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Liquid Chromatography–Isotope Dilution–Mass Spectrometry as a New Basis for the Reference Measurement Procedure for Hemoglobin A_{1c} Determination

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BACKGROUND: Standardization of hemoglobin (Hb)A_{1c} measurements is a process of considerable interest for quality assurance in diabetes management. To contribute to continuous quality improvement and fulfillment of the requirements for reference measurement procedures according to the standards of the International Organization for Standardization, we developed a calibration system of highest metrological order using isotope dilution–mass spectrometry with a reference material.

METHOD: Samples were prepared by enzymatic cleavage based on the IFCC reference measurement procedure for LC-MS analysis. After digestion the samples were spiked with [D7]-labeled glycated and nonglycated hexapeptides as internal standards for quantification. LC-MS analysis was performed by using a C12 reversed-phase column and a gradient of acetonitrile/H_2O containing 0.1% formic acid.

RESULTS: Calibration systems for HbA_{1c} determination based on liquid chromatography–isotope dilution–mass spectrometry (LC-ID-MS) and on the IFCC reference measurement procedure were compared. A linear regression analysis demonstrated a correlation of $r^2 = 1.00$ between the 2 different calibration systems. Mean deviation was 5.5% for the calibration and 3.3% for hemolysate samples, with a mean expanded uncertainty of 4.9%.

CONCLUSIONS: This LC-ID-MS procedure allows the current IFCC reference measurement procedure for HbA_{1c} to be raised to a higher order of accuracy.

Reference measurement procedures have the highest accuracy and lowest imprecision possible and allow the determination of target values for calibrators. The traceability of reference measurement procedures to SI units is specifically required and must be guaranteed *(1, 2)*.

In the past, the calibration of hemoglobin A_{1c} (HbA_{1c})[4] analyses was a special problem in clinical studies, because of the poor comparability of different analytical procedures. Reference material was not available because a reference measurement procedure for HbA_{1c} had not been established. Therefore a consensus standard based on an HPLC peak had been created in the Diabetes Control and Complications Trial study to obtain comparability of the analytical results. After the Diabetes Control and Complications Trial study, the calibration of HbA_{1c} systems was continued with the National Glycohemoglobin Standardization Program (NGSP) procedure, which had neither the required accuracy nor the traceability to SI units.

Later the IFCC reference measurement procedure for HbA_{1c} was established. Calibration was performed by mixing pure HbA_{1c} and HbA_0 preparations. This procedure for HbA_{1c} was developed by an IFCC Working Group on the basis of this calibration *(3)*. The original IFCC reference measurement procedure was improved by Kaiser et al. *(4, 5)*, but the calibration remained the critical point.

Our aim was to reestimate the existing calibration of the IFCC reference measurement procedure by introducing a new calibration system traceable to SI units. This goal was realized by the use of synthetic N-terminal hexapeptides of the β-chain as reference materials.

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[4] Nonstandard abbreviations: HbA_{1c}, hemoglobin A_{1c}; NGSP, National Glycohemoglobin Standardization Program; ID-MS, isotope dilution–mass spectrometry; LC, liquid chromatography; ESI, electrospray ionization.

Materials and Methods

MATERIALS

Acetonitrile (LiChrosolv) was purchased from Merck. Formic acid (98% purity) was obtained from Sigma-Aldrich. Endoproteinase Glu-C (sequencing grade, EC 3.4.21.19) was from Roche Diagnostics. The Jupiter™ Proteo column (C12 reversed-phase column, 2.0 × 150 mm, 4 μm) was purchased from Phenomenex. Water was prepared by using the purification system Direct-Q™ 5 (Millipore). Calibrators and hemolysate samples for accuracy control were obtained from the IFCC Working Group on HbA_{1c} Standardization.

REFERENCE MATERIALS

The VHLTPE and 1-deoxyfructosyl-VHLTPE hexapeptides used for calibration of the isotope dilution–mass spectrometry (ID-MS) procedure were purchased from ORPEGEN Pharma. The deuterated hexapeptides used as internal standards ([D7]-VHLTPE and [D7]-1-deoxyfructosyl-VHLTPE) were labeled at the isopropyl group of leucine. The calibration stock solutions were prepared by dissolving 6 mg of calibration material in 1 mL digestion buffer (50 mmol/L ammonium acetate buffer, pH 4.3). Aliquots were stored at −20 °C.

One aliquot of each unlabeled hexapeptide was used for peptide quantification by the Physikalisch Technische Bundesanstalt (Braunschweig, Germany). This procedure was done by hydrolysis of the peptides in 6 mol/L HCl at 120 °C for 65 h and subsequent quantification of the amino acids leucine, proline, and threonine with liquid chromatography (LC)-ID-MS. The amino acids were quantified on an Agilent 1100 LC/MSD by using isotope-labeled amino acids as internal standards (double ID-MS) as previously described *(6)*.

ID-MS PROCEDURE

Samples were prepared according to the IFCC reference measurement procedure for HbA_{1c} *(3)*. For measurement and quantification of the samples the calibration stock solutions were diluted in the digestion buffer and spiked to the samples after digestion, resulting in a deuterated/nondeuterated isotope ratio for the glycated and nonglycated hexapeptides of 1:1; 2 μL of the samples were injected into the LC-MS system.

LC-MS INSTRUMENTS

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

LC-MS SETTINGS

Separation was performed on a 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) *(4)*. The flow rate was 300 μL/min. The column temperature was 50 °C. The elution profile is provided in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue5. The injection volume was 2 μL. To avoid contamination of the ESI source we introduced a switching valve system (rotary valve A). 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.

The settings shown in online Supplemental Table 2 were used for the ESI-MS measurements. The doubly protonated β-N-terminal unlabeled hexapeptides were monitored at *m/z* 348.3 (nonglycated) and *m/z* 429.3 (glycated) and the deuterium labeled forms at *m/z* 351.8 (nonglycated) and *m/z* 432.8 (glycated). Dwell time was 1 s. Settings of the needle position were horizontal axis 5 mm and vertical axis 10 mm.

VALIDATION OF THE LC-ID-MS PROCEDURE

For accuracy control, calibrators and hemolysate samples with target values assigned by the IFCC Working Group on Standardization of HbA_{1c} were used. In accordance with the IFCC protocol for HbA_{1c} determination for each sample, 2 digestions were performed and 2 series were measured from each. The 4 values obtained were averaged and the results presented as statistical means.

Results

For comparison of the 2 different calibration systems, the IFCC calibrators and IFCC control samples were analyzed by the internal standard calibration procedure by using the isotope-labeled standards and by the external standard calibration procedure as described in the IFCC reference measurement procedure *(3)*.

ID-MS was performed by using reference materials with defined uncertainty of measurement. For the quantification of HbA_{1c}, expressed as $HbA_{1c}/(HbA_{1c} + HbA_0)$ (in mmol/mol), the measurement of 2 different analytes was required, namely the glycated and nonglycated VHLTPE hexapeptides. Each hexapeptide was spiked with its corresponding deuterium-labeled hexapeptide internal standard, and the exact concentration of both hexapeptides was calculated following the ID-MS principle. From these values the HbA_{1c} ra-

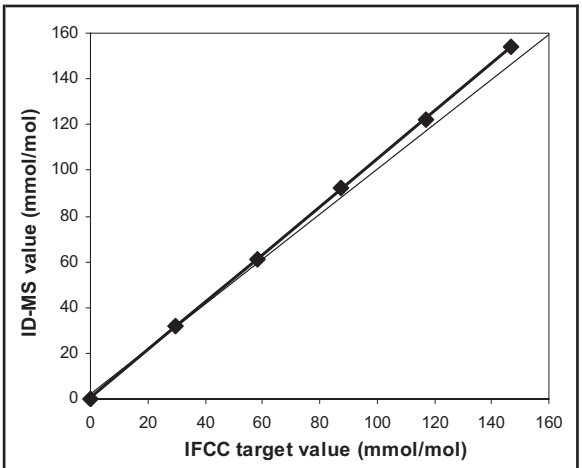

Fig. 1. Correlation of HbA_{1c} target values of IFCC calibrators with ID-MS values.

The HbA_{1c} target values of the IFCC calibrators set by the IFCC Working Group on HbA_{1c} Standardization plotted against their values determined by ID-MS. The regression equation is: $y = 1.044x - 0.482$ ($r^2 = 1.00$).

tios were calculated. After this procedure matrix effects and other systematic errors were minimized *(7)*.

The comparative measurements of the existing calibrators and hemolysate samples with the LC-ID-MS method produced the results shown in Fig. 1 and Tables 1 and 2.

The HbA_{1c} values of the IFCC calibrators ranged from 0 to 147.0 mmol/mol, corresponding to 0% and 15.6% (NGSP units) according to the master equation: NGSP [%] = 0.0915 × IFCC [mmol/mol] + 2.15.

The values obtained for the IFCC calibrators by the proposed LC-ID-MS were plotted against their target values assigned by the IFCC network for HbA_{1c} (Fig 1). A linear regression analysis demonstrated a correlation of $r^2 = 1.00$ between the 2 different calibration systems. The values obtained by LC-ID-MS were higher than the target values, both for the calibrators and hemolysate samples. The mean relative difference between the calibrators and the assigned IFCC target values was 5.5% (4.3%–7.7%), with a mean relative expanded uncertainty of U = 4.8% (Table 1). The absolute differences ranged from 2.3 to 6.8 mmol/mol (0.21%–0.62%, NGSP units).

Hemolysates in the concentration range between 32.1 and 100.2 mmol/mol (5.09%–11.3%, NGSP units) were examined. The results obtained are provided in Table 2. The mean relative difference between these samples and the assigned IFCC target values was 3.4% (1.7%–5.4%), with a mean relative expanded uncertainty of U = 4.9%, leading to absolute differences from 0.50 to 3.9 mmol/mol (0.05%–0.35%, NGSP).

Table 1. LC-ID-MS measurements (n = 4) of IFCC calibrators.

Target, mmol/mol	IDMS mean, mmol/mol	U, mmol/mol[a]	Bias,%[b]
HbA_{1c}	HbA_{1c}		
0	0		0
29.5	31.8	1.4	7.7
58.4	61.2	3.1	4.8
87.4	92.6	4.1	5.9
117.1	122.2	6.1	4.3
147.0	153.8	7.8	4.6

[a] Expanded uncertainty with k = 2.
[b] Relative percentage deviation from target value.

Table 2. LC-ID-MS measurements (n = 4) of hemolysate samples.

Target, mmol/mol	IDMS, mean mmol/mol	U, mmol/mol[a]	Bias,%[b]
HbA_{1c}	HbA_{1c}		
32.1	32.6	1.5	1.7
35.6	36.8	2.0	3.2
58.6	60.4	2.8	3.0
65.1	68.6	3.2	5.4
100.4	104.3	5.1	3.9

[a] Expanded uncertainty with k = 2.
[b] Relative percentage deviation from target value.

Discussion

Quantification in proteomics with stable isotope-labeled peptide standards is becoming increasingly important in clinical laboratories *(8–12)*. The use of reference materials in combination with isotope-labeled internal standards was the basis for the ID-LC-MS procedure presented here. This analytical procedure fulfills the requirements of reference measurement procedures "to be comparable and ultimately traceable to measurement standards of the highest metrological level" according to the International Organization for Standardization (ISO) Standards 15193 *(1)* and 15194 *(2)*.

The existing IFCC calibration has the disadvantage of using external standards. A prerequisite for an external calibration procedure is high long-term stabil-

ity of the LC-MS system *(4, 5)*. The stability of the LC-MS system can be critical for long-term measurements *(4)*, particularly HbA_{1c}, which is a ratio of 2 different analytes with different physicochemical properties. Besides the vulnerability of the current reference measurement procedure toward possible transient changes in mass spectrometer performance, loss of signal due to loss of carbohydrate or dehydration of the fructosylamine group on the glycated form of the peptide may cause analytical errors. Such errors may explain the difference between the values determined by ID-MS and the IFCC calibration method. The use of internal standards in the proposed ID-MS method corrects for these sources of error. In this way lower imprecision and improved accuracy of measurements can be achieved. A further advantage of the proposed ID-MS calibration is that the synthetic peptides are chemically clearly defined and make the calibration independent from the imponderability of protein standards prepared from whole blood.

In the existing IFCC reference measurement procedure the 2 measurands, namely the glycated and nonglycated hexapeptides, are derived from the β-chains of hemoglobin by proteolytic cleavage. During the proteolytic process the ratio of the glycated and nonglycated hexapeptides released remains constant independent of the extent of peptide cleavage *(3)*. We observed that it made no difference whether the labeled hexapeptides were added at the beginning or at the end of the proteolytic process, a finding that indicates that the hexapeptides are stable during this period of time (data not shown). This stability allows the addition of the isotope-labeled hexapeptides to the digestion mixture at the end of incubation.

As demonstrated in Fig. 1, the values obtained with the 2 different calibration systems showed a correlation of $r^2 = 1.00$, but also showed a mean relative difference of 5.5% (Table 1). The difference was 3.4% for the hemolysate samples (Table 2). Similar experiments comparing the IFCC calibration with LC-ID-MS calibration on HbA_{1c} were performed by Nakanishi et al. *(13)*. These authors observed a mean relative difference of 6.5% between their results and the existing IFCC calibration. In view of the mean expanded uncertainty of 4.8% of our values and the uncertainty of the assigned IFCC values, the calculated difference requires further evaluation.

In contrast to our method, the ID-MS procedure of Nakanishi et al. was based on material of which the purity was ascertained only by HPLC without stated uncertainty and traceability. This procedure does not fulfill the basic requirements for reference material according to the ISO Standards 15193 *(1)* and 15194 *(2)*. Nakanishi et al. prepared their standards by weighing the peptide powder, a process that may introduce inaccuracies due to contamination by water. In our work the risk of hygroscopic effects during the handling of synthetic peptides was eliminated by direct use of those aliquots of the peptide stock solutions for ID-MS calibration that had been analyzed for the peptide from its constituent amino acids. This peptide quantification was performed by LC-ID-MS, which is a method of the highest metrological order.

In general, working with peptide standards for quantification entails problems concerning batch-to-batch variability. Tracing back the calibration to amino acid analysis makes our method independent from impurities originating from the peptide preparation procedure. Amino acid analysis may suffer from imprecision due to variability in peptide hydrolysis before quantification.

Necessary precautions to avoid incomplete hydrolysis were taken, as previously discussed *(6, 12)*.

The present LC-ID-MS procedure offers an alternative to the current IFCC calibration system. The amino acid standards are well defined and are traceable to the SI units. Thus, the required "unbroken chain of comparison all having stated uncertainties" *(2)* is realized with this ID-MS calibration system *(14)*. Repeated separations of HbA_0 and HbA_{1c} with ion exchange and affinity chromatography *(15)* may not result in the required purity of reference standard preparation (e.g., due to postsynthetic modifications). In addition, the homogeneity of a protein preparation may be problematic because limited proteolytic attack on the target molecule may lead to modifications of the analyte. The detected relative difference of about 5% between the calibration of the current IFCC reference measurement procedure and the proposed ID-MS calibration supports our view that the traceability to reference protein preparations is less reliable than to certified pure amino acid standards. We think that this difference results from several preparative and analytical sources of error.

The difference of approximately 2–3 mmol/mol HbA_{1c} (0.2%–0.3% NGSP units) between the current and proposed calibration systems, in the clinically relevant HbA_{1c} range, may be small for a single patient, but may be of importance in clinical and epidemiological studies. Reference intervals have to be adapted if necessary. The statistical significance of this deviation in the calibration can be fully evaluated when all data of uncertainty of the other procedures are available.

In summary, we have demonstrated that fully SI-traceable HbA_{1c} quantification can be achieved when β-N-terminal glycated and nonglycated hexapeptides are used for calibration. The presented LC-ID-MS procedure allows the current IFCC reference measure-

ment procedure for HbA_{1c} to be raised to a higher order of accuracy.

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