Improved Diagnosis of Classical vs Atypical Phenylketonuria by Liquid Chromatography

Yolchi Matsubara, James A. Heininger, and Yong Y. Lin

Atypical phenylketonuria among infants with hyperphenylalaninemia must be promptly diagnosed and differentiated from classical phenylketonuria, because these patients require different treatment to prevent irreversible neurological damage. Measurement of pteridines in urine by liquid chromatography has been widely used for this purpose. Here we report a rapid, simplified liquid-chromatographic method for simultaneously measuring urinary biopterin, neopterin, and creatinine. Unlike previous methods, this method involves neither tedious cleanup procedures nor special equipment.

Additional Keyphrases: hereditary disorders - neonates - creatinine - fluorometry

Hyperphenylalaninemia in infants is caused not only by defective phenylalanine-4-monooxygenase (PM) (EC 1.14.16.1), as in classical phenylketonuria (PKU), but also by abnormalities in the metabolism of tetrahydrobiopterin, a cofactor for PM, tyrosine-3-monooxygenase (EC 1.14.16.2), and tryptophan-5-monooxygenase (EC 1.14.16.4). Atypical PKU patients do not respond to a low phenylalanine diet but do respond favorably to treatment with neurotransmitters or tetrahydrobiopterin (1, 2). Therefore, it is important that all neonates with hyperphenylalaninemia be checked promptly for atypical PKU.

Of the various techniques reported, liquid-chromatographic methods have been most widely used for this purpose (3–10), because they are non-invasive, are relatively simple, and can discriminate among classical PKU (PM deficiency), dihydropteridine reductase (EC 1.6.99.7) deficiency, and dihydrobiopterin deficiency by measuring urinary biopterin, neopterin, and creatinine values (11). The liquid-chromatographic methods proposed to date, however, require either cumbersome sample-cleanup procedures, such as two-step cation and anion ion-exchange columns (3), or special equipment (8).

We describe here a rapid, simple liquid-chromatographic method, involving neither tedious sample preparation procedures nor special equipment, by which biopterin, neopterin, and creatinine are simultaneously determined, thus enabling discrimination of classical and atypical PKU.

Materials and Methods

Biopterin and 6,7-dimethylpteridine were purchased from Vega Biochemicals, Tucson, AZ 85714, and neopterin from Fluka Chemical Corp., Hauppauge, NY 11787. For liquid-chromatographic analyses we used a Radial-PAK μBondapak C18 radial compression column (8 mm × 100 mm, 10-μm particle size), preceded by a C18-Coralis guard column (4 mm × 23 mm) purchased from Waters Associates Inc., Milford, MA 01757, and integrated with two Model 6000A solvent delivery pumps, a Model 660 solvent programmer, a Model 710B automatic sampler, a Model 420 fluorescence detector set at 338-nm excitation and 425-nm excitation, a Model 450 ultraviolet detector at 235 nm, all from Waters Associates. Chromatographic data were collected with a Sigma 115 console (Perkin-Elmer Corp., Norwalk, CT 06856).

Urine specimens were obtained from 28 patients (15 with classical PKU, six with dihydropteridine reductase deficiency, and seven with dihydrobiopterin deficiency), stored at −20 °C, and kept from direct light.

To 1.0 mL of urine, add 1.0 mL of an oxidizing solution containing 10 g of I2 and 20 g of KI per liter of 0.1 mol/L HCl (3), and let stand for 1 h in the dark. Then add 100 g/L ascorbic acid solution until it is in excess (by one or two drops), as indicated by the disappearance of the brown iodine color. Filter the sample through a 0.45-μm (nominal pore size) filter (Millipore Corp., Bedford, MA 01730) and inject 10 μL directly onto the column. Elute the column at a flow rate of 1.5 mL/min with KH2PO4-NaOH buffer (15 mmol/L, pH 7.0) prepared in water/methanol (50/1 by vol) for 6 min, then with the same buffer prepared in water/methanol (75/25 by vol) for 6 min, and finally with the starting solution for 10 min, to re-equilibrate the column for the next injection. At the end of each day, wash the column thoroughly with water and then with methanol. Replace the guard column after 50–60 analyses.

To verify the accuracy of this method, urinary biopterin and neopterin were also measured by the method described by Fukushima and Nixon (3), which requires two ion-exchange cleanup steps before analysis. We used 6,7-dimethylpteridine as an internal standard. The comparison method for determining creatinine was a slight modification of the alkaline-picrate reaction originally described by Jaffe (12).

Results

A chromatographic profile of urine from a healthy infant is illustrated in Figure 1. Neopterin and biopterin are detected by fluorescence and elute at 4.8 min and 8.4 min, respectively. Creatinine is concurrently detected by ultraviolet absorption at 235 nm and elutes at 4.3 min. Each compound is well separated from other interfering peaks.

The accuracy of the biopterin and neopterin measurements has been demonstrated by comparison with the method of Fukushima and Nixon (3) (Figure 2). Although values determined by the current method are somewhat higher than those by the comparison method, the correlation coefficients are very good: 0.98 for biopterin and 0.97 for neopterin. Especially noteworthy is the good correlation at low concentrations of biopterin, essential for the detection of dihydrobiopterin deficiency. Similarly, the accuracy of creatinine measurement was determined by comparison with the alkaline-picrate reaction: the correlation coefficient, the slope, and the intercept are 0.98, 0.92, and −0.013 g/L, respectively. The linearity of detection of each compound is maintained over a wide concentration range: 1.25–250 pmol per injection for biopterin, 1.25–150 pmol per injection for...
neopterin, and 0.15–150 μg per injection for creatinine. This enables us to analyze urine samples at a fixed injection volume regardless of concentration. The precision of the current method, summarized in Table 1, is good in both within-day and day-to-day studies. As for column lifespan, we have not observed loss of resolution or increasing back pressure since we introduced daily column cleanup and frequent replacement of precolumns.

Using this method, we have analyzed urine samples from 28 hyperphenylalaninemic patients. The results are evaluated by plotting urinary biotin/creatinine in "mol%" [biotin × 100] vs the biotin/creatinine ratio as described by Niederwieser et al. (11) (Figure 3). In agreement with their report, the three types of hyperphenylalaninemia fall into well-separated groups: samples from dihydriobiotin±deficient patients contained less than 7 "mol% biotin" and showed significantly lower biotin/creatinine ratios than the other groups; samples from dihydrioderidine reductase-deficient patients had more than 80 "mol% biotin," and 15 urine samples from classical PKU patients had between 20 and 80 "mol% biotin."

**Discussion**

Many liquid-chromatographic methods for pteridines have been developed and are considered to be efficient means of identifying patients with atypical PKU. Most of these methods, however, require time-consuming sample pretreatment under dim light (pteridines are light-sensitive) and remain special laboratory techniques.

Niederwieser et al. (8) reported an automated liquid-chromatographic system for pteridines that could eliminate sample pretreatment, but their method involved special equipment for automatic column switching that is not commercially available. Hausen et al. (13) reported a method for neopterin and creatinine in which a disposable SepPak C18 cartridge (Waters Associates) was used for sample cleanup. We have found (data not shown) that biotin can

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**Table 1. Precision (CV, %) of the Method**

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<thead>
<tr>
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<th>B×100</th>
<th>B*</th>
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<tr>
<td>Biotin</td>
<td>Neopterin</td>
<td>Creatinine</td>
</tr>
<tr>
<td>Within-day (n=15)</td>
<td>1.8</td>
<td>4.0</td>
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<tr>
<td>Day-to-day (n=17)</td>
<td>5.8</td>
<td>7.8</td>
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* Biotin (mol) × 100 [biotin (mol) + neopterin (mol)]
* Neopterin (mol)/creatinine (mol)

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**Fig. 1. Elution profile of urine from a healthy infant**

Upper, ultraviolet absorbance at 235 nm; lower, fluorescence (excitation at 338 nm, emission at 425 nm). Peaks: 1 = iodine and ascorbic acid, 2 = uric acid, 3 = creatinine, 4 = neopterin, 5 = biotin, ▽ = change in mobile phase

**Fig. 2. Accuracy of biotin (left) and neopterin (right) determinations, assessed by comparison with the method of Fukushima and Nixon (3)**
also be analyzed after this pretreatment by selecting an appropriate mobile phase; however, we have found that the same chromatographic profiles can be obtained without this cleanup procedure, which is rather costly. Woolf et al. (10) reported a liquid-chromatographic method involving direct injection of urine but did not present information regarding column life, accuracy, precision, and patients' data. Moreover, their method does not include buffer in the mobile phase. We have observed, however, that buffered mobile phase is needed for a reproducible chromatographic separation; in addition, maintaining the mobile phase at pH 7 is a prerequisite to prevent creatinine from appearing as a doublet, owing to its tautomeration.

The present method is simple, rapid, and precise. It permits simultaneous determination of bioppterin, neopterin, and creatinine, enabling us to distinguish three types of hyperphenylalaninemia. The response is linear over a wide concentration range, permitting fully automated analyses. Thus the proposed method is both useful for the diagnosis of atypical PKU and suitable for routine clinical laboratory use.

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