Labile glycated haemoglobin and carbamylated haemoglobin are still critical points for HbA_{1c} measurement

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Abstract

Introduction: Haemoglobin A_{1c} (HbA_{1c}) is a key analyte for the monitoring of glycemic balance in diabetic patients and is used for diabetes diagnosis in many countries. The potential interference of carbamylated haemoglobin (cHb) and labile glycated haemoglobin (LA_{1c}) on HbA_{1c} assays must remain a matter of vigilance. Such a situation has occurred in our laboratory with a kit replacement on the Bio-Rad Variant™ II testing system, a cation-exchange high performance liquid chromatography (HPLC) system. With this method, LA_{1c} and cHb coeluted in a same peak which may have different consequences on HbA_{1c} values.

Materials and methods: The influence of increasing LA_{1c} and cHb values on HbA_{1c} results was studied with *in vitro* glycation and carbamylation of samples. Samples from patients with high and normal blood urea concentrations were assayed by HPLC and immunological assay.

Results: We observed that the degree of interference greatly varied depending on the nature of the interfering Hb fractions found under the so-called "LA_{1c} peak". Thus, we have decided to apply a decision tree using "LA_{1c}" thresholds depending on: (i) the retention time, (ii) the shape of the peak, (iii) other analytes, like urea. If the peak recognized as "LA_{1c}" is mainly formed by $LA_{1c,}$ we consider that there is no interference until 4%. If the peak is mainly formed by cHb, we consider an interference threshold equal to 2%.

Conclusions: This situation reminds that cHb and LA_{1c} remain critical issues in chromatography-based HbA_{1c} assays and that adapted criteria must be set up for result interpretation.

Key words: carbamylated haemoglobin; labile glycated haemoglobin; HbA_{1c}; analytical interference

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Introduction

Since its discovery as unusual haemoglobin fraction in patients with diabetes mellitus, HbA_{1c}, the major component of glycated haemoglobin, has proved to be a useful analyte for the monitoring of glycemic balance in diabetic patients, and for diabetes diagnosis in many countries (1–3). HbA_{1c} is also an important predictive marker of long term complications of diabetes (4,5). HbA_{1c} assays can be performed using different analytical principles including separative methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis, but also immunological and enzymatic methods.

The quality of assay methods for HbA_{1c} determination has greatly improved over the last decades, and most of analytical interferences have been controlled (1,6). This is the case of carbamylated haemoglobin (cHb), which results from the binding of urea-derived isocyanic acid and is increased in patients with chronic kidney disease. However, the potential interference of cHb on HbA_{1c} assays must remain of matter of vigilance (7,8). Furthermore, interference may be caused by labile glycated haemoglobin (LA_{1c}), a labile and reversible fraction (Schiff base) formed at early stages of glyca-

tion reaction, which directly depends on blood glucose concentration. Such a situation is reported here.

We have recently changed the kit for HbA_{1c} determination in our laboratory, by replacing the NU Kit™ by the Dual Kit Program™ on the Bio-Rad Variant™ II testing system, based on cation-exchange HPLC. The analyser used for determination of HbA_{1c} is the same, but the chromatography program (column, buffer gradient) and the peak integration software (column, buffer gradient) are different. The main advantage of the method with the new kit is the possibility to use the same method for HbA_{1c}, haemoglobin A_2, and haemoglobin F measurements.

Most frequent interferences encountered with such HPLC-based methods have been evaluated (9). Whereas previous evaluations of this system did not report any interference studies (10), the instruction notes of the manufacturer mention a potential interference of cHb and LA_{1c} over 3%. Using the Dual kit program™, LA_{1c} and cHb coeluted in a same peak called LA_{1c}, contrary to the former program NU Kit™ which allowed the separation in two peaks. As these two minor Hb fractions coelute in a single peak before HbA_{1c} peak, the consequences on HbA_{1c} value may differ according to the increased fractions (cHb or LA_{1c}). Indeed, besides reporting the differences of chromatographic separation of cHb and LA_{1c} between the two kits, the aim of this study was to evidence that the influence of cHb or LA_{1c} on HbA_{1c} measurements remains a critical issue and to propose a decision tree for managing these interferences in routine practice with this method.

Materials and methods

Subjects

This study was performed from September to October 2016 in the Laboratory of Pediatric Biology and Research at the University Hospital of Reims.

Blood samples used for this study were received in the routine workload of the laboratory for HbA_{1c} assay as part of the follow-up of 19 patients. The interference noticed with the new kit was evidenced during regularly ordered HbA_{1c} assays. No other biological investigation than HbA_{1c} assay was performed, and additional experiments exclusively aimed at providing an interpretable HbA_{1c} result in patients.

Among these samples, those from patients with physiological (< 7.15 mmol/L) or increased blood urea (> 7.15 mmol/L) were assayed by two methods (HPLC and immunological assay).

Blood samples

Blood samples sent to the laboratory for HbA_{1c} assays using Variant II™ analyser were collected in 5 mL ethylenediamine tetra-acetic acid dipotassium salt (K_2EDTA)-containing tubes (ref. 367962, Becton Dickinson, Franklin Lakes, New Jersey, USA) and stored at + 4°C before analysis. The minimum required volume, for the primary tube, was 1 mL. For DCA Vantage® analyser, 1µL of capillary blood, obtained by finger skin puncture or venous punction (acceptable anticoagulants being K_2EDTA, heparin, fluoride/oxalate and citrate) was required. Capillary holders, which contain glass capillary, are provided by Siemens in DCA™ Systems Hemoglobin A_{1c} Reagent Kit.

For urea measurement, blood samples were collected on lithium heparin-containing tubes (ref. 367884, Becton Dickinson, Franklin Lakes, New Jersey, USA). The minimum required volume was 1 mL for primary tubes.

Methods

Blood urea was assayed on Cobas® 8000 modular analyser series on c701 and c502 modules (Roche Diagnostics, Mannheim, Germany). Determination of blood urea was based on kinetic test using urease and glutamate dehydrogenase.

Variant II Hemoglobin Testing System™ (Bio-Rad, Marnes-la-Coquette, France) is a completely automated system for HbA_{1c} measurement based on cation-exchange HPLC. Samples are automatically mixed, diluted on the station and injected into the column. Haemoglobins are separated based on their ionic interactions with the stationary phase. The mobile phase is composed of phosphate buff-

er; the chromatographic station delivers a gradient of increasing ionic strength to the cartridge. The separated haemoglobins pass through the flow cell of the filter photometer, and changes in the absorbance are measured at 415 nm, and at 690 nm for background correction.

Intra-assay coefficients of variation (CVs), calculated from patient samples, were 0.4%, 0.7% and 0.4% for HbA_{1c} values (expressed in percentages) of 5.4%, 7.3% and 12.8%, respectively. Between-assay CVs, calculated from quality control results, Bio-Rad Lyphocheck Diabetes Controls (Bio-Rad Laboratories Inc, Hercules, CA, USA), were 1.1% and 1.3% for HbA_{1c} values of 5.7% and 9.4%, respectively. Measurement uncertainties represented 0.27% and 0.38% for HbA_{1c} values of 5.6% and 9.4%, respectively. Bio-Rad Lyphocheck Diabetes Controls were used as quality control (QC) materials and assayed at the beginning and at the end of each assay series. Between-assay CVs were 1.0% and 1.2% for expected values at 5.2% and 10.1% of HbA_{1c} respectively.

DCA Vantage® (Siemens Healthcare, St Denis, France) system relies on an immunological assay. The concentrations of HbA_{1c} and total haemoglobin are both measured in parallel and the ratio reported as percent. For total haemoglobin measurement, haemoglobin is oxidized in methaemoglobin, then methaemoglobin complexes with thiocyanate to form thiocyan-methaemoglobin, the absorbance of coloured species being measured at 531 nm. The measurement of HbA_{1c} is based on a latex agglutination inhibition method using monoclonal antibodies. An agglutinator, containing immunoreactive portion of HbA_{1c}, causes the agglutination of latex particles coated with HbA_{1c} monoclonal antibody. The agglutination reaction increases scattering of light measured as an increase of absorbance at 531 nm. HbA_{1c} in patient blood sample inhibits the interaction between the agglutinator and latex particles and decreases scattering of light, measured as a decrease in absorbance at 531 nm.

Intra-assay CVs, calculated from patient samples, were 1.5% and 2.7% for HbA_{1c} values (expressed in percentages) of 5.2% and 7.7%, respectively. Between-assay CVs, calculated from quality control results (Bio-Rad Lyphocheck Diabetes Controls) were 1.8% and 1.7% for HbA_{1c} values of 5.6% and 10.2%, respectively. Measurement uncertainties represented 0.61% and 0.67% for HbA_{1c} values of 5.6% and 10.2%, respectively. Bio-Rad Lyphocheck Diabetes Controls were used as QC materials and assayed at the beginning and at the end of each assay series. Between-assay CVs were 1.8% and 2.3% for expected values at 5.6% and 9.9% of HbA_{1c}, respectively.

In the Dual kit program™, LA_{1c} and cHb coeluted in a same peak called LA_{1c}, contrary to the former program NU Kit™ which allowed the separation in two peaks. This peak, which elutes just before HbA_{1c} peak, may represent a potential interference on HbA_{1c} quantification.

Procedures for in vitro carbamylation and glycation

Whole blood samples were selected according the following criteria: haematocrit value in reference ranges, low LA_{1c} value (< 1.5%), and two HbA_{1c} levels (within reference range and over 7%).

Two mL of whole blood were centrifuged (4 °C, 8 minutes, 3000g), plasma removed, and three washes (4 °C, 8 minutes, 3000 x g) of red blood cells (RBC) performed with NaCl 0.15 M (saline). 200 µL of KCNO (Sigma-Aldrich, St Louis, Missouri, USA), prepared at different concentrations: 0.125 mM, 0.250 mM, 0.375 mM, 0.500 mM, 0.750 mM, were added and incubated for 3 hours at 37 °C. A control series was performed by incubating 200 µL KCl instead of KCNO. After centrifugation (room temperature (RT), 30 seconds, 20,000 x g), supernatant was removed and 2 washes were performed (RT, 30 seconds, 20,000 x g) with saline. 200 µL of saline were added, and HbA_{1c} assayed on Variant II™ system.

Procedure for in vitro glycation

Two mL of whole blood were centrifuged (4 °C, 8 minutes, 3000 x g), plasma removed, and three washes of RBC performed with saline. 200 µL of 0.250 mM glucose (alpha-D-glucose anhydrous 96%; Sigma-Aldrich, St Louis, Missouri, USA) solution in saline were added to RBC pellet and incu-

bated for 0, 15, 17, 22, 27, 30, 45 minutes at 37 °C. After centrifugation (RT, 30 seconds, 20,000 x g), supernatant was removed and replaced by 200 µL of 5 mM glucose, and HbA_{1c} assayed on Variant II™ system.

Statistical analysis

In vitro glycation or carbamylation experiments were analysed in triplicate. Results were expressed as means.

Relative and absolute biases were obtained with following formulas:

$$Relative\ bias = \left(\frac{x-y}{y}\right) \times 100$$

$$Relative\ bias = x - y$$

where x = HbA_{1c} measured with Variant II ™ analyser and y = HbA_{1c} measured with DCA Vantage® analyser.

Uncertainty measurement (U) was calculated using the following formulas:

$$U = 2 \times u(C)$$

$$u(C) = \sqrt{u^2\,(IQC) + u^2\,(EQE)}$$

$$u(IQC) = \frac{CV \times m}{100}$$

$$u(EQE) = \sqrt{\left(\frac{|\bar{E}|}{\sqrt{3}}\right)^2 + \hat{\sigma}_E^2}$$

$$\hat{\sigma}_E^2 = \frac{\Sigma i\,(E_i - \bar{E})^2}{n-1}$$

$$\bar{E} = \frac{\Sigma i\,(x_{lab} - x_{ref})i}{n}$$

$$E_i = (x_{lab} - x_{ref})$$

where IQC represents internal quality control, EQE external quality control, CV coefficient of variation, m represents the mean; x_{lab} laboratory result; x_{ref} reference value.

The measurement uncertainty of the Variant II™system, calculated with the above formulas and equal to 0.3%, was used as a threshold to determine a significant difference between HbA_{1c} results.

Results

First, we studied the influence of increasing LA_{1c} values on HbA_{1c} results, and showed that the increase of LA_{1c} peak led to a decrease of HbA_{1c} values (Table 1), the threshold of 0.3% being used as acceptance criterion to determine a significant difference between HbA_{1c} results. Thus, a significant interference was noticed when LA_{1c} exceeded 4%. Second, we studied the influence of cHb interference and found a significant interfering effect when cHb exceeded 2% (Table 2).

Thus, both LA_{1c} and cHb interfered with HbA_{1c} determination, but it was noteworthy that the influence of the peak recognized as LA_{1c} on chromatogram depended on the nature of the interference. When the interference was caused by cHb, HbA_{1c} decrease was more prominent than when caused by LA_{1c}. For example, for an initial level of HbA_{1c} equal to 5.2% (33 mmol/mol) with a LA_{1c} peak at 1.2%, the increase of LA_{1c} peak to 3.4 % due to glycation (LA_{1c} increase) led to a decrease of HbA_{1c} value to 4.9% (30 mmol/mol), whereas it was 4.4% (25 mmol/mol) when the interference was due to carbamylation (cHb). Similar results were found using a sample with initial HbA_{1c} value of 8.1% (65 mmol/mol): a decrease of HbA_{1c} to a value at 7.7% (61 mmol/mol) was observed for a cHb value of 2.2% whereas it was observed only at 4.6% of LA_{1c} (Tables 1 and 2). The impact of cHb and LA_{1c} on the HbA_{1c} measurements (Tables 1 and 2) was also highlighted using a graphical presentation (Figure 1).

Interestingly, we observed that chromatograms differed regarding retention time (higher for cHb than for LA_{1c}) and peak shape depending on the origin of the interference (LA_{1c} or cHb), even though the peak was only recognized as LA_{1c} by the software (Figure 2).

Table 1. Interference of labile glycated hemoglobin (LA_{1c}) on HbA_{1c} measurements by Variant II equipped with Dual kit program™

Incubation time (min) with 0.25 mol/L glucose	0	15	17	22	27	30	45
HbA_{1c}†, mmol/mol	33	31	30	30	28	27	25
HbA_{1c}†, %	5.2	5.0	4.9	4.9	4.7*	4.6*	4.4*
Peak recognized as «LA_{1c}»†, %	1.2	3.1	3.4	4.0	4.5	4.8	6.3
HbA_{1c}†, mmol/mol	65	64	64	62	61	60	56
HbA_{1c}†, %	8.1	8.0	8.0	7.8	7.7*	7.6*	7.3*
Peak recognized as «LA_{1c}»†, %	1.3	3.1	3.4	4.0	4.6	4.8	6.2

*Differences higher than 0.3% compared to initial value of HbA_{1c}. †mean of triplicate measurements.

Table 2. Interference of carbamylated hemoglobin (cHb) on HbA_{1c} measurements by Variant II equipped with Dual kit program™

3h-incubation with KCNO (mmol/L)	0.000	0.125	0.250	0.375	0.500	0.750
HbA_{1c}†, mmol/mol	33	32	31	30	28	25
HbA_{1c}†, %	5.2	5.1	5.0	4.9	4.7*	4.4*
Peak recognized as «LA_{1c}»†, %	1.2	1.4	1.7	2.1	2.5	3.4
HbA_{1c}†, mmol/mol	65	64	61	59	55	50
HbA_{1c}†, %	8.1	8.0	7.7*	7.5*	7.2*	6.7*
Peak recognized as «LA_{1c}»†, %	1.3	1.6	2.2	2.8	3.5	4.8

*Differences higher than 0.3% compared to initial value of HbA_{1c}. †Mean of triplicate measurements.

To go further into the investigation of the impact of cHb on HbA_{1c} measurements, we performed a comparison between HbA_{1c} values obtained with the Dual Kit Program™ (Bio-Rad) device and with the immunoassay-based DCA Vantage® (Siemens Healthcare) which is not influenced by cHb (11). Because uraemia is the main cause of increased carbamylation (12), and consequently of cHb formation, samples from 7 patients with increased blood urea (> 7.15 mmol/L) were assayed by both methods. Negative absolute and relative biases were evidenced in all samples with increased blood urea. Mean relative and absolute biases calculated were - 8.2% and - 5 mmol/L (- 0.5%), respectively (Table 3). By contrast, a similar comparison performed in samples from patients without hyperuraemia (urea < 7.15 mmol/L) showed mean relative and absolute biases equal to + 0.9% and +1.1 mmol/mol (+ 0.1%) respectively (Table 4).

Discussion

Because HbA_{1c} is widely used for the diagnosis of diabetes and the monitoring of diabetic patients, its quantification must be performed using robust and high quality methods (2). HPLC is commonly used for assaying HbA_{1c}, and the potential interferences, like those generated by LA_{1c} and cHb, have generally been well described. However, these additional Hb fractions may still cause unexpected interferences (7,8,13). A previous study investigating the influence of cHb to HbA_{1c} measurements by Bio-Rad Variant II™ analyser equipped with NU Kit™ did not evidence such an impact of cHb but recommended an interpretation with caution when cHb was increased (14).

Using the Dual kit program™, LA_{1c} and cHb coeluted in a same peak called LA_{1c}, contrary to the former program NU Kit™ which allowed the separation in two peaks. This peak which elutes just before HbA_{1c} peak could represent a potential interference on HbA_{1c} quantification.

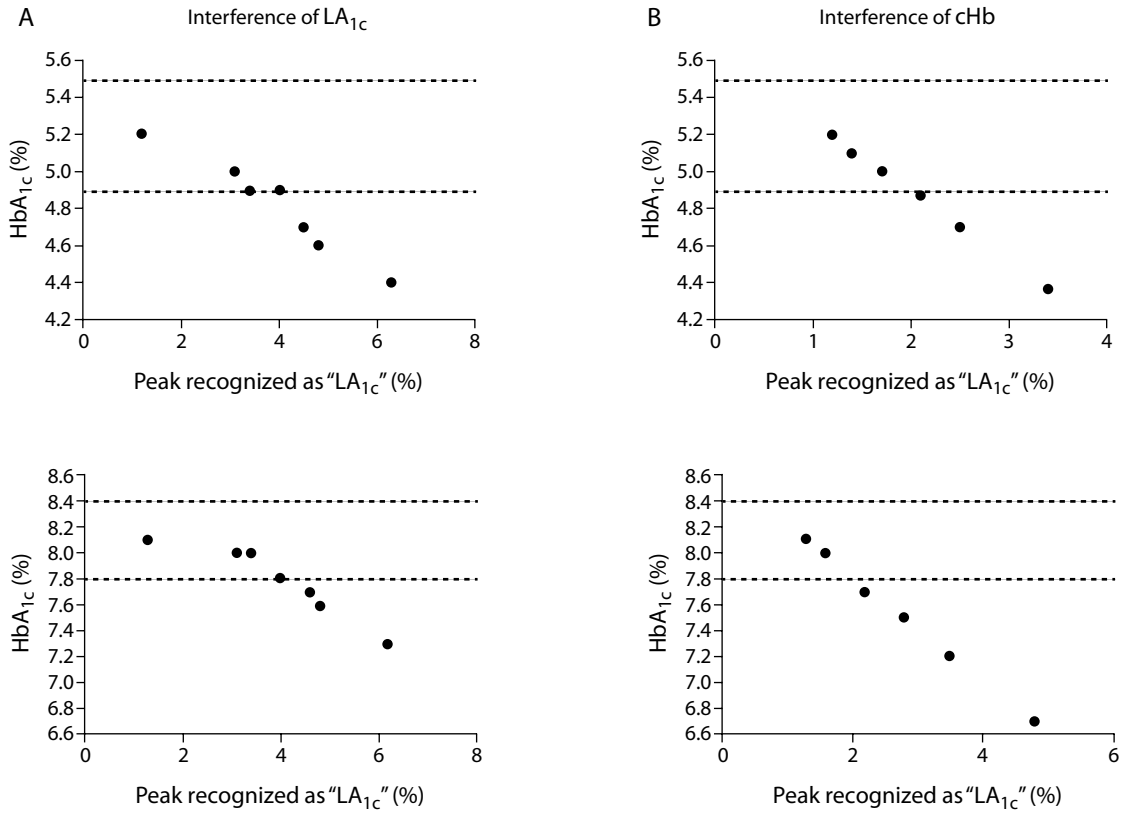

Figure 1. Interference of LA_{1c} (A) and cHb (B) on HbA_{1c} measurement by Variant II equipped with Dual Kit Program™.
For each interference, two samples with different levels of HbA_{1c} (5.2% and 8.1%) have been tested. The dashed lines represent the values above which the differences have been considered significant (initial HbA_{1c} values ± 0.3%, which corresponds to the measurement uncertainty of the method).

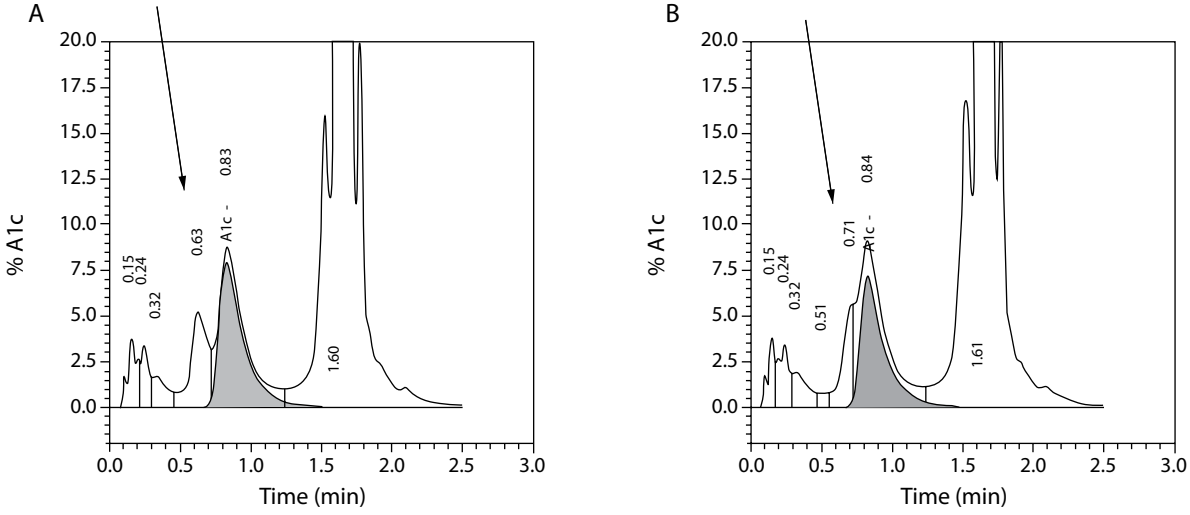

Figure 2. Chromatograms obtained in case of an increase of the "LA_{1c} peak" due to A: LA_{1c} (elution time: 0.63 min) or B: cHb (elution time: 0.71 min).

Table 3. Comparison of HbA_{1c} results obtained using Variant II Dual kit program™ and DCA Vantage®in patients with hyperuraemia

	HbA_{1c}, mmol/mol (%)*	HbA_{1c}, mmol/mol (%)†	Relative bias, %	Absolute bias, mol/mol (%)	Blood urea, mmol/L
Patient 1	31 (5.0)	40 (5.8)	- 13.7	- 9 (- 0.8)	27.8
Patient 2	53 (7.0)	58 (7.5)	- 6.6	- 5 (- 0.5)	25.0
Patient 3	38 (5.6)	42 (6.0)	- 6.6	- 4 (- 0.4)	15.3
Patient 4	55 (7.2)	58 (7.5)	- 4.0	- 3 (- 0.3)	29.0
Patient 5	27 (4.6)	33 (5.2)	- 11.5	- 5 (- 0.6)	9.0
Patient 6	30 (4.9)	34 (5.3)	- 7.6	- 4 (- 0.4)	30.9
Patient 7	44 (6.2)	48 (6.7)	- 7.5	- 4 (- 0.5)	24.0
Mean biases			- 8.2	- 5 (- 0.5)	

*Determined using Variant II. †Determined using DCA Vantage.

Table 4. Comparison of HbA_{1c} results obtained using Variant II Dual kit program™ and DCA Vantage® in non-uraemic patients

	HbA_{1c}, mmol/mol (%)*	HbA_{1c}, mmol/mol (%)†	Relative bias, %	Absolute bias, mol/mol (%)	Blood urea, mmol/L
Patient 1	37 (5.5)	37 (5.5)	0.0	0 (0.0)	4.2
Patient 2	88 (10.2)	79 (9.4)	8.5	9 (0.8)	7.0
Patient 3	50 (6.7)	46 (6.4)	4.7	4 (0.3)	7.0
Patient 4	34 (5.3)	34 (5.3)	0.0	0 (0.0)	7.0
Patient 5	58 (7.5)	57 (7.4)	1.4	1 (0.1)	7.0
Patient 6	53 (7.0)	53 (7.0)	0.0	0 (0.0)	5.4
Patient 7	37 (5.5)	39 (5.7)	- 3.5	- 2 (- 0.2)	5.7
Patient 8	41 (5.9)	41 (5.9)	0.0	0 (0.0)	7.1
Patient 9	40 (5.8)	42 (6.0)	- 3.3	- 2 (- 0.2)	7.1
Patient 10	62 (7.8)	61 (7.7)	1.3	1 (0.1)	7.0
Mean biases			+ 0.9	+ 1.1 (+ 0.1)	

*Determined using Variant II. †Determined using DCA Vantage.

In this study, we have evidenced that although migrating under the same peak; both LA_{1c} and cHb interfered differentially with HbA_{1c} measurements. Indeed, the degree of interference greatly varied depending on the nature of the interfering Hb fractions found under the so-called "LA_{1c} peak". In the case of the present method, manufacturer recommendations indicated the absence of interference of the peak recognized as LA_{1c} fewer than 3%. Our study evidenced that a significant interference was noticed when LA_{1c} exceeded 4% and found a significant interfering effect when cHb exceeded 2%. This interference caused by cHb could lead to underestimate the value of HbA_{1c} and have an impact on clinical classifications based on HbA_{1c} thresholds, or even on patient management (15).

Thus, we have decided to apply different thresholds depending on the nature of the interference, according to decision tree shown in Figure 3. For differentiating the origin of the interference, we consider (i) the retention time, (ii) the shape of the peak, and (iii) other biological results, especially uraemia. If the peak recognized as "LA_{1c}" is mainly formed by $LA_{1c,}$ we consider that there is no interference until 4% of LA_{1c}. When LA_{1c} is increased

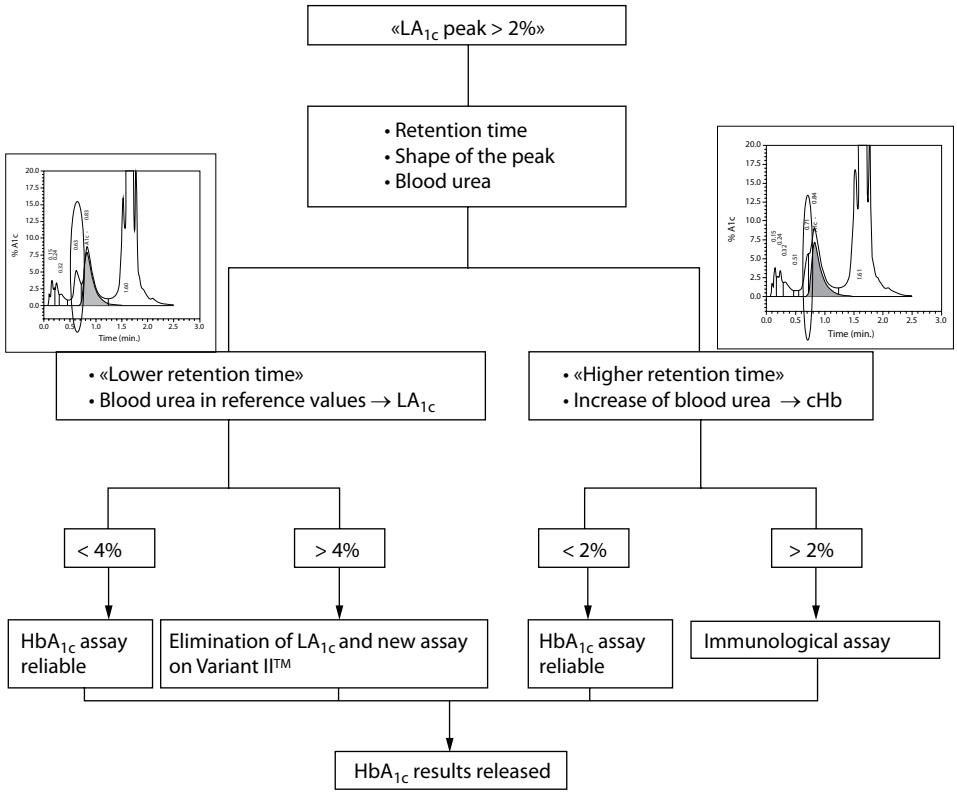

Figure 3. Decision tree in case of "LA_{1c} peak" higher than 2%

over 4%, this fraction is eliminated by incubation of red blood cells for 30 minutes at 37 °C, according to a previously described procedure (16). If the peak is mainly formed by cHb, we consider a lower interference threshold of 2%. When cHb values are higher than 2%, as cHb cannot be eliminated, we do not release values obtained by the HPLC method and perform HbA_{1c} assay using an immunological method, such as on DCA Vantage® System, not influenced by cHb.

Conclusion

This article reminds that cHb remains a critical issue in chromatography-based HbA_{1c} assays. The experimental verifications performed upon implementation of the method in our laboratory led us to change our practice, considering the coelution in a single peak of LA_{1c} and cHb. For that reason, the use of different thresholds (2% or 4% of LA_{1c} peak) has been established in our daily practice.

Potential conflict of interest

None declared.

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