Commercial Radioimmunoassay Kit for Measurement of Alpha-Fetoprotein Adapted for Use with Dried Blood Specimens from Newborns

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We adapted a commercial RIA kit to measure alpha-fetoprotein (AFP) in 0.75-µL portions of 60-µL spots of dried blood from newborns. We evaluated sample elution, temperature and time stability, between- and within-assay variability, sensitivity, and use of liquid vs dried specimens of blood. Also, we present normal AFP concentrations for healthy neonates during the first postnatal week. Our procedure permits measurement of AFP concentrations both in maternal liquid plasma and in spots of dried blood from the infant with the same RIA kit reagents and standards. The sensitivity, precision, stability, and simplicity of this procedure makes more practical the routine measurement of AFP in dried blood specimens from newborns than measurement in liquid plasma or serum. Blood-sample collection by heel stick suffices for this simple, efficient, inexpensive determination of AFP concentration in the newborn.

Additional Keyphrases: reference interval • "kit" methods • neural tube defects • monitoring tumor growth • screening • changes during the first postnatal week

Alpha-fetoprotein (AFP), a tumor-associated fetal (oncotelic) protein that is present early in the development of humans and other mammals (1, 2), has mainly been measured for prenatal detection of open neural-tube defects and postoperational monitoring of tumor growth in adults (3, 4). But detection, measurement, or differentiation of AFP in infants and newborns also shows promise as a diagnostic aid and marker for certain congenital disorders (5–9).

Mass screening of newborns is now possible by using biochemical, radioimmunological, and microbiological methods to quantitate constituents of blood samples spotted on strips of filter paper. Dried blood specimens, obtained by heel-stick and delivered by conventional surface mail, are now widely used in neonatal metabolic screening programs. The measurement of AFP in dried blood spots from newborns has previously been described for electroimmunoassay (5) and immunoradiometric (10) assays.

Our attempts to assess the potential use of AFP as a diagnostic aid in the newborn screening program made it necessary to measure AFP concentrations in dried blood specimens. Because no commercial radioimmunoassay (RIA) kits are available for this, we adapted a kit intended for use with liquid specimens. Here we present our findings on the development of this technique, and some preliminary estimates of AFP concentrations during the first postnatal week.

Materials and Methods

Specimens. About 800 dried blood specimens from newborns, submitted by hospitals throughout New York State for neonatal blood screening, were used in this study. These samples, obtained by heel-stick, were applied as 1.2-cm spots onto filter paper (no. 503; Schleicher and Schuell, Keene, NH 03431). Heparinized cord-blood (plasma) specimens were obtained from an additional 100 newborns. AFP concentrations were measured in both blood spot and plasma, and the results were compared with those for 48 cord-blood samples. Hematocrits were also determined. Dried blood specimens for use as quality controls were prepared by spotting single 60-µL drops of the cord blood from a micropipet directly onto filter paper. Liquid samples were obtained from about half of these blood collections. The dried blood specimens were air dried at room temperature and routinely stored in plastic bags at 4 °C. For the assay, a disc 1.5 mm in diameter, punched from between the center and edge of each blood spot, was placed directly into a 12 × 75-mm polystyrene test tube.

Sample preparation. All dried blood specimens were eluted with "zero standard" buffer (Amersham Corp., Arlington Hts., IL 60005) before assay. The punched disc (containing 0.75 µL of blood) was eluted by allowing it to stand for 1 h at room temperature with 100 µL of a "zero standard" buffer (whole human plasma; AFP concn. 0 µg/L) supplied with the RIA kit.
Sonication of the specimen did not increase the amount of AFP eluted. Experiments with 125I-labeled human AFP showed that the analytical recovery of AFP from dried blood spots was 90%. The RIA values (in μg/L) were converted to serum concentrations (in mg/L) by assuming a hematocrit of 0.50 (11) and a total blood volume of 0.75 μL per spot.

**Storage stability of AFP.** To determine whether AFP is more stable in the liquid or dry state, we spotted 60-μL aliquots of cord blood on filter paper as described above. Each sample was then centrifuged and the plasma removed for testing in parallel with a dried blood-spot disc. After the initial concentrations were obtained, the plasmas were stored at −20 °C and the blood spots at 4 °C, then retested four months later and the results compared.

We determined whether AFP in dried-blood spots was more stable when stored refrigerated or at room temperature. Two specimens representing low and high AFP concentrations were selected and tested at one- to two-month intervals for six months.

**Assay method and standards.** Nanogram concentrations of human AFP were determined in a single-antibody RIA in which nonlabeled AFP competes with radiolabeled AFP for antibody sites. The AFP RIA kits were purchased from Amersham Corp. To modify the assay for use with dried-blood specimens, each spot was eluted into 100 μL of “zero standard” buffer as described above, and 100 μL of 125I-labeled AFP was added to each tube, followed by 100 μL of rabbit antibody to human AFP. The tubes were then incubated at room temperature overnight (18 h). To precipitate the immune complexes, we added 1 mL of polyethylene glycol solution (140 g/L). After vortex-mixing, the tubes were centrifuged (1000 × g, 10 min, 4 °C). The supernate was decanted and its radioactivity counted (GAMMA-4000; Beckman Instruments, Irvine, CA 92713) with 71% efficiency. The precision (CV) stated by the supplier for the Amersham kit was 7% intra-assay and 8% inter-assay.

The liquid standards provided in the kit were dispensed into 100 μL of “zero standard” buffer and assayed to produce the standard curve. These standards represented 0, 20, 50, 100, 200, and 400 μg of AFP per liter, as determined by comparison with two WHO AFP reference preparations: 72/225 and 72/227. We derived quality-control values for dried-blood-spot samples by using separate specimens containing high and low concentrations of AFP.

A dried-blood-spot standard for use as a quality control in each assay was prepared as follows. A large specimen of blood from a male laboratory worker volunteered was centrifuged at 4 °C at 1000 × g for 15 min and the plasma removed. After three consecutive washes with phosphate-buffered saline, sufficient human amniotic fluid (15 mg AFP per liter) was mixed with the washed erythrocytes to compensate for the loss of plasma. Dried blood specimens were then prepared as described above, air-dried, and stored in plastic bags at 4 °C until further use.

**Statistical analysis.** The method of averages was used throughout this study. Discrete quantitative data were recorded as the mean (X), standard deviation (SD), and standard error (SE). Points differing by more than 1 SD from the mean were considered significant. We used the Pearsonian correlation coefficient for linear regression analysis.

**Results**

**Analytical recovery of AFP after elution.** We evaluated recovery of AFP from dried-blood spots as a function of time, directly eluting four specimens with various AFP concentrations (Figure 1). For blood specimens from newborns, within the limits of precision of the assay, all AFP that could be eluted was eluted from a disc within 30 to 60 min. AFP concentrations, measured thereafter at hourly intervals to 24 h, remained essentially constant.

**Assay sensitivity and precision.** The standard curve of the assay for plasma samples extends from 0 to 400 μg/L, as stated in the manufacturer’s instructions. Using a value of 95% for radioactivity percent bound as the upper limit of sensitivity for the dried-blood spots, this translated to a sensitivity of 1.5 mg/L in our assay. Correspondingly, we determined the lower limit of sensitivity to be 101 mg/L if we used a value of 5%.

A within-run CV of 12.2% was determined by testing 20 discs each from each of three spots containing 7.1, 25.3, and 29.4 mg of AFP per liter. Testing of duplicate discs from two samples with 12.3 and 22.8 mg of AFP per liter in 13 runs (13 different days) showed a between-run CV of 12.3%.

**Stability of AFP in cord blood and blood spots.** AFP stability was determined for plasma and dried blood spots obtained from 10 cord-blood specimens and stored for four months at −20 °C and +4 °C, respectively (Table 1). AFP concentrations decreased 8% in cord plasma stored at −20 °C, but increased 3% in cord-blood spots stored at 4 °C. However, this difference was not statistically significant.

AFP in dried blood spots with low (10.1 mg/L), medium (29.3 mg/L), and high (54.6 mg/L) concentrations was remarkably stable for up to six months of storage (Figure 2). Storage at refrigerator temperature appeared to be satisfactory for spots from samples with higher concentrations.

**AFP concentrations in cord plasma and in dried-blood spots from newborns.** The mean concentrations of AFP in 95 cord plasma samples was 50.8 (SD 40.8) mg/L. The hematocrit for 14 specimens from this group averaged 48.5%. This biological variation is consistent with previous reports (12, 13).

![Blood Spot ELUTION/Time Study](image)

**Figure 1.** Analytical recovery of AFP from dried blood specimens as a function of time

Four AFP concentrations studied (bottom to top): low (3.6 mg/L), medium (39.45 mg/L), high (88.42 mg/L), and excessively high (119.01 mg/L). Elution was essentially complete in 30–60 min. Each point (mean) represents six determinations. Brackets represent standard error.

<table>
<thead>
<tr>
<th>Storage temp, °C</th>
<th>Storage time, mo.</th>
<th>AFP, mg/L</th>
<th>Change, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>-20</td>
<td>0</td>
<td>47.84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>44.16</td>
<td>25.53</td>
</tr>
<tr>
<td>Blood spots</td>
<td>4</td>
<td>0</td>
<td>28.96</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>29.60</td>
</tr>
</tbody>
</table>

n = 10 for each storage condition.

Table 1. Stability of AFP in Frozen Plasma and Refrigerated Spots of Cord-Blood Specimens
In a comparative study (Figure 3) on cord bloods, AFP values for spots ($\bar{x} = 41.5$, SD = 29.7) and for plasma correlated closely ($r = 0.89$, slope = 0.71). The blood spot/plasma ratio of AFP concentrations averaged 96.38% for 40 such samples tested.

During the first 24 h after birth, AFP blood concentrations declined sharply from 41.5 mg/L in cord blood to 18.0 mg/L in blood from day-old newborns (Table 2). After a slight increase on days 2 to 4, AFP concentrations remain fairly constant.

The correlation coefficient between plasma and dried blood spots from one- to seven-day-old infants (Figure 3) was 0.96 (slope, 0.75). The blood spot/plasma ratio of AFP concentrations averaged 92.10% for 30 such samples tested. Thus, AFP concentrations measured in plasma and the corresponding dried blood spots correlated well.

**Discussion**

Our adaptation of a commercially available RIA kit for AFP serum determination to measure AFP in dried blood spots offers an advantage to the clinical laboratory that plans to measure AFP concentrations in both maternal plasma and dried blood specimens from newborns. The same kit and the same AFP standards can be used for both. Other benefits include the high sensitivity (1.5 mg/L) for dried blood spots, acceptable precision (12% within and between runs), good correlation ($r = 0.96$) between AFP concentrations in plasma and dried blood spots, ease of elution, storage stability, and the small sample volume needed (0.75 μL).

Heel-stick blood collection is efficient and inexpensive, with obvious advantages over venipuncture. AFP values for dried cord-blood specimens correlated well with those for plasma samples ($r = 0.89$, slope = 0.71). However, the values for plasma sometimes diverge from those for the spots. This discrepancy may be attributable to various factors, including hematocrit, viscosity, elution phenomena, partial denaturation, and interference of antibody–antigen interaction by blood cells and byproducts of hemolysis.

There appears to be a wide biological variation in AFP concentrations in cord blood and in blood from one- to seven-day-old normal infants, in agreement with previously published reports (12, 13). The overlap between normal ranges at the different age groups was especially evident in five- to seven-day-old infants. However, use of +2 SD cutoff values (Table 2) should aid in distinguishing highly above-normal values during the first postnatal week.

The rapid decline during the first hours after birth is in agreement with earlier studies done by rocket electroimmunoassay in agarose gels (14, 15). No detailed statistical account of these daily AFP blood values has hitherto been reported for the first postnatal week. Our findings agree well with values calculated from the data of Saito et al. (9) and Wu et al. (14).

**Table 2. AFP Concentrations in Serum Specimens Assayed as Dried Blood Spots**

<table>
<thead>
<tr>
<th>Age, days</th>
<th>No. samples</th>
<th>$\bar{x}$</th>
<th>SD</th>
<th>+2 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord</td>
<td>48</td>
<td>41.50</td>
<td>29.70</td>
<td>100.90</td>
</tr>
<tr>
<td>1</td>
<td>93</td>
<td>18.02</td>
<td>14.83</td>
<td>47.69</td>
</tr>
<tr>
<td>2</td>
<td>114</td>
<td>21.04</td>
<td>15.51</td>
<td>52.06</td>
</tr>
<tr>
<td>3</td>
<td>109</td>
<td>23.06</td>
<td>19.30</td>
<td>61.66</td>
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<td>4</td>
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<td>22.95</td>
<td>17.23</td>
<td>57.40</td>
</tr>
<tr>
<td>5</td>
<td>105</td>
<td>19.42</td>
<td>18.67</td>
<td>56.76</td>
</tr>
<tr>
<td>6</td>
<td>105</td>
<td>19.09</td>
<td>15.00</td>
<td>49.09</td>
</tr>
<tr>
<td>7</td>
<td>101</td>
<td>19.01</td>
<td>17.75</td>
<td>54.50</td>
</tr>
</tbody>
</table>
The present technique may be compared to the two-site immunoradiometric assay (IRA) previously described (10) to differentiate between newborn and hereditary tyrosinemia. Our method requires a 1.5-mm (0.75-μL) disc from a 60-μL blood spot; the IRA requires a 3.2-mm (3.1-μL) spot. Free and bound label are separated by the use of polyethylene glycol (centrifugation) in our method, by solid phase (non-centrifugation) in the IRA. Both techniques require no solution transfers and 24 h for completion. Finally, both methods are easily amenable to automation and large-scale analyses.

Our preliminary findings to date suggest that above-normal AFP concentrations may be related to or coexist with several newborn disorders. For example, we have been able to differentiate between confirmed cases of transitory (1/14 increased) and hereditary tyrosinemia (3/3 increased) (see Acknowledgement). However, the importance of relating AFP concentrations in newborns to perinatal disease is still in the exploratory stages. With notable exceptions (9, 14), few laboratories have even established normal AFP concentrations in newborns, because so many variables may be involved (gestational age, birthweight, sex, half-life, etc). We are currently investigating these variables and are attempting to correlate AFP concentrations with newborn disorders such as hereditary tyrosinemia (incidence = 1/685 in the Province of Quebec), hypothyroidism (incidence = 1/4000), and galactosemia (incidence = 1/74 000). The nature and scope of these and other relationships will be considered in detail in a forthcoming publication (16).

From the data presented here, it is apparent that measurement of AFP in dried-blood specimens from newborns is not only feasible but practical.

We thank André Grenier, M.Sc., a biochemist at Genetique Hu­maine, Le Centre Hospitalier, de l'Universite Laval, Quebec, for dried-blood-spot samples from neonatal and hereditary tyrosi­nemia.

References