Isocratic Reversed-Phase HPLC Method to Measure Pyrimethamine Extracted from Plasma of Infants Treated for Toxoplasmosis

T. H. Zytkovicz, Julia Salter, Laura Hennigan, Ralph Timperi, James Maguire, and Rodney Hoff

An isocratic HPLC method for measuring pyrimethamine extracted from infant plasma is reported. The method is an improvement over previously published methods by requiring lower volumes of plasma (100 μL) and having increased sensitivity to pyrimethamine at 210 nm. The procedure, which entails a basic organic extraction and subsequent HPLC chromatography of the reconstituted extract, can detect 1.4 ng and quantify 4.0 ng of pyrimethamine per 40-μL injection, with two analyses per 100-μL sample. Analytical recovery of pyrimethamine added to plasma at 10, 50, and 125 ng/100 μL averaged 80%, 92%, and 101%, respectively (n = 20). Within- and between-day CVs were <7%. Studies of various plasma samples from adults and infants (n = 15) revealed no interference from other plasma peaks with the analyte of interest.

Additional Keyphrases: infection · pediatric chemistry · acquired immunodeficiency syndrome

Toxoplasmosis, a common protozoal infection, can be life-threatening in infants who are infected congenitally (1) and in immunodeficient persons, especially those with acquired immunodeficiency syndrome (AIDS) (2). The preferred treatment for toxoplasmosis involves pyrimethamine, a basic, lipophilic, protein-bound drug with a half-life in adults of 23 to 175 h (3–7).

What concentration of pyrimethamine in blood is needed for effective treatment of congenital toxoplasmosis has not been thoroughly established. To monitor the blood concentrations of pyrimethamine in infants treated as part of a statewide screening program, we developed a sensitive analytical method for pyrimethamine that could be performed with the small volumes of plasma that are obtainable from newborn infants. A method to measure pyrimethamine in plasma would help define the appropriate treatment of congenital toxoplasmosis.

Pyrimethamine has been assayed by microbiological methods (8), gas–liquid chromatography (9–11), thin-layer chromatography (12), spectrophotometry (13) and HPLC (3–5, 14–16). Here we describe a modified HPLC method for quantifying pyrimethamine in blood samples from pediatric patients. The main advantages of the HPLC method described here are (a) greater sensitivity to pyrimethamine and (b) requirement for small volumes of plasma, which also allows duplicate analyses on the same sample. The analysis is done with a standard isocratic reversed-phase HPLC system with a 210-nm detector and a recorder/integrator.

Materials and Methods

Solutions. A 50 mg/L stock solution of pyrimethamine (Sigma Chemical Co., St. Louis, MO) was prepared in acetonitrile (J. T. Baker, Phillipsburg, NJ). Working standards of pyrimethamine (100, 250, 500, 937.5, and 1250 μg/L, corresponding to 4, 10, 20, 37.5, and 50 ng of pyrimethamine per 40-μL injection) were prepared from the stock solution by dilutions with the mobile phase. The mobile phase, sodium phosphate buffer (0.1 mol/L, pH 2.5) and acetonitrile (79/21, by vol), contained tetrabutylammonium hydroxide (Fisher Scientific, Fair Lawn, NJ), 0.5 mmol/L. Before use, we filtered the mobile phase through a 0.45-μm pore-size Nylon 66 filter under reduced pressure.

Instrumentation. The HPLC system consisted of a Model 2150 HPLC pump (LKB, Piscataway, NJ), a 250 × 4.6 mm (i.d.) 5-μm particle size Ultrasphere C18 Altex column (Alltech Assoc., Deerfield, IL), a Model 7126 sample injector (Rheodyne, Cotati, CA) fitted with a 100-μL loop, a Model 166 Programmable ultraviolet detector (Beckman Instruments, San Ramon, CA) operating at 210 nm and 0.01 absorbance unit full-scale, and a Model PC-8300 controller (NEC, Wood Dale, IL). The detector was connected to a Model LCI-100 computing integrator (Perkin-Elmer, Norwalk, CT) operating in the peak-height mode.

Extraction of pyrimethamine from plasma. To a 15-mL silanized screw-top glass centrifuge tube containing 100 μL of heat-inactivated plasma (at 56 °C for 30 min) (17), add 650 μL of distilled water and 250 μL of 2 mol/L sodium hydroxide reagent. Add 1 mL of acetonitrile to the sample, mix briefly, then add 5 mL of methylene chloride (Fisher Scientific). Vortex-mix for 1 min, then centrifuge at 450 × g for 10 min and remove the lower (organic) phase into a 15-mL silanized glass centrifuge tube. Evaporate the solvent at 37 °C under a stream of nitrogen. Re-extract the mixture as above, and combine the organic phase with the dried first extract, and again evaporate the solvent. If the extracted sample is not analyzed immediately, store it no longer than four days at 4 °C until analysis.

Reconstitution and analysis. To reconstitute the dried organic extract, add 21 μL of acetonitrile and vortex-mix, then add 79 μL of 0.1 mol/L sodium phosphate buffer (pH 2.5) and again vortex-mix. Analyze two 40-μL aliquots from each sample by HPLC with a flow
rate of 1.0 mL/min. For quality-control samples use plasma from adults with 50 ng of pyrimethamine added. Calculate the pyrimethamine concentration in samples and in controls by the external standard method, comparing the peak heights of unknown samples with those of the standards.

Results

Standard and calibration curve. The standard curve was constructed by plotting peak height vs nanograms of pyrimethamine. For six points, ranging from 0 to 50 ng, the standard curve was linear (y = 3.06x - 1.77, r = 0.99, S_{wix} = 1.7). The calibration curve, prepared from 100-μL samples of adult plasma supplemented with five different amounts of pyrimethamine (10 to 125 ng), was linear over the range of pyrimethamine added (y = 3.14x - 2.12, r = 0.99, S_{wix} = 2.1).

Detection and quantification limits. The limit of detection, defined as twice the standard error of estimate (S_u), (18) was estimated as 1.4 ng of pyrimethamine per 40-μL sample. The limit of quantification, defined as 6 S_u, was 4 ng of pyrimethamine per 40 μL.

Precision, accuracy, and interference. Within- and between-assay variations were evaluated by analyzing for pyrimethamine in 100-μL samples of adult plasma (n = 10) containing low (10 ng), intermediate (50 ng), and high (125 ng) amounts of added pyrimethamine (Table 1). The precision data were based on the average of two HPLC determinations, 40 μL each, per 100-μL sample. The mean recoveries of pyrimethamine at low, intermediate, and high amounts of added pyrimethamine were 79.9%, 91.9%, and 100.9%, respectively. Analysis of 11 different adult plasma blanks and four infant plasma blanks revealed no coeluting peaks with pyrimethamine. Figure 1 shows the chromatographic profile of normal plasma, normal plasma with 50 ng of added pyrimethamine, normal infant plasma, and plasma from an infant treated with oral pyrimethamine. The pyrimethamine was eluted as an isolated peak at 10.3 min. The other peaks in the chromatogram were not identified. The small peak eluting 1–2 min before pyrimethamine was occasionally seen in some chromatograms; when present, it did not interfere with the measurement of pyrimethamine.

Because infants’ samples sent to the laboratory may be of plasma or serum and often contain blood, we compared the recovery of pyrimethamine from whole blood, serum, and plasma. We added 50 ng of pyrimethamine to 100 μL of heparinized whole blood, serum, and plasma (n = 6 each) and assayed. Analytical recoveries of pyrimethamine were 90.0%, 90.2%, and 91.7%, respectively—all very similar to one another. No significant change in pyrimethamine concentration was observed in samples stored at 4 °C for one to four days. Additional drugs that are given to infants with pyrimethamine include leucovorin calcium and sulfadiazine; neither interfered with the measurement of pyrimethamine (data not shown).

Discussion

The described analytical method for pyrimethamine, with its ease of sample preparation, small specimen volumes, low limits of detection, and high percentage recoveries, provides a suitable HPLC method to measure pyrimethamine in plasma from infants. The assay is highly sensitive and specific for pyrimethamine in plasma over the range of plasma concentrations encountered with the usual oral dose of pyrimethamine. The retention time of pyrimethamine was generally ~11 min, but this changed slightly from day to day and with the lot of mobile phase used. Because the changing retention time could influence the slopes of the standard curve and the calibration curve, it is necessary to run these curves daily. About 10 extracted samples can be assayed each day, which allows sufficient time for equilibration and calibration of the instrument and analysis.

<table>
<thead>
<tr>
<th>Pyrimethamine concn, ng/40 μL</th>
<th>Mean (SD), ng/40 μL</th>
<th>CV, %</th>
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</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>3.16 (0.17)</td>
<td>5.38</td>
</tr>
<tr>
<td>20.0</td>
<td>18.62 (0.32)</td>
<td>1.72</td>
</tr>
<tr>
<td>50.0</td>
<td>50.51 (1.19)</td>
<td>2.35</td>
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</table>

* Each concentration was assayed on a different day (n = 10).

* Each analysis was done on 10 different days over 22 calendar days.

* Mean recovery of within- and between-day analyses.

![Chromatograms obtained from extracts of normal plasma (A), normal plasma with 50 ng of pyrimethamine (PYR) added (B), normal infant plasma (C), and plasma from infant treated with pyrimethamine (D).](image_url)
of blanks and quality-control samples. Pyrimethamine is very stable; standards have been stored for six months without noticeable changes in concentration. So far, 1500 biological samples have been analyzed on the C18 column without noticeable peak broadening and tailing of pyrimethamine. After each day’s analyses, we wash the C18 column and store it in aqueous acetonitrile, 500 mL/L. On a few occasions during the development of the assay, a thick emulsion formed during the vortex-mixing with the solvent methylene chloride. Adding 1 mL of acetonitrile before adding the methylene chloride reduced the incidence of emulsion formation.

In future studies with the reversed-phase HPLC method, we will investigate the pharmacokinetics of pyrimethamine in different infants.

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References

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Aluminum Phthalocyanine–Streptavidin: New, Sensitive Fluorescent Tracer for Immunoassay

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A new fluorescent reagent based on aluminum phthalocyanine has been developed for general application to immunoassay. This highly sensitive fluorophore, Ultralite 680, may be covalently attached to a variety of biological entities for use as a tracer. We report the use of an Ultralite 680–streptavidin–digoxin conjugate as a tracer in a direct, competitive, heterogeneous, fluorescent immunoassay of digoxin. The assay is performed manually with 50 µL of serum and no sample pre-treatment. Primary anti-digoxin antibody is incubated with a serum sample containing digoxin and Ultralite 680–streptavidin–digoxin. Bound and free tracer are separated, and the bound fluorescence is released and quantified with a commercially available spectrofluorometer. Assay precision was good (CV = 7.05% at a digoxin concentration of 2.00 µg/L) and results by our method correlated well with those by a conventional RIA (r = 0.9650, n = 24).

Additional Keyphrases: fluoroimmunoassay · digoxin

Fluorescence immunoassay (FIA) offers an attractive alternative to radioimmunoassay (RIA). In principle, the sensitivity of FIA should rival that of RIA. In practice, the utility of both direct and enzymatic FIA

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