Evaluation of Four "Kit" Immunoassay Methods for Determination of Alpha-Fetoprotein in Serum during Pregnancy

P. Y. Wong, T. A. Doran, F. F. K. Ho, and A. V. Mee

We evaluated four immunoassay methods for determination of alpha-fetoprotein in serum. All are commercially available in kit form in North America and we find them acceptable with respect to sensitivity, stability, precision, linearity, and analytical recovery. For the Dainabott method, the median values from 16 through 20 weeks of gestation are 34.4, 38.1, 42.1, 51.4, and 66.3 μg/L for each week, respectively. Corresponding values for the Amersham method are 27.9, 31.4, 34.9, 38.2, and 54.0 μg/L, respectively. For the Behring-RIA method they are 33.4, 37.4, 39.4, 49.9, and 66.3 μg/L, respectively. Results for four cases of confirmed neural tube defects are presented.

Additional Keyphrases: neural tube defects • "kit" methods • screening • cutoff value • development defects • fetal status

That alpha-fetoprotein (AFP) is present in the sera of pregnant women was initially demonstrated by Foy et al. (1); numerous subsequent investigations have delimited the normal values at different stages of pregnancy. All available current evidence suggests that fetal liver is the major source of the AFP.

During early pregnancy, serum AFP surpasses the concentrations present in the nonpregnant state as early as the seventh week of gestation, increases gradually to a maximum at the 36th to 38th week, then gradually decreases until the 42nd week of gestation. After delivery, AFP in the mother's serum diminishes promptly (2).

The availability of a sensitive radioimmunoassay (RIA) method for AFP determination since 1971 (3) and the discovery of an increase in AFP in both amniotic fluid and serum in a patient with a neural tube defect by Brock and Sutcliffe (4) in 1972 and by Brock et al. in 1973 (5) signalled the possibility of noninvasive mass screening in a low-risk population for neural tube defects. Several later studies (6–9) have indicated, however, that measurement of serum AFP in pregnant women is a comparatively crude screening procedure; it will identify some but not all the neural tube defects. Moreover, depending on the cutoff point, 1–5% of all pregnancies give values that are false positives. Those doing the screening therefore must rely on amniocentesis and ultrasonography for confirmation of the diagnosis.

A recent editorial (10) suggests that RIA's for AFP in sera of pregnant women were accurate and reproducible, could be automated, and were reasonably inexpensive—probably less than a dollar per case for screening.

We report our experience in the evaluation of four kit methods for determination of AFP in sera of pregnant women.

Materials and Methods

Materials

**AFP RIA kits:** The Dainabott AFP kit was obtained from Abbott Lab., Ltd., Montreal, Quebec, Canada. The Amersham AFP kit was supplied by Amersham Corp., Arlington Heights, IL 60005. The Pharmacia AFP kit was obtained from Pharmacia Diagnostics (Canada) Ltd., Dorval, Quebec, Canada.

**AFP international reference standard:** World Health Organization (WHO) preparation no. 72/225, 1975, was obtained from the National Institute for Biological Standards and Controls, London, England.

**Horse serum:** Obtained from Connaught Laboratories Ltd., Toronto, Ontario, Canada.

**Sera from pregnant women:** Obtained either from the Toronto General Hospital Blood Bank or from patients before amniocentesis.

**125I-labeled AFP:** Supplied by Radioimmunoassay Inc., Toronto, Ontario, Canada.

**AFP antiserum:** Supplied by Behring Diagnostics, Montreal, Quebec, Canada.

**Immunobead solid-phase antibody:** Solid-phase goat anti-rabbit gamma-globulin was supplied by Bio-Rad Lab., Mississauga, Ontario, Canada.

**Pipettor and dispenser:** Supplied by Micromedic Systems, Inc., Philadelphia, PA 19105.

**Gamma counter:** LKB Rackgamma II; LKB Instrumentation, Finland.

**Minicomputer:** PDP 11V03; Digital Equipment Corp., Maynard, MA 01754.

**Methods**

**Dainabott method:** Put 500 μL of borate buffer, 100 μL of standard or patient's sample, 100 μL of 125I-labeled AFP, and 100 μL of first antibody in sequence into 12 X 75 mm polystyrene tube. Vortex-mix and incubate at 4 °C for 24 h. At the end of the first incubation add 100 μL of the second antibody and incubate the mixture overnight at 4 °C. Then centrifuge all tubes at 1500 X g for 10 min. After decanting the supernate, count with the LKB gamma counter the radioactivity in the contents of tubes containing precipitate. Results are expressed in micrograms per liter.

**Amersham method:** In sequence, put 100 μL of standard or patient's sample, 100 μL of 125I-labeled AFP, and 100 μL of AFP antiserum into 12 X 75 mm polystyrene tubes. Vortex-mix, incubate at room temperature for 24 h, and add 1.0

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mL of polyethylene glycol. Mix again, then centrifuge all tubes at 1500 \( \times \) g for 10 min. Discard the supernate and measure the radioactivity of the tubes containing precipitate with a LKB gamma counter. Results are expressed in micrograms per liter.

**Pharmacia method:** Dilute the patient's samples fivefold with AFP-free diluent. Place one anti-AFP-disc on the bottom of 12 \( \times \) 75 mm polystyrene tubes, then add 50 \( \mu \)L of standard or diluted sample and incubate the mixture at room temperature for 3 h. Then aspirate all the liquid from each test tube and wash the anti-AFP-discs twice with 2.5-mL portions of NaCl solution (9 g/L). Add 50 \( \mu \)L of the anti-AFP-\(^{125}\)I solution to the bottom of all tubes and incubate overnight. Next day wash the anti-AFP-discs twice with 2.5-mL portions of saline. Count the anti-AFP-discs in the tubes with the LKB gamma counter. Results are expressed in kilounits/L (1 kilounit = 0.9 \( \mu \)g/L).

**Behring-RIA method:** In this method, the antiserum was supplied by Behring and \(^{125}\)I-labeled-AFP tracer was supplied by Radioimmunoassay Inc. Prepare standards (0 to 320 \( \mu \)g/L) by diluting the WHO reference standard with AFP-free serum. In sequence, place 50 \( \mu \)L of standard or patient's sample, 200 \( \mu \)L of 50 mmol/L phosphate–saline (9 g/L) buffer, 100 \( \mu \)L of \(^{125}\)I-labeled-AFP, and 100 \( \mu \)L of AFP antiserum (Behring AFP antiserum at 10 000-fold dilution) into 12 \( \times \) 75 mm polystyrene tubes. Vortex-mix, incubate overnight at room temperature, then add 100 \( \mu \)L of "Immunobead" antibodies (5000-fold dilution) and incubate at 37 °C for 1 h. After adding 1.0 mL of water, centrifuge all tubes at 1500 \( \times \) g for 10 min. Discard the supernate and count the radioactivity of the precipitates with the LKB counter. Express results in micrograms per liter.

Table 1 summarizes the above procedures.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Dainabott</th>
<th>Amer sham</th>
<th>Pharmacia</th>
<th>Behring-RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>100</td>
<td>100</td>
<td>10(a)</td>
<td>50</td>
</tr>
<tr>
<td>Sample volume, ( \mu )L</td>
<td>25 000</td>
<td>25 000</td>
<td>25 000</td>
<td>25 000</td>
</tr>
<tr>
<td>Total count, cpm</td>
<td>5 000</td>
<td>5 000</td>
<td>5 000</td>
<td>5 000</td>
</tr>
<tr>
<td>No. pipetting steps</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Washing step</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Separation technique</td>
<td>SA</td>
<td>PEG</td>
<td>SP</td>
<td>SA, LKB</td>
</tr>
<tr>
<td>Current cost per tube</td>
<td>$1.00</td>
<td>$1.10</td>
<td>$1.20</td>
<td>$0.50</td>
</tr>
</tbody>
</table>

*Abbreviations: RIA, radioimmunoassay; IRMA, immunoradiometric assay; SA, second antibody; SP, solid phase; PEG, polyethylene glycol. All methods involved overnight incubation at room temperature.*

\(a\) Predilution volume.

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**Fig. 2. Linearity of the four methods (2 SD shown)**

**Fig. 1. Representative standard curves for the four immunoassay methods**

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**Table 2. Precision Studies**

<table>
<thead>
<tr>
<th></th>
<th>Within-assay precision</th>
<th>Between-assay precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dainabott (20)(b)</td>
<td>Amer sham (10)</td>
</tr>
<tr>
<td>Mean, ( \mu )g/L</td>
<td>20.5</td>
<td>38.0</td>
</tr>
<tr>
<td>SD, ( \mu )g/L</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Mean, ( \mu )g/L</td>
<td>138</td>
<td>113</td>
</tr>
<tr>
<td>SD, ( \mu )g/L</td>
<td>7.4</td>
<td>3.7</td>
</tr>
<tr>
<td>CV, %</td>
<td>5.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

\(b\) Numbers in parentheses are numbers of assays. \(c\) \( n = 39 \) for lower concentration, 38 for higher concentration. \(d\) \( n = 8 \) for lower concentration, 7 for higher concentration.
Results

Analytical Variables

Standard curves: Representative standard curves for the four methods are shown in Figure 1.

Linearity: To determine the linearity of the methods, we diluted aliquots of sera from women in the third trimester with either horse serum, Amersham's zero standard, Pharmacia's AFP-free buffer, or AFP-free serum and assayed by all four methods. The results are summarized in Figure 2.

Analytical recovery: We added known amounts of AFP (up to 200 µg/L) to four sera from women in the first trimester and analyzed them. In the Dainabott method, analytical recovery averaged 93% (range 89 to 99%); the Amersham method, 101% (range 98 to 103%); the Pharmacia method, 106% (range 96 to 114%); and the Behring-RIA method, 104% (104 to 105%).

Precision: Within- and between-assay precision of these four methods at two concentrations of AFP are summarized in Table 2.

Calibration against WHO AFP reference standard no. 72/225, 1975: One ampoule of WHO AFP reference standard no. 72/225 was reconstituted according to WHO instructions. Reference standards of 100 int. units/mL in either horse serum or AFP-free serum were used to calibrate the standards of three methods. One microgram of Dainabott AFP standard was found to be equivalent to 1031 int. units of AFP. One microgram of Amersham AFP standard was equivalent to 1020 int. units of AFP. One unit of Pharmacia standard was equivalent to 0.971 int. unit of AFP. Because the standards of Behring-RIA method were made up directly from the WHO standard, we made no calibration with that method.

Intermethod Comparison

We compared results with the Dainabott kit (x) with those by the three other methods. Linear-regression analysis gave the following results. In 1644 comparisons with the Amersham method (y), $y = -3.63 + 0.92x$, $r = 0.97$, $p < 0.001$. In 87 comparisons with the Pharmacia method (y), $y = 4.47 + 1.01x$, $r = 0.96$, $p < 0.001$. In 1613 comparisons with the Behring-RIA method (y), $y = -1.35 + 1.08x$, $r = 0.94$, $p < 0.001$.

Clinical Studies

AFP in sera from women in the 8th to 40th week of gestation were determined with the Dainabott, Amersham, and Behring-RIA kits. Detailed data on the median and the upper 95th percentile confidence levels are available upon request from the authors or from the Editorial Office of this journal. Figure

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Table 3. Data from Four Cases of Confirmed Neural Tube Defects

<table>
<thead>
<tr>
<th>Weeks gestation</th>
<th>Amniotic fluid AFP</th>
<th>Dainabott a</th>
<th>Amersham a</th>
<th>Behring-RIA a</th>
<th>Followup</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>↑</td>
<td>128</td>
<td>130</td>
<td>130</td>
<td>TA b</td>
</tr>
<tr>
<td>18</td>
<td>↑</td>
<td>—</td>
<td>670</td>
<td>770</td>
<td>TA b</td>
</tr>
<tr>
<td>24</td>
<td>↑</td>
<td>1042</td>
<td>860</td>
<td>1002</td>
<td>LT c</td>
</tr>
<tr>
<td>18</td>
<td>↑</td>
<td>320</td>
<td>307</td>
<td>217</td>
<td>TA b</td>
</tr>
</tbody>
</table>

Ultrasound studies indicated anencephaly in each of these four cases.

a µg/L. b Therapeutic abortion. c Lost to followup.

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Fig. 3. The median value and 2X, 2.5X, and 3X the median value, for the Dainabott, Amersham, and Behring-RIA methods at 14 to 21 weeks of gestation.

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3 shows the median, 2 times, 2.5 times, and 3 times the median of all three methods for gestation of 14 to 21 weeks.

Four cases of neural tube defects were detected in this study. Results of confirmation tests, values for serum AFP as determined by three methods, and the followup are summarized in Table 3.

Discussion

Analytical Variables

The methods of Dainabott, Amersham, and Behring-RIA are quite similar, differing only in the separation step. The sensitivity of these three methods is approximately 0.5 ng per assay tube. The Pharmacia kit, in which I
t- labeled antibody is used, is much more sensitive than other methods (approximately 0.05 ng per assay tube); it requires no centrifugation step, but does include a predilution and several washing steps. For all four methods, the total counts are reasonably high; all four require overnight incubation.

Figure 2 shows the linearity of the methods. From this, we may assume that the material assayed in serum samples and the used in the standard curve are identical in their reactivity with the antibody.

Within-assay precision and between-assay precision are both within the allowable limit of RIA procedures (Table 2).

Stability

The Amersham and Pharmacia kits were both shipped in lyophilized form. Their standard curves did not change for a period of two months. For the Dainabott kit, shipped in solid CO2, separate experiment shows that the reagents were stable for at least a week, even when stored at 4 °C; the overall shelf-life of the Dainabott kit was about four weeks. The AFP tracer from Radioimmunoassay Inc. was stable for six weeks.

Clinical Studies

Figure 3 shows the distribution of AFP results for women in the 14th to 21st weeks of gestation for the Dainabott, Amersham, and Behring-RIA methods. AFP values for four cases of confirmed neural tube defects all exceeded 3 times the median value and agreed with results determined by ultrasound and amniotic fluid AFP (Table 3). As mentioned above, several studies (6–9) show that measurement of serum AFP in pregnant women will detect some but not all neural tube defects. The U.K. Collaborative Study (11), in which 19 centers studied 18,684 single pregnancies and 163 twin pregnancies, showed that 88% of the cases of anencephaly and 79% of the cases of open spina bifida could be detected by screening serum AFP at 15 to 18 weeks of gestation with a cutoff point of 2.5 times the median value for the population studied. The study concluded that screening pregnant women by measuring the concentration of AFP in their serum is an effective method of selecting women for ultrasoundography and amniocentesis so that neural tube defects can be diagnosed in utero.

A screening program such as this must be shown to be able to increase the proportion of affected pregnancies detected (12) and also be acceptable in terms of the number of amniocenteses needed and the possible hazards to normal pregnancies. Ferguson-Smith et al. (13) reported their experience with a pilot study designed to look at these problems. They found that the AFP test alone was sensitive enough to detect 93% of affected fetuses, and concluded that voluntary maternal screening for serum AFP had a valuable role in prenatal care. They emphasized (a) careful counselling before testing; (b) the voluntary nature of the program; (c) the need for high standards of obstetric ultrasonography to diagnose gestation correctly and rule out multiple pregnancy, threatened abortion, and other fetal abnormalities; and (d) the necessity for an efficient organization to follow up the patients tested.

We believe it is too early to introduce province-wide routine serum AFP screening in Ontario. Caution has been urged both here (14) and in the United Kingdom (15, 16). In a low-incidence area such as Ontario, the cutoff point of 2.5 times the median, as used in the U.K. studies, would result in an unacceptably high number of amniocenteses: 20 normals to one abnormal. By using 3 times the median as a cutoff point, we should be able to achieve a satisfactory diagnostic accuracy (84% of anencephalics and 60% of all cases of spina bifida) with an acceptable number of amniocenteses (10 amniocenteses for each neural tube defect detected).

In summary, we found that all four methods for detecting AFP in serum samples from pregnant women are acceptable on the basis of their sensitivity, stability, precision, linearity, recovery studies, and availability in North America.

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References