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Article

Standardization of method for determining glycosylated hemoglobin (Hb A_1c) by cation exchange high performance liquid chromatography

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Hemoblobin A_1c is the most important parameter for the monitoring of metabolic control of patients with diabetes mellitus. The purpose of this study was to adapt the Mono S method to a conventional HPLC system, allowing highly selective HbA_1c determination without the acquisition of kits or the use of dedicated systems The results obtained were compared to the Tinaquant® immune turbidimetric method and the Bio-Rad Variant® chromatographic method. The developed method presented intra-study precision (C.V. %) of 1.39-3.65 and inter-study precision (C.V. %) of 2.80-3.02%. The determination coefficients among methods were: HPLC Mono S x Tinaquant®: r^2: 0.9856 (n=60) and HPLC Mono S x HPLC Bio-Rad Variant®: r^2: 0.9806 (n=16). A conversion equation between HPLC Mono S and Bio-Rad Variant® was calculated allowing yielding comparable and interchangeable values. The HPLC Mono-S is a precise, low-cost method which yields similar values to the Bio-Rad Variant® method on conventional HPLC equipment.

Uniterms: High performance liquid chromatography. Mono S/method. Hemoglobin/A_1c. Hemoglobin/variants.

A hemoglobina A_1c é o parâmetro laboratorial mais importante no monitoramento do controle metabólico de pacientes portadores de diabetes melito. Dentre as metodologias existentes para a quantificação desta fração de hemoglobina, a cromatografia líquida de alta eficiência (CLAE) baseada em troca catiônica apresenta a melhor precisão, sendo o método de escolha. O objetivo deste trabalho foi adaptar o método Mono S a um sistema de CLAE convencional permitindo a disponibilidade da determinação altamente seletiva de Hb A_1c sem a aquisição de kits e comparar os resultados obtidos com o método imunoturbidimétrico Tinaquant® (Roche®) e com o método de cromatografia líquida Bio-Rad Variant®. O método desenvolvido apresentou precisão intra-ensaio de 1,39-3,65% e inter-ensaio de 2,80-3,02%. Os coeficientes de determinação entre os métodos foram: CLAE Mono S x Tinaquant®: r^2 = 0,9856 (n=60) e CLAE Mono S x Bio-Rad Variant®: r^2 = 0,9806 (n=16). Não foram observadas diferenças entre CLAE Mono S e Bio-Rad Variant® através de gráfico de Bland-Altman e regressão de Passing-Bablok. Foi obtida uma equação de conversão entre os valores do método teste e os valores obtidos por métodos rastreáveis ao National Glycohemoglobin Standardization Program (NGSP), permitindo a obtenção de valores comparáveis e intercambiáveis entre as metodologias com o uso de instrumentos convencionais de CLAE e com custo reduzido.

Unitermos: Cromatografia líquida de alta eficiência. Mono S/método. Hemoglobina/A_1c. Hemoglobina/variantes.

INTRODUCTION

Hemoglobin A_1c (Hb A_1c) is the most important laboratory parameter in monitoring metabolic control of patients with diabetes mellitus (Sacks *et al.*, 2002). The quantity of HbA_1c reflects the mean blood glucose concentration over the two or three preceding months and is therefore an independent parameter of carbohydrate metabolism. (Miedema, 2004). In 1993, *The Diabetes Control and Complications Trial Research Group* (DCCT) proposed target levels of Hb A_1c for diabetic control in order to minimize chronic complications characteristic of the disease such as retinopathy, nephropathy, and cardio-

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vascular disease. These values have since become widely used in clinical practice.

In view of the high worldwide incidence of diabetes mellitus and the complications resulting from uncontrolled circulating glucose levels, there is great demand for Hb A_1c determinations in clinical samples. As this parameter is used for long term control of patients, it is fundamental that the method used has a very high degree of reproducibility. In addition, in order to allow the use of universally agreed therapeutic target levels for this parameter, accuracy becomes paramount, given that the results of this test are standardized internationally through a variety of different national schemes.

The three nationally accepted methods for determination of Hb A_1c, based on cation exchange methods, are: the *National Glycohemoglobin Standardization Program* (NGSP) from the United States; *Japanese Diabetes Society* (JDS) from Japan, and the Mono S method from Sweden. Recently, the *International Federation of Clinical Chemistry and Laboratory Medicine* (IFCC) introduced a highly selective international reference standard for Hb A_1c, based on liquid chromatography associated in tandem with mass spectroscopy (LC-MS/MS), or alternatively, a dual method which involves initial purification of the fraction of Hb A_1c using high performance liquid chromatography, followed by determination using capillary electrophoresis (CE) (Jeppsson *et al.*, 2002). The IFCC reference method was extensively compared with national Standards, called Designated Methods of Comparison (DMC). Based on these studies, master equations for conversion between DMC and the IFCC reference standard were established and have shown high inter-laboratory reproducibility (Hoelzel *et al.*, 2004). The same study showed a high correlation among the DMCs, despite differences in specificities, allowing conversion of analytical findings among different methods.

The DMC used by the NGSP, which served as a basis for the therapeutic targets presently used for Hb A_1c determination by the DCCT, were initially described by Trivelli *et al.* (1971) and used a Bio-Rex 70 cation exchange column (produced by Bio-Rad). However, later studies found this separation technique to be limited and revealed that the peak quantified as Hb A_1c included a high proportion of other forms of hemoglobin (Miedema, 2004). The DMC of JDS also used a dedicated cation exchange chromatography system produced by Tosoh, which offered superior selectivity compared to the NGSP method. Both methods used dedicated, highly automated equipment for analyzing Hb A_1c and related compounds.

The Swedish DMC used a Mono S column, produced by Pharmacia (now GE HealthCare) and was initially proposed by Jeppsson *et al.* in 1986. The Mono S column is a strong, methyl sulphonate, based cation exchanger, with an extremely uniform granule distribution and compared to the other DMCs, yields values closer to the IFCC reference method (Hoelzel *et al.*, 2004). Values found by the Mono S method are always lower than those derived from the NGSP and JDS methods, whereby a master equation of the IFCC values for Mono S show higher slope and lower intercept, indicating improved chromographic separation and lower proportion of non-Hb A_1c components.

The Mono S method was originally developed for *Fast Protein Liquid Chromatography* (FPLC) units constructed to analyze proteins, and characterized by operating under pressures significantly lower than those of conventional HPLC units. Although Hb A_1c determination on traditional HPLC systems had been previously reported (Ellis *et al.*, 1984; Brunnekreeft, Eidhof, 1993; Turpeinen *et al.*, 1995), the unconverted values which resulted tended to limit clinical application, especially in view of the difficulty in comparing Hb A_1c values against standardized reference values for diabetes mellitus control. We found no studies which employed the Mono S column for Hb A_1c determination on HPLC systems, equipment that can be used for a broad range of other clinical analyses.

At present, the determination of glycosylated hemoglobin in Brazilian clinical laboratories is usually done with the dedicated Bio-Rad Variant® chromatography unit. The objective of this study was to develop a HPLC method for determining Hb A_1c using a Mono S column which could enable highly selective determination of Hb A_{1c} and represent a lower cost alternative that needs no commercial kits.

MATERIAL AND METHODS

Solutions, reagents and controls

Sodium maleonate and lithium chloride were acquired from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Sodium azide and mono basic potassium phosphate were purchased from Cromato Produtos Químicos Ltda (Diadema, Brazil), and Triton X-100 from Casa de Química (Diadema, Brazil). Citric acid was obtained from Dinâmica Química Contemporânea (Diadema, Brazil). Water was obtained by distillation and deionization and further purified using Elga Pure Lab Ultra SC from Elga Labwater (Lane End, United Kingdom).

Mobile phase A was prepared by diluting 3.05 g of sodium malonate and 0.2 g of sodium azide in 1 L of purified water. Mobile phase B was made by diluting 3.05 g

of sodium malonate, 0.2 g of sodium azide, and 12.85 g of lithium chloride in 1 L of purified water. The mean pH of both mobile phases was 5.7. The mobile phases were filtered with a 0.45 µm cellulose acetate membrane (Shleicher and Schuell, Germany). A hemolyzing solution was prepared by diluting 0.39 g of citric acid and 0.78 g of monobasic sodium phosphate in 100 mL of purified water, after adding 94 µL of Triton X-100. The mean pH of the hemolyzing solution was 5.4. The control for Hb A_1c was *Lyphochek Diabetes Control Levels 1 and 2* provided by Bio-Rad (Irvine, U.S.A), where mean values obtained from the Bio-Rad Variant were 5.6% (range 4.5-6.7%) and 9.2% (range 8.4-10.0%). This commercial control also contains Hb F and Hb A_0, although values are not declared quantitatively. Control samples for Hb F, S and C were obtained from Interscientific (Hollywood, U.S.A), with mean values on cellulose acetate electrophoresis of 21, 17 and 13%, respectively.

Chromatographic analysis

The chromatographic system was a quaternary system of LC-10AT pumps, a SCL-10-A control module, CTO10-AS column oven, SIL-10AF auto-injector and SPD-M10A diode arrangement detector (DAD) and Class VP 6.13 SP2 control program (Shimadzu, Kyoto, Japan). Separation was done in a 10 µm spherical phase column based on polystyrene cross linked with divinylbenzene, with methylsulphonate cation exchange groups in a glass tube of 5 by 50 mm internal diameter (Mono S GL 5/50) from GE Healthcare (Uppsala, Sweden). Chromatograms were monitored at 415 nm. The initial elution consisted of 80% mobile phase A and 20% mobile phase B, with the following gradient program: 50% B until 3.9 minutes; 75% B up to 4.26 minutes, 100% B until 4.58 minutes, and maintaining this proportion until a total of 11 minutes had elapsed, with a return to initial conditions in 11.8 minutes, which were maintained for a total elapsed time of 13 minutes. The equilibrium time between analyses was 2 minutes, and total chromatographic analysis time was 15 minutes. The samples were eluted at a rate of 1 mL/min and the column was kept at 30 ºC during analyses. The proportion of normal hemoglobin fraction (Hb F, Hb A_1c and Hb A_0), as well as the hemoglobin variants (Hb S and Hb C), were calculated as a percentage of the area of their peaks in relation to the total area of hemoglobin peaks on the chromatogram.

Sample preparation

Venous blood samples were collected in EDTA coated tubes and maintained at 4 °C until analysis, which occurred within a maximum of 7 days. Polypropylene micro tubes were used, containing 14 µL of whole blood and 700 µL of hemolyzing solution. This mixture was homogenized in a vortex agitator for 10 seconds, and then maintained in a water bath at 37 °C for 30 minutes in order to eliminate the labile fraction of Hb A_1c. Subsequently, the sample was centrifuged for 5 minutes in a micro centrifuge maintained at 4 ºC, with a velocity of 11,000 g. A 10 µL aliquot of the supernatant was injected into the HPLC-DAD system.

Quality control

Venous blood samples from three patients (Hb A_1c of 4.3, 5.9 and 10.2 %) were processed using the described method, in triplicate, on five different days, using the conversion equation for NGSP aligned values. The percentages of HbA_1c found for each patient were analysed using ANOVA (Analysis of Variance Test) with the days as a grouped variable, enabling calculation of intra and inter study precision. Precision was given as a coefficient of percentage variation (C.V%). Accuracy was calculated by comparing results of the analysis of 16 samples using the test method to results using the Bio-Rad Variant® method, and expressed as a percentage of the value obtained using the reference method. Additionally, 22 sample replicas of commercial quality controls for Hb A_1c (*Lyphochek Diabetes Control Levels 1 and 2)* were tested and the results obtained compared with those claimed by the manufacturer.

Comparison with other methods

Venous blood samples from 60 patients were processed by the test method and by the immunoturbidity method (Tinaquant®, Roche), calibrated according to IFCC/NGSP. A linear regression curve for the obtained values was calculated, in which the % Hb A_1c obtained by the present method was represented on the y (ordinal) axis, and the immunoturbidity values on the x axis (abscissa). Samples containing other hemoglobin variants were excluded. Correlation among the evaluated methods was determined using the correlation coefficients (r^2). Linear regression analysis was used to convert HPLC values to NGSP-aligned ranges. Calculations were performed using Microsoft Excel® (Microsoft Corporation, U.S.A).

In addition, a further 16 blood samples were evaluated using both the study method and a dedicated HPLC system (Bio-Rad Variant®). The Hb A_1c % values found using the test method and converted to NGSP values as

described above, were compared with the values obtained using the dedicated Bio-Rad Variant® system by means of Bland and Altman graphics (Bland, Altman, 1986) and Passing-Bablok regression (Passing, Bablok, 1983). These methods do not depend upon use of methods which classify variables as dependent or independent with calculation of slope and intercept and their respective confidence intervals.

RESULTS AND DISCUSSION

Chromatographic separation and detection

The composition of the mobile phase was the same as used by Jeppson et al (1986) in a HPLC system; with the elution conditions and modifying gradients modified for their HPLC system, and optimization of chromatographic resolution. The total analysis time was 15 minutes, during which the separation and quantification of the normal hemoglobin fractions (Hb F, Hb A_1c, Hb A_0) and variants (Hb S and Hb C), occurred with mean retention times of: 2.9, 3.6, 9.2, 10.1, and 11.8 minutes, respectively. Figure 1 shows typical control chromatograms for Hb A_1c and Hb F, Hb S and Hb C, as well as one sample for a patient with Hb S. The proportions of Hb A_1c and the other hemoglobins were calculated as a percentage of the area of their peaks in relation to the total area of all the hemoglobin peaks on the chromatogram. At the time of writing, more than 7,000 samples had been processed using the same Mono S column without loss of chromatographic resolution or the appearance of interfering peaks.

This method was developed to routinely measure levels of HbA_1c in the continuing care of patients with diabetes mellitus. However, it is also a useful tool for screening and measuring levels of hemoglobins F, S and C. Heterozygotes or homozygotes with these fractions can thus be found when testing for Hb A_1c. It is important to point out that HPLC, given its greater accuracy, is recommended over electrophoresis for hemoglobin analysis (Fernandes, Domingos, 2006).

Quality Control

The precision of the test method was evaluated by performing triplicate measurements on five different days on blood samples from patients with different levels of Hb A_1c%. The intra study variation in precision ranged from 1.39 to 3.65%, while inter study precision ranged between 2.80 and 3.02%. The validation parameters were in line with published values for bio analytic methods, as listed by Shah *et al.* (2000), and are shown in Tables I and II.

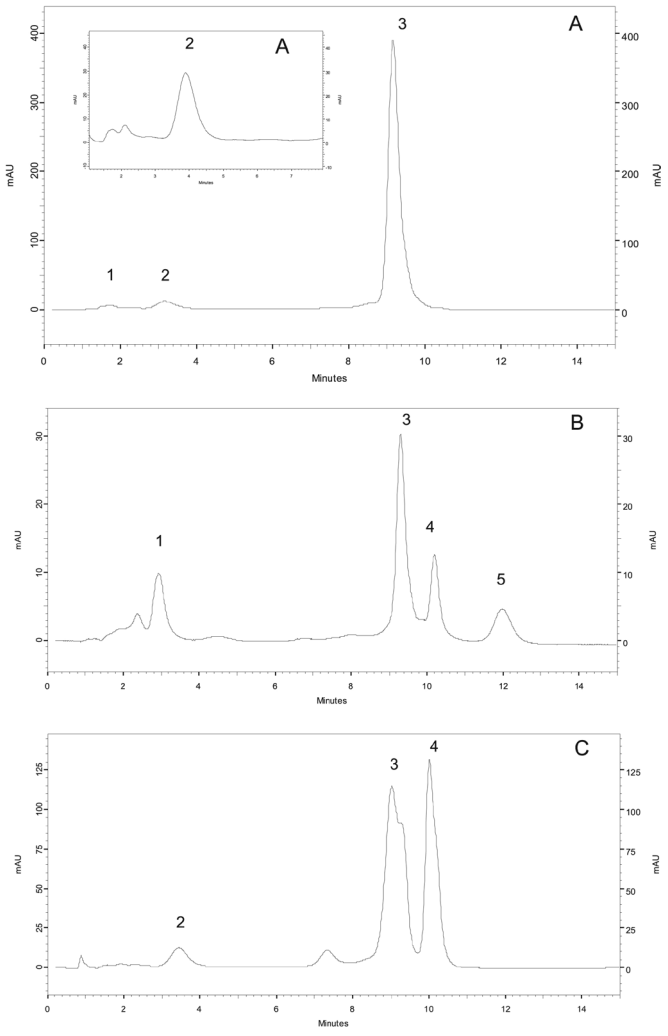


FIGURE 1 - Chromatograms monitored at 415 nm. **A:** Control sample of HbA_1c (9.2%), peak 1: Hb F (0.8%); peak 2: Hb A_1c (9.2%); peak 3: Hb A_0 (89%). **B:** Control sample of hemoglobin variants, peak 1: Hb F (17%), peak 3: Hb A_0 (39%); peak 4: Hb S (15%); peak 5: Hb C (11%). **C:** Patient sample; peak 2: Hb A_1c (6.8%); peak 3: Hb A_0 (51%), peak 4: Hb S (38%).

TABLE I - Inter and intra study precision of HbA_1c determinations in patient samples (n=15)

Hb A_1c (%) (mean)	**Intra-study** (C.V. %)	**Inter-study** (C.V. %)
4.3	3.65	2.80
5.9	1.50	2.96
10.2	1.39	3.02

Analysis of commercially available control samples of Hb A_1c using the test method are shown in Table II. Values obtained were all within recommended intervals, with values found to be very close to the central value stated by the supplier of the samples.

TABLE II - Results obtained from analyses of control samples of HbA_1c (n=22)

Concentration* Hb A_1c (%)	Concentration ranges found (%)	Mean of concentrations found (%)
5.6	5.3 – 5.9	5.6
9.2	9.4 – 10.3	9.7

* Median of range provided by the supplier

Hb A_1c levels based on the total area of the chromatogram were found to be very precise and neutralized the impact of any variations in the injected volumes. This strategy has been used extensively in the determination of Hb A_1c by HPLC, as the inter-study precision is superior to those protocols which use calibration curves (Gerlo and Gorus, 1997).

Comparison with other methods

Two comparative studies were done with other methods. Tinaquant® immunoturbidimetric method and HPLC Mono S methods were used to analyze 60 blood samples for Hb A_1c. Figure 2 shows the correlation between the values obtained using these two methods, which presented a high coefficient of determination (r^2 = 0.9856), although the values obtained using HPLC Mono S were, on average, 76.92% of the values of NGSP. The systematically lower values found with the HPLC Mono S method corroborated results of earlier studies, which indicated its higher specificity (Hoelzel *et al.*, 2004). The conversion equation was expressed as: $Hb\ A_1c_{NGSP} = (Hb\ A_1c_{Mono\ S} + 0.7866)/0.8675$. This conversion equation was used to transform the HPLC Mono S values to the reference standard NGSP values. In our comparison study, a clear gain in specificity of the chromatography method over the immunoturbidity method was found. One of the samples evaluated gave markedly discordant Hb A_1c % values between both methods (4.5% on HPLC Mono S versus 6.8% for Tinaquant®), which could be attributed to a higher proportion of Hb S (37.7%), which was readily detected by HPLC. The values for this particular sample were not included in the calculations for the conversion equation.

The comparison between the test method and the Bio-Rad Variant® system showed a high correlation between the two techniques. Using the conversion equation, the coefficient of determination was 0.9806 and absolute values were very similar (Figure 4). In this comparative study, the HPLC Mono S values, converted to NGSP values, showed a mean of 99.0% (range 97-104%) of the values found using the Bio-Rad Variant® system.

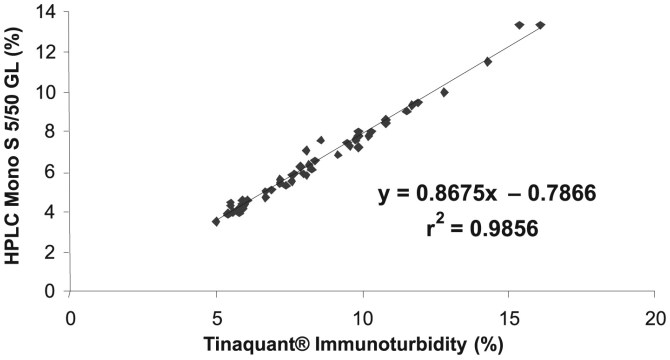
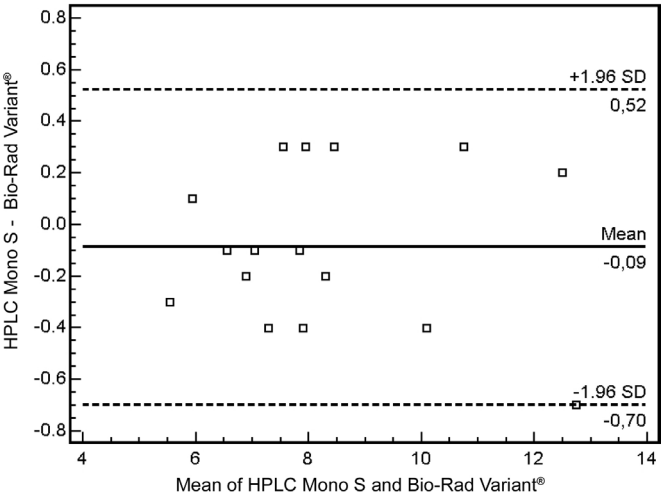


FIGURE 2 - Comparison between Hb A_1c% found by HPLC Mono S and Tinaquant® Immunoturbidity (n=60).

Using Bland and Altman graphs (Figure 3), the mean difference between the values obtained by the HPLC Mono S and the Bio-Rad Variant® was 0.09%, where all the values presented differences of between +0.30 and -0.70%, in relation to the mean. Differences were also distributed randomly around the mean, indicating the absence of systematic errors which were proportional to % Hb A_1c values.



Mean of HPLC Mono S and Bio-Rad Variant®
HPLC Mono S- Bio Rad Variant®

FIGURE 3 - Bland and Altman Graph comparing results of HPLC Mono S with Bio-Rad Variant®.

The values obtained using the HPLC Mono S and Bio-Rad Variant® methods were compared using the Passing-Bablok regression method (Figure 4). All values obtained were found to lie within the 95% confidence interval. The regression equation calculated was: $Hb\ A_1c_{Mono\ S} = Hb\ A_1c_{Bio\text{-}Rad\ Variant} - 0{,}1$. Regression analysis for the confidence intervals of the slope (0.9077 – 1.1224) and of the intercept (-1.0276 – 0.5654) show that the me-

thods do not differ by a constant value and that there are no proportional differences between the methods. The correlation between the data remains linear ($P > 0.10$).

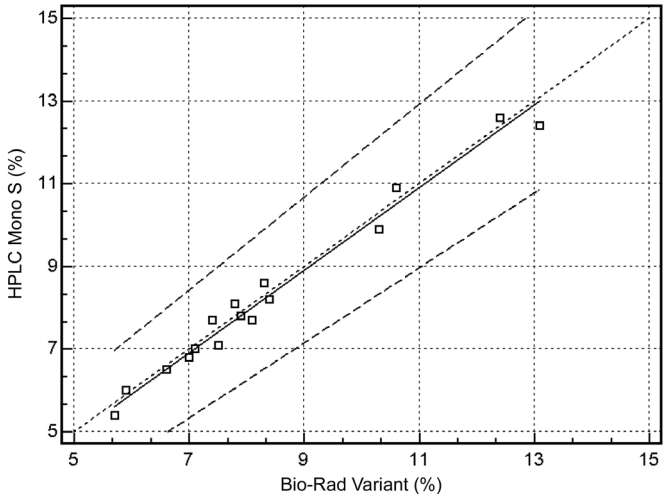


FIGURE 4 - Passing-Bablok regression, comparing results obtained by HPLC Mono S and Bio-Rad Variant® methods.

The slight differences between the results obtained using HPLC Mono S and the reference Bio-Rad Variant® method showed that the results are comparable, and thus interchangeable.

A disadvantage of the proposed protocol is the chromatographic analysis time, which is greater than that of dedicated systems for analyzing Hb A_1c. However, the use of the HPLC systems provides greater flexibility, allowing the equipment to be used for other laboratory applications. It should be noted that costs are significantly reduced using the protocol developed in this study, as all the reagents used in the analyses are prepared in-house by the laboratory at relatively low cost compared to the purchase price of specialized commercial kits.

CONCLUSIONS

A method was developed for determining Hb A_1c and hemoglobins F, S and C using a HPLC system, employing a Mono S5/50 GL cation exchange column. The method is precise and yields similar values to those of IFCC/DCCT. However, the values attained by the method are highly correlated with those of the dedicated Bio-Rad Variant® system. Besides being a low cost solution, the principal advantage of the method studied is the possibility of its inclusion in a routine laboratory setting using a high performance liquid chromatography system, where this same equipment can also be used with other techniques.

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REFERENCES

BLAND, J. M.; ALTMAN, D. G. Statistical-methods for assessing agreement between two methods of clinical measurement. *Lancet*, v.1, n.8476, p.307-310, 1986.

BRUNNEKREEFT, J. W. I.; EIDHOF, H. H. M. Improved rapid procedure for simultaneous determinations of hemoglobins A_1a, A_1b, A_1c, F, C, and S, with indication for acetylation or carbamylation by cation-exchange liquid chromatography. *Clin. Chem*., v.50, n.1, p.166-174, 2004.

DIABETES CONTROL AND COMPLICATIONS TRIAL RESEARCH GROUP. The effect of intensive treatment of diabetes on the development and progression of long term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med*., v.329, p.977-986, 1993.

ELLIS, G.; DIAMANDIS, E. P.; GLESBRECHT, E. E.; DANEMAN, D.; ALLEN, L. C. An automated "high-pressure" liquid-chromatographic assay for hemoglobin A_1c. *Clin. Chem*., v.30, n.11, p.1746-1752, 1984.

FERNANDES, A. R. C.; DOMINGOS, C. R. B. Metodologias laboratoriais para o diagnóstico de hemoglobinas variantes. *Rev. Bras. Hematol. Hemot.*, v.28, n.1, p.65-70, 2006.

GERLO, E.; GORUS, F. Calibration of ion-exchange HPLC measurements of glycohemoglobin: effect on interassay precision. *Clin. Chem*., v.43, n.12, p.2353-2357, 1997.

HOELZEL, W.; WEYKAMP, C.; JEPPSSON, J. O.; MIEDEMA, K.; BARR, J. R.; GOODALL, I.; HOSHINO, T.; JOHN, W. G.; KOBOLD, U.; LITTLE, R.; MOSCA, A.; MAURI, P.; PARONI, RITA; SUSANTO, F.; TAKEI, I.; THIENPONT, L.; UMEMOTO, M.; WIEDMEYER, H. M. IFCC reference system for measurement of hemoglobin A_1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison Study. *Clin. Chem*., v.39, n.12, p.2514-2518, 1993.

JEPPSSON, J. O.; JERNTORP, P.; SUNDKVIST, G.; ENGLUND, H.; NYLUND, V. Measurement of hemoglobin A_1c by a new liquid-chromatographic assay: methodology, clinical utility, and relation to glucose tolerance evaluated. *Clin. Chem*., v.32, n.10, p.1867-1872, 1986.

JEPPSSON, J. O.; KOBOLD, U.; BARR, J.; FINKE, A.; HOELZEL, W.; HOSHINO, T.; MIEDEMA, K.; MOSCA, A.; MAURI, P.; PARONI, R.; THIENPONT, L.; UMEMOTO, M.; WEYKAMP, C. Approved IFCC reference method for the measurement of Hb A_1c in Human blood. *Clin. Chem. Lab. Med*., v.40, p.78-89, 2002.

KOSKINEN, L. K. Specificity of hemoglobin A_1c measurement by cation exchange liquid chromatography: evaluation of a Mono S column method. *Clin. Chim. Acta*, v.253, p.159-169, 1996.

MIEDEMA, K. Towards worldwide standardization of Hb A_1c determination. *Diabetologia*, v.47, p.1143-1148, 2004.

PASSING, H.; BABLOK, W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. *J. Clin. Chem. Clin. Biochem*., v.21, p.709-720, 1983.

SACKS, D. B.; BRUNS, D. E.; GOLDSTEIN, D. E.; MACLAREN, N. K.; MCDONALD, J. M.; PARROTT, M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. *Clin. Chem*., v.48, p.436-472, 2002.

SALEH, A. K.; MOUSSA, M. A. A. An Automated Liquid-Chromatographic System for Convenient Determination of Glycated Hemoglobin A_1c. *Clin. Chem.*, v.31, n.11, p.1872-1876, 1985.

SHAH, V. P.; MIDHA, K. K.; FINDLAY, J. W. A.; HILL, H. M.; HULSE J. D.; MCGILVERAY, I. J.; MCKAY, G.; MILLER, K. J.; PATNAIK, R. N.; POWELL, M. L.; TONELLI, A.; VISWANATHAN, C. T.; YACOBI A. Bioanalytical method validation: a revisit with a decade of progress. *Pharm. Res.,* v.17, n.12, p.1551-1557, 2000.

TRIVELLI, L. A.; RANNEY, H. M.; LAI, H. T. Hemoglobin components in patients with diabetes mellitus. *N. Engl. J. Med*., v.284, p.353-357, 1971.

TURPEINEN, U.; KARJALAINEN, U.; STENMAN, U.-H. Three assays for glycohemoglobin compared. *Clin. Chem*., v.41, n.2, p.191-195, 1995.

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