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High performance liquid chromatographic measurement of iothalamate in human serum and urine for evaluation of glomerular filtration rate

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Abstract

A simple and sensitive HPLC-UV assay was developed for the measurement of iothalamate (IOT) in human serum and urine. Chromatographic separation was achieved using an embedded-carbamate-group bonded RP18 column and mobile phase consisting of 50 mM monobasic sodium phosphate and methanol (90:10, v/v) without the addition of ion-pair reagents. The assay demonstrated a high analytical reliability within the IOT concentration range of 1–150 μg/ml in serum and 25–1500 μg/ml in urine. The relative standard deviations (RSDs) for intra- and inter-day analysis were less than 5.1% in all cases. This method has been used for the evaluation of glomerular filtration rate (GFR) in subjects participating in a phase I clinical trial of a novel antimalarial medicine. The average baseline GFR was 100.41 ± 19.99 ml/min/1.73 m^2 in 119 healthy volunteers. The assay may also allow the simultaneous measurements of *p*-aminohippuric acid (PAH), *N*-acetyl PAH (aPAH), and IOT with some modification. PAH, IOT, aPAH, and β-hydroxyethyl-theophylline internal standard peaks appeared approximately at 2.5, 3.7, 5.9, and 11.8 min, respectively, in an isocratic run.
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Keywords: Iothalamate; BHET; GFR; HPLC-UV

1. Introduction

Glomerular filtration rate (GFR) has been widely used as an index for the evaluation of renal function in clinical practice, pre-clinical studies, and clinical trials. Among the endogenous and exogenous GFR markers, creatinine clearance or calculations based on serum creatinine concentration are the most widely used indicators for noninvasive assessment of GFR. Creatinine production depends on muscle mass, dietary intake, the use of certain medications, and is age and gender related. The elimination of creatinine is dependent on glomerular filtration, tubular secretion and reabsorption as well as extrarenal elimination. GFR estimated from serum creatinine using prediction equations, such as Modification of Diet in Renal Disease (MDRD) equations and Cockcroft–Gault (CG) formula, may result in unpredictable error imperceptibly when the diagnosis of chronic kidney disease is unknown [1–10]. Cystatin C, a low molecular weight protein that is produced by nucleated cells at a constant rate and is then filtered by glomeruli and reabsorbed and catabolized by tubular epithelial cells, has been suggested as an alternative for creatinine [11]. Cystatin C might be a more reliable endogenous GFR marker than serum creatinine, but several research reports have suggested that Cystatin C should not be interpreted as purely a marker of GFR, since it seems to be affected by factors other than renal function alone [12–19]. Direct measurement of GFR using inulin or radioactive labeled contrast media agents is considered to be the most accurate method of assessing kidney function. However, these procedures are not used routinely due to somewhat cumbersome techniques or radioactive safety considerations.

HPLC methods have been introduced for the quantification of iothalamate in human serum/plasma and urine for the measurement of GFR without the use of radioactive isotope ^{125}I-iothalamate [20–28]. The objective of the present study is to develop a highly reliable HPLC with ultraviolet detection assay for quantification of iothalamate concentration in human serum

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and urine to assess the changes between baseline and follow-up GFR in subjects undergoing safety and tolerability studies of tafenoquine, a promising 8-aminoquinolone antimalaria medicine [29,30]. Instead of using conventional reverse phase C18 or C8 columns and mobile phase containing ion-paired reagents [22–26], we selected an embedded-carbamate-group bonded SymetryShield RP18 column and applied mobile phase composed of 50 mM monobasic sodium phosphate and methanol (90:10, v/v) without the addition of ion pairing agent. The possibility of simultaneous analysis of *p*-aminohippuric acid (PAH), a marker for measurement of effective renal plasma flow, and acetyl-aminohippuric acid (aPAH), a metabolite of PAH [21–25], was also investigated in this report. Serum creatinine and iothalamate were measured using the same specimens.

2. Experimental

2.1. Chemicals and solvents

Iothalamic acid was purchased from Research Diagnostics (Flanders, NJ, USA). β-Hydroxyethyl-theophylline (BHET) and monobasic sodium phosphate were purchased from Sigma Chemical (St. Louis, MO, USA). All solvents were HPLC-reagent grade and were obtained from Fisher (Fair Lawn, NJ, USA).

2.2. Standard solutions

Standard solutions of iothalamic acid and BHET internal standard were prepared in methanol, stored at 4 °C. Calibration standards and quality controls were made by the addition of certain amounts of iothalamic acid standard solutions to 1.5 ml microcentrifuge tubes and evaporated to dryness and stored at 4 °C. Calibration standards and quality control samples were freshly prepared by addition of blank human serum and urine at the same day of analysis of subject specimen.

2.3. Sample preparation

Serum sample preparation: Human blood was collected in a serum separator tube at appropriately 60, 90, 120, and 150 min post 2-ml intravenous bolus dose of Conray® 30 (300 mg/ml iothalamate, Mallinckrodt) over 2.5 min and with a 5-ml sterile normal saline flush. After the blood clotted, the specimen was centrifuged and the serum was transferred to a labeled storage tube. Aliquots of 100 μl of subject serum were placed in clean labeled 1.5 ml micro centrifuge tubes followed by addition of 200 μl acetonitrile containing BHET internal standard (20 μg/ml BHET) to each sample, standard and quality control. The samples were mixed vigorously and centrifuged at 12,000 × *g* for 5 min at room temperature (18–25 °C). The supernatant was transferred into a clean, labeled glass test tube and evaporated to dryness using a Heto Speed-Vac apparatus (Heto Lab. Equipment A/S, Copenhagen, Denmark) at 45 °C. The residue was reconstituted with 100 μl of mobile phase and 10 μl was injected into the HPLC system.

Urine sample preparation: Urine samples were diluted 10-fold using mobile phase. The diluted urine (100 μl) was combined with 50 μl BHET internal standard (100 μg/ml BHET in mobile phase), vortex mixed and 10 μl was injected into HPLC system.

2.4. HPLC-UV detection

Compounds were separated on a SymmetryShield™ RP18 column, 150 mm × 4.6 mm I.D., 5 μm particle size, preceded by a SymmetryShield™ RP18, 3.9 mm × 20 mm, 5 μm particle size guard column (Waters, MA, USA). A Hewlett-Packard 1050 HPLC system (Wilmington, DE, USA) was used to deliver an isocratic run with in-line blending mobile phase consisting of 50 mM monobasic sodium phosphate (unadjusted pH 4.6) and methanol (90:10, v/v). The flow rate was 1.2 ml/min and the column temperature was kept at 30 °C. Injection of 10 μl serum extracts or diluted urine was accomplished using an autosampler. The IOT and BHET internal standards were detected using a 1046A ultraviolet detector at wavelength of 254 nm. Data analysis was performed using the peak height ratio of IOT to BHET to generate two separate calibration curves, one for serum and one for urine, using weighed $(1/y_{obs}^2)$ linear regression. Calibration curves were prepared freshly for each HPLC run. Unknown samples were quantified using the calibration curves obtained during the same HPLC analysis.

2.5. Validation

The analytical method was validated using the criteria suggested by FDA [32]. Ten distinct calibration curves were examined over a 6-month time period and the accuracy and precision of each of the IOT calibration standards (1, 2.5, 5, 10, 25, 50, 100, 150 μg/ml in serum and 25, 50, 100, 250, 500, 1500 μg/ml in urine) were determined by calculating the mean value and relative standard deviation (RSD) at each concentration. The intra- and inter-day precision and accuracy of this assay were determined by preparing three quality control samples of IOT at 2, 20, 120 μg/ml in serum and 40, 200, 1200 μg/ml in urine and analyzed in quintuplicate and determined on five separate occasions using five different sample calibration curves.

3. Results

3.1. HPLC analysis

High performance liquid chromatography effectively resolved IOT and BHET with retention times at 3.7 and 11.8 min, respectively (Fig. 1). Fig. 1 shows that selected chromatograms of blank human serum and urine, the spiked standards containing 2.5 μg/ml iothalamic acid in serum and 25 μg/ml in urine, and at measured IOT concentrations of 17.20 μg/ml in serum and 176.19 μg/ml in urine observed in a subject post an intravenous bolus injection of 2 ml Conray®30. The regression coefficient (r^2) of IOT serum calibration curves was 0.9995 ± 0.0006 (mean ± SD, $n = 10$) with a slope of 0.0123 ± 0.0108 and *y*-intercept of 0.0770 ± 0.0073 over a

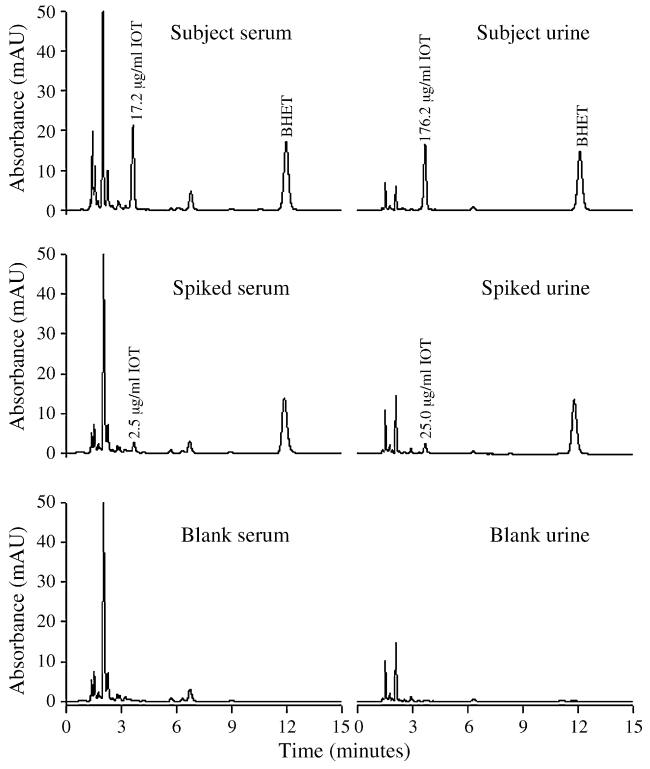

Fig. 1. Selected chromatograms of blank human serum and urine, the spiked standards containing 2.5 μg/ml iothamic acid in serum and 25 μg/ml in urine, and at measured iothalamate (IOT) concentrations of 17.20 μg/ml (in serum) and 176.19 μg/ml (in urine) observed in a subject after a single intravenous bolus injection of 2-ml Conray® 30. Identified peaks include IOT and β-hydroxyethyltheophylline (BHET) internal standard.

concentration ranged from 1 to 150 μg/ml. The average r^2 of urine standard curves over the validated range from 25 to 1500 μg/ml IOT was 0.9998 ± 0.0002 with a slope of 0.0248 ± 0.0160, and a *y*-intercept of 0.0065 ± 0.0007. The RSDs ranged from 0.85 to 2.66% for the IOT concentration of 25–1500 μg/ml in urine, and 1.39–3.58% over the concentration range of 1 to 150 μg/ml of IOT in serum.

The RSDs of the intra-day analysis of IOT were 1.28, 2.09, and 0.51% at the added concentrations of 2, 20, and 120 μg/ml IOT in serum, and 4.30, 1.79, and 1.05% at the IOT concentrations of 40, 200, and 1200 μg/ml in urine, respectively. The inter-day RSDs of IOT were 3.81, 5.07, and 4.84% in serum and 4.60, 2.50, 1.97% in urine. The deviation of the measured concentration from the added IOT concentration was less than 6% in all cases (Table 1).

3.2. Relative recovery from serum

Human serum and mobile phase (100 μl) standards were prepared in triplicate containing 2, 20, 120 μg/ml IOT and 20 μg/ml BHET internal standard. Human serum samples were extracted, processed and analyzed by HPLC as described above. Peak height of IOT and BHET in these samples were compared to unextracted standards. The percent relative recovery was defined as: (peak height of the serum standard/peak height of the unextracted standard) × 100. The average recoveries of IOT from serum were 101.1 ± 6.3, 93.4 ± 4.1, and $90.9 \pm 1.0\%$ ($n = 3$) for 2, 20, and 120 μg/ml IOT, respectively. The recovery of 20 μg/ml BHET internal standard was $92.3 \pm 2.3\%$ ($n = 9$).

Table 1
Intra- and inter-day precision and accuracy for the measurement of iothalamic acid (IOT) in human serum and urine

IOT concentration (μg/ml)		RSD (%)[a]	Percentage of the added concentration
Added	Measured (mean ± S.D.)		
In serum			
Intra-day ($n = 5$)			
2	1.92 ± 0.03	1.28	95.9
20	19.54 ± 0.42	2.09	97.7
120	119.20 ± 0.61	0.51	99.3
Inter-day ($n = 5$)			
2	2.04 ± 0.08	3.81	98.0
20	21.07 ± 1.01	5.07	94.6
120	125.43 ± 5.81	4.84	95.5
In urine			
Intra-day ($n = 5$)			
40	39.75 ± 1.72	4.30	100.6
200	193.62 ± 3.58	1.79	103.2
1200	1192.59 ± 12.64	1.05	100.6
Inter-day ($n = 5$)			
40	40.46 ± 1.84	4.60	98.8
200	204.99 ± 7.20	2.50	97.5
1200	1239.28 ± 23.66	1.97	96.7

[a] RSD, relative standard deviation.

3.3. Stability of iothalamic acid in human serum and urine

The stability of iothalamic acid (IOT) was tested in human serum spiked with 2 and 120 μg/ml IOT and urine spiked with 40 and 1200 μg/ml IOT in triplicate that were stored at −80 °C for 1 month. The stabilities of iothalamic acid in serum spiked with 2 and 120 μg/ml IOT and in urine spiked with 20 and 1200 μg/ml IOT, incubated at room temperature for 24 h or subjected to three freeze–thaw cycles, were examined in triplicates. Human serum and urine samples containing iothalamaic acid was stable when freshly prepared samples were compared to samples subjected to three freeze–thaw cycles, kept at ambient room temperature for 24 h, or after storage at −80 °C for at least 1 month.

The long-term stability of iothalamate in subject specimens and the reproducibility of the analytical assay were tested in two paired studies comparing IOT concentrations measured between 29 or 30 weeks apart using the same batches of urine and serum specimens ($n = 16$). The two batches of urine and serum specimen were collected from a subject administered a 2 ml intravenous bolus injection of Conray®30 at days 0 and 9 visits, respectively. The serum and urine specimens were analyzed on the day of collection and at 29 or 30 weeks after storage at −80 °C. The inter-assay precision was $2.38 \pm 1.46\%$ (mean ± SD, ranged from 0.44 to 4.89%) in serum samples, and $1.48 \pm 1.12\%$ (ranged from 0.02 to 2.95%) in urine specimens (Table 2). Subject specimens with iothalamate concentrations of 5.59–16.32 μg/ml in serum and from 66.56 to

Table 2
Assay reproducibility and long-term stability of iothalamate (IOT) in subject specimens

Sample times	IOT concentration (μg/ml)		
	Collection day	Stored at −80 °C[a]	% Deviation
Urine group-1			
60 min	241.06	237.91	1.30
91 min	180.31	176.27	2.24
121 min	96.75	94.32	2.51
151 min	66.56	64.60	2.95
Serum group-1			
63 min	14.83	14.89	0.44
93 min	10.16	10.48	3.20
125 min	7.16	7.40	3.44
153 min	5.59	5.87	4.89
Urine group-2			
60 min	378.01	385.67	2.03
90 min	208.69	209.51	0.39
120 min	113.48	113.07	0.36
150 min	74.27	74.26	0.02
Serum group-2			
63 min	16.32	16.50	1.09
92 min	11.22	11.50	2.56
122 min	8.33	8.43	1.21
152 min	6.43	6.57	2.17

[a] The two batches of urine and serum specimen were collected from a subject administered 2 ml intravenous bolus injection of Conray®30 at days 0 and 9 visits, respectively. The specimens were analyzed in duplicate on the day of collection and at 29 or 30 weeks after storage at −80 °C for urine/serum group-2 or urine/serum group-1 specimens, respectively.

378.01 μg/ml in urine measured at the day of sample collection were stable when stored at −80 °C for at least 6 months ($P \geq 0.967$).

3.4. Selectivity of the assay

There was no significant interference observed from endogenous substances on the chromatographic separation of iothalamate and BHET (Fig. 1). Potentially confounding chemicals such as *p*-aminohippuric acid and acetyl-PAH were examined. The spiked human serum and urine with PAH, IOT, aPAH and BHET internal standard were prepared and detected using the HPLC assay. Chromatographic peaks appeared at 2.5, 3.7, 5.9, and 11.8 min for PAH, IOT, aPAH, and BHET, respectively, in a simultaneous HPLC analytical run.

3.5. Analysis of subject samples

The study was conducted on a cohort of 119 eligible subjects after the screening evaluation. Subjects were assigned randomly to the study and placebo control groups. On the GFR evaluation, each subject visited the study center on day 0 (baseline), weeks 12 and 24, and in some cases additional visits. The subject received an intravenous bolus injection of 2-ml Conray®30 (600 mg iothalamte meglumine) and a 5-ml sterile normal saline flush. Blood and urine samples were collected after 60, 90, 120, and 150 min. Serum and urine IOT concentrations were measured in duplicates for each specimen. GFR was calculated as average of IOT clearance using four serum samples and three urine samples assayed for IOT concentration using the HPLC method developed in this study. IOT clearance = amount of IOT excreted in urine/AUC, where AUC is the area under the serum concentration–time curve, determined using the trapezoidal rule. The average baseline GFR was 100.41 ± 19.99 (mean ± SD, ranged from 57.13 to 184.53) ml/min/1.73 m^2 in 119 subjects aged 18–54 years. The baseline GFR in male subjects was 99.38 ± 18.18 ($n = 72$), and in female was 101.99 ± 22.59 ml/min/1.73 m^2 ($n = 47$). No significant difference was found between GFR in males and in females ($p = 0.441$).

4. Discussion

High performance liquid chromatography methods have been described for the detection of iothalamate in serum/plasma and urine without exposure to radioactive materials [20–28]. However, the existing assays have used conventional alkyl type reverse phase C18 or C8 columns that often required mobile phase containing ion-paired reagents to reduce the peak tailing and improve the peak shape for polar and basic compounds [22–26]. The use of ion-paired agents resulted in prolonged column equilibration time and complex mobile phase preparation. In our assay, we selected an embedded-carbamate-group bonded RP18 stationary phase, SymmetryShield™ RP18, column as the analytical column. The embedded-internal-carbamate-groups deactivated silanol interactions with basic analytes and improved peak shapes [31]. IOT and BHET had been eluted with retention times of 3.7 and 11.8 min by applying a flow rate of 1.2 ml/min and mobile phase composed of 50 mM monobasic sodium phosphate and methanol (90:10, v/v) without the addition of ion-pairing agents (Fig. 1). BHET was selected as an internal standard for this assay since it presented a chromatographic peak without significant interference from endogenous substances over the commonly used compound *p*-aminobenzoic acid (PABA) [23–25]. Precipitation of serum protein by the addition of acetonitrile was necessary to obtain an optimal assay sensitivity of 1 μg/ml IOT in this study where real serum IOT concentrations were often below 5 μg/ml in subjects post 2-ml intravenous bolus dose of Conray®30 (Fig. 2). This assay provides a significant improvement on sensitivity and reliability over previously published articles on the measurement of IOT. Most recent studies reported a lower limit of qualification of 10 μg/ml in serum or plasma [20,21]. Our assay demonstrated a high analytical reliability within the IOT concentration range of 1–150 μg/ml in serum.

Effective renal plasma flow (ERPF) as well as GFR are believed to be useful indexes for evaluation of renal function and have been measured simultaneously in prior studies [21–25]. Although ERPF estimation is not performed in our study, we have tested the possibility of simultaneous measurements of *p*-aminohippuric acid, *N*-acetyl PAH (aPAH), and IOT. PAH, IOT, aPAH, and BHET peaks appeared approximately at 2.5, 3.7, 5.9, and 11.8 min, respectively, in an isocratic run (data not shown).

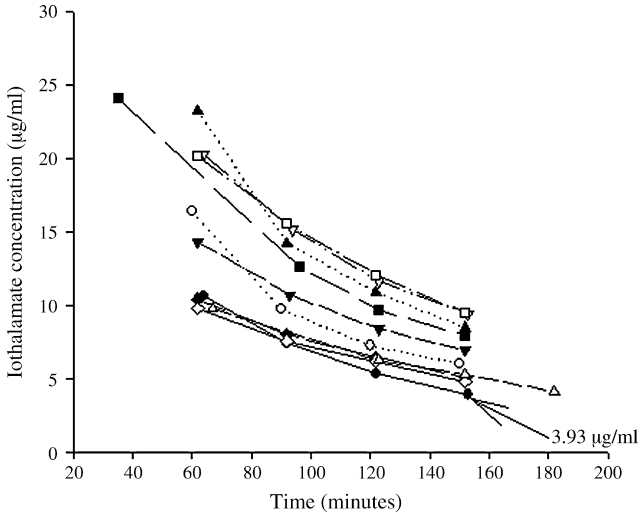

Fig. 2. Serum concentrations of iothalamate in 10 subjects after a single intravenous bolus injection of 2-ml Conray®30. Serum iothalamate concentrations ranged from 3.93 to 24.06 μg/ml in the10 subjects.

In initial assay development efforts, we employed the same sample treatment procedure for both urine and serum specimens by adding acetonitrile to precipitate protein. Considering the iothalamate levels in real subjects' specimen and our long-term follow-up objectives, we chose to dilute urine specimen by 10-fold with mobile phase and combined the freshly prepared urine samples with 50-μl internal standard solution to simplify the urine sample preparation procedure. This also brings an additional advantage of allowing us to monitor the performance of this validated assay by tracking the chromatographic response of the IOT standards. This, together with examination of quality control samples and the bias of duplicated analysis of each specimen, all demonstrated uniformly low analytic errors for the entire study period of more than two years.

In conclusion, we report a simple, rapid, sensitive, and highly reproducible HPLC assay for the measurement of iothalamic acid in human serum and urine for the reliable monitoring of glomerular filtration rate in 119 subjects undergoing a phase I safety and tolerability study of tafenoquine, a novel 8-aminoquinolone antimalarial drug. GFR was measured as the clearance of IOT using four serum samples and three urine samples assayed for IOT concentration by the HPLC method developed in this study. The assay is well suited for periodical renal function evaluations of subjects during clinical studies. If desired, this assay also allows the simultaneous measurements of *p*-aminohippuric acid, *N*-acetyl PAH, and iothalamate with minimal modifications. Further analysis of the effect of tafenoquine on GFR measured by serum creatinine and iothalamate clearance will be reported in the near future.

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