Rapid phenotype hemoglobin screening by high-resolution mass spectrometry on intact proteins

Clinica Chimica Acta 460 (2016) 220–226

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Rapid phenotype hemoglobin screening by high-resolution mass spectrometry on intact proteins

Floris Helmich [a,c,*], Joost L.J. van Dongen [a], Philip H.M. Kuijper [c], Volkher Scharnhorst [b], Luc Brunsveld [a], Maarten A.C. Broeren [c]

[a] Laboratory of Chemical Biology, Department of Biomedical Engineering, Institute of Complex Molecular Systems, Eindhoven University of Technology, Expert Center Clinical Chemistry Eindhoven, The Netherlands
[b] Expert Center Clinical Chemistry Eindhoven, Clinical Laboratory, Catharina Hospital, Eindhoven, The Netherlands
[c] Laboratory for Clinical Chemistry and Hematology, Máxima Medisch Centrum, Veldhoven, Expert Center Clinical Chemistry Eindhoven, The Netherlands

ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 7 July 2016
Accepted 8 July 2016
Available online 11 July 2016

Keywords:
Hemoglobin
Globulin
Hemoglobinopathy
Sickle cell
Thalassemia
Newborn screening
Mass spectrometry
Q-ToF
Macromolecular

ABSTRACT

Background: Given the excellent performance of modern mass spectrometers, their clinical application for the analysis of macromolecules is a growing field of interest. This principle is explored by hemoglobin analysis, which is a representative example by its molecular weight and clinical relevance in e.g. screening programs for thalassemia and hemoglobin variants. Considering its abundance and cellular containment, pre-analysis is significantly reduced allowing for essential rapid acquisitions.
Methods: By parallel analysis of routine diagnostics for hemoglobin variants and thalassemia, we acquired samples of adults who were consented for hemoglobinopathy screening in our clinical laboratory. The pre-analytical process comprised of red cell lysis only; without further digestion and purification steps, the samples were directly injected in an electrospray ionization quadrupole time-of-flight setup and the intact proteins were analyzed by flow injection analysis. After optimization of process parameters, the deconvoluted mass spectra revealed the presence of α- and β-globulins. The reference ranges for the average mass of both globulins and their intensity ratio (α/β-ratio) were deduced from a disease-free subgroup and patients with a hemoglobinopathy were compared.
Results: The α/β-ratio is a poor marker for thalassemia patients, yet deviant α/β-ratios are found for patients with a hemoglobin variant. Mass deviations down to 1 Da can be resolved; even if the patient suffers from a heterozygotic disorder, the average mass is found outside the established reference interval.
Conclusions: Although subjects with mild thalassemia were not detected, all patients with a hemoglobin variant were resolved by top-down mass spectrometry using the average globulin mass and the α/β-ratio as screening parameters.

1. Introduction

Human hemoglobin is a tetrameric structure based on two different subunits holding heme molecules for the transportation of oxygen and carbondioxide. Adult hemoglobin (HbA) consists of two α- and two β-subunits (α_2/β_2) and these globulins are held together by non-covalent interactions. Related hemoglobin disorders refer to abnormalities in the structure and synthesis of the α- and β-subunits; these hemoglobinopathies are the most common single-gene disorders worldwide [1]. Repressed production of individual globulins (thalassemia) or the synthesis of globulins with an abnormal amino acid sequence (hemoglobin variants) are clinically connected to a variety of possible symptoms such as inherited anemia, iron overload, heart failure and jaundice [2]. Few hemoglobin variants and thalassemia-related disorders are clinically relevant and they regularly coexist. Therefore hemoglobinopathy analysis is included in screening programs for newborns, while many clinical laboratories offer test combinations of α-thalassemia DNA analyses and high pressure liquid chromatography (HPLC) or slab-gel/capillary-zone electrophoresis for β-thalassemia and hemoglobin variants [3]. In the latter setting, routine analyses are performed for undiagnosed patients with a late onset of symptoms. Considering the coherence between a hemoglobin variants and thalassemia, symptomatological as well as biochemical, we explore an alternative diagnostic approach using top-down mass spectrometry (MS).

Hemoglobin analysis by MS is based on different features when compared with current techniques such as HPLC [4]. Herein, specificity is attained by the retention time of hemoglobin tetramers in a properly calibrated column, which may become less specific in the presence of interfering chromophores. Due to the ionization-induced disassembly of the hemoglobin tetramer, the mass of the different globulin types is

* Corresponding author at: Laboratory for Clinical Chemistry and Hematology, Máxima Medisch Centrum, De Run 4600, 5500 MB, Veldhoven, The Netherlands.
E-mail address: florishelmich@gmail.com (F. Helmich).

http://dx.doi.org/10.1016/j.cca.2016.07.006
0009-8981/

acquired, which is therefore a highly specific marker. Hence, MS has elaborately been recognized for the analysis of hemoglobin variants. Appropriate mass resolution has been attained by tryptic digestion and subsequent MALDI-ToF measurements of peptide fragments [5,6]. Advanced methodologies employing Electrospray Ionization (ESI) tandem MS on tryptic peptides allowed for sequence analysis, in which the location of a given amino acid substitution could be conferred [7–12]. By this method, ratios of specific peptide fragments have been applied in the screening for hemoglobinopathies in dried blood spots [13–16]. Though some of these bottom-up approaches have been automated, sample preparation may be time consuming especially when mass analysis is preceded by chromatography. Rapid top-down MS has also been applied for the screening of hemoglobin variants, in which intact globulin masses have been obtained by MALDI-ToF [17] and ESI triple-quadrupole MS [18]. Both approaches have been combined in two-tier methodologies for screening and confirmation; some methodologies have been clinically assessed [8,11,12,19,20]. Only in a sophisticated Orbitrap-based MS, the top-down approach allowed for the unambiguous diagnosis of clinically relevant phenotypes in dried blood spots by single ion monitoring of the intact globulin and subsequent fragmentation steps [20–22].

Given the resolution and sensitivity of currently accessible MS, we present an alternative and fast top-down approach in an ESI-Quadrupole Time-of-Flight (Q-ToF) MS. Considering their high abundance and cellular containment, we omit any type of sample preparation or purification prior to hemoglobin analysis. Hence, by Flow Injection Analysis (FIA), we directly inject lysed erythrocytes and use, after spectral deconvolution, the intensity ratio of the α- and β-subunit as well as their specific mass as screening parameters. We deduce the mass distributions of the α- and β-chain and the α/β-intensity ratio from a population comprising of disease-free adults that serve as a reference. Subsequently, healthy subjects and patients with hemoglobinopathies who have been analyzed by HPLC and DNA are compared.

2. Materials and methods

2.1. Chemicals and instrumentation

Blood was drawn using Li-heparin sample tubes (BD Vacutainer). HPLC-grade acetonitrile and water for sample dilution and FIA were obtained from Biosolve. Formic acid and ammonium formate were purchased from Sigma Aldrich. Solutions for analysis were stored in 1.5 ml glass vials (ALWSCI).

HPLC analysis was performed on a Waters 2695 Separations Module (Alliance) with a PolyCAT A column (200 × 4,6 mm, 5 μm, Alltech Applied Science BV). Detection was performed by in-line UV–vis detection (Waters 2487 Dual λ Absorbance Detector) and date was processed using Empower Pro 2. Ingredients for mobile phase A and B for HPLC (40 mM Bis-Tris + 2 mM KCN, pH 6.5 and 40 mM Bis-Tris + 2 mM KCN + 200 mM NaCl, pH 6.8) were obtained from Sigma Aldrich.

DNA analysis for α-thalassemia screening by GAP-PCR was performed on a Peltier Thermal Cycler (Biorad). Primers for five deletions were applied: 3.7, 4.2, 20.5, SEA and MED, which account for ±80% of α-thalassemia [23]. Quantification was performed by ImaGo Imaging (B&L systems).

UV–vis spectra for determination of the hemoglobin concentration were recorded on a Perkin Elmer Lambda 25.

Mass spectra were acquired from a Waters Xevo® G2-S Q-ToF with ESI connected to a Aquity UPLC I-class system. Operation of the setup was conducted with the software package MassLynx.

2.2. Sample preparation

For HPLC measurements, 100 μl heparin whole blood was washed with 1 ml 0.9% NaCl. After shaking, the material was centrifuged for 5 min at 15000G. The supernatant was removed by pipette and 200 μl double ionized water was added. The cells were lysed for 5 min and the material was stored in the freezer (−20 °C).

For MS measurements, 20 μl of the lysate for HPLC was added to a 1000 μl ammonium formate buffer (10 mM in water at pH = 4.5). The approximate concentration of the solutions was ±20 μM as established by UV–vis. After mixing by vortex, the samples were stored at 10 °C in sample carousel of the MS.

2.3. Mass spectrometry conditions

For injection, 0.2 μl of sample was injected by partial loop injection in an isocratic flow (0.2 ml/min) of 90/10 v/v water/acetonitrile with 0.1% formic acid. Samples were analyzed by FIA (no column), in which the run time was set at 1.5 min.

For the formation of ions by ESI, the following optimized settings were applied: Capillary voltage = 1.0 kV, Sampling cone = 20 V, Extraction cone = 4.0 V, Source temperature = 100 °C, Desolvation temperature = 500 °C, Cone gas flow = 10 l/h, Desolvation gas flow = 650 l/h.

For the acquisition of mass spectra in positive resolution mode, the MS was calibrated with phosphoric acid. In-run mass correction was attained using LockSpray® with a leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu, 556.2771 Da) standard every 10 s. The mass envelope was obtained in the range between 150 and 2000 *m/z* at a scan time of 0.1 s. An optimized profile of the first quadrupole was maintained at Mass1 (300 *m/z* at 10% scan time for dwell and ramp time), Mass2 (700 *m/z* at 20% scan time for dwell time) and Mass3 (1200 *m/z*).

2.4. Data processing

A raw mass spectrum was obtained from the Total Ion Current (TIC) chromatogram between 5.0 and 12.0 s. This cumulative spectrum, based on 70 individual mass spectra, was processed by the maximum entropy (MaxEnt1) deconvolution script in the MassLynx software. The mass envelope between 650 and 1200 *m/z* was processed and the following scripting parameters were used: Deconvolution output mass range: 14,000–17,000 Da, Deconvolution output resolution: 0.1 Da, Settings for the simulated isotope pattern damage model: width at half height: 0.25 Da and minimal intensity ratios: 33%. Deconvolution to convergence took ±25 s.

After deconvolution, accurate deconvoluted masses were obtained by peak centering in MassLynx (1, centroid top 80%, area spectrum).

Statistical analysis on the average α- and β-mass and the α/β-ratio was performed by Analyze-it in Microsoft Excel using a parametric method.

2.5. Sample selection

No patient selection was applied and samples were used from adult subjects who were consented for hemoglobinopathy analysis. In our laboratory, this includes a total blood count, HPLC and DNA analysis; thereby affording a routine-based diagnosis necessary for this comparison, in which parallel measurements were performed for a period of 3 months.

The baseline characteristics of subjects are shown in Table 1. Rather than selecting upon a healthy subgroup, a population of negative controls (or disease-free subjects) served as a reference. Subjects were considered negative in the absence of a diagnosis for thalassemia and a hemoglobin variant. Thalassemia is diagnosed by a positive DNA test for α-thalassemia and the diagnostic criteria for a β-thalassemia are a HbA_2-fraction > 3.5% with MCV < 80 fl and a normal iron status. The latter disorder is diagnosed by a HbA_1-fraction < 95% and the presence of other hemoglobin variants in HPLC.

Table 1
Baseline characteristics.

60 Subjects		Age	Gender [%]		Hb [mmol/l]		MCV [fl]	
			♂	♀	♂	♀	♂	♀
Negative	36	43	44	56	8.5	7.1	87	84
α-Thalassemia[a]	6	27	67	33	7.8	7.5	80	78
β-Thalassemia	8	26	63	37	6.2	6.3	62	66
Hb-variant[b]	10	40	40	60	7.5	6.5	85	75

[a] Patients with αα/−α3.7 (N = 4), −α3.7/−α3.7 (N = 1), αα/−−SEA (N = 1).
[b] Patients with HbAC (N = 1), HbAE (N = 1), HbE/β-thalassemia (N = 1), HbAF (N = 1), HbAS + αα/−α3.7 (N = 3), HbSS + αα/−α3.7 (1 patient, 3 samples drawn during treatment of sickle cell crisis).

3. Results

3.1. Optimization study

Prior to the measurement of patient samples, an optimization study was performed for the appropriate conditions for sample preparation, injection, ionization, mass acquisition and spectral deconvolution. For these investigations, we employed a hemoglobin sample of a healthy subject at an optimized concentration of ±20 μM. The shape and area under the TIC-chromatogram was used to access the efficiency of ionization, which was considered optimal when they were Gaussian shaped and the area related linearly with the amount of sample injected. In order to obtain the right conditions, the flow rate and injection volume were varied between 0.1 and 0.3 ml/min and 0.1–0.3 μl, respectively. In this process, ionization parameters such as desolvation gas flow/temperature, cone gas flow, sample/extraction cone voltage, capillary voltage and source temperature were optimized as well. At a flow of 0.2 ml/min and 0.2 μl sample injected, mass spectra were collected between 5.0 and 12.0 s resulting in the raw mass spectrum (Fig. 1A).

The raw mass spectrum reveals the presence of both α- and β-globulins at differently charged states and the single-charged heme molecule at 616.5 Da. The α-globulin is mostly abundant in its 21-fold charged state, while the 20-fold charged state of the β-globulin is mostly present. Due to their mass difference of 740.9 Da, the mass envelope of the heavier β-globulin is shifted to higher m/z-values. As a consequence, both mass peaks closely approximate at $721 < m/z < 723$

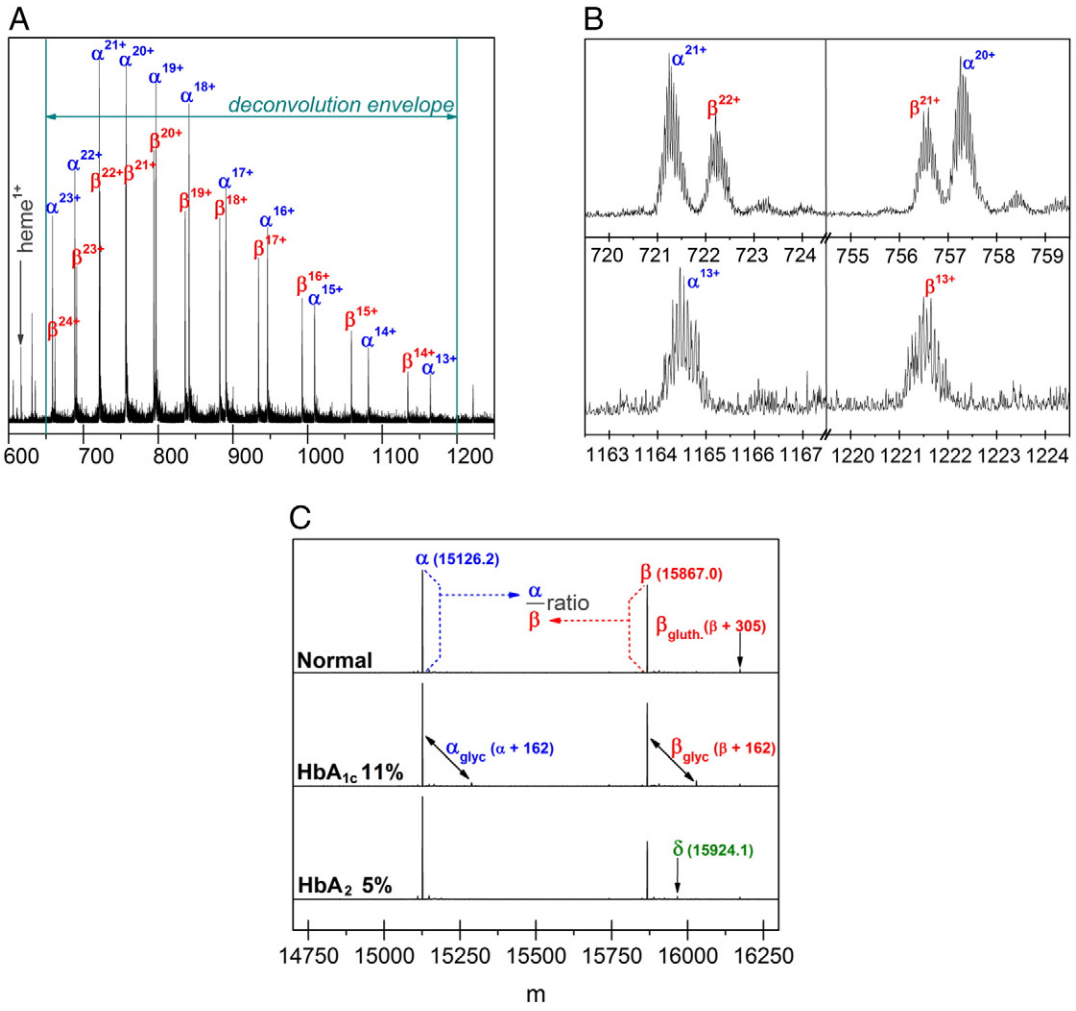

Fig. 1. (A) Raw mass spectrum after injection optimization of a normal hemoglobin sample at 20 μM. (B) The effect of the charged state on the α/β-ratio of the raw mass spectrum: a zoom-in for the normalized $[\alpha/\beta]^{21+}$-ratio in the partly overlapping α^{21+}–β^{22+} ($721 < m/z < 723$) and β^{21+}- α^{20+} ($756 < m/z < 758$) region (upper panel) and the normalized $[\alpha/\beta]^{13+}$-ratio in the $1160 < m/z < 1225$ region (lower panel). (C) Deconvoluted mass spectrum of a normal hemoglobin sample (upper panel), a diabetic patient (HbA_{1c} = 11%, 97 mmol/mol, center panel) and a patient with β-thalassemia (HbA_2 = 5%, lower panel).

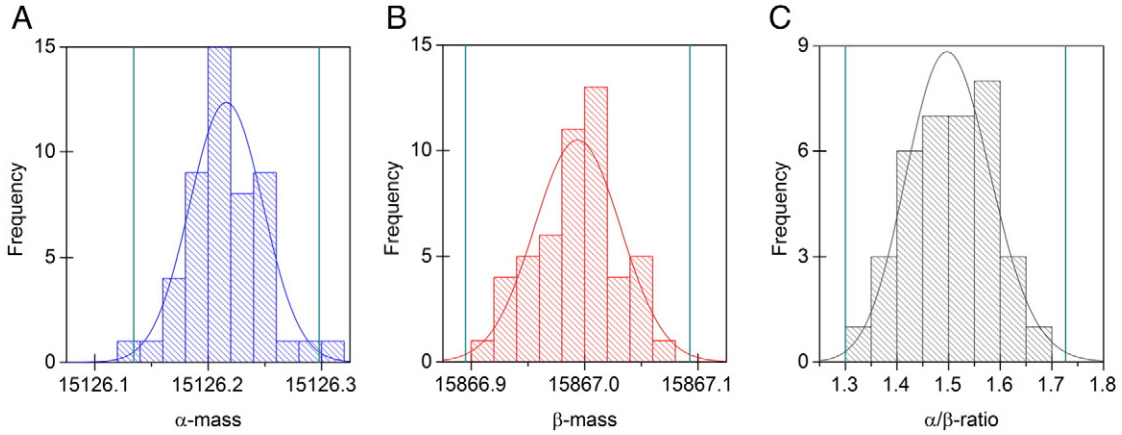

Fig. 2. Histogram and the 1–99th percentile reference interval for the negative population: (A) deconvoluted mass of the α-globulin. (B) Deconvoluted mass of the β-globulin. (C) The α/β-ratio deduced from the deconvoluted mass spectra.

(α^{21+}–β^{22+}) and $756 < m/z < 758$ (β^{21+}–α^{20+}) at which the resolution of the mass spectrometer is nevertheless sufficient (Fig. 1B, upper panel).

In order to obtain the final mass spectrum, a deconvolution script was used, which yields the deconvoluted mass peaks of the α- and β-globulin at 15,126.2 and 15,867.0 Da, respectively (Fig. 1C). As already

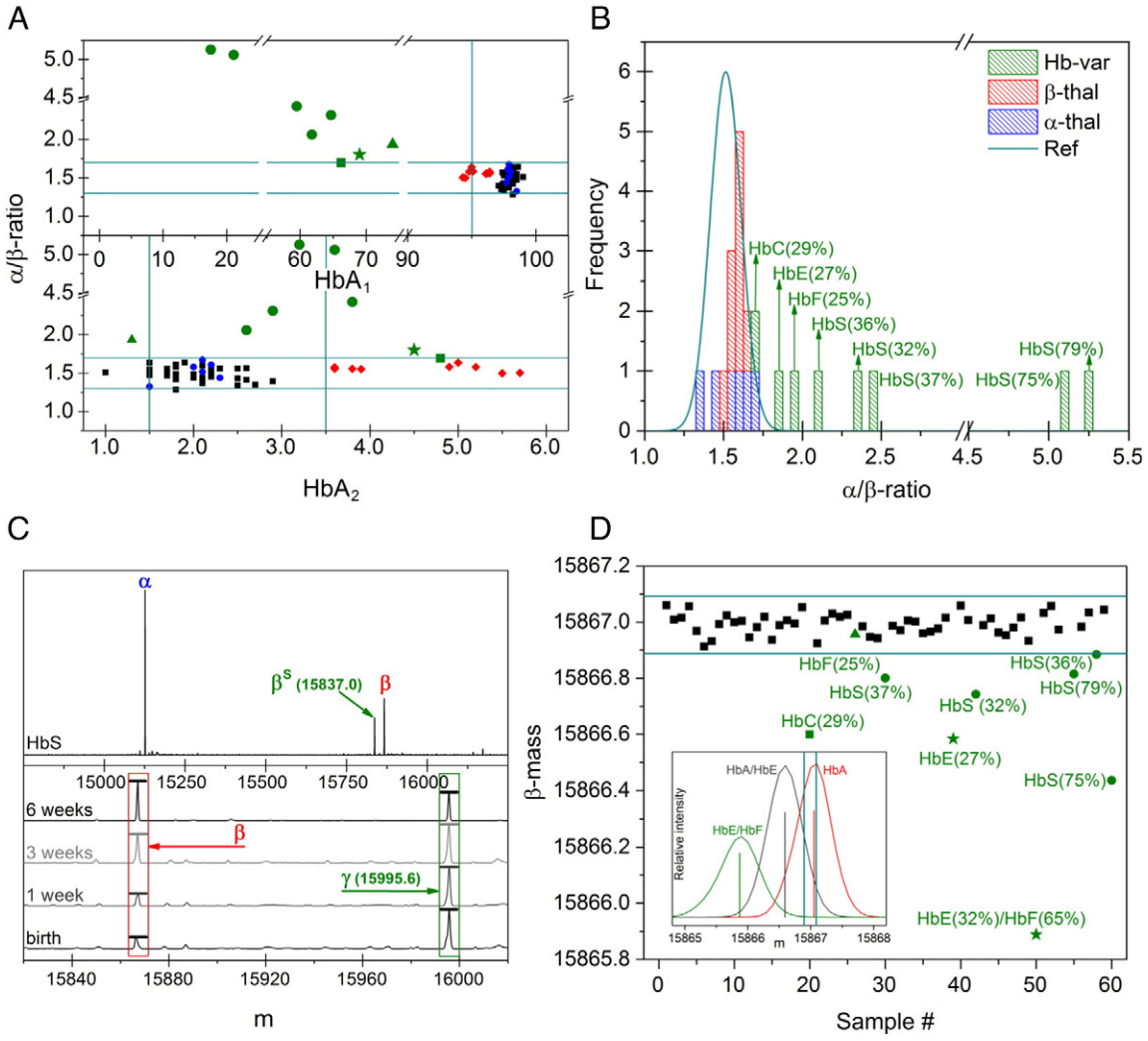

Fig. 3. (A) Concordance between α/β-ratio versus HbA_1 (upper panel) and HbA_2 (lower panel). (B) The α/β-ratio for the pathological population with an overlay of the distribution curve of negative controls. (C) Deconvoluted mass spectrum of a HbS sample (upper panel) and the β/γ-region of a newborn at different ages (normalized to the α-intensity, lower panel). (D) Deconvoluted mass of the β-globulin for detection of patients with different hemoglobin variants. Inset in (D) shows the deconvoluted mass peaks of the β-domain with the centered masses relative to the β-mass range. Patients with α-thalassemia (red symbols), β-thalassemia (blue symbols) and hemoglobin variant (green symbols; HbS ●, HbC ■, HbE *, and HbF ▲).

observed in the raw spectra, the intensity of the α-globulin is highest, hence the intensity ratio between the α- and β-peak of the deconvoluted spectrum exceeds unity (α/β-ratio > 1). By considering the amount mass peaks for α and β in the raw spectrum, it is obvious that the α/β-ratio depends on the selection which part of the raw spectrum is used for deconvolution. In addition, the intensity ratio of the raw mass peaks changes upon the charged state (e.g. $[\alpha/\beta]^{21+}$-ratio > $[\alpha/\beta]^{13+}$-ratio, Fig. 1B upper- and lower panel, respectively). In the optimization study, the highest precision of the α/β-ratio ($CV_{within\text{-}run} = 2.7\%$) was achieved upon selecting a large, rather than a small, mass envelope between $650 < m/z < 1200$ (β^{24+}–α^{13+}), in which all peaks were properly resolved.

The discrepancy between physiological α/β-ratios (≥1, at low HbA_2 levels) and higher physical values deduced from the mass spectrometer (α/β-ratio ± 1.5) is rationalized by the lower proton affinity of β-globulins due to conformational effects [24]. As part of the optimization study, selective influx of β-ions was investigated by the composition of the liquid flow and different buffering strategies of samples. Buffering with ammonium formate enhanced ionizability, in which acidic buffers (pH = 2.5) resulted in high ionizability and high α/β-ratios. Vice versa, the less acidic buffer (pH = 4.5) afforded α/β-ratios closer to unity at lower ionizability. Besides chemistry, the ESI settings were optimized, in which a similar trend was observed; α/β-ratios closer to unity compromising lower TIC signals. Compensation for lower TIC signals was achieved by increasing the injection volume, in which the linear relation between the area under the TIC-chromatogram and injection volume persisted. In addition, increasing the injection volume did not affect the α/β-ratio, hence ion suppression was not evidenced. Lastly, optimization of the mathematical parameters for deconvolution only revealed a marginal effect on α/β-ratio. However, peak centering in MassLynx strongly improved the reproducibility and lowered the value of the α/β-ratio.

3.2. Reference population

Under the optimized conditions, patient samples were measured shortly after routine analysis by HPLC. During 3 months parallel acquisition, 60 samples were analyzed including their DNA analysis. After an SQL-query on the data in our laboratory information system, all subjects without a hemoglobin variant (N = 50) were enrolled for the reference intervals of the deconvoluted α- and β-masses. A mean α-mass of 15,126.216 was determined at a 1–99th percentile reference interval of 15,126.136–15,126.292 Da (Fig. 2A). For the β-globulin, a mean mass 15,866.994 Da was deduced at a 1–99th percentile reference interval of 15,866.896–15,867.089 Da (Fig. 2B). The disease-free subgroup with negative results for a hemoglobinopathy (N = 36) was used to establish the reference interval for the α/β-ratio (Fig. 2C); the mean α/β-ratio was 1.50 at a 1–99th percentile reference interval of 1.30–1.70.

Occasionally, upon processing mass spectra of the reference population, other peaks than α and β were observed at small intensities. In many subjects, a glutathione adduct is found on the β-globulin at 16,172.2 Da (Fig. 1C, upper panel) [25] and glucose adducts on both α- and β-globulins were found in diabetic patients (Fig. 1C, center panel). Furthermore, δ-globulins originating from HbA_2 (α_2/δ_2) at 15,924.1 Da were weakly visible.

3.3. Patient comparison

After setting the reference for the negative controls, the α/β-ratio of all subjects was compared with the fraction HbA_1 and HbA_2, which were determined by HPLC (Fig. 3A). All patients with α-thalassemia (N = 6) had normal HbA_1 (>95%) and HbA_2 (1.5–3.5%) levels and their α/β-ratio was found in the reference range (Fig. 3B). β-thalassemia patients (N = 8 with HbA_2 levels >3.5%) also had α/β-ratios in the reference range, albeit all values were found above the 50th percentile. In this category, patients with HbA_1 < 95% and sufficiently high HbA_2 levels were analyzed for a concordance study between the δ/β-ratio and HbA_2 level [9]. Although δ-globulins were visible in these patients (Fig. 1C, lower panel), the mass peak was insufficient for reproducible quantitation.

Strong concordance, however, was found in patients with hemoglobin variants (N = 10). We analyzed 6 samples of patients with HbS, in which a mutated β-globulin (β^S) at 15,837.0 Da was found next to the normal β-globulin at 15,867.0 Da (Fig. 3C, upper panel). This 30 Da difference is due to the substitution of glutamic acid (147.13 Da) with valine (117.15 Da) at position 6 of the β-globulin. In these subjects (and the majority of patients with other hemoglobin variants), the β-globulin was compromised, thereby causing high α/β-ratios (Fig. 3B). An increased α/β-ratio was also found in a patient with persisting HbF (α_2/γ_2), in which the $^G\gamma$-globulin at 15,995.6 Da was apparent next to the β-globulin. Besides, with respect to fetal hemoglobin, we analyzed newborn samples, in which we occasionally found an additional $^A\gamma$-globulin at 16,009.3 Da [27]. In one subject, the γ/β-ratio was used to monitor the fetal-to-adult globulin gene switching (Fig. 3C, lower panel). The α/β-ratio could not be deduced from two patients who fully lacked HbA_1 in HPLC, which corroborated with the absence of a β-mass peak in MS. One homozygous HbSS ($\alpha_2/\beta^S{}_2$) patient in a sickle cell crisis only revealed the mass peak of the β^S-globulin in addition to the α-globulin. Another subject with HbE/β-thalassemia (HbE(35%)/HbF(65%)) revealed a mass peak of the $^G\gamma$-globulin next to the α-globulin and an 'apparent β-globulin' at 15,865.9 Da (Fig. 3D).

Since no HbA_1 was found in this particular patient, β-globulins were absent and the acquired mass solely depended on the β^E variant allele. The observed 1 Da mass difference originates from the substitution of glutamic acid (147.13 Da) by lysine (146.18 Da) at position 26 (for patients with HbC at position 6) of the β-globulin. In two heterozygotic patients with HbC(29%) and HbE(27%), HbA_1 fractions of >65% were found, in which regular β-globulins were more abundant than their β^C and β^E counterparts. Despite their mixed overlapping states, the resultant average mass was found outside the established 1–99th percentile reference range for the β-mass (Fig. 3D-inset). Remarkably, however, all HbS patients revealed deviant average masses for the β-globulin apart from deviant α/β-ratios established earlier. This was not expected considering the presence of regular β-globulins of residual HbA_1 in these subjects. Noteworthy, Wild and co-workers found a 1–99th percentile β-mass range of 0.42 and 0.84 Da for HbFA and HbFAS, respectively [18]. An ESI triple-quadrupole MS was used for the top-down analysis of neonatal samples and the β-mass was determined upon fixation of the α-mass at 15,126.38 Da. Thereby, this phenomenon is recognized, while a significantly smaller β-mass range is found in our higher resolution setup.

4. Discussion

The reference range of the α/β-ratio was deduced from disease-free subjects, which all revealed normal HbA_1 and HbA_2 levels in HPLC. When compared to MS, the spread of the α/β-ratio may be unexpectedly high. It is physically caused by the amounts of α- and β-ions entering the Q-ToF, in which a mild inter-patient variation is observed. With the aforementioned $CV_{within\text{-}run} = 2.7\%$ and an $CV_{within\text{-}laboratory} = 4.8\%$ (deduced from an EP05 protocol based on identical frozen lysates), the imprecision of the MS, rather than biologic matrix effects, predominantly attributes to this reference range. Hence, the applicability of the α/β-ratio as a screening parameter for thalassemia seems poor; after all, the majority of our thalassemia patients have normal HbA_1 levels in HPLC with only minor variation (Fig. 3A).

Regarding α-thalassemia, all patients were found in the established reference interval, while we hypothesize that patients with three/four affected α-alleles will have lower α/β-ratio due to the abundant presence of HbH and/or Hb Barts. Regarding β-thalassemia, we observed α/β-ratios of carrier phenotypes above the 50th percentile, which is likely caused by the presence of HbA_2. In our case, intact δ-globulins were poorly quantifiable and considering our top-down approach we

may be able to identify subjects with clinically relevant disorders such as β-thalassemia major. By using HPLC with optical detection, Wan et al. demonstrated that the ratio of intact globulins (α/β and β/δ) could be used to discriminate normal subjects from β-thalassemia carriers [26]. This was achieved by remarkably small variations of globulin intensity ratios between subgroups. By using MS, quantitation has been performed by bottom-up MS only, in which MRM transitions of globulin-specific tryptic peptides were quantified yielding peptide ratios in relatively broad reference ranges. Daniel and coworkers revealed strong concordance between HbA_2 levels and δ/β-ratios based on T2, T3i and T13 peptide fragments [9]. Boemer et al. identified newborns with β-thalassemia major by this method using the β(T1b1)/γ(T12b2) ratio [15], while Moat et al. discriminated normal subjects from newborns with β-thalassemia major/intermedia by low β(T1y4)/γ(T2y6) and high γ(T2y6)/β(T2y6) ratios in paper blood spots [13].

In contrast to the detection of thalassemia, deviant α/β-ratios were found for patients with hemoglobin variants. The incorporation of a different amino acid causes a mass shift of the globulin peak; for instance in case of HbS, the β-globulin is less abundant due to the presence of β^S and α/β-ratios higher than the upper reference limit are found. A prerequisite for this algorithm is proper mass differentiation between the wild type and mutant globulin, which is achievable by top-down MS as presented here. In the first place, mass-reproducibility is attained by within-acquisition mass corrections using LockSpray®; every 10 s, a leucine-enkephalin standard at 556.2771 Da corrects the raw mass spectrum towards mass accuracies within 0.8 ppm. After proper deconvolution and peak centering, this affords mass peaks with 1–99th percentile mass ranges of ±0.20 Da for both α- and β-globulins. Secondly, these deconvoluted peaks feature a mathematical peak width at half maximum abundance of ±1 Da. Hence, peak overlap is no issue for globulin mutations that account for mass differences >2 Da such as HbS and HbF that are straightforwardly detected. Mass differences <2 Da, however, overlap with the regular β-globulin and the adjacent mass peak induces a shoulder. In this case, peak centering generates an average mass, which is found outside the reference range for the β-mass. Our examples of patients with HbC and HbE (both −1 Da mass difference relative to the β-globulin) revealed that their average globulin mass was apparently deviant; even in presence of ±65% HbA_1. Mass deviations down to 1 Da can thus be resolved after peak centering in the β^A – 1 Da domain, even if the patient suffers from a heterozygotic disorder.

Top-down MS allows for rapid screening through quantification by the α/β-ratio and qualification of the β-mass. In contrast to screening, our approach does not allow for the unambiguous identification of hemoglobin variants. Firstly, since multiple mutations are based on identical amino acid substitutions at different sites, the average mass of the intact protein is not variant-specific. Secondly, identification is restrained by sub-dalton discrepancies between the measured mass and calculated mass due to different isotopic distributions allocated by different algorithms. With the established level of feasibility, a cohort expansion is underway for further validation purposes. This entails patients with thalassemia major, in which the α/β-ratio may be an applicable marker and other clinically relevant hemoglobin variants such as O^{arab}, D^{Punjab} and Lepore. In view of newborn screening, the transformation to fetal samples allows the verification of this diagnostic approach including the pre-analytic process concerning paper blood spots. Parallel to cohort expansion, the quantification process is being optimized by fine-tuning ESI, hereby aiming at more reproducible and uniform α/β-ratios in healthy subjects. Lastly, technical considerations are under investigation for a confirmatory method that allows for the identification of globulin variants.

5. Conclusions

The analysis of large molecules by mass spectrometry is clinically explored by parallel hemoglobin screening in a routine-based clinical laboratory. Rapid acquisitions with a run-time in the order of one minute are assured by omitting pre-analytical factors such as protein digestion and chromatography. Reference intervals derived from a relatively small cohort of negative controls have successfully been applied in patients with a hemoglobin variant. In our relatively small cohort, hemoglobin variants with mass deviations down to 1 Da can be resolved; even if the patient suffers from a heterozygotic disorder, the average mass is found outside the established reference interval for the globulin mass. In addition, deviant α/β-ratios are found in these patients. Accordingly, all hemoglobin variants established by routine-based analysis have been detected by rapid screening of the average β-globulin mass and subsequent determination of α/β-ratio.

Acknowledgments

Members of the Expert Center Clinical Chemistry Eindhoven, Ellen Schmitz and Daan van de Kerkhof are acknowledged for fruitful discussions. Gerard Coppens is acknowledged for enabling parallel analysis with routine diagnostics.

References

[1] D.J. Weatherall, J.B. Clegg, Inherited haemoglobin disorders: an increasing global health problem, Bull. World Health Organ. 79 (8) (2001) 704–712.
[2] J.C. Wood, Estimating tissue iron burden: current status and future prospects, Br. J. Haematol. 170 (1) (2015) 15–28.
[3] D.N. Greene, C.P. Vaughn, B.O. Crews, A.M. Agarwal, Advances in detection of hemoglobinopathies, Clin. Chim. Acta 439 (2015) 50–57.
[4] P. Kleinert, M. Schmid, K. Zurbriggen, O. Speer, M. Schmugge, B. Roschitzki, S.S. Durka, U. Leopold, T. Kuster, C.W. Heizmann, H. Frischknecht, H. Troxler, Mass spectrometry: a tool for enhanced detection of hemoglobin variants, Clin. Chem. 54 (1) (2008) 69–76.
[5] U.A. Kiernan, J.A. Black, P. Williams, R.W. Nelson, High-throughput analysis of hemoglobin from neonates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Chem. 48 (6 Pt 1) (2002) 947–949.
[6] P. Lacan, M. Becchi, I. Zanella-Cleon, M. Aubry, D. Quinsat, N. Couprie, A. Francina, Identification by mass spectrometry of a hemoglobin variant with an elongated beta-globin chain, Clin. Chem. 51 (1) (2005) 213–215.
[7] R. Das, G. Mitra, B. Mathew, C. Ross, V. Bhat, A.K. Mandal, Automated analysis of hemoglobin variants using nanoLC-MS and customized databases, J. Proteome Res. 12 (7) (2013) 3215–3222.
[8] A.K. Mandal, S. Bisht, V.S. Bhat, P.R. Krishnaswamy, P. Balaram, Electrospray mass spectrometric characterization of hemoglobin Q (Hb Q-India) and a double mutant hemoglobin S/D in clinical samples, Clin. Biochem. 41 (1–2) (2008) 75–81.
[9] Y.A. Daniel, C. Turner, R.M. Haynes, B.J. Hunt, R.N. Dalton, Quantification of hemoglobin A2 by tandem mass spectrometry, Clin. Chem. 53 (8) (2007) 1448–1454.
[10] Y.A. Daniel, C. Turner, R.M. Haynes, B.J. Hunt, R.N. Dalton, Rapid and specific detection of clinically significant haemoglobinopathies using electrospray mass spectrometry-mass spectrometry, Br. J. Haematol. 130 (4) (2005) 635–643.
[11] B.J. Wild, B.N. Green, E.K. Cooper, M.R. Lalloz, S. Erten, A.D. Stephens, D.M. Layton, Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry, Blood Cells Mol. Dis. 27 (3) (2001;Jun) 691–704.
[12] D.K. Rai, W.J. Griffiths, G. Alvelius, B. Landin, Electrospray mass spectrometry: an efficient method to detect silent hemoglobin variants causing erythrocytosis, Clin. Chem. 47 (7) (2001) 1308–1311.
[13] S.J. Moat, D. Rees, L. King, A. Ifederu, K. Harvey, K. Hall, G. Lloyd, C. Morrell, S. Hillier, Newborn blood spot screening for sickle cell disease by using tandem mass spectrometry: implementation of a protocol to identify only the disease states of sickle cell disease, Clin. Chem. 60 (2) (2014) 373–380.
[14] C.A. Haynes, S.L. Guerra, J.C. Fontana, V.R. DeJesús, HPLC-ESI-MS/MS analysis of hemoglobin peptides in tryptic digests of dried-blood spot extracts detects HbS, HbC, HbD, HbE, HbO-Arab, and HbG-Philadelphia mutations, Clin. Chim. Acta 424 (2013) 191–200.
[15] F. Boemer, Y. Cornet, C. Libioulle, K. Segers, V. Bours, R. Schoos, 3-years experience review of neonatal screening for hemoglobin disorders using tandem mass spectrometry, Clin. Chim. Acta 412 (15–16) (2011) 1476–1479.
[16] F. Boemer, O. Ketelslegers, J.M. Minon, V. Bours, R. Schoos, Newborn screening for sickle cell disease using tandem mass spectrometry, Clin. Chem. 54 (12) (2008) 2036–2041.
[17] M. Zehl, G. Allmaier, Ultraviolet matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of intact hemoglobin complex from whole human blood, Rapid Commun. Mass Spectrom. 18 (17) (2004) 1932–1938.
[18] B.J. Wild, B.N. Green, A.D. Stephens, The potential of electrospray ionization mass spectrometry for the diagnosis of hemoglobin variants found in newborn screening, Blood Cells Mol. Dis. 33 (3) (2004) 308–317.
[19] J.P. Williams, A.J. Creese, D.R. Roper, B.N. Green, H.J. Cooper, Hot electron capture dissociation distinguishes leucine from isoleucine in a novel hemoglobin variant, Hb Askew, beta54(D5)Val → Ile, J. Am. Soc. Mass Spectrom. 20 (9) (2009) 1707–1713.

[20] R.L. Edwards, A.J. Creese, M. Baumert, P. Griffiths, J. Bunch, H.J. Cooper, Hemoglobin variant analysis via direct surface sampling of dried blood spots coupled with high-resolution mass spectrometry, Anal. Chem. 83 (6) (2011) 2265–2270.
[21] R.L. Edwards, P. Griffiths, J. Bunch, H.J. Cooper, Top-down proteomics and direct surface sampling of neonatal dried blood spots: diagnosis of unknown hemoglobin variants, J. Am. Soc. Mass Spectrom. 23 (11) (2012 Nov) 1921–1930.
[22] R.L. Edwards, P. Griffiths, J. Bunch, H.J. Cooper, Compound heterozygotes and beta-thalassemia: top-down mass spectrometry for detection of hemoglobinopathies, Proteomics 14 (10) (2014) 1232–1238.
[23] C.L. Harteveld, State of the art and new developments in molecular diagnostics for hemoglobinopathies in multiethnic societies, Int. J. Lab. Hematol. 36 (1) (2014) 1–12.
[24] M.C. Kuprowski, B.L. Boys, L. Konermann, Analysis of protein mixtures by electrospray mass spectrometry: effects of conformation and desolvation behavior on the signal intensities of hemoglobin subunits, J. Am. Soc. Mass Spectrom. 18 (7) (2007) 1279–1285.
[25] T. Niwa, C. Naito, A.H. Mawjood, K. Imai, Increased glutathionyl hemoglobin in diabetes mellitus and hyperlipidemia demonstrated by liquid chromatography/electrospray ionization-mass spectrometry, Clin. Chem. 46 (1) (2000) 82–88.
[26] J.H. Wan, P.L. Tian, W.H. Luo, B.Y. Wu, F. Xiong, W.J. Zhou, X.C. Wei, X.M. Xu, Rapid determination of human globin chains using reversed-phase high-performance liquid chromatography, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 901 (2012) 53–58.
[27] H.F. Bunn, B.G. Forget, Hemoglobins A2, F, and A1c, Hemoglobin: Molecular, Genetic and Clinical Aspects, W.B. Saunders, Philadelphia, 1986 61–90.