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Determination of iothalamate in rat urine, plasma, and tubular fluid by capillary electrophoresis

Natalia N. Davydova[a], Roberto Gotti[c], William J. Welch[b], Irving W. Wainer[a,*]

[a]*Department of Pharmacology, School of Medicine, Medical Center, Georgetown University, Washington, DC 20007, USA*
[b]*Division of Nephrology and Hypertension, Medical Center, Georgetown University, Washington, DC 20007, USA*
[c]*Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy*

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Abstract

A method for the quantitative determination of iothalamate (IOT) in rat urine, plasma and tubular fluid by capillary zone electrophoresis (CE) has been developed and validated. Samples of urine and tubular fluids were diluted with water and samples of plasma were deproteinized with two volumes of acetonitrile containing the internal standard, *p*-aminobenzoic acid (PABA). A BioFocus 2000 system (Bio-Rad, Hercules, CA, USA) was used. The UV detector was set at 254 nm. The samples were loaded into uncoated fused-silica capillary (40 cm×50 μm) by pressure injection. A borate buffer [20 m*M*, pH 12 (pH adjusted with 1.0 *M* NaOH)] was used as the electrophoretic buffer. The typical analytical conditions were: voltage, 22 kV; injection, 9 psi×s; capillary and carousel temperatures were 20°C and 18°C respectively. The linear relationship was observed between time-corrected peak area of IOT in water and urine or the corrected peak area ratio of IOT to PABA in plasma and the nominal concentration of IOT with correlation coefficient greater than 0.999. The intra- and inter-day coefficients of variation (CV) were less than 8%. The concentration of IOT in plasma, urine and tubular fluid determined by CE can be used for estimation of whole kidney and single nephron clearances.

Keywords: Glomerular filtration rate; Iothalamate

1. Introduction

A rapid and facile method for the determination of glomerular filtration rate (GFR) is key to the study of renal disease in both clinical and research settings. Rigorous assessment of GFR requires measurement of the renal clearance of an exogenous marker that is freely filtered by the kidney, and that does not undergo metabolism, tubular secretion or absorption. Inulin is such a marker. However, inulin is expensive, in short supply and technically difficult to determine in urine and plasma. Therefore its usefulness in clinical practice is limited.

Iothalamate (IOT, Fig. 1) is a urographic contrast medium, which is excreted in a fashion similar to inulin [1,2] and ^{125}I-IOT has been used to evaluate the renal function [3,4]. However the cost of ^{125}I-IOT, the regulatory constraints on the purchase,

*Corresponding author. Tel.: +1-202-6871650; fax: +1-202-6875015.
E-mail address: waineri@gunet.georgetown.edu (I.W. Wainer)

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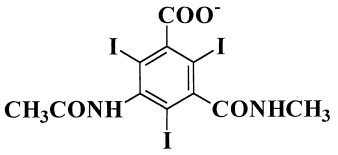

Fig. 1. Structure of iothalamate.

disposal and use of radioactive materials, and the exposure of the patients to radioactivity have led to the development of non-isotopic methods for the IOT-based measurement of GFR [5–13].

Several techniques have been published for the estimation of IOT in the serum and urine of humans, dogs and rats by high-performance liquid chromatography and X-ray fluorescence analysis [5–11]. While these methods avoid isotopes they are labor intensive, and not readily adaptable for routine clinical use. The development of capillary electrophoresis (CE) has offered new technology for the development of clinical assays. The CE conditions for quantitative measurement of the non-isotopic IOT in human urine and plasma were initially reported by Wilson et al. [12], and Bergert et al. [13]. Using CE, concentrations of iothalamate in urine and plasma can be determined and used to directly calculate GFR. CE has been shown to be a cost-effective approach for the clinical evaluation of GFR.

The CE technology has not been applied to measure the quantity of IOT in rat plasma, urine and tubular fluid. This paper reports development and validation of a CE-based method for determination of IOT in rat urine, plasma, and tubular fluid. The assay can be used for the estimation of whole kidney and single nephron GFR values.

2. Experimental

2.1. Reagents

Iothalamate (IOT) was obtained as Conray USP 66.8% from Malinckrodt Medical (St. Louis, MO, USA), *p*-aminobenzoic acid (PABA) was purchased from Sigma (St. Louis, MO, USA), sodium tetraborate was purchased from Aldrich (Milwaukee, WI, USA), sodium hydroxide was from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Apparatus and electrophoretic conditions

A BioFocus 2000 system (Bio-Rad, Hercules, CA, USA) was used for method development with UV detection at $\lambda = 254$ nm. The samples were loaded into uncoated fused-silica capillary (40 cm×50 μm) by pressure injection. A borate buffer [20 m*M*, pH 12 (pH adjusted with 1.0 *M* NaOH)] was used as the electrophoretic buffer. The typical analytical conditions were: voltage, 22 kV; injection, 9 psi×s; capillary and carousel temperature were 20°C and 18°C respectively. The capillary was rinsed with 0.1 *M* NaOH (60 s), with distilled water (90 s), and borate buffer [20 m*M*, pH 12] (90 s) between each injection. Plasma, urine and tubular fluid preparations were assayed under identical conditions, except for the rinse programs used. The time to rinse the capillary was increased for plasma over that used for urine and tubular fluid analysis. For plasma analysis, the capillary was rinsed with 0.1 *M* NaOH (120 s), with distilled water (180 s), and borate buffer [20 m*M*, pH 12] (180 s) between each injection.

2.3. Preparation of stock solutions.

Standard stock solution of IOT (66.8 mg/ml) was prepared in distilled deionized (DD) water. An internal standard solution containing 20 μg/ml PABA was prepared in acetonitrile.

2.4. Calibration standard preparation

2.4.1. In urine

Urine was diluted 10^3 times with water. The diluted urine was spiked with IOT stock solution to yield concentration 200 μg/ml for IOT. Calibration standards were prepared from this stock solution by serially diluting to yield concentrations of 100.00, 50.00, 25.00, 12.50, 1.25 μg/ml for IOT. The calibration curve was constructed by plotting the corrected peak area (area/migration time) versus the concentration of IOT in μg/ml.

2.4.2. In plasma

Spiked plasma standards for the calibration curve, were all obtained by serial dilution of an IOT stock solution (200 μg/ml in plasma). The plasma cali-

bration curve consisted of six standard at 200.00, 100.00, 50.00, 25.00, 12.50, 1.25 μg/ml of IOT.

2.4.3. In tubular fluid

The calibration curve for tubular fluid was made in DD water. IOT standard stock was diluted with water to yield a concentration 100 μg/ml and other standards were obtained by serial dilution with DD water. The water calibration curve consisted of five standards at IOT concentrations of 100.00, 50.00, 25.00, 12.50, 6.25, 1.25 μg/ml.

2.5. Sample preparation

2.5.1. Urine

The rat urine was diluted 10^3 times with water. The aliquot of the diluted urine (50 μl) was transferred into microcentrifuge tubes and centrifuged at 1500 *g* for 5 min. The clear supernatant was transferred to the EZ micro test tubes and used for analysis.

2.5.2. Plasma

Plasma was diluted ten times with water. The acetonitrile (200 μl) containing the internal standard *p*-aminobenzoic acid (20 μg/ml) was added into microcentrifuge tubes with 100 μl of plasma samples in it. The tubes were vortexed briefly and then centrifuged at 1500*g* for 10 min. The clear supernatant was transferred to other tubes and used for CE analysis.

2.5.3. Tubular fluid

The different amount of rat's tubular fluid (ranging from 13 nl to 42 nl) were diluted to 10 μl with DD water and without other treatment injected into CE system.

2.6. Intra-day and inter day validation

Intra-day and inter day validation studies for precision and accuracy were performed on five low-, medium-, and high-concentration quality control standards in urine and plasma. QC samples with following IOT concentration were prepared: 2.0, 40.0, 80.0 μg/ml (in urine), and 1.5, 75.0, 150.0 μg/ml (in plasma). The analyses were repeated on three separate days.

2.7. GFR study

2.7.1. Dosing

The Sprague-Dawley male rats weighing 250–370 g (Harlan Sprague-Dawley, Indianapolis, IN, USA) were used for studies of whole kidney and single nephron clearances. A maintenance fluid of 0.154 *M* NaCl solution containing bovine serum albumin (1%) and 16.4 mg/h IOT was infused at 0.5 ml/100 g body weight/h.

2.7.2. Plasma and urine samples

For whole kidney GFR, an equilibration period of 45 min was observed, followed by three study periods of 30 min each. For each period, urine was collected in a pre-weighed tube for 30 min and volume determined gravimetrically. At 15 min into each period, 0.5 ml of arterial blood was collected into a syringe with 50 units of heparin. Plasma was separated from red cells and stored at −20°C until analysis.

2.7.3. Tubular fluid samples

For single nephron GFR, free-flow micropuncture collections were made by inserting collection pipettes into proximal tubules (PT) and distal tubules (DT). Separate tubules were identified and selected. They were punctured in random order with glass micropipettes (OD 8–12 μm), containing dark-stained mineral oil, and connected by PE tubing to a glass syringe. Timed (2–5 min) samples of fluid were withdrawn following an initial injection of stained oil downstream, by aspirating upstream from the oil block. The sample was transferred into a constant bore capillary tube, whose length was measured with a micrometer to calculate the tubular fluid volume. The collection was transferred to a micro test tube, diluted to 10 μl with deionized water and stored at −20°C until analysis.

3. Results and discussion

3.1. Electropherograms

Electropherograms from a typical blank rat urine and plasma, ones spiked with IOT (urine) or IOT and PABA (plasma), and electropherograms of urine and

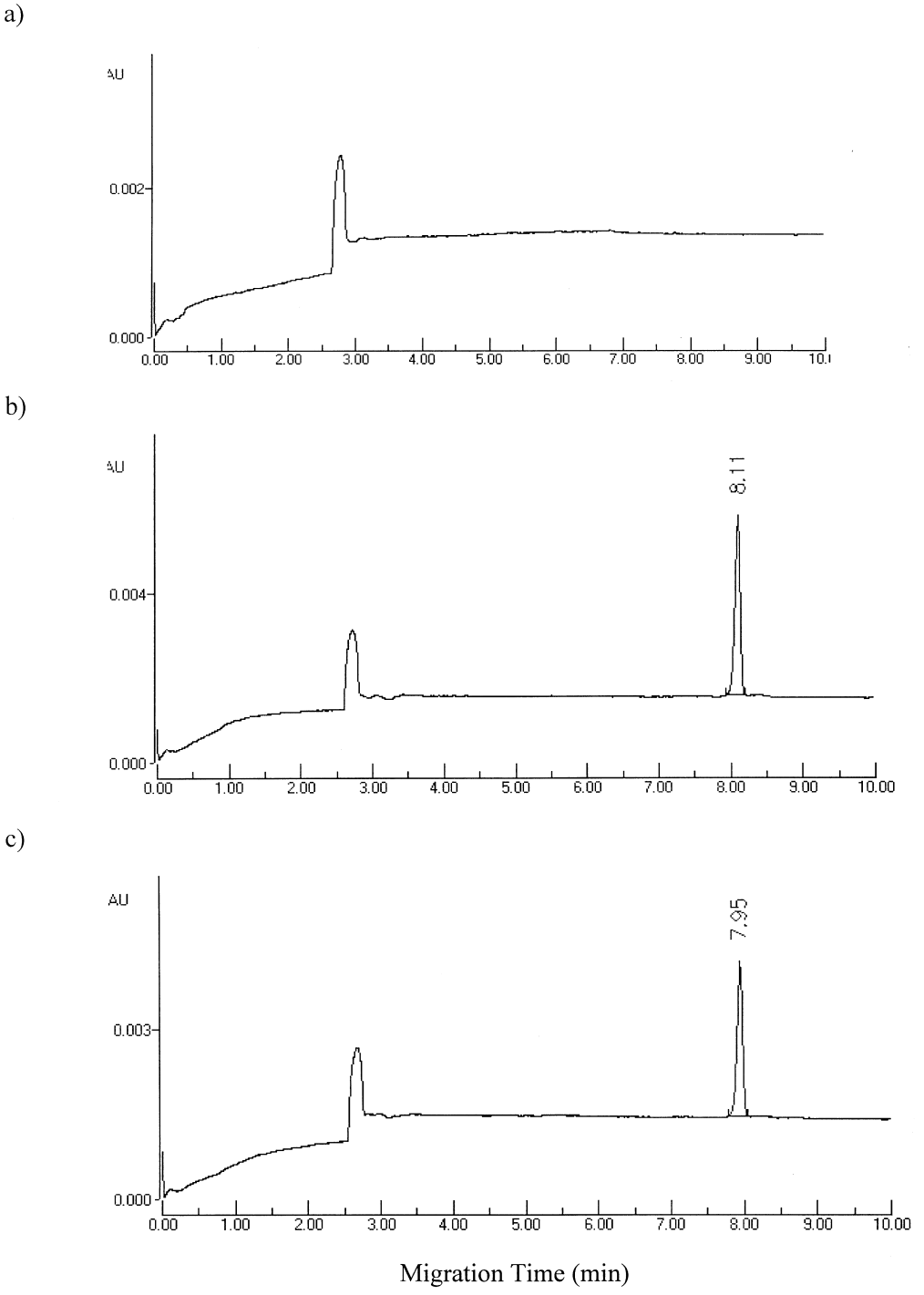

Fig. 2. Electropherograms of diluted urine samples. a) blank urine, b) urine spiked with IOT (8.11 min), c) diluted urine from rat administered IOT (7.95 min). See text for experimental details.

plasma from rat administered IOT are shown in Figs. 2 and 3 respectively. Electropherogram of a rat tubular fluid is shown in Fig. 4. The method for analysis IOT in urine, plasma, and tubular fluid described above was previously developed based on undiluted urine and plasma. The best results when blank urine and plasma samples were free of interfering peaks for IOT and PABA were achieved using

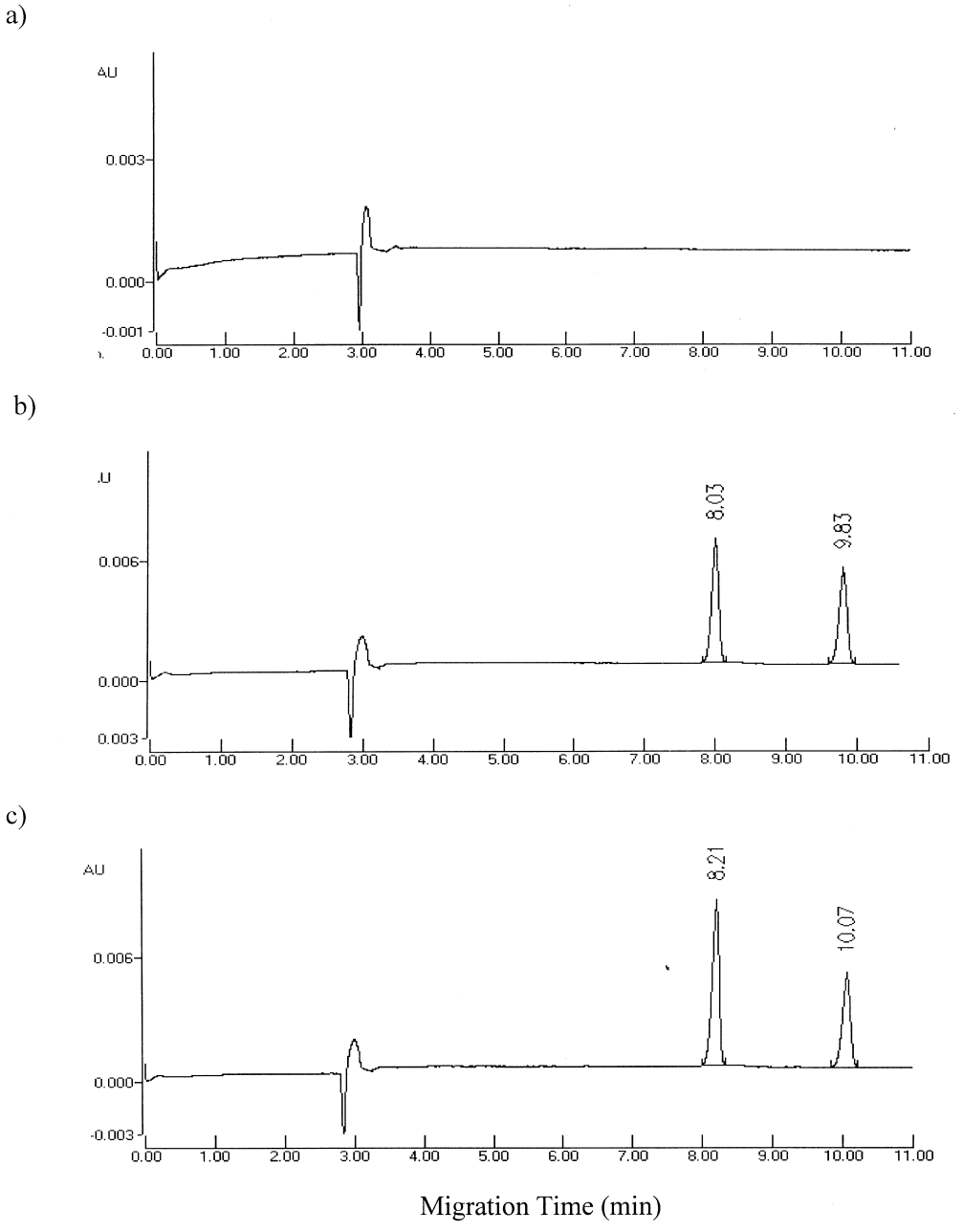

Fig. 3. Electropherograms of plasma samples. a) blank plasma; b) plasma spiked with IOT (8.01 min), PABA (9.83 min); c) plasma from rat administered IOT (8.21 min), PABA (10.07 min). See text for experimental details.

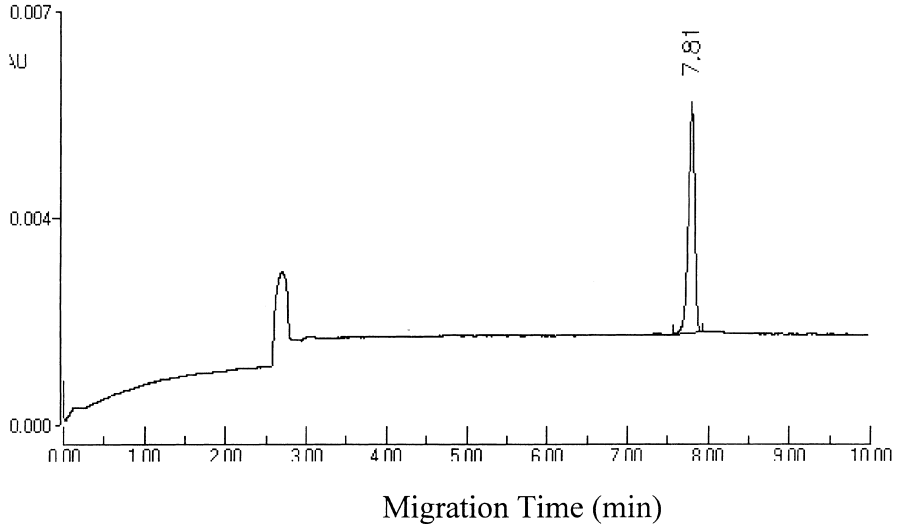

Fig. 4. Electropherogram of rat tubular fluid (IOT: 7.81 min).

borate buffer with pH 12. In case of the diluted urine and plasma, the electropherograms were very clean and there was no obvious interference. Therefore, a 20 m*M* borate buffer with pH 11 can be recommended to shorten the migration time for IOT and PABA from 8 min to 4 min and from 10 min to 6 min, respectively, reducing the total analysis time.

The electrophoretic conditions for analysis of IOT in human plasma and urine were previously published [12,13]. That method was developed with polyamide-coated fused-silica capillary. This study reports a CE-based methodology for analysis of nonisotopic IOT in rat plasma, urine and tubular fluid using uncoated fused-silica capillary which is less expensive for clinical application. In additional to that an internal standard was employed for determination of IOT level in plasma to improve the precision of the method during automatic injection.

3.2. Linearity

Calibration curves (1.25–200.00 μg/ml) were established in water, urine and plasma by plotting the corrected peak area (in water and urine) or the corrected peak area ratio of IOT to PABA (in plasma) versus the nominal IOT concentration. The correlation coefficient was 0.999 or better in urine, water and plasma. The equation for the line for IOT in diluted urine and water was $y=1000.1x+599.1$, where y represents the time normalized peak area, and x is the analyte concentration in μg/ml. For IOT in plasma, the equation for the calibration curve was $y=0.001x+0.01$, where y represents the corrected peak area ratio of IOT and PABA. Each set of standards was run in duplicate, as were the rat samples.

3.3. Validation

The results of intra-day and inter-day precision and accuracy for low-, medium-, and high-concentration of IOT in urine and plasma are reported in Tables 1 and 2 respectively. All CVs were less than 8%.

3.4. Determination of GFR

The whole kidney clearance of IOT, expressed in ml/min was calculated according to the formula:

$$\text{GFR (ml/min)} = \text{IOT}_{\text{Urine}}\,\text{(mg/ml)} \times \text{Urine flow-rate (ml/min)} / \text{IOT}_{\text{Plasma}}\,\text{(mg/ml)}$$

Whole kidney GFR data are presented in Table 3.

Table 1
Intra-day and inter-day precision and accuracy of IOT in urine

IOT	LoQC	MeQC	HiQC
Conc. (μg/ml)	2.0	40.0	80.0
Intra-day			
n	5	5	5
mean	1.9	41.5	81.2
S.D.	0.1	0.5	0.9
C.V.%	4.6	1.2	1.1
Accuracy	102.5	103.7	101.8
Inter-day			
n	15	14	14
mean	2.1	42.5	81.4
S.D.	0.1	1.5	1.5
C.V.%	6.5	3.5	1.9
Accuracy	104.2	106.2	101.8

Table 2
Intra-day and inter-day precision and accuracy of IOT in plasma

IOT	LoQC	MeQC	HiQC
Conc. (μg/ml)	1.5	75.0	150.0
Intra-day			
n	5	5	5
mean	1.5	76.6	149.6
S.D.	0.1	3.3	1.2
C.V.%	6.4	4.3	0.8
Accuracy	98.0	102.1	99.7
Inter-day			
n	13	11	12
mean	1.5	76.2	147.0
S.D.	0.1	3.7	2.8
C.V.%	7.5	4.9	1.9
Accuracy	102.2	101.6	98.0

Table 3
GFR values calculated by CE assay compared with [^{125}I]-IOT assay

Rats	Corrected GFR (ml/min/100 b.w.)	
	CE assay	[^{125}I]-IOT [14]
WKY ($n=2$)	0.80±0.1	0.72±0.1
SHR ($n=5$)	0.48±0.2	0.55±0.1

Table 4
CE analysis of rat tubular fluid

Tubular fluid	Conc. (ng/nl)
1. 22 nl diluted to 10 μl with water	8.1
2. 36 nl diluted to 10 μl with water	1.5
3. 42 nl diluted to 10 μl with water	1.8
4. 38 nl diluted to 10 μl with water	6.8
5. 13 nl diluted to 10 μl with water	6.5

The results obtained by CE were consistent with data obtained from the same species of rats utilizing ^{125}I-IOT as the GFR marker [14].

The results of the analysis of rat tubular fluid collected from selected tubules are shown in Table 4. The concentration of IOT in tubular fluid determined by CE, demonstrate that this method can be used for estimation of single nephron clearance. The results from studies to determine single nephron clearances will be reported elsewhere.

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