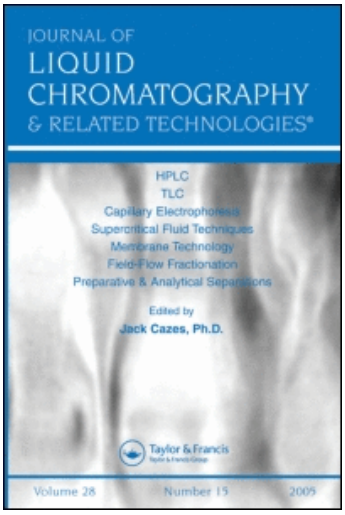

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QUANTITATION OF P-AMINOHIPPURIC ACID AND N-ACETYL-P-AMINOHIPPURIC ACID FROM BLOOD BY HPLC

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ABSTRACT

P-aminohippuric acid, N-acetyl-p-aminohippuric acid, and p-aminobenzoic acid can be separated by a reverse-phase, isocratic high-pressure liquid chromatographic procedure in less than 4 minutes. The eluent is 10 mM sodium phosphate buffer, pH 3.5, containing 30% methanol. Detection is by absorbance at 270 nm; quantitation is accomplished by peak height. Linear response was obtained from 3 to 1600 nmoles of each compound in aqueous standards and in blood deproteinized with perchloric acid.

INTRODUCTION

Interest in p-aminohippuric acid (PAH) for assessment of effective renal filtration of plasma or blood, for determination of blood flow, and for assessment of renal damage has led to a variety of methods for quantitating PAH from biological fluids. Bratton and Marshall (1) used N-(1-naphthyl)-ethylenediamine dihydrochloride to react with diazotizable aryl amines such as PAH to form azo dyes, which then were quantitated colorimetrically

Their method subsequently was modified (2). Brun (3) suggested a colorimetric method utilizing p-dimethylaminobenzaldehyde that forms a Schiff base when reacted with primary aromatic amines. Waugh and Beall (4) deproteinized and acidified samples of plasma in one step and modified the procedure of Brun. All these methods lack reagent stability and specificity and require careful timing between addition of reagents (5).

N-acetyl-p-aminohippuric acid (aPAH), the major metabolite of PAH in several species (6, 7, 8), can be detected by colorimetric procedures only after removing the N-acetyl group by acid hydrolysis before analysis for PAH. Hydrolysis of N-acetyl metabolites to primary aromatic amines is not always specific, however, in producing the desired compound (9). Therefore, secondary decomposition products must be considered when colorimetric, chemical, or separation techniques are employed after hydrolysis.

High-pressure liquid chromatography (HPLC) has been used to quantitate PAH by UV-absorbance (9, 10, 11) or by electrochemical detection (5). HPLC methods offer advantages because aPAH can be detected in samples, along with PAH, without prior hydrolysis, which removes a major potential source of error in blood-flow determinations where PAH is used as the marker. However, previous HPLC methods have poor resolution, require expensive electrochemical detectors, require elution times of at least 10 min., or use 254 nm absorbance for detection. Possible interference from other blood constituents could occur at 254 nm, and PAH, aPAH, and p-aminobenzoic acid (PAB) have greater absorbance at 270 nm (present report).

We developed a rapid, sensitive method of PAH and aPAH determination so that they could be used to accurately determine blood flow in animals to measure fluxes of metabolites in and out of organs. With PAB as an internal standard, the method

quantitates PAH and aPAH in less than 4 min. by using a reverse-phase column, isocratic mobile phase, and UV-absorbance detection.

MATERIALS AND METHODS

Analyses were performed on a Waters Associates HPLC system equipped with a model 6000A pump, a U6K continuous-flow injector, and an RCM-100 radial compression module containing a Microbondapak C^{18} Radial Pak cartridge. The mobile phase of 30% methanol (V/V) in 10 mM NaH_2PO_4 buffer was mixed and the pH adjusted to 3.5. Degassing and particle removal was by vacuum filtration through a 0.45-μM Millipore filter. Eluent flow of 2.5 ml/min. produced pressure of 800 to 1000 psi. Detection was at 270 nm by a Perkin-Elmer model 55 variable-wavelength spectrophotometer. Recordings were with a Houston Instruments Omniscribe recorder set at 0.1, 1.0, or 10.0 mV, providing 0.003, 0.03, or 0.3 absorbance units full scale.

Lyophilized PAH from Sigma Chemical Co. was neutralized with NaOH so that standard solutions had a pH of approximately 7.0. aPAH was prepared from PAH (12), and purity was monitored by HPLC and by a melting point between 198 and 200° C. PAB was obtained from J. T. Baker Chemical Co. Aqueous standards of PAH, aPAH, and PAB were prepared, adjusted to pH 7.0 with NaOH, and injected as 25-μl aliquots. Peak height was used for quantitation.

Whole-blood and plasma samples were prepared by mixing 2.5 ml of 1.6 μmoles/ml PAB internal standard with 5 ml of blood or plasma. To this mixture, 5 ml of 1.0 M perchloric acid was added and placed in ice for 10 min. After centrifugation to precipitate protein, the supernatant was filtered through tissue paper to another tube, and 1.25 ml of 2.0 M KOH was added to precipitate unreacted perchlorate. After centrifugation to remove potassium perchlorate, the supernatant was transferred to a vial that was capped and stored at 4° C. Unpublished experiments have shown that

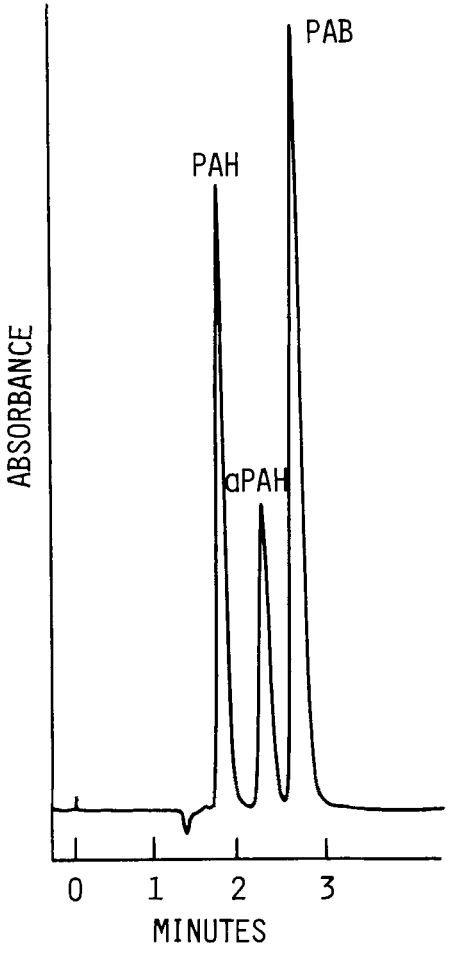

Figure 1. Chromatogram of a 20 µg/ml standard of PAH, aPAH, and PAB. Chromatographic conditions: Microbondapak C_{18} Radial PAK cartridge (10 cm x 0.8 cm i.d.); mobile phase of 30% methanol in 0.01 M NaH_2PO_4, pH 3.5 with a flow rate of 2.5 ml/min.; injection volume of 25 µl; detection at 270 nm.

the compounds in these basic supernatants are stable at 4° C for 1 month and longer. Within 24 hours, 25-µl aliquots were injected onto the HPLC column, and PAH and aPAH were quantitated by using PAB as an internal standard. Perchloric acid as a deproteinizing

agent gave quantitative recovery and baseline resolution of PAH, aPAH, and PAB. Sulfosalicyclic acid, $BaOH/ZnSO_4$, and trichloroacetic acid did not.

RESULTS AND DISCUSSION

A chromatogram of aqueous standards of PAH, aPAH, and PAB is shown in figure 1. Baseline resolution was achieved for all three compounds from 3 to 1600 nmoles. Linearity of response was maintained when the recorder was at 0.1, 1 or 10 mV full scale, which provides the detection range necessary for quantitation of PAH, and aPAH in blood-flow experiments. Figure 2 depicts the

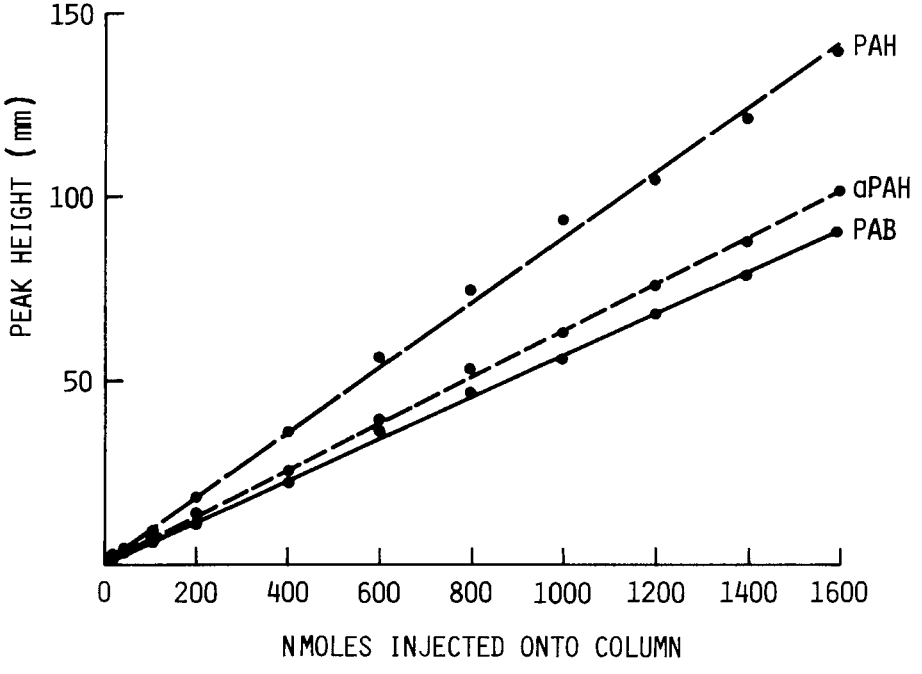

Figure 2. Standard curve of PAH, aPAH and PAB. Separate solutions of each compound were prepared in various concentrations and injected as 25 µl aliquots. The lines represent least-square estimates of the linear equations: PAH, Y=0.087X + 2.02; aPAH, Y=0.063X + 1.07; and PAB, y=0.56x + 1.0 (X=nmoles injected, Y=mm peak height).

TABLE 1.

Peak Height Ratios of PAH:PAB and aPAH:PAB from Standards

Amount Injected (nmoles)	PAH:PAB Ratios*	aPAH:PAB Ratio*
20	1.45 (0.06)	0.98 (0.03)
40	1.46 (0.02)	1.00 (0.04)
100	1.61 (0.01)	1.12 (0.02)
200	1.53 (0.01)	1.21 (0.01)
400	1.58 (0.01)	1.12 (0.01)
600	1.57 (0.01)	1.11 (0.01)
800	1.58 (0.004)	1.12 (0.006)
1200	1.54 (0.002)	1.12 (0.002)
1400	1.54 (0.003)	1.12 (0.003)
1600	1.55 (0.004)	1.13 (0.004)
$\bar{x}$ =	1.54 (0.05)	1.10 (0.07)

*± Standard Error

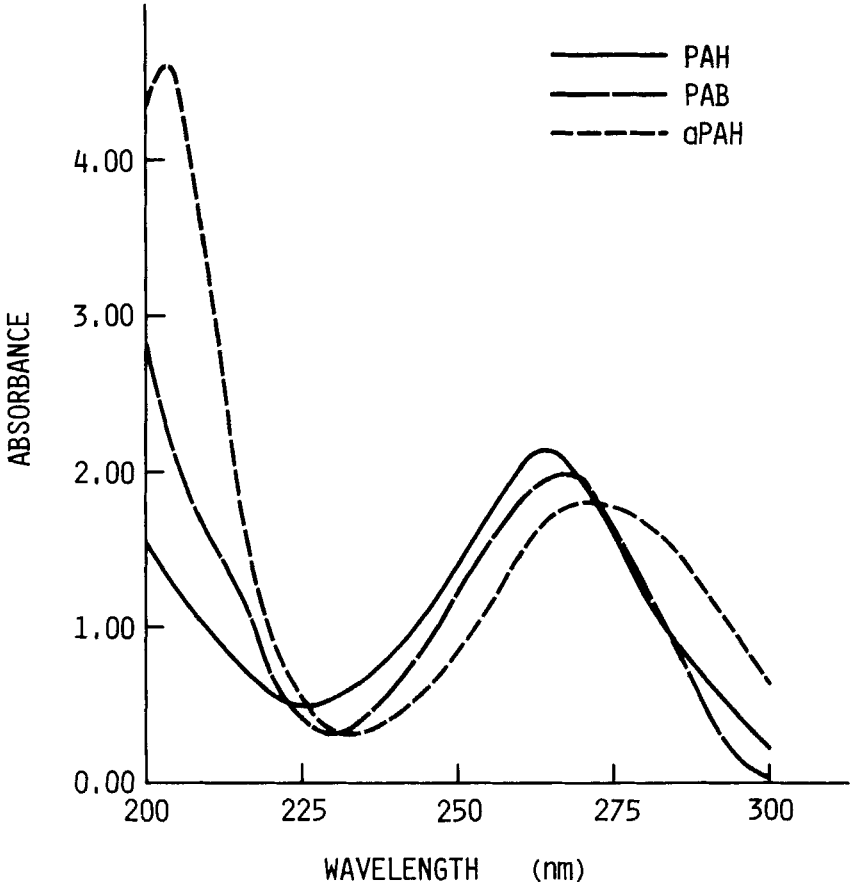

Figure 3. Absorption spectra of equal-molar concentration solutions of PAH, aPAH, and PAB. The pH of the solutions was 3.5.

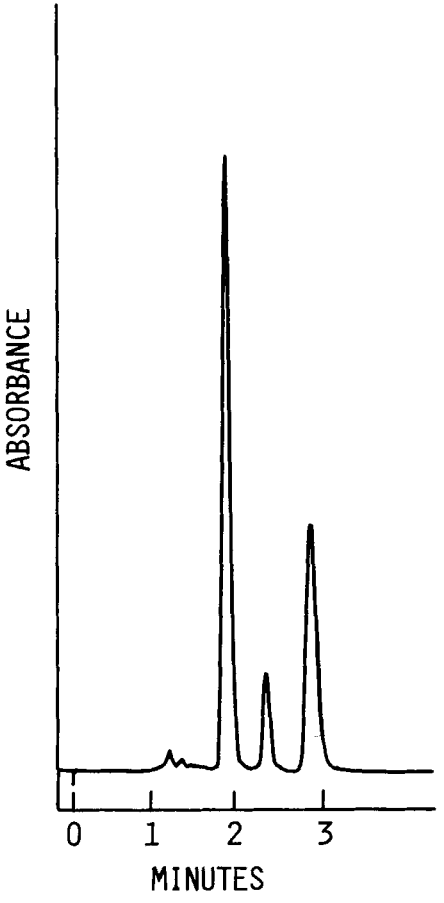

Figure 4. Chromatogram of a perchloric acid filtrate of whole blood. The steer was injected with 7 mg/kg of PAH and aPAH 15 minutes prior to the blood sampling.

linearity of detector response over an extended standard curve. For blood-flow experiments, the 10-mV recorder setting can give adequate sensitivity to quantitate the concentrations found in blood. ^{14}C-PAH and ^{14}C-aPAH confirmed 98 to 101% recovery in the two respective peaks.

Peak-height ratios for PAH:PAB and aPAH:PAB were essentially constant over a wide range of concentrations. Table 1 depicts

this constancy over a 500-fold concentration range. The constant ratios allow use of PAB as an internal standard for PAH and aPAH quantitation and negate the need for quantitative transfers of supernatants and filtrates during sample preparation.

Use of 270 nm maximizes aPAH detection and offers advantages over 254 nm used previously (9, 10). Interference from other compounds is minimized, and absorbance of PAH, aPAH, and PAB is greater (figure 3).

Figure 4 shows a chromatogram of PAH, aPAH, and PAB from a perchloric acid filtrate of whole blood taken from a steer injected with 7 mg/kg of body weight of PAH and aPAH 15 minutes preceeding blood sampling. There is consistent peak resolution and no interference from other blood constituents.

The adaptability of our HPLC procedure provides an attractive method for quantitation of PAH, aPAH, and PAB in wide concentration ranges in whole blood. Its use for determination of PAH in blood-flow experiments removes questionable pre-analysis sample treatment and thereby is more accurate and dependable than previously described methods.

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