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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF *p*-AMINOBENZOIC ACID AND SOME OF ITS METABOLITES

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SUMMARY

The development of a relatively simple high-performance liquid chromatographic method for the analysis of *p*-aminobenzoic acid and some of its metabolites is reported. The method is specific for detecting and quantifying *p*-aminobenzoic acid, *p*-aminohippuric acid, N-acetyl-*p*-aminobenzoic acid, and N-acetyl-*p*-aminohippuric acid. Urine and serum samples can be analyzed directly without solvent extraction or pretreatment. The method has a lower detection limit of 5 ng on column. All the compounds are eluted within 20 min. The method is suitable for routine clinical determinations.

INTRODUCTION

The potassium salt of *p*-aminobenzoic acid (PABA), POTABA, has enjoyed some popularity in the past 15–20 years as a therapeutic modality in the treatment of systemic sclerosis. Zarafonetis[1–3] recommended the long-term administration of 12 g of POTABA daily and gave it in divided doses of 2.5 g, 5 times a day.

Drucker *et al.*[4] have studied some of the factors affecting the acetylation of PABA by humans. PABA and metabolites (Fig. 1) were measured by the Bratton–Marshall reaction[5], a method that is quite specific and sensitive in quantifying primary aromatic amines but has little value in differentiating one aromatic amine from another. The presence of two or more of these compounds produces colored substances whose absorbances are additive in the determination. If either or both of the N-acetylated metabolites are present, acid hydrolysis is required for the detection. The amount of the acetylated compound is estimated by the difference between the absorbance before and after hydrolysis. However, this method makes no distinction between the two possible metabolites.

High-performance liquid chromatography (HPLC) has been shown to be an innovative technique for chromatographing many types of organic and biochemical

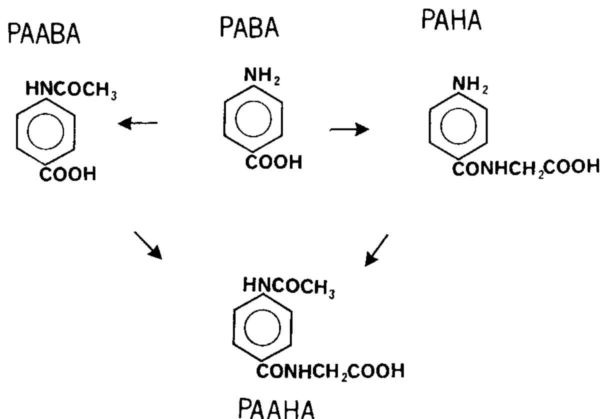

Fig. 1. Schematic of PABA and three of its metabolites. The glycine conjugated and acetylated analogues shown were all separated using the HPLC method.

compounds in physiological fluids[6–8]. Many of the analyses performed involve the separation and identification of metabolic end products chromatographed over an extended period of time[9–11]. It has been observed that within certain areas of these graphic profiles, specific groups of metabolites can be readily determined using other operating parameters. A method has been developed for the separation and detection of PABA, *p*-aminohippuric acid (PAHA), N-acetyl-*p*-aminobenzoic acid (PAABA), and N-acetyl-*p*-aminohippuric acid (PAAHA) using a formate buffer. The technique offers many advantages over current methodologies. Each metabolite of the untreated physiological sample was detected and determined as the unaltered by-product of PABA. Since all the compounds are benzenoid, detection was possible at 254 nm.

MATERIALS* AND METHODS

A Varian LC Series 4100 liquid chromatograph was used throughout this investigation. The system consisted of a positive displacement syringe pump, a 254-nm UV detector, a circulating preset 25° water-bath, and an A-25 recorder.

Analytical-grade sodium formate (Mallinckrodt, St. Louis, Mo., U.S.A.) and purified 90% formic acid (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were used to prepare the buffer. Standard solutions of each compound were prepared using 99% pure PABA (Aldrich, Milwaukee, Wisc., U.S.A.), 97% pure PAHA (Aldrich), and PAABA (City Chemicals, New York, N.Y., U.S.A.).

PAAHA was prepared using the method of Newman *et al.*[12]. Identity was confirmed by its melting point, (198–200°) and by gas chromatography–mass spectroscopy of its methyl ester.

Procedure

A 1 m × 2 mm I.D. 316 stainless-steel column was dry-packed with AS-PELLIONEX-SAX (Reeve Angel, Clifton, N.J., U.S.A.) and converted to the formate

* The manufacturer's name and products are given as scientific information only and do not constitute an endorsement by the United States Government.

form. AS-PELLIONEX-SAX pellicular packing media is a strong anion exchanger. It can withstand operating temperatures up to 85°. Cross-linked polystyrene and trimethylbenzylammonium groups form the active stationary and ionogenic phases of the exchanger, respectively. The resin capacity is approximately 10 mequiv. per gram of packing medium.

A degassed buffer solution of 0.005 *M* sodium formate, adjusted to pH 3.50 $\pm$ 0.05, with 90% formic acid was prepared as the eluent. The pH of the buffer was a critical parameter, with slight variations resulting in loss of resolution. At pH 3.45, the PABA starts to merge with the unknown peaks of the urine or serum. At a pH of 3.55, PAHA and PAABA lose resolution. Molarity was varied between 0.005 and 0.001. However, the 0.005 *M* concentration produced optimum separation. An unstable baseline can be avoided by preparing the buffer 12–24 h ahead of time and storing it in the pump until used.

Two microlitres of sample were injected into the column at a stop flow condition. The injection port pressure was reduced to atmospheric conditions. The flow-rate was 40 ml/h, producing pressures of 400–500 p.s.i. The four metabolites were eluted within 20 min.

Standard solutions of each compound were chromatographed at 5 absorbance ranges (0.005–0.08). The detection limit was 5 ng on column with a signal-to-noise ratio of 3 to 1.

Samples

Urine and serum specimens were collected from one normal human volunteer following a single oral dose of 40 mg/kg of PABA dissolved in water. Blank urine and serum specimens were taken just prior to the administration of the drug. Samples were then collected at 15-min intervals for 1 h and 30-min intervals for the remaining 9 h. All samples were frozen at −20° until analyzed.

In another experiment, a PAHA clearance study was run. A normal human volunteer was given a 100-mg bolus of PAHA and then a continuous infusion of 20 mg/min for a 4-h period. Urine and serum samples were collected on a schedule identical to the oral dose PABA study. The specimens were kept at −20° until analyzed. The physiological samples were run at the appropriate absorbance range required by the concentration of the metabolite being determined. The control serum and urine specimens produced no significant interference. This included the normally occurring urinary hippuric acid.

RESULTS AND DISCUSSION

Acetylated metabolites of PABA are very difficult to determine in physiologic fluids. The determination of primary aromatic amines usually associated with clinical chemistry procedures has been based on measuring their concentration by the Bratton–Marshall reaction. Several automated methods have been developed around this technique, but they all suffer from the inability to distinguish between these acetylated products and the corresponding primary aromatic amines produced after hydrolysis.

The HPLC method used for this study was highly specific for each of the four metabolites. Hippuric acid, which is a normally occurring urinary end product, did not create a problem of any significance. The hippuric acid concentration present in

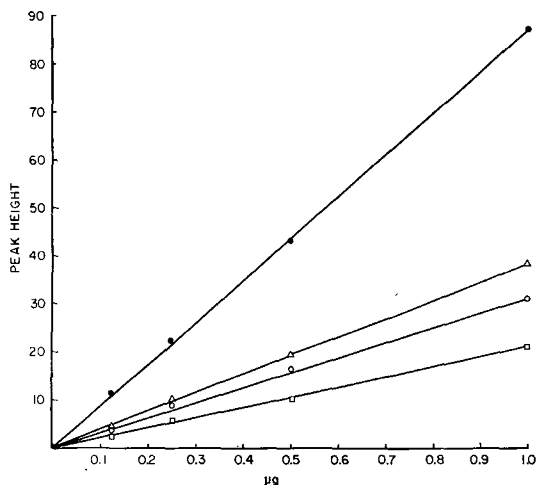

Fig. 2. Calibration curve of PABA (●), PAHA (○), PAABA (△) and PAAHA (□), detected at 254 nm, 0.08 A. Column, 1000 × 2 mm AS-PELLIONEX-SAX.

2-µl aliquots was too low to be detected. Standard calibrations were made by plotting concentration *versus* peak height. A series of standard solutions was prepared to check reproducibility and sensitivity. Four different concentrations were used for each compound, with five chromatographic runs recorded for each concentration. The points were averaged and plotted. No individual point differed from the mean by more than ±3%. Linearity was observed at all absorbance ranges studied. Fig. 2 shows a typical

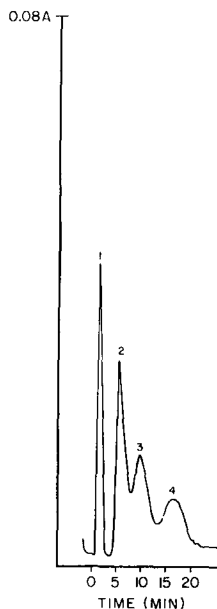

Fig. 3. Separation of a standard solution of (1) PABA, (2) PAHA, (3) PAABA and (4) PAAHA. Mobile phase, 0.005 *M* sodium formate at pH 3.50; Column temperature, 25°; flow-rate, 40 ml/h.

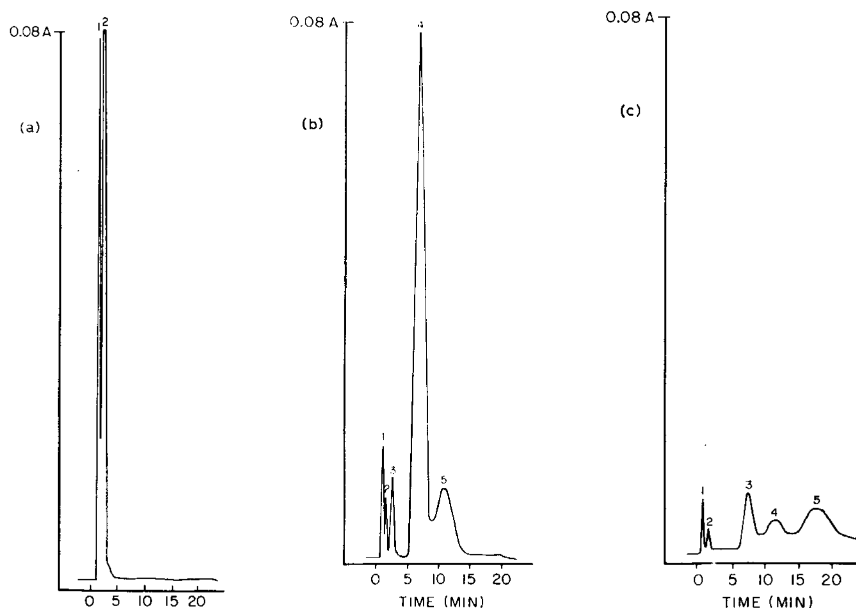

Fig. 4. (a) Chromatogram of a blank urine specimen (0 time) detected at 0.08 A. Unknown peaks (1 and 2) were always observed in urine specimens. (b) Chromatogram of an undiluted urine specimen, 45 min after an oral dose of PABA. Five peaks were observed: 1 = unknown; 2 = unknown; 3 = PABA; 4 = PAHA; 5 = PAABA. (c) Chromatogram showing the glycine conjugated and acetylated metabolites of PABA in urine (180 min aa). 1 = Unknown; 2 = unknown; 3 = PAHA; 4 = PAABA; 5 = PAAHA.

calibration, plotted at the 0.08 absorbance range. PABA, PAHA, PAABA, and PAAHA were well resolved when all four compounds were present. Fig. 3 depicts a standard run containing all four metabolites.

In the three chromatograms obtained following the oral dose of PABA shown in Figs. 4a–c, separation of the metabolites present in urine proved that the method could be used for clinical determinations of PABA and its acetylated analogues. As noted in Fig. 4a (zero time), the compounds of interest were not present. Only two unknown peaks were observed. These peaks were always eluted with the solvent front. In Fig. 4b, 45 min after administration (aa), five peaks were chromatographed from urine. It was noted that PAAHA was not present in the specimen. As shown in Fig. 4c, 180 min aa, PABA has completely disappeared, and PAAHA appears in significant amounts. The chromatograms shown in Figs. 5a and b represent control and experimental serum specimens, respectively. A much lower concentration of the metabolites was present in these specimens requiring a lower absorbance range. No interfering peaks were observed in the blank serum. The oral dose clearance curves for urine and serum are shown in Figs. 6a and b, respectively. PABA and its N-acetyl and glycine conjugates were detected in both urine and serum. PABA was not detected after 1.5 h in the urine and after 2 h in the serum. PAAHA was not detected in urine until 1 h aa and was completely absent in serum. All detected metabolic products disappeared completely within 3 h aa.

This method has been used in a PAHA clearance study with a single normal

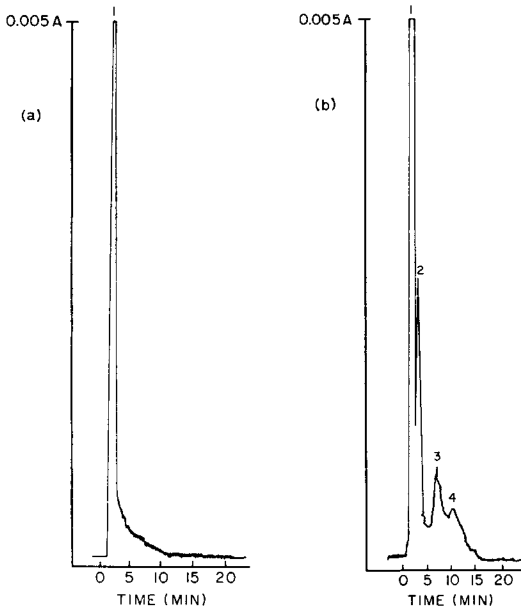

Fig. 5. (a) Control serum sample (0 time). Chromatographed at 40 ml/h; 2-μl sample; detector 254 nm, 0.005 A. One unknown peak was observed. (b) Separation of (1) unknown, (2) PABA, (3) PAHA and (4) PAABA in a 2-μl serum sample (45 min aa).

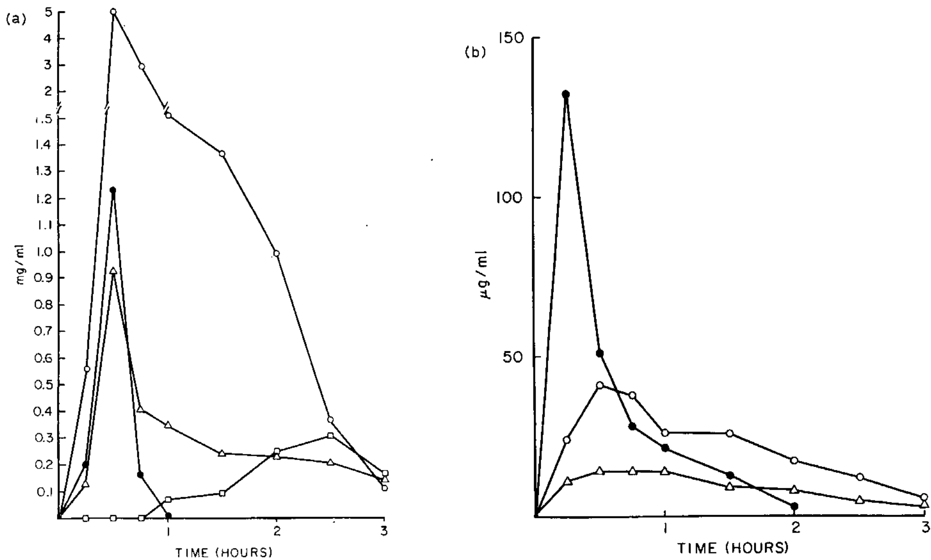

Fig. 6. (a) Clearance curves of PABA (●), PAHA (○), PAABA (△) and PAAHA (□), in urine. Maximum excretion of PABA, PAHA and PAABA occurred 30 min after oral administration. (b) Comparative clearance curves of serum from the same human subject. No PAAHA was detected in any of the serum specimens.

volunteer. Fifteen minutes after intravenous infusion, PAHA and PAAHA were seen in the urine. No PAAHA was present in the infused PAHA. PAHA was the only compound detected in the serum.

A specific method has been developed for evaluating PABA, PAHA, PAABA, and PAAHA present in physiologic specimens.

Since the pressures are low and gradient elution is not required, sophisticated chromatographic instrumentation such as we used is not needed. The simplicity of the method and instrumentation required makes the determintaion of these metabolites feasible as a routine clinical test.

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