Ned Tijdschr Klin Chem Labgeneesk 2010; 35: 175-177

Analytical validation of the screening for glutathionylated haemoglobin (HbX_{1d3}) by MALDI-TOF-MS in order to monitor oxidative stress

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Introduction

Besides traditional techniques based on electrophoresis and chromatography, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) also has potential to screen for haemoglobin (Hb) variants (1). Whereas separation approaches detect symmetric pairs of dimers of holo-subunits and assigns signals to Hb variants based on retention time (e.g. HbA = $\alpha_2\beta_2{}^A$; HbA_{1c} = $\alpha_2\beta_2{}^A{}_{glyc}$; HbA_{1d3} = $\alpha_2\beta_2{}^A{}_{SSG}$; HbS = $\alpha_2\beta_2{}^S$; HbS_{1c} = $\alpha_2\beta_2{}^S{}_{glyc}$; HbS_{1d3} = $\alpha_2\beta_2{}^S{}_{SSG}$; HbF = $\alpha_2\gamma_2{}^A$ and/or $\alpha_2\gamma_2{}^G$; HbF_{1c} = $\alpha_2\gamma_2{}^A{}_{glyc}$ and/or $\alpha_2\gamma_2{}^G{}_{glyc}$), the MALDI-TOF-MS approach detects apo-subunits based on *m/z* values (e.g. $\Sigma\alpha$; $\Sigma\beta^A$; $\Sigma\beta^S$; $\Sigma\gamma^A$; $\Sigma\gamma^G$; $\Sigma\beta^A{}_{glyc}$; $\Sigma\beta^S{}_{glyc}$; $\Sigma\gamma^A{}_{glyc}$; $\Sigma\gamma^G{}_{glyc}$; $\Sigma\beta^A{}_{SSG}$; $\Sigma\beta^S{}_{SSG}$). In both distinctive approaches, the profiles of symmetric pairs of dimers of holo-subunits and of separate apo-β-subunits can be translated into comparable profiles of Hb variants. This study validates the determination of $\%HbX_{1d3}$ in haemolysates of erythrocytes as measured by MALDI-TOF-MS, where the notation X_{1d3} stands for glutathionylated β-subunit of Hb ($\beta^X{}_{SSG}$) and X for A, S, C, D, E, O, etc (table 1).

Glutathionylated wild type haemoglobin (HbA_{1d3}; X=A) is gaining interest, because it is being recognized as a probable marker of oxidative stress in chronic diseases such as diabetes mellitus, hyperlipidemia and chronic renal failure (2-5). Similar to $\%HbA_{1c}$, which measures exposure to glucose, $\%HbA_{1d3}$ measures exposure to glutathione disulfide (GSSG). GSSG is the oxidized form of reduced glutathione (GSH) and is either continuously reduced by the glutathione reductase system or actively transported out of the erythrocyte so as to maintain a high intracellular GSH/GSSG ratio (2, 4, 5). In addition to certain chronic diseases, the oxidative stress is also of interest for certain Hb disorders. Therefore within erythrocytes, $\%HbX_{1d3}$ may serve as a useful clinical marker of oxidative stress in all relevant syndromes.

Methods

Subjects and samples

Anonymous human whole blood samples for routine laboratory analysis or for certain scientific projects were used to prepare haemolysates [n = 64]. Approval to use such samples was according to the policy of the Maastricht University Medical Centre and granted by a local ethics committee. The quality control (QC) sample for Hb variants level 3 containing a mixture of erythrocytes with a certified level of %HbA [58-66%], %HbS [14-22%] and of %HbC [16-24%], a non-certified level of $\%HbX_{1d3}$ and non-detectable HbF was obtained from Eurotrol [batch number 20-3-B642].

Sample preparation and MALDI-TOF-MS analysis

Haemolysate of erythrocytes was prepared according to de Boer et al. (7) and mass spectra of the pseudo-molecular ions of apo-subunits were obtained. After internal calibration the measured *m/z* values were compared with the theoretical *m/z* values of the respective apo-subunits (table 1). Ions were assigned if the theoretical *m/z* value was within ± 0.8 Th of the mean of the *m/z* value measurements in 5 spots, which corresponds to a mass accuracy of 50 ppm. $\%HbX_{1d3}$ was calculated as the percentage of the mean intensity of $\Sigma\beta^X{}_{SSG}$ with respect to the sum of all mean intensities of assigned apo-β-subunits (e.g. $\Sigma\beta^{A/C/D/E/O}$; $\Sigma\beta^S$; $\Sigma\gamma^A$; $\Sigma\gamma^G$; $\Sigma\beta^{A/C/D/E/O}{}_{glyc}$; $\Sigma\beta^S{}_{glyc}$; $\Sigma\gamma^A{}_{glyc}$; $\Sigma\gamma^G{}_{glyc}$; $\Sigma\beta^{A/C/D/E/O}{}_{SSG}$; $\Sigma\beta^S{}_{SSG}$) [n = 5 measurements].

Results and discussion

Specificity

Although the assigned *m/z* value may be considered as characteristic, under the conditions studied the apo-$\beta^A{}_{SSG}$-subunit cannot be distinguished from the apo-$\beta^X{}_{SSG}$-subunit of HbC_{1d3}, HbD_{1d3}, HbE_{1d3} or HbO_{1d3}, all which have merely a mass of 1 Da less than of HbA_{1d3} (table 1). Because of that, assignments in such situations must be done carefully. Furthermore, results must be expressed as $\%HbX_{1d3}$, unless the subject is a homozygote with a known genotype or a heterozygote/homozygote for HbS.

Due to the lack of a certified HbA_{1d3} QC sample, we performed the analytical validation using a QC sample containing a non-certified level of $\%HbX_{1d3}$. We assumed that the bulk was composed of HbA_{1d3} (see specifications of QC sample). Accordingly and considering the mass resolution of the instrument, we assigned in that QC sample the *m/z* value of 16173 to the apo-$\beta^A{}_{SSG}$-subunit (theoretical pseudo-molecular ion is at *m/z* 16173.3). This assignment was verified and confirmed by synthesizing HbA_{1d3} through the reaction of reduced glutathione with HbA in the presence of hydrogen peroxide.

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Table 1. Selection of haemoglobin variants and characteristics of the respective apo-β-subunits

Haemoglobin variants and some of their post-translational modifications	Non-standard abbreviation	Homologous subunit composition	Mass apo-β-subunit in Da[1] [respective *m/z* value of MH^+ in Th[2]]
Wild type haemoglobin	HbA	$\alpha_2\beta_2{}^A$	β^A: 15867 [15868]
β globin chain, pos 6: Glu→Val (sickle cell)	HbS	$\alpha_2\beta_2{}^S$	β^S: 15837 [15838]
β globin chain, pos 6: Glu→Lys	HbC	$\alpha_2\beta_2{}^C$	β^C: 15866 [15867]
β globin chain, pos 121: Glu→Gln	HbD (Punjab)	$\alpha_2\beta_2{}^D$	β^D: 15866 [15867]
β globin chain, pos 26: Glu→Lys	HbE	$\alpha_2\beta_2{}^E$	β^E: 15866 [15867]
β globin chain, pos 121: Glu→Lys	HbO (Arab)	$\alpha_2\beta_2{}^O$	β^O: 15866[15867]
Fetal haemoglobin, pos 136: Gly	HbF	$\alpha_2\gamma_2{}^G$	γ^G: 15995 [15996]
Fetal haemoglobin, pos 136: Ala	HbF	$\alpha_2\gamma_2{}^A$	γ^A: 16009 [16010]
Wild type glycated HbA	HbA_{1c}	$\alpha_2\beta_2{}^A{}_{glyc}$	$\beta^A{}_{glyc}$: 16029 [16030]
Sickle cell glycated HbS	HbS_{1c}	$\alpha_2\beta_2{}^S{}_{glyc}$	$\beta^S{}_{glyc}$: 15999 [16000]
Glycated HbC	HbC_{1c}	$\alpha_2\beta_2{}^C{}_{glyc}$	$\beta^C{}_{glyc}$: 16028 [16029]
Glycated HbD	HbD_{1c}	$\alpha_2\beta_2{}^D{}_{glyc}$	$\beta^D{}_{glyc}$: 16028 [16029]
Glycated HbE	HbE_{1c}	$\alpha_2\beta_2{}^E{}_{glyc}$	$\beta^E{}_{glyc}$: 16028 [16029]
Glycated HbO	HbO_{1c}	$\alpha_2\beta_2{}^O{}_{glyc}$	$\beta^O{}_{glyc}$: 16028 [16029]
Glycated HbF, pos 136: Gly	HbF_{1c}	$\alpha_2\gamma_2{}^G{}_{glyc}$	$\gamma^G{}_{glyc}$: 16157 [16158]
Glycated HbF, pos 136: Ala	HbF_{1c}	$\alpha_2\gamma_2{}^A{}_{glyc}$	$\gamma^A{}_{glyc}$: 16171 [16172]
Wild type glutathionylated HbA	HbA_{1d3}	$\alpha_2\beta_2{}^A{}_{SSG}$	$\beta^A{}_{SSG}$: 16172 [16173]
Sickle cell glutathionylated HbS	HbS_{1d3}	$\alpha_2\beta_2{}^S{}_{SSG}$	$\beta^S{}_{SSG}$: 16142 [16143]
Glutathionylated HbC	HbC_{1d3}	$\alpha_2\beta_2{}^C{}_{SSG}$	$\beta^C{}_{SSG}$: 16171 [16172]
Glutathionylated HbD	HbD_{1d3}	$\alpha_2\beta_2{}^D{}_{SSG}$	$\beta^D{}_{SSG}$: 16171 [16172]
Glutathionylated HbE	HbE_{1d3}	$\alpha_2\beta_2{}^E{}_{SSG}$	$\beta^E{}_{SSG}$: 16171 [16172]
Glutathionylated HbO	HbO_{1d3}	$\alpha_2\beta_2{}^O{}_{SSG}$	$\beta^O{}_{SSG}$: 16171 [16172]
Glutathionylated HbF, pos 136: Gly	HbF_{1d3}	$\alpha_2\gamma_2{}^G{}_{SSG}$	$\gamma^G{}_{SSG}$: 16300 [16301]
Glutathionylated HbF, pos 136: Ala	HbF_{1d3}	$\alpha_2\gamma_2{}^A{}_{SSG}$	$\gamma^A{}_{SSG}$: 16314 [16315]

Da[1] stands for Dalton and is an atomic mass unit, while Th[2] stands for Thomson and is a mass-to-charge ratio unit.

Another limitation in respect with the specificity of the *m/z* value, is that the *m/z* value of the apo-$\beta^X{}_{SSG}$-subunit if X = A, C, D, E or O in theory is very similar or identical to the *m/z* value of the apo-$\gamma^A{}_{glyc}$-subunit of HbF_{1c} (table 1). In practice, we observed in case of homozygotes for HbS combined only with %HbF > 15%, that indeed detectable signals could be found in the range at *m/z* 16172-16173. As no HbA due to for example blood transfusions was detected in those homozygotes for HbS and consequently no HbA_{1d3} could be present, it must be attributed to the apo-$\gamma^A{}_{glyc}$-subunit of HbF_{1c}. Accordingly, in the case of an elevated HbF combined with a heterozygote or homozygote for HbA, in which both HbF_{1c} and HbX_{1d3} can be expected, the specificity of the *m/z* value may be insufficient. As we only observed the apo-$\gamma^A{}_{glyc}$-subunit of HbF_{1c} if %HbF > 15%, we established the %HbF cut-off value for specificity of $\%HbX_{1d3}$ as 15%. This implies that the $\%HbX_{1d3}$ MALDI-TOF-MS method lacks specificity if %HbF > 15% and if X = A, C, D, E or O in the case of a heterozygote or of a homozygote for HbX with an unknown genotype.

Accuracy, repeatability and reproducibility

Because the assignment of a peak in the mass spectrum to HbX_{1d3} is purely based on the *m/z* value, the first part of the validation was focussed on the absolute *m/z* value of the apo-$\beta^A{}_{SSG}$-subunit of HbA_{1d3} in the QC sample. The internal calibration proved to be accurate and robust, as the mass accuracy ± SD for the apo-$\beta^A{}_{SSG}$-subunit was *m/z* 16173.4 ± 0.1 (theoretical *m/z* 16173.3) [n = 14], while repeatability [n = 5] and reproducibility [n = 14] were < 0.0008%.

The second part of the validation concerned the reproducibility of $\%HbX_{1d3}$, which in the QC sample was 8.3% [n = 14]. The absolute $\%HbA_{1d3}$ was 2.5% ± 0.2 [n = 14], which in our case was in the low range of measured values (figure 1). Compared to literature, our reproducibility of $\%HbA_{1d3}$ is according to the state-of-the-art of MALDI-TOF-MS analysis (1).

Example of applications

In order to present examples in application areas, we measured profiles of apo-$\beta^X{}_{SSG}$-subunits in subjects with suspected pathological Hb disorders [n = 21] (figure 1A)

and in subjects participating within a project studying oxidative stress in Chronic Obstructive Pulmonary Disease [n = 41] (figure 1B). The suspicion for pathological disorders was based on observed abnormal Hb profiles after cation exchange high-performance liquid chromatography analysis combined with ultraviolet detection. The overall frequency histogram of $\%HbX_{1d3}$ (figure 1C) of this heterogeneous population [n = 62] showed besides one extreme outlier at 12.9% in the Hb disorder group, two distinct populations (frequency tops at 4.5% and 6.3%, respectively). Two samples of subjects belonging to suspected Hb disorders were excluded because %HbF > 15%, the cut-off value for specificity of our $\%HbX_{1d3}$ MALDI-TOF-MS method. The exact interpretation of these physiological and pathological results is still in progress and will be published elsewhere.

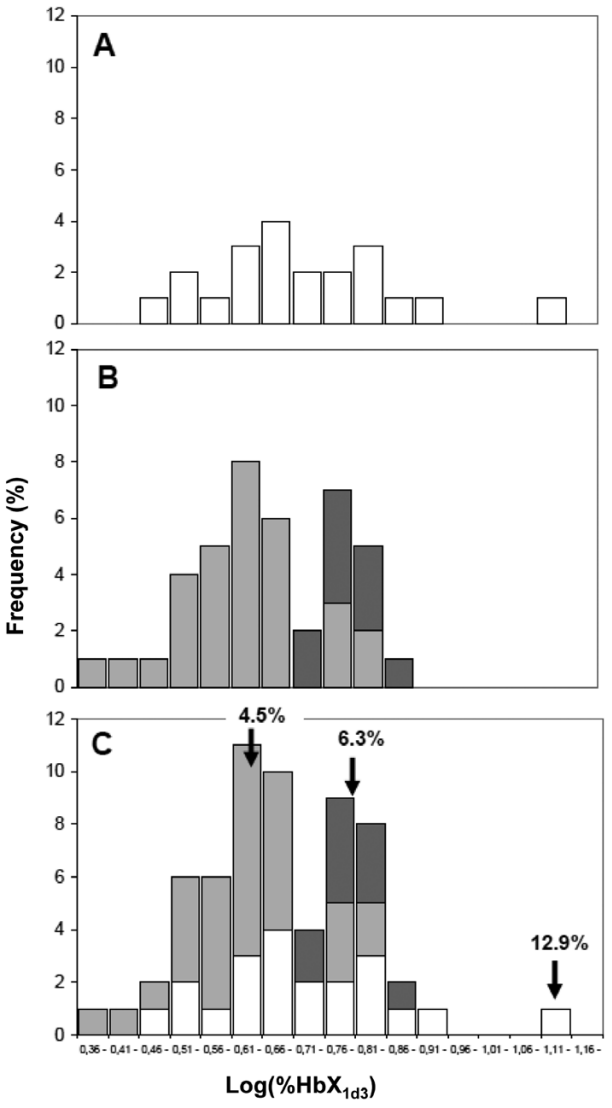

Figure 1. Frequency histogram of logarithmic transformed $\%HbX_{1d3}$ A) in subjects with suspected pathological haemoglobin (Hb) disorders, B) in subjects participating within a project studying oxidative stress in Chronic Obstructive Pulmonary Disease (COPD) and C) of all subjects involved.
□ Hb disorders; ■ COPD control subjects; ■ COPD patients.

An important challenge for the future will be the definition of $\%HbX_{1d3}$ as only recent insight into its formation is available (8) and thus far no consensus description exists for the sample preparation (9) as well as for the calculation of $\%HbX_{1d3}$ (1, 3, 6). Assuming that $\%HbX_{1d3}$ in fact correlates with oxidative stress and is independent from the definition of $\%HbX_{1d3}$, it is obvious that MALDI-TOF-MS may be an adequate analytical tool to measure $\%HbX_{1d3}$.

Conclusions

A MALDI-TOF-MS method was developed and validated to distinguish the Hb apo-β^{X}_{SSG}-subunit from other apo-subunits. Profiles of apo-β^{X}_{SSG}-subunits indicated two populations for $\%HbX_{1d3}$, which apparently were subjected to distinct kind of oxidative stress. Also observed was an outlier, which actually must have been subjected to extreme oxidative stress.

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