Liquid-Chromatographic Measurement of p-Aminobenzoic Acid and Its Metabolites in Serum

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This is a high-performance liquid-chromatographic method for measuring p-aminobenzoic acid (PABA) and its metabolites in plasma or serum. Samples are deproteinized, then extracted with organic solvents before chromatography. For quantification, the peak height of the individual compound is compared with that of the internal standard. Analytical recoveries ranged from 41% to 100%, depending on the compound studied. Comparison of patients' samples after oral administration of either N-benzoyl-L-tyrosyl-p-aminobenzoic acid or free PABA revealed that PABA is extensively metabolized and conjugated to either p-acetamidobenzoic acid, p-aminohippuric acid, or p-acetamidohippuric acid. PABA concentrations in serum as measured with the Bratton-Marshall ultraviolet spectrophotometric procedure would appear predominantly to reflect measurements of metabolites, with only a minor contribution from PABA itself.

Additional Keyphrases: cystic fibrosis · pancreatic insufficiency · pediatric chemistry · Bratton-Marshall procedure compared

There are no simple, practicable diagnostic tests to determine exocrine pancreatic function. Current tests involve gastrointestinal intubation followed by the stimulation of exocrine pancreatic secretion with hormones (secretin, secretin—cholecystokinin) or a test meal (e.g., the Lundh meal) and subsequent measurement of bicarbonate concentration and pancreatic enzyme output. These invasive direct pancreatic-function tests can be performed only in specialized hospital departments and usually are expensive and time consuming. The need for a simple, accurate, indirect test of pancreatic function has led to efforts to develop an oral procedure that is more affordable, convenient, and acceptable to patients, especially children (1–3).

In 1972, Imondi et al. (4) first described reliable results for a pancreatic function test in animals, which involved the oral administration of a synthetic peptide, N-benzoyl-L-tyrosyl-p-aminobenzoic acid (Bz-ty-PABA) (3). In the small bowel, this peptide is specifically cleaved to N-benzoyl-L-tyrosine and p-aminobenzoic acid (PABA) by the pancreatic endopeptidase, chymotrypsin (EC 3.4.21.1). The PABA is rapidly absorbed from the gut—probably by passive diffusion—and undergoes conjugation and further metabolism in the liver to p-acetamidobenzoic acid (PAABA), p-aminohippuric acid (PAHA), and p-acetamidohippuric acid (PAHAA) (Figure 1) before being excreted in the urine. The amount of PABA and its metabolites found in serum or plasma and urine can be used as an index of the intraluminal activity of chymotrypsin and thus provides an indirect measurement of exocrine pancreatic function (5, 6).

Shwachman's syndrome and cystic fibrosis are the major causes of exocrine pancreatic insufficiency in children. Studies on cystic-fibrosis patients evaluated the diagnostic importance of this test in cases of exocrine pancreatic insufficiency, by means of a nonspecific spectrophotometric procedure (7–10). The PABA recovered in urine was significantly lower in cystic fibrosis patients than in a normal control group (7–9), and there was a good correlation between PABA recovery, fecal chymotrypsin activity, and coefficient of fat absorption. Evidently, PABA recovery is significantly decreased in patients with cystic fibrosis and steatorrhea, and thus PABA might be a reliable marker of pancreatic insufficiency in other patients as well.

At present, in most published procedures the PABA collected in a timed urine specimen is measured. Determinations in plasma or serum would, however, provide a more direct evaluation of the enzymatic hydrolysis of the peptide (10, 11), and blood samples are much easier to obtain from

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Fig. 1. (Top) Chemical formula of the synthetic peptide N-benzoyl-L-tyrosyl-p-aminobenzoic acid (the peptide bond cleaved by chymotrypsin is indicated by a broken line); (Bottom) Structures of p-aminobenzoic acid and its metabolites.
children than are timed urine specimens. The amount of PABA and its metabolites present at a certain time after administration of the compound should allow assessment of both the exocrine pancreatic function and the conjugating ability of the liver. As measured with a nonspecific spectrophotometric procedure (Bratton–Marshall method), the purported concentration of PABA in plasma or serum of patients with exocrine pancreatic insufficiency was significantly lower than in healthy controls (12–14). Some reports also indicate that the determinations of PABA in plasma or serum are a more sensitive indicator of pancreatic insufficiency than is steatorrhea, and more specific than the results from determination only in urine (12–17).

Our goal in this study was to develop a micro-scale HPLC procedure for measuring PABA and its metabolites in serum or plasma, to obtain greater and more reliable information than is possible with the conventional Bratton–Marshall method (18, 19).

Materials and Methods

Reagents. Throughout these studies, we used PABA supplied from J. T. Baker Chemical Co., via Johns Scientific Co., Toronto, Canada, and PABA, PAABA, and m-hydroxybenzoic acid from Sigma Chemical Co., St. Louis, MO. PAAHA was synthesized in house as follows: add 5 g of PAAHA to a mixture of 60 mL water and 12.5 mL concentrated hydrochloric acid. Dissolve 3 mL of acetic anhydride and 3.85 g of sodium acetate in 12 mL water, and add this to the PAAHA-HCl mixture. Stir at 25 °C for about 10 min until the precipitation of white crystals is complete. Wash the crystals with cold distilled water and air-dry. PAAHA’s melting point is 236–238 °C.

Phosphoric acid was obtained from Fisher Scientific, Toronto, Canada. Methanol, “Accusolv,” and dichloromethane were from Anachemia, Mississauga, Ontario, Canada. Sulfosalicylic acid, 50 g per liter of methanol/water (50/50 by vol), was obtained from Abbott Diagnostics, Toronto, Canada. Ethyl acetate—Omnisolv was purchased from BDH Chemicals, Toronto, Canada.

Chromatographic system. The HPLC system consisted of a Model U-6-K injector, Model 6000 pump, a Lambda-Max LC spectrophotometer 481, a Servogor 120 recorder, and an LC pre-column filter, all from Waters Associates, Milford, MA. The column, selected from several C18 columns of various lengths and particle sizes, was a 4.6 mm (i.d.) 25-cm Altex Ultrasphere column (Beckman Instruments, Inc., Fullerton, CA), packed with C18 material bonded to 5-μm silica particles. This column best resolved PABA and its metabolites from the solvent front and from the other peaks in the chromatogram. The chromatography was performed at room temperature (20–25 °C).

The mobile phase was 15/85 (by vol) methanol/phosphate buffer (10 mmol/L, pH 3.0). Solvent delivery was set to a flow rate of 2.0 mL/min. Absorbance of the column effluent was monitored at 290 nm and a sensitivity setting of 0.05 A full scale. The Servogor 120 recorder was set at a chart speed of 0.5 cm/min.

Extraction procedure. Combine, then vortex-mix for 15 s, 100 μL each of serum or plasma and sulfosalicylic acid containing the internal standard, m-hydroxybenzoic acid, to precipitate protein. Extract PABA and its metabolites by combining the protein-free filtrate with 400 μL of ethyl acetate, vortex-mixing for 1 min, and centrifuging for 2 min. Remove the organic (top) phase and evaporate it under a stream of nitrogen. Add 80 μL of doubly distilled water and 200 μL of dichloromethane, vortex-mix for 30 s, then centrifuge for 6 min. Inject 20 μL of the aqueous extract (top layer) onto the column.

Calculations. Quantify the analytes by comparing the peak-height ratio for the compound of interest and the internal standard with that obtained for three standards of known concentrations processed through the entire procedure.

To verify the identification of the compounds we compared the retention times of the peaks in the chromatograms of serum samples with those obtained by direct injection of standards.

Results

Figure 2 depicts typical chromatograms for a blank serum sample and a patient’s sample.

The order in which the compounds eluted was pH dependent over the pH range 3.0–7.0, the retention time of PABA and its metabolites increasing at lower pH. Resolution was optimal at pH 3.0.

Linearity. We prepared PABA, PAABA, PAHA, and m-hydroxybenzoic acid standards to cover a wide concentration range and analyzed them as described. The standard curves were linear up to 80 mg/L in all cases, well exceeding the highest concentration likely to be encountered in patient’s samples.

Precision. Between-day precision of the method was assessed by repeated analyses of serum controls containing various concentrations of PABA, PAABA, PAHA, and PAAHA. The results are shown in Table 1.

Recovery. Known concentrations of PABA and its metabolites were added to serum, the extractions and assays were performed as described, and the peak heights obtained were compared with those obtained by direct injection of aqueous standards. Table 2 summarizes the results.

Interference. We obtained 20 plasma samples from the blood bank (normal population) and 20 serum samples from patients receiving various medications (e.g., digoxin, anticonvulsants, ticarcillin, theophylline, chloramphenicol).
These were analyzed as described. We saw no co-elution with the compounds of interest.

Preliminary clinical studies. A standard dose of Bz-ty-PABA containing the equivalent of 5 mg of free PABA per kilogram of body weight was administered to two normal subjects and to a patient with cystic fibrosis. In a second study, free PABA, 5 mg/kg, was administered to a normal volunteer and another patient with cystic fibrosis. Table 3 shows the concentrations of PABA and its metabolites found 60 and 90 min after the dose. The HPLC results are compared with those obtained by the Bratton-Marshall procedure.

Discussion

To evaluate exocrine pancreatic function, most PABA analyses have been done on urine samples by the Bratton-Marshall spectrophotometric method, which does not differentiate between PABA and its metabolites (20-23). Later reports described HPLC techniques using ion-pairing reagents to detect PABA and its metabolites in urine (24, 25). A method for measuring PABA in serum was described based on the procedure of Bratton-Marshall (12-14, 17) with the disadvantage that it again does not differentiate between PABA and its metabolites, and the result produced is presumably a combination of PABA and metabolite concentrations (Table 3). The present HPLC method has many advantages over the classical Bratton-Marshall procedure in that it allows for individual quantification of PABA and each of its metabolites and is not interfered with by those commonly prescribed drugs that we tested.

It is difficult to establish conditions that will produce optimal recoveries for all of the PABA metabolites in serum. Although the recovery figures for PABA and PABA are relatively low compared with those for the other metabolites, the recoveries are consistent and provide adequate sensitivity for the assay.

After an oral dose of Bz-ty-PABA, a patient with cystic fibrosis and pancreatic insufficiency showed no detectable PABA, or any of its metabolites, in serum, whereas all these compounds were seen in patients with normal pancreatic function (Table 3). In contrast, a dose of PABA produced measurable concentrations of PABA and (or) its metabolites in both the normal subject and the patient with cystic fibrosis (Table 3).

Evidently, very little of the "PABA" measured in the Bratton-Marshall procedure is in fact PABA. Rather, the measurement primarily is of PABA metabolites, and possibly of other nonspecific substances. Whether the ability to quantify PABA and its metabolites separately by HPLC will be more helpful than PABA measurement by nonspecific spectrophotometric procedures and allow for improved clinical assessment of the patient's condition is the subject of another study. We will assess the diagnostic capability of this test by analyzing plasma PABA and its metabolites in both patients with cystic fibrosis and those with liver disease.

Table 2. Analytical-Recovery Studies

<table>
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<tr>
<th>Mean, mg/L</th>
<th>m-Hydroxybenzoic acid (%)</th>
<th>PABA</th>
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<tr>
<td>2</td>
<td>49.7</td>
<td>40.6</td>
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<td>5</td>
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<td>10</td>
<td>45.3</td>
<td>46.5</td>
<td>78.4</td>
<td>77.2</td>
<td>88.4</td>
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Table 3. Metabolites in Serum of Two Normal Persons and a Patient with Cystic Fibrosis Compared After an Oral Dose of Bz-Ty-PABA or Free PABA

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time after dose, min</th>
<th>&quot;PABA&quot; by Bratton-Marshall procedure</th>
<th>PABA</th>
<th>PABA</th>
<th>PABA</th>
<th>PABA</th>
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<tr>
<td>After Bz-Ty-PABA</td>
<td></td>
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<td>1.0</td>
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<td>Normal (no. 1)</td>
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<td></td>
<td>0.54</td>
<td>0.54</td>
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<tr>
<td>Cystic-fibrosis patient</td>
<td>60</td>
<td></td>
<td>0.65</td>
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<td>0.65</td>
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<tr>
<td>After free PABA</td>
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<td></td>
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<td></td>
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<tr>
<td>Normal (no. 1)</td>
<td>60</td>
<td></td>
<td>0.93</td>
<td>0.93</td>
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<td>0.93</td>
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<tr>
<td>Cystic-fibrosis patient</td>
<td>60</td>
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<td>0.89</td>
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References


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