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

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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

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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.