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Determination of Glycated and Acetylated Hemoglobins in Cord Blood by Time-of-Flight Mass Spectrometry

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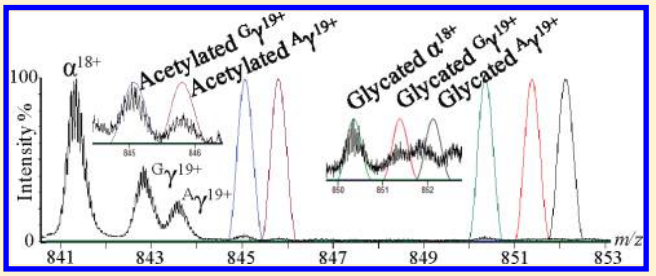
Supporting Information

ABSTRACT: The characterization of cord blood hemoglobin at the molecular level is a daunting challenge because hemoglobin F (HbF) and hemoglobin A (HbA) coexist in neonatal blood. We developed and validated a method using electrospray time-of-flight mass spectrometry (ES-TOF-MS) that measures, in a single analysis, relative levels of glycated and acetylated hemoglobin and allows the calculation of relative proportions of HbA, HbF_0, and HbF_1 in cord blood. Specific sections of acquired spectra were deconvoluted using a maximum entropy-based approach to true mass scale spectra . Mass precisions were less than 3 ppm with similar accuracies. Intra−interday precisions for α- and γ-chain glycation levels were 2.10%/3.72% and 2.75%/6.79%, respectively. The linearity of the α-chain glycation response was excellent ($r^2 = 0.9990$). We performed sample analysis on 39 cord blood specimens and found that the glycated α- and γ-chain levels were 2.27 ± 0.21% and 2.38 ± 0.29%, respectively, while the acetylated $^{G}\gamma$ and $^{A}\gamma$-chain levels were 8.48 ± 0.53% and 7.14 ± 0.74%, respectively. We observed three types of HbF distinguishable by the intensities of γ-chain variants. Two-thirds of cord blood specimens were classified as HbF^{I} with an intensity ratio $^{G}\gamma/^{A}\gamma$ of 1.90 ± 0.12. For HbF^{II} type (10/39 neonates), the intensity ratio of $^{G}\gamma/^{A}\gamma$ was 3.71 ± 0.28. For three neonates with HbF^{III}, no $^{A}\gamma$-chain was detected.



Gestational and unidentified pregestational diabetes mellitus (GDM and pGDM) are major worldwide public health problems. Their prevalence, using the newest American Diabetes Association (ADA) screening and diagnosis thresholds, was recently estimated at 9.4% of pregnancies in Caucasian women and up to 38% in Arab and Indian women.[1] The lifetime risk of obesity, type 2 diabetes, prediabetes, or cardiometabolic diseases is significantly increased in offspring of women who had GDM and pGDM.[2] This seems to be linked to the fetal environment, which plays an etiologic role in the development of several cardiometabolic diseases, according to the so-called "fetal programming" theory.[3] However, no biomarker is available to evaluate its impact.

Professional associations recommend routine measurement of glycated hemoglobin (GHb) in subjects with diabetes because it reflects the average blood glucose over the preceding three months (adult Hb life span is 120 days).[4] The measurement of GHb in cord blood at delivery is a potential biomarker of fetal exposure to glucose during the last 4−8 weeks of gestation (fetal Hb life span is 60−80 days).[5]

Hemoglobin A (HbA), composed of two α- and two β-chains, is the predominant form of hemoglobin in adults, whereas hemoglobin F (HbF_0), composed of two α- and two γ-chains ($^{G}\gamma$ and $^{A}\gamma$ variants), is the predominant form in newborns. At about 32−36 weeks of gestation, the proportion of HbA rises (increase in β-chain synthesis) while the fetal form declines (decrease in γ-chain).[6] From a molecular point of view, cord blood hemoglobin is a mixture of α-, β-, and γ- subunits and glycation may occur on all of them. Therefore, the amount of HbA and HbF_0 is variable in neonates, and the measurement of GHb in cord blood must be selective in order to determine the glycation levels of the different hemoglobin chains. Moreover, acetylated HbF_0, an enzymatic post-translational modification, referred to as HbF_1, is present in significant amounts (11.6 ± 2.8%) in cord blood and needs to be taken into account during measurements of GHb.[7] Koga et al.[8] analyzed cord blood samples to evaluate the possibility of measuring GHb chains using various techniques: high performance liquid chromatography, latex-immunoturbidimetry, and enzymatic and affinity methods. They demonstrated that current methods are not suitable for GHb measurements in cord blood, because of a lack of selectivity.

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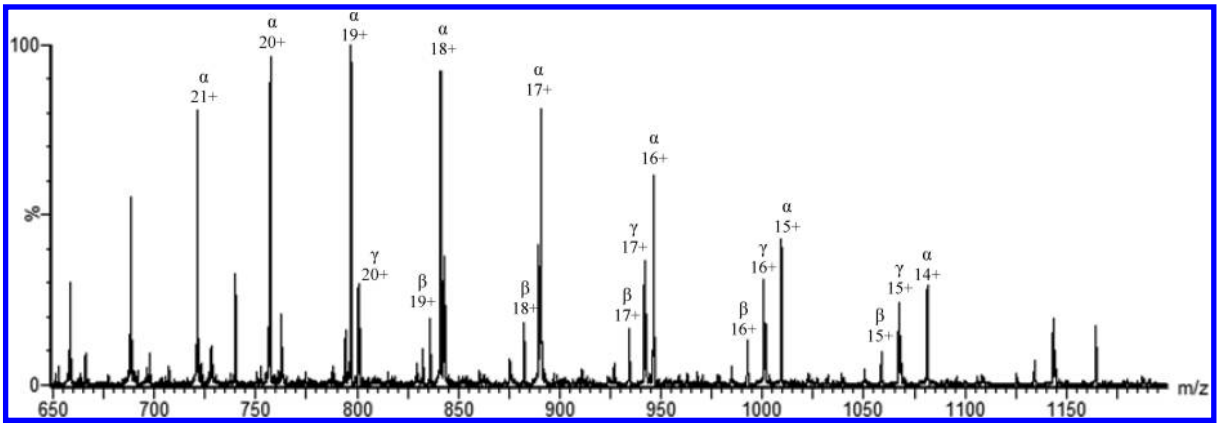

Figure 1. Mass spectrum of cord blood hemoglobin: m/z 650−1200.

On the other hand, Davison et al. reported that electrospray ionization mass spectrometry (ESI-MS) may be useful for the estimation of glycated and acetylated Hb levels in cord blood.[9] However, the linearity of the response, selectivity, and data deconvolution processing were not clearly established.

The objectives of our study were to develop and validate a method to measure glycated and acetylated hemoglobin relative levels in umbilical cord blood by electrospray time-of-flight mass spectrometry (ES-TOF-MS) and to determine relative proportions of HbA, HbF_0, and HbF_1. Moreover, we also report and emphasize the different steps required for the deconvolution process, because incorrect data analysis could lead to erroneous interpretation of spectra and false results. Finally, we performed the analysis of 39 cord blood specimens to corroborate our method validation.

EXPERIMENTAL SECTION

Ethics Approval. This project was approved by the Research Ethics Board (REB) of the Faculty of Medicine and Health Sciences and the Centre hospitalier universitaire de Sherbrooke (CHUS).

Sample Collection. After informed consent was obtained from diabetic (n = 19) and nondiabetic women (n = 20), umbilical venous cord blood specimens of term neonates (>37th weeks of gestation) were collected immediately after birth in BD Vacutainer tubes (13 mm × 75 mm × 2.0 mL, Fisher Scientific, Montreal, QC, Canada) with sodium fluoride/Na_2EDTA as an additive. Within 30 min of specimen collection, samples were homogenized by shaking and stored in multiple aliquots at −80 °C until analysis.

Reagents. LC-MS grade acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ, U.S.A.), and 99.9+% formic acid was purchased from Acros (Morris Plains, NJ, U.S.A.). Cation exchange resin Dowex 50WX8 hydrogen form (100−200 mesh) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Water was purified to ultrapure grade using a Nanopure Infinity water purification system (Ultrapure, 18.3 MΩ, Barnstead, Dubuque, IA, U.S.A.).

Cation Exchange Resin Preparation. The resin was washed prior to use as follows: 0.5 g of cation exchange resin Dowex 50WX8 hydrogen form (100−200 mesh) was deposited in microcentrifuge tubes, and mixed for 30 s with 1 mL of water. The aqueous supernatant was removed with a micropipet after the beads had deposited in the tube. The procedure was performed twice.

Sample Preparation. Whole cord blood (20 μL) was diluted 50-fold to 1000 μL with deionized water and mixed by vortex until a clear solution was obtained; 25 μL of the resulting solution was diluted with 475 μL of acetonitrile−water (5:4) containing 0.2% (v/v) of formic acid. The final solution was treated with ∼20 mg of cation exchange resin by shaking for approximately 20 s prior to injection in the mass spectrometer. This latter step is critical in order to minimize sodium and potassium adducts and to ensure robust measurements.[9]

Mass Spectrometry. Analyses were performed on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (QTOF-MS) (SYNAPT MS, Waters Corporation, Milford, MA, U.S.A.) using an electrospray ionization source working in positive mode and W-Optics. The system was calibrated with a solution of 0.5 M of sodium formate in 2-propanol−water (9:1) over the m/z range 600−1400. Samples were injected in the system at a constant flow rate of 5 μL/min using a built-in syringe pump. After signal stability was obtained, data were acquired over 3 min in a continuum mode with a scan time of 1 s over the m/z range 600−1400. Voltages for the capillary, sampling cone, and extraction cone were set to 2.1 kV, 30 V, and 5 V, respectively. Desolvation temperature and flow rate were set to 200 °C and 700 L/h, respectively. These parameters allow signal response between 90 and 100 counts/s at m/z 797, corresponding to the $[\alpha + 19H]^{19+}$ ion. To obtain reliable signal-to-noise spectra, acquired data were combined over a 3 min window. Background was subtracted with polynomial order set to 90, below curve set to 2%, and tolerance set to 0.01%. A typical mass spectrum of cord blood hemoglobin is shown in Figure 1.

Deconvolution. Specific spectrum ranges (Figure 2) were deconvoluted (MaxEnt 1 Software, Waters Corporation) onto a true mass scale spectrum using the following output parameters: resolution 0.05 Da/channel and simulated isotope pattern damage model with a spectrometer blur width set to 0.2 Da. The minimum intensity ratio left/right was 70%/70%. The resulting MaxEnt spectra were then smoothed (2 × 3 Da, Savitsky−Golay smooths) and centered (top 90%, area). The peak at 16071.74 Da in Figure 2c represents the deconvolution of the $[\alpha + 16H]^{16+}$ ion at m/z 946.4. This represents a false assignation of the α-chain in the MaxEnt spectrum as a result of the selected output mass (15800−16400 Da), only specific to the γ-chain.

In the MaxEnt spectrum, we targeted the following calculated Hb chain average masses from the protein sequence according to the relative atomic mass of each element (C = 12.0110, H = 1.0079, N = 14.0067, O = 15.9994, and S = 32.066): α = 15 126.3807 ($C_{685}H_{1071}N_{187}O_{194}S_3$); β = 15 867.2406 ($C_{724}H_{1119}N_{195}O_{201}S_3$); $^{G}\gamma$ = 15 995.2736 ($C_{723}H_{1127}N_{193}O_{211}S_3$); $^{A}\gamma$ = 16 009.3005

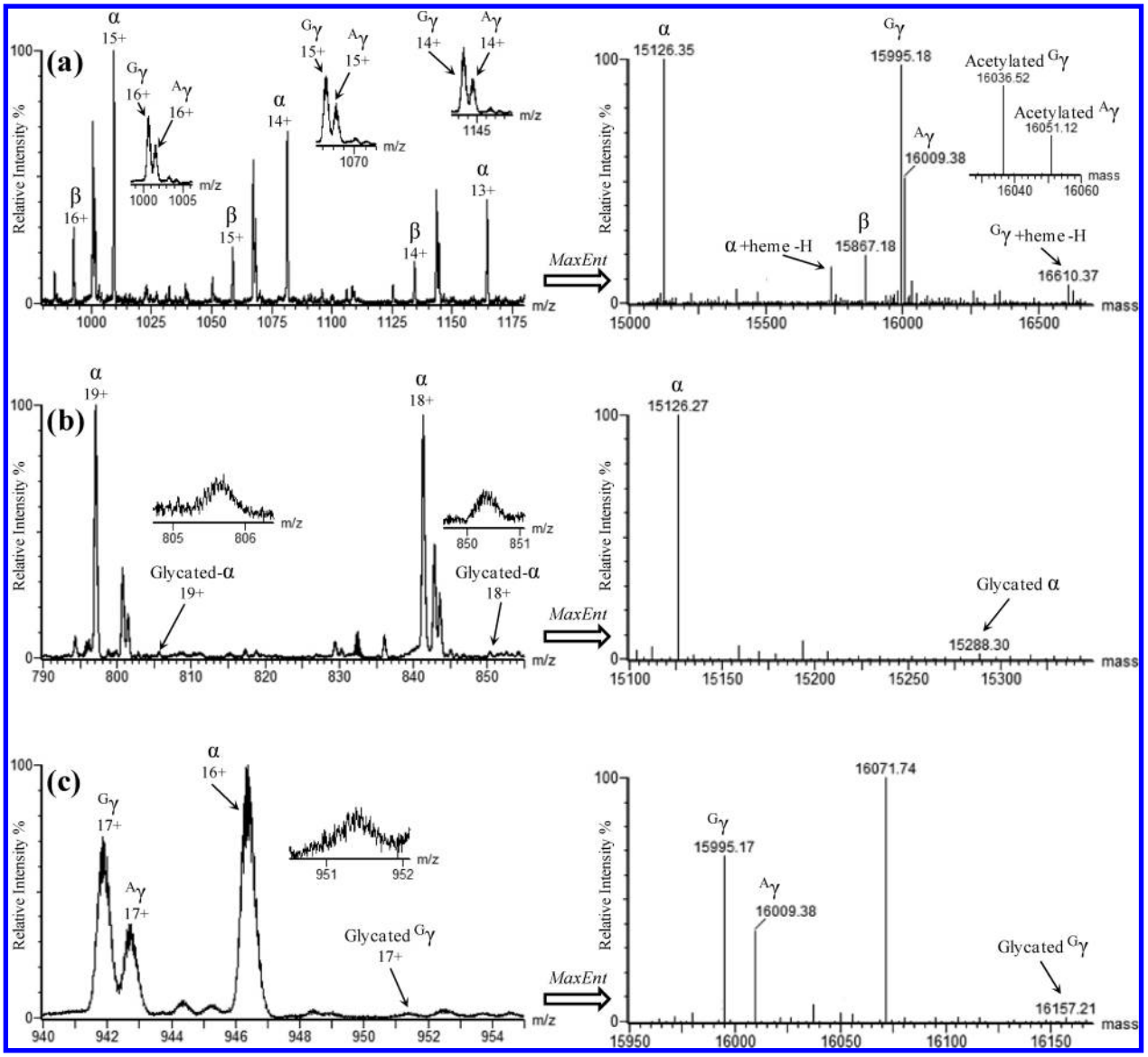

Figure 2. Specific TOF mass spectrum regions (left section) of cord blood hemoglobin selected for the determination of the following: (a) mass measurements, relative proportions of HbA, HbF_0, HbF_1, and γ-chain acetylation level; (b) α-chain glycation level; (c) γ-chain glycation level. These ranges were chosen to avoid signal interferences. Representative centroid (average masses) MaxEnt spectra are shown on the right section.

($C_{724}H_{1129}N_{193}O_{211}S_3$); glycated α = 15 288.5231 ($\alpha + C_6H_{10}O_5$); glycated $^{G}\gamma$ = 16 157.4160 ($^{G}\gamma + C_6H_{10}O_5$); acetylated $^{G}\gamma$ = 16 037.3109 ($^{G}\gamma + C_2H_2O$); acetylated $^{A}\gamma$ = 16 051.3378 ($^{A}\gamma + C_2H_2O$).[10] The reported experimental masses correspond to the average mass of the protein. In order to obtain full accuracy and precision, the masses of the final centroid MaxEnt spectra were corrected by calibrating the α-chain equal to its sequence mass (15 126.3807 Da). The glycated α- and γ-chain (Glα and Glγ) levels, the acetylated γ-chain ($Ac^{G}\gamma$ and $Ac^{A}\gamma$) levels (percent ion signals), and the relative proportions of hemoglobin types (HbA, HbF_0, and HbF_1) in cord blood were calculated according to the specific formulas described below. A summary of processing parameters, formulas (Table S-1), and theoretical masses calculated from the protein sequence (Table S-2) are presented as Supporting Information.

Relative Proportions of HbA, HbF_0, HbF_1. Ranges corresponding to m/z 980−1180 were deconvoluted using MaxEnt 1, with the output mass range 15 000−16 700 (Figure 2a). HbA, HbF_0, and HbF_1 proportions are measured as a fraction of total Hb by using the peak intensity (I) of the corresponding Hb chain according to the following equations:

$$\%\mathrm{HbA} = I\beta/(I\beta + I^{A}\gamma + I^{G}\gamma + I\mathrm{Ac}^{A}\gamma + I\mathrm{Ac}^{G}\gamma)100 \quad (1)$$

$$\%\mathrm{HbF_0} = I^{A}\gamma + I^{G}\gamma/(I\beta + I^{A}\gamma + I^{G}\gamma + I\mathrm{Ac}^{A}\gamma + I\mathrm{Ac}^{G}\gamma)100 \quad (2)$$

$$\%\mathrm{HbF_1} = I\mathrm{Ac}^{A}\gamma + I\mathrm{Ac}^{G}\gamma/(I\beta + I^{A}\gamma + I^{G}\gamma + I\mathrm{Ac}^{A}\gamma + I\mathrm{Ac}^{G}\gamma)100 \quad (3)$$

It is not necessary to include the α-chain in the calculation since HbA is defined by a 1:1 α/β dimer, HbF is defined by a 1:1 α/γ dimer, and HbF_1 is essentially the γ acetylated counterpart.

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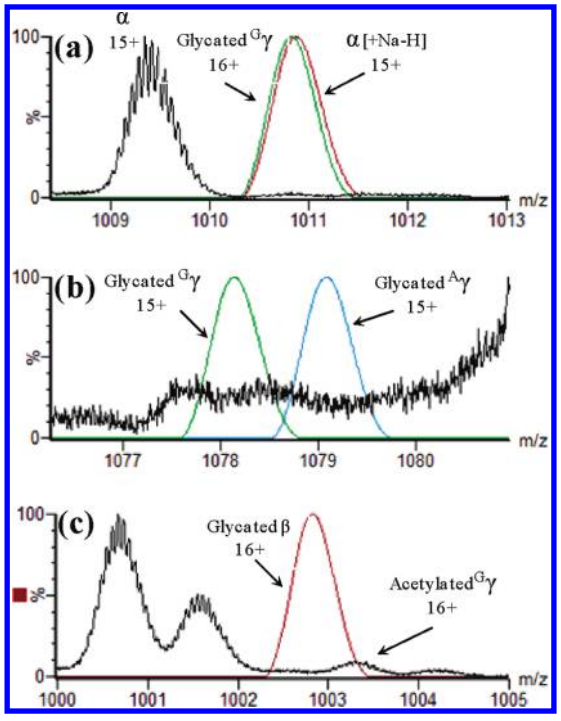

Figure 3. Isotope-modeling peaks (smoothed lines) reveal possible interferences that can lead to false results: (a) glycated- $^{G}\gamma$ signal overlaps with the sodium adduct of the α-chain; (b) interferences lead to an unresolved glycated γ-chain signal; (c) a glycated β-chain signal partially overlaps with the acetylated $^{G}\gamma$-chain.

$^{G}\gamma$- and $^{A}\gamma$-Chain Acetylation Levels. Ranges corresponding to m/z 980−1180 were deconvoluted using MaxEnt 1, with the output mass range 15000−16700 (Figure 2a). Percentages of acetylated $^{G}\gamma$- and $^{A}\gamma$-chains ($Ac^{G}\gamma$ and $Ac^{A}\gamma$ %) were calculated by using the peak intensity (I) of the corresponding Hb chain according to these equations:

$$\%Ac^{G}\gamma = IAc^{G}\gamma/(I^{G}\gamma + IAc^{G}\gamma)100 \quad (4)$$

$$\%Ac^{A}\gamma = IAc^{A}\gamma/(I^{A}\gamma + IAc^{A}\gamma)100 \quad (5)$$

α-Chain Glycation Levels. Ranges corresponding to m/z 790−855 were deconvoluted with MaxEnt 1 software, with the output mass range 15 000−15 400 (Figure 2b). The percentage of α-chain glycation (Glα %) was calculated by using the peak intensity (I) of the corresponding Hb chain according to the following equation:

$$\%Gl\alpha = I\alpha/(I\alpha + IGl\alpha)100 \quad (6)$$

γ-Chain Glycation Levels. Ranges corresponding to m/z 940−955 were deconvoluted with MaxEnt 1, with an output mass range 15 800−16 400 (Figure 2c). The percentage of glycated hemoglobin $^{G}\gamma$-chain ($Gl^{G}\gamma$ %) was calculated by using the peak intensity (I) of the corresponding Hb chain according to this equation:

$$\%Gl^{G}\gamma = IGl^{G}\gamma/(I^{G}\gamma + IGl^{G}\gamma)100 \quad (7)$$

RESULTS AND DISCUSSION

MaxEnt produces relative intensity data providing a zero-charge spectrum in which areas under the peaks are a measure of the sum of the intensities of the peaks in the original multiply charged spectrum. Using the known amino acid sequences[10] of HbA and HbF_0 subunits (α, β, $^{G}\gamma$, $^{A}\gamma$), theoretical spectra were created using an isotope modeling tool (MassLynx). These spectra-models were then compared with recorded multiple-charged spectra, enabling the identification of each Hb chain. We extended this strategy to the modified Hb chains by the addition of one glucose moiety (glycation, $+C_6H_{12}O_6-H_2O$, 162.14 Da) or one acetyl moiety (acetylation, $+C_2H_2O$, 42.04 Da) to the chemical composition of α-, β-, $^{G}\gamma$-, and $^{A}\gamma$-chains. By overlaying the created spectrum with the original m/z spectrum, we confirmed the position of the multiple-charged ions. Careful evaluation of the multiple-charged data is essential to ensure selectivity and to avoid false results. In fact, the quality of the deconvolution of the results depends mainly on the selected range in the original multiply charged spectrum. For example, the isotope-modeling reveals that the selected m/z 980−1180 range published by Davison et al.[9] was not appropriate to determine α- and γ-chain glycation because of the presence of interferences as seen in Figure 3. Even after desalting, we observed sodium adducts of the α-chain (~2%, data not shown). This leads experimentally to possible interference of the $[\text{glycated } ^{G}\gamma + 16H]^{16+}$ ion at m/z 1010.84 by the $[\alpha + Na + 14H]^{15+}$ ion at m/z 1010.90 and will eventually overestimate the $^{G}\gamma$-chain glycation (Figure 3a). Moreover, defined peaks for glycated $^{G}\gamma$ and $^{A}\gamma$ chains are not observed in the selected range as a result of unresolved interferences (Figure 3b). The isotope-modeling also reveals that it is not possible to measure adequately the β-chain glycation (Figure 3c), as partial overlap between the modeled $[\text{glycated } \beta + 16H]^{16+}$ ion at m/z 1002.84 and the experimental $[\text{acetylated } ^{G}\gamma + 16H]^{16+}$ ion at m/z 1003.34 is observed. In addition, a possible peak corresponding to the $[^{A}\gamma + Na + 15H]^{16+}$ ion at m/z 1002.96 overlaps with the glycated β-chain ion (modeling not shown). We found that no area of the spectrum was free of interference for the glycated β-chain.

Regarding the determination of the glycated α-chain levels (%), we obtained the best results by deconvoluting the m/z range from 790 to 855 (Figure 2b). This range includes essentially $[\alpha + 18H]^{18+}$ and $[\alpha + 19H]^{19+}$ ions. The percentage of glycated γ-chain was only determined on the most abundant $^{G}\gamma$ variant by deconvoluting the range m/z 940−955 corresponding to the $[^{G}\gamma + 17H]^{17+}$ ion (Figure 2c), whereas, for the $^{A}\gamma$ variant, we found by isotope-modeling that glycation m/z signals are not free of interferences in the multiply charged spectrum.

Method Validation. In order to evaluate the overall quality of our analytical method, we performed precision, accuracy, selectivity, and linearity assessments. The precision of the assay was calculated by repeating measurements (intraday, n = 5 and interday, n = 10) of the same sample.

Interday mass measurements (mean ± 1SD) for β-, $^{G}\gamma$-, and $^{A}\gamma$-chains were 15 867.191 ± 0.048 Da (±3.0 ppm), 15 995.200 ± 0.016 Da (±1.0 ppm), and 16 009.415 ± 0.016 Da (±1.0 ppm), respectively. These mass measurements were considered to be accurate, as the errors (Da/ppm) observed were small: β-chain (−0.049/−3.1); $^{G}\gamma$-chain (−0.074/−4.6); and $^{A}\gamma$-chain (+0.114/+7.1). Intraday and interday coefficients of variation (CVs) for relative proportions (%) of HbA were 1.8%/2.3%. Intraday and interday CVs for glycated α- and $^{G}\gamma$-chain levels were 2.10%/3.72% and 2.75%/6.79%, respectively. Finally,

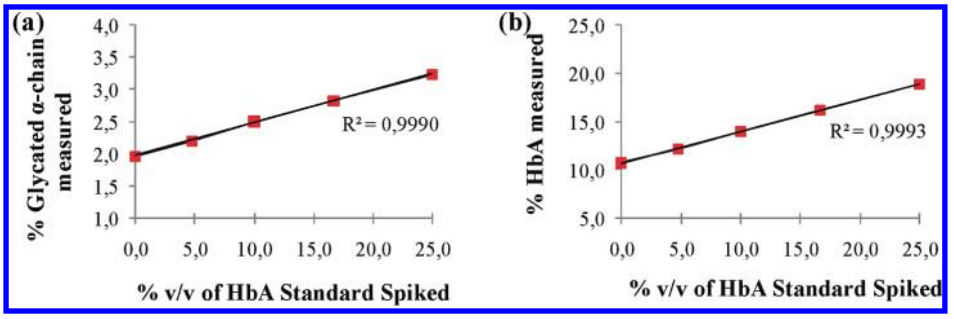

Figure 4. Linearity assessments in the determination of (a) glycated α-chain levels (%) and (b) the relative % of HbA in cord blood. The *x*-axis corresponds to the calculated volume of the HbA standard solution added to the HbF solution (20:1, 9:1, 5:1, and 3:1 (HbF−HbA) equivalent to an addition of 4.8, 10.0, 16.7, and 25% v/v of HbA, respectively).

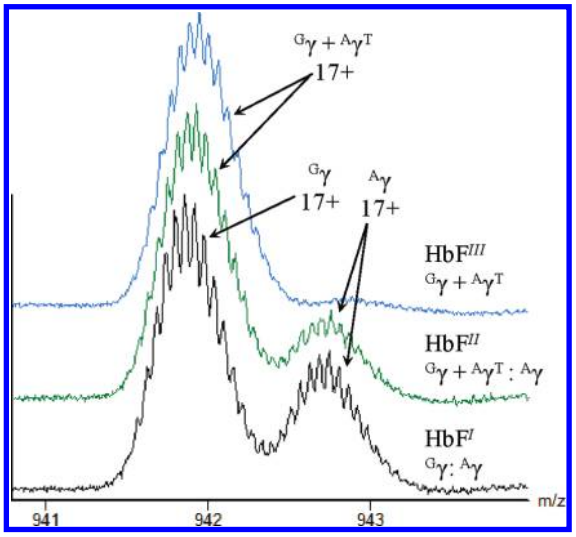

Figure 5. Overlaid representative spectra for three types of HbF (I, II, III) detected in 39 cord blood specimens. The difference in the apparent intensity ratio of $^{G}\gamma$- and $^{A}\gamma$-chains is due to the substitution of $^{A}\gamma$ by $^{A}\gamma^{T}$ ($^{A}\gamma$75Ile → Thr).

intraday and interday CVs for acetylated $^{G}\gamma$- and $^{A}\gamma$-chains were 2.66%/4.18% and 4.26%/6.27%, respectively.

We tested selectivity and linearity responses for the α-chain glycation level and the relative proportion of HbA (Figure 4). With a certified glycated HbA sample (A1c 14−17%, Bio-Rad Laboratories, Montréal, Canada), we did four different cross-dilutions (20:1, 9:1, 5:1, and 3:1, v/v) using a cord blood sample and the HbA standard sample. Linearity for the α-chain glycation level over five measurements (unspiked cord blood sample included) was excellent (R^2 = 0.9990) (Figure 4a). Similarly, measurement of the increase of the HbA proportion (Figure 4b) showed good linear regression data (r^2 = 0.9993). These results also demonstrate that the variation of HbA and HbF amounts in cord blood does not affect linearity and selectivity. This is of major importance because the HbF level in cord blood varies from 65 to 90% for term neonates.[6]

Sample Analysis. Mass measurement of Hb chains, levels (%) of α- and γ-chain glycation, and levels (%) of $^{G}\gamma$- and $^{A}\gamma$-chain acetylation were determined in 39 cord blood specimens. In addition, proportions of HbA, HbF_0, and HbF_1 in cord blood were also calculated. When applicable, data are reported as mean ± 1SD.

We observed three types of HbF (Figure 5), referred to as HbF^{I}, HbF^{II}, and HbF^{III}, distinguishable by the ratio of γ-chain variant intensities. This ratio is influenced by the presence of the less common but normal variant $^{A}\gamma^{T}$. Table 1 summarizes mass measurements and intensities related to the γ-chain for 39 cord blood samples from neonates. Two-thirds (26/39 neonates) of cord blood specimens analyzed were classified as HbF^{I} with a mean intensity ratio $^{G}\gamma/^{A}\gamma$ of 1.90 ± 0.12; the mean masses measured were 15 995.213 ± 0.020 Da for $^{G}\gamma$ and 16 009.420 ± 0.021 Da for $^{A}\gamma$. For HbF^{II} type (10/39 neonates), the mean intensity ratio of $^{G}\gamma/^{A}\gamma$ (3.71 ± 0.28) was higher than that in HbF^{I} type and the mean mass measured of $^{G}\gamma$ (15 995.564 ± 0.026 Da) was significantly higher. For HbF^{III} type (3/39 neonates), no $^{A}\gamma$-chain was detected but only single peaks at 15 995.835, 15 995.827, and 15 995.831 Da for the three samples analyzed. These observations are in agreement with the results reported by Davison et al.[9] where the $^{A}\gamma$-chain of HbF^{I} is replaced by the $^{A}\gamma^{T}$-chain ($^{A}\gamma$75Ile → Thr). The calculated masses of $^{G}\gamma$ (15 995.2736 Da) and $^{A}\gamma^{T}$ (15 997.2461 Da) only

Table 1. Experimental Masses for Hb Chains and Relative Intensities Classified According to Their HbF Types (I, II, III).

	HbF^{I} n = 26			
	mean molecular masses			intensity ratio
	β	$^{G}\gamma$	$^{A}\gamma$	$^{G}\gamma/^{A}\gamma$
mean	15867.208	15995.213	16009.420	1.90
SD	0.036	0.020	0.021	0.12
CV %	0.0002	0.0001	0.0001	6.27
	HbF^{II} n = 10			
	mean molecular masses			intensity ratio
	β	$^{G}\gamma+{}^{A}\gamma^{T}$	$^{A}\gamma$	$^{G}\gamma+{}^{A}\gamma^{T}/^{A}\gamma$
mean	15867.200	15995.564	16009.454	3.71
SD	0.025	0.026	0.034	0.28
CV %	0.0002	0.0002	0.0002	7.53
	HbF^{III} n = 3			
	mean molecular masses			
	β	$^{G}\gamma+{}^{A}\gamma^{T}$		
mean	15867.200	15995.831		
SD	0.053	0.004		
CV %	0.0003	0.00002		

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Table 2. Proportions of Hemoglobin Types Determined Experimentally in 39 Cord Blood Specimens Reported as Mean (% ± 1SD) (*Range*).

	HbA	HbF_0	HbF_1
HbF^{I} (n = 26)	13.5 ± 3.7 *(8.5−23.5)*	79.5 ± 3.3 *(70.8−84.5)*	7.0 ± 0.6 *(5.7−8.0)*
HbF^{II} (n = 10)	15.9 ± 5.9 *(8.0−27.1)*	77.5 ± 5.6 *(66.7−85.6)*	6.6 ± 0.5 *(6.0−7.4)*
HbF^{III} (n = 3)	16.5 ± 4.5	77.6 ± 3.9	7.1 ± 0.5
total	14.2 ± 4.4 *(8.5−27.1)*	78.8 ± 4.0 *(66.7−85.6)*	6.9 ± 0.6 *(5.7−8.0)*

Table 3. Glycated and Acetylated Levels (% ± 1SD) of α- and γ-Chain Hemoglobin for 39 Cord Blood Specimens Classified According to Their HbF Types (I, II, III)

	glycated-α	HbF^{I} n = 26 glycated-$^{G}\gamma$	acetylated-$^{G}\gamma$	acetylated-$^{A}\gamma$
mean level	2.28 ± 0.24	2.44 ± 0.31	8.60 ± 0.56	7.27 ± 0.68
level range	(1.90−2.71)	(1.96−3.09)	(7.62−9.45)	(5.75−8.34)
$m_1 - m_0$[a]	162.03 ± 0.10	161.62 ± 0.63	41.32 ± 0.11	41.80 ± 0.17
	glycated-α	HbF^{II} n = 10 glycated-$^{G}\gamma$+$^{A}\gamma^{T}$	acetylated-$^{G}\gamma$+$^{A}\gamma^{T}$	acetylated-$^{A}\gamma$
mean level	2.21 ± 0.16	2.26 ± 0.25	8.19 ± 0.43	6.80 ± 0.84
level range	(2.01−2.46)	(1.97−2.82)	(7.37−8.93)	(5.38−8.08)
$m_1 - m_0$[a]	162.12 ± 0.18	161.61 ± 0.51	41.52 ± 0.12	42.22 ± 0.32
	glycated-α	HbF^{III} n = 3 glycated-$^{G}\gamma$+$^{A}\gamma^{T}$	acetylated-$^{G}\gamma$+$^{A}\gamma^{T}$	
mean level	2.37 ± 0.12	2.34 ± 0.05	8.37 ± 0.29	
$m_1 - m_0$[a]	162.15 ± 0.13	162.04 ± 0.34	41.63 ± 0.11	
		Total n = 39		
mean % ± SD	2.27 ± 0.21	2.38 ± 0.29	8.48 ± 0.53	7.14 ± 0.74
range	(1.90−2.71)	(1.96−3.09)	(7.37−9.45)	(5.38−8.34)
$m_1 - m_0$[a]	162.06 ± 0.13	161.65 ± 0.59	41.40 ± 0.16	41.92 ± 0.16

[a] $m_1 - m_0$ = mass difference between modified (m_1) and native (m_0) chains.

differ by 2 Da and are not resolved in the multiply charged spectrum; therefore, the experimental masses of HbF^{II} and HbF^{III} represent the abundance-weighted mean of the two chains.[11] The average measured mass for the β-chain was 15 867.205 ± 0.034 Da.

Our methodology using ES-TOF-MS exhibited excellent mass precision and accuracy. Compared to results from Davison et al.[9] obtained by ESI-MS, the mass precision achieved using the QTOF-MS was twice the value obtained for the $^{G}\gamma$-chain with a SD on the mean measured mass of 0.020 Da, 5-fold that for the $^{A}\gamma$-chain with a SD of 0.021 Da, twice that for $^{G}\gamma$+$^{A}\gamma^{T}$-chain with a SD of 0.026 Da, and almost 6-fold that for the $^{A}\gamma$-chain with a SD of 0.034 Da. The coefficient of variation (<0.0003%) was also superior to MALDI-TOF MS results already published (0.005%).[11,12]

HbA, HbF_0, and HbF_1 in Cord Blood. A summary of the proportions of different types of hemoglobin determined experimentally in 39 cord blood specimens is presented in Table 2. The HbF_0, HbA, and HbF_1 mean levels were 78.8 ± 4.0, 14.2 ± 4.4, and 6.9 ± 0.6 (% ± SD), respectively. In a previous study, using an isoelectric focusing (IEF) method, the HbF_0, HbA, and HbF_1 mean levels were 70.1, 20.2, and 9.5% for 30 term infants.[13] Using a HPLC method, in eight cord blood samples from neonates born at 38 weeks of gestation, Shiao et al. found levels of HbF_0, HbA, and HbF_1 to be 73.0 ± 3.8, 15.3 ± 1.4, and 11.6 ± 2.8, respectively.[7] It is important to mention that our reported values are not quantitative but relative-based. The MS signal response is not necessarily equimolar for the α-, β-, and γ-chains, as their respective amino acid sequences are different. However, the method described here has excellent potential for measurements of hemoglobin variant proportions. With proper standards, we think that quantitative data can be obtained.

γ-Chain Acetylation. It is well-known that a variable fraction of the γ-chain is acetylated at its N-terminus glycine.[14] Acetylation of HbF_0, also referred to as HbF_1, seems to modulate the strength of its subunit interaction.[15] The levels (%) of γ-chain acetylation measured on $^{G}\gamma$- and $^{A}\gamma$-chains are summarized in Table 3. We found in 39 cord blood specimens that the acetylated $^{G}\gamma$- and $^{A}\gamma$-chain levels were 8.48 ± 0.53% and 7.14 ± 0.74%, respectively. For HbF^{II} and HbF^{III} the variant $^{A}\gamma^{T}$ was not resolved from $^{G}\gamma$ in the mass spectrum, as they only differ by 2 Da. Therefore, acetylation of the major counterpart $^{G}\gamma$, in HbF^{II} and HbF^{III} types, is reported as the acetylation of $^{G}\gamma + {}^{A}\gamma^{T}$.

The examination of HbF^{I} data (n = 26) shows that a significant difference is observed between the acetylated $^{G}\gamma$- and $^{A}\gamma$-chain levels (8.60% ± 0.56, 7.27% ± 0.68, $P < 0.0001$). This divergence is relatively surprising, since both chains differ only by the presence of Gly or Ala at position 136. We hypothesize that the higher level of the acetylated $^{G}\gamma$-chain might be due to an interference effect. In fact, the measured mass difference between the acetylated and nonacetylated $^{G}\gamma$-chains (41.3 ± 0.1 Da) is significantly less than that for the $^{A}\gamma$-chain (41.8 ± 0.2 Da).

α- and γ-Chain Glycations. We have determined that the glycated α-chain level (% ± 1SD) was 2.27 ± 0.21% (range 1.90−2.71%) and that the glycated γ-chain level was 2.38 ± 0.29% (range 1.96−3.09%) in 39 cord blood specimens (Table 3). Glycation of the α-chain occurs essentially at the lysine position 61 located at the helical external position E10,[16] and glycation of the γ-chain to its N-terminus glycine.

The differences between masses of glycated (m_1) and nonglycated (m_0) α- and γ-chains were 162.06 ± 0.13 Da and 161.65 ± 0.59 Da (mean ± 1SD), respectively. With such good precision, $m_1 - m_0$ values can be used as a confirmation threshold for data reliability. This is supported by the fact that our experimental values are close to the calculated glucose moiety of 162.14 Da. However, the $m_1 - m_0$ values observed for the γ-chain are lower than those for the α-chain. This can be explained by the fact that only one ion was chosen in the deconvolution step for the γ-chain versus two ions for the α-chain (Figure 2).

In HbA, α-chain glycation increases proportionally to β-chain glycation.[17] We think that the same phenomenon might be applicable for α/γ pairing to form HbF_0. Using 26 cord blood samples of HbF^{I} type, we found that glycation of α- and $^{G}\gamma$-chains (Figure 6a) was significantly correlated (R^2 = 0.4784, $P <$ 0.0001). However, these samples contained an amount of HbA

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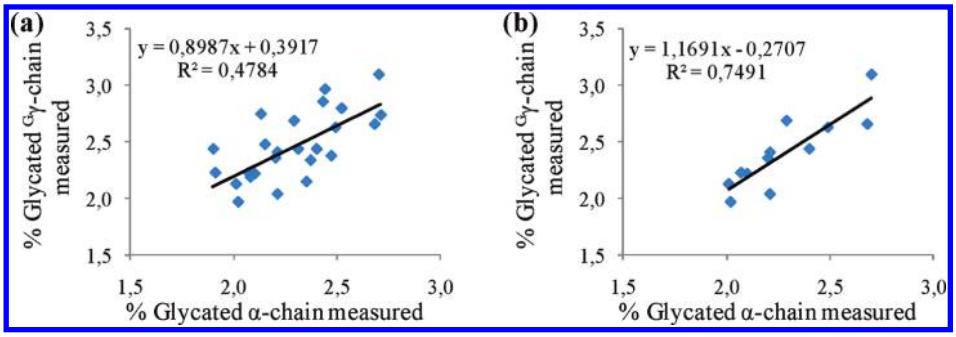

Figure 6. Correlations of α- and $^{G}\gamma$-chain glycation of cord blood hemoglobin (type I HbF): (a) samples with a relative amount of HbA varying between 8% and 27%; (b) samples with a relative amount of HbA varying between 8% and 13%.

varying from 8 to 27%, thus, measured α-chain levels represent the sum of both form HbA and form HbF. By selecting samples with lower amounts of HbA, we can decrease the proportion of α-chain deriving from HbA. As expected, the correlation shown in Figure 6b (n = 12, R^2 = 0.7491, P < 0.0003) is enhanced for cord blood specimens containing lower amounts of HbA (8−13%). This suggests that the rate of α-chain glycation for HbF may be different from that of HbA, even though both chains have identical amino acid sequences. In fact, Nacharaju and Acharya demonstrated that the Amadori rearrangement activity of the glycation site is influenced by its three-dimensional structure rather than by the amino acid sequence.[18]

CONCLUSIONS

This study demonstrates the feasibility of the quantification of post-translational modifications, such as glycation and acetylation, of intact hemoglobin in whole cord blood by the combination of ES-TOF-MS and a maximum entropy method producing true molecular mass spectra. We validated the method and have shown that it is accurate, precise, and selective, regardless of the relative proportions of HbA and HbF in the cord blood sample. Our methodology enabled us to determine variants of hemoglobin on the basis of the excellent mass accuracy, which is less than 3 ppm. We demonstrated that the selection of a specific region of the hemoglobin spectrum is crucial to avoid interferences, which can lead to erroneous interpretation and spurious results. To our knowledge, this is the first demonstration of the linear relationship between the glycation of both the α- and γ-subunits for HbF.

The importance of glycated hemoglobin in adults is well established as a reliable indicator of the average glucose index.[19] Considering the evidence that the intrauterine environment contributes to adult health,[3] we think that this method, which provides high selectivity and precision for measurements of glycated and acetylated hemoglobin in cord blood, paves the way to several new fields of research on fetal physiology and fetal metabolic programming.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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