Rapid microplate method for PAH estimation

RAJIV AGARWAL
(With the Technical Assistance of Shawn D. Chase)
Division of Nephrology, Department of Medicine, Indiana University and Richard L. Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana 46202

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Agarwal, Rajiv, and Shawn D. Chase. Rapid microplate method for PAH estimation. Am J Physiol Renal Physiol 283: F236–F241, 2002.—Evaluation of renal hemodynamics requires estimation of effective renal plasma flow, which is commonly measured by the renal clearance of p-aminohippuric acid (PAH). There are many existing methods for PAH assay that are complicated, expensive, or time consuming. We describe a rapid, precise, and accurate microplate-based assay of PAH using p-dimethylaminocinnamaldehyde, which produces a red color on reaction with PAH, and compare it with a reference HPLC method. Renal PAH clearances were measured in 10 volunteers, and clearances were calculated by using the new and HPLC methods. There was excellent agreement between the HPLC and the microplate method of PAH assay. The average ratio of microplate to HPLC method was nearly 1.0, and the limits of agreement were calculated by using the new and HPLC methods. There was excellent agreement between the HPLC and the microplate method of PAH assay. The average ratio of microplate to HPLC method was nearly 1.0, and the limits of agreement (2 SD) for plasma, urine, and clearances were 17.2, 19.3, and 25.5%, respectively. Intraday coefficient of variation for urine and plasma was <7%; interday coefficient of variation was <6% for urine and plasma samples. The microplate method is a reliable alternative to a reference HPLC method and can be performed for a fraction of the cost, time, and reagents.

high-performance liquid chromatography; colorimetry; renal plasma flow; p-aminohippuric acid

METHODS

Reagents. p-Dimethylaminocinnamaldehyde (DACA) was purchased from Sigma (St. Louis, MO). PAH was purchased from Merck (West Point, PA). HPLC-grade ethanol and trichloroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). A 1% solution of DACA was made in ethanol (stable if stored at 4°C for several weeks), and a 15% solution of trichloroacetic acid was made in distilled deionized water (DDW).

Methods described by Newman et al. (14) were used to prepare N-acetyl-PAH for interference studies. In brief, 10.4 M acetic anhydride was added to a 10% solution of PAH in a molar ratio of 2:1. The mixture was gently rotated for 1 h at room temperature. A thick white amorphous mass formed; this was vacuum filtered with a 0.22-μm filter (Millipore, Bedford, MA), and the paste was dried in a light-protected desiccating chamber overnight. Other drugs used for the interference studies were obtained from the hospital pharmacy.

Standards and quality controls. A stock solution of PAH was prepared as 10 mg/ml PAH in DDW. Standard curve and quality control (QC) samples were prepared, aliquoted, and stored at −85°C until analysis. These standards were then used for all experiments.

Eight standards were made for plasma and urine. Drug-free blank urine was diluted 1:10 with DDW and used in all standards. Dilutions of the stock PAH solution were made to yield concentrations of 1,000, 800, 600, 500, 400, 300, 200, and 100 μg/ml PAH in diluted urine. The plasma standard curve was prepared with expired fresh-frozen plasma obtained from the blood bank. PAH stock solution was diluted with blank donor plasma to 30 μg/ml. This was added in varying volumes to blank plasma to yield concentrations of 30, 24, 18, 15, 12, 9, 6, and 3 μg/ml PAH.

Nominal concentrations of 21 and 9 μg/ml in serum and 700 and 300 μg/ml in urine were used to assess low and high QCs, respectively.

Fifty samples from study patients that contained unknown amounts of PAH were analyzed in duplicate on the same day to assess the intraday coefficient of variation (CV). Plasma QC samples were analyzed on 13 separate days and urine QCs on 14 separate days to assess interday CV.

Sample preparation. Urine from research subjects and standards were diluted 1:10 with DDW. One hundred microliters of diluted urine were transferred to microcentrifuge tubes. To this were added 100 μl of 15% trichloroacetic acid to precipitate proteins. The tubes were vortexed briefly, then centrifuged at 14,000 g for 4 min. The clear supernatant (50
was transferred to a 96-well ELISA plate (Echostar, Corning, NY), to which were added 150 μl of ethanolic 1% DACA solution. The plate was incubated for 20 min at room temperature and read at 550 nm with a Spectramax 190 plate reader with the PathCheck feature turned on (Molecular Devices, Sunnyvale, CA). Turning on the PathCheck feature yields absorbances equivalent to 1-cm path length despite considerably shorter path lengths. Plasma samples were prepared identically, except that samples were not diluted and were centrifuged for 8 min. All analyses were performed in duplicate.

Calculations and statistical methods. Standard curves were created by linear regression of optical density of PAH vs. nominal concentrations of PAH. Concentrations of QC and unknown samples were estimated by applying the standard curve linear regression equation to the sample optical density. Recovery was determined by calculating the mean difference between expected and observed concentrations of QCs expressed as a percentage of expected concentration as well as its 95% confidence intervals (CIs). Precision of the assay was assessed over two concentrations, 9 and 21 μg/ml in plasma and 300 and 700 μg/ml in urine. Interday and intraday CV were calculated by one-way ANOVA with the method described by Chinn (8). The lower limit of detection was calculated as described by Anderson (2). Commonly used drugs that may interfere with the colorimetric assay were tested. These included acetyl-p-aminophenol (acetaminophen), p-aminobenzoic acid, sulfadiazine, sulfanilamide, and sulfaacetamide. In addition, because PAH is metabolized to N-acetyl-PAH in the kidney, interference was also tested. Finally, to determine the limits of agreement, bias, and precision, a Bland-Altman analysis (4) was performed by using a previously established HPLC method as a reference standard (1).

PAH clearance studies in volunteers. The study was approved by the Institutional Review Board for Human Studies of Indiana University. Written informed consent was obtained from each volunteer. A water load of 10 ml/kg body wt was given orally, and 5 ml/kg water was given every hour to maintain urine flow. A loading dose of 10 mg/kg of 20% PAH was administered intravenously. This was followed by infusion of a solution of PAH in normal saline at a rate calculated to give a serum PAH concentration between 10 and 20 μg/ml. Infusion rate was 1 ml/min to match insensible losses. Urine and blood were sampled every 30 min for four consecutive periods after 1 h of PAH infusion. Each urine collection period was bracketed by serum sample collection. Each volunteer was studied on 2 consecutive days. The clearances of PAH were calculated by the traditional UV/P method whereby U is the urinary concentration of PAH, P is the geometric mean of the bracketing PAH plasma concentrations, and V is the urine flow rate. All samples were stored at −85°C until analyzed.

RESULTS

Analyzing PAH over a range of clinically relevant concentrations in urine and plasma demonstrated a time-dependent development and deepening of a red color. However, on testing a range of concentrations of DACA between 1 and 10% in ethanol, we found that

### Table 1. Interday assay characteristics for microplate method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nominal Concentration, μg/ml</th>
<th>Obtained Concentration, μg/ml (95% CI)</th>
<th>Recovery, % (CI)</th>
<th>Coefficient of Variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-quality control</td>
<td>21</td>
<td>20 (19.6, 20.5)</td>
<td>95.4% (93.3, 97.4%)</td>
<td>4.03%</td>
</tr>
<tr>
<td>Low-quality control</td>
<td>9</td>
<td>8.6 (8.4, 8.9)</td>
<td>95.9% (93.2, 98.7%)</td>
<td>5.26%</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-quality control</td>
<td>700</td>
<td>695.5 (677, 714)</td>
<td>99.4% (96.7, 102%)</td>
<td>5.10%</td>
</tr>
<tr>
<td>Low-quality control</td>
<td>300</td>
<td>294.1 (289, 299.3)</td>
<td>98.0% (96.3, 99.8%)</td>
<td>3.35%</td>
</tr>
</tbody>
</table>

Plasma samples were run on 13 separate days and urine samples on 14 days. CI, confidence interval.
the lowest concentration was the least sensitive to the
time-dependent changes in color but produced a deep
color at 20-min incubation at room temperature. Thus
the assay was performed with 1% DACA solution in
ethanol and incubated with protein-free, trichlorace-
tic acid-precipitated plasma or urine samples. Al-
though the color tended to deepen over time, because
all samples (standards and unknown) underwent a
similar change, no substantial differences were seen in
results for plates read between 15 and 25 min.

Figure 1 shows that for urine and plasma standard
curves, the coefficient of determination was 0.99 or
better. The standard error of estimate of PAH concen-
tration using this standard curve was between 1.5 and
3.6 \mu g/ml. None of the intercepts was significantly
different from zero. The lower limits of detection for
plasma and urine samples were 1 and 3.3 \mu g/ml, re-
spectively. The small positive intercept is likely due to
primary amines that react to give a low level of non-
specific chromogens.

Intraday CV was 3.8% for plasma and 6.3% for urine.
Table 1 shows the interday precision (% of CV) and
accuracy (% of recovery) for high and low concentra-
tions of PAH in urine and plasma. Accuracy was >95%
and precision was within 6% for all analyses. Studies
performed with six compounds demonstrated no inter-
ference with acetaminophen, \(N\)-acetyl-PAH, or sul-
salazin (all <1%). Interference was noted with \(p\)-ami
benzoic acid (120%), sulfacetamide (121%), and
sulfisoxazole (43%). Additional experiments were con-
ducted in which plasma and urine (diluted 1:10) were
each supplemented with urea (200 mg/dl) or creatinine
(13.5 mg/dl urine, 7.5 mg/dl plasma). PAH was added
in concentrations of 20 and 50 \mu g/ml, respectively. No
interference was seen with creatinine in urine or
plasma at baseline or after PAH was added. However,
blank plasma supplemented with urea yielded concen-
trations of PAH shown in Table 2.

Ten volunteers [8 men and 2 women, aged 69 ± 10
(SD) yr] were recruited from the renal clinic for the
 clearance study. Calculated creatinine clearances
(Cockcroft-Gault) ranged from 22 to 72 ml/min (mean,
48; SD, 18 ml/min).

Figure 2 shows the correlation between HPLC and
DACA techniques for plasma, urine, and clearance
results. Coefficients of determination \(r^2\) for all three
methods were excellent, and the standard error of
estimates was small.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obtained Concentrations of PAH, \mu g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>Plasma Blank</td>
<td>-0.48 ± 0.22</td>
</tr>
<tr>
<td>Supplemented with PAH (20 \mu g/ml)</td>
<td>18.41 ± 0.24</td>
</tr>
<tr>
<td>Urine Blank</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>Urine supplemented with PAH (50 \mu g/ml)</td>
<td>52.60 ± 1.50</td>
</tr>
</tbody>
</table>

Values are means ± SD. PAH, \(p\)-aminohippuric acid.

Fig. 2. Plasma (A) and urine (B) \(p\)-aminohippuric acid (PAH) concentrations and renal PAH clearances (PAH Clr; C) calculated by using the DACA method vs. the HPLC method. SEE, standard error of estimate; \(n\), number of samples or clearances.
Figure 3 shows the ratio plot of PAH in plasma with the two methods. The x-axis shows average PAH concentration with the two techniques, and the y-axis shows the ratio of PAH concentration obtained with DACA/HPLC methods. The average ratio was 1.019 (95% CI; 1.002, 1.036; P < 0.04), indicating that there was between 0.2 and 3.6% overestimation of plasma PAH with the new method. The limits of agreement shown by the dotted line were within 20% (±2 SD). Only 4 of 98 samples are outside these limits of agreement. Overall, the CV between the two methods was 5.98%.

Figure 4 shows the ratio plot of PAH in urine with the two methods. The average ratio was 1.0 (95% CI; 0.981, 1.189; P > 0.2), indicating that there was no bias in the results obtained with the new method. The limits of agreement shown by the dotted line are within 20% (±2 SD). Only 6 of 99 samples are outside these limits of agreement. Overall, the CV between the two methods was 7.52%.

Figure 5 shows the ratio plot of PAH clearances obtained with the two methods. The average ratio was 1.0 (95% CI; 0.972, 1.028; P = 0.2), indicating that there was no bias in the results obtained with the new method. The limits of agreement shown by the dotted line are within 25% (±2 SD). Only 4 of 78 clearances are outside these limits of agreement. Overall, the CV between the two methods was 9.66%.

DISCUSSION

Bratton and Marshall (6), more than 60 years ago, reported a colorimetric technique for the assay of sulphanilamide in urine and blood. In this technique, a trichloroacetic acid filtrate of the sample was mixed in a stepwise fashion with sodium nitrite, ammonium sulfamate, and N-(1-naphthyl)-ethylenediamine to yield a colored dye, the intensity of which was read at 545 nm. Smith et al. (19) modified this procedure by producing a cadmium sulfate filtrate of plasma or urine that was first acidified by 0.2 volume of 1.2 N-hydrochloric acid before the sequential steps noted above. It was several decades later that it was realized that chloride and temperature were critical for color development in the above assays (22). The above assay is not specific for p-aminohippuric acid. Free primary o- and m-aminoaromatic compounds (19), phenols, tryptophan, and indican also give color reactions (23). Interference from some sulfa drugs, such as sulfamethoxazole, can be overcome with isoamyl acetate extraction (17), and this assay has even been automated (11, 13). Nevertheless, fresh preparation of reagents (nitrite, sulfamate), sequential addition of three reagents, the timing of which is critical, and temperature dependence of these assays make these techniques cumbersome.

Some simplification of the PAH assay technique was obtained when Brun (7) created a two-step procedure. In this technique, plasma protein is precipitated by...
Somogyi's zinc sulfate and sodium hydroxide, followed by addition of p-dimethylanobenzaldehyde (Ehrlich's reagent) in acid alcohol to form a yellow dye read at 465 nm in a colorimeter. However, different analytical procedures are required for high and low plasma concentrations. More recently, Waugh and Beall (23) reported a two-step procedure in which buffered 1.0 M dichloroacetate and 0.3 M p-toluenesulfonate reagent are used for deproteinization and acidification of the sample; a yellow product is then obtained by adding 57% ethanolic 1% p-dimethylanobenzaldehyde. Even in this assay, blank plasma chromogen ranges between 21 and 73%, and urea and other sulfonamides containing free p-amino radical interfere with the assay.

Initially described by Japanese investigators, DACA has been used for simplified determination of PAH using DACA in 0.17 mmol hydrochloric acid (24), and even this method has been automated with an analyzer by Parekh et al. (15). Others have reported that substituting hydrochloric acid with ethanol gives a deeper and more persistent color and improves the assay performance (18). Because of the simplicity of this assay, we adapted it to the microtiter plate.

The established standard for estimation of PAH is HPLC. Baranowski and Westenfelder (3) found an excellent correlation between an HPLC method and a colorimetric technique. Our laboratory also reported an HPLC method that simultaneously assays PAH and iothalamate, which does not require the micropartition system described by Baranowski and Westenfelder (1). However, HPLC requires a large capital investment and is cumbersome and time-consuming. Therefore, our laboratory developed a rapid colorimetric method and compared it with our reference HPLC method.

Our results show that the new method using DACA in microplates for measurement of PAH is rapid, accurate, precise, and less expensive and compares favorably with the HPLC method. The limits of sensitivity for our assay is at least 50 ng/well for plasma and 165 ng/well for urine. As expected, some sulfonamides with the free p-amino group give color reactions, but not substituted amino groups such as acetaminophen or N-acetyl-p-aminohippuric acid, a metabolite of PAH. Our data demonstrate ~10–15% interference with urea, at most, when the plasma urea concentrations are in the uremic range. Because there are numerous compounds that can potentially give color reactions with DACA, it is recommended to run blank samples to screen for these potentially interfering compounds. The interday reproducibility of the new assay was superior to the HPLC method. Moreover, excellent agreement exists between the reference and new methods for plasma and urine concentrations as well as calculated PAH clearances performed in nearly 100 samples. We speculate that wider adoption of such simple and precise methods for estimation of renal plasma flow may improve our ability to determine the pathophysiology and better characterize the progression of renal diseases.

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REFERENCES


