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Note

An improved high-performance liquid chromatographic method for quantifying *p*-aminobenzoic acid and some of its metabolites

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We have recently developed an improved high-performance liquid chromatographic (HPLC) method for determining *p*-aminobenzoic acid (PABA) and some of its metabolites in physiologic fluids. The new method has several advantages over our previously reported procedure[1]. Aside from the simplicity and specificity, the procedure offers increased sensitivity, higher resolution and a shorter analysis time.

p-Aminobenzoic acid, N-acetyl-*p*-aminobenzoic acid (PAABA), *p*-aminohippuric acid (PAHA) and N-acetyl-*p*-aminohippuric acid (PAAHA) are eluted from an anionic silica gel micro-Partisil column with a single sodium formate–formic acid buffer. Serum and urine specimens are analyzed directly without pretreatment.

We are currently using this procedure to characterize excretion patterns in normal and reduced renal function patients.

EXPERIMENTAL*

Apparatus

A Varian LC Series 4100 liquid chromatograph was used for separating and quantifying standards and experimental samples. The system consisted of a positive displacement syringe pump, a 254-nm UV detector, a circulating water-bath maintained at 25°, and an A-25 recorder.

Reagents

Aqueous standards stock solutions containing 1 μg/μl 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 synthesized in our laboratory, using the method of Newman *et al.*[2]. Analytical-grade sodium formate (Mallinckrodt, St. Louis, Mo., U.S.A.) and 90% formic acid (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were used to prepare the buffer.

Procedure

A prepacked 25-cm × 1/4-in.-O.D. × 4.6-mm-I.D., 10-μm-size Partisil SAX column (Whatman, Clifton, N.J., U.S.A.) was used for all separations. Partisil-10

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

SAX is a strong microparticulate anion exchanger. It can withstand operating temperatures exceeding 70° and a pH range from 1.5–10. The organo-chlorosilane ion exchanger has a quaternary nitrogen functional group and is prepared in the $H_2PO_4^-$ form. The exchanger is stable over a wide range of aqueous or organic solvent gradients.

An isocratic mode of elution was used to separate PABA and its metabolites. The simple buffer system consisted of a 0.1 *M* sodium formate solution, adjusted to pH 3.50 with 90% formic acid.

In our previous method[1], the pH of the buffer was a critical factor for obtaining excellent resolution. We found this not to be the case for this new method. Slight variation in pH and molarity did not noticeably influence the separation.

The flow-rate of the buffer system was 40 ml/h. Column pressures ranged between 350 and 400 p.s.i. Samples were introduced into the injection port at atmospheric conditions and in a stop-flow mode of operation. Two microlitres of serum and urine were analyzed without solvent extraction or pretreatment. The four metabolites were eluted within 12 min. Excellent resolution was obtained from both types of physiologic specimens.

The detection limit of the method was 1 ng on column. A low signal-to-noise ratio was observed for all absorbance ranges (0.005–0.08 A).

RESULTS AND DISCUSSION

The introduction of the microparticulate exchange columns into the field of HPLC has greatly improved the arts and sciences of chromatography. Aside from the vast number of compounds that now can be separated, the chromatographic performance has been elevated by one great quantum leap. We have been able to make a 100% improvement in the performance of separating PABA and its metabolites using this method as compared to our previous method[1]. The improvements in sensitivity, selectivity and resolution were all in evidence by this new procedure.

A series of standards, serum and urine samples were analyzed. Three of the chromatograms obtained from the study are displayed in Figs. 1–3.

The chromatogram depicted in Fig. 1 represents PABA and three of its metabolites detected at 0.04 A. The separation of these four standard compounds at various absorbance ranges (0.005–0.08 A) produced peak heights proportional to their concentrations. A standard calibration curve was made by plotting concentrations *versus* peak heights. The linearity of the curves was duplicated for each absorbance range used in this study.

In the previous study[1], the unknown peak No. 6 seen in Fig. 2 was not resolved when the 1-m AS-Pellionex SAX column was used for separation. The unknown peak has not yet been identified.

The chromatography of serum (Fig. 3) increased the resolution between peaks. This new improvement increases the probability of "finding" PAAHA in serum specimens, especially since our previous method[1] failed to detect this compound.

The method described in this paper offers a vast improvement over our previous procedure[1]. The increased simplicity of the method, the lower operating pressures and less sophisticated instrumentation make it all the more feasible as a routine test procedure for the clinical laboratory.

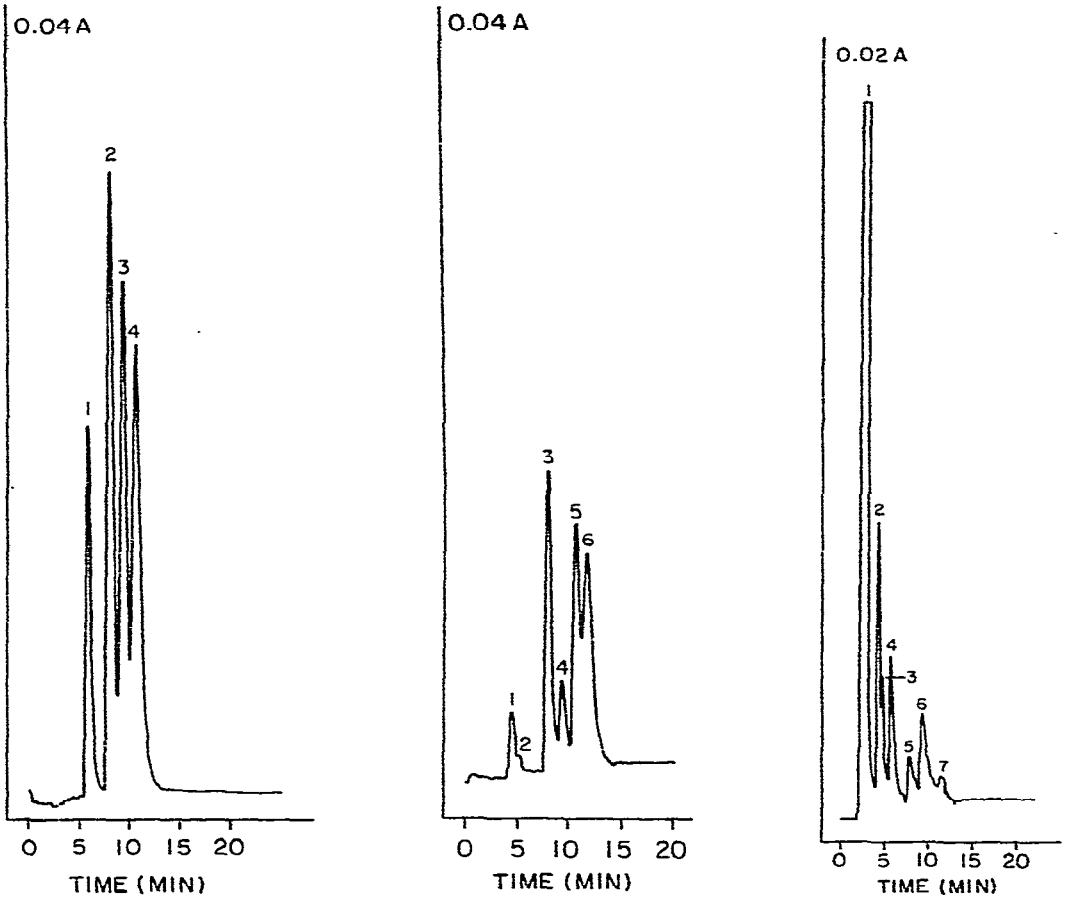

Fig. 1. Chromatogram of a standard solution of (1) PABA, (2) PAABA, (3) PAHA and (4) PAAHA. Mobile phase, 0.1 *M* sodium formate, pH 3.50; column temperature, 25°; flow-rate, 40 ml/h.

Fig. 2. Chromatogram showing the glycine conjugated and acetylated metabolites of PABA in urine, 180 minutes after administration. (1) unknown; (2) traces of PABA, (3) PAABA, (4) PAHA, (5) PAAHA, (6) unknown.

Fig. 3. Separation of (1) unknown, (2) unknown, (3) unknown, (4) PABA, (5) PAABA, (6) PAHA, and (7) PAAHA in a 2-μl serum sample, 45 min after oral administration of PABA.

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REFERENCES

1 N. D. Brown, R. T. Lofberg and T. P. Gibson, *J. Chromatogr.*, 99 (1974) 635.
2 E. Newman, A. Kattus, A. Genecia, J. Genest, E. Calkins and J. Murphy, *Bull. John Hopkins Hosp.*, 84 (1949) 135.