did not interfere in the determination of HbA_{1c} (Table 4).

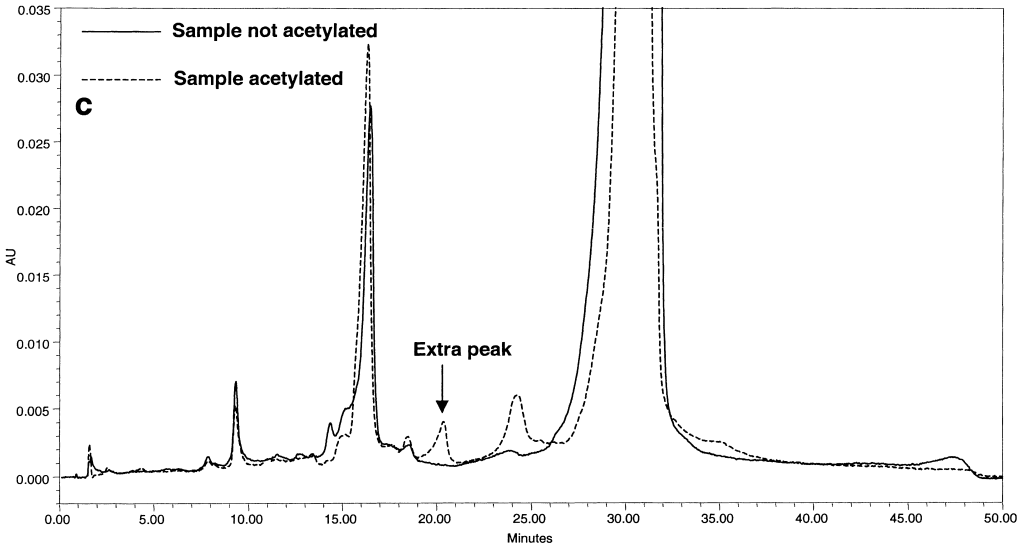

Fig. 3. (*continued*)

3.3. Studies with HbS and HbA_2

Thus far, more than 800 haemoglobinopathies have been described [27]. The performance of the method was examined when applied to a common haemoglobin variant, HbS, and to a naturally occurring physiological type of haemoglobin, HbA_2, which in normal individuals accounts for 2.5–3.5% of total Hb. Fig. 4 shows the chromatograms obtained for Hb from an individual without variant Hb, HbA_2 and HbS at concentrations of 1 mg/mL. As can be observed, HbS does not interfere with the determination of HbA_{1c}, or of HbA_2, as it can in cation-exchange-based methods. The chromatograms corresponding to HbA_2 and HbS have the same pattern as that of non-variant Hb, but with longer retention times, due to the difference in charge induced by the presence of different amino acids with regards to non-variant Hb. The method could, in principle, be applied not only to the determination of HbA_{1c}, but also to simultaneously detect haemoglobinopathies. Indeed, cation-exchange chromatography and capillary electrophoresis are the techniques most commonly used to detect thalassemias and haemoglobinopathies [19].

4. Discussion

Different principles form the basis of the methods used in clinical laboratories to detect glycated haemoglobin. As a consequence, different targets are actually determined: HbA_{1c}, total glycated haemoglobin, which makes it difficult to compare the

Table 4
Percent HbA_{1c} obtained in the presence or absence of some likely interferences: pre-HbA_{1c}, carbamylated haemoglobin and acetylated haemoglobin

Sample	Pre-HbA_{1c}		Carbamylated haemoglobin		Acetylated haemoglobin		
	Without	With	Without	With	Without	High conc.[a] With	Low conc.[b] With
Non-diabetics	3.31±0.03	3.36±0.05	3.37±0.02	3.44±0.06	3.01±0.05	4.36±0.17	3.12±0.04
Diabetics	4.17±0.02	4.14±0.02	–	–	4.39±0.07	5.17±0.05	4.45±0.04

[a] High concentration: 4 mmol/L salicylic acid.
[b] Low concentration: 0.8 mmol/L salicylic acid.

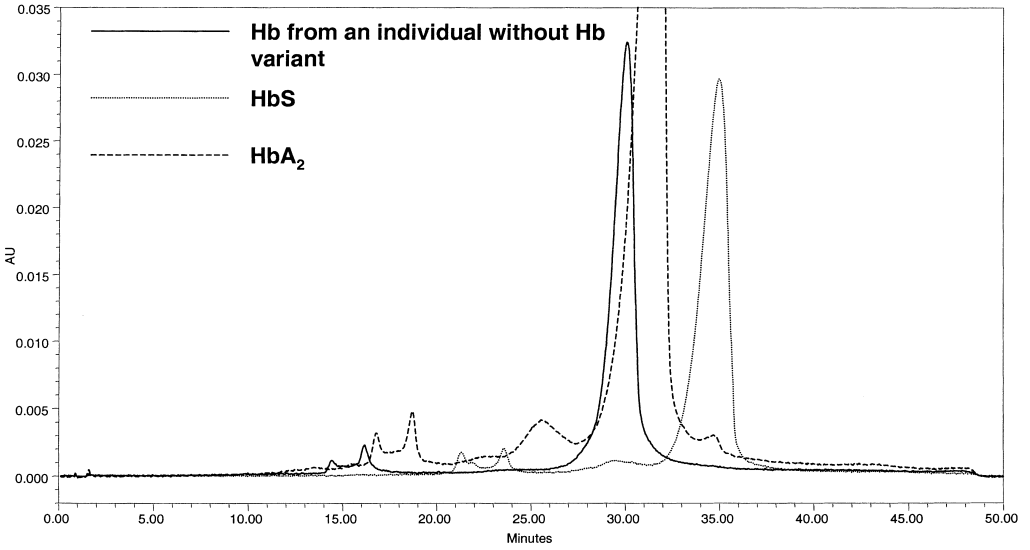

Fig. 4. Chromatograms corresponding to Sigma–Aldrich standards: Hb from an individual without variant Hb, HbA_2 and HbS.

results obtained with the different techniques. Due to known and unknown interferences (mainly in the case of cation-exchange-based methods) the presently accepted average mean for the percentage glycated haemoglobin in non-diabetics is probably too high, as recently demonstrated by the IFCC method. Most likely, the ranges of the percentage glycated haemoglobin used to classify the degrees of severity of diabetes would also be systematically too high. Cation-exchange columns used in standardization studies, such as Mono S and Bio-Rex 70, have some drawbacks associated with the occurrence of interferences.

The average mean $\%HbA_{1c}$ value for non-diabetics obtained using the ProPac SCX-10 column was in good agreement with the value obtained by Jeppsson et al. [20] applying the IFCC method. This seems to indicate that the method described here is free of some of the interferences that frequently affect separations by cation-exchange chromatography. Indeed, no significant variation in the $\%HbA_{1c}$ values was detected after removing the labile pre-HbA_{1c} fraction. No variation was observed either when the blood samples were incubated with urea concentrations in the same range as those found in patients undergoing dialysis, which indicates that interference due to carbamylation does not take place. However, the method suffers from a positive interference due to acetylated haemoglobin, which hampers the use of the ProPac SCX-10 column when determining glycated haemoglobin in patients treated with high doses of aspirin or in alcoholics. Nevertheless, the presence of an extra peak in the chromatogram can be used as an indicator of the interference.

It is also known that some variants of haemoglobin, such as HbS, can interfere with the determination of HbA_{1c} by cation exchange. With the proposed method, no interference from HbS was observed. We did not have access to a fresh blood sample containing HbS, which would provide us with more reliable results concerning the performance of the method when applied to that haemoglobin variant. Thus, we had to rely on results obtained with commercially available lyophilized standards (obtained from Sigma–Aldrich) of that particular haemoglobin variant, even if it has been reported that the analysis of lyophilized samples can produce erroneous results [28]. Numerous aspects have to be kept in mind when trying to determine glycated haemoglobin variants [29]. To evaluate if the method described here can be applied to the detection of diabetes in patients with sickle cell anaemia goes beyond the scope of this work and our capabilities, due to the lack of representative samples from that group of patients.

The determination of HbA_2 in the presence of

HbS seems to be possible (Fig. 4) due to the good resolution provided by the type of column used, however further experiments with fresh blood should be performed before making any final statement.

The elimination of well-known interferences that affect other cation-exchange-based methods and perhaps also some unknown interferences, which could explain the lower reference values achieved using the the ProPac SCX-10 column, is most likely due to the special characteristics of the column packing. Detailed information on why this type of support increases the selectivity and the resolving power of the column can be found in Ref. [21].

Due to the lack of a large number of blood samples from non-diabetics and from diabetic patients covering the whole range of severity of the illness, the performance of the method and its ability to detect diabetes was studied by making an analysis of outliers using the test of Hampel. This test has been reported to be robust and able to detect outliers that remain masked when other tests are applied [25]. Applying the method to a population of 59 blood samples, an average value for $\%HbA_{1c}$ in non-diabetics was obtained that was lower than the values reported thus far by most of the commonly used methods. The value, $3.29 \pm 0.44\%$, is comparable to that reported in Ref. [20]. The method allowed the examination of three blood samples from non-severe diabetic patients, as confirmed by the Medical Service of the European Commission. Both the proposed method and the immunoassay method used by the Medical Service, when applied to sample 2 (Table 2), agreed in that the blood corresponded to a non-severe diabetic. However, it is difficult to establish a more direct comparison with the immunoassay or with most of the accepted methods. As previously stated, most of them, including the DCCT/NGSP selected method, have reference values which are higher than those obtained with the method presented here. Indeed, data obtained from one method to another are not directly comparable, unless methods are standardized to reference systems, and calibrated accordingly [30]. Our values can only be compared directly with the reference values provided by the IFCC method. The latter method is currently being implemented in our laboratory. When applied to sample 1 (Table 2) the result obtained was in good agreement with that obtained using the ProPac SCX-10 column. This result is not given and can only be considered as preliminary because the IFCC method has not yet been completely implemented.

Obviously, the detection of three non-severe diabetics out of 59 samples is not sufficient to claim that the developed method can be used to detect diabetes with equal or better proficiency than other existing methods. Further analyses need to be performed with a large number of samples of both normal and diabetic subjects across the likely test range in order to establish the real precision, trueness and robustness of the method. Nevertheless, the results achieved so far are extremely promising.

It is important to stress the need of having a certified reference for HbA_{1c} in human blood, certified using the IFCC reference method, allowing the evaluation of the analytical characteristics of a method without the need to compare it with other existing methods.

Acknowledgements

The Medical Service of the European Commission and, in particular, Dr. Jadot, Mr. Vandevelde and Mrs. De Bruyn are kindly acknowledged for their collaboration. We also thank the members of the staff of IRMM who volunteered as blood donors. R.W. thanks the European Commission for financing his contribution to this work through a post-doctoral Marie Curie fellowship.

References

[1] H. King, R.E. Aubert, W.H. Herman, Diabetes Care 21 (1998) 1414.
[2] The Diabetes Control and Complications Trial Research Group, New Engl. J. Med. 329 (1993) 977.
[3] A. Mosca, R. Paleari, A. Trapolino, F. Capani, G. Pagano, M. Plebani, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 243.
[4] J.O. Jeppsson, P. Jerntorp, G. Sundkvist, H. Englund, V. Nylund, Clin. Chem. 32 (1986) 1867.
[5] T. Hoshine, M. Okahashi, H. Arai, J. Pharm. Biomed. Anal. 15 (1997) 1551.
[6] I.M. Stratton, A.I. Adler, H.A. Neil, D.R. Matthews, S.E. Manley, C.A. Cull, D. Hadden, R.C. Turner, R.R. Holman, Br. Med. J. 321 (2000) 405.

[7] R.C. Turner, H. Millns, H.A.W. Neil, I.M. Stratton, S.E. Manley, D.R. Matthews, R.R. Holaman, Br. Med. J. 316 (1998) 823.
[8] K.T. Khaw, N. Wareham, R. Luben, S. Bingham, S. Oakes, A. Welch, N. Day, Br. Med. J. 322 (2001) 15.
[9] H. Reinauer, Klin. Lab. 39 (1993) 984.
[10] B.J. Gould, S.J. Davie, J.S. Yudkin, Clin. Chem. Acta 260 (1997) 49.
[11] R.R. Little, D.E. Goldstein, Anal. Chem. 67 (1995) 393R.
[12] R.L. Garlick, J.S. Mazer, P.J. Higgins, H.F. Bunn, J. Clin. Invest. 71 (1983) 1062.
[13] R.R. Little, H.M. Wiedmayer, J.D. England, H.K. Naito, D.E. Goldstein, Clin. Chem. 37 (1991) 1725.
[14] G. Bolli, P. Compagnucci, M.G. Carthechini, P. De Feo, R. Santeusiano, P. Brunetti, Diabetologia 35 (1981) 93.
[15] J.W.I. Brunnekreeft, H.H.M. Eidhof, Clin. Chem. 39 (1993) 2514.
[16] M.P. Cohen, J. Witt, V.Y. Wu, Ann. Clin. Biochem. 30 (1993) 265.
[17] U. Turpeinen, U. Karjaleinen, U. Stenman, Clin. Chem. 41 (1995) 191.
[18] M. Campbell, J.S. Henthorn, S.C. Davies, Clin. Chem. 45 (1999) 969.
[19] G.M. Clarke, T. Higgins, Clin. Chem. 46 (2000) 1284.
[20] J.O. Jeppsson, U. Kobold, J. Barr, A. Finke, W. Hoelzel, T. Hoshino, Clin. Chem. Lab. Med. 40 (2002) 78.
[21] M. Weitzhandler, D. Farnan, J. Horvath, J.S. Rohrer, R.W. Slingsley, N. Avdalovic, C. Pohl, J. Chromatogr. A 828 (1998) 365.
[22] D.W. Allen, W.A. Schroeder, J. Balog, J. Am. Chem. Soc. 80 (1958) 1628.
[23] C.W. Weykamp, K. Miedema, T. de Haan, C.J.A. Doelman, Clin. Chem. 45 (1999) 438.
[24] D.M. Nathan, T.B. Francis, J.L. Palmer, Clin. Chem. 29 (1983) 466.
[25] P.L. Davies, Fresenius J. Anal. Chem. 331 (1998) 513.
[26] D.B. Sacks, D.E. Bruns, D.E. Goldstein, N.K. Maclaren, J.M. McDonald, M. Parrott, Clin. Chem. 48 (2002) 436.
[27] On the web (http://globin.cse.psu.edu).
[28] C.W. Weykamp, T.J. Penders, K. Miedema, F.A.J. Muskiet, W. van der Slik, Clin. Chem. 41 (1995) 82.
[29] L. Bry, Ph.C. Chen, D.B. Sacks, Clin. Chem. 47 (2001) 153.
[30] N. Schneider, P. Ruiz, Ph. Gillery, Clin. Chim. Acta 325 (2002) 47.