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Paired ion reversed-phase HPLC assay for the determination of iothalamic acid and para aminohippuric acid in urine

ANANDA K. SENEVIRATNE, ANURA L. JAYEWARDENE and JOHN G. GAMBERTOGLIO*

Clinical Research Unit, Division of Clinical Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143-0622, USA

Abstract: A paired ion reversed-phase high performance liquid chromatographic method for simultaneous determination of iothalamic acid (Io) and para aminohippuric acid (PAH) in urine is described. The method uses a single internal standard for both drugs. The only sample preparation required is dilution of urine (1:100 or 1:500) with deionized water. The internal standard is added to a small aliquot of the diluted specimen and injected. For HPLC, a C_8 column and a mobile phase consisting of potassium phosphate buffer with dodecyl triethylammonium phosphate IP reagent, 25% organic modifier with UV detection at 254 nm was used. Within day and between day variation for the assay were in the range of 1.48–9.46% for iothalamic acid and 1.84–10.36% for para aminohippuric acid for four levels of concentration. Limits of quantitation were 50.0 μg ml^{-1} for iothalamic acid and 75.0 μg ml^{-1} for para aminohippuric acid. Mean recovery was 98.55% for Io and 97.79% for PAH. This isocratic HPLC assay is simple, rapid and relatively inexpensive.

Keywords: *Iothalamic acid; p-aminohippuric acid; ion paired high-performance liquid chromatography; glomerular filtration rate (GFR); effective renal plasma flow (ERPF); renal blood flow (RBF).*

Introduction

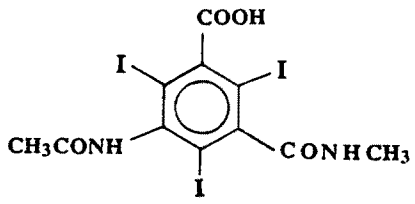
Iothalamic acid (Io, Fig. 1a, 3-(acetylamino)-2,4,6-triiodo-5-[(methylamino) carbonyl]-benzoic acid) is a well known radiographic contrasting agent used in renal and urological procedures [1]. It is also used as a marker, to replace inulin for measuring glomerulin filtration rate (GFR). Para aminohippuric acid (PAH, Fig. 1b, N-(4-aminobenzoyl)-glycine) can be used to determine the effective renal plasma flow (ERPF) and is considered an ideal marker for measuring renal blood flow (RBF) [2–4]. The current methods used for these measurements are considered relatively invasive requiring IV infusion of markers and accurate urine collections, while creatinine clearance though widely used is prone to errors and cannot detect minor changes in GFR [5].

Studies employing Io clearance for estimating GFR indicate, good correlation with inulin clearance and this method is well tolerated by children and infants. Since the use of radiolabelled iothalamic acid in patients is risky and subject to strict health and safety guidelines during usage, a method for measuring non-radioactive Io as well as PAH in urine and plasma is advantageous.

Most of the methods reported in the literature determine either Io or PAH [1, 6–8], and only one method reports the simultaneous assay for Io and PAH using reversed-phase HPLC [9]. Many of the methods assaying for PAH use colorimetry while several of the HPLC methods use immiscible organic solvent extractions to isolate the drugs from plasma and urine [1, 6, 8, 10]. Only two methods used a paired-ion mobile phase with reversed-phase columns for HPLC separation of either Io or PAH [4, 5].

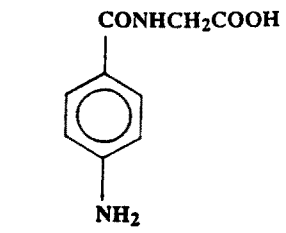
One significant problem with isocratic elution using the mobile phases reported in the literature where the organic phase is below 10% is that residual plasma and urine constituents are strongly retained on the column. Over time, this could cause column performance to deterioriate. Usually under these conditions a second pump coupled through a gradient controller is used to flush the column with a polar mobile phase for 2–5 min after

* Author to whom correspondence should be addressed.



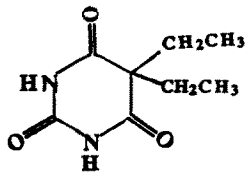
IOTHALAMIC ACID

$C_{11}H_9I_3N_2O_4$
MW. 613.9



PARA AMINOHIPPURIC ACID

$C_9H_{10}N_2O_3$
MW. 194.2



BARBITAL

$C_8H_{12}N_2O_3$
MW. 184.2

Figure 1
Chemical structures of (a) iothalamic acid, (b) *p*-aminohippuric acid and (c) 5,5 diethyl barbituric acid (barbital, internal standard).

each sample run followed by equilibration with the running mobile phase for at least 10 min. This technique requires either two pumps and a controller or a switching valve and a single pump coupled to a controller. A simple and less costly method was needed to determine Io and PAH simultaneously. To achieve the best separation of compounds from urine constituents it was necessary to increase retention times beyond 10 min. To realize this, an ion-pairing reagent was incorporated into the mobile phase containing up to 23.5% methanol. This high concentration of organic modifier ensured that endogenous urine constituents were eluted during the HPLC run. Accordingly, there is no build up of residual urine components. However *p*-aminobenzoic acid (PABA) was not adequately resolved from the two compounds, therefore barbital was used as the internal standard.

We decided to work in an isocratic mode with higher amounts of organic modifier in order to elimiante build up of urine residue on the column. An octyl C_8 reversed-phase column was selected in place of a C_{18} and the detector wavelength was fixed at 254 nm.

A complete assay validation, reported in this paper was performed followed by determination of Io and PAH in a set of clinical samples.

Experimental

Apparatus

The HPLC system used consisted of a Waters M-510 HPLC pump, a model 712 WISP autoinjector, and a model 441 UV detector (Waters Assoc., Milford, MA, USA). An HP 3396A-integrator (Hewlett–Packard, Avondale, PA, USA) was used for collecting the chromatographic data. The detector wavelength was 254 nm and the absorbance was set at 0.01 aufs. Separation was achieved with an octyl (C-8), 5 μm particle size, 25 cm × 4.6 mm i.d. reversed-phase column (Beckman–Ultrasphere, Beckman Instruments Inc., Fullerton, CA, USA).

Reagents

All solvents were of HPLC grade (Fisher Scientific Co., Fair Lawn, NJ, USA). Dodecyl triethylammonium phosphate [(Q-12, Ion pair

reagent, 0.5 M solution), Regis Chemical Co., Morton Grove, IL, USA], potassium diacid phosphate, potassium mono acid phosphate (Fischer Scientific Co., Fair Lawn, NJ, USA), iothalamic acid (Mallinckrodt Chemicals Inc., St Louis, MO, USA), *p*-aminohippuric acid sodium salt and barbital (The Sigma Chemical Co., St Louis, MO, USA), were used as received. Deionized distilled water was obtained from "Barnstead Nanopure" purification system (Barnstead Co., Boston, MA, USA). Drug free human urine was obtained from healthy volunteers.

Mobile phase

The mobile phase consisted of potassium phosphate buffer (pH 7.5, 10 mM)–methanol–acetonitrile–dodecyl triethylammonium phosphate (Q-12 IP reagent 0.5 M) (300:94:6:0.6, v/v/v/v) at an isocratic flow rate of 1.0 ml min^{-1}. The mixture was filtered through a 0.22 μm filter and degassed by sonication under vacuum.

Sample preparation

Calibration curve samples were prepared by spiking iothalamic acid, *p*-aminohippuric acid and the internal standard (barbital) into urine which was then diluted. The calibration curve samples, quality assurance (QA) controls or aliquots of clinical samples were treated identically. The thawed samples were diluted either 1:100 or 1:500 and 200 μl were spiked with internal standard (50.0 μl). Samples were vortexed for 15 s and 20–30 μl samples were injected directly onto the HPLC column for analysis. All clinical samples, QA samples and stock solutions of compounds were stored at −20°C until analysis. Spiked samples for calibration curves and controls at four different compound concentrations were prepared with drug free urine and were spiked with separately prepared drug solutions.

Results

Figure 2(A) represents a chromatogram of blank urine from a renally impaired patient, with internal standard. Figures 2(B) and (C) represent typical chromatograms of the internal standard in blank urine from a healthy

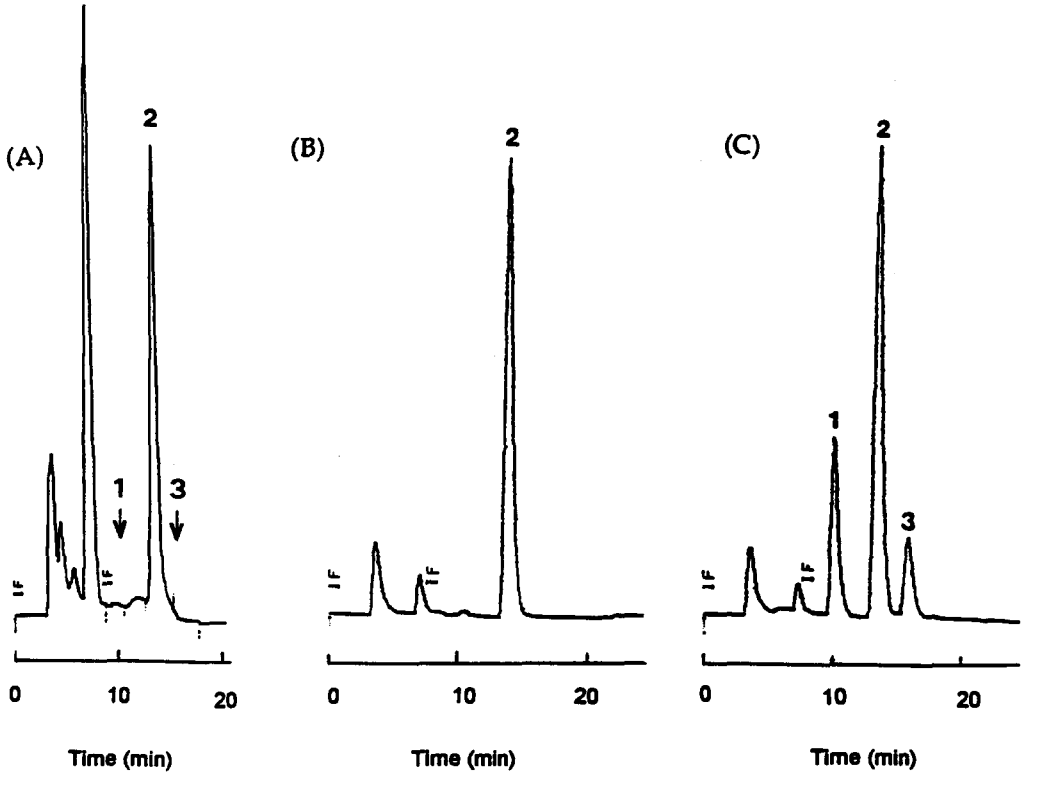


Figure 2
(A) Chromatogram of urine (renal impaired) with internal standard, (barbital) 2. (B). Chromatogram of urine (healthy)· with internal standard (barbital). 2. (C). Chromatogram of *p*-aminohippuric acid (PAH) 1; barbital; 2 and iothalamic acid (Io) 3, in urine (healthy).

volunteer and the two compounds and internal standard in urine (healthy), respectively. The mean retention times of *p*-aminohippuric acid, barbital and iothalamic acid are 10.4, 14.5 and 16.8 min and their relative standard deviations (RSD) are 8.6, 6.6 and 5.3%, respectively. The concentration data used for the calibration curves are presented in Table 1 (100.0–6000.0 μg ml^{-1} for Io and 125.0–8000.0 μg ml^{-1} for PAH). Even though the limits of quantiation were 50.0 μg ml^{-1} RSD 5.77%) and 75.0 μg ml^{-1} (RSD 9.04%) for the two compounds, these lowest points were omitted from the calibration curves.

Slope, standard error of the slope, and the intercept of calibration curves used for the calculation of iothalamate and *p*-aminohippurate concentrations are 7.47×10^{-4}, 9.07×10^{-6}, -9.53×10^{-3} and 1.28×10^{-3}, 4.18×10^{-5}, 1.19×10^{-3}, respectively. Linear regression of compound concentration against peak height ratio gives typical coefficients of determination (r^2) of 0.998–0.999 for both iothalamic acid and *p*-aminohippuric acid.

Variability studies

Interday and intraday variability was studied using frozen controls at four concentrations, extra-low, low, medium and high. Five samples from each concentration were assayed for both interday and intraday studies. For interday variability, six calibration curves on six different days were used and one calibration curve was used for the intraday variability study. The ranges for the RSD were 2.79–9.46% for iothalamic acid and 4.43–10.36% for *p*-aminohippuric acid for the interday studies as shown in Table 2. Relative standard deviation ranged from 1.48 to 3.53% for iothalamic acid and 1.84–4.27% for *p*-aminohippuirc acid for the intraday study as shown in Table 3.

Recovery

Assay recovery was measured by comparing the calculated concentration of the two compounds, at four different concentrations in urine and in aqueous solutions compound spiked at the same concentration. The mean % recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Calculated conc of compound in urine}}{\text{Calculated conc of compound in water}} \times 100$$

Table 1
Data used for calibration curves of iothalamic acid and *p*-aminohippuric acid in human urine

Iothalamic acid			*p*-Aminothippuric acid		
Conc. Io μg ml^{-1}	Peak height ratio (Io/Barb)	Calc. conc. Io, μg ml^{-1}	Conc. PAH μg ml^{-1}	Peak height ratio (PAH/Barb)	Calc. conc. PAH, μg ml^{-1}
0.00	0.000	0.0	0.00	0.000	0.00
100.00	0.018	116.2	125.00	0.027	148.80
250.00	0.042	268.7	300.00	0.071	324.90
500.00	0.083	522.8	700.00	0.161	725.00
1000.00	0.173	1057.0	1500.00	0.377	1582.90
2000.00	0.331	1994.9	3000.00	0.738	3016.50
4000.00	0.636	3805.3	5000.00	1.170	4732.20
6000.00	1.026	6120.2	8000.00	2.029	8143.60

Table 2
Interday variation of iothalamic acid and *p*-aminohippuric acid in urine

	Concentration* (μg ml^{-1})							
	Iothalamic acid				*p*-Aminohippuric acid			
	Extra-low [500.0]	Low [875.0]	Medium [1500.0]	High [3125.0]	Extra-low [500.0]	Low [1250.0]	Medium [2250.0]	High [3750.0]
Mean	469.02	892.79	1536.56	3181.10	483.73	1204.69	2265.07	3766.87
SD	44.28	42.00	55.91	88.95	49.54	94.05	115.32	167.65
RSD%	9.46	4.70	3.64	2.79	10.36	7.86	5.13	4.43

*Each value represents mean of $n = 4$.

The mean recovery was 98.6% for iothalamic acid and 97.8% for *p*-aminohippuric acid (Table 4).

Stability

The stability of the two compounds in urine during storage at room temperature for 24 h and during heat deactivation at 56–58°C for 55 min were evaluated. Five samples each from the four controls were used for these stability studies. Both compounds were found to be stable at RT, however heat deactivation caused a slight drop in concentration of both compounds (Table 5). Further, the stability of the two compounds during three freezing and thawing cycles was investigated. These results showed that the two drugs were very stable during three cycles (Table 5).

Table 3
Intraday variation of iothalamic acid and *p*-aminohippuric acid in urine

	Concentration* (μg ml^{-1})							
	Iothalamic acid				*p*-Aminohippuric acid			
	Extra-low [500.0]	Low [875.0]	Medium [1500.0]	High [3125.0]	Extra-low [500.0]	Low [1250.0]	Medium [2250.0]	High [3750.0]
Mean	494.27	887.52	1520.12	3154.24	512.54	1264.87	2326.58	3857.64
SD	17.43	17.42	37.28	46.80	14.47	54.01	69.62	71.04
RSD%	3.53	1.96	2.45	1.48	2.82	4.27	2.99	1.84

*Each value represents mean of $n = 6$.

Table 4
Recovery of iothalamic acid and *p*-aminohippuric acid from urine

Iothalamic acid	Conc. μg ml^{-1}	Calc. conc.* μg ml^{-1}	RSD (%)	Recovery (%)
	500.0	490.40	1.55	91.7
	875.0	922.70	1.75	98.0
	1500.0	1619.40	3.05	102.6
	3125.0	3285.75	1.61	101.9
p-Aminohippuric acid	500.0	530.25	1.43	93.55
	1250.0	1293.65	1.07	95.99
	2250.0	2372.15	1.38	97.59
	3750.0	3832.85	1.29	104.02

*Each value represents mean of $n = 5$.
Overall recovery of iothalamic acid 98.6%. RSD% 5.07.
Overall recovery of *p*-aminohippuric acid 97.8%. RSD% 4.58.

Table 5
Storage (24 h at RT), heat deactivation and freeze–thaw stability of iothalamic acid and *p*-aminohippuric acid in urine

		Percentage change*		
	Conc. μg ml^{-1}	Storage	Heat deactiv.	Freeze–thaw†
Iothalamic acid	500.0	−3.80	−2.61	−3.71
	875.0	5.52	−2.26	3.78
	1500.0	10.33	−4.36	5.36
	3125.0	6.96	−3.68	3.96
Overall mean		4.75	−3.23	2.35
p-Aminohippuric acid	500.0	−6.23	0.00	3.95
	1250.0	2.25	−11.22	3.65
	2250.0	2.03	−8.62	2.19
	3750.0	1.03	0.65	−0.47
Overall mean		−0.23	−4.80	2.33

*Each value represents mean of $n = 5$.
†After three cycles.

Discussion

The foregoing method is the first reported for determining iothalamic acid and *p*-aminohippuric acid in the same urine sample using an isocratic paired ion reversed-phase HPLC assay. The use of barbital to replace PABA as the internal standard has the advantage of eliminating interferences by metabolites of PAH, including PABA.

The recently marketed ion pairing reagent dodecyl trimethylammonium phosphate is needed only in small quantities. The long 12-carbon chain bonds more effectively to the C-8 group on the column packing thus giving better retention properties for acidic drugs. The main advantage of our method is that gradient elution or dual pump operations are eliminated by having a polar mobile phase containing 20–25% methanol. This results in improved column performance over long periods of time without frequent washing of the column to remove interfering substances usually present in biomatrices.

The assay method has a lower limit of quantitation of 100.0 μg ml^{-1} for iothalamic acid and 120.0 μg ml^{-1} for *p*-aminohippuric acid. These concentrations are acceptable for a urine assay because the drug concentrations are much higher than in plasma. The assay recovery of the compounds was very satisfactory and their stability in urine was within acceptable limits.

Using this method, urine samples from 24 subjects with normal and impaired renal function were analysed for Io and PAH. During the course of this pharmacokinetic study the renal clearances of iothalamic acid and *p*-aminohippuric acid were determined [11]. The plasma samples of this study were assayed with a method developed and validated previously by the same authors [12].

The validation of this assay was carried out according to the guidelines put forward by the conference on Analytical Methods Validation; Bioavailability, Bioequivalence and Pharmacokinetic Studies sponsored by the FDA, AAPS and AOAC among others [13]. It is noteworthy that, this reversed-phase ion pair HPLC method with isocratic elution is a simple, rapid and accurate method for simultaneous determination of these two compounds in human urine.

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