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Chromatographic estimation of iothalamate and *p*-aminohippuric acid to measure glomerular filtration rate and effective renal plasma flow in humans

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Abstract

Iothalamate (IOT) clearance and *p*-aminohippuric acid (PAH) clearance are used for estimation of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF). A simple and rapid method is described for simultaneous determination of IOT and PAH in the same chromatogram in the serum and urine of humans. The mobile phase consisted of methanol–50 m*M* sodium monobasic phosphate with 0.5 m*M* tetrabutyl ammonium hydrogen sulfate (18:82, v/v), pumped at a rate of 0.8 ml/min on a C_{18} reversed-phase column. Samples of serum and urine were deproteinized with two volumes of acetonitrile containing the internal standard, *p*-aminobenzoic acid (PABA). The UV detector was set at 254 nm and peak height ratios of PAH or IOT to PABA were calculated with an integrator. Precision and accuracy were within 15% for both PAH and IOT. The recovery of PAH in urine and serum were 94% and 91%, respectively. For IOT the corresponding recoveries were 93% and 92%, respectively. This method clearly distinguishes acetyl-PAH from PAH and has been validated in healthy volunteers.

Keywords: Iothalamate; *p*-Aminohippuric acid

1. Introduction

Measurement of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) are essential for the study of renal hemodynamics. Serum creatinine and creatinine clearance are poor markers of GFR and can be influenced by a variety of drugs and glomerular diseases [1–5]. Traditionally, inulin [6,7] or iothalamate (IOT) clearance [8–13] are used to measure GFR and *p*-aminohippuric acid (PAH) clearance is used to estimate ERPF [14–16]. Inulin is expensive, is in short supply, and its colorimetric assay is nonspecific and has technical difficulties [17]. PAH estimation by modified Bratton Marshall reaction is cumbersome, unreliable in the presence of glucose and certain drugs such as sulfonamides, procainamide and some local anesthetics, and the colorimetric reaction is nonspecific [18]. Frequently, IOT and PAH clearances are performed with radioactive compounds [12,13,19], which creates additional problems such as obtaining permission from institutional review boards for use in healthy volunteers, disposal of waste and radiation exposure of subjects and workers.

In the last decade, high-performance liquid chromatography (HPLC) techniques have been automated with the availability of autoinjectors, integrators and online processing of data. In parallel,

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several techniques have been published for the estimation of IOT and PAH by HPLC but are not without problems. These problems include extensive extraction procedures [20], peak splitting with large volume injections [21], lack of internal standard [22] and the need for dual-wavelength UV detection [23]. Some techniques do not have the capability to measure PAH and IOT simultaneously [22,24,25]. Herein is reported a method that allows estimation of PAH and IOT in the same chromatogram. The method is a modification of that of Bell et al. [26], who used the technique to measure GFR and ERPF in rats.

2. Experimental

2.1. Reagents

Sodium monobasic phosphate, *p*-aminobenzoic acid (PABA), acetic anhydride and tetrabutyl ammonium hydrogen sulfate were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and 4-aminohippuric acid (PAH) purchased from Aldrich (Milwaukee, WI, USA). Iothalamic acid was a gift of Malinckrodt Medical (St. Louis, MO, USA).

Acetyl-PAH was prepared by the method of Newman et al. [27]. Briefly, a 10.4 *M* solution of acetic anhydride, was added to a 10% solution of PAH (Merck, Rahway, NJ, USA), in a molar ratio of 2:1. The solution was gently rotated for 1 h 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.

2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of a Hewlett-Packard Chromatographic Series 1100, consisting of a variable-wavelength UV detector with wavelength set at 254 nm and an isocratic pump set at a flow-rate of 0.8 ml/min. The mobile phase was methanol–buffer (18:82, v/v). The buffer consisted of 50 m*M* sodium monobasic phosphate with 0.5 m*M* tetrabutyl ammonium hydrogen sulfate with an unadjusted pH of 4.11. The column was a 25 cm C_{18} Primesphere reversed phase column (particle size 5 μm) purchased from Phenomenex (Torrance, CA, USA). The guard column containing 5 μm, C_{18} Ultrasphere packing was purchased from Alltech Associates (Deerfield, IL, USA). A Waters Intelligent Sample Processor (WISP, Model 712, Waters, Milford, MA, USA) was used to inject 20 μl of sample each with a run time of 22 min. Peak areas were determined using a Spectra-Physics ChromJet integrator set at a chart speed of 0.25 cm/min. All analysis were performed at room temperature.

2.3. Solution preparation

Stock solutions of PAH and IOT were prepared as 2 mg/ml in distilled deionized water. Stock solutions of acetyl-PAH (1 mg/ml) and PABA (1 mg/ml) were also made in distilled deionized water. Standard curve and quality control (QC) samples were freshly prepared for each run.

2.4. Standards and quality controls

Ten standards ranging from 5 to 200 μg/ml were made for each matrix-distilled deionized water, urine and serum. Standard stock solution containing 2 mg/ml IOT and PAH was diluted 1:10 with water to yield a concentration of 200 μg/ml. Subsequently, 200 μl of the concentrate was added serially to 100 μl of water to yield standards containing 133.33, 88.89, 59.26, 39.51, 26.34, 17.56, 11.71, 7.80 and 5.20 μg/ml of PAH and IOT. To prepare the standard curve in urine, a subject's urine, prior to infusion of PAH or IOT, was diluted 10-fold with water. This was used in place of water to prepare standards in urine yielding the same concentrations as above. Similarly, standards were made using serum from a subject that did not contain PAH or IOT.

Quality controls were prepared from a separate standard stock solution of IOT and PAH (2 mg/ml). Nominal concentrations of 10 and 40 μg/ml in serum and 20 and 100 μg/ml in urine were used to assess low- and high-quality controls, respectively. Five low- and five high-quality controls were prepared in serum and urine. High (40 μg/ml serum or 100 μg/ml urine) and low (10 μg/ml in serum and

20 μg/ml in urine) quality controls were run to assess intra-assay coefficient of variation.

Serum containing 10 and 40 μg/ml PAH and IOT and urine containing 20 and 100 μg/ml PAH and IOT were run on five days to assess inter-day coefficient of variation. For each of these runs, a nine-point standard curve for serum and six-point standard curve for urine was generated.

2.5. Sample preparation

The subject's urine was diluted 1:10 with water. 100 μl of the diluted urine was transferred into microcentrifuge tubes. To this was added 200 μl of acetonitrile containing the internal standard *p*-aminobenzoic acid (20 μg/ml). The tubes were vortexed briefly and then centrifuged at 12 000 *g* for 4 min. The clear supernatant was transferred to WISP vials and 20 μl injected into the HPLC system. Sera were treated identically to urines, except they were not diluted.

2.6. Calculations

Standard curves were created by linear regression of peak area ratios of PAH and IOT to PABA versus known concentrations of PAH and IOT. Concentrations of QC and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown sample peak-area ratio.

2.7. Recovery of PAH and IOT

The recovery of PAH and IOT from urine and serum was determined by calculating the ratio of slopes of IOT and PAH standard curves against the slope of the same standards prepared in distilled deionized water.

2.8. Precision and accuracy

Precision of the assay was assessed over two concentrations, 10 and 40 μg/ml in serum and 20 and 100 μg/ml in urine. Five separate samples at each concentration were assayed. Precision and accuracy are reported as percent coefficient of variation and percent accuracy [(observed−expected)×100/expected concentration], respectively.

2.9. Subjects

The study was approved by the Institutional Review Board for Human Studies of Indiana University. Written informed consent was obtained and retained from each volunteer. Nine healthy volunteers aged 20 to 50 years were recruited for the study. In four healthy volunteers a 3-g dose of IOT meglumine (5 ml of Conray 60, Malinkrodt, St.

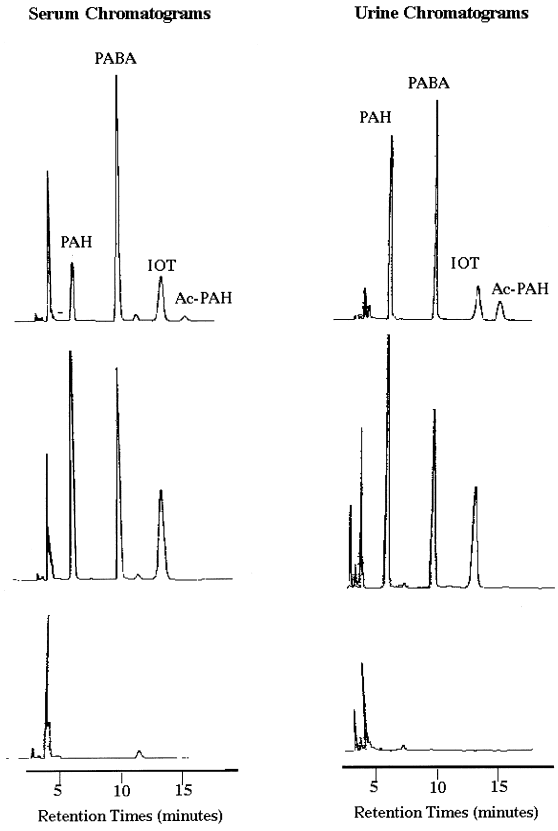

Fig. 1. Chromatograms obtained after 20 μl injection of serum and diluted urine. The left panels show the serum chromatograms and the right panels exhibit the urine chromatograms. The bottom panels are nonspiked serum and diluted urines, the middle panels represent serum and diluted urine spiked with PAH, PABA and iothalamate. The top panels illustrate serum and diluted urine from a healthy volunteer administered PAH and iothalamate. Note the acetyl-PAH peak in the top panels is absent in the bottom and middle panels.

Louis, MO, USA) and a loading dose of 10 mg/kg of 20% PAH (Merck) was administered intravenously. A water load of 10 ml/kg body mass was given orally and to maintain urine flow, the urine output was replaced ml for ml with oral distilled water. IOT was not infused after the bolus injection. This was followed by an infusion of a solution of PAH in normal saline at a rate calculated to give a serum concentration of PAH of between 10 and 20 μg/ml. In a healthy water loaded volunteer, IOT was given in the same dose but without PAH. The study lasted over 12 h with frequent blood and urine sampling. In four separate healthy volunteers, PAH was given in a loading dose of 10 mg/kg, followed by an infusion of 20 mg/min as a 20 mg/ml solution in saline. Urine and blood were sampled every 30 min for three consecutive periods after 1 h of PAH infusion. Each urine collection period was bracketed by serum samples. All samples were stored at −20°C until analyzed (within one month).

3. Results and discussion

3.1. Chromatography

Chromatograms from a typical blank human serum and urine, and ones supplemented with PAH, IOT and PABA, are shown in the bottom and middle panels of Fig. 1, respectively. A representative chromatogram, in urine and serum, after injecting PAH and IOT in a human subject, is shown in the top panel of Fig. 1.

The chromatographic conditions were adapted from a previously published report [26]. Some changes of the previous method included reduction in the molarity of the buffer, reduction of the ion pairing reagent and increase of the organic phase concentration in order to improve the peak shape and retention characteristics of the analytes. The molarity of sodium monobasic phosphate was reduced to 50 m*M* from 100 m*M* reported by Bell et al. [26] to prolong the life of the column and HPLC system. Then the molarity of ion pairing agent was altered. The influence of altering the molarity of tetrabutyl ammonium hydrogen sulfate in the aqueous phase on the retention times of each compound is shown in Fig. 2. Since ion pairing affected the retention time

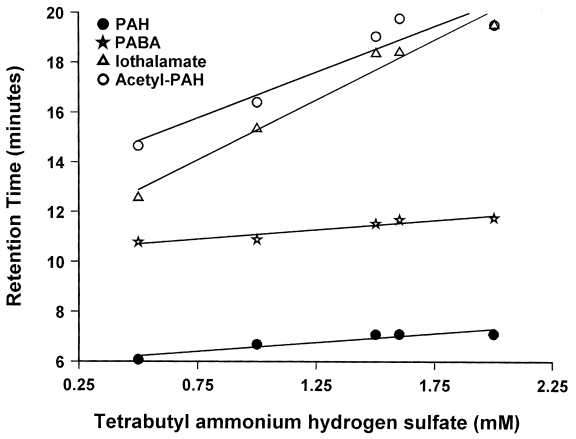

Fig. 2. Retention times of PAH, PABA, iothalamate and acetyl-PAH plotted against the concentration of tetrabutyl ammonium hydrogen sulfate present in the aqueous part of the mobile phase.

of PAH metabolite less than that of IOT it was possible to separate the PAH metabolite from the IOT peak. As can be seen in Fig. 2, a concentration of 0.5 m*M* tetrabutyl ammonium hydrogen sulfate is optimal in separating the IOT peak form that of the PAH metabolite. Finally, the concentration of methanol was increased in the mobile phase to accelerate elution times. It was found that a concentration of 18% methanol provided the best peak separation and retention times (data not shown).

Modification of the HPLC conditions were critical to an accurate analysis of IOT and PAH in humans. In contrast to the results of Bell et al. [26], there was a peak noted in the urine and serum of humans after infusion of PAH alone which coeluted with IOT under previously reported HPLC conditions. Because this peak occurred only after injection of PAH and not IOT it was likely a metabolite of PAH. To test this hypothesis, acetylated PAH was prepared (100 μg/ml in water) and 10 μl was injected on to the column. A single peak was seen with a retention time of approximately 16 min, identical to that seen in serum and urine of the PAH infused subject.

The presence of this acetylated metabolite of PAH was known as early as 1945 when Smith et al. [18] reported its presence in urine. Separation of the acetyl-PAH peak from that of PAH is clinically relevant. Infused PAH is removed from the serum both by renal clearance as well as acetylation. Some

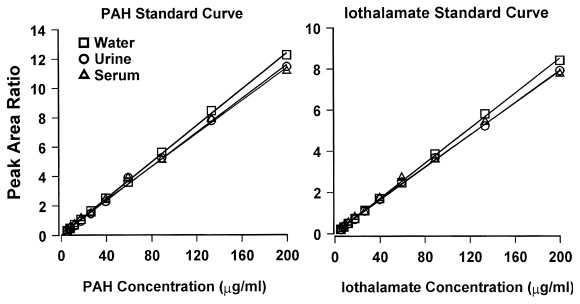

Fig. 3. Standard curve of PAH and iothalamate in water, serum and diluted urine. The peak area ratio is the peak area of PAH or iothalamate to the peak area of PABA. Coefficient of determination was 0.99 for all curves.

investigators have estimated renal plasma flow by serum sampling alone after a continuous infusion of PAH. The infused PAH is assumed to be fully excreted in the urine; urine collections are not made and ERPF inferred from steady state serum levels of PAH. The latter technique is prone to overestimation of ERPF [15]. Therefore, both serum and urine sampling are important for accurate estimation of ERPF.

3.2. Linearity

Calibration curves were created for 10 different concentrations (5–200 μg/ml) in water, urine and serum by plotting the peak area ratio, of PAH or IOT to PABA, versus the nominal drug concentration. For both PAH and IOT, the coefficient of determination was 0.99 or better in water, serum and urine (Fig. 3). The standard error of estimate of PAH and IOT concentration using this standard curve was between 1.5 to 3.6 μg/ml. None of the intercepts were significantly different from zero.

3.3. Recovery of calibration standards

The recoveries of PAH in urine and serum were 94% and 91%, respectively. For IOT the corresponding recoveries were 93% and 92%, respectively. To compare the recoveries in the water, urine and serum, the standard curves are plotted on the same graph for PAH and IOT (Fig. 3).

3.4. Intra-day and inter-day precision and accuracy

Within-assay and between-assay precision and accuracy for both high- and low-concentration of PAH and IOT in urine and serum are reported in Table 1.

When urine and serum samples were allowed to sit at 4°C for two weeks and samples analyzed, no difference in repeated results were noted.

3.5. Human studies

The clearances of PAH and IOT were calculated by the traditional UV/P method where U and P are the urinary and serum concentrations of PAH or IOT, respectively and V the urine flow-rate. Fig. 4 shows

Table 1
Intra-day and inter-day precision and accuracy of iothalamate and PAH estimation in serum and urine

	Precision (C.V., %)		Accuracy (R.E., %)	
	PAH	IOT	PAH	IOT
Intra-day precision and accuracy				
Serum 10 μg/ml	3.7	3.8	3.8	−3.6
Serum 40 μg/ml	5.5	6.2	11.2	12.4
Urine 20 μg/ml	5.9	4.3	−8.3	−7.9
Urine 100 μg/ml	0.7	1.3	−3.8	−2.6
Inter-day precsion and accuracy				
Serum 10 μg/ml	11.8	10.6	−10.9	−14.5
Serum 40 μg/ml	7.2	6.4	1.2	2.5
Urine 20 μg/ml	6.0	11.1	7.8	3.7
Urine 100 μg/ml	2.8	2.1	−0.7	−2.5

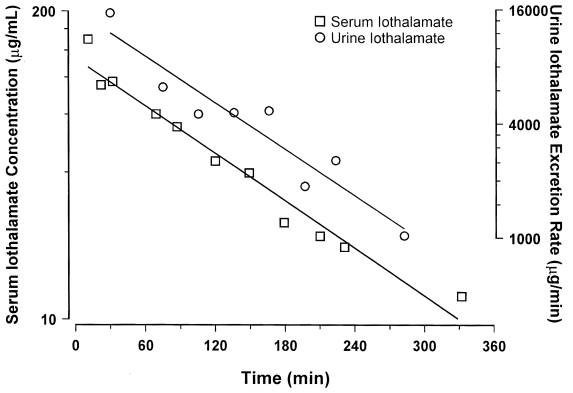

Fig. 4. Serum iothalamate concentration–time profile and urinary excretion–time profile from a volunteer who was administered PAH and iothalamate.

the urinary excretion rate and the serum concentration–time profile of IOT after a single injection. Since IOT clearances were calculated by a single bolus injection, urine IOT excreted per collection interval was plotted against serum area under curve within the same sampling interval (Fig. 5). Slope of

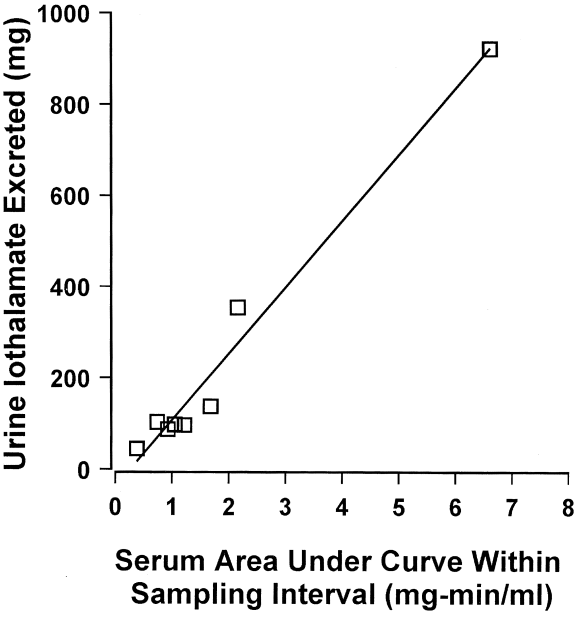

Fig. 5. Urine iothalamate excretion plotted against serum area under the curve within the sampling interval to calculate GFR. The slope of the line is 145 ml/min and when normalized for body surface area is 125 ml/min/1.73 m^2. The coefficient of determination is 0.98.

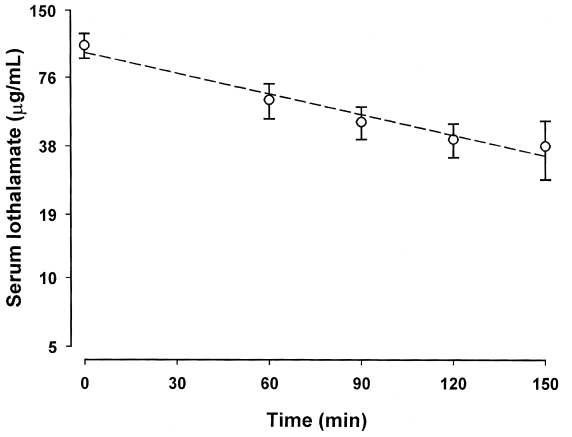

Fig. 6. Serum concentration–time profile of iothalamate in five healthy volunteers. Data represent mean and standard errors after intravenous administration of 3 ml Conray 60. No infusion was given.

this line would be the GFR. This was calculated to be 145 ml/min. When adjusted for body surface area, the GFR is calculated to be 124 ml/min/1.73 m^2. In the five subjects studied the GFRs were 145, 107, 74, 132 and 119 ml/min. The serum concentration–time profile of IOT in these five volunteers is shown in Fig. 6. ERPFs for the eight subjects were 553, 601, 521, 591, 544, 718, 725, 765 and 631 ml/min, respectively. These values are generally what would be expected in healthy volunteers.

In conclusion, we report a new method for measurement of IOT and PAH to estimate GFR and ERPF simultaneously. The current method employs an internal standard, PABA and has the added advantage of clearly distinguishing between acetyl-PAH and PAH. The method has been validated in healthy volunteers and shows that both GFR and ERPF can be rapidly estimated with minimal blood and urine sampling.

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