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Alternative high-performance liquid chromatographic assay for *p*-aminohippuric acid (PAH): effect of aging on PAH excretion in the isolated perfused rat kidney

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Abstract

Para-aminohippuric acid (PAH), an indicator of renal plasma flow, is a commonly used marker of organic anion transport by the kidney. An analytical method for PAH using HPLC was developed. The method is simple, fast and requires a minimum amount of organic solvent. Sample preparation involved protein precipitation with zinc sulfate. Para-amino benzoic acid was utilized as an internal standard (IS). Chromatography was performed using a reversed-phase phenyl column with UV detection at a wavelength of 254 nm. Mobile phase consisted of 0.1 M acetic acid and acetonitrile (99:1) at a flow rate of 1 ml/min. The assay was validated over a standard concentration range from 1 to 25 μg/ml. Accuracy, precision, reproducibility and specificity of the method was established with coefficients of variation $< 10\%$. The method was sensitive and showed linear response in peak height ratio (analyte:IS) over the concentration range studied ($r^2 > 0.99$). The assay was used to study the effect of aging on PAH excretion in the isolated perfused rat kidney model. Experiments were conducted in kidneys from young (2–3 months, $n = 6$), adult (6–9 months, $n = 5$) and aged (12–16 months, $n = 3$) male Sprague–Dawley rats at an initial drug concentration of 20 μg/ml. Significant differences in kidney function (e.g. glomerular filtration rate and glucose reabsorption) were observed in aged kidneys. Despite a 5-fold reduction in glomerular filtration rate, PAH renal clearance (kidney weight-corrected) decreased by only 2-fold in aged (2.2 ± 0.42 ml/min per gram) compared to young (4.6 ± 0.70 ml/min per gram, $P < 0.05$) rats. Furthermore, renal excretion ratio was significantly higher in aged rats (27 ± 8.0 vs. 15 ± 5.0, $P < 0.05$). These preliminary findings challenge the 'Whole Nephron Hypothesis' that assumes parallel reductions in renal filtration and secretory capacity secondary to disease or aging.

Keywords: Para-aminohippurate; Reversed-phase chromatography; Perfused rat kidney; Aging

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1. Introduction

The kidney is a primary organ of drug clearance from the body. There are three mechanisms of renal drug handling: glomerular filtration, tubular secretion and tubular reabsorption [1]. It is well documented in the literature that, with increasing age, there is a corresponding decrease in renal function. Davies and Shock reported that glomerular filtration rate (GFR), measured by inulin clearance, declines with age independent of cardiovascular disorders, renal diseases and other acute illnesses [2]. The clearance of creatinine, the most commonly used indicator of GFR, declines by 10% per decade of life after 40 years of age [3]. As much as a 50% reduction in GFR has been observed in subjects over 70 years of age [4,5]. However, there is a paucity of data regarding the differential effects of aging on the various components of renal function and elimination capacity of the kidney.

Changes in renal function are often assessed through GFR or its clinical marker, serum creatinine. The 'Whole Nephron Hypothesis' assumes that reductions in renal filtration (reflected in GFR) are accompanied by parallel diminutions in secretory and reabsorptive capacity of the nephron [6]. Consequently, for medications eliminated primarily via renal mechanisms, dosage adjustments in elderly patients are frequently based on GFR. The results of previous studies in both human [7–10] and animal models [11–14] indicate that renal drug excretion may not correlate with GFR in cases of renal insufficiency. This would suggest that GFR-based dosing in elderly subjects could predispose patients to therapeutic failure secondary to insufficient dosage administration or toxicity due to administration of an excessive dose.

p-Aminohippuric acid (PAH) is a commonly used marker of renal plasma flow [15]. A compound of high extraction across the kidney, PAH has also been utilized extensively in the study of organic anion renal secretion [16–18]. There are many assay methods for PAH reported in the literature including colorimetry [19,20], gas chromatography [21] and high performance liquid chromatography (HPLC) [22–31]. In the present investigation, an alternative HPLC method for PAH was developed and validated. Comparison of the method with those previously reported in the literature is presented. The method was used to support in vitro studies of the effect of aging on PAH excretion in an isolated perfused rat kidney (IPK) model. The objective of these studies was to test the validity of the 'Whole Nephron Hypothesis' for a compound that is actively secreted by the kidney.

2. Experimental

2.1. Materials

PAH (sodium salt), *p*-aminobenzoic acid (PABA), sodium pentobarbital, creatinine, bovine serum albumin (Fraction V), clinical grade dextran, sodium chloride, potassium phosphate, calcium chloride, magnesium chloride, L-amino acids, glucose and diagnostic test kits for glucose (Kit No. 315) and creatinine (Kit No. 555-A) were purchased from Sigma (St. Louis, MO). Zinc sulfate ($ZnSO_4*7H_2O$) was obtained from Alfa Aesear Chemical Company (Ward Hill, MA). Solvents used for HPLC were obtained from J & H Berge Co. (Plainfield, NJ).

2.2. HPLC assay and validation

2.2.1. Stock solutions and standard curves

Stock solutions of PAH were prepared at 0.1 and 1 mg/ml. Stock solutions (aqueous) of PABA (internal standard, IS) and zinc sulfate used in the study were 0.5 and 10 mg/ml, respectively. All stocks and standard solutions were prepared in HPLC grade water.

Standard curves were prepared for a concentration range of PAH from 1 to 25 μg/ml. Six concentrations were used to construct each standard curve: 1, 2.5, 5, 10, 15 and 25 μg/ml. Blank samples (matrix only) were prepared and analyzed with each standard curve.

2.2.2. Sample preparation

A total of 25 μl of internal standard was added to a 250 μl aliquot of sample. 250 μl of zinc

sulfate solution (1%) was added to precipitate proteins and the mixture vortexed for 30 s and centrifuged (VWR Microcentrifuge, South Plainfield, NJ,) at 10 000 rpm for 10 min. Of the resultant supernatant, 20 μl was injected into the HPLC.

2.2.3. HPLC system

The HPLC system consisted of a Thermo Separation Products P1000 isocratic pump and a UV1000 ultraviolet detector (Thermo Separation Products, Riviera, FL). Output from the detector was processed using an IBM P75 personal computer (IBM, Research Triangle Park, NC) with PC1000 software (Thermo Separation Products). The mobile phase consisted of 0.1 M acetic acid and acetonitrile (99:1). Separation was achieved using a Waters μ Bondapak Phenyl Column (3.9 × 300 mm, 10 μm particle size). The detection wavelength and mobile phase flow rate were 254 nm and 1 ml/min, respectively.

2.2.4. Assay validation

The assay was validated in IPK perfusate (see perfusate composition below) and Krebs–Henseleit buffer (for IPK urine analysis). The selectivity of the method was determined by comparing the chromatograms obtained from the samples containing PAH and PABA with those obtained from blank samples. Method precision was determined by analyzing five identical samples (prepared through identical sample treatment) of PAH at three concentrations (2, 12 and 20 μg/ml). Inter-day precision of the method in perfusate and KHS buffer was evaluated by assaying calibration standards at 5 consecutive days. System precision was assessed by analyzing six injections from the same sample (PAH concentration 12 μg/ml).

The limit of detection (LOD) was defined as the analyte concentration that produced a 3-fold signal:noise ratio compared to blank samples. Likewise, the limit of quantitation was defined as the analyte concentration that produced a 10-fold signal:noise ratio.

The percentage of analyte recovered (% recovery) from the matrix was calculated as follows:

$$\%\ \text{Recovery} = \left(\frac{\text{PH}_{\text{matrix}}}{\text{PH}_{\text{aqueous}}}\right) \times 100$$

where $\text{PH}_{\text{matrix}}$ and $\text{PH}_{\text{aqueous}}$ represent peak height of PAH in matrix and water, respectively.

2.2.5. Stability of PAH

The stability of PAH in perfusate and buffer was determined at two concentrations (2 and 20 μg/ml) following storage at room temperature for 1 day and after storage at −20°C for 1 month. PAH concentrations in these samples were determined by HPLC. Each stability study was performed in triplicate.

2.3. IPK experiments

2.3.1. Isolated perfused rat kidney preparation

The Institutional Animal Care and Usage Committee of Long Island University approved the experimental protocol for this investigation. The surgical technique employed was the Bowman modification [32] of the Nishiitsutsuji-Uwo procedure [33]. Male Sprague–Dawley rats were used for perfusion experiments. Animals were housed in stainless steel cages and fed a standard diet including water ad libitum. Anesthesia was induced with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). A midline incision was made and the renal segment of the aorta exposed. A ligature was passed under the right renal artery close to the aorta and distal and proximal ligatures placed around the superior mesenteric artery. The right ureter was catheterized with PE-10 tubing, in order to facilitate urine collection. A cannula was then threaded through the mesenteric artery, across the aorta and into the right renal artery in situ. The ligatures were tied, securing the cannula in place. The right kidney was then excised from the animal, trimmed of adhering tissue and transferred to the in vitro recirculating perfusion apparatus.

Perfusion of the kidney proceeded according to the method described by Bekersky [34]. Perfusate consisted of the following: Krebs–Henseleit buffer (pH 7.4), 4.00% Fraction V bovine serum albumin, 1.67% clinical grade dextran, 100 mg/dl glucose, 54.3 mg/dl creatinine and a 13 mM amino acid solution. Inclusion of amino acids

improves viability of the preparation [35]. The volume of recirculating perfusate was 80 ml.

The composition of Krebs–Henseleit buffer is as follows: NaCl (0.117 M), $NaHCO_3$ (0.025 M), KCl (0.0047 M), $CaCl_2$ (0.0025 M), $MgSO_4$ (0.0021 M) and KH_2PO_4 (0.0012 M).

2.3.2. Study groups

Perfusion experiments (three to six per study group) were conducted with kidneys obtained from rats representing three age groups (Table 1): 2–3 months (termed young rats), 6–9 months (termed adult rats) and 12–16 months (termed aged rats). PAH was administered as a bolus dose (1.6 mg) to reach an initial perfusate concentration of 20 μg/ml that is less than the reported Km for PAH secretory transport in the rat (Km $\approx$ 40–50 μg/ml) [36,37]. Control (drug-naïve) perfusions were conducted for all age groups to identify any drug-induced changes in kidney function.

2.3.3. Study design

Following kidney excision and transfer to the recirculating perfusion system, a stabilization period (10–20 min) preceded any pharmacokinetic experimentation. Drug was then added and perfusate (0.7 ml) sampled at 2, 5, 7, 10 and 15 min and every 10 min thereafter. Urine was collected at 10-min intervals over the entire experiment (2 h). Volume lost due to sampling or urine excretion was replaced as needed with a 50:50 mixture of perfusate and deionized water. All samples were stored frozen at -20°C prior to analysis.

Aliquots of both perfusate and urine were analyzed for sodium (Glass Micro-Sodium Combination Electrode, Model No. 9811BN, ATI Orion, Boston, MA), creatinine and glucose. The creatinine method employed a picric acid colorimetric assay. Picric acid solution was formed by combining a colorimetric reagent (Kit 555A, Sigma) with 1 N NaOH in a ratio of 5:1. Perfusate (30 μl) was added to 270 μl of water (1:10 dilution) in a disposable cuvette. For urine analysis, 5 μl of sample was added to 295 μl of water (1:60 dilution). Picric acid solution (3 ml) was added and the samples were allowed to stand at an ambient temperature for 15 min. Samples were then analyzed for absorbance using a Perkin–Elmer Lamba 3A UV/VIS Spectrophotometer (Perkin–Elmer, Norwalk, CT) at a wavelength of 500 nm. The observed rate of complex formation was directly proportional to the sample creatinine concentration as reflected in absorbance.

Glucose concentrations in perfusate and urine were determined using a glucose oxidase reaction. Glucose (Trinder) reagent (Kit 325-A Sigma) was prepared by adding a vial of Trinder powder to 500 ml of deionized water. A total of 10 μl of sample (perfusate or urine) was added to a cuvette containing 3 ml of Trinder reagent. The cuvette was then allowed to stand for 18 min at ambient temperature. At that time, samples were assayed for glucose concentration by measuring absorbance at a wavelength of 505 nm.

Parameters monitored to assess kidney function throughout an IPK perfusion included glomerular filtration rate (GFR, estimated as creatinine clearance), reabsorption of glucose and sodium, urine flow rate and urine pH. Perfusion pressure was maintained at 100 ± 10 mmHg by adjusting perfusate flow rate as necessary.

The perfusate binding of PAH was determined by ultrafiltration. Aliquots (1 ml) of perfusate were sampled during each perfusion (at 45 and 115 min) and were added to Amicon Centrifree™ Micropartition Systems (Millipore Corp., Bedford, MA) and centrifuged at $1500 \times g$ for 15 min. The resultant ultrafiltrate contained free drug and was stored as described previously. Preliminary experiments determined that binding of the drug to the membrane was negligible.

Table 1
Anatomic data for IPK kidney donors[a]

Parameter	Young	Adult	Aged
Body weight (g)	292 ± 30.3	622 ± 40.0	644 ± 64.0
Kidney weight (g)	1.36 ± 0.114	2.60 ± 0.223	2.78 ± 0.377
N[b]	10	9	6

[a] Data are expressed as mean $\pm$ S.D.

[b] Number of perfusion experiments conducted. This includes both control (drug-naïve) and drug-treated perfusions.

2.3.4. Data analysis

PAH renal clearance (Cl_{total}) was calculated as the ratio of dose and AUC ($0-\infty$). AUC ($0-\infty$) was calculated using trapezoidal rule. Secretion clearance ($Cl_{secretion}$) was defined as the difference between Cl_{total} and filtration clearance ($Cl_{filtration}$), the product of glomerular filtration rate (GFR) and unbound fraction of PAH in perfusate (f_u). Because of the increased body weight of the adult and aged rats, clearance parameters were corrected for kidney weight. Excretion ratio (XR), an indication of net mechanisms of elimination, was calculated as the ratio of Cl_{total} and $Cl_{filtration}$.

2.3.5. Statistical analysis

Mean parameter estimates of IPK viability criteria (e.g. GFR, urine flow rate) for control and drug treatment groups were compared using analysis of variance (ANOVA). Multiple comparison tests (Student–Newman–Keuls) were performed to identify those study groups that differed in terms of viability criteria. Consequently, alterations in kidney function as a function of kidney age and/or drug administration were determined. Kidney viability was monitored during each urine collection period. Therefore, a total of ten collection periods were performed for each perfusion.

Mean values for PAH clearance parameters were also compared by ANOVA. Significant differences among the various treatment groups were identified in an effort to elucidate the consequences of aging on PAH excretion.

3. Results

Table 2 contains the slopes and intercepts of standard curves representing the concentration range utilized for quantitation and detection of PAH in perfusate and buffer. The response function employed was peak height ratio (analyte:IS). Included in this table are the average values from five standard curves that were constructed over 5 days.

Example chromatograms are provided in Fig. 1. Presented in that figure are chromatograms for a blank perfusate sample and a sample containing analyte and internal standard. The retention times for PAH and PABA was 6.37 and 9.78 min, respectively. The LOD of PAH was 0.5 μg/ml.

Table 2
Summary of calibration curves[a] for PAH HPLC assay in perfusate and KHS buffer (standard concentration range 1.0–25 μg/ml)

Matrix	Correlation[b] (r^2)	Slope[b]	Intercept[b]
Perfusate	0.997 ± 0.00195	0.024 ± 0.0028	−0.0072 ± 0.024
KHS buffer	0.997 ± 0.00164	0.027 ± 0.0021	−0.012 ± 0.034

[a] Peak height ratio (PAH:IS) versus concentration.
[b] Data presented as mean ± S.D. of five calibration curves.

Accuracy and precision of the analytical method were assessed through replicate sets of analysis of three concentrations within the standard curve range: 2, 12 and 20 μg/ml. The results are presented in Table 3. Data are presented as the mean (S.D.) concentration of five samples analyzed for each concentration studied. The LOQ was 1 μg/ml.

Table 4 contains the results of recovery studies for PAH in perfusate and buffer. Percent recovery of PAH from plasma was calculated through comparison with analysis of aqueous drug samples. Mean percent recovery ranged from 88.3 to 101% across all concentration studied (R.S.D. $< 3.01\%$).

Results of the stability studies are presented in Table 5. PAH was stable in perfusate following storage at room temperature for 24 h. Drug was also stable in the matrix when stored for up to 1 month at −20°C. Stability studies beyond that storage period were not performed in this investigation.

Table 6 contains a summary of kidney functionality across all study groups. Included in this table are values for the various criteria used to assess functionality of the IPK. Application of the analytical method is displayed in Fig. 2, plots of PAH perfusate concentrations and cumulative urinary excretion over time in the IPK. The effect of aging on PAH excretion is summarized in Table 7 (renal excretion parameters) and Fig. 3, a graph that demonstrates the nonlinear relation-

ship between PAH clearance and GFR across all study groups. Perfusate f_u of PAH was 0.28.

4. Discussion

A number of analytical methods for detection and quantitation of PAH in biological fluids (plasma, urine) by HPLC have been reported [22–31] and are summarized in Table 8. Possible disadvantages of these methods include the need for multiple extraction steps [23], inclusion of an ion-pairing agent or high organic component in the mobile phase [25,27,29–31] or the use of a heated column [26]. In the present study, an HPLC assay for PAH in human plasma was developed and validated. The variability of the method with regard to reproducibility (Table 2), accuracy and precision (Table 3) was within acceptable limits. Nearly complete recovery of drug from the matrix was obtained (Table 4). The LOQ (1 μg/ml) equals or is lower than that obtained with other methods (Table 8). Furthermore, the drug was found to be stable in each matrix for at least 1 month when stored frozen (stability beyond this time was not tested).

The method compares favorably with those listed in Table 8. However, notable differences exist between this assay and those previously reported methods. A phenyl column was used for separation of analyte. Other methods have utilized a C-8 [29,30] column, a C-18 column [23–28,31] or an ion exchange column [8]. With regard to the assay reported in this communication, it can be hypothesized that the phenyl column interacts with a benzyl ring in the PAH molecule though electronic interaction forces, weak bonds in comparison to the expected interaction between the ion-pair and a C-18 or C-8 column, thereby significantly lowering the amount of organic sol-

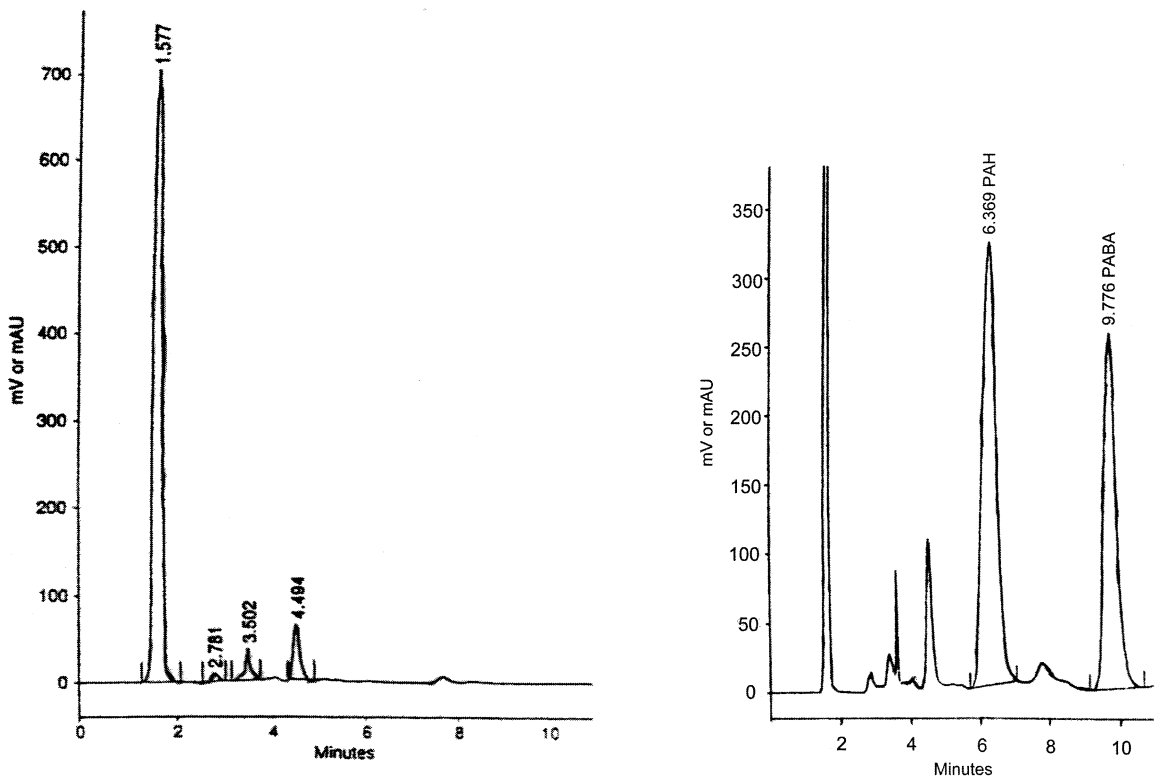

Fig. 1. Sample chromatograms of an extract of blank perfusate (left panel) and perfusate containing PAH (50 μg/ml) and PABA (IS).

Table 3
Determination of accuracy and precision[a] of HPLC assay for PAH in perfusate and KHS buffer

Method precision		
Perfusate		
Sample[b] (μg/ml)	Concentration[c] (μg/ml)	MPE%
2	2.15 ± 0.040	7.7
12	12.0 ± 0.421	2.5
20	20.6 ± 1.72	7.6
KHS buffer		
Sample[b] (μg/ml)	Concentration[c] (μg/ml)	MPE%[d]
2	1.89 ± 0.0115	0.30
12	11.3 ± 0.173	5.7
20	19.1 ± 0.115	4.7
System precision[e]		
Matrix	Concentration (μg/ml)	RSD%[f]
Perfusate	12.2 ± 0.089	0.733
KHS buffer	12.4 ± 0.089	0.721

[a] Replicate sets of analysis of three concentrations within standard curve range studied.
[b] Standard concentration used to assess method precision.
[c] Data presented as mean ± S.D. of five replicate samples.
[d] Mean prediction error.
[e] System precision based on six replicate injections of 12 μg/ml sample.
[f] Relative S.D.

vent required in the mobile phase. Comprised of acetic acid (0.1 M) and 1% acetonitrile, the organic component of this mobile phase is significantly reduced in comparison to most of the methods listed in Table 8. Moreover, the use of an ion-pairing agent is avoided. The sample size employed in our validation procedures was 250 μl. However, in consideration of the modest sample preparation involved (protein precipitation with equal volume of $ZnSO_4*7H_2O$), small injection volume (20 μl) and complete sample recovery, the assay could readily be modified to accommodate much smaller sample volumes (50 μl or less). Thus, this method could be employed as an alternative microassay for PAH. A potential disadvantage of the present method is that it does not allow for simultaneous determination of PAH and iothalamate, a marker of glomerular filtration rate. Several of the methods listed in Table 8 offer this feature [24,29–31]. Nevertheless, the assay is a simple, inexpensive method for quantitation of PAH. It should be noted that this HPLC method has also been validated in human plasma [38].

Table 4
Data for recovery of PAH from perfusate and KHS buffer[a]

Concentration (μg/ml)[b]	Recovery (%)[c]	RSD%
Perfusate		
2	101 ± 3.05	3.01
20	90.1 ± 2.69	2.99
KHS buffer		
2	88.3 ± 1.15	1.30
20	91.3 ± 0.577	0.632

[a] Comparison between plasma and aqueous samples spiked with drug.
[b] Standard concentrations used to determine recovery.
[c] Data presented as mean ± S.D.% recovery of three replicate samples.

Table 5
Stability data for PAH in perfusate and KHS buffer following storage for 1 day (room temperature) and 1 month (−20°C)

Storage condition	Concentration (μg/ml)	% Change in concentration[a]
Room temperature (1 day)	2	(+) 4.52
	20	(+) 0.317
−20°C for 1 month	2	(+) 7.83
	20	(−) 1.22

[a] Data presented as mean of three samples.

The analytical method was used to investigate the effect of aging on PAH excretion in the perfused kidney model. A comparison of physiologic kidney function among the three age groups is presented in Table 6. In perfusions performed with kidneys from young rats, good renal function was maintained. The values listed in Table 6 (young rats) are comparable to or exceed those reported previously [39–41]. However, significant differences in kidney function were noted among study groups.

In IPK methodology, the perfusion flow rate is adjusted as needed during each perfusion experi-

ment. This is performed in order to maintain an optimal perfusion pressure at 100 ± 10 mmHg. As demonstrated in Table 2, significant differences in weight-corrected perfusion flow rates were noted. Likewise, urine pH and sodium reabsorption were also age-dependent, although these differences did not likely influence study results. GFR, estimated by creatinine clearance, was significantly lower in adult and aged rats. Glucose reabsorption, presumably an indicator of proximal tubular function, was also significantly decreased in aged kidneys.

According to the 'Whole Nephron Hypothesis', diminished renal function (defined as reduced GFR) as a consequence of aging should produce a proportional decrease in the renal excretion of PAH. As shown in Table 7, weight-corrected PAH clearance was reduced by roughly 2-fold in aged kidneys. However, this difference was less than expected from the nearly 5-fold decrease in GFR. Additionally, renal excretion ratio, the ratio of total drug clearance to filtration clearance, was nearly doubled in aged rats (27 ± 8.0 vs. 15 ± 5.0, Table 2). Further evidence of this glomerulo-tubular imbalance in aging kidneys is provided in Fig. 3, a plot of PAH clearance versus GFR for individual perfusion experiments. The plot demonstrates the nonlinearity between PAH excretion and GFR. Included in Fig. 3 are data analysis results using linear regression (line 1) and nonlinear regression (line 2). Not only was the data best described by a hyperbolic function (line 2, $r^2 = 0.68$) in comparison to linear regression (line 1, $r^2 = 0.40$), but linear regression analysis also yielded a significant y-intercept, indicating that renal clearance is maintained in the absence of filtration (when GFR = 0). Collectively, these findings suggest the 'Whole Nephron Hypothesis' may not apply to compounds that undergo extensive tubular secretion, such as PAH.

Overall, these experiments yielded a number of interesting findings. For example, glucose reabsorption was significantly reduced in aged kidneys, yet sodium reabsorption remained relatively constant across all study groups. It is unclear how the aging process elicits differential effects on

Table 6
IPK viability parameters as a function of kidney age[a]

Parameter	Young		Adult		Aged	
	Control	Drug	Control	Drug	Control	Drug
Perfusion flow rate (ml/min/g)	17 (3.1)	14[b] (4.0)	13[b] (5.0)	13 (3.8)	17[b] (4.9)	14[b,c] (5.0)
Urine pH	6.9 (0.18)	7.1[c] (0.23)	6.9 (0.28)	6.6[b] (0.33)	6.9 (0.24)	6.5[b] (0.23)
Urine flow rate (ml/min/g)	0.056 (0.020)	0.091[b,c,d] (0.033)	0.027[b] (0.0089)	0.023[c] (0.012)	0.023[b] (0.0073)	0.010[b] (0.0054)
GFR (ml/min/g)	0.51 (0.15)	0.56 (0.25)	0.38[b] (0.20)	.36[b] (0.26)	0.23[b] (0.15)	0.11[b,c] (0.057)
Glucose reabsorption (%)	93 (2.1)	91 (5.8)	96 (3.0)	95 (3.9)	75[b,c] (21)	75[b,c] (22)
Sodium reabsorption (%)	95 (2.3)	93[c] (5.2)	96 (2.0)	96[d] (3.1)	93[c] (4.5)	93 (8.0)
Number of perfusions	4	6	4	5	3	3

[a] Data reported as mean (S.D.) for control (drug-naïve) and drug (treated) perfusions. A total of ten urine collection intervals were performed for each perfusion.
[b] Significantly different from young control group ($P < 0.05$).
[c] Significantly different from adult control group ($P < 0.05$).
[d] Significantly different from aged control group ($P < 0.05$).

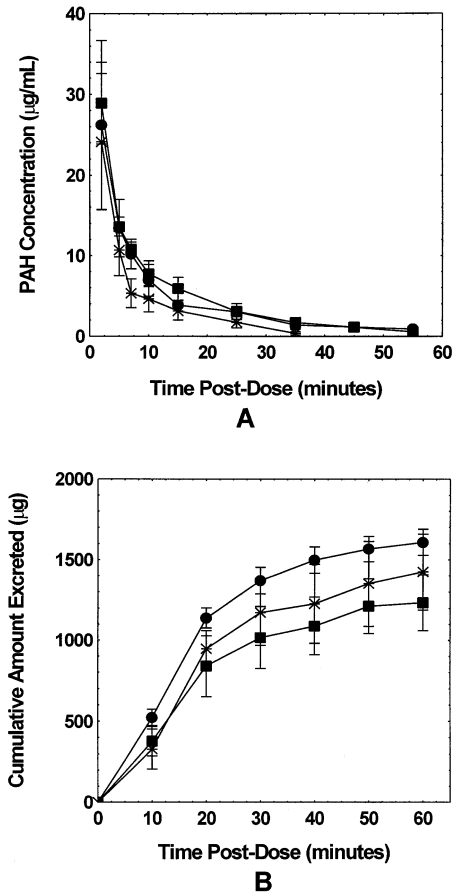

Fig. 2. Plots of PAF perfusate concentrations (A) and cumulative urinary excretion on PAH (B) versus time in the IPK. (■ = young; ● = adult; * = aged).

reabsorption mechanisms, although a reduction in glucose transport by the brush border membrane of the proximal tubule has been reported [42]. Since glucose reabsorption by the kidney proceeds via a sodium-dependent co-transport system, this observation is consistent with a reduction in proximal tubule function in aged kidneys. Diminished proximal tubule function would likewise explain the differences in PAH clearance among study groups. Nevertheless, these differences are much less than predicted based on GFR. These results indicate that secretory capacity does not necessarily correlate to GFR in cases of diminished renal function (despite reductions in GFR).

Previous studies in both humans and animal models have investigated the relationship between renal drug excretion and GFR. Reidenberg et al., studying the correlation between aging and the renal excretion of procainamide and its metabolite *N*-acetylprocainamide, found that the tubular secretion of both compounds (reflected as decreased drug:creatinine clearance ratio) was reduced in the elderly [8]. This study suggested that tubular se-

Table 7
PAH clearance parameters as a function of kidney age in the IPK[a]

Parameter	Young	Adult	Aged
Cl_{total} (ml/min/g)	4.6 (0.70)	3.5 (1.0)	2.2[b] (0.42)
$Cl_{secretory}$ (ml/min/g)	4.3 (0.74)	3.2 (1.1)	2.1[b] (0.52)
Excretion ratio	15 (5.0)	20 (12)	27 (8.0)
GFR (ml/min/g)	0.56 (0.25)	0.38[b] (0.2)	0.11[b,c] (0.057)
Number of perfusions	6	5	3

[a] Data reported as mean (S.D.).
[b] Significantly different from young group ($P<0.05$).
[c] Significantly different from adult group ($P<0.05$).

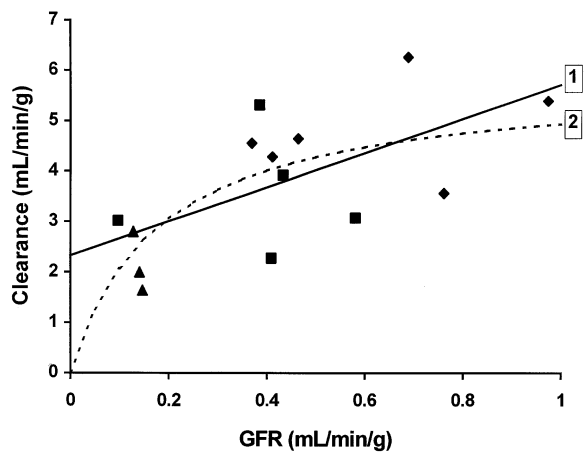

Fig. 3. Plot of PAH clearance versus GFR for individual perfusion experiments. Data is corrected for kidney weight. (◆ = young; ■ = adult; ▲ = aged). The solid line (1) is the predicted relationship based on linear regression analysis ($r^2 = 0.40$). The dashed line (2) represents the 'best fit' of a hyperbolic function to the data using nonlinear regression ($r^2 = 0.68$).

Table 8
Summary of published HPLC assays for quantitation of PAH in biological matrices

Authors	Matrix	Sample size (μl)	Chromatography		Retention time (min)	Limit of quantitation (μg/ml)	Comments
			Column	Mobile phase			
Shoup and Kissinger [22]	Serum	200	Anion exchange	Acetate buffer (0.08 M)	10	5	Electrochemical detection
	Urine	1000					
Boschi and Marchensini [23]	Plasma	500–1000	C-18	Acetonitrile (4%)	5.6	1.0	Multiple extraction steps
	Urine	1000		Phosphoric acid			
Prueksaritanont et al. [24]	Plasma	100	C-18	Acetonitrile (3.5%)	4.5	2.5	PAH/iothalamate simultaneous assay
	Urine	100		Phosphoric acid (0.04%)			
Baranowski and Westenfelder [25]	Plasma	50	C-18	Acetonitrile (50%)	4.1	Not determined	Validation not reported
	Urine	50		Ammonium acetate (0.05 M)			
Kiguchi and Sudo [26]	Plasma	10	C-18 (40°C)	Acetonitrile (5%) KH_2PO_4 (0.05 M)	5.2	1.0	Good method for microassay
	Urine	10					
Chan et al. [27]	Urine	100	C-18	Ion pairing agent	12	2.5	Metabolism study
Jenny et al. [28]	Serum	50	C-18	Ion pairing agent phosphate buffer	7.4	1	Good method for microassay
Jayewardene et al. [29]	Plasma	100	C-8	Ion pairing agent methanol 22.5%; acetonitrile 2.5%	12	5	PAG/iothalamate simultaneous assay
Bell et al. [31]	Serum	100	C-18	Ion pairing agent; methanol 15%	6.4	1.3	PAH/Iothalamate simultaneous assay
	Urine	100		NaH_2PO_4 (0.1 M)		1.5	

cretion declines more dramatically than filtration in the elderly. Based upon these findings, the authors proposed individualized dosing of procainamide in elderly patients. In a subsequent investigation, Lin et al. reported that the ratio of renal clearance to creatinine clearance of famotidine decreased as creatinine clearance decreased [43]. The authors concluded that the age-mediated deterioration in the secretion process was much faster than glomerular filtration and was incompatible with 'The Whole Nephron Hypothesis'.

Studying the pharmacokinetics of pindolol, Ujhelyi et al. demonstrated that pindolol clearance was similar in elderly and young subjects despite a lower creatinine clearance in the elderly subjects [7]. Although an inhibitor of organic cation secretion was able to affect transport equally in both study groups (elderly and young), a stimulator of organic cation secretion had a less pronounced effect in the elderly subjects, suggesting that aging affects the capacity of the transporter, but not the ability of one agent to inhibit the transport of another. Non-parallel changes in filtration and secretion have been demonstrated in patients with renal insufficiency who received chlorpropamide, ampicillin and cephalexin [9,10].

Studies using various animal models support these clinical findings. Maiza and Yates have investigated renal drug clearance in several models of experimental renal failure [6–8]. Among various types of induced renal failure (proximal tubular necrosis, glomerulonephritis and papillary necrosis), GFR and tubular function were not affected to the same extent. This observed glomerulo-tubular imbalance, which is incompatible with the 'Whole Nephron Hypothesis' resulted in variable clearance of several medications, including cimetidine and cephalexin.

The present study of PAH excretion in the IPK support these findings of other investigators that challenge the validity of the 'Whole Nephron Hypothesis' as it pertains to renal disposition of medications. However, several potential drawbacks to this study should be noted. First, the IPK is an artificial system. Other studies have demonstrated the utility of the IPK for predicting renal drug disposition in vivo [34,44,45]. Consequently, the IPK is a potentially useful model to study the effects of aging on renal drug excretion. However, studies in whole animals are needed to confirm the findings of this research. Second, perfusion experiments were limited to three study groups, with the aged group representing 12–16 months. In the rat species, senescent rats generally fall within the 24–28 month range. Therefore, a future direction is to expand the research to this age range and to increase the number of perfusion experiments conducted for each study group ($n = 3$ for the aged treatment group in the present investigation). Third, the potential contribution of increased perfusion flow rates to PAH excretion in kidneys from aged rats must be considered. Being a high extraction ratio compound across the kidney, PAH clearance is expected to be influenced by flow rate. Since the perfusion flow rate is significantly increased in IPK methodology (a limit of this 'artificial' model), this may be the reason for the surprisingly high renal clearance values despite diminished kidney filtration. However, Redegeld et al. have reported that PAH clearance was independent of flow rate in the IPK [46], which supports our theory that secretory transport can be maintained despite decreased filtration (as reflected through GFR). Moreover, since PAH does display flow dependent clearance in vivo, the observed decrease in PAH excretion in geriatric subjects [2,47] may be more reflective of compromised cardiovascular function rather than altered intrinsic secretory clearance.

5. Conclusions

An HPLC assay for PAH in human plasma was validated in the present study. The assay displayed excellent reproducibility and was linear over standard concentrations of 1–25 μg/ml. The method is simple, rapid and requires a minimum amount of organic solvent. Consequently, it is a less expensive alternative to those previously reported.

IPK studies demonstrated an age-dependent reduction in PAH excretion. However, the limited correlation between PAH clearance and GFR observed in the investigation contradicts the 'Whole Nephron Hypothesis'. Further studies are needed to address this clinically important topic.

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