Interference of the most frequent haemoglobin variants on quantification of HbA1c: Comparison between the LC–MS (IFCC reference method) and three routinely used methods


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Abstract

Aim. – Assaying HbA1c in patients with haemoglobin variants has long been a technical challenge, despite methodological advances that have progressively limited the problem. The purpose of this study was to evaluate the impact of the most frequent haemoglobin variants on three routine separation methods compared with the IFCC reference method.

Patients. – Blood samples from heterozygous patients (AS, AC, AD, AE) were analyzed using the IFCC reference method (LC–MS), and the results compared with those obtained by capillary electrophoresis (CAPILLARYS 2 Flex Piercing, Sebia) and two HPLC methods using cation-exchange (Variant II, Bio-Rad) and affinity chromatography (Ultra², Primus).

Results. – HbA1c values obtained by the IFCC reference method were comparable to those obtained by the three tested methods whatever the haemoglobin variant. Mean relative biases did not exceed the threshold of 7% (above which differences are generally considered clinically significant), although some individual values were above this limit with Variant II in samples with HbS and for all three methods in samples with HbE.

Conclusion. – This comparative study of the LC–MS reference method and three field methods has demonstrated that these assays are not clinically influenced by the presence of the most common haemoglobin variants. The present results also confirm that the interpretation of HbA1c values in patients with Hb variants remains complex and depends on the assays used and should, in some cases, take into account parameters other than analytical ones (such as differences in glycation rates and half-lives of haemoglobin variants).

Keywords: HbA1c; Reference method; Haemoglobin variants; Diabetes mellitus

Résumé

Interférence des variants de l’hémoglobine les plus fréquents sur le dosage de l’HbA1c : comparaison entre la LC-MS (méthode de référence IFCC) et trois méthodes de routine.

But. – La présence d’un variant de l’hémoglobine est connue depuis longtemps pour être à l’origine d’interférences analytiques lors du dosage de l’HbA1c, même si les avancées technologiques ont permis de limiter progressivement ce problème. Le but de cette étude était d’évaluer l’impact des variants les plus fréquemment rencontrés sur trois méthodes séparatives utilisées en pratique courante et de les comparer à la méthode de référence IFCC.

Patients. – Des échantillons de sang de patients hétérozygotes (AS, AC, AD, AE) ont été analysés à l’aide de la méthode de référence IFCC (LC-MS) et les résultats ont été comparés à ceux obtenus soit par électrophorèse capillaire (Capillarys 2 Flex Piercing – Sebia), soit à l’aide de deux méthodes CLHP au principe différent : la chromatographie échangeuse de cations (Variant II – Bio-Rad) et la chromatographie d’affinité (Ultra² – Primus).

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Résultats. — Les résultats d’HbA1c obtenus avec la méthode de référence IFCC étaient comparables à ceux obtenus par les trois méthodes testées, quel que soit le variant présent. Les biais relatifs moyens n’ont pas dépassé 7 % (seul au-dessus duquel les différences sont généralement considérées comme cliniquement significatives), même si certains échantillons ont donné des valeurs supérieures à ce seuil, comme par exemple en présence d’HBs (pour l’automate Variant II) et d’HbE (pour les trois automates).

Conclusions. — Cette étude comparative entre la méthode de référence LC-MS et trois méthodes de routine a démontré que ces méthodes ne sont pas influencées pour l’interprétation clinique par la présence des variants de l’hémoglobine les plus fréquents. Cependant, cet article rappelle que l’interprétation des résultats d’HbA1c en présence d’un variant reste délicate, dépend de la méthode utilisée, et doit aussi, dans certains cas, tenir compte d’autres paramètres relatifs aux variants, comme une cinétique de glycation ou une demi-vie différentes.

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Mots clés : HbA1c ; Méthode de référence ; Variants de l’hémoglobine ; Diabète sucré

1. Introduction

HbA1c is a widely used biomarker in the management of diabetes because it provides information on the monitoring of long-term glycaemic control and an assessment of the risk of developing complications [1–3]. Its use has also been recently proposed for the diagnosis of diabetes [4,5]. Consequently, HbA1c quantification now has to meet defined quality criteria to ensure reliable results and optimal clinical use. In this regard, many technological advances have been made by manufacturers to limit analytical errors and interference, and an international standardization process has been completed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [6,7] to ensure the traceability of methods to an internationally accepted reference system [8].

Nevertheless, HbA1c use remains controversial in pathological situations that can generate analytical interference and also alter its informational value. One such case is chronic renal failure (CRF), which leads to the accelerated formation of carbamylated haemoglobin (Hb) due to the increase in uraemia, which can interfere with HbA1c separation and quantification [9,10]. CRF is also responsible for an anaemic state, thus frequently necessitating erythropoietin therapy, which can distort the interpretation of HbA1c because of modification of red blood cell and Hb half-lives [11]. Similar limitations are encountered in patients with an Hb variant that could also lead to haemolysis, especially in a homozygous state. In addition, Hb variants can interfere with HbA1c determination. Several studies have been devoted to determining the impact of Hb variants on the analytical performance of HbA1c assays [12–14], but few have emphasized the impact of the presence of Hb variants on the informational value of HbA1c [15].

For this reason, the present study has evaluated the impact of the most frequent Hb variants (HbS, C, D and E) on the analytical performance of common field HbA1c methods compared with the IFCC liquid chromatography–mass spectrometry (LC–MS) reference method [16]. Three systems using distinct principles for the separation and quantification of HbA1c were tested: a device recently introduced into the market using capillary electrophoresis for HbA1c separation and quantification (CAPILLARYS 2 Flex Piercing, Sebia, Lisses, France) [17]; a cation-exchange high-performance liquid chromatography (HPLC) method (Variant II NU kit, Bio-Rad Laboratories, Hercules, CA, USA); and a boronate affinity HPLC method (Ultra², Primus Corporation, Kansas City, MO, USA). The ultimate purpose of this study was to determine whether, beyond the potential analytical problems that are nowadays limited, the interpretation of HbA1c results in patients with Hb variants remains a critical issue because of the impact of other parameters (such as differences in glycation kinetics and quantification of a glycated variant as HbA1c) on the informational value of HbA1c still needs to be taken into account.

2. Patients

2.1. Samples

Whole blood samples collected in EDTA-containing tubes (Greiner Bio-One, Courtaboeuf, France) from subjects homozygous for HbA (n = 8), or heterozygous for S (n = 9) or C, D or E (n = 10) Hb variants, were sent to the laboratory for routine HbA1c assay or were provided by Sebia (Lisses, France). The analyses covered a wide range of clinically relevant HbA1c values (from 4.9–13.5%, or 30–124 mmol/mol). All samples were frozen at −80 °C before analysis. No additional samples were necessary for this study, and no sample was kept after the assays.

2.2. HbA1c assays

Samples were assayed using the following analyzers: CAPILLARYS 2 Flex Piercing; Variant II NU kit; and Ultra². All assays were performed according to the manufacturer’s instructions. Results were compared with those obtained by the LC–MS IFCC reference method. This method is based on the quantification of the N-terminal hexapeptide of Hb β-chains obtained after the digestion of Hb by a specific endoproteinase Glu-C (endo-Glu-C). The generated peptides (whether glycated or not) were then quantified by LC–MS [16]. Our laboratory belongs to the IFCC network of reference laboratories for HbA1c [18] and, as such, regularly participates in the external quality-assessment scheme for reference laboratories in laboratory medicine (RELA–IFCC) [19].

2.3. Data analysis

Results obtained for each type of sample (homozygous AA and heterozygous AS, AC, AD and AE) by each method were subtracted from the values obtained by the IFCC reference
method to obtain absolute biases. The HbA1c values obtained for each Hb variant were compared using the Wilcoxon matched-pairs test to determine whether the presence of the variant led to a statistically significant difference \((P < 0.05)\) from the reference method. In addition, relative biases due to the presence of Hb variants were also calculated. Differences were considered clinically different when the relative bias was >7% (0.42% at 6% HbA1c and 0.63% at 9% HbA1c), the threshold usually applied in the literature for similar studies [20].

### 3. Results

#### 3.1. Analytical interference of haemoglobin (Hb) variants on HbA1c measurement

Hb variants can interfere in different ways with HbA1c determination depending on the technical features of the method used. The main expected analytical interferences due to Hb variants are summarized in Table 1.

Using the IFCC reference method, no analytical interference was expected, as the method specifically quantifies peptides (glycated or not) generated by the enzymatic digestion of Hb by endo-Glu-C (Fig. 1A). However, depending on the position of the mutation within the Hb β-chain genes (Table 2), the quantified hexapeptide may come from either only HbA or HbA with an Hb variant. Indeed, for HbS and HbC, the hexapeptide sequences affected by the mutation, only the peptides derived from HbA were quantified, as the peptides obtained from the Hb variant exhibited a different molecular mass and were therefore not quantified by MS. In contrast, for HbD and HbE, the mutation did not involve the hexapeptide, so the peptides generated from HbA and the variant exhibited the same molecular mass and were thus quantified by MS as the same entity.

### Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Measured analyte</th>
<th>Expected analytical interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC–MS</td>
<td>HbA1c (glycated N-terminal hexapeptide of Hb β-chain)</td>
<td>No interference; the hexapeptide generated from glycated variants is recognized as HbA1c hexapeptide except when the mutation involves the hexapeptide</td>
</tr>
<tr>
<td>Capillary electrophoresis</td>
<td>HbA1c</td>
<td>Potential modification of separation profile, thus perturbing quantification of HbA1c peak</td>
</tr>
<tr>
<td>Ion-exchange HPLC</td>
<td>HbA1c</td>
<td>Potential modification of separation profile, thus perturbing quantification of HbA1c peak</td>
</tr>
<tr>
<td>Boronate affinity HPLC</td>
<td>All glycated forms of Hb</td>
<td>No interference; the glycated variant is recognized as the same as any other glycated form of Hb</td>
</tr>
<tr>
<td>Immunoeassay</td>
<td>HbA1c</td>
<td>No interference, except when the mutation involves the epitope recognized by the antibody</td>
</tr>
</tbody>
</table>

LC–MS: liquid chromatography–mass spectrometry (used in this study as the IFCC reference method); HPLC: high-performance liquid chromatography.

With the CAPILLARYS 2 Flex Piercing analyzer, the quantification of HbA1c is based on the separation of Hb fractions by capillary electrophoresis. The potentially expected interference is modification of the separation profile that might prevent correct integration of the HbA0 and HbA1c peaks. The tested variants (HbS, C, D and E) did not modify the separation profile (an example of the electropherogram in the presence of HbS is shown on Fig. 1B) except for HbC, where the glycated form migrated just after HbA0, as reported elsewhere [17]. However, the software uses a specific algorithm that subtracts the expected area of glycated HbC peak from the HbA0 peak area. Accordingly, in the presence of all the Hb variants studied, CAPILLARYS 2 Flex Piercing was able to specifically quantify HbA1c without analytical interference.

With the Variant II analyzer, Hb fractions are separated by cation-exchange HPLC. The potential interference of Hb variants may arise from modification of the separation profile in the first part of the chromatogram (before elution of HbA0) [21]. All tested Hb variants were well separated from HbA0 and were thought not to interfere with the quantification of HbA1c (Fig. 1C shows an example of HbS). None of the two fractions of the variant (glycated and non-glycated forms) were included in the calculation of HbA1c value.

With boronate affinity HPLC, all glycated forms of Hb can interact with the column, so no analytical problem was observed in the presence of Hb variants, all of which were considered identical to HbA (Fig. 1D) [13].

#### 3.2. Comparison of methods

Using the IFCC reference method as the comparative method, the absolute differences among the tested assays have been shown in box plots (Fig. 2) and as relative biases (Fig. 3).

In the absence of Hb variants, HbA1c values obtained in homozygous (AA) samples were not significantly different with LC–MS vs. the CAPILLARYS 2 Flex Piercing analyzer nor vs. either of the other two systems (Variant II and Ultra2).

Also, in the presence of Hb variants, no significant differences in HbA1c values were found between the CAPILLARYS 2 Flex Piercing analyzer and LC–MS reference method. Absolute differences in HbA1c ranged from −0.7% (−8 mmol/mol) to +0.8% (+9 mmol/mol), whereas the mean relative biases ranged from −4.7% to +1.7%. For HbE, some of the individual values of relative bias were >7%.

### Table 2

<table>
<thead>
<tr>
<th>Hb</th>
<th>Chain</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>β</td>
<td>VHLTPE</td>
</tr>
<tr>
<td>S</td>
<td>β</td>
<td>VHLTPVE</td>
</tr>
<tr>
<td>C</td>
<td>β</td>
<td>VHLTPKE</td>
</tr>
<tr>
<td>D</td>
<td>β</td>
<td>VHLTPE</td>
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<tr>
<td>E</td>
<td>β</td>
<td>VHLTPE</td>
</tr>
</tbody>
</table>

Primary sequences of peptides released upon haemoglobin (Hb) digestion by endoprotease Glu-C.
Fig. 1. Typical patterns obtained from samples containing the HbS variant (32% HbS): chromatographic and electrophoretic patterns obtained by LC–MS (IFCC reference method) (A); CAPILLARYS 2 Flex Piercing (B); Variant II (C); and Ultra2 (D). GHb: glycated haemoglobin.

With the Variant II analyzer, no significant differences in HbA1c values were observed in comparison to the LC–MS reference method for HbC, D and E variants, whereas HbA1c values were significantly different in the presence of HbS. Indeed, in the latter case, absolute HbA1c differences ranged from 0% to −0.8% (−9 mmol/mol), and the mean relative bias reached +6.9%. Although the mean relative bias was not > 7%, the values were widely dispersed, with half of them exceeding the 7% threshold. In addition, several individual values of relative bias were > 7% for HbE.

Comparison of HbA1c results with the LC–MS reference method vs. boronate affinity chromatography (Ultra2) demonstrated that these methods were well correlated for all variants except for HbE, for which values were significantly different ($P=0.033$). These differences were confirmed by the relative biases, which were usually negative and ranged from 2.7% to −11.0%.

4. Discussion

HbA1c has long been considered the gold standard for assessing glycaemic control in patients with diabetes and has recently been proposed as a valuable diagnostic tool [5]. It is therefore important to guarantee the reliability of HbA1c results, and to evaluate all parameters likely to interfere with its measurement and interpretation in clinical practice. The presence of Hb variants was identified years ago as one of the major issues in HbA1c quantification [22]. Initially the presence of variants led to analytical pitfalls such as the co-elution of HbA and the variant that prevented the proper quantification of HbA1c. Over
the past decade, however, many improvements in analytical systems have been made to reduce the impact of Hb variants on HbA1c assays.

The present study aimed to evaluate the behavior of three analytical systems applying distinctly different principles of HbA1c quantification as regards such interference—namely, capillary electrophoresis, cation-exchange chromatography and boronate affinity chromatography. The originality of our work is based on the comparison of these systems to the IFCC reference method, which uses enzymatic digestion of Hb followed by LC–MS analysis and is considered free of any analytical interference [16]. In fact, most published studies have used the boronate affinity method for comparisons as it is said to be unaffected by the presence of Hb variants. Yet, the IFCC reference method appears to be more suitable for this purpose in theory, as it specifically quantifies HbA1c, whereas boronate affinity chromatography quantifies all glycated forms of Hb. A previous study has suggested that the two methods are well correlated for samples containing HbS and HbC variants [23], but such a comparison has never been performed using HbD and HbE variants which, unlike HbS and HbC, are recognized as HbA by the LC–MS method after enzymatic digestion of peptides (Table 2). Interestingly, our present study showed that HbA1c values obtained by the two methods were significantly different in the presence of the HbE variant, with relative biases being overall negative.

In addition, our study has demonstrated that, for each type of Hb variant, HbA1c values obtained by CAPILLARYS 2 Flex Piercing were not significantly different from those obtained with the LC–MS method. For the other techniques, a significant absolute difference was observed only in the presence of HbS using Variant II and HbE using Ultra2. These data confirm our previous results on comparing CAPILLARYS 2 Flex Piercing and Ultra2 [20], and demonstrate that the presence of Hb variants has only limited analytical impact on the three assays tested here. Indeed, the mean relative biases were all <7%, the threshold above which the presence of a variant is considered to have led to a clinically significant modification of HbA1c results [20]. However, a 7% relative bias represents a variation of 0.42%, 0.49% and 0.63% at 6%, 7% and 9% HbA1c, respectively. Accordingly, a 0.5% variation in HbA1c values at the decisive threshold may have led to misclassification of patients to a normoglycaemic or diabetic population. This problem is also encountered as a function of coefficients of variation (CVs) reproducibility of a given method and between-method variations.

The relative biases obtained in the presence of HbS showed that this variant had a greater impact on Variant II than on the CAPILLARYS 2 Flex Piercing and Ultra2 analyzers. The relative biases obtained for HbE samples also showed some differences between the LC–MS method and the three other assays, which were closely correlated. This might be explained by the fact that HbE is considered HbA on LC–MS.

A secondary aim of the present study was to discuss the interpretation of HbA1c results in the presence of Hb variants while emphasizing the need to know the characteristics and limitations of the method used. Indeed, although the presence of an Hb variant is no longer an analytical problem for HbA1c determination in most cases, as confirmed by the present study, its impact on the semiological value and reliability of this biomarker still needs to be taken into account. This means that when the Hb variant behaves like HbA in a given assay (for example, with boronate affinity chromatography or LC–MS for HbD and HbE), a crude
interpretation of the results would presuppose that the glycation rates for the variant and for HbA are similar. However, in the absence of conclusive studies, it might be expected that their glycation rates are different for a number of reasons, such as different protein half-lives, or modification of protein-folding or aggregation of Hb (as in HbS), both limiting the accessibility of N-terminal valine residues for glucose [24,25]. These questions need to be addressed and experimental answers provided before it can be considered that a given variant behaves like HbA. When the variant is not included in the calculation of HbA1c (as with CAPILLARYS 2 Flex Piercing and Variant II), it could be hypothesized that the glycation rate of HbA is not affected by concomitant glycation of the variant. Other confusing parameters may intervene, such as the reduction of erythrocyte lifespan, but this phenomenon is mainly observed in homozygous patients (for example, HbCC) [13]. As a consequence, it appears to be of greater interest to prefer the latter type of method.

Nevertheless, these conclusions are restricted to the Hb variants tested in the present comparative study and cannot be extended to less frequently seen variants, which may yet constitute an analytical problem for HbA1c quantification, as recently demonstrated in the case of Hb Hope [26].

In conclusion, the presence of Hb variants is no longer responsible for major analytical interference in HbA1c assays, although some biases have been noted with HbE and HbS with some methods. However, it remains a critical issue in the interpretation of results as the kinetics of the glycation process and the Hb half-life may vary, thereby diminishing the informational input of HbA1c. This implies that specialists in laboratory medicine need to be aware of the limitations of their assay method and so should provide enough information to clinicians as to the potential interferences in order to obtain useful interpretations of HbA1c results. In addition, no generalizations can be made based on the type of method used [20]. Thus, in some cases, HbA1c determination should be completed using other markers of glycation such as fructosamines or glycated albumin [27–29], even though these tests are still seldom used because of the current lack of precise clinical guidelines.

Fig. 3. Plots of the relative biases obtained for each Hb variant show deviations from results obtained by the LC–MS method expressed as relative biases for each method: CAPILLARYS 2 Flex Piercing (circles); Variant II (squares); and Ultra2 (triangles). Dotted lines represent the accepted threshold of 7%, above which differences are considered clinically significant [20].
Disclosure of interest

This study was supported by Sebia (Lisses, France), which provided the CAPILLARYS 2 Flex Piercing® analyzer as well as reagents and blood samples with Hb variants. This study benefited from an evaluation contract granted by Sebia to the University Hospital Centre of Reims.

References


