Newborn Screening for Sickle Cell Disease Using Tandem Mass Spectrometry

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BACKGROUND: Neonatal screening programs for sickle cell disease are now widespread in North American and European countries. Most programs apply isoelectric focusing or HPLC to detect hemoglobin variants. Because tandem mass spectrometry (MS/MS) is being used for screening of inherited metabolic disorders and allows protein identification, it was worth testing for hemoglobinopathy screening.

METHODS: We minimized sample preparation and analysis times by avoiding prior purification, derivatization, or separation. We developed a tryptic digestion methodology to screen for the main clinically important variants (Hb S, Hb C, and Hb E) and β-thalassemia. To ensure proper discrimination between homozygote and heterozygote variants, we selected 4 transitions with good signal intensities for each specific peptide and calculated variant/Hb A ratios for each. Method validation included intra- and interseries variability, carryover, and limit of detection. We also performed a comparative study with isoelectric focusing results on 2082 specimens.

RESULTS: Intraassay imprecision values (CVs) varied between 2.5% and 30.7%. Interassay CVs were between 6.3% and 23.6%. Carryover was <0.03%, and the limit of detection was fixed at 1% of Hb S. According to the MS/MS settings (detection of Hb S, Hb C, Hb E, and β-globin production defects), the comparative study did not yield any discrepant results between the 2 techniques.

CONCLUSIONS: MS/MS is a reliable method for hemoglobinopathy neonatal screening.

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Hemoglobin disorders are the most common monogenic diseases encountered around the world. It has been estimated that approximately 7% of the world population are heterozygous carriers of these disorders. As a consequence about 300 000 babies with severe hemoglobinopathies are born each year (1). More than 900 different variants have now been identified (2), but most of them are not clinically significant.

Sickle cell disease refers to a group of genetic disorders characterized by production of hemoglobin S, anemia, and acute and chronic tissue damage secondary to blood vessel occlusions produced by the abnormally shaped red cells. The disease is associated with high morbidity and mortality in early childhood (3). In 1986, it was demonstrated that prophylactic penicillin markedly reduces the incidence of pneumococcal sepsis in sickle cell patients (4), providing a powerful incentive for the widespread implementation of neonatal screening programs for this disease (5). Subsequent screening experiences demonstrated that newborn screening, when linked to timely diagnostic testing, parental education, and comprehensive care, reduces morbidity and mortality from sickle cell disease in infancy and early childhood (6–8).

Any newborn screening program should allow the detection of all the clinically significant conditions for which there is evidence that early intervention is likely to be beneficial: the relevant hemoglobin (Hb)3 mutants thus include Hb SS, Hb SC, Hb SD-Punjab, Hb S/β-thalassemia (β+, β0, δβ, Lepore), Hb SO-Arab, and Hb S/HPFH. In addition to these sickle cell disorders, other conditions that could be detected at birth with a benefit for the patient include major β-thalassemia, intermediate β-thalassemia, Hb H disease, Hb E/β-thalassemia, and Hb SE (9). Nowadays, the majority of screening programs use isoelectric focusing (IEF) on cord blood or eluates of dried blood spots; a few programs perform HPLC as the initial screening method. Most programs retest abnormal specimens with a second electrophoretic technique, HPLC, immunologic tests, or DNA-based assays (10, 11).
Neonatal Screening for Sickle Cell Disease by MS/MS

Tandem mass spectrometry (MS/MS) is increasingly being used for newborn screening of inherited metabolic disease. Numerous published reports describe the utility of MS/MS for the detection of amino, organic, and fatty acid disorders (12–15). Because MS/MS is already effectively being used for traditional neonatal screening, it would be convenient if the same equipment could also be employed for hemoglobinopathy screening. Indeed, mass spectrometry has been previously used for the characterization of hemoglobin mutations (16–18). Different strategies have been assessed to identify variants, using either whole-protein scan to measure the masses of intact globin chains (19) or analysis of tryptic peptide fragments (20, 21). Some protocols combined the 2 approaches (22, 23).

We describe here an original screening methodology based on a tryptic digest approach to identify clinically significant hemoglobinopathies.

Materials and Methods

Materials
We purchased ammonium bicarbonate and tosyl-phenylalanyl-chloromethyl-ketone (TPCK)-treated trypsin from Sigma-Aldrich. TPCK-treated trypsin was dissolved in 1 mol/L ammonium bicarbonate to a final concentration of 5 g/L. We obtained MS-grade water, formic acid, and acetonitrile (ACN) from Biosolve.

Sample Preparation
The sample treatment was modified from a previously described method (20). From each dried blood card, 1 3.2-mm diameter disc was punched into 96-well microplates, and we added 200 µL deionized water to each well. Elution was carried out by gentle rotation for 60 min, and 100-µL aliquots were transferred to fresh 96-well microtiter plates. We added ACN (17 µL) and 12 g/L aqueous formic acid (17 µL) to each well before vigorous mixing. After 10-min incubation at room temperature, denatured proteins were treated with 10 µL TPCK-treated trypsin solution until the solution was clear. After centrifugation and incubation overnight at 37 °C, 20 µL digested solution was diluted with 180 µL ACN/deionized water (1:1) with 1.2 g/L formic acid and centrifuged again. This working solution was then ready for injection.

Mass Spectrometry
We loaded 20-µL aliquots of working solution via a 50-µL loop injector into the electrospray source of a Quattro Premier triple quadrupole mass spectrometer (Waters) operating in the positive ionization mode. Samples were injected into the mobile phase stream [ACN:H₂O (50:50) with 1.2 g/L formic acid] using a Waters Acquity UPLC system and directly introduced into the source without prior chromatographic separation.

Analysis was performed in the multiple-reaction-monitoring (MRM) mode with a dwell time of 0.2 s per channel. The total acquisition time was 150 s.

MRM Acquisition
Tryptic digestion generates β-globin fragments that have been well characterized. Among these, 2 peptides present a mutation in major clinically significant hemoglobin variants (Hb S, Hb C, and Hb E) and therefore were considered as informative for the neonatal screening application (see Supplemental Tables 1 and 2, which accompany the online version of this article at http://www.clinchem.org/content/vol54/issue12). The first tryptic peptide is common for β- and δ-globin chains; however, this interference can be neglected since hemoglobin A2 concentrations in newborns are insignificant.

Information about the sequence of a specific tryptic fragment can be obtained by interpretation of its product mass scan. A comprehensive analysis of generated ions (an,b n,c n,x n,y n,z n) within the collision-induced dissociation (CID) cell give indications of the amino acid sequence. Therefore, we subjected informative peptides to fragmentation to precisely determine their specific product masses. Application of low fragmentation energies generates mainly b n and y n ions (Supplemental Fig. 1).

Comparative Study
We compared results obtained by IEF and MS/MS on the same newborns. IEF screening was performed on cord blood samples from neonates born in the maternity hospital CHR Citadelle (Liège, Belgium) with the PerkinElmer Neonatal Hemoglobin Test kit, according to the manufacturer’s instructions. Abnormal specimens were further submitted to capillary zone electrophoresis (CZE) with the Analis HbA2-CE and HbAlc-CE kits. Maternal consent was obtained before collecting blood samples. This program is part of a collaborative project with the Réseau des hémoglobinopathies (24).

All samples tested by MS/MS were obtained from the same 5-day old newborns. Whole blood dried on filter paper (Whatman 903 card) was collected as part of the mandatory neonatal screening program in Belgium, and the study was performed on residual blood spots after the requested analyses.

Results

MS/MS Detection of Mutated Peptides
To ensure good discrimination between homozygote and heterozygote variants, we selected 4 transitions
with good signal intensities for each informative peptide and calculated ratios of variant/Hb A for each.

Additionally, a hemoglobin F specific transition was recorded. As only 3 rare variants are described for the 12th tryptic fragment of the γ chain, LHVD PENFK (2), this peptide was selected to check the efficiency of digestion and to calculate the Hb A/Hb F ratio. This proportion gives information on the production of the normal β chain and thus could allow identification of newborns with major β-thalassemia.

All acquired transitions and ratios are presented in Table 1, and examples of MRM acquisition profiles are illustrated in Fig. 1.

**METHOD VALIDATION**
We evaluated repeatability and reproducibility on cumulative calculated ratios (Table 1). To estimate within-series variability, we considered 16 consecutive measurements in the same run for the following samples: neonatal Hb AA, Hb AS, Hb SS, Hb AC, and adult Hb CC.

### Table 1. Acquired MRM transitions for the first tryptic peptide (T1) and third tryptic peptide (T3) of β-globin.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>F, peptide T12</th>
<th>A, peptide T1</th>
<th>S, peptide T1</th>
<th>C, peptide T1</th>
<th>A, peptide T3</th>
<th>E, peptide T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>b2-ion</td>
<td>550 &gt; 251</td>
<td>477 &gt; 237</td>
<td>462 &gt; 237</td>
<td>694 &gt; 237</td>
<td>1315 &gt; 214</td>
<td>916 &gt; 214</td>
</tr>
<tr>
<td>y2-ion</td>
<td>694 &gt; 244</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y3-ion</td>
<td></td>
<td>694 &gt; 345</td>
<td></td>
<td></td>
<td>916 &gt; 261</td>
<td></td>
</tr>
<tr>
<td>y4-ion</td>
<td>477 &gt; 502</td>
<td>462 &gt; 472</td>
<td></td>
<td>694 &gt; 458</td>
<td>1315 &gt; 416</td>
<td>916 &gt; 360</td>
</tr>
<tr>
<td>y5-ion</td>
<td>477 &gt; 603</td>
<td>462 &gt; 573</td>
<td></td>
<td></td>
<td>916 &gt; 489</td>
<td></td>
</tr>
<tr>
<td>y6-ion</td>
<td>477 &gt; 716</td>
<td>462 &gt; 686</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y8-ion</td>
<td></td>
<td></td>
<td></td>
<td>1315 &gt; 758</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y9-ion</td>
<td></td>
<td></td>
<td></td>
<td>1315 &gt; 887</td>
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<table>
<thead>
<tr>
<th></th>
<th>A/F</th>
<th>S/A</th>
<th>C/A</th>
<th>E/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>T1/T12</td>
<td>T1 ratio</td>
<td>T1 ratio</td>
<td>T3 ratio</td>
</tr>
<tr>
<td>Cumulated</td>
<td>b2/b2</td>
<td>(b2 + y4 + y5 + y6)/ (b2 + y4 + y5 + y6)</td>
<td>(b2 + y2 + y3 + y4)/ (b2 + y4 + y5 + y6)</td>
<td>(b2 + y3 + y4 + y5)/ (b2 + y3 + y4 + y5)</td>
</tr>
</tbody>
</table>

\(^a\) The 12th tryptic fragment (T12) of γ-chain allows checking the efficiency of digestion and calculating Hb A/Hb F ratio. Additionally, for each informative peptide ratios, variant/Hb A was calculated. Formation of simply and multiply charged peptides occurs in the source and affects the mass/charge ratio (m/z). Therefore, the specific fragments for normal and sickle hemoglobin are observed on both forms on a scan spectrum. Because doubly charged ions fragment at lower collision energies, they result in better sensitivity; thus, m/z of 477 (Hb A) and 462 (Hb S) were selected for MRM acquisitions.
Hb AE (spotted on filter paper). We assessed interassay variability on 10 measurements of different neonatal Hb AA, Hb AS, and Hb AC specimens. According to the considered ratio, results for intraassay CV varied between 2.5% and 30.7%. Interassay CV fluctuated between 6.3% and 23.6% (see online Supplemental Table 3). The high intraassay CV of the homozygote Hb SS sample (30.7%) correlates with the fluctuation of instrument noise. The acquired signal for Hb A first tryptic peptide is negligible owing to the absence of normal hemoglobin. However, slight fluctuations of background intensities can largely influence the S/A ratio, and we therefore calculated relative high CV.

We assessed carryover by 3 consecutive measurements of concentrated purified Hb S (H1, H2, H3) followed immediately by 3 measurements of a normal sample (L1, L2, L3). Five such series were performed. Carryover was calculated as \((L1 - H1)/H3\) and expressed as a percentage. Results for each series were <<0.03%.

We measured the limit of detection (LOD) of Hb S by preparing sequential dilutions of an adult pathologic sample (Hb AS) in an adult normal sample (Hb AA) at the same hemoglobin concentration. We analyzed 5 series of the following concentrations: 0%, 1%, 2%, 5%, 10%, 20%, and 30% of Hb S. Because samples containing 1% of Hb S showed results statistically different from those of normal samples (Mann–Whitney test, \(P < 0.0001\)), the LOD was fixed at this concentration.

COMPARATIVE STUDY
We carried out a retrospective study to compare results obtained by MS/MS and IEF on the same newborns, analyzing 2082 specimens by both techniques. The IEF and MS/MS results are summarized in Table 2. According to the MS/MS settings (limited to the identification of Hb S, Hb C, Hb E, and \(\beta\)-globin production defects), we did not find any discrepant results; however, 17 samples with Hb-Barts were observed by IEF as well as 1 \(\alpha\) and 1 unidentified variant.

Median values of the cumulated ratios for normal samples tested during the comparative study were near 0 (S/A 0.004, range 0 – 0.06; C/A 0.018, range 0.006 – 0.08; E/A 0.019, range 0.002 – 0.17). Conversely, ratios observed for heterozygote patients were close to 1, and those of homozygotes (Hb SS or Hb S/\(\beta\)-thalassemia) were at least 10-fold higher (Table 3). Additionally, the A/F ratio was markedly decreased for the homozygote SS and S/\(\beta\)-thalassemia patients \((P < 0.0001)\). The A/F ratio variability encountered for normal phenotypes (Table 3) is related to sample heterogeneity (i.e., transfused newborns or babies older than 5 days resulting in highly increased proportion values).

**Table 2. Summary of results obtained with IEF and MS/MS.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>IEF</th>
<th>MS/MS</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>1961</td>
<td>1978</td>
</tr>
<tr>
<td>AS</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>AS + Hb-Barts</td>
<td>2</td>
<td>2a</td>
</tr>
<tr>
<td>AC</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>AE</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Hb-Barts</td>
<td>15</td>
<td>NSb</td>
</tr>
<tr>
<td>SS(^b)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>S/(\beta)-thalasemia(^c)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(\alpha) Variant</td>
<td>1</td>
<td>NSc</td>
</tr>
<tr>
<td>Unknown variant</td>
<td>1</td>
<td>NSc</td>
</tr>
</tbody>
</table>

\(^a\) Hb AS phenotype was correctly identified. Hb-Barts is not screened by MS/MS.

\(^b\) NS, not screened.

\(^c\) Hb SS and S/\(\beta\)-thalassemia were discriminated at the age of 6 months on hemogram parameters and hemoglobin S and A\(_2\) levels.

**Table 3. Cumulative ratios for the different phenotypes observed.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Ratio</th>
<th>n</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>A/F</td>
<td>1961</td>
<td>0.40 (0.188)</td>
</tr>
<tr>
<td>SS or S/(\beta)-thalassemia</td>
<td>A/F</td>
<td>5</td>
<td>0.01 (0.005)</td>
</tr>
<tr>
<td>AS</td>
<td>S/A–T1</td>
<td>80</td>
<td>0.80 (0.143)</td>
</tr>
<tr>
<td>SS or S/(\beta)-thalassemia</td>
<td>S/A–T1</td>
<td>5</td>
<td>20.6 (4.730)</td>
</tr>
<tr>
<td>AC</td>
<td>C/A–T1</td>
<td>13</td>
<td>1.16 (0.281)</td>
</tr>
<tr>
<td>AE</td>
<td>E/A–T3</td>
<td>4</td>
<td>1.14 (0.612)</td>
</tr>
</tbody>
</table>

Discussion

Most screening programs for hemoglobinopathies use IEF or HPLC as the initial testing method, but the interpretation of results (i.e., IEF migration profiles) may be unclear.

Because tandem mass spectrometry is currently the reference method for neonatal screening of inborn errors of metabolism (IEM), we decided to assess its ability to screen for sickle cell disease. The emergence of mass spectrometry technologies brings a new opportunity in the identification of protein mutations. Technically, MS/MS is a more analytically sensitive and specific method than IEF or HPLC, and theoretically, an optimized method could allow the identification of most hemoglobin mutants. Most variants have little clinical relevance, however, and the objectives of the United Kingdom NHS Sickle Cell and Thalassaemia
Screening Program mainly focused on the identification of infants at risk of sickle cell disorders and cases of β-thalassemia (9).

To implement tandem mass spectrometry for a routine screen of sickle cell disease, we needed to minimize sample preparation and analysis times by avoiding prior purification, derivatization, or separation. Therefore, we did not consider approaches combining whole-protein analysis and trypsin digestion, since they require time-consuming protocols. Whole-protein analysis lacks specificity, as the sickle hemoglobin (β6 Glu>Val) is 30 Da lighter the normal protein, and such a mass shift is also observed for other amino acid substitutions (see online Supplemental Table 4). Other common hemoglobin variants (Hb C, Hb E, Hb D-Punjab and Hb O-Arab) with mass decreases of <1 Da highlight the inability of this approach to identify important variants (see online Supplemental Table 5). Moreover, high-sensitivity equipment and deconvolution software have had difficulty in resolving 2 globin chains that differ in mass by <6 Da (18, 25). Finally, we developed an approach based on a tryptic peptide analysis and limited our method to the detection of Hb S, Hb C, and Hb E and the identification of β-globin production defects (β-thalassemia).

Previous publications evaluating MS/MS for hemoglobinopathy detection showed insufficient analytical sensitivity for discriminating homo- and heterozygote patients (20). Therefore, we focused the acquisition parameters on 4 specific transitions for each informative peptide; the calculation of ratios variant/normal peptides gave unambiguous information on the hetero- or homozygote status of the newborns.

We tested 2082 specimens by IEF and MS/MS; no discrepancies were registered with our MS/MS parameters. However, the 17 Hb-Barts detected by IEF were missed by MS/MS screening, since our method was not focused on the identification of α-thalassemia. Nevertheless, α-thalassemia screening should be adaptable for this purpose by evaluating the production ratio between α- and γ-globin chains.

Moreover, the presented methodology could lead to misinterpretations for rare β-globin variants. The pattern of a clinically insignificant T1-peptide homozygote mutation (other than Hb S or Hb C) may be interpreted as a major β-thalassemia profile, since the concentrations of T1 peptide would be dramatically reduced. Compound heterozygote T1-peptide variant/Hb S could be confused with homozygosity for hemoglobin S.

Although a larger series of samples needs to be compared by both approaches (MS/MS vs IEF or HPLC) to increase and validate the detection of rare variants, we demonstrated that MS/MS is an efficient method for performing a primary screen for main hemoglobinopathies. HPLC, IEF, and/or molecular analysis will in all cases remain essential as second-line tests to verify the suspected abnormality or discriminate between equivocal phenotypes (e.g., Hb SS and Hb S/β0-thalassemia).

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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