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Modified HPLC-Electrospray Ionization/ Mass Spectrometry Method for HbA_{1c} Based on IFCC Reference Measurement Procedure

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BACKGROUND: Monitoring of hemoglobin A_{1c} (HbA_{1c}) is important in the management of diabetes. The IFCC reference measurement procedure for HbA_{1c} is based on the ratio of glycated to nonglycated N-terminal hexapeptides of the β-chains of hemoglobin after digestion with Glu-C endoproteinase. We developed a modification of the original reference measurement procedure with HPLC-electrospray ionization/mass spectrometry (ESI/MS).

METHOD: We performed chromatographic separation of the hexapeptides using a C12 reversed-phase column and a binary gradient system consisting of a mixture of H_2O/acetonitrile/formic acid.

RESULTS: Using this method, we obtained higher signal intensities and improved system stability compared with the reference measurement procedure. In the range of 3% to 14% HbA_{1c}, intralaboratory CVs were 0.71% to 1.86%. Deviations from IFCC target values were −0.87 to 1.00 relative %. These values fulfill acceptability criteria for HbA_{1c} determination set by the IFCC Working Group on HbA_{1c} Standardization.

CONCLUSIONS: This procedure for the determination of HbA_{1c} improves the existing reference measurement procedure.

Hemoglobin A_{1c} is an important parameter for the assessment of diabetes management. Standardized determination of HbA_{1c}[3] is the basis for clinical studies and evaluation of the performance of diabetes centers *(1, 2)*. The IFCC reference measurement procedure for determination of HbA_{1c} *(3, 4)* is used for calibration of kits and instruments and ensures traceability and comparability of the analytical results. In a recent study, Kaiser et al. *(5)* discussed analytical problems concerning the current IFCC reference measurement procedure for determination of HbA_{1c} by HPLC-electrospray ionization/mass spectrometry (ESI/MS). One of the main difficulties was a lack of robustness due to insufficient reproducibility of separation characteristics between different batches of cyanopropyl stationary-phase columns. The method was modified by increasing the trifluoroacetic acid (TFA) concentration in the elution buffer and by changing the gradient elution profile *(5)*. The improved robustness of the method for HbA_{1c} determination needs special attention, since the HbA_{1c} target values for IFCC calibrators and controls are set by a network of international reference measurement laboratories that use different equipment. We present an improvement of the reference measurement procedure by changing the column type and elution conditions, increasing system stability and reducing the risk of ion suppression in the HPLC-ESI/MS measurements.

Materials and Methods

MATERIALS

We purchased trifluoroacetic acid (25% solution in water) and acetonitrile (LiChrosolv) from Merck, formic acid (98%, puriss. p.a.) from Sigma-Aldrich, endoproteinase Glu-C (sequencing grade, EC 3.4.21.19) from Roche Diagnostics, and the Jupiter™ Proteo column (C12 reversed phase column, 2.0 by 50 mm, 4 μm) from Phenomenex. Water was prepared using the purification system Direct-Q™ 5 (Millipore GmbH). We obtained calibration material and secondary reference material for accuracy control from the IFCC Working Group on HbA_{1c} Standardization and lyophilized whole blood samples from Recipe.

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[3] Nonstandard abbreviations: HbA, hemoglobin A; ESI/MS, electrospray ionization/mass spectrometry; TFA, trifluoroacetic acid.

SAMPLE PREPARATION

We prepared samples according to the IFCC reference measurement procedure for HbA_{1c} *(3)*. Briefly, an aliquot of the sample containing approximately 1 mg hemoglobin was digested as follows. We added 50 μL Glu-C enzyme solution (200 mg/L) and brought the mixture to a final volume of 500 μL with digestion buffer (50 mmol/L ammonium acetate buffer, pH 4.3). The mixture was incubated for 18 h at 37 °C. Glycated and nonglycated β-N-terminal hexapeptides were generated during the enzymatic cleavage of the β-chains of hemoglobin. Proteolysis was stopped by freezing the samples at −20 °C for 2 h. The samples were thawed, centrifuged at 8000*g* for 2 min, and injected in the HPLC-ESI/MS system. We calculated the peak area ratios for the β-N-terminal hexapeptides for HbA_{1c} and HbA_0, performed external standard calibration by HPLC-ESI/MS measurements of the IFCC calibrators, and constructed calibration curves by linear regression.

HPLC-ESI/MS

APPARATUS

The HPLC system (Shimadzu) consisted of a SCL-10A system controller, 3 LC-10ADvp pumps (A, B, C), a DGU-14A degasser, a SIL-10AD autoinjector, a CTU-10AS column oven, and a FCU-12A flow switch (rotary valve). The mass spectrometer was an API 4000 equipped with a TurboV™ ESI source with TurboIon Spray™ probe (Applied Biosystems, MDS-Sciex).

HPLC-ESI/MS CONDITIONS

Elution was performed on the C12 reversed-phase Jupiter Proteo column with a binary gradient consisting of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile) at a flow rate of 300 μL/min, column temperature of 50 °C, and injection volume of 1 μL. The elution profile is given in Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue6. A postcolumn flow splitting system (1:6 split ratio) was installed, and to avoid contamination of the ESI source, a switching valve system (rotary valve A) was introduced. At position 0 of rotary valve A the eluate passed into the ESI source. At position 1 the eluate was discarded, during which the ESI source was supplied with 50% acetonitrile (eluent C) delivered by pump C (auxiliary pump) at a flow rate of 300 μL/min.

For the ESI/MS measurements, we used the settings shown in Supplemental Table 2 in the online Data Supplement. The doubly protonated β-N-terminal hexapeptides were monitored at *m*/*z* 348.3 for HbA_0 and *m*/*z* 429.3 for HbA_{1c}. Dwell time was 1 s, and settings of the needle position were horizontal axis 5 mm and vertical axis 10 mm.

LINEARITY

For calibration, we used calibrators containing mixtures of chromatographically purified HbA_{1c} and HbA_0 obtained from the IFCC Working Group on HbA_{1c} Standardization. After digestion, we analyzed the calibrators by HPLC-ESI/MS and plotted the peak area ratios of the β-N-terminal hexapeptides against the ratios of HbA_{1c} and HbA_0 concentrations.

IMPRECISION AND INACCURACY

For quality control, we used hemolysate samples with target values assigned by the IFCC Working Group on Standardization of HbA_{1c}. For lyophilized whole blood samples, we determined the target values with the original IFCC reference measurement procedure. Following the IFCC regulations for HbA_{1c} determination, 2 digestions were performed and 2 sequences were measured from each. The 4 values obtained were averaged and the results presented as statistical means.

Results

CYANOPROPYL VS C12 COLUMNS

In the IFCC reference measurement procedure for determination of HbA_{1c}, the HPLC-ESI/MS analysis makes use of a cyanopropyl column and a TFA-containing elution buffer. As pointed out in previous work *(5)*, this analytical system shows peak tailing, lack of reproducibility of retention times, and different peak shapes depending on column batch. A typical chromatographic elution profile following the IFCC conditions is given in Fig. 1A. An improvement of the peak shapes of the HbA_0- and HbA_{1c}-derived β-N-terminal hexapeptides and the separation performance is achieved by introduction of a C12 reversed-phase Jupiter Proteo column (Fig. 1B).

REPLACEMENT OF TFA BY FORMIC ACID

It is known that TFA may have adverse effects in liquid chromatography–ESI/MS *(6–8)*, so we examined replacement of TFA by formic acid in the elution buffer (Fig. 2). At 0.1% formic acid, peak sharpness is enhanced and signal intensities highly improved, allowing reduction of the injection volume from 3 to 1 μL. Approximately 15-fold higher absolute signal intensities are obtained with formic acid compared with TFA.

LINEARITY AND CHROMATOGRAPHIC REPRODUCIBILITY

As a measure for system stability, we determined the peak area ratios and the retention times of the β-N-terminal hexapeptides for HbA_{1c} and HbA_0 in a digested hemolysate by repeated measurements. The C12

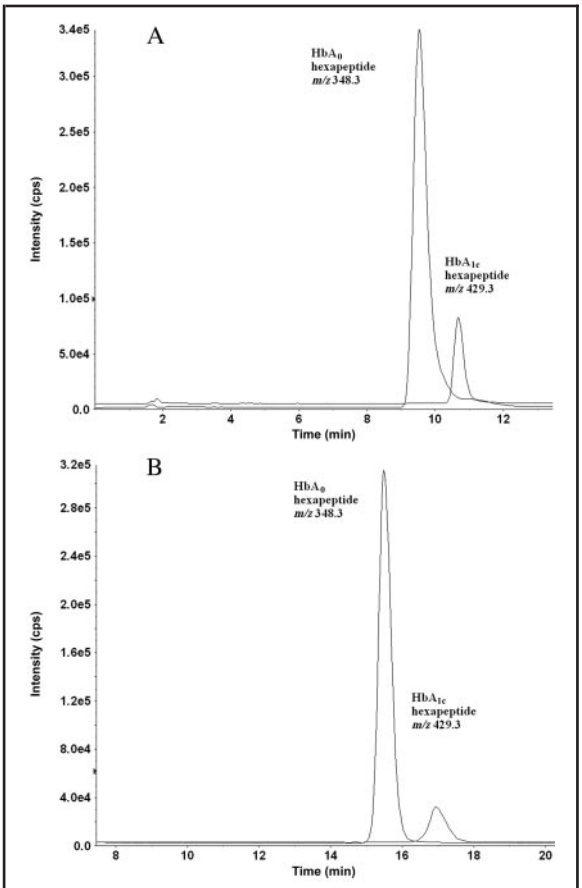

Fig. 1. HPLC-ESI/MS chromatograms of a digested mixture of HbA_{1c} and HbA_0 using a cyanopropyl column (A) and a C12-reversed phase column (B).

Elution of 3 μL IFCC calibrator F (14% HbA_{1c}) performed with eluent A (0.025% TFA in water) and eluent B (0.023% TFA in acetonitrile) as follows: 0 min, 0% B; 3 min, 0% B; 9 min, 5% B; 20 min, 5% B. Flow rates were 300 μL/min (A) and 350 μL/min (B). Overlaid chromatograms of the doubly charged β-N-terminal hexapeptides of HbA_0 (*m/z* 348.3) and HbA_{1c} (*m/z* 429.3) are presented. cps, counts per second.

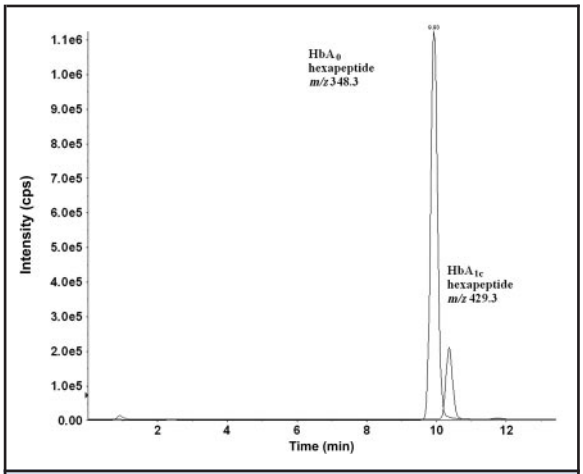

Fig. 2. HPLC-ESI/MS chromatogram of a digested mixture of HbA_{1c} and HbA_0 using a C12 reversed-phase column and formic acid in the elution buffer.

Elution of 1 μL IFCC calibrator F (14% HbA_{1c}) with eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile): 0 min, 0% B; 3 min, 0% B; 9 min, 6% B; 16.5 min, 6% B at a flow rate of 300 μL/min. Overlaid chromatograms of the doubly charged β-N-terminal hexapeptides of HbA_0 (*m/z* 348.3) and HbA_{1c} (*m/z* 429.3) are presented. cps, counts per second.

reversed-phase column showed a highly reproducible elution profile for the 2 peptides. For the peak area ratio, we observed a within-run CV of 1.34% (n = 48) under the selected conditions. The within-run CV for retention time was 0.17% (n = 41).

Fig. 3 shows that linear calibration curves for the IFCC calibrators are obtained in the concentration range between 3% and 14% HbA_{1c}.

According to the IFCC protocol for HbA_{1c} measurements *(3)*, the analysis of samples has to be performed in a defined sequence such that the calibrators are arranged before and after controls and the samples to be determined. This sequence requires long-term stability of the analytical system. Fig. 3 shows the congruence of the calibration curves at the beginning and the end of 1 measuring sequence, demonstrating system stability.

IMPRECISION AND INACCURACY

The values of the IFCC quality control samples are presented in Table 1. From these data, we calculated between-run CV values of between 0.71% and 1.86% and deviations from the IFCC target values of between −0.87 and 1.00 relative %.

For lyophilized whole blood samples, the deviations from the target values were between −1.45 and 1.41 relative %, with between-run CV values between 1.08% and 1.90% (Table 2).

Discussion

The method presented in this study proved to be a stable analytical procedure for the determination of HbA_{1c} in human blood. Improvement of the reference measurement procedure promotes comparability of results in standardization of HbA_{1c} measurements.

An important advance is the substantial improvement of chromatographic performance of the ana-

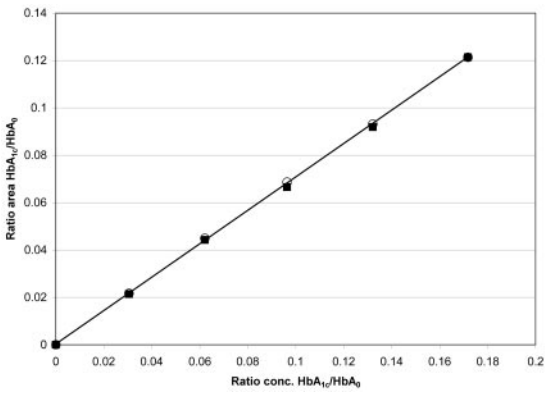

Fig. 3. Calibration curves of 6 digested IFCC calibrators, measured at the beginning (■) and the end (○) of one measuring sequence.

The target values set by the IFCC Working Group on HbA_{1c} Standardization of the IFCC calibrators (expressed as ratio concentration of HbA_{1c} vs HbA_0) are plotted against the peak area ratios of the glycated and nonglycated β-N-terminal hexapeptides of HbA_{1c} and HbA_0. The regression equation for calibration curve 1 (■) is: $y = 0.705x - 0.0004$ ($r^2 = 1.00$) and for calibration curve 2 (○): $y = 0.702x - 0.0001$ ($r^2 = 1.00$).

lytical system. Considerable peak tailing for the nonglycated hexapeptide has been observed with some batches of the cyanopropyl columns *(5)*, which makes adequate quantification difficult. In contrast to the cyanopropyl stationary phase originally proposed by the IFCC Working Group on Standardization of HbA_{1c} *(3)*, a symmetric peak shape, without significant tailing, is achieved with the C12 reversed-phase column used here. The use of formic acid as an organic acid modifier also contributes to the sharp and symmetric peaks with high signal intensities and stable retention times (Fig. 2). When 4 batches of columns were tested,

Table 1. Imprecision and inaccuracy of measurements ($n = 4$) of IFCC quality control samples.

Target % HbA_{1c}	Mean % HbA_{1c}	CV, %	Bias, %[a]
3.21	3.19	1.86	−0.78
3.24	3.27	0.84	1.00
8.48	8.47	0.71	−0.09
8.58	8.51	1.41	−0.87

[a] Relative % deviation from target value.

Table 2. Imprecision and inaccuracy of measurements ($n = 4$) of lyophilized whole blood samples.

Target % HbA_{1c}	Mean % HbA_{1c}	CV, %	Bias, %[a]
3.28	3.23	1.08	−1.45
5.43	5.51	1.34	1.41
7.87	7.76	1.90	−1.40

[a] Relative % deviation from target value.

no problems with batch-to-batch reproducibility were observed.

The use of a C18 guard column instead of an analytical column for HbA_{1c} analysis has been described *(9)*. We prefer to take advantage of a highly reproducible packed analytical column specially produced for peptide analysis. The C12 reversed-phase column shows a well-suited hydrophobic/hydrophilic inter-action between analyte and bonded phase. A higher degree of endcapping of the free silanol groups of the C12 reversed-phase column, which shields the analytes from nonspecific interactions, leads to higher peak symmetry.

In HPLC analysis of peptides, trifluoroacetic acid is used for better chromatographic performance owing to its ion-pairing properties. It is well known, however, that the use of TFA in HPLC-ESI/MS is prone to problems with ion suppression *(6)*. Signal suppression has been explained by the combined effect of unstable spray generation due to high conductivity and surface tension *(7)* and ion pair formation between analyte and the TFA anion *(8)*. Other studies reported that higher signal intensities were obtained when TFA was replaced by organic acid modifiers such as acetic acid *(7, 8)*, possibly owing to changes in the charge state of the peptide analytes *(10)*. The replacement of TFA by formic acid in the new method led to signal intensities increased by a factor of about 15.

A further aspect of spray stability is dependence on the concentration of organic solvents in the elution buffer due to changes in conductivity and surface tension *(11)*. To reach stable electrospray ionization, it is therefore important that the analytes elute in an isocratic phase within the gradient elution profile *(5)*. This is realized under the conditions used in our assay.

Care should be taken to avoid the possible occurrence of in-source fragmentation during electrospray ionization. Cleavage of the glycation site underestimates values for the glycated hexapeptide; this phenomenon would be visible as an additional signal on the mass trace of the nonglycated hexapeptide

and was verified by measurements of synthetic glycated hexapeptides *(5)*. Under our conditions, such in-source fragmentation was not observed. The retention times for the β-N-terminal hexapeptides are very reproducible, as shown by the low within-run CV values of 0.17% for both analytes. With the original IFCC reference measurement procedure, much higher CV values for retention times have been observed.

The IFCC reference measurement procedure for HbA_{1c} employs a measuring period of about 24 h per sequence, and quantification of HbA_{1c} is performed by the use of external standards. Stable peak area ratios over the whole measuring sequence are a prerequisite for high accuracy and precision. A within-run CV value of 1.34% (n = 48) for peak area ratios calculated from repeated injections of a digested hemolysate demonstrates adequate system stability during this period of time.

Acceptability criteria given by the IFCC Working Group on Standardization of HbA_{1c} are intralaboratory CV <3% and maximal deviation from the target value <2 relative % *(3)*. In our method, the imprecision varied from 0.71% to 1.86% and the bias related to the IFCC target value from −0.87 to 1.00 relative %.

Reference measurement procedures and their modifications must be confirmed by comparison studies. The transferability of the modified method to other laboratories using mass spectrometry is very important and needs to be evaluated. Laboratories may be using different instrumentation, for which these modifications may or may not be optimal.

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