Determination of p-Aminobenzoic Acid in Urine by Room-Temperature Phosphorimetry, with Application to the Bentriomide Test for Pancreatic Function

H. Thomas Kanes,1 Rickey P. Bateh,2 J. D. Winefordner,2 and S. G. Schulman1

Analytical recovery of urinary p-aminobenzoic acid liberated from bentriomide (N-benzoyl-L-tyrosyl-p-aminobenzoic acid) was determined by room temperature phosphorimetry as an index to exocrine pancreatic function. Metabolites were hydrolyzed to the parent compound in 4 mol/L NaOH, then quantified, after pH adjustment, by spotting 3 μL of this solution onto filter paper treated with potassium iodide and measuring the resulting phosphorescence relative to a standard curve. The method is sufficiently precise, and results compare well with those by the Bratton–Marshall colorimetric method (r = 0.993) for determining p-aminobenzoic acid in urine from patients undergoing the bentriomide test. The present procedure is rapid and is more selective than are colorimetric procedures.

The bentriomide test has been proposed as a reliable screening test for diagnosis of pancreatic exocrine insufficiency (1–3). In the test, orally administered N-benzoyl-L-tyrosyl-p-aminobenzoic acid (bentriomide) is specifically cleaved to benzoxytyrosine and p-aminobenzoic acid (PABA) by the pancreatic enzyme chymotrypsin (EC 3.1.21.1). The PABA is absorbed from the small bowel, metabolized by the liver, and excreted in the urine. The concentration of PABA in urine therefore reflects chymotrypsin activity in the gut, and exocrine pancreatic insufficiency is indicated by low values for PABA.

PABA has been determined in urine by hydrolysis followed by quantification of PABA and certain of its metabolites. Methods currently used include the Bratton–Marshall diazo-coupling (4) or dimethylaminocinnamaldehyde (5) colorimetric methods and, more recently, liquid chromatography with electrochemical detection (6). Both colorimetric methods lack specificity for aromatic amines, several of which may be present in urine as a result of drug therapy or the consumption of certain foods (7). Liquid chromatography is less subject to such interferences, but is more expensive and less convenient because of the additional post-hydrolysis steps required. Room-temperature phosphorimetry of organic compounds adsorbed onto various solid surfaces is a relatively recent development in trace analysis and has received considerable attention for drug analysis during the past decade (8). Its successful application depends on several factors, including the selection of appropriate support material to maximize analyte binding, use of a suitable heavy-atom species to enhance phosphorescence intensity, and an optimal sample-drying procedure to minimize the quenching effects of water and oxygen.

Room-temperature phosphorimetry as used to quantify PABA in vitamin tablets (9) is adaptable to use with biological fluids. We describe here a method for urinary PABA based on this technique as an alternative to existing procedures. The method is highly selective, fairly precise, and suited to clinical laboratory use, because neutralization, sample application, and measurement are the only post-hydrolysis steps required.

Materials and Methods

Instrumentation

We used a modified spectrophotofluorimeter (Aminco-Bowman, Model SPF 100) equipped with a rotating can phosphorescope and a ratio photometer (all from American Instrument Co., Silver Spring, MD) and a IP21 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ 08846). We used a specially constructed solid-sample bar, as described by Ward et al. (10), to position samples within the sample compartment. An Aminco 150 W xenon arc lamp was used as the excitation source.

Reagents and Materials

"Nanopure" de-ionized water (Barnstead system of Sybron Co., Boston, MA) was used for analyte dilution. PABA, p-aminophippuric acid, and p-acetamidohippuric acid were all purchased from Sigma Chemical Co., St. Louis, MO. p-Acetamidohippuric acid was synthesized by reduction of acetic anhydride with p-aminophippuric acid (11). Diethylaminoethylcellulose (DE-81) anion-exchange filter paper (Whatman Inc., Clifton, NJ 07014) was used as the solid-support material. All other reagents were AR grade.

Procedures

Pancreatic function test. To each of 24 healthy adult volunteers, five patients with small-bowel malabsorption, six patients with chronic pancreatitis, and three patients with exocrine pancreatic insufficiency, 500 mg of bentriomide (Adria Labs, Inc., Columbus, OH) was orally administered in 250 mL of water after an overnight fast. Just before dosing, the subjects were instructed to empty their bladders and consume 500 mL of water. An additional 500 mL of water was given and subjects continued to fast until the test was completed. Urine was collected for 6 h after drug administration, the total volume was measured, and the specimen was divided into aliquots and stored at −4 °C until analysis.
Sample preparation. Add 0.5 mL of an 8 mol/L NaOH solution to 0.5 mL of sample urine and aqueous PABA standards (400, 300, 200, 100, and 50 mg/L) in 10 × 150 mm screw-capped tubes calibrated at 5 mL. Replace the caps loosely and heat all tubes for 1 h in a heating block (Lab-Line Instruments Inc., Melrose Park, IL) set at 120 °C. Allow the tubes to cool at room temperature for 2–3 min and add 4 mL of approximately 1.0 mol/L H2SO4 containing 1.0 mol of KH2PO4 per liter; this is a combination neutralization and buffering reagent. (This reagent is titrated beforehand with the NaOH solution, and the H2SO4 concentration must be adjusted so that the pH is about 6.4 when the 4 mL is added.) Now adjust the total volume of this mixture to the 5-mL calibration mark with distilled water. This corrects for any evaporation during the heating at 120 °C.

Room temperature phosphorimetry. Apply 2 µL of a 1.0 mol/L aqueous KI solution followed by 3 µL of the sample solution to a filter paper disc, 3.1 mm in diameter, mounted on the sample bar. Place the sample bar in the phosphorimeter sample compartment and allow it to dry in a stream of dry nitrogen for 15 min. With the excitation and emission wavelengths set at 295 and 432 nm, respectively, measure the phosphorescence intensity from each of the four sample positions on the bar. Repeat this procedure until all samples have been measured. Evaluate sample concentrations by comparison with a best-fit standard curve.

Colorimetry. We also determined urinary PABA concentrations by the Bratton–Marshall diazo-coupling method (4), for comparison. In this method, hydrolysis was in 1.2 mol/L HCl for 1 h, in a bath of boiling water. The hydrolyzed urine samples were diluted according to the urine collection volume and color was developed according to the usual procedure.

Results

Analytical Recovery of PABA Metabolites

PABA (250 mg/L) and its metabolites—p-aminobenzolic acid (354 mg/L), p-acetamidohippuric acid (431 mg/L), and p-acetamidobenzoic acid (326 mg/L)—were diluted with blank urine, taken through the alkaline hydrolysis procedure, and evaluated by the present method (Table 1). These metabolite concentrations are such that an equivalent amount of PABA (250 mg/L) is liberated for each compound if hydrolysis is complete. Preliminary experiments demonstrated that the phosphorescence intensities produced by these metabolites studied were all less than 1.0% of that produced by PABA. Therefore, any appreciable signal detected from the hydrolyzate solutions was due to that liberated from PABA and not from metabolite phosphorescence. The data in Table 1 demonstrate that hydrolysis of PABA metabolites was essentially complete and the parent compound was stable under the conditions used.

| Table 1. Analytical Recovery of PABA and Its Metabolites Added to Drug-Free Urine |
|-----------------------------------|----------------|----------------|----------------|----------------|
|                                  | n              | Concentration, mg/L | Recovered | Percent recovered | Mean | SD | Mean | SD |
|                                  |                | p-Aminobenzoic acid |          |                |      |    |      |    |
|                                  | 10             | 250               | 253     | 7.1            | 101  | 2.8|
|                                  |                | p-Aminohippuric acid |         |                |      |    |      |    |
|                                  | 10             | 354               | 265     | 8.3            | 106  | 3.3|
|                                  |                | p-Acetamidobenzoic acid |       |                |      |    |      |    |
|                                  | 10             | 326               | 245     | 4.2            | 98   | 1.7|
|                                  |                | p-Acetamidohippuric acid |       |                |      |    |      |    |
|                                  | 10             | 431               | 244     | 8.9            | 98   | 3.6|

Room Temperature Phosphorimetry

The pH of hydrolyzed solutions was adjusted since phosphorimetry signals declined sharply above pH 12.0. The mean pH of 35 adjusted solutions was 6.42 (SD 0.04), indicating successful control of this potential source of inaccuracy. The iodide perturber was used because a 654 relative intensity unit (CV 12.0%) increase in signal, with a relatively small increase in blank signal (292, CV 51.8%), greatly enhanced the sensitivity (n = 10 samples). After a 15-min drying time the phosphorescence intensity from samples remained constant for 9 min, then began declining at a rate of 1.8 relative intensity units per minute.

Analytical Variables

Linearity. The standard curve was linear through the range of 0 to 40 mg/L, which corresponds to original urinary PABA concentrations of 0 to 400 mg/L. Only six of 75 patients' samples required dilution with an equal volume of distilled water to bring them within that range.

Sensitivity. The limit of detection for the present method—i.e., the concentration of PABA resulting in a signal three times the noise level—is 0.67 mg/L. This is far below the concentrations ordinarily found in patients' samples.

Precision. Precision was evaluated by repeated analysis of aliquots taken from patients' samples (stored frozen) on both a within-run and day-to-day basis (Table 2). The three samples chosen provided concentrations reflecting the entire range of the assay.

Selectivity. Drug-free urine samples, collected from fasting subjects after voiding their first morning specimen, were subjected to the entire analytical procedure. The average blank signal from 13 such subjects was 6.9 (SD 4.2) relative intensity units, corresponding to an apparent PABA concentration of 3.6 (SD 2.2) mg/L in urine.

We also tested some commonly used drugs for interferences, each in a 500 mg/L concentration: chlorothiazide, sulfadiazine, atropine, neomycin, chlorpropamide, tolbutamide, methoclorpramide, hydrochlorothiazide, acetaminophen, lidocaine, caffeine, and xylose. All of these gave signals smaller than those of corresponding blanks. Indomethacin, procaine, acetylsalicylic acid, chloramphenicol, and sulfanilamide exhibited apparent interferences of 4.1, 40.6, 1.1, 0.7 and 8.0%, respectively, tested at the same concentration.

Patients' Sample Correlation

We compared data on urinary PABA concentration as measured by the present method with those obtained by Bratton–Marshall colorimetry. Results by the two methods agreed well, as indicated by a linear relationship between Bratton–Marshall (x) and the present method (y) of y = 0.997x + 1.651. The correlation coefficient (r) for 75 samples in the range 58 to 786 mg/L was 0.993. The standard deviations of the slope and intercept were 0.002 and 0.300, respectively.

| Table 2. Precision of the Present Method |
|------------------------------------------|---|---|---|
| Conc, mg/L                               | Mean | SD | CV, % |
| Within-run                               | 10 | 93.2 | 7.9 | 8.5 |
| Day-to-day                                | 10 | 162.0 | 8.6 | 5.3 |
| Day-to-day                                | 10 | 347.2 | 21.8 | 6.3 |
| Day-to-day                                | 10 | 75.5 | 10.9 | 14.4 |
| Day-to-day                                | 10 | 163.6 | 9.0 | 5.5 |
| Day-to-day                                | 10 | 335.5 | 19.2 | 5.7 |
Discussion

Ito et al. (6) have shown that acid hydrolysis, as used in both the Bratton–Marshall and dimethylaminocinnamaldehyde colorimetric methods, does not completely convert p-acetamidohippuric acid (a major PABA metabolite) to the parent compound. Fortuitously, this metabolite is converted to the primary aromatic amine p-aminohippuric acid and therefore reacts to form a chromophore similar to that of the PABA chromogen complex. However, differences in the absorbancies of these complexes and variable PABA metabolism could lead to erratic results. Alkaline hydrolysis is a better alternative, because it is advantageous to measure a specific analyte rather than the additive contributions of two analytes. Additionally, colorimetric methods are non-specific, and contamination with urinary aromatic amines would cause falsely positive results. Extreme care must be taken to avoid these interferences, and so interfering drugs must be discontinued at least three days before the test—something not always possible. Electrochemically detected liquid chromatography is seemingly not subject to such interferences, but this technique is more expensive and less convenient.

Our method is relatively specific with regard to the compounds we tested, except for procaine, which does not pose a therapeutic problem if discontinued. Also, metabolite-recovery studies and sample-blank analysis indicate that endogenous urinary components and hydrolysis by-products do not interfere with our method. The accuracy, precision, and linearity are within acceptable limits for clinical analysis of urine samples. The detection limit is more than adequate and suggests that the procedure might be adapted to analysis for PABA in blood.

Although the sample holder used is not commercially available, it is easily built, and commercially available front-surface attachments could be used with slight modification (10).

Phosphorescence equipment also requires less maintenance than does "high-performance" liquid chromatography. The present procedure is technically simple and requires no expensive reagents or materials. This favors routine use, and the same equipment can potentially be used for other clinically important analytes (8). Vo-Dinh et al. (12) have demonstrated the capability for automation of the technique, and their system could be applied to high-volume clinical analysis. Finally, analysis time could be shortened by using organic solvents for sample application and heat lamps could hasten sample drying.

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References