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Liquid chromatography for iothalamate in biological samples

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Abstract

We have previously reported an iothalamate assay for the assessment of the glomerular filtration rate (GFR) that required a long column equilibration time and 22 min run time per sample. We now report a simpler assay that requires a run time of only 5.5 min and is more precise and accurate than the earlier technique. The mobile phase consisted of methanol–acetonitrile–50 m*M* sodium monobasic phosphate (10:5:85, v/v) at pH 4.4, pumped at a rate of 1.5 ml/min on a C_{18} reversed-phase column. Samples of plasma and urine were deproteinized with 1 volume of 4% perchloric acid or 9 volumes of 2% perchloric acid, respectively. No internal standard was used. The diode array detection system collected absorbance at 240 nm and the peak height areas of iothalamate were determined. The iothalamate peak appeared at 3.5 min. Detector response was linear over the range tested (10–2000 μg/ml). Within-run precision was <3% for both plasma and urine and accuracy was 96–102%. Between-day precision for plasma and urine analyses were <7%. The recovery of iothalamate in urine and plasma were 102% and 91%, respectively. There was excellent thermal and pH stability of iothalamate. No interference was found with *para*-amino hippuric acid (PAH) or *N*-acetyl PAH, which can be simultaneously assayed, if desired.

Keywords: Iothalamate

1. Introduction

Assessment of the glomerular filtration rate (GFR) requires the measurement of urinary clearance of a solute that is neither reabsorbed nor secreted by the renal tubules and is neither protein bound nor metabolized. Inulin qualifies as such a marker but is in short supply, is expensive and is difficult to assay. Alternatively, ^{125}I-Iothalamate which has been extensively used in clinical trials [1], has been validated as a marker of GFR by direct comparison with inulin [2]. Nevertheless, use of a radioactive marker is also compromised by the short shelf life, expense and radiation exposure. Thus there is a need to develop methodology that can be used in clinical trials without the use of a radioactive marker, yet provides precise estimates of GFR.

There are several methods, both HPLC [3–9] and non-HPLC [10], that are available for estimation of iothalamate levels in plasma and urine. We have previously reported an iothalamate assay for the assessment of GFR that required a long column

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equilibration time and 22 min run time per sample [4]. We now report a simpler assay that requires a run time of only 5.5 min and is more precise and accurate than the earlier technique. This rapid technique provides the added advantage of coinfusion of *para*-amino hippuric acid for the simultaneous determination of renal plasma flow.

2. Methods

2.1. Reagents

Sodium monobasic phosphate, HPLC grade methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ, USA) and perchloric acid from Mallinckrodt (Phillipsburg, NJ, USA). Chemical solutions were prepared using distilled deionized water unless otherwise indicated.

2.2. Chromatographic apparatus and conditions

The chromatographic system consisted of a Hewlett-Packard Chromatographic Series 1100 autosampler, diode array detection system, and isocratic pump, all computer controlled via the CHEMSTATION software (Agilent Tech., Palo Alto, CA, USA). The autosampler was programmed to inject 5 μl of each sample with run time of 5.5 min. The mobile phase was comprised of methanol–acetonitrile–buffer (10:5:85, v/v/v) and was pumped at a flow-rate of 1.5 ml/min. The buffer was 50 m*M* potassium monobasic phosphate (dihydrate) with an unadjusted pH of 4.4. The diode array detection system recorded absorbance spectrum from 190 to 600 nm with the signal at 240 nm stored for determination of iothalamate levels. Peak areas were obtained using the CHEMSTATION software.

The column was a 25 cm, 5 μm C_{18} Alltima reversed-phase column (Alltech Associates, Deerfield, IL, USA) attached to an Ultrasphere 5 μm, C_{18} guard column (Alltech Associates). The column and guard were placed in a column warmer with temperature set to 37 °C.

2.3. Standards and quality controls

Seven IOT standards in a drug-free urine were prepared by serial dilution of a 2000 μg/ml IOT stock solution to yield 2000, 1500, 1000, 800, 400 and 200 μg/ml IOT. Drug-free urine from a healthy volunteer was diluted 1:10 with water and an aliquot of this served as a blank standard. Urine quality controls (QCs) were prepared from a 2000 μg/ml IOT stock solution to give nominal concentrations of 1200 and 300 μg/ml IOT.

Expired, drug-free, fresh frozen plasma from the hospital blood bank was used to prepare IOT standards in a plasma matrix. Serial dilution of a 100 μg/ml IOT stock solution produced seven standards having concentrations 100, 75, 50, 30, 20, 10 and 0 (blank) μg/ml IOT. QC samples of IOT in plasma were prepared from a 100 μg/ml stock solution to yield 80 and 25 μg/ml IOT.

2.4. Sample preparation

Urine specimens and urine standards were diluted 1:10 with 2% perchloric acid to precipitate protein present in the sample. Following addition of perchloric acid, the specimens were vortex mixed and centrifuged for 4 min at 14 000 *g*. Plasma specimens were combined 1:1 (v/v) with 4% perchloric acid to precipitate proteins with subsequent vortex mixing and centrifugation for 8 min at 14 000 *g*. For both urine and plasma samples, an aliquot of the supernatant was loaded into vials for injection by the autosampler.

2.5. Calculations

Standard curves were created by linear regression of peak area of iothalamate versus known concentrations of iothalamate. Concentrations of QCs and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown sample peak area.

2.6. Drug interference study

Because assessment of GFR is often made in conjunction with renal plasma flow, *p*-amino hippuric acid (PAH) and *N*-acetyl-*p*-aminohippuric acid (*N*-acetyl-PAH) were tested for interference with the assay. Plasma and urine of volunteers infused with PAH as well as stock solutions of PAH and *N*-acetyl-PAH were tested for interference with the assay. Acetaminophen, hydralazine, atenolol, metoprolol,

metformin, trazodone, ibuprofen and *para*-aminobenzoic acid were also tested for interference because of a reasonable possibility of coelution with the peaks. Patients with chronic kidney disease often take multiple drugs including antihypertensives, oral hypoglycemic agents, statins and hematinics. We tested the urine and plasma of 50 volunteers with chronic kidney disease who were taking these drugs. We injected plasma and urine blanks on each patient to evaluate interference with ingested drugs.

2.7. Recovery and limit of detection

The recovery of iothalamate from urine and plasma was determined by calculating the ratio of slopes of iothalamate standard curves to the slope of the same standards prepared in distilled deionized water. Data were analyzed by analysis of covariance to test the significance of difference in slopes. The limit of detection was calculated by the method of Anderson as three times the slope divided by the standard error of the estimate [11]. Iothalamate was always dosed in a way to obtain well detectable levels, therefore we did not assess the limit of quantification of the assay.

2.8. Precision, accuracy and stability

The precision of the assay was assessed over two concentrations, 25 and 80 μg/ml in plasma and 30, 120, 300 and 1200 μg/ml in urine. Four to five aliquots of plasma at each concentration were assayed on 5 days and three to seven aliquots of urine at each of the concentrations on 2–4 days. The precision of the assay was also assessed by running twenty-three plasma samples and twenty urine samples from two subjects on 2 different days. Precision is reported as relative standard deviation calculated from random effects analysis of variance and percent accuracy as [(observed−expected)/expected concentration]×100. System precision was assessed by repeated injections of the same sample of plasma or urine at two different nominal concentrations.

Iothalamate stability was examined in relation to thermal and chemical stress using plasma, urine, or water samples spiked with iothalamate. To assess thermal stability, samples were either repeatedly frozen and thawed 5–10 times, stored at 4 °C for 4 days, stored at room temperature for 4 days, or exposed to 50 °C heat for 1 h. To measure chemical stability, additional samples were exposed to either 0.1 *M* NaOH or 0.1 *M* HCl for 1 h at room temperature. Iothalamate concentration in samples was determined and compared with the concentration of a reference standard stored at −86 °C.

2.9. Subjects

Fourteen patients with chronic kidney disease between the ages of 18 and 80 years were recruited from the outpatient renal clinics of the Richard L. Roudebush Verterans Administration, Indianapolis. All these subjects were participating in a study comparing two diuretics. Subjects with previous allergy to radiocontrast or iodine or unstable creatinine were excluded. We measured IOT clearance by the single bolus technique in which 3 ml of Conray 60 (1.8 g of iothalamate meglumine) was administered intravenously at 8 a.m. after an overnight fast. Plasma samples were collected 2, 3, 6, 9 and 12 h after injection in a heparinized tube. We waited 2 h prior to collecting plasma samples because we were only interested in the terminal elimination rate constant and not the distribution rate constant. We repeated the GFR measurement after an interval of 3 days. GFR was calculated as plasma iothalamate clearance by the product of volume of distribution of iothalamate by the terminal elimination rate constant assuming first order kinetics. To calculate the inter-day relative standard deviation (RSD) in GFR we first calculated the natural log of GFR for each subject over the two visits. We then performed a one-way random effects ANOVA with subjects as random variable and calculated the RSD as square root of the mean square error of the log transformed clearances [12]. Variability is reported as standard deviations unless otherwise stated. All analyses were performed with STATISTICA for Windows 5.5 (Statsoft, Tulsa, OK, USA). The study was approved by the Institutional Review Board of Indiana University and all subjects provided written informed consent.

2.10. Drug interference

There was no interference noted with PAH, *N*-acetyl PAH or acetaminophen that can commonly interfere with chromatography. The retention times

for PAH, *N*-acetyl PAH was 2.5 and 4.1 min, respectively, with good resolution of baselines. The present technique can potentially be used to assay PAH and *N*-acetyl PAH although there are several other methods available for simultaneous assay of these compounds.

Although our subjects were on antihypertensives, oral hypoglycemic agents, statins and diuretics, no interference with these commonly ingested drugs was observed in the blank plasma specimen. Some patients had late eluting peaks that did not interfere with the assay. Retention times of metformin, atenolol and acetaminophen were noted to be 2.0, 5.8 and 6.3 min, respectively. Trazodone, ibuprofen and tolbutamide produced no peaks. Hydralazine at therapeutic concentrations in plasma would produce a peak below detectable level in plasma at 3.7 min.

2.11. Chromatography

Chromatograms of typical blank human plasma and urine, ones supplemented with iothalamate and from a subject administered iothalamate are shown in Fig. 1. The retention time of iothalamate was noted to be 3.5 min.

The chromatographic conditions were optimized with respect to the pH, and the ratio of acetonitrile and methanol in the mobile phase to yield rapid

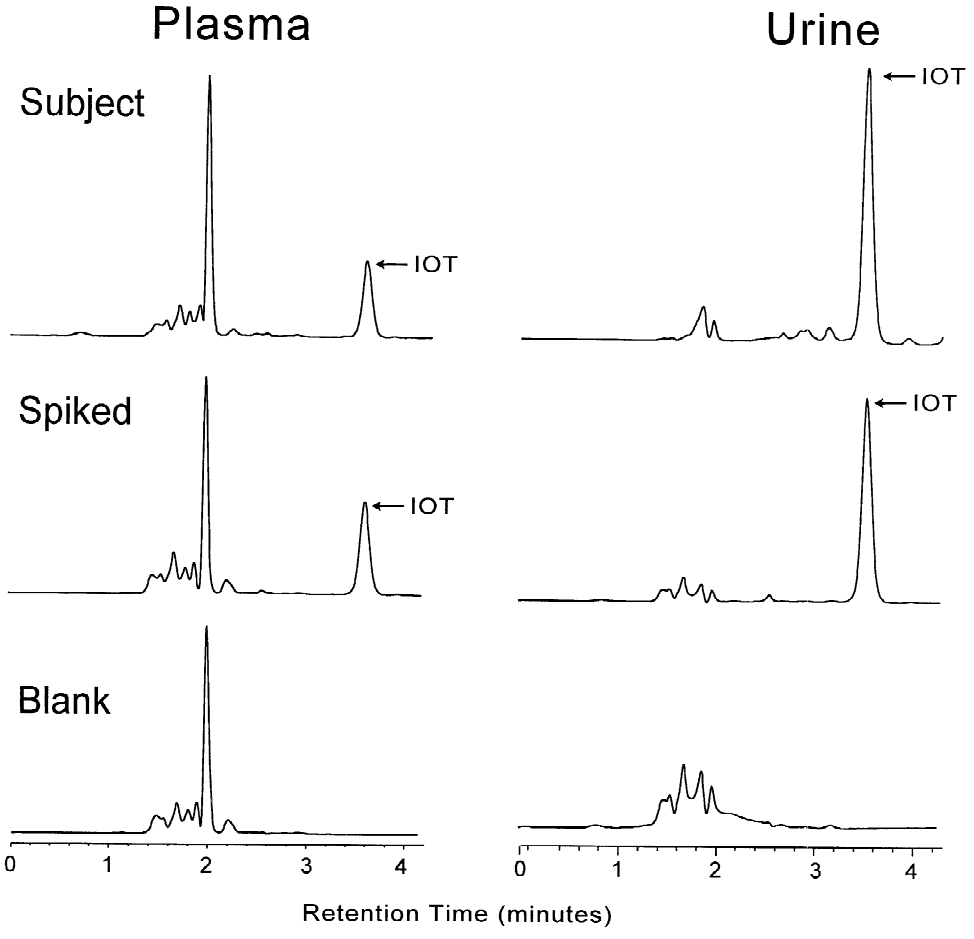

Fig. 1. Chromatograms obtained from 5 μl injection of plasma or dilute urine specimens. The left panels show plasma chromatograms and the right panels exhibit urine chromatograms. The bottom panels are blanks, the middle panels display samples spiked with iothalamate and the top panels represent specimens from study subjects administered iothalamate.

elution of iothalamate. This allowed the run time to be reduced to 5.5 min from the previously reported run time of 22 min [4]. Use of acetonitrile as a deproteinizing agent resulted in peak tailing and the resultant loss of symmetry necessitating the use of perchloric acid. We found 240 nm to be the optimal detection wavelength after spectral analysis of the iothalamate peak. The 1.5 ml/min flow-rate, though higher than usual, permitted greater throughput while maintaining acceptable peak resolution and system pressure.

2.12. Linearity

Calibration curves created for six different concentrations (20–200 μg/ml) in water, urine and plasma demonstrated coefficient of determination of 0.999 or better in any matrix (Fig. 2). None of the intercepts were significantly different from zero. In further experiments, the standard curve maintained linearity to 2000 μg/ml, which was the maximum concentration tested.

2.13. Recovery of calibration standards and limit of detection

The recovery of PAH was 90.8% in plasma and 102.3% in urine (Fig. 2). The recovery from plasma was significantly lower in comparison to urine or water, probably due to loss of <10% of iothalamate with precipitation of protein and was similar to what has been reported previously [4,13]. Recovery of iothalamate from urine was not significantly different from water. The limit of detection was calculated conservatively to be 4.2 μg/ml in diluted urine and 1.7 μg/ml in plasma.

2.14. Assay performance

Intra-day and inter-day precision for any concentration of iothalamate in urine or plasma were <3% and <7% (Table 1). Samples of plasma from two patients (n=23) yielded an inter-day precision of 3% and samples of urine from two patients (n=20) showed an inter-day precision of 13%. Accuracy ranged between 96 and 102%. There was excellent

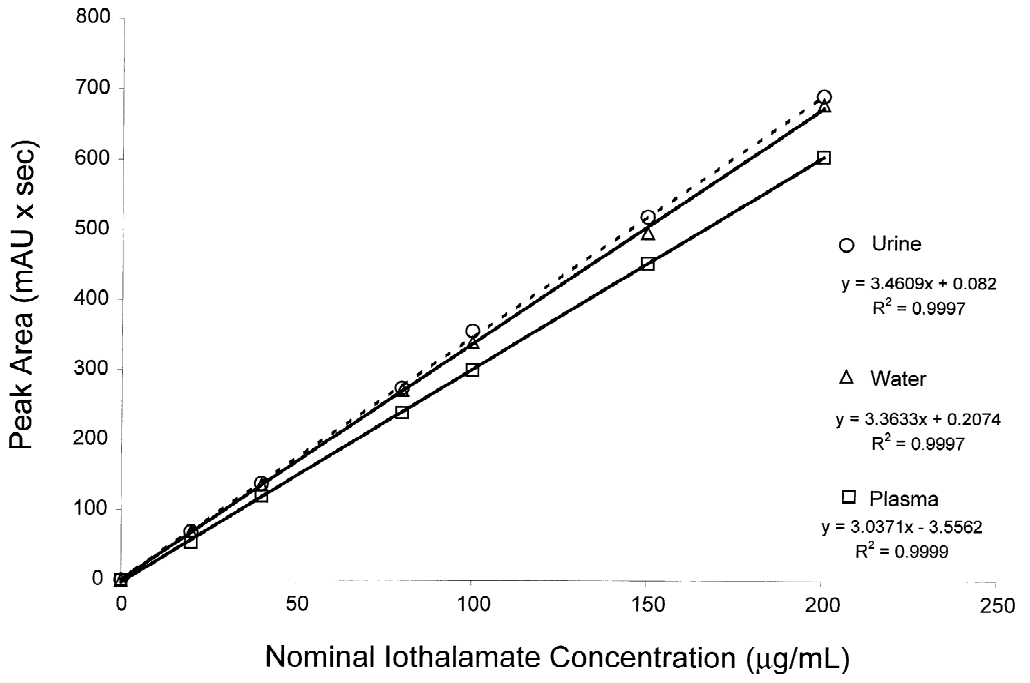

Fig. 2. Standard curve of iothalamate in water, urine and plasma. Recovery of iothalamate from plasma was 91% which was statistically lower compared to water.

Table 1
Within- and between-day assay characteristics for iothalamate estimation

Sample type and nominal concentration[a,b]	RSD (%)		Accuracy, % (95% CI)
	Intra-day	Inter-day	
Plasma 80 μg/ml	1.79	1.79	101.5 (100.7–102.2)
Plasma 25 μg/ml	2.95	2.95	101.0 (99.5–102.4)
Urine 1200 μg/ml	0.32	0.75	98.8 (98.3–99.2)
Urine 300 μg/ml	0.45	0.88	99.4 (98.9–99.9)
Urine 120 μg/ml	0.95	3.81	97.1 (95.1–99.0)
Urine 30 μg/ml	0.84	6.42	95.9 (92.9–99.0)

RSD, relative standard deviation; CI, confidence interval.
[a] Plasma samples were run on 5 separate days in three to five replicates.
[b] Urine samples were run on 2–3 days in three to seven replicates.

thermal and pH stability of iothalamate (Table 2). System precision at two different concentrations of iothalamate in plasma, 25 and 80 μg/ml yielded RSDs of 0.22 and 0.13%, respectively. For urine iothalamate at 30 and 120 μg/ml final concentration, the RSDs were 0.18 and 0.26%, respectively.

2.15. Human studies

We studied fourteen subjects with a mean age 66.5±10.6 years, thirteen males, nine whites and five blacks, with chronic kidney disease due to hypertension (ten), diabetes (three) or nephrectomy (one) and calculated creatinine clearance of 59±27 ml/min [14]. Seated BP averaged 153±9/81±5 mmHg. Subjects tolerated the bolus injection well except one subject experienced transient flushing of the skin after the first injection and was not exposed to the iothalamate again. Fig. 3 shows plasma concentration time profile in the remaining thirteen subjects with chronic kidney diseases who had measurements of plasma iothalamate clearance by a single dose bolus technique 3 days apart. The regression coefficients for the curve fits were uniformly high (>0.95) for each subject and GFR averaged 44.3±16.4 and 43.6±15.9 ml/min, respectively on the 2 days for subjects who completed both phases of the study. The inter-day RSD for GFR measurements was 5.7%. It is notable that the average GFR as measured by plasma iothalamate clearance was substantially lower than that calculated by the Cockcroft Gault formula and likely reflects the secretion of creatinine [15].

The automated, reproducible, accurate and sensitive HPLC system offers several advantages. First, in contrast to the fluorescent excitation assay for

Table 2
Stability of iothalamate to thermal stress

Sample type	Change in iothalamate, % (95% confidence intervals)					
	Freeze–thaw (5–10 cycles)	4 °C storage (for 3 days)	Room temp. storage (for 3 days)	50 °C stress for 1 h	Alkali stress (0.1 *M* NaOH for 1 h)	Acid stress (0.1 *M* HCl for 1 h)
Plasma	−0.20 (−0.69 to 0.30)	−0.75 (−2.60 to +1.10)	−1.75 (−3.7 to 0.2)			
Urine	−0.91* (−0.45 to −1.37)	−1.6 (−0.48 to 0.5)	−1.9 (−0.47 to 0.9)			
Water				+3.77* (2.29 to 5.25)	−0.47 (−2.29 to 1.36)	−0.36 (−1 to 0.27)

For freeze–thaw experiments, % change per freeze–thaw cycle is reported (negative values reflect degradation). None of the results were statistically significant ($P<0.05$) except for those marked with an asterisk (*). The increase in iothalamate concentration with heat likely reflects evaporation of water. See text for details.

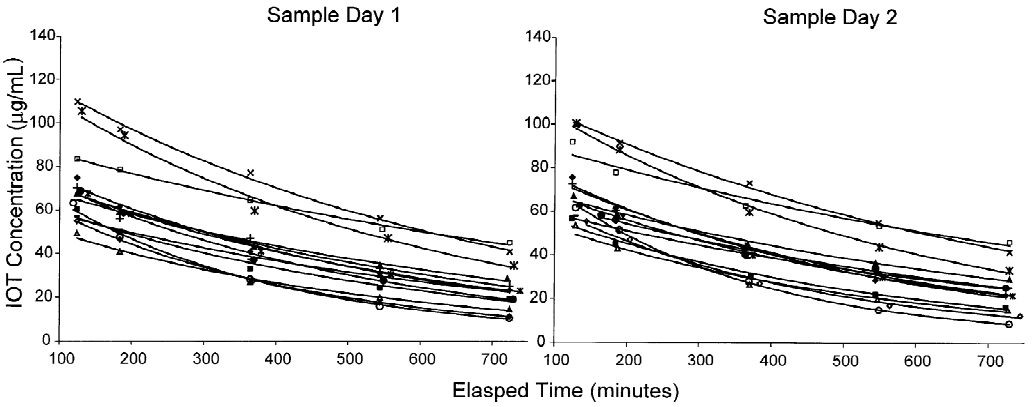

Fig. 3. Log linear decline is seen in plasma iothalamate concentration time profiles in 13 patients with chronic kidney disease each studied after 3 days showing a RSD between studies of only 5.7%.

radiocontrast, the HPLC system provides improved sensitivity. Typically, 25–50 ml of radiocontrast is injected for estimation of GFR with the fluorescent excitation analysis [16]. In our method, we administered a fixed dose of 3 ml to obtain the GFR, thus avoiding potential injury from large volume of radiocontrast. In comparison to our previous HPLC assay, we have reduced the run time from 22 to 5.5 min and have eliminated prolonged column equilibration due to use of the ion-pairing reagent [4]. Optimization of the HPLC condition increased the throughput of this automated technique and increased its accuracy and precision [4]. Although several HPLC methods are published, none is as rapid as that reported here. The existing methods typically have a 20-min run time or longer [3–6]. Even the most rapid method reports peak elution at 8 min [9] in contrast to 3.5 min noted in this study. Most methods require more extensive sample processing than reported here [6–9]. Furthermore, ion pairing used in several reports necessitates prolonged column equilibration prior to sample injection [3,4,6]. Even the least time-intensive method with simple sample deproteinization with acetonitrile requires a 10-min run time per sample. Thus, the present method represents a substantial advance in the HPLC estimation of iothalamate. Although, a 4-min run time may be reasonable, we recommend using a 5–5.5 min run time for subjects receiving an infusion of PAH for estimation of renal plasma flow.

In conclusion, we report a new method for measurement of iothalamate that is rapid, reproducible, and does not require extensive sample preparation. Our data show that the single shot bolus plasma clearance technique to measure GFR can be done with great precision. The procedure involved blood sampling for up to 12 h after the iothalamate bolus. Although our method for GFR measurement was prolonged and required multiple blood sampling, limited sampling after 1–2 h of equilibration may allow shorter studies with single shot bolus clearance technique. However, when prolonged sampling is possible, plasma clearance after a single bolus of iothalamate may allow an excellent choice for measurement of GFR. Implementation of this rapid method in clinical laboratories is possible and will allow accurate and precise measurement of GFR without exposure to radioactivity.

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