Liquid-Chromatographic Measurement of Biopterin and Neopterin in Serum and Urine

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We report an improved "high-performance" liquid-chromatographic (HPLC) method for measuring biopterin and neopterin in serum and urine. Specimens are acidified, treated with iodine in 0.2 mol/L trichloroacetic acid, partly purified on Bio-Rad MP-50 cation-exchange columns, and analyzed by reversed-phase HPLC with fluorometric detection. The minimal concentration of biopterin detectable is 0.3 μg/L in a 50-μL injection. The total CV is ≤10%. Improvements over other reported methods include the use of a single, simplified sample-preparation step with a Baker-10 SPE System, and a guard column to increase analytical column stability and analyte recovery. The assay is semiautomated to reduce technician time and improve precision. Mean observed values for biopterin and neopterin in sera of normal human adults were 1.64 and 5.52 μg/L, respectively. The mean ratio of neopterin to biopterin in acidified adult urine samples was lower than that found in matched nonacidified samples (n = 10). Serum specimens from diagnosed phenylketonuric (PKU) and hyperphenylalaninemias patients were also analyzed for biopterin and neopterin; the findings agreed with reported values for similar patients. One patient, previously identified as an atypical PKU patient, showed serum values of neopterin and biopterin suggestive of a defect in biopterin synthesis.

Additional Keyphrases: chromatography, reversed-phase - phenylketonuria - hyperphenylalaninemia

Classic phenylketonuria (PKU) is associated with decreased activity of phenylalanine hydroxylase (EC 1.14.16.1).1 The identification of biopterin as an essential cofactor for this enzyme and for the hydrolyses of tyrosine and tryptophan led to the study of the importance of biopterin in related metabolic errors (7). Biopterin is an obligatory cofactor for the enzymatic hydroxylation of phenylalanine to tyrosine in mammalian tissue. The reduced form of biopterin, trihydrobiopterin (BH₃), functions as an electron donor in the hydroxylation reaction. Lack of BH₃ may occur because of a deficiency of biopterin itself (a synthesis defect) or because of an inability to reduce oxidized biopterin to its active tetrahydro-form (a deficiency of dihydropyridine reductase, DHPR; EC 1.6.99.7) (2). Each defect impairs the normal metabolism of phenylalanine and results in a PKU-like disorder that requires definitive diagnosis. An important early indicator common to these related disorders is the abnormal, increased concentration of phenylalanine. Biopterin-associated disorders, however, require a unique course of treatment that only in part parallels conventional PKU therapy. Thus, special diagnostic methods are required to differentiate biopterin-related defects from classic PKU and hydroxylase-associated hyperphenylalaninemas.

Neopterin is an important intermediate in the de novo synthesis of biopterin from guanosine triphosphate (3-6). The synthesis block that causes biopterin deficiency can occur after the formation of neopterin, causing abnormally high concentrations of this pteridine (7). More recently, deficiency of GTP cyclohydrolase I (EC 3.5.4.16), in which the conversion of GTP to d-erythro-dihydrobiopterin triphosphate is blocked, has been reported (8, 9).

The differential diagnosis of cofactor-related hyperphenylalaninemas depends on the following observations. An above-normal concentration of total biopterin with normal or slightly increased neopterin concentrations is associated with deficiency of DHPR. An increased neopterin/biopterin ratio (increased neopterin and decreased biopterin concentrations) suggests a block in the synthesis of BH₄. GTP cyclohydrolase I deficiency is characterized by extremely low concentrations of both neopterin and biopterin (10).

The determination of both neopterin and biopterin is necessary to differentiate defects of biopterin synthesis from deficiencies of DHPR, each of which requires unique courses of treatment. Moreover, quantification of neopterin has become useful for monitoring cell-mediated immunity in vivo and in malignant tumors, viral infections, and various other diseases (11-15).

HPLC techniques have been used extensively for the study of pteridines, in particular biopterin and neopterin, and of their role in various pathological conditions. Fukushima and Nixon (16) described a rapid reversed-phase HPLC procedure for detecting biopterin and other pteridines that enabled quantification at picomole quantities. They measured alkaline-stable biopterin after oxidizing the specimen by addition of an alkaline iodine solution. Total biopterin, reduced and oxidized, was then measured after oxidizing the specimen with an acidic solution. Salts, nonspecific fluorescent compounds, and high-molecular-mass molecules that would interfere with the separation and detection of the analytes were removed by passage through a Dowex 50 column and then through a Dowex 1 column. After these purification steps, the eluates were subjected to HPLC analysis with fluorometric detection. Neopterin was also measured with this method. This and other reported methods are lengthy and tedious; specimen throughput is slow (16, 17).

We report an improved method that requires smaller volumes of serum or urine and apply it to the measurement of biopterin and neopterin in normal volunteers and in patients diagnosed as hyperphenylalaninemic.

Materials and Methods

Aqueous biopterin and neopterin standards. We dissolved 10 mg of crystalline biopterin (Calbiochem, La Jolla, CA) in 800 mL of de-ionized distilled water, by gently heating and stirring it constantly and then cooling it slowly to room

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1 Nonstandard abbreviations: PKU, phenylketonuria; BH₃, trihydrobiopterin; BH₄, dihydrobiopterin; DHPR, dihydropteridine reductase; QC, quality control; and ODS, octadecylsilane.

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Reduced biopterin standards. We synthesized BH₄ and dihydrobiopterin (BH₂) as described by Kaufman (18), using a Supelco Micro-hydrogenator (Supelco, Inc., Bellefonte, PA) for the BH₂ reaction. The products were placed in screw-cap amber vials, flooded with N₂, and stored at −40 °C. We determined the identity and purity of the products as described by Kaufman (18).

Pteridine-deficient serum. Fresh-frozen serum from adults was thawed at 4 °C and centrifuged at 3000 × g for 30 min at 4 °C. The supernate was filtered sequentially through 0.45- and 0.20-μm (pore size) Millipore filters in Nalgene sterilization filtration units. We diazylated the final filtrate for 48 h against the following buffer (per liter): 0.02 mol of Tris·HCl, 0.15 mol of NaCl, and 0.2 g of NaN₃. The buffer was adjusted to pH 8.0, de-aerated, and bubbled with N₂. About 400 mL of the serum filtrate was diazylated in 4 L of buffer with constant slow stirring, and the starting buffer was decanted and replaced with fresh buffer after 24 h. We monitored the removal of pteridines by HPLC. Dilutions of the aqueous stock standards were added to diazylated serum to prepare serum-based assay calibrators and quality-control (QC) materials containing various concentrations of biopterin and neopterin. BH₄ and BH₂ serum calibrators were prepared as needed.

Specimen collection. Venous blood specimens were drawn and allowed to clot in the dark at room temperature for 10–15 min. After clotting, the sample was centrifuged and the supernatant serum frozen at −40 °C. Plasma specimens, collected into heparinized tubes, were treated in the same manner as serum specimens. Clean-catch urine specimens were collected from 10 apparently healthy adult volunteers at the time of serum collection and frozen without delay at −40 °C. To evaluate urine collection methods, a 5-mL portion of each freshly collected specimen was acidified with 0.1 mL of 4 mol/L HCl and stored at −40 °C. All urine samples were diluted 500-fold in de-ionized distilled water for analysis. Two milliliters of diluted sample was required for each analysis.

The human serum and urine specimens from healthy adults were assayed for biopterin and neopterin by the HPLC assay method, as were seven blind-coded serum or plasma specimens from PKU-affected patients (ranging in age from five weeks to 25 years), obtained from Dr. Harvey Levy of the Massachusetts State Laboratory (Boston, MA).

Oxidation of reduced forms of biopterin. Acid oxidation of reduced biopterin with I₂, 5 g/L in 0.2 mol/L trichloroacetic acid solution, and alkaline oxidation with 2.0 mol/L NaOH reagent and 5 g of I₂ and 10 g of KI per liter of 0.1 mol/L NaOH reagent were evaluated by analyzing by HPLC 2-mL serum samples containing 5 to 10 μg of BH₂ or BH₄ (per milliliter).

HPLC system. We used the Series 7800 modular HPLC system from Laboratory Data Control (LDC, Division of Milton Roy, Riviera Beach, FL), which included two Constatem III mobile-phase pumps, a Rhodyne injector, a Fluoromonitor III fluorometer, a two-channel printer/plotter, and a Chromatographic Control Module minicomputer. An autosampler with a Rhodyne injector was also included in the system. The continuous-flow fluorometer was equipped with a 360-nm black fluorescent excitation lamp, a 370-nm excitation filter, and a 418–700-nm emission filter. We used 250 × 4.6 mm Excalibur 5-μm octadecysilane (ODS) reversed-phase columns (Applied Science Division, Milton Roy Co., State College, PA) for pteridine analysis. A 70 × 4.6 mm guard column, to protect the analytical column from contamination by impurities in the processed specimen, was made by slurry-packing the short column with a methanol suspension of 5-μm spherisorb ODS (Applied Science Division, Milton Roy Co.).

Ion-exchange chromatography. Small (column bed = 5 × 10 mm) ion-exchange columns (1-mL syringe barrels) were packed with a loose, aqueous slurry of Bio-Rad AGMP-50, 200–400 mesh (H⁻) cation-exchange resin (Bio-Rad, Richmond, CA). We processed 10 columns at a time with a Baker-10 SPE apparatus (J.T. Baker Chemical Co., Phillipsburg, NJ 08865) to partially purify oxidized specimens before HPLC analysis. We also prepared anion-exchange columns, as described by Fukushima and Nixon (16), in an attempt to further purify and concentrate serum samples.

Experimental protocol. Calibrators containing neopterin and biopterin (1–5 μg/L each) and biopterin QC materials (0.5, 1.5, 3.5, and 4.5 μg/L) were analyzed in duplicate, along with single 2-mL aliquots of each thawed specimen (serum or urine). We added 0.5 mL of 300 g/L trichloroacetic acid reagent and 0.25 mL of iodine solution (5 g of I₂ and 10 g of KI in 0.2 mol/L trichloroacetic acid solution) to each tube, mixed the samples thoroughly, and incubated them for 1.0 h at room temperature under reduced light. We then centrifuged the samples at 3100 × g for 30 min at 4 °C. We used an Eppendorf pipette to transfer 1.75 mL of supernate to separate, disposable cation-exchange columns. After attaching the system to reduced pressure, we washed each column with 5 mL of de-ionized distilled water, positioned collection vials in the extraction system, and eluted the biopterin and neopterin with 2 mL of 1 mol/L NH₄OH reagent. We added 30 μL of glacial acetic acid to each collection vial to neutralize the eluate. The tubes were capped and vigorously vortex-mixed for 2–3 s. We analyzed the neutralized eluate by HPLC with the following chromatographic conditions: mobile phase, methanol and water (15/85 by vol); flow rate, 1 mL/min; detector sensitivity, range = 2; temperature, ambient; loop, 50-μL injector type; and chart speed, 60 s/cm.

Initially, we manually injected 50 μL of eluate onto the analytical column. In later studies we transferred 1-mL portions of each neutralized eluate to injection vials (Varian Instrument, Inc., Walnut Creek, CA) for use with the autosampler option of the HPLC system.

Phenylalanine (Phe), tyrosine (Tyr), and creatinine determinations. Urine specimens for creatinine determination were collected and stored as described earlier. Portions of nonacidified urine specimens were analyzed for creatinine with a Gilford 3500 system and Worthington/Gilford creatinine reagents (Worthington Diagnostics, Freehold, NJ). Serum specimens were analyzed for Phe and Tyr by an HPLC procedure (19).

Calculations. We used Statistical Analysis System (SAS) procedures for linear regression and nested analysis of variance (20) to evaluate the performance characteristics of the reported HPLC assay.

Results

Chromatograms of the analysis of paired acidified/nonacidified urine samples from split specimens and of the corresponding serum specimens are shown in Figure 1.
Neopterin and biopterin were eluted at about 5.8 and 8.3 min, respectively.

Calibration curves were derived from peak-height measurements for each calibrator by linear regression (SAS GLM–least-squares analysis). Coefficients of determination ($r^2$) for the calibration curves of six assays were >0.941. Y-intercepts ranged from -404 to 667 peak-height counts and did not vary significantly from zero (Student's $t$-test, $P = 0.05$). The mean slope was 3000 counts (SD = 610) per micrograms per liter.

Detection limits for the assay (the apparent concentrations 3 SD from the minimal measurable concentrations of biopterin and neopterin) were <0.3 μg/L each in a 50-μL sample injection. Fluorescence peak height was proportional to the concentration of pteridine present ($r^2$ = 0.9988, y-intercept not significantly different from zero).

We determined the analytical recovery for the assay by diluting stock solutions of aqueous biopterin from 100 to 5 μg/L in distilled water and in pteridine-free pooled serum. Two-milliliter portions of fortified serum were assayed in 10 independent runs; 10 samples of the aqueous biopterin solution were injected directly onto the analytical column. Peak heights for biopterin were recorded and serum values were adjusted for dilution during the assay procedure. Based on peak-height measurements, the percent of added biopterin recovered from the sera pool was 99%. This compared favorably with the mean percent recovery of 103% observed for the QC materials. The analytical recoveries of biopterin for QC materials 1–4 were 116%, 102%, 97%, and 100%, respectively (Table 1).

Table 1 also shows that total CVs for all but QC 1 are about 10%. Acceptable assay precision is further indicated by the analytical variability of the calibrators, which is similar to that obtained for QC materials. An analysis of variance (SAS ANOVA) is presented in this table. The data show that the mean values obtained for the QC materials are within 3% of expected recovery values at all four concentrations.

Table 2 presents the findings in biopterin and neopterin assays of serum specimens obtained from 10 healthy adult volunteers. Urine specimens collected at the time of serum collection from the same volunteers were also analyzed (Table 3). We split each urine specimen, acidified one portion, and left the remainder untreated. HPLC assay results showed the biopterin and neopterin concentrations were 200 to 600 times greater in urine than in serum. Acidifying the urine caused an 18% decrease in measured biopterin concentration and a 41% decrease in measured neopterin concentrations. This effect was consistent from specimen to specimen. In a linear-regression analysis of acidified vs nonacidified assay values, $r^2$ was 0.993 for biopterin and 0.997 for neopterin. For all 10 urine specimens, acidification caused a significant decrease in measured pteridine (Student's $t$-test, $P < 0.01$).

Table 3 also shows biopterin and neopterin values adjusted for urinary creatinine content. When adjusted for creatinine content, pteridine concentrations measured in 10 individual specimens were less variable than those measured in matched, unadjusted samples. Coefficients of variation for mean values of biopterin and neopterin, measured from both acidified and nonacidified urine specimens, were reduced from about 90% to <26% (n = 10). Pteridine concentrations and creatinine content are strongly correlated; the coefficients of determination were 0.939 and 0.858 for biopterin and neopterin, respectively.

In Table 4 we show assay results of serum specimens from six patients diagnosed for PKU and one patient suspected of having tyrosinosis. Except for one patient diagnosed as an abnormal phenylketonuric and the patient with increased tyrosine, all patients had increased Phe concentrations (i.e., >40 mg/L). When compared with the data in Table 2, all but one of the PKU patients had increased biopterin concentrations, normal neopterin concentrations, and depressed neopterin-to-biopterin ratios.

### Table 1. Results of HPLC Analyses for Biopterin in Serum Quality-Control Materials QC 1–4

<table>
<thead>
<tr>
<th>QC</th>
<th>Target value, μg/L</th>
<th>Observed mean value, μg/L</th>
<th>Bias from target value, %</th>
<th>Among-run CV, %</th>
<th>Within-run CV, %</th>
<th>Total CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC 1</td>
<td>0.5</td>
<td>0.58</td>
<td>+1.5</td>
<td>26.0</td>
<td>4.3</td>
<td>26.4</td>
</tr>
<tr>
<td>QC 2</td>
<td>1.5</td>
<td>1.53</td>
<td>+1.7</td>
<td>9.9</td>
<td>3.3</td>
<td>10.4</td>
</tr>
<tr>
<td>QC 3</td>
<td>3.5</td>
<td>3.40</td>
<td>-2.9</td>
<td>9.3</td>
<td>4.0</td>
<td>10.2</td>
</tr>
<tr>
<td>QC 4</td>
<td>4.5</td>
<td>4.52</td>
<td>+0.04</td>
<td>5.4</td>
<td>5.2</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Number of runs = 6; number of replicates per run = 2.

### Table 2. HPLC Assay Results of Healthy Adult Serum Specimens

<table>
<thead>
<tr>
<th>Biopterin</th>
<th>Neopterin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
<td>1.64</td>
</tr>
<tr>
<td>SD</td>
<td>0.42</td>
</tr>
<tr>
<td>CV, %</td>
<td>25</td>
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</table>

* Ratio of neopterin and biopterin measurements.
**Table 3.** HPLC Results for 10 Paired Acidified (A) and Nonacidified (NA) Urine Specimens from Healthy Adults

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>Mean (SD) % change due to acidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/C</td>
<td>1168</td>
<td>957</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>463</td>
<td>261</td>
<td>194</td>
<td></td>
</tr>
<tr>
<td>N/C</td>
<td>1280</td>
<td>745</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>293</td>
<td>194</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>N/B</td>
<td>1.14</td>
<td>0.80</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.28</td>
<td>0.18</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

* Biocerin, ng/mg creatinine.
* Neopterin, ng/mg creatinine.
* Ratio of creatinine-adjusted values of neopterin and biocerin (calculated from N/B values).
* All changes were significant (P < 0.01, Student's t-test); calculation of mean values was based on pairwise comparison of 10 samples.

**Table 4.** HPLC Results for Serum Specimens from Seven Patients with Hyperphenylalaninemia

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age, y</th>
<th>Biocerin, µg/L</th>
<th>Neopterin, µg/L</th>
<th>N/B*</th>
<th>Phe, mg/L</th>
<th>Tyr, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU (on diet)</td>
<td>5</td>
<td>6.70</td>
<td>3.8</td>
<td>0.55</td>
<td>151.3</td>
<td>7.1</td>
</tr>
<tr>
<td>PKU (on diet)</td>
<td>5</td>
<td>5.69</td>
<td>3.58</td>
<td>0.63</td>
<td>98.6</td>
<td>5.9</td>
</tr>
<tr>
<td>PKU</td>
<td>8</td>
<td>9.23</td>
<td>3.04</td>
<td>0.33</td>
<td>215.0</td>
<td>8.8</td>
</tr>
<tr>
<td>PKU</td>
<td>15</td>
<td>6.82</td>
<td>3.21</td>
<td>0.47</td>
<td>288.0</td>
<td>8.3</td>
</tr>
<tr>
<td>PKU</td>
<td>17</td>
<td>10.45</td>
<td>8.55</td>
<td>0.82</td>
<td>222.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Atypical PKU (on diet)</td>
<td>5 wk</td>
<td>1.45</td>
<td>6.24</td>
<td>4.30</td>
<td>8.5</td>
<td>10.4</td>
</tr>
<tr>
<td>Tyrosinosis (Type II)</td>
<td>25</td>
<td>1.19</td>
<td>1.88</td>
<td>1.58</td>
<td>8.5</td>
<td>224.0</td>
</tr>
</tbody>
</table>

* Ratio of neopterin to biocerin.

**Discussion**

We used specially prepared, disposable cation-exchange columns with the Baker-10 SPE system for sample preparation and a short guard column with a reversed-phase analytical column in an HPLC method with fewer intermediate preparation steps and significantly improved effective column life. We improved sample preparation by using a single extraction step with small, easily prepared columns packed with Bio-Rad AGMP cation-exchange resin. Subsequent extraction steps with fragile, anion-exchange columns to further concentrate and (or) purify specimens (as described by others) were not necessary for this method. Eluates were collected directly into small storage vials with no transfer step. Both biocerin and neopterin were quantitatively measured in both serum and urine with excellent analytical recovery (>98%).

Parallel alkaline oxidation of prepared serum pools did not yield satisfactory recovery of reduced forms of biocerin as fully oxidized, fluorescent biocerin. In fact, whereas about 80% less BH₄ was recovered as fully oxidized biocerin by alkaline oxidation than by acid oxidation, in agreement with Fukushima and Nixon (16), only about 42% of BH₄ was recovered as biocerin when oxidized under alkaline conditions. Similar recoveries were demonstrated for neopterin. As a result of these findings, only an acid-oxidation step was used to measure biocerin content.

Spherisorb ODS analytical columns, which gave satisfactory resolution of all pteridines tested, initially gave erratic results for processed serum and urine samples. This was attributed to slight pH differences in the neutralized eluates and, as a result, limited the number of effective runs to less than ten. The method of Fukushima and Nixon (16) required washing the analytical column with absolute methanol after analyzing about 30 specimens. Column regeneration, a time-consuming step, affected the reproducibility of subsequent analytical analyses. Altering the mobile phase did not yield satisfactory results. To resolve the problem, we added a precolumn, also packed with 5-μm Spherisorb ODS, with no significant adverse effects on the retention time, resolution, or quantitation of biocerin and neopterin. The degenerative effects of the processed samples on the analytical column were eliminated, and up to 200 successive injections without column regeneration were possible with a 2-min wash of standard mobile phase after each run. As a result, the pH balance of the final extraction eluates was not as critical: a simple, isocratic mobile phase could be used; and injection of samples and calibrators was easily automated.

All specimens were frozen (−40 °C) after collection; the effect of acidification of urine specimens, as recommended by some (21, 22), was examined. To normalize urine values for each analyte, we also made creatinine measurements (23). These correlated well with biocerin and neopterin values. We found that the creatinine-adjusted neopterin-to-biocerin ratio of the acidified urine samples (0.80) varied significantly from that of the nonacidified samples (1.14) at P = 0.01. Assayed measurements of biocerin and neopterin in acidified urine samples were generally lower than those of nonacidified samples. It is difficult to evaluate the practical effects of specimen acidification on analytical results as reported by others (21, 22, 24, 25). Age pairing or other matching approaches of results may be important in establishing normal values of measured pteridines (22, 24). We also found that serum and urine values of biocerin and neopterin for paired specimens from normal individuals were not strongly correlated (for biocerin, r² values were 0.058 and 0.146 for nonacidified and acidified urine specimens, respectively). These observations suggest that specimen selection, collection, and handling before analysis are critical in establishing normal values and making clinical assessments in pteridine-associated disorders.

Using the reported method, we assayed serum or plasma specimens from six patients with hyperphenylalaninemia and one with tyrosinemia. The results for biocerin and
neopterin are slightly lower than those from similar subjects reported elsewhere (22, 24), perhaps because of relative assay specificity. However, the neopterin-to-biopterin ratios reported here are in close agreement with those previously reported (22, 24). This supports using the ratio of these analytes in the diagnosis and characterization of cofactor-related hyperphenylalaninemas and perhaps other disorders marked by abnormal concentrations of these pteridines (12, 26, 27).

The measurement of biotinoid and neopterin in serum and urine specimens is an accepted approach in the detection and evaluation of hyperphenylalaninemia and other disorders. The reported method uses simplified sample preparation steps to measure biotinoid and neopterin and is easily automated for unattended operation. Reliable analytical results confirm the identification of a hyperphenylalaninemic variant. Reproducibility of results and high-volume specimen-throughput capacity easily permit the differentiation of various pteridine-associated disorders and the determination of normal reference ranges for biotinoid and neopterin values.

We are grateful to Dr. Harvey Levy of the New England Regional Screening Program and Dr. Edwin W. Naylor of Western Pennsylvania Hospital for their interest and contributions of special materials for this project.

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

References