Iothalamate Quantification by Tandem Mass Spectrometry to Measure Glomerular Filtration Rate

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BACKGROUND: Glomerular filtration rate (GFR) can be determined by measuring renal clearance of the radiocontrast agent iothalamate. Current analytic methods for quantifying iothalamate concentrations in plasma and urine using liquid chromatography or capillary electrophoresis have limitations such as long analysis times and susceptibility to interferences. We developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to overcome these limitations.

METHODS: Urine and plasma samples were deproteinized using acetonitrile and centrifugation. The supernatant was diluted in water and analyzed by LC-MS/MS using a water:methanol gradient. We monitored 4 multiple reaction monitoring transitions: \( m/z \ 614.8 – 487.0, 614.8 – 456.0, 614.8 – 361.1, \) and \( 614.8 – 177.1 \). We compared the results to those obtained via our standard capillary electrophoresis (CE-UV) on samples from 53 patients undergoing clinical GFR testing.

RESULTS: Mean recovery was 90%–110% in both urine and plasma matrices. Imprecision was \( \leq 15\% \) for the \( m/z \ 614.8 – 487.0 \) and \( 614.8 – 456.0 \) transitions over a 10-day period at 1 mg/L. Method comparison for 159 patient samples (53 clearances) provided the following Passing–Bablok regressions: plasma iothalamate LC-MS/MS \((y)\) vs CE-UV \((x)\), \( y = 0.99x + 0.36 \); urine iothalamate LC-MS/MS vs CE-UV, \( y = 1.01x + 0.31 \); corrected GFR LC-MS/MS vs CE-UV, \( y = 1.00x + 0.00 \). Interfering substances prevented accurate iothalamate quantification by CE-UV in 2 patients, whereas these samples could be analyzed by LC-MS/MS.

CONCLUSIONS: Iothalamate can be quantified by LC-MS/MS for GFR measurement. This method circumvents potential problems with interfering substances that occasionally confound accurate GFR determinations.

The precise and accurate measurement of glomerular filtration rate (GFR) has value in chronic kidney disease, since GFR measurements are used for diagnosis, to monitor progression, to provide insight for proper medication dosing, and to assist in planning for renal replacement therapies (dialysis and transplantation) \((1)\). Many studies have highlighted the importance of an accurate estimate of GFR to determine prognosis and predict mortality \((2–4)\). GFR measurement is also an important component for assessing persons wishing to donate a kidney (living kidney donation).

GFR is commonly estimated on the basis of the serum creatinine concentration, or measured creatinine clearance. However, creatinine is not an ideal marker for GFR owing to several well-documented limitations. For example, tubular secretion of creatinine typically leads to overestimation of GFR, especially in patients with chronic kidney disease. GFR can be more precisely and accurately measured using exogenous compounds that are freely cleared by the kidney. Iothalamate is a radiopaque imaging media that has been used extensively in research and clinical practice for GFR determination \((5–8)\). \(^{125}\)I-labeled iothalamate has been used for GFR measurement \((9, 10)\). To avoid the use of radioactive materials, laboratory methods have been developed to quantify nonradioactive iothalamate using HPLC \((5, 6, 11–13)\) or capillary electrophoresis (CE) \((7, 8)\) coupled with ultraviolet (UV) detection systems. The HPLC-UV methods require long run times, limiting sample throughput, and the CE-UV methods can be susceptible to interference \((7, 8)\).
To minimize interference with iothalamate quantification, the previously reported CE-UV method used in our laboratory (7, 8) has since been modified to run in reverse polarity. Interferences are still encountered in approximately 1% of patient samples, however. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is an alternative method that typically enhances specificity when quantifying small molecules in biological samples. Therefore, we developed an LC-MS/MS method to quantify iothalamate in plasma and urine and calculate GFR. We compare this new method with our current CE-UV technique.

**Materials and Methods**

**REAGENTS**

All chemicals used were of reagent grade or better. Ethylenediamine dihydrochloride, phenyl phosphate, cetyltrimethylammonium bromide, sodium hydroxide, and formic acid were purchased from Sigma Aldrich; hydrochloric acid, phosphoric acid, acetonitrile, methanol (HPLC grade), and water (HPLC grade) from Fisher Scientific; and Conray® iothalamate meglumine injection USP 60% from Malinkrodt Inc. d3-Iothalamic acid was synthesized by the Mayo Clinic Chemical Synthesis Core Facility in Jacksonville, Florida.

**STUDY POPULATION**

We collected urine and blood specimens from 53 patients undergoing clinically indicated GFR testing in the Mayo Clinic Renal Function Laboratory. Patient preparation and protocols for iothalamate (Conray) administration and subsequent timed blood and urine collections were as described (8). We quantified creatinine using the Roche Creatinine Plus enzymatic assay performed on the Roche/Hitachi 912 per the manufacturer’s instructions. On the day of the renal clearance study, we performed the CE-UV method currently used in our Renal Function Laboratory to quantify iothalamate in urine and plasma samples. Blood and urine samples were stored refrigerated at 4 °C for up to 7 days before repeat iothalamate analysis by LC-MS/MS. Blood and urine samples were stored ambient, refrigerated at 4 °C, or frozen at −20 °C before iothalamate analysis by LC-MS/MS on days 0, 1, 3, and 7 to complete stability studies. We calculated GFR as described (8). This protocol was approved by the Mayo Clinic Institutional Review Board.

**CE-UV**

**Sample preparation.** For plasma analysis, we collected 2 timed 5-mL whole blood specimens in sodium heparin tubes and centrifuged them at 650g for 5 min, after which the plasma was removed for analysis. We combined 300 μL of the plasma obtained at the beginning (P1) and end (P2) of the GFR testing period, and deproteinized 200 μL pooled plasma by centrifugation for 10 min at 12 100g using an Amicon Micron Ultrafilter 10-kDa molecular weight cutoff filter (Millipore). We then added 25 μL filtrate to 25 μL internal standard (0.01 mol/L phenyl phosphate in 0.1 mol/L HCl).

For urine analysis, we deproteinized 300 μL of the timed urine collection (U1) by centrifugation for 10 min at 12 100g using an Amicon Micron Ultrafilter. We diluted the samples 1:10 with 0.1 mol/L HCl and added 25 μL of the diluted filtrate to 25 μL internal standard.

**Methodology.** Separation buffer for capillary electrophoresis consisted of 100 mmol/L phosphoric acid, 20 mmol/L ethylenediamine dihydrochloride, and 0.35 mmol/L cetyltrimethylammonium bromide, adjusted to pH 2 using sodium hydroxide pellets. The capillary column was 39 cm in length and consisted of polyimide-coated fused silica with an inner diameter of 50 μm and an outer diameter of 365 μm (Polymicro Technologies). We made a 2-mm detection window at 29 nm using a MicroSolv Window Maker (MicroSolv Technology). Samples were run on a P/ACE™ MDQ capillary electrophoresis system (Beckman Coulter) with the capillary temperature held at 20 °C and signal detection at 254 nm. We performed electrophoretic runs using a 20-s preparation rinse at 20 psi with fresh separation buffer followed by sample injection for 20 s at 0.5 psi, and applying a constant voltage of 602.5 V/cm (23.5 kV; reversed polarity) for 122 s. After each sample run, we rinsed the column with 0.1 mol/L NaOH at 50 psi for 1 min followed by a 1-min separation buffer rinse at 50 psi.

**Calibration.** We calibrated the CE-UV method using 3, 6, 12, 30, and 60 mg/L calibrators prepared using a 600 g/L stock solution of injectable USP grade Conray iothalamate meglumine. The 3, 6, 12, 30, and 60 mg/L calibrators contained 0.1 mol/L HCl. We made working calibrators by mixing equal volumes of the calibrator with the internal standard.

**LC-MS/MS**

**Sample preparation.** For plasma analysis, we pooled 50 μL of both P1 and P2 and added 20 μL internal standard consisting of 38 mg/L d3-iothalamic acid solution. We deproteinized the samples by adding 100 μL acetonitrile followed by vortex-mixing (5 s) and centrifugation (650g for 5 min). We diluted a 10-μL aliquot of the resulting supernatant in 490 μL water from a NANOpure® DIamond™ ultrapure water system (Barnstead Thermolyne).
For urine analysis, we diluted U₁ 1:10 with water and added 20 µL internal standard to 100 µL diluted urine. We deproteinized the samples by adding acetonitrile (100 µL) followed by vortex-mixing (5 s) and centrifugation (650g for 5 min). We diluted a 10-µL aliquot of the resulting supernatant with 490 µL water.

Methodology. We performed LC-MS/MS on an Aria TLX2 LC system (Thermo Scientific) coupled to an API 5000 triple-quadrupole mass spectrometer (Applied Biosystems). For each run, we injected 20 µL processed sample onto a 33 × 4.6 mm Supelcosil™ LC-18 column (Supelco). Chromatography was performed at 25 °C with a flow rate of 500 µL/min and a total run time of 6.25 min. Mobile phases consisted of solvent A (water, 0.1% formic acid) and solvent B (methanol, 0.1% formic acid). The chromatographic run started at 3% B and was held for 0.75 min, followed by a linear gradient to 95% B over 2 min, held at 95% B for 0.5 min, and then returned to 3% B over 0.5 min and held there for 2.5 min. The first 0.75 min of the LC run was diverted to waste, and data were collected for the next 4.15 min, before again diverting the last 1.25 min of the LC run to waste. The API 5000 instrument was used in positive ion mode with the following source parameters: CAD 12, CUR 40, GS1 25, GS2 40, IS 5500, TEM 600, and EP 10. Eight multiple reaction monitoring (MRM) transitions were acquired during this study, with the singly-charged precursor ion of iothalamate at the mass-to-charge ratio (m/z) of 614.8 monitored in Q1 (DP 138) and the following product ions monitored in Q3: m/z 487.0, 456.0, 361.1, and 177.1. The internal standard MRM transitions monitored the precursor mass of m/z 617.9 for the d3-iothalamate in Q1 (DP 138) and the following +3 mass units for the corresponding product ions in Q3: m/z 490.0, 459.0, 364.2, and 180.1.

All MRM transitions were monitored continuously throughout the MS/MS run, with an 80-ms dwell time. Only data from the m/z 614.8–456.0 transition are reported here and used for the GFR determinations reported in this article. We report validation data for all 4 MRM transitions in the supplemental tables, which accompany the online version of this article at http://www.clinchem.org/content/vol56/issue4.

Calibration and control material. We calibrated the LC-MS/MS method using 3, 6, 12, 30, and 60 mg/L Conray stock diluted in water. We also made a 1-mg/L specimen using Conray and water. This 1-mg/L specimen was not used in the calibration curve, however, but only to assess assay imprecision and limit of quantification.

We made controls by adding Conray 60% USP to plasma and urine samples that did not contain iothalamate. We prepared 1 plasma (11.3 mg/L) and 3 urine controls (5.6, 11.3, and 20.5 mg/L) to represent 3 GFR values denoted low, medium, and high.

Data reduction. We calculated iothalamate concentration by area under the curve using 1/x weighing with respect to the internal standard and linear regression to extrapolate calibration curves with 32 Karat™ software (Beckman Coulter) for CE-UV and Analyst® software (Applied Biosystems) for LC-MS/MS.

We performed Passing–Bablok and Bland–Altman analyses using Analyze-it® Software.

Limit of quantification and limit of detection. We calculated the limit of quantification (LOQ) for iothalamate as the response resulting in a CV ≤ 20% and recovery of 80%–120% and the limit of detection (LOD) using 3 SDs from the background signal of a zero calibrator.
Results

The CE-UV and LC-MS/MS data for a 12-mg/L calibrator are shown in Fig. 1. Elution times were 1.62 min for phenyl phosphate and 1.73 min for iothalamate in the CE-UV method (Fig. 1A). For the LC-MS/MS method, we observed a retention time of 1.80 min for iothalamate and d3-iothalamate (Fig. 1B; d3-iothalamate not shown).

We measured intraassay imprecision for all 4 MRM transitions by assaying the 3, 6, 12, 30, and 60 mg/L calibrators along with a 1 mg/L sample made in water a total of 10 times (see Supplemental Table 1A). All 4 MRM transitions displayed acceptable imprecision, <6% CV, and recoveries of 85%–115% (80%–120% at the LOQ) at all concentrations with the exception of the m/z 614.8–361.1 MRM transition, where the mean concentration deviated at 1 mg/L by >120%. These same samples were also used to determine interassay imprecision over a 10-day period (see online Supplemental Table 1B). All 4 MRM transitions displayed acceptable recoveries of 85%–115% (80%–120% at 1 mg/L). Imprecision over these 10 days was <15% CV for all concentrations assayed and <20% CV at the LOQ using the m/z 614.8–456.0 and 614.8–487.0 MRM transitions. Imprecision using the m/z 614.8–361.1 and 614.8–177.1 MRM transitions increased >20% at 1 mg/L. We determined the LOQ using imprecision data over the course of 10 days. Repeated measurements at 3 mg/L using CE-UV (4.89 μmol/L)
resulted in a CV of 9.3%. At the 1 mg/L LOQ of the LC-MS/MS method, (1.63 μmol/L; on-column 652 fmol), the CVs were 15.0% and 15.5% for the m/z 614.8–487.0 and 614.8–456.0 MRM transitions, respectively.

We also determined the intra- and interassay imprecision for all 4 MRM transitions by assaying controls a total of 10 times each over 10 days. Controls consisted of 1 plasma specimen and 3 urine specimens and were formulated to monitor the imprecision across 3 GFR values (low, medium, and high; summarized in online Supplemental Table 2). All controls displayed CVs <10% CV for all 4 MRM transitions for both the measured iothalamate concentration and the calculated GFR (see online Supplemental Tables 2A and 2C).

Mean recovery data for all 4 MRM transitions are summarized in online Supplemental Table 3 for plasma and urine, where the recoveries were 90%–110% for all 4 transitions in either urine or plasma matrices. The LOD was found to be 0.51 and 0.15 mg/L, respectively, for CE-UV and LC-MS/MS.

Stability studies demonstrated that the iothalamate measurements by LC-MS/MS did not vary by more than 15% when 10 unprocessed plasma and urine specimens were stored under ambient, refrigerated, and frozen conditions for up to 7 days. To assess ion suppression, a water matrix containing 12 mg/L iothalamate and d3-iothalamate was continuously infused at 0.6 mL/h. Plasma and urine samples (n = 10), to which 20 μL of water was added in place of the internal standard and which did not contain iothalamate or d3-iothalamate, were then prepared and injected via the standard chromatographic conditions described above, and the signal was monitored throughout the run. Although some ion suppression was observed, it was in a region of the chromatogram clearly distinct.

![Passing–Bablok regression comparison plots of plasma iothalamate (A), urine iothalamate (B), and surface area–corrected GFR (C) and Bland–Altman plot of the GFR comparison (D).](image-url)
from the elution of iothalamate and d3-iothalamate, and therefore was not a factor for our analysis. Online Supplemental Fig. 1, A and B, shows the ion suppression traces for plasma and urine, respectively.

An example of CE-UV analysis for a patient plasma specimen containing an unknown interfering substance is shown in Fig. 2A. This same sample was assayed by LC-MS/MS using the m/z 614.8–456.0 MRM (Fig. 2B). The LC-MS/MS method was not affected by iothalamate cross-talk with the interfering substance using this MRM; however, cross-talk was apparent with m/z 614.8–361.1 MRM. Furthermore, this interfering substance was detected in the patients urine (U0 sample) obtained before iothalamate administration (Fig. 2C), confirming that this unidentified substance was contained in a patient plasma and urine and not related to the iothalamate administration. The interference was not observed when the U0 sample was analyzed using the m/z 614.8–456.0 MRM (Fig. 2D). Therefore, although all transitions were acquired, the m/z 614.8–456.0 MRM was selected for quantification of iothalamate in plasma and urine. This interfering substance in the CE-UV method produced a high GFR value of 314 mL·min⁻¹·(1.73 m²)⁻¹, compared to the true GFR value of 21 mL·min⁻¹·(1.73 m²)⁻¹ obtained using iothalamate concentrations measured by LC-MS/MS.

Comparison plots of LC-MS/MS (m/z 614.8–456.0) vs CE-UV are shown in Fig. 3. Of the 53 patient GFR measurements evaluated in this study, 2 were found to have interfering substances where a coeluting substance was observed using the CE-UV method and were therefore excluded from the figures. The plasma, urine, and GFR iothalamate comparisons are provided in Fig. 3A, B, and C, respectively, and demonstrate agreement across all concentration ranges. A Bland–Altman plot comparing GFR calculated by iothalamate concentrations measured using CE-UV and LC-MS/MS is shown in Fig. 3D, showing low bias between the 2 methods. Fig. 4 shows the comparison of iothalamate clearance and creatinine clearance in our patient group. The bias at lower GFR values can be attributed to tubular creatinine secretion.

Discussion

In the current study, we have shown that iothalamate quantification can be achieved in plasma and urine specimens using either CE-UV or LC-MS/MS. The resulting calculations of GFR were highly correlated between the 2 methods, supporting LC-MS/MS as an acceptable alternative platform to quantify iothalamate in biological samples for determining GFR.

Urine and plasma sometimes contain compounds that interfere with iothalamate quantification by CE-UV. Even after modification of our previously published CE-UV protocol (7, 8), approximately 1% of specimens still contain unknown compounds that confound results. Although a creatinine clearance can be measured from the collected samples, it is well known that creatinine clearance may significantly overestimate true GFR. The LC-MS/MS technique is less prone to sample interferences, thereby increasing specificity for iothalamate quantification. We illustrated this using a sample that contained an interfering substance by CE-UV analysis as well as in 1 of the transitions monitored in the LC-MS/MS assay. However, the other MRM transitions were unaffected by this substance. It should be possible to use analysis software to compare the area ratios from all 4 transitions and set a threshold (e.g., 20%) of the expected area ratio to flag the sample for manual review. However, we did not validate this approach in the current study.

A previous report suggested that iothalamate is secreted by the kidney, and therefore iothalamate clear-
ance could overestimate GFR to the same extent that creatinine clearance does (14). Other studies however, suggest that iothalamate clearance is very tightly correlated with inulin clearance, whereas creatinine clearance consistently exceeds iothalamate clearance (6, 15, 16). As shown in Fig. 4, when creatinine clearance was obtained concurrently with iothalamate clearance, there was marked bias at lower GFRs.

In summary, we describe a method for GFR measurement by iothalamate clearance via LC-MS/MS analysis and have shown applicability of the method with actual biological samples. Because this LC-MS/MS method is less susceptible to interfering substances present in patient samples, it provides a more robust clinical GFR testing platform.

References

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