A rapid microplate method for PAH estimation

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Short title: Rapid PAH estimation

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Abstract
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**Background.** 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 either complicated, expensive or time consuming.

**Methods.** We describe a rapid, precise and accurate microplate based assay of PAH using p-dimethylaminocinnamaldehyde that gives a red color on reacting with PAH and compare it to a reference HPLC method. Renal PAH clearances were measured in 10 volunteers and clearances calculated using the new and HPLC methods.

**Results.** 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 (2SD) for plasma, urine and clearances were 17.2%, 19.3% and 25.5%, respectively. Intraday coefficient of variation (CV) for urine and plasma were <7%, interday CV was <6% for urine and plasma samples.

**Conclusions.** 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.

Keywords: HPLC, colorimetry, renal plasma flow.
**Introduction**

Effective renal plasma flow, a major determinant of glomerular filtration rate (GFR), is required for evaluating renal hemodynamics and is traditionally measured by p-aminohippuric acid (PAH) clearance \((9;10;20)\). PAH estimation by modified Bratton Marshall reaction is cumbersome \((19)\) while performance of measurements with radioactive compounds \((12),(16),(21)\), creates additional problems such as disposal of waste, and radiation exposure of subjects and workers. Techniques such as HPLC require large capital investment and establishment of methodology which can take several months. Additional procedures including extensive and arduous extractions may be involved when estimating PAH via HPLC \((5)\). Microplate methods have not been reported for PAH quantification assays. Herein is reported a microplate method allowing rapid, accurate and precise estimation of PAH in comparison to the established standard of HPLC.

**Methods**

**Reagents**

\(p\)-Dimethylaminocinnamaldehyde (DACA) was purchased from Sigma Chemical Company (St. Louis, MO, USA). PAH was purchased from Merck and Co. (West Point, PA, USA). HPLC grade ethanol and trichloroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A 1% solution of DACA was made in ethanol (stable if stored at \(4^\circ\)C for several weeks) and 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 performing interference studies. In brief, 10.4 molar acetic anhydride was added
to a 10% solution of PAH, in a molar ratio of 2:1. The mixture was gently rotated for one hour at room temperature. A thick white amorphous mass formed; this was vacuum filtered using a 0.22 µm filter (Millipore, Bedford, MA, USA) and the paste 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**

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 1000, 800, 600, 500, 400, 300, 200 and 100 µg/mL PAH in diluted urine. The plasma standard curve was prepared using 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 quality controls respectively.

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

**Sample preparation**

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

**Calculations and Statistical Methods**

Standard curves were created by linear regression of optical density of PAH versus nominal concentrations of PAH. Concentrations of QC and unknown samples were estimated by applying standard curve linear regression equation to the sample optical density. Recovery was determined by calculating the mean difference between expected and observed concentration of quality controls expressed as a percent of expected as well as its 95% confidence intervals. 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 using
the method described by Chinn (8). 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 acetaminophen, p-aminobenzoic acid, sulfadiazine, sulfanilamide and sulfacetamide. 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 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 weight 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 4 consecutive periods after one hour of PAH infusion. Each urine collection period was bracketed by serum sample collection. Each volunteer was studied on two consecutive days. The clearances of PAH were calculated by the traditional UV/P method where U is the urinary concentration of PAH, P the geometric mean of the bracketing PAH plasma concentrations and V 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% to 10% in ethanol, we found that the lowest concentration was the least sensitive to the time dependent changes in color, yet gave a deep color at 20 minutes incubation at room temperature. Thus, the assay was performed with 1% DACA solution in ethanol and incubated with protein free, tricholoracetic acid precipitated, plasma or urine samples. Although the color tends to deepen over time, because all samples (standards and unknown) undergo a similar change, no substantial differences will be seen in results for plates read between 15-25 minutes.

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 concentration using this standard curve was between 1.5 to 3.6 µg/mL. None of the intercepts were significantly different from zero. The lower limits of detection for plasma and urine samples were 1 and 3.3 µg/mL respectively. The small positive intercept is likely due to primary amines that react to give a low level of nonspecific chromogens.

Intraday coefficient of variation was 3.8% for plasma and 6.3% for urine. Table 1 shows the interday precision (CV%) and accuracy (recovery%) for high and low concentration of PAH in urine and plasma. Accuracy was >95% and precision within 6% for all analyses. Studies performed with six compounds demonstrated no
interference with acetyl p-aminophenol (acetaminophen), n-acetyl p-aminohippuric acid, or sulfasalazine (all <1%). Interference was noted with p-aminobenzoic acid (120%), sulfacetamide (121%), and sulfisoxazole (43%). Additional experiments were conducted in which plasma and urine (diluted 1:10) were supplemented with urea (200 mg/dL) or creatinine (13.5 mg/dL urine, 7.5 mg/dL plasma) each. PAH was added in concentration of 20 $\mu$g/mL and 50 $\mu$g/mL respectively. No interference was seen with creatinine in urine or plasma at either baseline or after adding PAH. However blank plasma supplemented with urea yielded concentrations of PAH shown in Table 2.

Ten volunteers aged 69 ± 10 (SD) years, 8 males, 2 females 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 for all three methods ($r^2$) were excellent and the standard error of estimates (SEE) were small.

Figure 3 shows the ratio plot of PAH in plasma by the two methods. The x-axis shows average PAH concentration by the two techniques and the y-axis the ratio of PAH concentration obtained by 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% to 3.6% overestimation of plasma PAH by the new method. The limits of agreement shown by the dotted line were within 20% (2 SD = 17.2%). Only 4 out of 98 samples lie outside these limits of agreement. Overall, the coefficient of variation between the
two methods was 5.98%.

Figure 4 shows the ratio plot of PAH in urine by 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 by the new method. The limits of agreement shown by the dotted line were within 20% (2 SD = 19.3%). Only 6 out of 99 samples lie outside these limits of agreement. Overall, the coefficient of variation between the two methods was 7.52%.

Figure 5 show the ratio plot of PAH clearances obtained by 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 by the new method. The limits of agreement shown by the dotted line are approximately 25% (2 SD =25.5%). Only 4 out of 78 clearances lie outside these limits of agreement. Overall, the coefficient of variation between the two methods was 9.66%.

Discussion
Bratton and Marshall, more than 60 years ago, reported a colorimetric technique for the assay of sulfanilamide in urine and blood (6). In this technique, trichloroacetic acid filtrate of 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. Homer Smith and associates 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 prior to the sequential steps noted above (19). It was several decades later that it was realized that chloride
and temperature were critical for the color development in the above assays (22).

The above assay is not specific for p-amino hippuric acid. Free primary \(\alpha\)- and \(m\)-aminoaromatic compounds (19), phenols, tryptophan and indican also give colored 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, timing of which is critical, and temperature dependence of these assays makes these techniques cumbersome.

Some simplification of PAH assay technique was obtained when Brun created a two-step procedure (7). In this technique plasma protein is precipitated by Somogyi’s zinc sulfate and sodium hydroxide followed by addition of \(p\)-dimethylaminobenzaldehyde (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 reported a two-step procedure in which buffered 1.0 M dichloroacetate and 0.3 M \(p\)-toluenesulfonate reagent is used for deproteinization and acidification of the sample; a yellow product is then obtained by adding 57% ethanolic 1% \(p\)-dimethylaminobenzaldehyde (23). Even in this assay, blank plasma chromogen ranges between 21-73%, and urea and other sulfonamides containing free \(p\)-amino radical interfere with the assay.

Initially described by Japanese investigators, \(p\)-dimethylaminocinnamaldehyde (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 using an autoanalyzer by Parekh and associates (15). Others have reported that substituting hydrochloric acid with ethanol gives a deeper and more persistent color and improves the assay performance (18). Due to the simplicity of this assay, we adapted it to the microtiter plate.

The established standard for estimation of PAH is high performance liquid chromatography (HPLC). Baranowski and Westenfelder found an excellent correlation between an HPLC method and a colorimetric technique (3). We have 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, is cumbersome and time consuming. Therefore, we developed a rapid colorimetric method and compared it to our reference HPLC method (1).

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 50ng/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 acetyl p-aminophenol (acetaminophen) or n-acetyl p-amino hippuric acid, a metabolite of PAH. Our data demonstrate about 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.
Reference List


Table 1: Interday Assay Characteristics for the Microplate Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nominal Concentration (µg/mL)</th>
<th>Obtained Concentration (µg/mL) and 95% CI</th>
<th>Recovery (%) and 95% CI</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 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>Plasma 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 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>Urine 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, urine samples on 14 days., CI= confidence interval
Table 2: Interference Studies with Urea and Creatinine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obtained Concentrations of PAH (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>Blank Plasma</td>
<td>-0.48 ± 0.22</td>
</tr>
<tr>
<td>Plasma Supplemented with PAH (20 µg/mL)</td>
<td>18.41 ± 0.241</td>
</tr>
<tr>
<td>Blank Urine</td>
<td>0.94 ± 0.103</td>
</tr>
<tr>
<td>Urine Supplemented with PAH (50 µg/mL)</td>
<td>52.6 ± 1.5</td>
</tr>
</tbody>
</table>
Figure Legends:

Figure 1: Plasma and urine standard curves using the DACA assay show nearly a perfect correlation.

Figure 2: Plasma and urine PAH concentrations and renal PAH clearances calculated using the DACA method vs. HPLC method. SEE = standard error of estimate, n = number of samples or clearances.

Figure 3: Bland-Altman plot of average plasma PAH concentration by DACA and HPLC methods and ratio of DACA/HPLC plasma PAH concentration. Horizontal line is the bias, which in this case is between 0.2% and 3.6%, as shown by the horizontal dotted lines. Horizontal dashed lines (±2 SD) that show limits of agreement are within 20%.

Figure 4: Bland-Altman plot of average urine PAH concentration by DACA and HPLC methods and ratio of DACA/HPLC urine PAH concentration. Horizontal line at 1.0 indicates no bias. Horizontal dashed lines (±2 SD) that show limits of agreement are within 20%.

Figure 5: Bland-Altman plot of average renal PAH clearances calculated by DACA and HPLC methods and ratio of DACA/HPLC renal PAH clearances. Horizontal line at 1.0 indicates no bias. Horizontal dashed lines (±2 SD) that show limits of agreement are within 25%.
Urine Standard Curve

Nominal PAH Concentration (µg/mL)

Optical Density

\[ Y = 0.188X + 0.656 \]

\[ r^2 = 0.999 \]

Plasma Standard Curve

Nominal PAH Concentration (µg/mL)

Optical Density

\[ Y = 0.198X + 0.533 \]

\[ r^2 = 0.999 \]
Figure 2

**Plasma PAH (µg/mL) HPLC Method**

- **Y = 0.99X + 0.425**
- \( r^2 = 0.87, \ p<0.001 \)
- SEE = 1.34
- n=98

**Urine PAH (µg/mL) HPLC Method**

- **Y = 1.01X - 8.9**
- \( r^2 = 0.98, \ p<0.0001 \)
- SEE = 128
- n=99

**PAH Clr (mL/min) HPLC Method**

- **Y = 0.97X + 5.03**
- \( r^2 = 0.94, \ p<0.0001 \)
- SEE = 37
- n = 78
Figure 4

Average Urine PAH Concentration (µg/mL) vs. Ratio of Urine PAH concentration [DACA/HPLC]
Figure 5

Average PAH Clearances (mL/min)

Ratio of PAH clearances
[DACA/HPLC]