Screening Cord Blood for Hemoglobinopathies and Thalassemia by HPLC

Fey P. L. van der Dijl,1 Gita A. van den Berg,2 John G. Schermer,3 Fred D. Muskiet,4 Hans Landman,4 and Frits A. J. Muskiet1

We evaluated the use of an HPLC method for screening hemoglobin in cord blood. We studied the genotype frequencies of the structural hemoglobin variants HbS and HbC and the synthesis variants α- and β*-thalassemia in babies born on Curaçao. During three months, 67.2% of all (748) newborns were screened: 122 (24.3%) had an abnormal hemoglobin pattern, of which 53 (43.4%) had a hemoglobinopathy (HbS or HbC), 64 (52.2%) had α-thalassemia (HbBarts >0.5%, corresponding to heterozygous or homozygous α-thalassemia-2), and 5 (4.1%) had a hemoglobinopathy plus α-thalassemia. None of the newborns with heterozygous HbS and HbC had concomitant β*-thalassemia. The population genotype frequency of heterozygous α-thalassemia-2 was calculated to be 30.7%. The data are in excellent agreement with those previously established for the adult population and those available from the black population in the United States and Jamaica. Based on the HPLC results, we estimate that 67.1% of newborns with heterozygous α-thalassemia-2 remain undetected. A coincidental finding was a relation between demonstrable α-thalassemia and short gestation. Because of its superior separating power and high sensitivity for quantifying relatively low percentages of hemoglobins in the presence of HbF, the HPLC method was preeminently suitable for screening cord-blood samples.

1 Central Laboratory for Clinical Chemistry, University Hospital Groningen, and 2 Stichting Klinisch Chemisch Laboratorium, Leeuwarden, The Netherlands.
3 Public Health Laboratory, and 4 St. Elisabeth Hospital and Maternity Clinic, Curaçao, The Netherlands.

Received January 27, 1992; accepted April 10, 1992.

References
Accurate identification and quantification of hemoglobins are essential for the diagnosis and understanding of hemoglobinopathies and thalassemia (1–7). Hemoglobin profiling of cord blood is greatly complicated by the presence of relatively high amounts of fetal hemoglobin (HbF). Screening by electrophoresis requires a combination of the alkaline (cellulose acetate) and acidic (citrate agar) methods (8, 9). Although the common β-chain variants hemoglobin S (HbS) and C (HbC) are readily observed, differentiation among conditions such as heterozygous hemoglobin S (HbAS), HbAS-a-thalassemia, HbS-β+-thalassemia, and even homozygous hemoglobin S (HbSS) is sometimes difficult (3).

Recently Ou et al. (10) described a high-performance liquid-chromatographic (HPLC) method, using a cation-exchange column and a combined pH and NaCl gradient for separation and quantification by spectrophotometric detection. To evaluate this method for the screening of cord blood, we studied the gene frequencies of the structural variants HbS and HbC and the synthesis variant α-thalassemia (by the assay of HbBarts) in cord blood of babies born on the Caribbean island of Curacao (The Netherlands Antilles). The vast majority of the population on Curacao is of West African descent (11). For the structural variants, comparative data from population studies among adults were available from the Public Health Laboratory on Curacao. Although the frequency of α-thalassemia is unknown, it seemed reasonable to expect data similar to those reported for the black population in the United States and Jamaica.

Materials and Methods

Study design. From July through September 1990, we attempted to screen all newborns for hemoglobin disorders demonstrable by HPLC analysis (see below) of cord blood. The vast majority of babies are born in the Maternity Clinic and the hospital (St. Elisabeth Hospital). Information on sex and gestational age, usually determined by recording the time of last menstruation, was collected as completely as possible.

Samples. Cord-blood samples were collected by midwives under supervision of a pediatrician in the Maternity Clinic and by one of us in the St. Elisabeth Hospital. After clamping, cord blood was sampled into a Vacutainer Tube containing EDTA (Becton Dickinson Vacutainer Systems Europe, Meylan, France) and transported to the Public Health Laboratory in ice. Samples were centrifuged for 5 min at 1500 × g and ambient temperature (24°C). After removing the plasma we lysed the erythrocytes by adding an equal volume of doubly distilled water. We then added 5 μL of the lysate to 1.0 mL of buffer A (see below), mixed, and centrifuged this at 6000 × g for 5 min (sample solution).

Chemicals. (Bis[2-hydroxyethyl]amino)tris(hydroxymethyl)methane (Bis Tris) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Potassium cyanide, sodium chloride, and Triton X-100 were obtained from Merck (Darmstadt, FRG). Hemoglobin control solution (AFCS control), specified to contain almost equal amounts of HbA, HbF, HbC, and HbS, was purchased from Helena Labs. (Beaumont, TX).

Hemoglobin profiling. We used the integrated HPLC “System Gold” from Beckman Instruments (Brea, CA), consisting of a Model 126 solvent-delivery module, a Model 166 spectrophotometer, and a Model 427 integrator. The system was equipped with a 200 × 4.6 (i.d.) mm column packed with 5-μm microparticulate poly(aspartic acid) silica (Poly Cat A) from Custom LC (Houston, TX).

The mobile phase, composed of buffers A and B, and the gradient program were essentially the same as developed by Ou et al. (10). In this system, separation of hemoglobins is accomplished by a combined NaCl and pH gradient. Buffer A (pH 6.5) contained 40 mmol of Bis Tris, 4 mmol of KCN, and 0.5 mL of Triton X-100 per liter. Buffer B (pH 6.8) contained 40 mmol of Bis Tris, 4 mmol of KCN, 200 mL of NaCl, and 0.5 mL of Triton X-100 per liter. The pH of the buffers was adjusted with 4 mol/L HCl. Before use, the buffers were filtered through a 0.45-μm pore-size membrane. For hemoglobin analyses, we injected 20-μL portions of the sample solution (see above) at a flow rate of 1.0 mL/min. The column effluent was monitored at 420 nm.

To identify hemoglobins A, F, C, and S in cord-blood samples, we compared the HPLC peak retention times with those obtained for the AFCS control. HbBarts was identified by analyzing a cord-blood sample containing a large percentage of HbBarts, as demonstrated by the electrophoretic method routinely performed in the Central Laboratory for Hematology of the University Hospital of Groningen, The Netherlands. (The diagnosis of α-thalassemia in this sample was confirmed by DNA analysis.) Peak area percentages were used for calculating the hemoglobin composition, on the assumption that equal masses analyzed give rise to equal peak areas.

Interpretation and statistics. An HbBarts percentage between 0.5% and 2.0% was assumed to be associated with heterozygous α-thalassemia-2 (α-thalassemia-2 trait or silent carrier), a condition with three active α-genes (ααα−). An HbBarts percentage between 2.0% and 10.0% was considered indicative for homozygous α-thalassemia-2, a condition with two active α-genes (α−αα) (12, 13). Heterozygous α-thalassemia-1, also a condition with two active α-genes (αα−−), has not been taken into account, because this type of thalassemia is prevalent in individuals from the Far East and the Mediterranean area.

For α-thalassemia, the population genotype frequency was estimated from the sample phenotype frequency, according to the Hardy–Weinberg law (14), described by the formula \( p^2 + 2pq + q^2 \), where \( p \) is the frequency of an autosomal recessive gene (−), \( q = 1 - p \): the frequency of the normal gene (αα), \( 2pq \) = the frequency of heterozygotes (ααα−), \( p^2 \) = the frequency of homozygotes (α−α−), and \( q^2 \) = the frequency of normal homozygotes (αααα).

Odds ratios (15) were calculated to compare the frequency of prematurity (gestational age <38 weeks) between newborns with HBAA plus HbBarts ≤0.5%,
Fig. 1. Typical HPLC patterns of cord-blood samples: (a) HbAA; (b) HbAA with 3.0% HbBarts; (c) HbAS; (d) HbAC; (e) HbSS; (f) HbCC; (g) HbSC; (h) HbAS with 1.5% HbBarts; (i) HbAC with 1.5% HbBarts

CLINICAL CHEMISTRY, Vol. 38, No. 9, 1992
HbAA plus HbBarts between 0.5% and 2.0%, and HbAA plus HbBarts between 2.0% and 10.0%.

Quality control. The AFCS hemoglobin control was used to estimate the within-day precision and day-to-day reproducibility.

Results

According to the Population Registry Office of Curanão, 748 children were born during the observation period of three months. We collected samples from 503 newborns, 67.2% of all newborns. The vast majority of infants on Curanão are born in the Maternity Clinic in St. Elisabeth Hospital. The racial composition and gestational age distribution of the group for which no samples were collected or received were not considered to differ from those that were actually screened.

The chromatograms in Figure 1 show the separation efficiency of the HPLC method for cord-blood samples of newborns with HbAA, HbAA with 3.0% HbBarts, HbAS, HbAC, HbSS, HbCC, HbSC, HbAS with 1.5% HbBarts, and HbAC with 1.5% HbBarts. Relative retention times of the hemoglobins were comparable with those given by Ou et al. (10). The results of the quality-control analyses are listed in Table 1.

The hemoglobin patterns of the 503 newborns studied, grouped according to hemoglobin and α-thalassemia type, are shown in Table 2. In all, 122 (24.3%) displayed an abnormal hemoglobin pattern, of which 53 (43.4%) had a hemoglobinopathy (HbS or Hbc), 64 (52.2%) had α-thalassemia (heterozygous or homozygous α-thalassemia-2), and 5 (4.1%) had a hemoglobinopathy plus α-thalassemia. The HbS/HbA0 + HbS) ratio in all newborns with HbAS (n = 23) amounted to 0.44 ± 0.04 (range: 0.36–0.52). For newborns with HbAC (n = 32), the HbC/HbA0 + HbC) ratio was 0.46 ± 0.05 (range: 0.34–0.56). The population genotype frequency of heterozygous α-thalassemia-2 in the population of Curanão was calculated as 30.7%, based on the frequency of newborns with homozygous α-thalassemia-2.

From the newborns (see Table 2) with HbAA (n = 381), HbAA with heterozygous α-thalassemia-2 (n = 47), HbAA with homozygous α-thalassemia-2 (n = 17), HbAC (n = 28), and HbAS (n = 22), the gestational ages were known for 237, 38, 13, 11, and 18 babies, respectively. In all cases, labor was initiated by natural causes. No gestational age data were available for the newborns with HbAC plus α-thalassemia (n = 4), HbAS plus α-thalassemia (n = 1), HbCC (n = 1), HbSS (n = 1), and HbSC (n = 1). The frequency of premature delivery in newborns with HbAA was compared on the basis of differences in the percentage of HbBarts. In comparison with the group with HbAA plus HbBarts ≤0.5% (n = 237), prematurity occurred 1.99 (odds ratio) times as often among newborns with HbAA plus HbBarts between 0.5% and 2.0% (n = 38) and 4.79 times as often among newborns with HbBarts between 2.0% and 10.0% (n = 13). The respective 95% confidence intervals were 1.43–2.77 and 2.45–9.36. No significant difference in prematurity was observed between the two groups with HbAA plus HbBarts >0.5%. Figure 2 shows the smoothed frequency distribution curves for gestational ages of the three groups of newborns with HbAA and various percentages of HbBarts. The data suggest that, with a decreasing number of active α-genes, gestational age tends to be shorter.

Figure 3 shows the means and 95% confidence intervals for the percentages of HbA0 and HbF0 as a function of gestational age for newborns with HbAA plus HbBarts ≤0.5%. The HbF0/HbA0 ratio did not change as a function of gestational age. Percentages of HbA0 and HbF0 for newborns with HbAA plus α-thalassemia and heterozygous hemoglobinopathies are depicted as individual points. The data suggest that, corrected for ges-

---

Table 1. Within-Day and Day-to-Day Quality-Control Data

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean ± SD, %</th>
<th>CV, %</th>
<th>Mean ± SD, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA0</td>
<td>23.0 ± 0.7</td>
<td>3.3</td>
<td>22.0 ± 1.6</td>
<td>7.4</td>
</tr>
<tr>
<td>HbF0</td>
<td>20.3 ± 0.9</td>
<td>4.5</td>
<td>20.8 ± 1.6</td>
<td>7.6</td>
</tr>
<tr>
<td>HbC</td>
<td>24.1 ± 0.5</td>
<td>1.9</td>
<td>23.0 ± 2.1</td>
<td>10.7</td>
</tr>
<tr>
<td>HbS</td>
<td>22.3 ± 1.0</td>
<td>4.3</td>
<td>23.0 ± 1.3</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* Determined with Helma Labs. AFCS control.

---

Table 2. Hemoglobin and α-Thalassemia Types in 503 Consecutive Cord-Blood Samples on Curanão

<table>
<thead>
<tr>
<th>Type</th>
<th>M</th>
<th>F</th>
<th>U</th>
<th>No.</th>
<th>%</th>
<th>HbBarts</th>
<th>HbF0</th>
<th>HbF0</th>
<th>HbF0</th>
<th>Tot. HbF</th>
<th>HbA0</th>
<th>HbC</th>
<th>HbS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbAA</td>
<td>159</td>
<td>164</td>
<td>56</td>
<td>381</td>
<td>75.7</td>
<td>≤0.5</td>
<td>9.4 ± 3.0</td>
<td>72.5 ± 5.8</td>
<td>81.9 ± 6.0</td>
<td>15.4 ± 5.8</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAA-α-thal.*</td>
<td>22</td>
<td>20</td>
<td>7</td>
<td>47</td>
<td>8.3</td>
<td>1.1 ± 0.3</td>
<td>9.0 ± 3.5</td>
<td>72.9 ± 6.8</td>
<td>81.9 ± 6.8</td>
<td>14.7 ± 6.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAA-α-thal.*</td>
<td>8</td>
<td>9</td>
<td>0</td>
<td>17</td>
<td>3.4</td>
<td>5.8 ± 2.9</td>
<td>10.1 ± 2.5</td>
<td>69.7 ± 6.0</td>
<td>79.8 ± 5.8</td>
<td>12.9 ± 4.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAC</td>
<td>14</td>
<td>11</td>
<td>3</td>
<td>28</td>
<td>5.6</td>
<td>≤0.5</td>
<td>8.9 ± 1.5</td>
<td>72.1 ± 6.1</td>
<td>81.0 ± 5.9</td>
<td>8.7 ± 3.1</td>
<td>7.3 ± 2.8</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAC-α-thal.*</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0.6</td>
<td>0.9 ± 0.1</td>
<td>9.2 ± 2.5</td>
<td>81.5 ± 2.8</td>
<td>90.7 ± 2.6</td>
<td>4.2 ± 1.4</td>
<td>3.3 ± 1.1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAC-α-thal.*</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.2</td>
<td>3.0</td>
<td>4.5</td>
<td>80.5</td>
<td>85.0</td>
<td>4.2</td>
<td>4.0</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAS</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>22</td>
<td>4.4</td>
<td>≤0.5</td>
<td>10.0 ± 3.0</td>
<td>73.1 ± 8.8</td>
<td>83.2 ± 9.8</td>
<td>8.2 ± 5.6</td>
<td>6.3 ± 3.0</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbAS-α-thal.*</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.2</td>
<td>1.5</td>
<td>9.8</td>
<td>80.0</td>
<td>89.0</td>
<td>4.7</td>
<td>n.d.</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>HbCC</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.2</td>
<td>≤0.5</td>
<td>7.8</td>
<td>70.7</td>
<td>78.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>HbSS</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.2</td>
<td>≤0.5</td>
<td>7.7</td>
<td>80.4</td>
<td>88.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>HbSC</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.2</td>
<td>≤0.5</td>
<td>15.1</td>
<td>67.8</td>
<td>82.9</td>
<td>6.0</td>
<td>8.0</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

* Male, female, and unknown. a Acetylated hemoglobin. b Heterozygous α-thalassemia-2 (based on HbBarts between 0.5% and 2.0%). c Homozygous α-thalassemia-2 (based on HbBarts between 2.0% and 10.0%).

---

CLINICAL CHEMISTRY, Vol. 38, No. 9, 1992 1867
tational age, newborns with heterozygous or homozygous α-thalassemia-2 have lower percentages of Hbf0 and somewhat higher percentages of Hba0 than infants without thalassemia. Corrected for gestational age, newborns with heterozygous hemoglobinopathies (HbAC or HbAS, without coexisting α-thalassemia) had relatively low percentages of Hba0.

Discussion

The frequencies of the structural variants (Table 2) are in excellent agreement with the adult prevalences (HbS = 5% and Hbc = 8%) previously found in a population study carried out by the Public Health Laboratory on Curacao in 1977–8. The higher frequency (6) of Hbc than HbS can be explained by the predominant descent of the population from West Central Africa, particularly Ghana (11).

The calculated percentage of the genotype frequency (30.7%) for heterozygous α-thalassemia-2 on Curacao corresponds with the percentages found in the black populations of the United States and Jamaica (30%) (3, 16). However, our estimate is based on the frequency of newborns with homozygous α-thalassemia-2, as calculated by the Hardy–Weinberg formula. The observed frequency of heterozygous α-thalassemia-2 in the screened newborns (10.1%; Table 2) does not correspond with the calculated frequency. Previous studies in the black population have also demonstrated that the deletion of one α-globin gene is not necessarily associated with a detectable amount of Hbbarts at birth (4, 12, 13).

On the basis of our results, we predict that 67.1% of the newborns with heterozygous α-thalassemia-2 will remain undetected with HPLC screening.

Because in the newborns with heterozygous HbAS and HbAC the upper limits of Hbs/(Hba0 + Hbs) and Hbc/(Hba0 + Hbc) were 0.52 and 0.56, respectively, we conclude that none of them had concomitant β*-thalassemia. Concomitant β*-thalassemia would have led to Hbs/(Hba0 + Hbs) and Hbc/(Hba0 + Hbc) values of 0.75–0.80 (3). In general, hemoglobin profiling methods for screening cord blood cannot distinguish Hba0, Hbs, and Hbc by Hba-β, Hbs-β, and Hbc-β, respectively.

The finding of somewhat higher percentages of Hba0 and lower percentages of Hbf0 for newborns with heterozygous or homozygous α-thalassemia-2 than in unaffected infants (Figure 3) can be explained by the higher affinity of the limited number of α-chains for the β-chains, rather than for the γ-chains (3). The incidental finding of an association between demonstrable α-thalassemia and duration of gestation (Figure 2) suggests that the shorter gestation of black newborns (17, 18) is related to their high frequency of α-thalassemia rather than to race per se. It is tempting to speculate that the lower percentage of Hbf0, and consequently a decreased capacity to extract oxygen from the maternal circulation, is a factor in the somewhat earlier birth of newborns with α-thalassemia.

Given its superior separating power and high sensitivity for quantifying relatively low percentages of hemoglobins in the presence of high percentages of Hbf0, we consider the HPLC method preeminently suitable for screening cord-blood samples. The early diagnosis of sickle cell disease (HbsS and HbSC) provides the possibility of adequate counseling of the parents by the family doctor. Increased parental awareness may lead to adequate action when the first crisis occurs. The early
identification of α-thalassemia may prevent institution of iron therapy after an unexplained microcytosis is established at a later age.

We thank J. M. Eustatia (Head of the Public Health Laboratory) for the opportunity to perform the study on Curacao, the nurses of the St. Elisabeth Hospital and the midwives of the Maternity Clinic for collecting the cord-blood samples, and M. A. Kibbele (Head) and R. S. Simoes-Barrow of the Public Health Department of Curacao for providing us with the necessary population data. We are indebted to H. Oehler-Hammen and her coworkers of the Department of Clinical Chemistry and Hematology (Public Health Laboratory), H.J.R. Velvia and A.M.A. Borbboom-Meyer (Central Laboratory for Clinical Chemistry), and R. Petronia (Head) and his coworkers of the Department of Toxicology and Pharmaceutical Quality Control (Public Health Laboratory) for their excellent assistance, and M. Volmer (Central Laboratory for Clinical Chemistry) for performing the statistical analyses.

References

CLIN. CHEM. 38/9, 1869-1873 (1992)

Clostebol-Positive Urine after Consumption of Contaminated Meat

G. Debruyckere,1 R. de Sagner,2 and C. Van Peteghem1

We examined the relationship between the consumption of meat from animals treated with anabolic steroids and the detection of these steroids in the athletes consuming this meat. We proved that clostebol metabolites (e.g., 4-chloro-Δ4-androstene-3α-ol-17-one) in the urine of one of the volunteers involved in a feeding experiment were due to accidental consumption of meat contaminated with clostebol acetate. When two volunteers consumed meat (100 g) containing either 1 or 0.1 mg of clostebol acetate, the same metabolite was found in their urine.

Additional Keyphrases: anabolic steroids • athletic drug testing • gas chromatography/mass spectrometry

Routine screening for illegal use of anabolic steroids to fatten cattle in Belgium is regulated by the Institute of Veterinary Inspection, which depends on the Ministry of Public Health. The screening tests, carried out at our laboratory and other accredited laboratories, revealed that the use of illegal additives in cattle fattening, which contain anabolic steroids prohibited by the International Olympic Committee for use by athletes, has ramifications for humans (1).

Through research on the metabolism of nandrolone in men, we (2) showed that nandrolone metabolites were sometimes excreted in urine by untreated men. We were not able to determine the origin of those compounds but strongly suspected ingestion of meat from animals treated with nandrolone esters. Here we examine the relationship between the consumption of meat from retail sources and the detection of anabolic steroids in urine after meat consumption.

Materials and Methods

Urine and meat samples. For feeding experiment 1, 10 members (men and women) of the laboratory staff bought a 150-g portion of minced beef from their usual retail trade butcher. Before the meat was consumed, a 5-g portion was stored at -20 °C until analysis. Urine was collected just before the raw meat was consumed

Laboratories of 1 Food Analysis and 2 Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

Received July 16, 1991; accepted December 24, 1992.