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Short communication

Rapid determination of *p*-aminohippuric acid in serum and urine by high-performance liquid chromatography

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Abstract

We report a simple and rapid HPLC procedure for measuring *p*-aminohippuric acid in serum and urine. After deproteinization with acetonitrile and addition of *p*-aminobenzoic acid as an internal standard, the chromatographic run is performed on a C_{18} column with the absorbance detector set at 275 nm. The separation is carried out in 10 min at a flow-rate of 1.0 ml/min with a mobile phase composed of 7 mmol/l 1-decanesulfonic acid, pH 3.70, and acetonitrile (82:18, v/v). The relationship between *p*-aminohippuric acid concentration and the *p*-aminohippuric acid/internal standard peak area is linear up to 100 μg/ml. Within-run precision measured at three different *p*-aminohippuric acid concentrations ranges from 1.73 to 1.98% in serum and from 0.72 to 1.32% in urine. Between-run precision varies from 1.80 to 4.06% in serum and from 1.05 to 2.66% in urine. Analytical recovery is between 98.26 and 99.44% in serum and from 99.57 to 100.45% in urine. The method is very simple, sensitive, and requires small volumes of sample for the assay (100 μl). Therefore, it could be a useful tool for the analysis of *p*-aminohippuric acid in the evaluation of renal plasma flow.

Keywords: *p*-Aminohippuric acid

1. Introduction

Renal blood flow (RBF), comprising 20–30% of cardiac output, is maintained in a narrow range under physiological conditions. However, in the presence of cardiac failure, reduced circulating volumes or after administration of drugs acting on renal vessels (i.e. antihypertensive drugs, indomethacin and cyclosporin) the homeostatic mechanisms may be impaired and the RBF reduced. The most commonly used method to determine variations of RBF and effective renal plasma flow (ERPF) is based on the clearance of *p*-aminohippuric acid (PAH). This is an exogenous non-toxic molecule, neither bound by proteins in plasma, nor permeable by erythrocyte membranes, that is almost completely extracted from the blood after single passage by the kidney.

The analytical reliability of colorimetric assays for measuring PAH using a Bratton and Marshall reaction [1–3] is often unsatisfactory due to interference from glucose and exogenous compounds such as sulfonamides, procainamides and local anesthetics [4]. Several HPLC procedures have been proposed in the last years in an attempt to improve analytical reliability and to overcome interferences. Some of them are performed without any extraction of the sample; however, a rapid deterioration of the column is reported after a small number of analyses [5,6].

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Other methods require a long time to perform the chromatographic separation [7] or give an inadequate resolution of the recorded peaks [8]. Several methods employ organic solvents for deproteinization or sample extraction. Unfortunately, most of them are characterized by a short life of the analytical column [9], poor precision [10], extensive sample preparation [11] or limitation of the volume of injection without *p*-aminohippuric acid peak splitting [12].

We report here an ion-pair HPLC procedure for the measurement of PAH which has the advantages of being rapid, precise and accurate both in serum and urine.

2. Experimental

2.1. Chemicals

1-Decanesulfonic acid (sodium salt), *p*-aminohippuric acid and *p*-aminobenzoic acid were obtained from Sigma (St. Louis, MO, USA); orthophosphoric acid, (89%) and acetonitrile HPLC grade were purchased from Merck (Darmstadt, Germany) and Carlo Erba (Milan, Italy), respectively. Water was obtained from a Milli-Q Waters purification system (Millipore, Milford, MA, USA). Micro-Spin Centrifuge Filters Nylon 66 were obtained from Alltech Assoc. (Deerfield, IL, USA).

2.2. Samples

Blood and urine samples were obtained from children and adults with normal renal function and with different nephropathies who underwent an evaluation of ERPF using the clearance of PAH. Serum and urine were also obtained from renal transplant patients ($n=23$).

2.3. Standard solutions

Stock standard solutions of both PAH and *p*-aminobenzoic (PABA) were prepared in acetonitrile to yield a final concentration of 1 mg/ml. A series of dilutions of PAH (5, 10, 25, 50 and 100 μg/ml) were prepared in water. Working standard solution of PABA was prepared daily at the concentration of 12.5 μg/ml in acetonitrile.

2.4. HPLC apparatus

The liquid chromatograph consisted of a Model 510 pump (Waters Assoc., Milford, MA, USA), a Model 430 detector, a Model 465 autosampler and a Model 450-MT chromatography data system, all from Kontron Instruments (Milan, Italy).

2.5. Samples preparation for HPLC

One hundred microliters of serum or 20-fold diluted urine were added to 200 μl acetonitrile containing 12.5 μg/ml of PABA, vortex-mixed, and centrifuged at 3000 *g* for 5 min in a Biofuge A (Heraeus) to remove precipitated proteins. One hundred microliters of supernatant were filtered with a 0.2-μm Micro-Spin centrifuge Nylon 66 filter, and 5-μl aliquots were analyzed directly by HPLC.

2.6. HPLC analysis

Chromatography was performed using a Ultracarb ODS 30 column (150×4.6 mm ID), containing 5-μm particles (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 7 mmol/l 1-decanesulfonic acid adjusted to pH 3.70±0.05 with orthophosphoric acid (89%) and acetonitrile (82:18, v/v). The chromatographic separation was carried out at room temperature in 10 min at the flow-rate of 1.0 ml/min. The eluent was filtered through a 0.45-μm Millipore filter and degassed with helium before use. The absorbance of the effluent was monitored at 275 nm with detector sensitivity set at 0.01 a.u.f.s.

3. Results

The chromatographic profiles of serum (b) and urine (c) samples prior to and after PAH addition are compared in Fig. 1 with a chromatogram of an aqueous standard solution (a). Both PAH and PABA peaks are well resolved with a retention time of 1.91±0.11 ($n=30$) and 3.78±0.15 min ($n=30$), respectively. The chromatographic profiles did not show any interfering substance at the retention time corresponding to the recorded peaks in serum (mean PAH concentration 13.5 μg/ml, range 4.4–31.8) and urine (mean PAH concentration 334 μg/ml, range 96–1234).

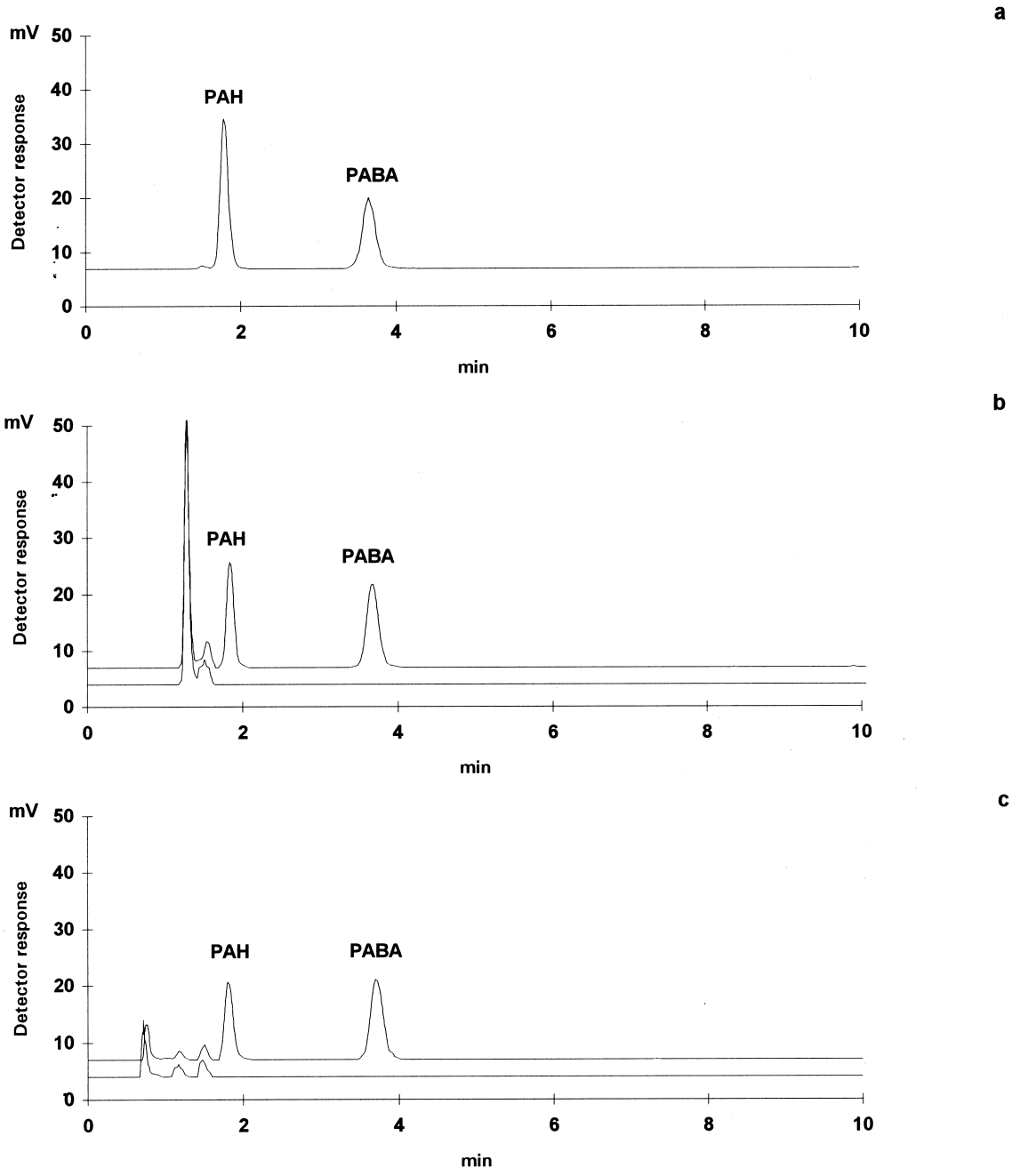

Fig. 1. Representative chromatograms of (a) a PAH (50 μg/ml) standard solution compared with overlapped chromatograms of (b) serum and (c) urine samples collected before and after PAH administration.

3.1. Calibration curves and linearity

Standard curves show a linear relationship between the ratio of PAH/I.S. peak areas and PAH over a wide range of concentrations (5, 10, 25, 50, 100 μg/ml) both in serum and urine. Each point was established from an average of five determinations. The regression equation of curve for plasma samples

Table 1
Precision of the method in serum and urine

Serum (n=10)		Urine (n=10)	
Mean±S.D. (μg/ml)	C.V. (%)	Mean±S.D. (μg/ml)	C.V. (%)
Within-run			
7.58±0.15	1.98	9.89±0.16	1.32
16.23±0.28	1.73	21.37±0.24	1.14
31.90±0.55	1.73	40.33±0.29	0.72
Between-run			
7.57±0.31	4.06	9.81±0.18	2.66
16.21±0.29	1.80	21.32±0.45	2.09
31.70±0.66	2.09	40.08±0.42	1.05

was $y=0.01527+0.07924x$, where y is the peak area and x is the *p*-aminohippuric acid concentration. The standard error of the slope was 0.02029 and the correlation coefficient (r) was 0.9999. The regression equation of the curve for urine samples was $y=0.064849+0.07807x$. The standard error of the slope was 0.01159 and the correlation coefficient (r) was 0.9999. The lowest concentration of PAH detected at a signal-to-noise ratio of 3 was 0.18 μg/ml.

3.2. Precision

The within-day precision was determined by running three serum and urine samples with different PAH content 10 times each. Between-day precision of the method was obtained from 10 runs performed on 10 different days (Table 1).

3.3. Analytical recovery

The recoveries were calculated by comparing the measured values of supplemented samples of each medium with those of aqueous calibration solutions. Analytical recovery ranged from 98.26 to 99.44% in serum and from 99.57 to 100.45% in urine, respectively (Table 2).

3.4. Interferences

To examine potential interferences, the drugs commonly taken by patients with renal diseases and listed in Table 3 were added to serum and urine samples. No interferences with either *p*-aminohippuric acid or internal standard peaks were observed in the chromatograms.

4. Discussion

A reliable procedure for measuring *p*-aminohippurate is essential for performing a clearance of PAH which represents a simple and inexpensive method for estimating ERPF in the clinical practice.

We present here an HPLC assay which has the advantages of being sensitive and economical. The use of acetonitrile for protein precipitation, with a 1:2 volume ratio of sample–organic solvent, as previously described [12], allows a rapid and satisfactory deproteinization and the supernatant may be injected without interfering substances with both PAH and internal standard peaks. The sample preparation step comprising deproteinization and filtration is very simple. It requires only 15 min, and a plate of 24 samples can be prepared simultaneously.

In the preliminary phase of this work, we adopted also the chromatographic procedure proposed by Prueksaritanont et al. [12]. However, the unsatisfactory resolution of *p*-aminohippuric peak found in several plasma samples of renal transplant patients required a modification of the mobile phase. The best performances were obtained using a mobile phase containing 7 mmol/l 1-decanesulfonic acid–acetonitrile (82:18, v/v), pH 3.70±0.05. The addition of an ion-pairing agent with long chain (C_{10}) and the choice of a shorter column allows a better separation

Table 2
Analytical recovery of PAH in serum and urine

PAH added (μg/ml)	Serum (n=10)		PAH added (μg/ml)	Urine (n=10)	
	Found (Mean±S.D.)	Recovery (%)		Found (Mean±S.D.)	Recovery (%)
5	4.91±0.16	98.26	10	9.96±0.18	99.57
10	9.88±0.24	98.79	25	25.11±0.31	100.45
25	24.90±0.57	99.44	50	50.20±0.31	100.40

Table 3
Compounds tested for interference

Compound	Concentration (mg/l)	Retention time (min)
Acyclovir	10 000	7.21
Acetylsalicylic acid	3600	7.42
Ampicillin	10 000	6.48
Azathioprine	500	3.11
Methylprednisolone	260	3.21
Joscine butylbromide	4000	n.d.
Cephazolin	1000	1.32
Cephtazidim	4000	5.56
Chlormethyldiazepam	2000	n.d.
Cyclosporin	5000	8.97
Diazepam	10 000	7.44
Digoxin	500	1.04
Furosemide	5000	3.15
Gentamicin	10 000	n.d.
Ketoprophen	5000	1.05
Labetalol	5000	9.98
Methamizol	10 000	1.07
Methochlopramide	5000	n.d.
Ranitidine	5000	n.d.
Theicoplanine	13 000	n.d.

n.d., not detected.

of *p*-aminohippuric acid and internal standard while keeping a short analysis time.

The small volume (5 μl) injected allows analysis of more than 50 plasma and urine samples, consecutively, without any variation of column efficiency. Column clean-up with water for at least 1 h and successive reequilibration with mobile phase for the same time is required every 100–120 injections. In addition, the mobile phase used, containing 18% of acetonitrile, avoids retention of other residual serum and urine components which may damage or reduce the performance of the column; we carried out more than 2000 injections without any variation of retention time or shape of the recorded peaks. Moreover, overnight clean-up with water is necessary to preserve the column from salt precipitation.

It is noteworthy that the proposed method does not require a control of the temperature since its variation does not change the performances of the column. Precision and accuracy are increased by the use of an internal standard even though the analytical recovery is satisfactory ranging from 98.26 to 100.45%.

The small volumes of serum required for the assay (100 μl) are also very important when RBF and GFR have to be monitored together particularly in infants and anemic patients or in small animals for experimental purposes. The time needed to carry out the procedure is very short (10 min for each injection), particularly when an autosampler is available. The specificity of method was evaluated by analyzing plasma and urine samples after supplementing with the compounds frequently administered to patients with renal failure. None of the compounds listed in Table 3 interfered with the *p*-aminohippuric acid and the I.S. peaks. The absence of interferences was also assessed by the analysis of plasma and urine samples of all patients before PAH infusion.

In conclusion, this technique is sensitive, easy to automate and could represent a useful tool for measuring PAH in biological fluids.

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