ELSEVIER

Journal of Chromatography A, 979 (2002) 201–207

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Direct monitoring of glycohemoglobin A_{1c} in the blood samples of diabetic patients by capillary electrophoresis Comparison with an immunoassay method

Heli Sirén[a,*], Päivi Laitinen[b], Ursula Turpeinen[c], Päivi Karppinen[d]

[a]*Laboratory of Analytical Chemistry, P.O. Box 55, University of Helsinki, FIN-00014 Helsinki, Finland*
[b]*Oulu University Hospital, P.O. Box 500, FIN-90029 OYS, Finland*
[c]*Helsinki University Central Hospital, P.O. Box 140, FIN-00029 HUS, Finland*
[d]*Ordior Ltd., P.O. Box 86, FIN-01601 Vantaa, Finland*

Abstract

The capillary electrophoresis (CE) study was focused on quantifying glycohemoglobin A_{1c}, HbA_{1c}, in whole blood samples of patients suffering diabetes mellitus. The results showed that dynamic polyionic coating of the capillary made the method very reproducible. The precision evaluation, method comparison and bias estimation in CE were performed during 20 days with patient blood samples and with four control samples. The influence of the storage time and temperature on the glycohemoglobin levels were also tested. High resolution in CE could be used to show evidences of the ageing of the samples stored at −70 °C. The results showed that the ageing peak was not originated from HbA_{1c}, because it did not affect the HbA_{1c} level which was in balance with the results of fresh samples measured with immunoassay. The HbA_{1c}% values of blood samples of 105 patients measured with the CE technique varied between 3.6 and 11.8 and they were approximately 2–3% lower than measured with immunoassay. The correlation (R^2) of CE results with immunoassay and HPLC results were 0.962 and 0.781, respectively.

Keywords: Glycohemoglobin; Hemoglobins; Proteins

1. Introduction

The determination of glycohemoglobins is extremely important when monitoring the glucose levels in diabetics. Glycohemoglobin A_{1c}, HbA_{1c} is the highest fraction, which correlates best with the diabetes equilibrium. The percentage of HbA_{1c} reflects blood glucose concentrations of the previous 1–2 months [1]. It is therefore a valuable indicator of long-term diabetic control [2].

HbA_{1c} is routinely measured by high-performance liquid chromatography (HPLC) with cation-exchange column materials [3–6]. The different HPLC methods provide precision and long-term stability but may lack separation efficiency. The separations may also suffer from the coelution of, e.g., fetal hemoglobin F, HbF, the amount of which increases from 1 to 3–4% during pregnancy. The measurement of HbA_{1c} must be reliable enough to eliminate wrong medication. Especially, high HbA_{1c} (above 8.5%) must be certified as early as possible, since it

*Corresponding author. Tel.: +358-9-1915-0264; fax: +358-9-1915-0253.
E-mail address: heli.siren@helsinki.fi (H. Sirén).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01403-6

correlates to the probability of the child having a developmental disorder [1,7–9].

International comprehensive Diabetes Control and Complications Trials (DCCTs) have been set up to provide evidence about micro vascular complications that are directly related to the degree of hyperglycemia of patients suffering from insulin-dependent diabetes. The tests have been done using over 20 different techniques in national laboratories worldwide, since no internationally accepted reference system is yet available. Therefore, the work in order to standardize analysis methods to correlate with each other is going on. Capillary electrophoresis (CE) methods, in that respect, have been tested to find out if they may give possibilities of standardization [10–12].

In capillary electrophoresis technique the capillary surface can be coated with chemicals to prevent the adsorption of amino acid containing bio-macromolecules on the surface walls. In addition, coated capillaries are used to control electroosmosis. When the coating is disposable, the chemicals used to modify the surface should be water-soluble. Dynamic coating has the following features: it forms non-covalent bonds, physical adsorption is the fixing mechanism on the surface, and coating reagents can be used to make the coated layer in on-line mode in the presence of the buffer reagents in the electrolyte solution.

The additives possible in dynamic coatings are organic amines like Tris buffer or other biochemical buffers. They make a cationic layer (e.g., albumin [12]) on the surface in acidic pH. A second layer can be used to reverse the direction of electroosmosis. In that case, e.g., modified polyaspartic acid or chondroitin sulfate [12] may be used to modify the cationic amine coating in CE. The formed bilayer prevents the adsorption of hemoglobin on silica.

Janssens and co-workers [12,13] have shown that CE separations of the hemoglobin variants within 4 min are possible. The CE method separates HbA_{1c} not only from hemoglobin A, HbA, but also from other hemoglobins HbA_{1a}, HbA_{1b}, HbA_{1d}, labile Hb, carbamylated Hb, fetal-Hb, meth-Hb, acetylated Hb, and from the genetic variants of HbS and HbC. Especially, separation of carbamylated Hb [13,14] from HbA_{1c} is important. It is the indicator of uremia, which can result from renal disease due to diabetes.

Reproducibility of the HbA_{1c} determination is usually below 2% and an excellent correlation with the Goldstein reference method (American Association of Clinical Chemistry, subcommittee on glycohemoglobin standardization) has been obtained [12].

The aim of the work was to use a bilayer surface coating in the CE separation of HbA variants. We have used an earlier developed method to separate the HbA_{1c} from HbA and other post-translation modifications of the Hb molecules from whole blood samples and to quantify HbA_{1c}. Method validation (intra-day and inter-day) was performed with samples of diabetes patients containing low, normal, elevated and high HbA_{1c} levels. The results obtained with CE and immunoassay were compared. In addition, the results of HbA_{1c} determination of 10 patient samples were compared with those measured with the ion-exchange HPLC method.

2. Experimental

2.1. Instrumentation

The CE data were measured with a Beckman-Coulter P/ACE MDQ (Beckman-Coulter Instruments, Fullerton, CA, USA) capillary electrophoresis instrument. Mainly visible absorption detection at 415 nm (105 samples) but also at 405 nm (10 samples) were used to detect HbA_{1c} and HbA variants. The MDQ system was equipped with 32 Karat Software. The P/ACE macro programme was used to calculate the peak areas and relative peak areas. Other experimental conditions are listed in Table 1.

The immunological measurements were performed with a Cobas Integra 700 analyzer (Roche Diagnostics, Indianapolis, IN, USA). A HPLC instrument with a weak cation-exchange column, PolyCAT A (PolyLC, Columbia, MD, USA) was used. An elution gradient containing 10 m*M* bis-Tris–1.0 m*M* KCN (pH 6.9) in water and 10 m*M* bis-Tris–1.0 m*M* KCN–200 m*M* NaCl (pH 6.6) was used to obtain the reference data for CE results. The runs were per-

Table 1
The conditions of separations in dynamic coated capillaries made with polyionic reagents

Capillary electrophoresis equipment (Beckman-Coulter, Palo Alto, CA, USA)	P/ACE MDQ
Capillary (Composite Metal Services, The Chase, UK)	33 cm (separation length 10 cm)×25 μm×375 μm
Detection	UV 415 nm (high resolution)
Voltage	24 kV (reverse polarity)
Temperature	26 °C (cartridge), 17 °C (sample)
Injection (outlet)	5 kV (voltage reverse) for 6 s
Electrolyte solution	27 g/l malic acid, pH 4.6
Volume of the electrolyte	1.4 ml for rinsing and 2×30 ml for separation (one at the inlet, the other in the outlet end of the capillary)
Dynamic cation coating (Analis, BE)	33 g/l malic acid containing polycations (malic acid–arginine mixture)
Dynamic anion coating (Analis, BE)	Polyanions in malic acid–argine mixture
Total analysis time (2.4 min) with capillary washings and surface coating replacements is 3.70 min	
Sample volume in analyses	50 μl
Initiator	Malic acid
No. of patient samples	105 (for immunoassay) and 10 (for HPLC)
No. of determinations	700
The samples must be analyzed within 8 days, when they are stored at +4 °C. When stored at −20 °C and −70 °C HbA profile gives an ageing peak, which did not originate from HbA_{1c}	

formed at room temperature. More detailed information about the system is described elsewhere [3,4].

2.2. Reagents

The CE reagents were obtained from Analis (Namur, Belgium). 0.2 *M* sodium hydroxide, distilled water, 33 g/l malic acid with arginine and polycations (pH 4.6), 27 g/l malic acid with arginine and polyanions (pH 4.6) and malic acid solution (pH 5.6) were used for capillary conditioning, coating and sample separation, respectively. The solutions were stabilized with 0.1 g/l sodium azide [11].

2.3. Blood samples and tests

The blood samples from diabetes patients were collected in EDTA-treated tubes and mixed. They were stored at −20 °C. When kept at 4 °C, the samples were stable only for 7 days. For multicenter evaluation and precision tests an aliquot of the samples were stored at −70 °C.

All the patient's blood samples in the study were analysed with immunoassay or with HPLC before the CE measurements.

2.4. Quantification of the samples

2.4.1. Measurements with capillary electrophoresis

HbA_{1c} was determined from a whole blood sample hemolysate. A 50-μl volume of venous blood was hemolysed with 150 μl of malic acid. The samples were analysed by CE in dynamically coated capillary. The HbA variants were separated within 2.5 min. HbA_{1c} was calculated as a relative percentage area.

2.4.2. Immunoassay measurements

Total Hb and HbA_{1c} concentrations were determined after hemolysis of the anticoagulated whole blood specimen by the Cobas Integra 700 instrument using a cyanide-free colorimetric method based on the formation of a brownish–green chromophore (alkaline hematin D-575) in alkaline detergent solution. The color intensity was proportional to the Hb concentration in the sample and it was determined by monitoring the increase of absorption at 552 nm. The

test results were calculated using a response factor determined with the primary calibrator chlorohemin.

In contrast, HbA_{1c} was determined immuno-turbidimetrically. The ratio of both concentrations yields the final relative content (percentage) of HbA_{1c}. The anticoagulated whole blood specimen was first manually pretreated with HbA_{1c} hemolysis reagent. Erythrocytes were lysed by osmotic pressure. The released Hb was proteolytically degraded by pepsin, to make N-terminal structures of hemoglobin β-chains more accessible for the immunoassay. Additionally, the heme portions were oxidized for the Hb assay. HbA_{1c} was measured by attaching monoclonal antibodies to latex particles. The antibodies bound the B-N-terminal fragments of HbA_{1c}. Remaining free antibodies against HbA_{1c} were agglutinated with a synthetic polymer carrying multiple copies of the B-N-terminal structure of HbA_{1c}. The change in turbidity was inversely related to the amount of bound glycopeptides of HbA_{1c} and was measured turbidimetrically at 552 nm.

The final result was expressed as HbA_{1c} content and calculated from the HbA_{1c}/Hb ratio, including a conversion equation to match a HPLC reference method with the following equation: $HbA_{1c}\% = (HbA_{1c}/Hb)\cdot 177.3 + 2.09$.

3. Results and discussion

The CE method is based on ion-pair formation. The ion pairs are formed between anionic malic acid and the cationic hemoglobin amino groups in acidic pH. The electropherograms (Fig. 1a and b) show good resolution between HbA_{1c} and other HbA peaks. In Fig. 1b the ageing peak between the two HbA peaks is caused by the long storage time at −70 °C, which was necessary due to the 20-day analysis period.

According to these results, the levels of HbA_{1c} were above normal level (>4.0%) in all patient samples. On the basis of the analyses, two of the samples were chosen to represent the lowest (5.11%) and the highest (9.62%) HbA_{1c} levels of the patient

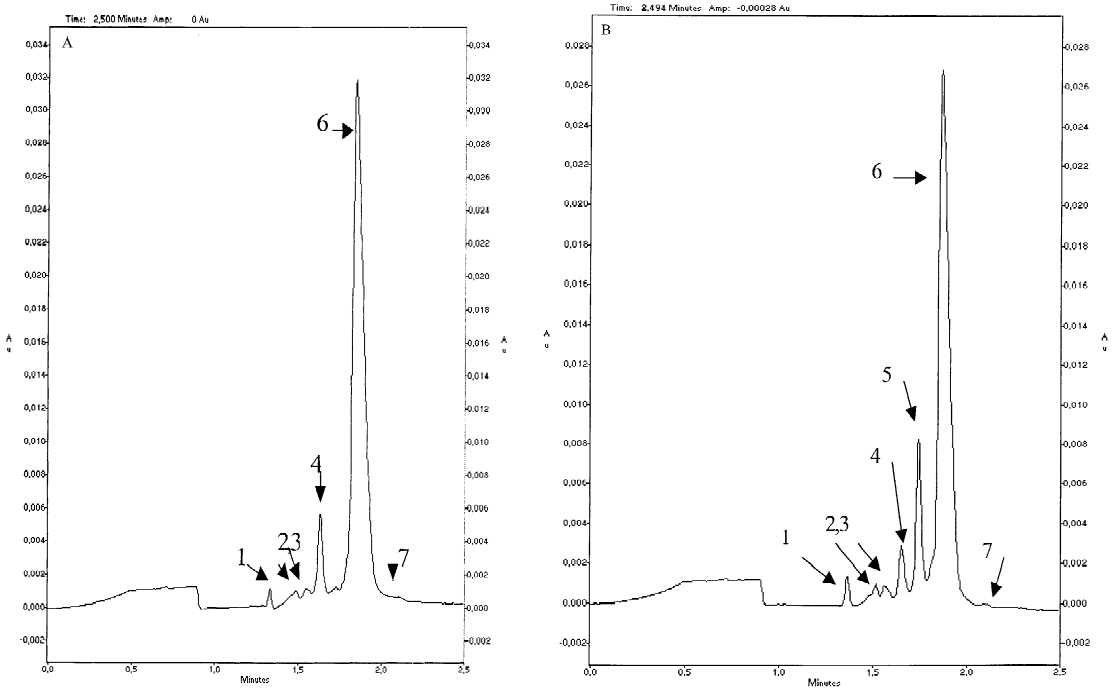

Fig. 1. Electropherogram of a patient sample: profiling the glycohemoglobin fractions of (A) 10.2 and (B) 8.8 HbA_{1c}%. Peaks: 1=hemolyzer marker, 2=HbF, 3=Carb-Hb, 4=$HbA1_c$, 5=HbA_{1d2}, 6=HbA_0 and 7=HbA_2.

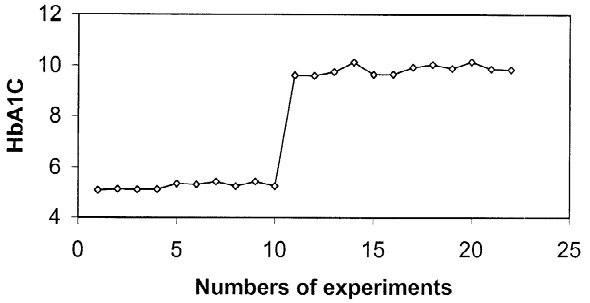

Fig. 2. Reproducibility of the measurements (NCCLS EP5-A tests) at low and high HbA_{1c} concentrations.

material (Fig. 2). Precision evaluation (NCCLS-EP5-A test [15]) was performed with two analyses with two blood samples, one with normal and the other with >9% HbA_{1c} content for 20 days. The samples were frozen as aliquots at −70 °C. Method comparison and bias estimation (NCCLS-EP9-A test [16]) with CE were performed using 8, 12, 12 and 2 patient samples containing 4–6.5, 6.5–8.5, 8.5–10.5 and 10.5–14% of HbA_{1c}, respectively. With each specimen duplicate analyses were performed. The results were used for concentration calibration (Fig. 3), i.e., multicenter evaluation of HbA_{1c}% [15–17].

Migration time, t_m, of HbA_{1c} in the validated CE method was 1.77 min (SD±0.01, RSD 0.56%, n= 40). RSDs for its area and corrected area (i.e., area related to migration velocity) were 15 and 7.9%, respectively. The NCCLS EP5-A tests to evaluate precision are listed in Table 2. It can be noticed that within a day in one sequence the RSD of all concentration levels is below 1.4%. Fig. 2 shows the reproducibility data with low and high HbA_{1c} concentrations. The samples were kept when not analyzed in −70 °C. No ageing of the HbA_{1c} was noticed. Fig. 3 shows concentration calibration of HbA_{1c} made with CE during 11 days. Our tests show that the repeatability of the CE method within a day was good. The RSD values from repeated analyses were 6.3, 4.6, 4.0 and 4.4%, respectively, for low, normal, elevated and high levels of HbA_{1c} in our patient material. Inter-day analysis showed 8.5, 3.2, 3.3 and 6.3% RSDs for the low, normal, elevated and high levels of HbA_{1c}, respectively. Table 3 lists the analysis results of randomly taken patient sample: within a day HbA_{1c} was 5.21% (SD 0.09, RSD 1.80%) and from day to day it was 5.09% (SD 0.32, RSD 6.2%, 11 days). One hundred and 5 patient samples (Fig. 4) were analysed within the CE method validation and performance tests. The HbA_{1c} levels in the samples were 3.51–10.8% according to the CE measurements. It was noticed that better correlation between CE and immunoassay results had been obtained at elevated HbA_{1c} concentrations.

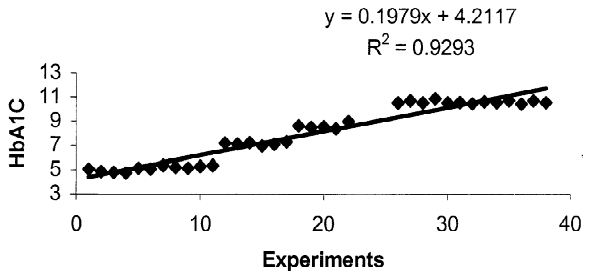

Fig. 3. Concentration level calibration and reproducibility tests using NCCLS EP9-A tests—a methodology with capillary electrophoresis.

Table 2
Reproducibility of the method measured with diabetics blood samples

Sample* (HbA_{1c} content)	HbA_{1c} (%) (min, max)	HbA_{1c} (%) (average)	SD	RSD (%)
Low	5.09, 5.13	5.11	0.02	0.34
High	9.62, 9.61	9.62	0.01	0.05
Normal	6.63, 6.64	6.64	0.01	0.08
Elevated	8.30, 8.49	8.40	0.10	1.13
Elevated	8.56, 8.46	8.51	0.05	0.59
Normal	6.42, 6.32	6.37	0.05	0.78
High	9.76, 10.03	9.90	0.14	1.41
Low	5.11, 5.12	5.12	0.05	0.10

*The sample order in the column is identical with the analysis order of the sample. *Note: The content of HbA_{1c} of a healthy person is 3.2–4.80%. Here the sample names are according to the specific patient material obtained in this study.

In spite of the derivatization step in the immunoanalysis technique, correlation between CE and immunoassay results could be noticed, although the values by CE were 2–3% lower than those obtained with the immunoassay technique (Fig. 5). It was also noticed that correlation between the HPLC and CE was not as good (Fig. 6).

For standardization of the methodology of hemoglobin measurements, the capillary electrophoresis technique has less sample preparation steps than immunoassay and can therefore be used for real-time

Table 3
Results of the ''low'' content of HbA_{1c} in the patient blood samples

No.	Date	First analysis in the sequence (first replicate of the blood sample)		Second analysis in the sequence (second replicate of the blood sample)		Average value of the first analysis	Average value of the second analysis	Average value intra-day
1	2 October 2001	4.61	5.10	5.05	4.61	4.86	4.83	4.84
2	3 October 2001	5.04	4.68	4.97	5.10	4.95	5.04	4.95
3	4 October 2001	5.85	4.05	4.08	6.35	4.95	5.22	5.08
4	5 October 2001	4.80	4.74	4.63	4.93	4.77	4.78	4.78
5	10 October 2001	5.13	5.30	4.51	5.95	5.20	5.23	5.22
6	11 October 2001	5.33	5.45	4.95	5.20	5.39	5.08	5.23
7	12 October 2001	5.30	5.10	5.95	4.28	5.20	5.12	5.16
8	17 October 2001	5.25	5.13	5.25	5.00	5.19	5.10	5.16
9	18 October 2001	5.09	5.13	5.11	5.12	5.11	5.12	5.11
10	18 October 2001	5.12	5.34	5.31	5.42	5.23	5.37	5.30
11	19 October 2001	5.24	5.43	5.19	5.25	5.33	5.22	5.28

The results are calculated according to the NCCLS-EP5-A test methodology.

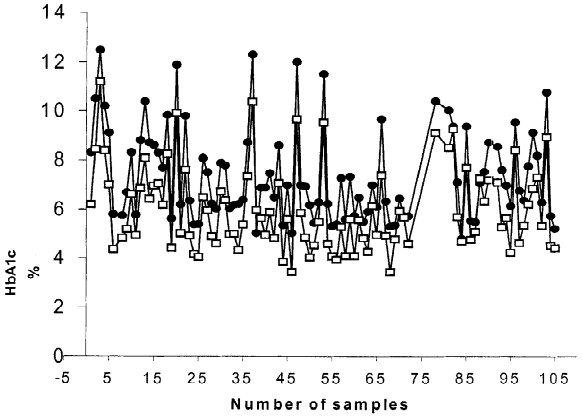

Fig. 4. HbA_{1c} concentration in patient blood samples used in the study. Measurements with (triangle) immunoassay (Roche Integra 700) and (square) CE.

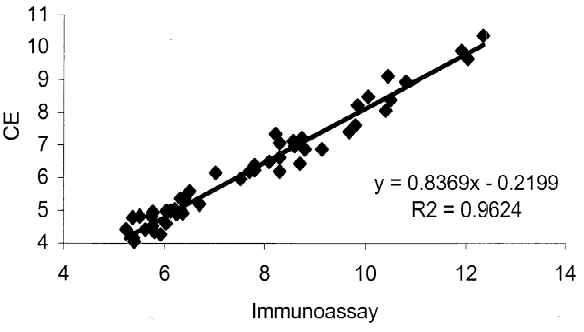

Fig. 5. Correlation results with immunoassay and CE.

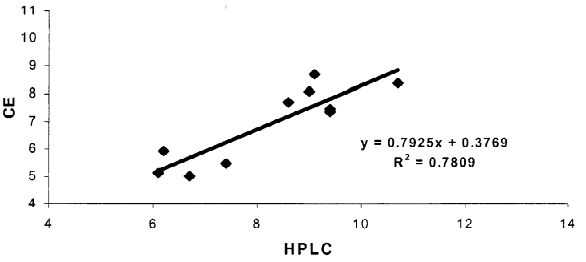

Fig. 6. Correlation results with HPLC and CE.

monitoring of the blood profile. Without manipulation of human blood samples the CE results of HbA_{1c} content obtained in our study were 2–3% lower than immunoassay values. Other advantages of CE measurement, which could be noticed, were repeated analyses in sequence, good repeatability and reproducibility, high throughput and low cost. The method without changing the electrolyte solutions could be performed for 1 month using sequences of 18 h. In routine analyses advantages of the optimized CE method are high running speed, good resolution, easy sample preparation to micro vials, small volumes of sample and buffer, consecutive separation enabling higher throughput and low cost of singular analysis.

The results showed that the capillary electrophoresis method was reproducible (Figs. 3 and 4, Table 3) on the basis of a stable current (56 μA) in each run and the repeatable migration time of the HbA_{1c}

peak. The electropherograms show that glycohemoglobin screening from different patient blood samples is easy due to the unambiguous fingerprint profile. Simultaneous screening of HbA variants and quantification of HbA_{1c} speaks favorably of its use in medical diagnosis.

4. Conclusions

A fast, sensitive and automated capillary electrophoresis method was compared to an immunoanalytical method in high-throughput screening and simultaneous quantifying of glycohemoglobin A_{1c}. The performance of the CE method was estimated with capacity, repeatability, precision and bias estimation. Whole blood samples of patients having diabetes mellitus were analyzed with the optimized CE–Vis technique, with a commercialized immunoassay technique and with HPLC. The samples were hemolysed before analyses. Other pretreatment steps were done in-line as a pre-analysis in CE. However, pretreatment to visualize glycohemoglobins in immunoassay was needed. The HbA_{1c} levels in the patient samples were 3.51–10.8%. The values were lower than those measured with immunoassay technique. It was noticed that the best correlation between CE and immunoassay results were obtained from patient bloods having elevated HbA_{1c} concentrations.

Acknowledgements

The Academy of Finland (H.S.) and Analis Belgium are gratefully acknowledged for the support.

References

[1] M.-L. Lamberg, Kliiniset Laboratoriotutkimukset, Diacor, Otava, Helsinki, 1991.
[2] D.E. Goldstein, K.M. Parker, J.D. England, J.E. England Jr., H.-M. Wiedmeyer, S.S. Rawlings, Diabetes 31 (1982) 70.
[3] U. Turpeinen, U.-H. Stenman, in: H.Y. Aboul-Enein (Ed.), Analytical and Preparative Separation Methods of Biomacromolecules, Marcel Dekker, New York, 1999, p. 1.
[4] U. Turpeinen, U. Karjalainen, U.-H. Stenman, Clin. Chem. 41 (1995) 190.
[5] B. Landin, J.O. Jeppson, Hemoglobin 17 (4) (1993) 303.
[6] U. Turpeinen, U.-H. Stenman, M. Syrjälä, J.B. Wilson, K.M. Gooding, in: Chromatographic Science Series, Vol. 87, Marcel Dekker, New York, 2002, p. 589.
[7] C.J.A. Doelman, C.W.M. Siebelder, W.A. Nijhof, C.W. Weykamp, J. Janssens, T.J. Penders, Clin. Chem. 43 (4) (1997) 644.
[8] U. Kobold, J.-O. Jepsson, T. Dülffer, A. Finke, W. Hoelzel, K. Miedema, Clin. Chem. 43 (10) (1997) 1944.
[9] D.M. Nathan, New Engl. J. Med. 328 (1993) 1676.
[10] J. Gerritsma, D. Sinnige, C. Drieze, B. Sittrop, P. Houtsma, N. Hulshorst-Jansen, W. Huisman, Ann. Clin. Biochem. 37 (2000) 380.
[11] J. Janssens, R. Chevigné, US Pat. 5 611 903 (1997).
[12] C. Doelman, C. Siebelder, W. Nijhof, C. Weykamp, J. Janssens, T. Penders, Clin. Chem. 43 (1997) 644.
[13] C. Lin, F. Cotton, B. Fontaine, B. Gulbis, J. Janssens, F. Vertongen, Hemoglobin 23 (1999) 97.
[14] C. Weykamp, K. Miedema, T. de Haan, C. Doelman, Clin. Chem. 45 (1999) 438.
[15] Evaluation of Precision Performance of Clinical Chemistry Devices; Tentative Quideline, NCCLS Publication EP5-A, National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA, 1999.
[16] Evaluation of Precision Performance of Clinical Chemistry Devices; Tentative Quideline, NCCLS Publication EP9-A, National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA, 1995.
[17] R.R. Little, C.L. Rohlfing, H.-M. Wiedmeyer, G.L. Myers, D.B. Sacks, D.E. Goldstein, Clin. Chem. 47 (2001) 1985.