Hemoglobin A_{1c} measurement with Mono S method

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Abstract

Mono S 5/50 GL (Tricorn™) columns are suitable for the measurement of HbA_{1c} and the calibration of healthcare instruments that analyze HbA_{1c}. The Mono S method is the Swedish designated method since it is effective in separating components that do not represent HbA_{1c}. With a greater resolution than both the Japanese and US designated methods, Mono S gives results that are closer to the HbA_{1c} standard set by the International Federation for Clinical Chemistry.

The performance of the Tricorn column, Mono S 5/50 GL, was compared with the older design Mono S HR 5/5 column for the analysis of HbA_{1c}. No differences were found between the results from the old and new columns. The Tricorn columns exhibited a high degree of precision in tests both within and between assays.

Diabetes and HbA_{1c}

Measurement of Hemoglobin-A_{1c} (HbA_{1c}) in diabetic patients is the most established procedure used for evaluating the long-term control of diabetes (1, 2). Hemoglobin-A_{1c} reflects the average blood glucose concentration for the past three months, which is the approximate life span of red blood cells. Hemoglobin, like many other proteins in blood, links up with sugars, such as glucose. In healthy individuals the HbA_{1c} peak represents 4%-5% of the total hemoglobin compared to 10%-15% in untreated diabetics. The goal of successful treatment is to keep the level of HbA_{1c} as close to the non-diabetic conditions as possible depending on the situation for the individual patient.

HbA_{1c} is a stable product where glucose is attached to the amino acid valine at the N-terminal of the β-chains of the molecule. Other possible glycation sites are the N-terminals of the α-chains as well as some lysine residues that can be glycated to some extent depending of the blood glucose level. The glycation of the N-terminals changes the charge of the amino groups, which results in a clear separation with the Mono S method of the glycated (HbA_{1c}) and the non-N-terminal glycated form (HbA0). The hemoglobin molecule dissociates into α-β-dimers at pH 5.7. Thus the major content of the HbA_{1c} peak contains glycated-β-chain-non-glycated-α-chain dimer and a minor amount of non-glycated-β-glycated-α-chain dimer. A small amount, 0.3%, of carbamylated β-chains is also present during these separation conditions (Fig 1).

Column:	Mono S 5/50 GL
Sample:	hemolyzed EDTA blood
Sample volume:	10 µl
Buffer A:	20 mM sodium malonate, 0.2 g/l sodium azide, pH 5.7
Buffer B:	buffer A + 0.3 M LiCl
Gradient:	20%-50% B for 3 min, 50%-75% for 1.1 min, 75%-100% for 0.3 min, 100% for 2.6 min, 20% for 1 min.
Flowrate:	2 ml/min
System:	HPLC system
Detection:	415 nm

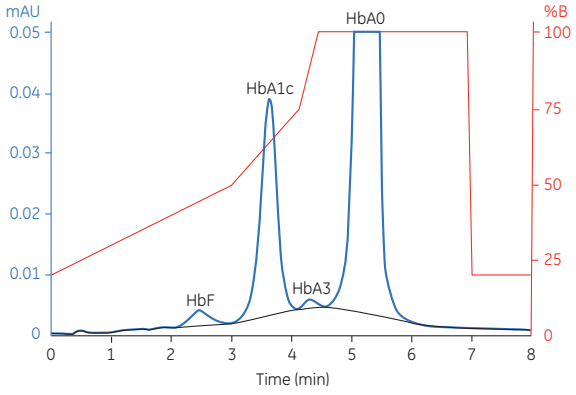

Fig 1. Mono S ion exchange chromatography of HbA1c. HbA3 represents the Hb-glutathione adduct and the HbF peak also contains some minor hemoglobins.

Mono S and calibration

Regular calibration of instruments used to measure HbA_{1c} is necessary due to the wide variety of instruments and techniques used in healthcare services. Since national standardization programs are based on arbitrarily chosen methods, there are problems in comparing results regionally and internationally. Therefore a methodologically sound, international reference measurement system has been developed by the International Federation for Clinical Chemistry (IFCC) for worldwide calibration (3).

Figure 2 shows how well three nationally designated comparison methods compare with the 'true' proportion of HbA_{1c} as defined by the IFCC. Many countries and instrument manufacturers calibrate their techniques with a method from the United States National Glycoprotein Standardization Program (NGSP [4]). The NGSP method overestimates the proportion of HbA_{1c} in blood samples since the chromatography used identifies a proportion of components that do not represent HbA_{1c}. The method recommended by the Japanese Diabetes Society (JDS), is more specific and lies closer to the IFCC standard (Fig 2). The Swedish designated comparison method is based on cation exchange chromatography and uses Mono S. This method has the highest resolution for the HbA_{1c} top and therefore lies closest to the standard set by the IFCC.

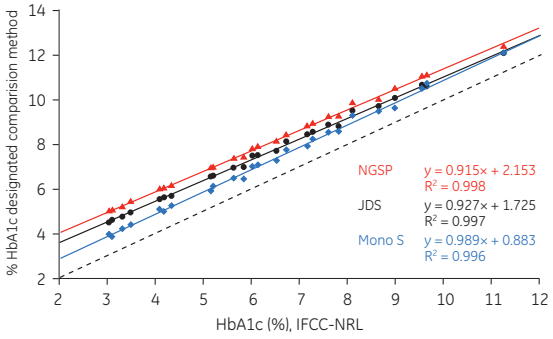

Fig 2. A comparison of HbA_{1c} measured with nationally designated methods and HbA_{1c} as defined by the IFCC method (5).

From HR to Tricorn

For almost two decades the Mono S method was conducted with Mono S HR 5/5 columns from GE Healthcare (6, 7). These columns were replaced with Mono S 5/50 GL in the Tricorn column design.

Tricorn columns incorporate features designed to meet the demands of today's laboratories, while retaining the standards set by HR columns. The principle modifications include Valco™ fittings for simple connection to ÄKTA™ design systems. A clear coating on the glass column protects users and the column without impairing visibility of the media bed. Tricorn columns have a smaller dead volume than HR columns and no fixed capillary tubing that allows optimization of capillary tube length and resolution. Finally, Tricorn columns have fewer components than HR columns, making them robust and simple to operate and maintain. The analysis of HbA_{1c} on Tricorn columns has been compared with the old Mono S HR 5/5 at the Department of Clinical Chemistry, Malmo University Hospital, Sweden.

Materials and methods

Preparation of samples

Blood was collected in EDTA-containing tubes. 14 µl of the mixed whole blood was diluted and hemolysed with 700 µl of a buffer containing Citric acid, 0.02 mol/L, $Na_2HPO_4(2H_2O)$, 0.055 mol/l and Triton™ X-100, 0.1% w/v, pH 5.4. After incubating the hemolysed cells at 37°C for 30 min to eliminate pre-HbA_{1c} the tubes were then centrifuged at 3000 × g for 10 min. 10 µl of the supernatant was injected into the column.

Columns and procedures

Analysis of HbA_{1c} was conducted on HPLC with following columns, Mono S 5/50 GL (Media lot nr. 286415) and Mono S HR 5/5 (Media lot nr. 288385). HbA_{1c} was separated from other hemoglobin forms by a LiCl-gradient. Buffer A contained 20 mmol/l of sodium malonate and 0.2 g/l of sodium azide, pH 5.7. Buffer B was buffer A plus 0.3 mol/l of lithium chloride. Before use, the buffers were degassed and filtered through a 0.45 µm pore filter. Flow rate was 2 ml/min. 10 µl was injected on the column.

Column equilibration

To equilibrate the columns for first time use before HbA_{1c} analysis a number of injections are necessary before the chromatography yields acceptable results. Forty samples were loaded and injected overnight to dispose of tailing caused by the HbA_0 fraction.

Results and Discussion

To determine whether Mono S 5/50 GL (Tricorn) columns were comparable with Mono S HR 5/5, 30 blood samples from diabetic patients and non-diabetics were assayed for HbA_{1c} (Fig 3). Assays with the Tricorn column had a near-perfect correlation to assays with the Mono S HR 5/5 column ($R^2 = 0.999$).

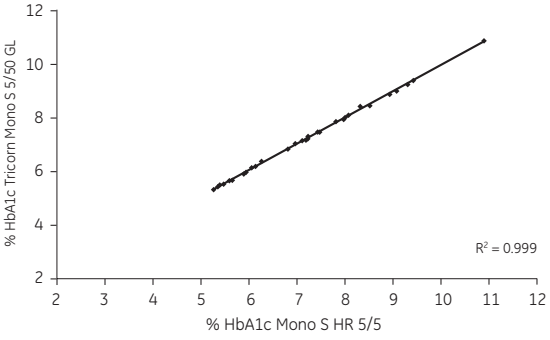

Fig 3. Comparison of HbA1c separated with Tricorn and HR columns.

The within-assay precision was examined with a Tricorn column. This was done by repeatedly assaying HbA_{1c} in two series of 21 samples from a non-diabetic and diabetic patient. The results revealed high reproducibility as indicated by the low coefficient of variance (CV) for assays (Table 1).

Table 1. Precision within assay

Within Assay	n	Mean	SD	CV (%)
Non-diabetic	21	5.0	0.02	0.40
Diabetic	21	8.9	0.02	0.27

Between-assay precision with a Tricorn column was assessed by repeatedly assaying samples from three test groups with low, medium, and high HbA_{1c} content over a five-day period. The results all had CV values that were < 1.5% (Table 2), indicating a good analytical procedure on a well-maintained instrument. The higher CV values found in the between-assay test (Table 2) when compared to the within assay test (Table 1) were probably due to changing reagents between the assay runs.

Table 2. Precision between assays

Between Assay	n	Mean	SD	CV (%)
Low	15	5.0	0.08	1.50
Medium	25	7.0	0.08	1.14
High	15	8.9	0.09	1.05

Conclusion and discussion

The Mono S method is a reliable and well-tested method for the measurement and calibration of HbA_{1c} instruments. The method is currently used by network of accredited laboratories that are responsible for calibration of all Swedish hospital and point of care equipment independent of manufacturers (8).

A comparison of Tricorn Mono S 5/50 GL with Mono S HR 5/5 columns using the Mono-S method showed no noticeable effect on the quality of HbA_{1c} results as indicated by the high degree of correlation. The results confirm Tricorn columns have the same high chromatographic performance, stability and reproducibility as HR columns.

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Ordering information

Article	Code No.
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