Nonradioactive Quantification of Low Concentrations of Hemoglobin A by HPLC for Mid trimester Prenatal Diagnosis of β-Thalassemia

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The usual methods for prenatal diagnosis of β-thalassemia and other hemoglobinopathies by assay of fetal blood erythrocytes are either complex (analysis of globin chains synthesis by carboxymethylcellulose chromatography) or only semiquantitative [isolectric focusing of hemoglobin (Hb)]. To further simplify the diagnostic procedure and to obtain quantitative data, we measured the small concentrations of Hb A in fetal erythrocytes by using a high-pressure liquid chromatography (HPLC) instrument (DIAMAT-TM; Bio-Rad) equipped with the new column proposed for measuring Hb A₂. We analyzed 212 uncontaminated fetal blood samples obtained by cordocentesis between the 18th and 22nd weeks of pregnancy, using the HPLC procedure, and compared the results with those obtained by the above-named methods. The Hb A values obtained ranged between 0% and 8.5%; they were ≤1.8% in 44 fetuses affected by homozygous β-thalassemia and ≥2.5% in 188 unaffected fetuses. The method was simple, rapid, and reproducible (CV 3.2%) and there was good correlation between Hb A concentrations determined by HPLC and the β/γ ratio determined by carboxymethylcellulose chromatography (r = 0.7687; P <0.0001). No false-negative or false-positive results were observed, and there was no overlap of values between affected and unaffected fetuses.

Additional Keyphrases: fetal status • erythrocytes • hemoglobinopathies • hemoglobin variants

Prenatal diagnosis of β-thalassemia and other hemoglobinopathies by assaying fetal blood samples obtained by cordocentesis between the 18th and 22nd weeks of pregnancy is still important when DNA testing during the first trimester is not feasible. The methods usually used in our laboratory for this purpose are isoelectric focusing (IEF) of hemoglobin (Hb) (1, 2) and carboxymethylcellulose (CMC) chromatography (3, 4).5 The latter method, although considered the method of choice for this kind of investigation, is quite complex and requires the use of radioactive isotopes. IEF, in our experience, is simple and rapid and is conclusive in ~80% of the diagnoses. Its main limitations are the need for fetal blood samples uncontaminated with maternal blood and also some preliminary sample preparation steps. Moreover, IEF is only semiquantitative, the result being based on the intensity of the Hb A band.

The introduction of a new simple HPLC method allowing precise quantification of the various Hb fractions (5) prompted us to evaluate its reliability in prenatal diagnosis of hemoglobinopathies. This method, which utilizes a commercial column designed for Hb A₂ measurement, allows much more rapid and precise evaluation of fetal blood samples in the mid trimester of pregnancy than do the above methods. Here, we demonstrate that measurement of the Hb A fraction in fetal blood samples completely discriminates between affected and unaffected fetuses. The only requirement is the availability of uncontaminated fetal blood.

Materials and Methods

Between January 1988 and January 1990 we examined 229 fetal blood samples obtained by cordocentesis (gestation weeks 18–22), 212 of which were uncontaminated with maternal blood. These samples were from couples at risk for β-thalassemia (n = 193), β-thalassemia/β-thalassemia (n = 6), and β-thalassemia/abnormal hemoglobins (Hb S, Hb C, Hb Lepore, Hb J Sardinia) (n = 13). All samples were evaluated for possible contamination by maternal erythrocytes by using the volume distribution curve for erythrocytes (Coulter Channelizer; Coulter Corp., Hialeah, FL) and the test of Kleihauer et al. (6). Fetal blood samples containing even a small percentage of maternal erythrocytes were examined by CMC chromatography only. In the presence of a macroscopically evident amniotic fluid contamination, samples were washed twice in saline before being processed by HPLC, thereby avoiding any adverse effects due to such contamination.

Hb A was measured with a DIAMAT-TM HPLC analyzer (Bio-Rad Labs, Richmond, CA), with the instrument settings modified as described before (5). The hemoglobins were eluted at the following retention times: Hb A₁₄ and Hb A₁₉ at 1.7 min, A₁₆ at 2.5 min, F at 3.6 min, A at 8.5 min, A₂ at 11.7 min, and S at 13.5 min. Each run took ~15 min. The reproducibility (CV) of the Hb A quantification, evaluated by assessing the differences between duplicates, was 3.2% for a mean Hb A content of 2.5%.

We also studied the fetal blood samples by IEF (2) and in a few cases also by CMC chromatography (4). The typical imprecision (CV) of the CMC method is 18.3% (mean β/γ mass fraction 0.043). Fetal blood samples

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6 Nonstandard abbreviations: IEF, isoelectric focusing; CMC, carboxymethylcellulose; and Hb, hemoglobin.

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from unaffected subjects or from subjects homozygous for \( \beta \)-thalassemia were used as controls.

In agreement with fetal diagnosis, none of the 115 newborns that we checked at birth was affected by \( \beta \)-thalassemia.

**Results**

Of the 212 cases considered, 44 were affected by \( \beta \)-thalassemia major or related hemoglobinopathies and 168 were not affected, as determined with the comparison methods used in our laboratory (191 cases with IEF, 21 with both IEF and CMC chromatography). The distribution histogram of the relative Hb A concentrations is shown in Figure 1. A clear cutoff between the 44 affected fetuses (Hb A values ≤1.8%) and the 168 unaffected fetuses is evident. No false-negative or false-positive results for \( \beta \)-thalassemia major or related hemoglobinopathies were obtained, as determined by comparing the HPLC data with the data obtained by the other methods.

A good correlation \( (P <0.0001) \) between the Hb A concentrations and the \( \beta/\gamma \) ratio was found (Figure 2). In our experience, \( \beta/\gamma \) values <0.025 identify patients homozygous for \( \beta \)-thalassemia.

Thirteen fetuses were at risk for \( \beta \)-thalassemia and \( (or) \) abnormal hemoglobins (Hb S, Hb Lepore, Hb J Sardinia, and Hb C). A small Hb S peak can be associated with the Hb A peak in a fetus heterozygous for Hb S. Also in these cases, the IEF and CMC results always agreed with each other and with the HPLC results. None of the cases at risk showed inheritance of Hb Lepore, Hb C, and Hb J Sardinia from the parents, so that approach for evaluating these hemoglobin variants in HPLC during the midtrimester of pregnancy was impossible. However, adults heterozygous for these hemoglobins are easily identified by the HPLC method (data not shown).

**Discussion**

Since 1974, prenatal diagnosis of \( \beta \)-thalassemia in the midtrimester of pregnancy by means of CMC chromatography has been an important tool for the prevention of this disease (7). In 1984, introduction of IEF in prenatal diagnosis of \( \beta \)-thalassemia offered a valid alternative to CMC chromatography in our center by being a simpler, rapid, inexpensive method for examining uncontaminated fetal blood samples (2). However, IEF is a semiquantitative method, and absolute discrimination is impossible in borderline cases (e.g., faint Hb A band), which need to be confirmed by CMC chromatography. An HPLC method involving radioactive isotopes and globin chain separation has also been proposed as an alternative to CMC chromatography (8) for prenatal diagnosis of \( \beta \)-thalassemia.

The HPLC technique we used does not use radioactive materials and represents a new application of a commercially available system for Hb A\(_2 \) measurement. This HPLC method is advantageous in terms of simplicity, speed (15 min per run), and cost (about the same as that of IEF). With regard to the comparison between Hb A measured by HPLC and CMC chromatography of globin chains, the correlation, although statistically significant, is not total. A possible explanation for this could be the large difference in analytical imprecision of the two techniques.

Our results indicate that the method proposed is very reliable, with no overlap between results for affected and unaffected fetuses and producing no false-positive or false-negative results. The possibility of differentiating \( \beta^0 \) from \( \beta^+ \) homozygotes seems beyond the resolution limits of the technique; in fact, our recent experience with couples undergoing first-trimester fetal diagnosis indicates a high incidence of \( \beta^0 \) carriers (54%), whereas very low amounts of Hb A, probably representing the minimum background of the technique, were usually found in affected fetuses (both \( \beta^0 \) and \( \beta^+ \) homozygotes) at the midtrimester fetal diagnosis. However, a possible overlap between \( \beta^0 \) heterozygotes and \( \beta^+ \) homozygotes may be excluded, considering that the incidence of affected fetuses in this series is only slightly below that expected and that thalassemia major was excluded at follow-up in 115 cases.
We conclude that Hb A measurement by the HPLC technique proposed here should be the method of choice for prenatal diagnosis of thalassemia and related disorders in the midtrimester of pregnancy. The much more complex and time-consuming CMC chromatography is needed only for those few cases (7.5% in our experience) in which some degree of contamination of fetal samples by maternal erythrocytes is present.

References