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Note

Quantitation of *p*-aminohippuric acid in biological fluids by high-performance liquid chromatography and dual-wavelength ultraviolet detection

PATRICK D. JENNY*, ALLAN WEBER and ARNOLD L. SMITH

Department of Pediatrics, University of Washington, Division of Infectious Disease, Children's Hospital and Medical Center, 4800 Sand Point Way N.E., Seattle, WA 98105 (U.S.A.)

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Determination of renal blood flow (RBF) is often useful in both research and clinical settings. Clearance of *p*-aminohippuric acid (PAH) has been widely used in determining effective renal plasma flow (ERPF) [1,2]. PAH is actively secreted in the proximal tubules as well as being filtered in the glomeruli. Extensive physiological and biochemical studies have been performed on PAH extraction and dynamics in the kidney [3–5] making it an ideal marker for RBF.

Determination of PAH concentration was first published by Smith et al. in 1946 [2] using a modified Bratton Marshall reaction, and this assay is still in use [3,4]. The assay is time-consuming and is unreliable in the presence of glucose, after the administration of sulfonamides, procainamide and certain local anesthetics [6]. An autoanalyzer method for quantitation, introduced in 1970 by Louye [7], is also unreliable in the presence of these interfering compounds. Radionuclides, especially [^{125}I]iodohippurate [4,5], an analogue of PAH with a similar renal clearance, has been used to simplify ERPF estimations. Disadvantages of using radionuclides are radiation exposure to the patient and medical care personnel to radiation, problems with handling, storage, and disposal of radioactive materials, and the change in specific activity over a short period of time.

Several high-performance liquid chromatographic (HPLC) methods for PAH

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quantitation have been developed over recent years. Two methods employed direct injection of biological samples but are associated with rapid deterioration of the column [8,9]. Another method circumvented column deterioration but required an extensive extraction procedure [10]. In a more recent publication, a simple and rapid methodology was developed; however, the assay limited the injection volume because of PAH peak splitting [11].

We have developed an assay which employs dual-wavelength monitoring to ensure selectivity and by addition of an ion-pair reagent, have eliminated peak splitting without limiting the injection volume. Peak splitting may be related to the balance of ionized/non-ionized forms of PAH as the addition of the ion-pair reagent resulted in single peak formation. The simple, rapid extraction of samples by the assay avoided complex extraction procedures and direct injection of the biological fluid.

EXPERIMENTAL

A 10 μm particles size μBondapak C_{18} reversed-phase column (10 cm$\times$8 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) was used in the separation with a mobile phase delivered by a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) at a flow-rate of 2.0 ml/min. The 20-μl sample was injected by a Waters Intelligent Sample Processor (WISP Model 710B, Waters Assoc.). The mobile phase was monitored by a variable-wavelength UV detector set at 275 nm and 0.02 a.u.f.s. (absorbance units full scale) (Spectro Monitor III, Laboratory Data Control) and a fixed-wavelength UV monitor set at 254 nm and 0.008 a.u.f.s. (UV III Monitor, Laboratory Data Control). Signals from the above detectors were recorded by a dual-pen strip chart recorder (Omniscribe, Houston Instrument, Houston, TX, U.S.A.) at 0.5 cm/min on the 10-mV scale.

The mobile phase was a 67 mmol/l phosphate buffer (KH_2PO_4–Na_2HPO_4, 97.5:2.5, w/v) pH 2.8 in type I water with tetrabutylammonium phosphate added as an ion-pair reagent (low-UV Pic A reagent, Waters Assoc.) to a final concentration of 5 mmol/l. The mobile phase was filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo daily.

PAH (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in water to yield concentrations of 40, 20, and 5 μg/ml; these were stored at -70°C and used as assay standards.

p-Aminobenzoic acid (PABA) (Sigma, St. Louis, MO, U.S.A.) was dissolved in acetonitrile (HPLC grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) to give a 20 μg/ml solution and stored at 4°C. This compound was used as an internal standard.

Samples were prepared by mixing 50 μl of standard or patient's specimen and 50 μl of internal standard. The mixture was vortexed for 15 s and centrifuged at 10 000 *g* for 5 min at room temperature (Eppendorf centrifuge). A 20-

μl volume of the supernatant was injected into the column. Ratio of peak heights of PAH to PABA from the 275-nm UV detector was measured. Concentrations were calculated from a curve derived by a least-squares linear regression of peak-height ratios (PAH/PABA) versus concentration of standards. Specificity was checked by measuring the 275/254 ratio of the PAH peaks.

Linearity

An aqueous stock solution of PAH (100 μg/ml) was serially diluted with water to 100, 60, 40, 20, 5, and 2.5 μg/ml. These were assayed and the peak-height ratios to the internal standard calculated. The resulting curve (peak-height ratio versus concentration) was analyzed by least-squares regression.

Precision

Stock solutions were diluted with pooled normal, drug-free human serum to 20 and 2 μg/ml and stored at −70°C. Eight aliquots of each of the concentrations were assayed in one run to calculate intra-assay precision. One aliquot of the high and low concentration was assayed with each run to calculate inter-assay precision.

Stability

An aqueous solution of PAH (100 μg/ml) was diluted to 15 μg/ml in pooled normal, drug-free human serum and aliquots were stored at 22, 4, −20, and −70°C. The samples were assayed on 24 h, 48 h, 1 week and 4 weeks.

Accuracy

Eight samples with known amounts of PAH were prepared in pooled normal, drug-free human serum and assayed in an encoded fashion (the samples were prepared by a person not performing the assay and they were labelled with no indication of concentration).

Sensitivity

Standards were serially diluted with water until a concentration was reached in which the peak height of PAH was twice background noise of the chromatogram.

Specificity

Peak heights from 275 nm were routinely used for calculation of concentration. The ratio of the 275 nm peak height to the 254 nm peak height was calculated and found to be constant. A value less than or greater than 2 standard deviations from this value indicated an interfering substance.

RESULTS

Retention times of PAH and PABA were 7.4 and 13.0 min respectively. Chromatograms of representative samples are shown in Fig. 1. Initially a standard curve was calculated using seven concentrations, including a zero point. The square of the correlation coefficient was 0.9993. The y-intercept of the regression line was -0.6492 and the slope was 45.0624. Due to this high correlation only three standards and a zero point were used in daily calculations, 40, 20, and 5 μg/ml. The sensitivity of the assay for PAH was 1.0 μg/ml with a 20-μl injection and a signal-to-noise ratio of 2.5 at 275 nm and 2.0 at 254 nm.

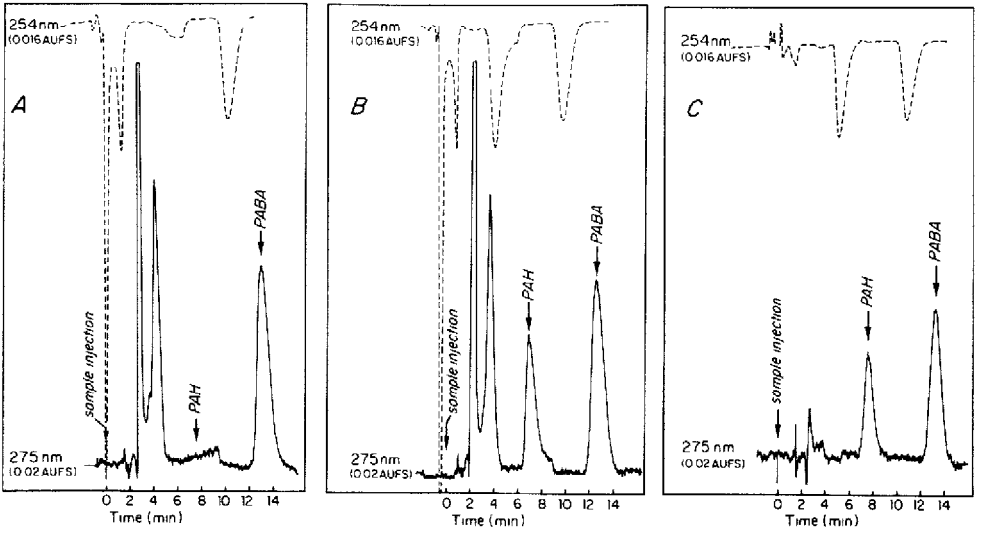

Fig. 1. Chromatogram of (A) a drug-free serum sample, (B) a serum sample with a calculated PAH concentration of 29.15 μg/ml, and (C) an aqueous PAH standard (40 μg/ml). In all chromatograms, the concentration of internal standard (PABA) was 20 μg/ml. AUFS = absorbance units full scale.

TABLE I

PRECISION DATA

Assay		Concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-assay ($n=8$)	High	20.7 ± 0.71	3.4
	Low	2.1 ± 0.17	8.3
Inter-assay ($n=14$)	High	20.9 ± 0.79	3.8
	Low	2.2 ± 0.21	9.8

TABLE II

ACCURACY DATA

Actual concentration (μg/ml)	Assayed concentration (μg/ml)	Recovery (%)
3 0	2.7	90.0
3.0	2.6	86.9
6 0	5.4	90.6
6.0	5.5	91.5
6.0	5.5	91.5
6.0	5.5	91.7
8.0	7.4	92.9
8.0	7.8	96.9
8.0	7.4	93.1
8.0	7.6	95.0
13.0	12.0	92.3
25 0	24.1	96.5
25.0	24 1	96.5
30 0	30.7	102.3
30.0	30 8	102.8
37.50	36.1	96.3
37.50	36 5	97.4
50.0	52.7	105.4
50.0	55 0	110.1
		Mean 95.7
		S.D. 5.9
		Range 86.9-110.1

The results of assay precision are as follows. The coefficient of variation was 3.4 and 8.3% for intra-assay run precision for the high and low controls, respectively. The inter-assay precision (coefficient of variation) was 3.83 and 9.82% for the high and low controls, respectively. Data are shown in Table I. PAH in serum was stable up to one month at temperatures between −70 and 4°C. The accuracy data are shown in Table II. The mean recovery is 95.7%, with a range of 86.9–110.1%.

DISCUSSION

A selective and sensitive assay of PAH is useful in the determination of RBF and ERPF. The method described here does not limit the injection volume and sensitivity may be increased by increasing injection volume. The small sample volume required for routine assays (20 μl) allows multiple assays from a small sample, an important consideration in pediatric populations. The column used in this assay had a life of approximately 300 injections. No interfering substances have been found to co-elute with PAH by dual-wavelength monitoring

when clinical specimens were assayed, and it has been reported that there is no detectable PABA in urine or serum due to the injection of PAH [8].

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