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Simultaneous determination of *p*-aminohippuric acid, acetyl-*p*-aminohippuric acid and iothalamate in human plasma and urine by high-performance liquid chromatography

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Abstract

A sensitive and specific high-performance liquid chromatographic assay was developed for the simultaneous determination of *p*-aminohippuric acid (PAH), acetyl-*p*-aminohippuric acid (aPAH), and iothalamate in human plasma and urine. Plasma samples were prepared by protein precipitation with acetonitrile followed by evaporation, reconstitution in mobile phase, and injection onto a C_{18} reversed-phase column. Urine samples were diluted with 3 volumes of mobile phase prior to injection. Column effluent was monitored by UV detection at 254 nm. The lower limits of quantification in plasma were 0.5 mg/l for PAH and aPAH, and 1.0 mg/l for iothalamate. The within-day and between-day coefficients of variation in plasma and urine were $\leq$7.8% for all analytes. This method is well suited for renal function studies using iothalamate and PAH, whether administered as a bolus dose or by continuous infusion, to measure glomerular filtration rate and effective renal plasma flow, respectively.

Keywords: *p*-Aminohippuric acid; Acetyl-*p*-aminohippuric acid; Iothalamate

1. Introduction

Accurate measurement of glomerular filtration (GFR) and effective renal plasma flow (ERPF) are valuable for evaluating kidney function and nephrotoxicity in a variety of clinical research and patient care settings. Accurate GFR determinations are recommended to appropriately assess the value of pharmacotherapeutic interventions aimed at improving glomerular hemodynamics and slowing the progression of renal disease [1,2]. Marker compounds such as iothalamate, iohexol, or inulin have been used extensively to estimate GFR, while ERPF is most commonly estimated using *p*-aminohippuric acid (PAH) or *ortho*-iodohippurate (I^{131}). Although renal clearance of these markers during continuous infusion is considered the gold standard, simplified methods using limited sampling after intravenous bolus administration with and without urine collection have been proposed [3,4].

PAH is an ideal marker for estimating ERPF since it is freely filtered at the glomerulus and undergoes extensive secretion and negligible reabsorption within renal tubules. However approximately 20% of PAH is metabolized to acetyl-*p*-aminohippuric acid (aPAH), which is also extensively secreted [5,6].

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Competition for anionic tubular secretion between PAH and aPAH has been observed after bolus dose administration and continuous infusion of PAH, however the clinical significance of this competitive interaction on ERPF estimation is unknown [5,7].

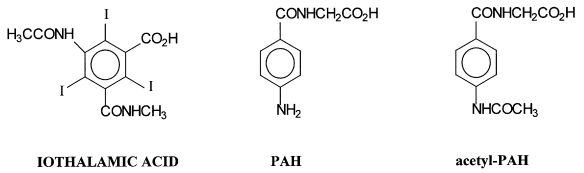
Radiolabeled iothalamate (I^{125}) or *ortho*-iodohippurate (I^{131}) permit accurate determination of GFR and ERPF, however issues such as radiation exposure, storage and disposal of hazardous materials, as well as cost have limited the use of these procedures [8,9]. To circumvent these hazards, HPLC methods for detection of iothalamate in plasma and urine were introduced. The major limitations of these methods include large sample volume requirements (0.5–1.0 ml), tedious extraction procedures or, most importantly, insufficient sensitivity to measure iothalamate following bolus dose administration [10–13].

Detection of PAH in plasma and urine has historically employed a nonspecific colorimetric method that is susceptible to many spectral interferences including sulfanilamide, procainamide and *p*-aminobenzoic acid (PABA) [14]. When using a colorimetric method, detection of aPAH in plasma and urine requires acid hydrolysis to PAH, sample reanalysis and subsequent calculation of total PAH. This cumbersome process has rarely been used [7,15]. However, methods that permit determination of PAH alone or with iothalamate, included only plasma analysis [12] or did not quantify aPAH in plasma and urine [13,16–18]. Only two HPLC methods for quantification of both PAH and aPAH in plasma and urine have been reported, however the assays did not incorporate a GFR marker such as iothalamate [5,19]. In this report, we describe a sensitive and reproducible HPLC method for determining iothalamate, PAH and aPAH in human plasma and urine. This method has been used extensively to quantify GFR and ERPF in clinical investigations.

2. Experimental

2.1. Reagents and chemicals

PAH (Fig. 1), β-hydroxyethyltheophylline (BHET) and tetrabutylammoniumbromide (TBA) were purchased from Sigma (St. Louis, MO, USA). Iothalamate was obtained from Mallinkrodt (St. Louis, MO, USA). All organic solvents (HPLC grade methanol, acetonitrile, tetrahydrofuran), sodium acetate and acetic anhydride were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized, distilled water was acquired from a Barnstead Nanopure purification system (Barnstead, Boston, MA, USA). Acetyl-PAH (aPAH) is not available commercially and therefore was synthesized according to the method of Newman et al. [7]. Briefly, 20 ml of a 1% PAH solution was combined with 1.9 ml acetic anhydride and allowed to stand at room temperature for 30 min with occasional shaking. The mixture was cooled in an ice bath, filtered by suction through a Buchner funnel and washed several times with ice-cold deionized, distilled water followed by 95% ethanol. The precipitate was spread out on filter paper to dry in room air overnight. The purity and identity of aPAH was verified by HPLC and gas chromatography–mass spectrometry. Drug free human plasma was obtained from the Blood Bank.



IOTHALAMIC ACID PAH acetyl-PAH

Fig. 1. Molecular structures of iothalamate, PAH and aPAH.

2.2. Instrumentation

The HPLC system consisted of a Waters model 501 HPLC pump, a model 712 WISP autoinjector and a model 481 tunable absorbance detector set at 254 nm (Waters, Milford, MA, USA). The chromatographic data was collected and analyzed using MILLENIUM chromatography manager (Waters, version 2.15). Separation was achieved with an Alphabond C_{18}, 10 μm particle size, 25 cm×3.9 mm I.D., reversed-phase HPLC column preceded by an Alltech guard column packed with C_{18} Bondapak/Corasil 37–50 μm column packing. The mobile phase used for plasma analysis consisted of 12% methanol, 3% acetonitrile, and 0.65 m*M* TBA in 20 m*M* sodium acetate buffer. Slight adjustments were made to the composition and flow-rate of the mobile phase used for urine analysis to eliminate interference from a urine constituent observed in patients

with severe renal disease. The mobile phase used for urine analysis consisted of 6.5% methanol, 2% acetonitrile, 0.2% THF and 3.0 m*M* TBA in 70 m*M* sodium acetate buffer. Mobile phases were filtered through a 0.45 μm filter and delivered at a rate of 1.0 and 1.4 ml/min for plasma and urine analysis, respectively. The total analysis time for each sample was 20 min.

2.2.1. Metabolite characterization

The structure of aPAH was verified on a Finnigan GCQ mass spectrometer (Finnigan Corp., San Jose, CA, USA) equipped with a direct exposure probe. One microliter of a methanolic solution of PAH or aPAH (1 mg/ml) was evaporated onto the wire tip of the probe. The probe was inserted into the ion source (maintained at 175°C) and was volatilized by rapid heating of the wire. A full scan spectra from 50–650 a.m.u. was run for each compound.

2.3. Preparation of stock solutions and spiked standards

Stock solutions of 1.0, 10.0 and 100.0 mg/ml for PAH, 1.0 and 10.0 mg/ml for iothalamate, 1.0 and 20.0 mg/ml for aPAH and 1.0 mg/ml for BHET were made in methanol and stored at 4°C. These solutions were stable for at least 1 month at 4°C and were used to spike plasma and urine samples in the preparation of standards. Standards and quality control samples were made by addition of the specified quantity of stock solution to drug free plasma and urine and stored in 100-μl aliquots at −20°C.

2.4. Preparation of plasma samples

Aliquots (100 μl) of plasma (standards, quality controls or patient samples) were pipetted into 1.5-ml microcentrifuge tubes followed by the addition of 300 μl of the working internal standard solution (BHET 12.5 μg/ml in acetonitrile). After capping, each tube was vortex-mixed briefly and centrifuged at 16 000 *g* for 8 min. A 200-μl aliquot of the supernatant was then transferred to a clean 1.5-ml microcentrifuge tube, and evaporated to dryness at 37°C under a stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase, which was transferred to a WISP microinsert; 20-μl aliquots were injected into the HPLC system.

2.5. Preparation of urine samples

Urine samples were prepared by adding 300 μl of mobile phase spiked with internal standard (120 μg/ml) to 100 μl of urine (standard, quality control or patient samples). After capping, each tube was vortex-mixed briefly. An aliquot of 100 μl of each sample was transferred to a WISP microinsert and 10 μl was injected into the HPLC system.

2.6. Calibration and linearity

Calibration curves were constructed using duplicates of six standards of each analyte in plasma and urine and were obtained daily for 3 days by calculating the peak-height ratios of each analyte to the internal standard against the corresponding concentration of each analyte. Linear calibration curves were generated by weighted ($1/y^2$) linear regression analysis and obtained for each analyte over the respective standard concentration range. The lower limit of quantitation (LOQ) for each analyte and matrix was selected as the concentration at which the assay precision was within 20% and the signal-to-noise ratio exceeded 3:1. In plasma, the standard concentration ranges for PAH, aPAH and iothalamate were 0.50–75.0, 0.50–5.0 and 1.0–75.0 μg/ml, respectively. In urine, the standard concentrations ranges for PAH, aPAH, and iothalamate were 5.0–2500.0, 5.0–500.0 and 25.0–1500.0 μg/ml, respectively. All standards and QC samples were stored at −20°C until analysis.

2.7. Precision and accuracy

The precision and accuracy of the assay was determined through analysis of plasma and urine QC samples for each analyte. Plasma QC sample concentrations for iothalamate and PAH were 3, 20 and 50 mg/l and for aPAH were 0.75, 1.75 and 4.0 mg/l. Urine QC sample concentrations were 40, 250 and 1250 mg/l for iothalamate, 7.5, 250 and 1500 mg/l for PAH, and 15, 80 and 300 mg/l for aPAH. Seven replicate QC samples at each concentration were analyzed on 3 consecutive days, after which inter-

and intra-day means, standard deviations, and coefficients of variation (C.V.) were calculated.

2.8. Stability

Plasma and urine QC samples were subjected to three freeze–thaw cycles (−20°C and room temperature) and a 48-h stability test at room temperature. The concentrations of each analyte in the samples were then determined.

2.9. Application to renal function studies

Blank plasma and urine samples from six different sources including normal healthy volunteers, patients with severe or end stage renal disease, and patients with liver disease were evaluated for interference with the analytes and internal standard. PAH and iothalamate clearances were determined in volunteers with varying degrees of renal function. Patients allergic to iodine or shellfish were excluded from participation. PAH and iothalamate were administered by both single bolus injection and continuous infusion methods. For the single bolus injection studies, PAH (5 mg/kg) and iothalamate (434 mg, 2 ml Conray-30) were given consecutively over 5 min. Blood samples were collected prior to drug administration and for 180 min after. Urine was collected prior to drug administration and at 30-min intervals throughout the 180-min study. After a 120-min wash-out period, PAH and iothalamate were given as loading doses (3 mg/kg and 217 mg, respectively) followed by a continuous infusion (approx. 15 and 4 mg/min, respectively) designed to achieve steady-state plasma concentrations of 20–25 mg/l. Plasma and urine were collected prior to drug administration and at 30-min intervals throughout the 150-min study. Plasma was separated by centrifugation at 3000 *g* for 10 min. Plasma and urine aliquots were stored at −20°C until analysis.

The PAH and iothalamate concentration–time data obtained following administration by single bolus injection were fitted to a two-compartment model ($C_p = Ae^{-\alpha t} + Be^{-\beta t}$). Plasma clearance ($CL_P$) was calculated as dose/$AUC_{0-\infty}$, where $AUC_{0-\infty}$ is the area under the concentration–time curve extrapolated to infinity, calculated as $AUC_{0-\infty} = A/\alpha + B/\beta$. To calculate CL_P during the constant infusion, data were fitted to a one-compartment model using the zero-order infusion rate as the input function. Renal clearance (CL_R) for both administration methods was calculated as $CL_R = \Sigma Au_{t_1-t_2}/\Sigma AUC_{t_1-t_2}$, where $Au_{t_1-t_2}$ is the amount of drug recovered in the urine during each urine collection interval from time t_1 to time t_2 and $AUC_{t_1-t_2}$ is the AUC during the same time interval calculated using the trapezoidal rule.

3. Results and discussion

Since aPAH was not commercially available it was necessary to synthesize it and subsequently verify the structure of the synthetic product. Structure verification was carried out with a Finnegan GCQ mass spectrometer run in the full scan mode. The mass spectra of authentic PAH and synthesized aPAH are presented in Fig. 2. The spectrum of PAH (Fig. 2A) corresponds to published reference spectra [20] in

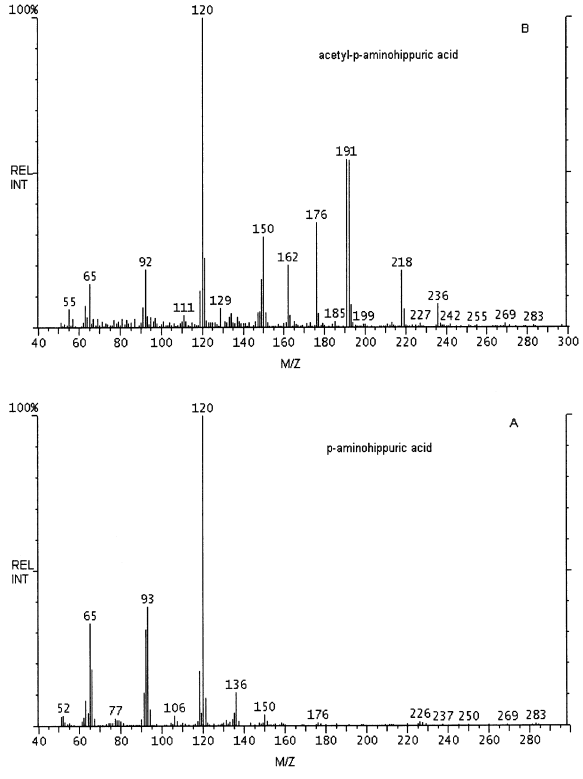


Fig. 2. Mass spectrum of PAH and aPAH.

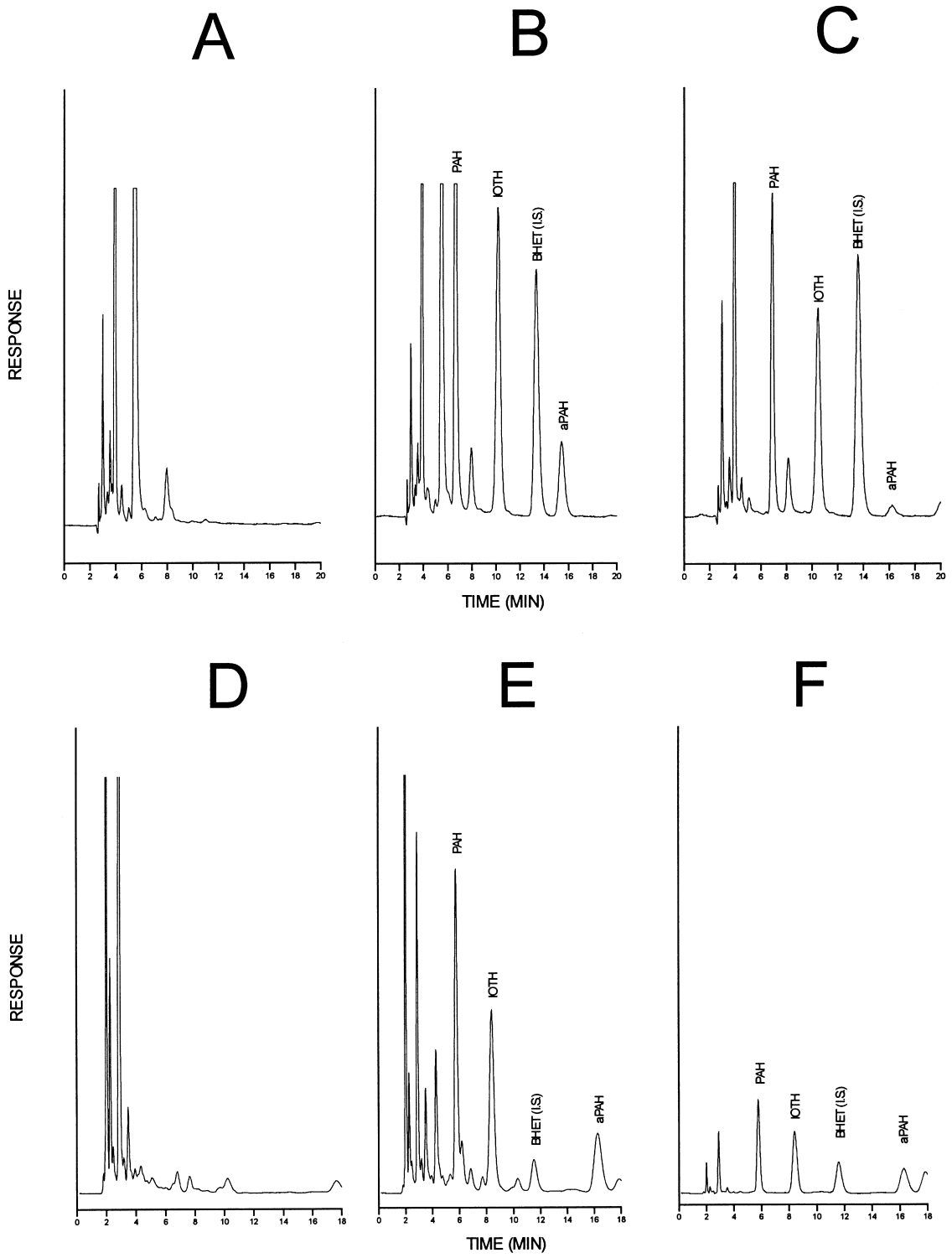


Fig. 3. Representative chromatograms of (A) blank plasma, (B) plasma quality control (HC), (C) patient sample, (D) blank urine, (E) urine quality control (MC), and (F) patient sample. Patient samples were obtained after bolus administration (see text).

Table 1
Mean parameters of the calibration curves for PAH, aPAH, and iothalamate

	$y=mx+b$		Regression coefficient (r)
	m (mean±S.D.)	b (mean±S.D.)	
Plasma ($n=6$)			
PAH	0.100±0.002	−0.008±0.007	0.998
aPAH	0.066±0.002	−0.001±0.001	0.997
Iothalamate	0.054±0.001	−0.005±0.008	0.996
Urine ($n=6$)			
PAH	0.005±0.001	−0.0007±0.003	0.999
aPAH	0.003±0.001	−0.0004±0.001	0.998
Iothalamate	0.002±0.0002	0.0012±0.003	0.998

terms of base peak and prominent fragments. Little parent ion ($m/z=194$) is present, however. The spectrum of the synthesized aPAH is presented in Fig. 2B. The parent ion at $m/z=236$ corresponds to the molecular mass of authentic aPAH. The fragment at $m/z=150$ in the PAH spectrum represents the loss of CO_2 (44). The corresponding fragment in the aPAH spectrum is at $m/z=192$, representing the loss of CO_2 from aPAH. The presence of the acetylated parent and acetylated fragment in the spectrum of the synthesized aPAH verify its structure. HPLC analysis of authentic aPAH demonstrated a single symmetrical peak with a retention time corresponding to the peak observed in plasma and urine samples from subjects given PAH.

Representative chromatograms from plasma and urine QC and subject samples are shown in Fig. 3. In plasma, the absolute and relative (%) retention times for PAH, iothalamate, BHET and aPAH were 6.7 min (52), 10.2 min (78), 13.0 min and 15.3 min (118), respectively. In urine, the absolute and relative (%) retention times for PAH, iothalamate, BHET and aPAH were 5.9 min (50), 8.5 min (72), 11.8 min and 16.2 min (137), respectively. Acceptable column performance based on peak shape and retention times was maintained for approximately 1000 injections. Linear calibration curves were obtained for each analyte with correlation coefficients (r) greater than 0.99; mean standard curve parameters for each

Table 2
Intra- and inter-day precision and accuracy for PAH, iothalamate and aPAH in plasma

Analyte	Concentration (mg/l)		C.V. (%)	% Deviation (found versus added)
	Added	Found (mean±S.D.)		
Intra-assay reproducibility[a]				
PAH	3	3.1±0.1	2.8	1.5
	20	20.3±1.2	5.8	1.5
	50	48.7±0.9	2.3	−6.3
Iothalamate	3	2.9±0.1	2.3	−5.1
	20	21.3±1.3	6.0	6.5
	50	48.4±0.8	1.7	−3.3
aPAH	0.75	0.74±0.03	3.7	−1.7
	1.75	1.71±0.04	2.3	−2.5
	4	4.3±0.1	3.2	6.4
Inter-assay reproducibility[b]				
PAH	3	3.2±0.2	6.7	5.7
	20	21.1±1.1	5.2	5.6
	50	47.8±1.3	2.8	−4.5
Iothalamate	3	2.9±0.2	5.5	−3.4
	20	21.1±1.1	5.2	5.6
	50	47.6±1.2	2.4	−4.7
aPAH	0.75	0.79±0.05	6.1	4.8
	1.75	1.69±0.04	2.6	−3.4
	4	4.3±0.2	4.8	7.6

[a] Seven samples per concentration.
[b] Seven samples per day per concentration per 3 days.

analyte in each matrix are reported in Table 1. The intra- and inter-day precision and C.V. were determined for each analyte in plasma (Table 2) and urine (Table 3) and were 7.8% or less. At the LOQ, the signal-to-noise ratio was greater than 4:1 and intra- and inter-day C.V. was less than 15% for each analyte in both plasma and urine. Plasma and urine QC samples were stable at room temperature for 48 h and through three freeze–thaw cycles. No analytical interference was found in plasma or urine samples from different populations including healthy volunteers, patients with renal or liver disease or from the coadministered drugs (Table 4).

Simplified sample processing, increased sensitivity, and detection of aPAH are significant advantages of this assay over previously reported methods. Sample processing consists of only protein precipitation (plasma) or dilution (urine) without extensive extraction, thus minimizing sample processing time. The increased sensitivity of this method relative to those previously reported permits quantitation of PAH and iothalamate for at least 180 min following bolus dose administration. This is essential for adequate characterization of PAH and iothalamate disposition and accurate clearance estimates when bolus dose administration is used. Previously reported limits of quantitation (greater than 2.5 mg/l) [13,18,19] would have yielded measurable PAH concentrations for only approximately 60 min, thereby preventing adequate characterization of the terminal plasma elimination phase resulting in an overestimation of clearance. A representative plasma con-

Table 4
Alphabetical listing of concurrent medications used by patients evaluated

Alendronate	Disopyramide	Prednisone
Allopurinol	Enalapril	Propranolol
Ampicillin/sulbactam	Famotidine	Sertraline
Atenolol	Furosemide	Spironolactone
Calcitriol	Metoprolol	Sulfamethoxazole
Clonazepam	Nifedipine	Tacrolimus
Conjugated estrogens	Omeprazole	Trazeodone
Cyclosporine	Pentoxifylline	Trimethoprim
Diltiazem	Phenytoin	

Table 3
Intra- and inter-day precision and accuracy for PAH, iothalamate and aPAH in urine

Analyte	Concentration (mg/l)		C.V. (%)	% Deviation (found versus added)
	Added	Found (mean±S.D.)		
Intra-assay reproducibility[a]				
PAH	7.5	7.2±0.2	3.3	−3.9
	250	248.3±3.3	1.3	−0.7
	1500	1486.3±11.4	0.8	−0.9
Iothalamate	40	40.1±0.7	1.8	0.2
	250	235.6±2.9	1.2	−5.8
	1250	1254.1±10.4	0.8	0.3
aPAH	15	14.7±0.26	1.8	−2.1
	80	77.5±1.1	1.5	−3.1
	300	300.3±2.6	0.9	0.1
Inter-assay reproducibility[b]				
PAH	7.5	6.8±0.5	7.8	−9.5
	250	245.9±4.1	1.7	−1.6
	1500	1482.7±32.0	2.2	−1.2
Iothalamate	40	40.2±0.8	1.9	0.6
	250	233.9±4.0	1.7	−6.4
	1250	1235.3±31.0	2.5	−1.2
aPAH	15	15.0±0.6	3.7	−0.1
	80	77.2±1.8	2.3	−3.5
	300	295.8±9.0	3.0	−1.4

[a] Seven samples per concentration.
[b] Seven samples per day per concentration per 3 days.

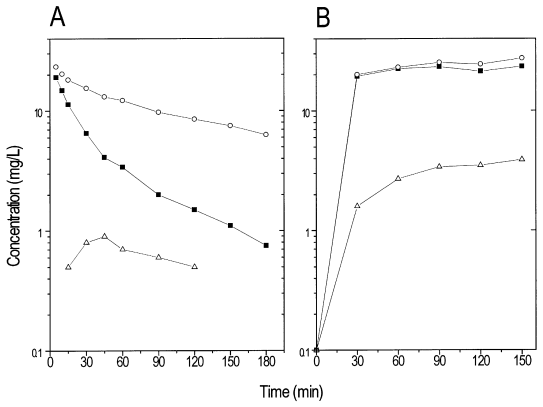


Fig. 4. Plasma concentrations of iothalamate (○), PAH (■), and aPAH (△) following single intravenous bolus (A) and continuous infusion (B) doses of iothalamate and PAH (see text). Plasma clearance estimates in this patient are 136 and 582 ml/min for iothalamate and PAH, respectively.

centration versus time curve obtained following administration by single bolus and continuous infusion is shown in Fig. 4. With the method described, adequate characterization of PAH and iothalamate following single bolus and continuous infusion dosing was achieved with clearance estimates corresponding to ERPF and GFR measurements, respectively. In addition to traditional clearance studies with multiple plasma and urine samples, we and others, are evaluating the potential for renal function evaluation in the clinic with studies aimed at identifying limited plasma sampling schemes that are validated against renal clearance measurements [21–23].

The internal standard used in this assay may also provide some advantages over the more commonly used PABA, which is structurally similar to PAH. Interference with PABA as an internal standard may occur since it is found naturally in some foods (i.e., cereals, meats, etc.), is part of a number of B-complex and multivitamin preparations, and is excreted in the urine [24,25]. Therefore, we chose BHET as the internal standard to minimize any potential for interference in patient samples.

Although PAH renal clearance is an accepted measure of ERPF, both renal and extrarenal acetylation of PAH occurs in man [5–7,15,18,19]. Prior methods of analysis were unable to differentiate PAH from aPAH in plasma and urine yielding the invalid assumption that PAH is not significantly metabolized. Furthermore, when compared to specific HPLC methods of analysis, nonspecific (colorimetric) methods yield consistently higher PAH concentrations in urine and lower concentrations in plasma, which results in 35% higher estimates of renal clearance [19,26]. In addition, we and others have shown that approximately 20% of a bolus dose is excreted as metabolite and that urinary aPAH excretion is a concentration-dependent process [5,17]. However, the clinical significance of acetylation with respect to the total body and renal clearances, as well as, the concentration-dependent interactions between PAH and aPAH within the kidney remain to be elucidated. Therefore, these interactions may be further evaluated using the present assay to quantify PAH and aPAH in plasma and urine, coupled with the ability to characterize glomerular filtration rate using iothalamate.

In conclusion, the method reported here is a sensitive and reproducible assay for the simultaneous determination of PAH, iothalamate and aPAH in plasma and urine. This method is currently being used in clinical pharmacokinetic studies for quantification of renal function.

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