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Verification of separate measurement procedures where analytical determinations influence the clinical interpretation of GFR: Iohexol quantitation by HPLC and LC-MS/MS

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ABSTRACT

Background: The glomerular filtration rate (GFR) is monitored clinically to follow renal function of a patient. This is commonly performed using endogenous compounds, which estimate GFR (eGFR). However, several conditions exists which may confound or render the eGFR inaccurate. In such cases, it is appropriate to perform a procedure to directly measure GFR (mGFR). Iohexol plasma disappearance is a procedure to determine mGFR and is typically performed using bolus injection of contrast media followed by timed plasma collections. The iohexol plasma concentrations are referenced to the dose given and the elimination rate of iohexol is reflective of the mGFR. Therefore, analytical bias or interference in the iohexol analytical measurement procedure will directly impact the mGFR result.

Methods: Plasma sample iohexol concentrations were measured using both high performance liquid chromatography-ultraviolet detection (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MS/MS) measurement procedures. Results were compared on 50 patients where the mGFR was calculated from the iohexol plasma disappearance on two collection time points.

Results: Bland-Altman analysis illustrated < 1% mean bias when comparing iohexol concentration determinations from the measurement procedures. Passing-Bablok regression revealed $y = 1.028x - 0.9420$ (slope 95% CI: 1.011, 1.041; Y-intercept 95% CI: −1.606, −0.1638) when comparing LC-MS/MS to HPLC-UV.

Conclusions: Comparison studies of the LC-MS/MS and HPLC-UV measurement procedures displayed a mean bias of < 1% by Bland Altman analysis. Measurement of iohexol by LC-MS/MS and HPLC-UV produced similar results and suggests there should be minimal bias in concentration or computed mGFR solely due to the measurement procedure employed.

1. Introduction

Kidney function is an essential component in human physiology. The glomerular filtration rate (GFR) is generally considered the best diagnostic for overall kidney health and function [1]. Therefore, an accurate measure of GFR is desirable in clinical practice. An ideal marker of GFR must possess the following characteristics: be solely eliminated through the kidney via glomerular filtration, freely filtered by the glomerulus, not subjected to protein binding, not eliminated via renal tubular secretion, and not reabsorbed after glomerular filtration. There have been several endogenous and exogenous filtration markers, which have shown utility for GFR determinations. Endogenous creatinine is the most commonly used marker to assess and estimate GFR (eGFR). It is known creatinine is flawed for true GFR determinations as it is subject to tubular secretion, which has been demonstrated in studies with direct comparison to inulin [2]. Inulin is an exogenously administered inert fructose polymer, and inulin renal clearance is considered the “gold standard” in measuring GFR (mGFR) as it possesses all of the characteristics of an ideal GFR marker [3]. However, due to inulin's sporadic availability and lack of injectable inulin in many geographic locations along with procedural nuances, inulin measurement procedures (MPs) are almost never used in clinical laboratories. Numerous other exogenous GFR markers have been studied, and literature is quite confounded with studies showing both agreement and varying degrees of discordance with inulin mGFR [4,5]. This prompts scientific inquiry from practitioners and laboratories on the

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role the analytical MP presents in some of these comparison studies.

A common exogenous marker used to perform mGFR studies is the nonionic contrast agent iohexol. Iohexol has demonstrated plasma disappearance correlation with inulin renal clearance [6]. An interesting chemical feature of iohexol is it exists in different isomeric forms, and these forms slowly interconvert in aqueous solution [7]. The measurement of iohexol has been performed using a variety of MPs including high performance liquid chromatography-ultraviolet detection (HPLC-UV) [6,8,9], liquid chromatography tandem mass spectrometry (LC-MS/MS) [10–12], and X-ray fluorescence [13,14]. The isoforms (*endo-* and *exo-*) of iohexol can be separated chromatographically, and is common practice for most HPLC-UV MPs to separate and quantitate using only the major (*exo*-iohexol) isomeric peak. It is noted researchers have shown independent integration of either the endo or exo form accurately quantitates iohexol in specimens [6].

Proper calibration is essential for every clinical assay, and is critical when quantifying iohexol for mGFR purposes in plasma disappearance protocols since the laboratory result is directly referenced back to the calculated iohexol dose administered. This necessity for proper calibration was highlighted in a multicenter iohexol comparison study where analytical biases of iohexol concentrations between laboratories and/or MPs were found to be related to analytical calibration, and this directly influenced mGFR calculations from plasma disappearance studies [15]. Other potential complications for plasma disappearance procedures and protocols are filtration agent dose, sample collection time, duration, and analytical MP used. For example, investigators performed a method comparison study between HPLC-UV and LC-MS/MS MPs and found a difference of approximately 10% lower mGFR when assessing plasma by LC-MS/MS [16]. No reason for the discrepancy was provided in this report. Presently there is limited research documenting method comparison studies for iohexol. Given questions surrounding differences in mGFR determinations, why some studies suggest concordance while other studies suggest bias, we sought to focus on two laboratory MPs for iohexol, HPLC-UV and LC-MS/MS, and examine the potential methodological differences for the clinical determination of mGFR.

2. Materials and methods

2.1. Reagents

All chemicals used were of reagent grade or better. Acetonitrile (HPLC grade), methanol (HPLC grade), water (HPLC grade), and formic acid were purchased from Fisher Scientific (Hampton, NH); zinc sulfate (2 mol/L in water), glacial acetic acid (analytical grade), granular sodium acetate, and iohexol (USP reference material) were purchased from MilliporeSigma (Burlington, MA); tetrabutylammonium phosphate (0.5 mol/L) was purchased from Regis Technologies, Inc. (Morton Grove, IL); Saline (0.9%) was purchased from Allegiance Healthcare Corporation (McGaw Park, IL); iohexol (Omnipaque™ 300; 300 mg I/mL) was purchased from GE Healthcare (Chicago, IL); iohexol-d5 was purchased from Santa Cruz Biotechnology (Dallas, TX); and fresh frozen plasma was obtained from University of Minnesota Medical Center Blood Bank (Minneapolis, MN).

2.2. Study population

The study population consisted of 50 patients with clinically indicated mGFR testing using iohexol plasma disappearance. Each patient was given 5 mL of Omnipaque 300™ (GE Healthcare) followed by 10 mL of normal saline solution intravenously. Blood collections in lithium heparin tubes were obtained at 120 and 240 min with the mGFR calculated from iohexol concentrations using the Brochner-Mortensen correction [17]. Samples were prepared and iohexol measured as detailed below by both HPLC-UV and LC-MS/MS [10] MP's. Of this population, 98% ($N = 49$) were being evaluated for potential kidney donation, and 2% ($N = 1$) was being evaluated for potential pancreatic islet transplantation at the University of Minnesota Medical Center. The population mean age was 47.6 ± 14.3 years with minimum and maximum ages of 21 and 74 respectively. There were 29 females (58%), 21 males (42%), where all patients were Caucasian. This study was approved by the University of Minnesota Institutional Review Board.

2.3. HPLC-UV measurement procedure

2.3.1. Calibration curve

A standard stock of 10,000 μg/mL iohexol was made by diluting 773 μL of Omnipaque™ 300, (647.1 mg/mL iohexol) to a total volume of 50 mL with 0.9% saline solution. This standard stock solution was further diluted with saline to make a 100 μg/mL calibrator. The 100 μg/mL calibrator was then diluted further with 0.9% saline to make 50, 20, 10, 5, 2, 0.5 μg/mL calibrators. A calibration curve of three points was used consisting of 10, 5, and 2 μg/mL.

2.3.2. Quality controls (QCs)

Two levels of controls were prepared by adding standard stock to iohexol-free plasma and values of each QC sample were determined over 21 consecutive days yielding mean values of 11.1 and 40.7 μg/mL. Both QC samples are injected in duplicate, and checked at the beginning and end of each batch.

2.3.3. Sample preparation

Calibrators were injected as is and undiluted throughout the sample batches. Patient and QC samples were processed by taking 100 μL of each specimen and diluting with 900 μL of 0.9% sodium chloride solution in a 12 × 75 mm glass culture tube from Allegiance Healthcare Corporation (McGaw Park, IL). Diluted specimens were then subjected to vortex-mixing prior to addition to a 30 kDa molecular cutoff filter Vivafree™ 2 from Vivaproducts (Littleton, MA) for the purpose of protein removal. Specimens were then centrifuged at 4500 ×*g* for 15 min. The filter was discarded, and the filtrate briefly vortexed. A 150 μL aliquot of the filtrate from the lower portion of the filter was transferred to a 9 mm autosampler vial from Chrom Tech (Apple Valley, MN) prior to analysis.

2.3.4. HPLC-UV chromatographic conditions

HPLC was performed using a 2000 series with an ECD 2000 detector from ECOM (Prague, Czech Republic) connected to an L-3320 autosampler from RIGOL Scientific, Inc. (Beijing, China). For each run, 20 μL of sample was injected onto a 7.5 cm × 4.6 mm Supelcosil LC-18-DB HPLC column from MilliporeSigma (Burlington, MA) fitted with a 2 μm precolumn filter from Idex Health & Science (Lakeforest, IL). A 100 mmol/L sodium acetate buffer was prepared in water, and the pH was adjusted to 5.15 ± 0.05 with glacial acetic acid. The mobile phase consisted of 100 mmol/L sodium acetate buffer with 0.25 mmol/L of ionate tetrabutylammonium phosphate (TBAP) [18]. The isocratic elution was run at 2.0 mL/min for a total run time of 7 min, and UV absorbance was monitored at 254 nm.

2.3.5. Data reduction

Iohexol concentration was calculated by Clarity advanced chromatography software from DataApex (Prague, Czech Republic).

2.4. LC-MS/MS measurement procedures

2.4.1. Calibration curve

A 10,000 μg/mL iohexol stock solution was made by diluting 773 μL of Omnipaque™ 300 (647 mg/mL, iohexol), with diluent (5% acetonitrile in saline (0.9%)) to 50 mL. The amount of Omnipaque™ 300 added was also verified gravimetrically and referenced to density (1.342 g/mL at 25 °C) prior to final dilution. This stock solution was used to make a

200 μg/mL and 100 μg/mL calibrators by dilution with diluent. The 100 μg/mL calibrator was used to make the 50, 20, 10, 5, 2, 1 μg/mL calibrators with the same 5% acetonitrile in 0.9% saline diluent. This eight point calibration curve was processed and injected for each batch.

2.4.2. Internal standard

The working internal standard (50 μg/mL) was made by diluting 500 μL of iohexol-d5 (1.0 mg/mL) to a total volume of 10 mL using 10% acetonitrile in water.

2.4.3. Quality control (QC) samples

Five plasma QC samples were prepared by spiking iohexol-free fresh frozen plasma with stock iohexol solution and the values of each QC sample were determined over 21 days yielding mean concentrations of 4.85, 29.55, 68.8, 84.4 and 87.0 μg/mL.

2.4.4. Sample preparation

All specimens, controls, and calibrators were processed by adding 20 μL of internal standard to 100 μL of each sample. Samples were deproteinized with the addition of 100 μL 0.1 M zinc sulfate solution in methanol:water (70:30), followed by vortex-mixing (30 s) and centrifugation (9875 × g for 2 min). A 20 μL aliquot of the resulting supernatant was diluted with 1.0 mL of water in a 1.8 mL autosampler vial from Chrom Tech (Apple Valley, MN) and the solution used for analysis.

2.4.5. LC-MS/MS total iohexol analysis

This chromatographic approach was used for the elution of all iohexol isomers in a single peak for integration and quantitation of total iohexol. 10 μL of processed sample was injected onto a 30 × 4.6 mm Pursuit XRs 3 C18 column from Agilent (Santa Clara, CA). Chromatography was performed at 40 °C at a flow rate of 0.90 mL/min with a run time of 5.5 min. Mobile phases consisted of solvent A (water, 0.1% formic acid) and solvent B (methanol, 0.1% formic acid). Gradient chromatography was employed with the profile starting at 3% B held for 0.50 min followed by a linear gradient to 35% B over 2.0 min, ramped to 95% B over 0.50 min and held for 0.50 min, before being returned to 3% B over 0.10 min and equilibrated for 1.9 min. The first 0.50 min and last 1.5 min of the LC run was diverted to waste.

2.4.6. LC-MS/MS endo- and exo-iohexol analysis

This chromatographic approach was employed to separate the *endo*- and *exo*-iohexol isomers into two separate peaks for independent quantitation of the endo and exo forms. Each run consisted of a 10 μL injection of processed sample onto a 30 × 2.1 mm Kinetex 2.6 μm Biphenyl column from Phenomenex (Torrance, CA). Chromatography was performed at 40 °C with a flow rate of 0.70 mL/min, and a run time of 5.5 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 2.0 min and then ramped to 95% B over 0.10 min, held at 95% B for 1.0 min, and then returned to 3% B over 0.10 min and equilibrated for 2.3 min. The first and last 0.50 min of the LC run were diverted to waste.

2.4.7. LC-MS/MS system

LC-MS/MS analysis was performed using a LC-20XR integrated liquid chromatography system Shimadzu (Kyoto, Japan) coupled to a QTRAP 6500 triple quadrupole mass spectrometer SCIEX (Framingham, MA). The mass spectrometer was operated in positive electrospray ionization mode with the following source parameters in both chromatographic procedures: CAD medium, CUR 35, GS1 50, GS2 55, IS 5500, TEM 650, EP 10. Multiple reaction monitoring (MRM) transitions for iohexol were obtained using the singly-charged precursor ion of iohexol at the mass-to-charge ratio (*m/z*) of 821.9 monitored in Q1 and the following product ions monitored in Q3: *m/z* 656.7 and 602.8. Internal standard MRM transitions used a precursor mass of *m/z* 826.9 for the d5-iohexol in Q1 and the following product ions in Q3: *m/z* 657.5 and 607.8.

All MRM transitions were monitored throughout the MS/MS run using a 30-ms dwell time. We only used data from the iohexol *m/z* 821.9 – 602.8 transition for iohexol quantitation and GFR determinations.

2.4.8. Data reduction

Iohexol concentration was calculated by area under the curve using 1/x weighting with respect to the internal standard and linear regression to extrapolate calibration curves with MultiQuant™ software from SCIEX.

2.4.9. Data analysis

Bland-Altman analysis was performed using Microsoft Excel software (Redmond, WA). The percent bias for iohexol concentrations was calculated using Bland-Altman analysis as the difference between iohexol concentrations calculated from HPLC-UV and LC-MS/MS divided by the mean concentration from the HPLC-UV and LC-MS/MS results. The percent bias was calculated for mGFR determinations using Bland-Altman analysis from the difference between mGFR derived from HPLC-UV and LC-MS/MS divided by the mean GFR from the HPLC-UV and LC-MS/MS results. Passing-Bablok analysis was performed using Analyse-It (Analyse-It Software, Ltd., Leeds, LS3 1HS, United Kingdom) Microsoft Excel add-in software. Glomerular filtration rates were calculated using the two time-points (120 and 240 min) using Microsoft Excel software (Redmond, WA) as per Brochner-Mortensen [17].

3. Results

Limit of Quantification (LOQ) was determined for the HPLC-UV MP chromatographic protocol by evaluating the *exo*- iohexol peak for the 0.5 μg/mL calibrator by running for 21 days where the %CV was found to be 9.0%. Other calibrators assessed during this 21 day period displayed %CV between 0.3% and 1.8%. Limit of detection (LOD) was calculated using a signal-to-noise (S/N) ratio of 3 and was determined to be 9.7 ng/mL. Within-batch imprecision was assessed by 10 replicates for 2 samples in the same batch and found to be < 0.4%CV with mean concentrations of 10.4, and 42.9 μg/mL. The between-day imprecision was determined over 21 separate days for plasma controls with mean concentration of 11.2 and 40.7 μg/mL where the %CV all for each level was < 3.9%. The MP was found to linear throughout analytical measurement range (AMR) 0.5–100 μg/mL and could be extended to 500 μg/mL employing a 5 fold sample dilution. Representative chromatograms for the HPLC-UV measurement procedure are illustrated in Fig. 1.

The total iohexol analysis LC-MS/MS MP chromatographic protocol LOQ was determined by running the 1 μg/mL calibrator for 21 days and the %CV was found to be 4.2%. Other calibrators assessed during this 21 day period displayed %CV between 1.2% and 3.9%. LOD was calculated using a S/N ratio of 3 and was determined to be 2.8 ng/mL. Within-batch imprecision was assessed by 20 replicates for 3 controls in the same batch and found to be < 3%CV with mean concentrations of 4.65, 67.9 and 85.7 μg/mL. The between-day imprecision was determined over 21 separate days for plasma controls with mean concentration of 4.85, 29.6, 68.8, 84.4 and 87.0 μg/mL where the %CV all for each level was < 3.8%. The MP was found to linear throughout analytical measurement range (AMR) 1–200 μg/mL could be extended to 2000 μg/mL by employing a 20 fold sample dilution. The chromatographic traces for total iohexol by LC-MS/MS are shown in Fig. 1.

Comparison of iohexol concentrations and computed mGFR's from LC-MS/MS (Total Iohexol) vs. HPLC-UV (Exo-Iohexol) MP's for iohexol is shown in Fig. 2. Both the iohexol concentration and mGFR determination derived from the respective MP results were obtained for 100 plasma samples from 50 patients undergoing kidney evaluation studies. Passing-Bablok regression (Fig. 2A) revealed y = 1.028x − 0.942 (slope 95% CI: 1.011, 1.041; Y-intercept 95% CI:

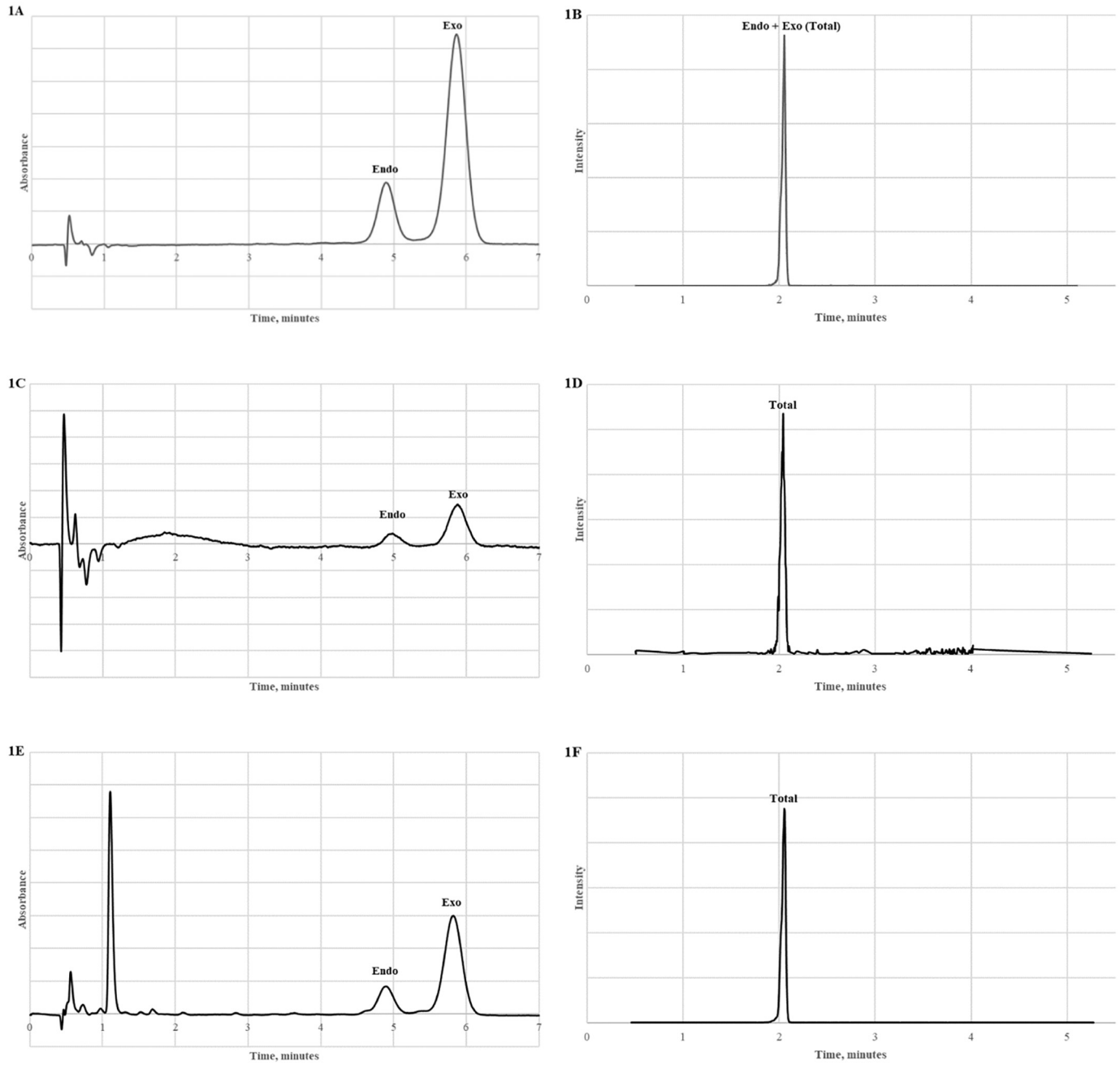


Fig. 1. A. Representative HPLC-UV chromatogram from a 10 μg/mL calibrator illustrating separation of the endo and exo iohexol isomers. B. Representative LC-MS/MS Total Iohexol chromatogram from a 10 μg/mL calibrator with simultaneous elution of the endo and exo iohexol isomers for quantification of "Total" iohexol. C. HPLC-UV chromatorgram at the 0.5 μg/mL level. D. LC-MS/MS Total Iohexol chromatrogram at the 1 μg/mL level. E. HPLC-UV representative patient sample. F. LC-MS/MS Total Iohexol representative patient sample.

−1.606, −0.1638) for the iohexol concentrations ($N = 100$), and $y = 1.005x - 1.539$ (slope 95% CI: 0.9646, 1.050; Y-intercept 95% CI: −5.438, 2.125) for the mGFR plasma disappearance (Fig. 2C) ($N = 50$) when comparing the two MPs. A mean bias < 1% was observed by Bland-Altman analysis between both MP's for the iohexol concentration (Fig. 2B) and mGFR determination (Fig. 2D) comparison studies. The overall study mean iohexol concentrations determined by the HPLC-UV and LC-MS/MS MPs were 59.4 ± 26.3 and 60.0 ± 27.1 μg/mL, respectively; and the mGFR overall study mean was 91 ± 16 mL/min/1.73m^2 for HPLC-UV and 90 ± 16 mL/min/1.73m^2 for LC-MS/MS MPs.

Chromatographic separation and quantification of iohexol was also investigated in the MPs. Three chromatographic separation profiles were examined: 1) the HPLC-UV MP Endo- Exo-Iohexol isomeric separation, 2) the LC-MS/MS MP Total Iohexol with no isomer separation, and 3) a LC-MS/MS Endo- and Exo-Iohexol with separation the *endo*- and *exo*-isomers.

A study was performed by assessing a 38 μg/mL iohexol solution, which was derived from iohexol powder dissolved in 0.9% saline. This powder derived solution of iohexol was measured that same day (Day 0) by two MPs HPLC-UV Endo- Exo-Iohexol and LC-MS/MS Total Iohexol and this solution was aliquoted for storage at different temperatures (room temperature, 4 °C, and −20 °C), for a total of 16 days. Iohexol concentrations of the solutions were again measured on Days 1, 7, and 16. The individual values measured at each day after storage at room temperature (Fig. 3A), 4 °C (Fig. 3B), and −20 °C (Fig. 3C). The

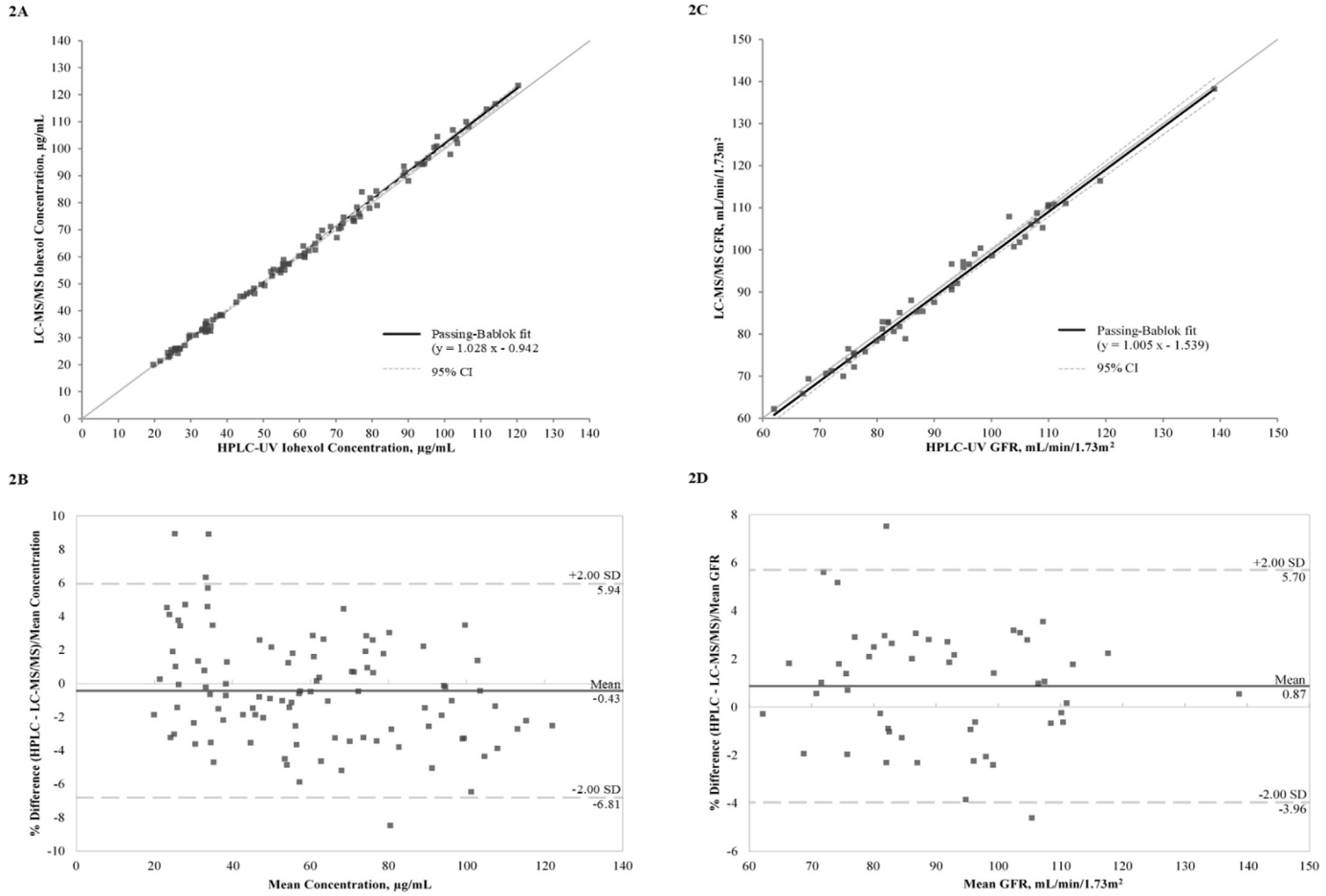


Fig. 2. A. Passing-Bablok regression and B. Bland-Altman analysis: Comparison of iohexol concentrations measured by HPLC-UV (Exo-Iohexol) and LC-MS/MS (Total Iohexol). C. Passing-Bablok regression and D. Bland-Altman analysis: Comparison of iohexol derived GFR measured by the two MPs. Passing-Babloks: HPLC-UV Exo-Iohexol (x-axis) vs. LC-MS/MS -Total Iohexol (y-axis); and regression (black), with 95% confidence intervals (dashed), and line of identity (grey).

iohexol concentration was measured as a single peak using LC-MS/MS Total Iohexol and both of the HPLC-UV Endo- Exo-Iohexol peaks. Quantitation in all MP's was achieved by referencing the chromatographic results to a calibration curve constructed from Omnipaque as described in the methods section.

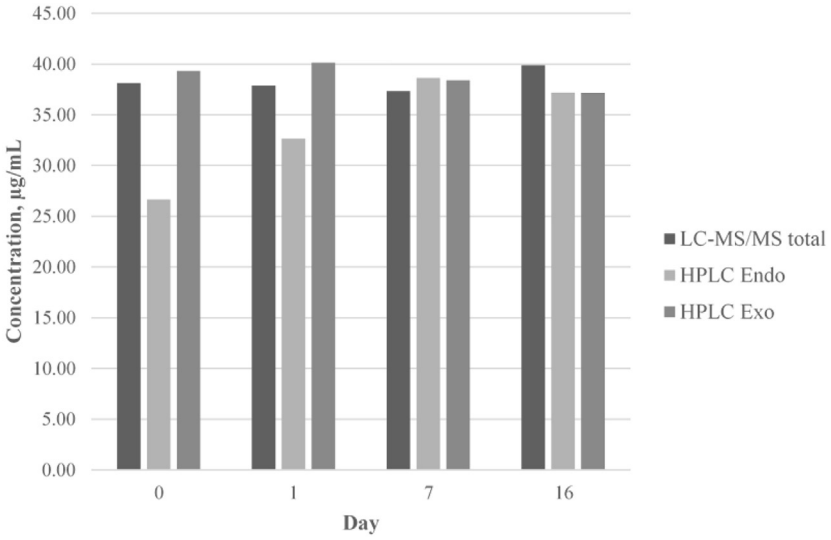
Monitoring mGFR using either the *endo*- or the *exo*-iohexol by both HPLC-UV and LC-MS/MS was also examined on a subset of 12 patients (24 samples). For this a LC-MS/MS MP that separates the isomers of iohexol was developed (Endo- and Exo-Iohexol) to allow for independent integration of the isomers by LC-MS/MS. Passing-Bablok regressions for the computed mGFR's are shown for HPLC-UV Endo-Iohexol vs. HPLC-UV Exo-Iohexol peaks (Fig. 4A) $y = 0.946\ x + 1.548$ (slope 95% CI: 0.6033, 1.350; Y-intercept CI: −39.88, 29.54) and LC-MS/MS Endo-Iohexol vs. LC-MS/MS Exo-Iohexol peaks (Fig. 4B) $y = 1.023\ x - 1.608$ (slope 95% CI: 0.9675, 1.110; Y-intercept 95% CI: −9.312, 3.703).

4. Discussion

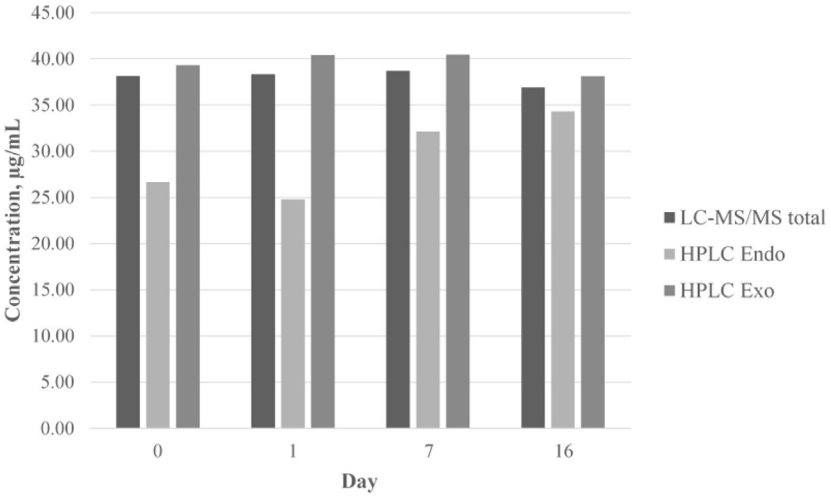
The data derived from the HPLC-UV (Exo-iohexol) and LC-MS/MS (Total Iohexol) method comparisons suggests the ability for these two separate MPs to have a minimal bias (< 1%) between them when assessing both the iohexol concentration and determining mGFR as illustrated in Fig. 2. This would suggest that no platform derived large measurement difference or bias should be observed in results obtained between these two MPs. Since both MPs assess physical properties of iohexol and a minimal bias was observed in measured iohexol concentrations between the MPs, we sought to investigate how larger bias could have potentially been found between these different methodological approaches [16].

Iohexol exists as a mixture of conformational *exo*- and *endo*-isomers, and the isomer ratio gradually equilibrates in solution, and this interconversion rate is temperature dependent [7]. Most published HPLC MP's separate these isomers, and quantify iohexol using only the major (Exo-Iohexol) isomer [6,15,19]. Most LC-MS/MS MPs do not separate the isomers and quantify all forms of iohexol (Total Iohexol) in a single integration event [10,11]. Thus, any interconversion that occurs, the "Total" approach would be unaffected, and results obtained using the LC-MS/MS MP for total iohexol should remain constant with isomer interchange. It was found the initial peak area ratios of *endo*- (~13%) and *exo*-(~87%) isomers in aqueous solutions from freshly prepared iohexol powder is quite different from the final endo-(~26%) and exo-(~74%) peak ratios in the aqueous solution, which is similar to what is observed in Omnipaque derived calibrators. The quantitative effects of this are shown in in Fig. 3. Both peaks from the HPLC-UV Endo- and Exo-Iohexol results move toward unity as isomer equilibration occurs over time from stock solutions made from iohexol powder. This equilibration time is dependent on the storage temperature. Whereas, the results generated by the LC-MS/MS Total Iohexol integration remained constant over time at every temperature investigated, which is to be expected as the total concentration of iohexol is not changing. This time-dependent equilibration is important for laboratories preparing calibrators or controls from powdered iohexol and assessing these samples on MPs, which separate the *endo*- and *exo*-iohexol forms. This

3A



3B



3C

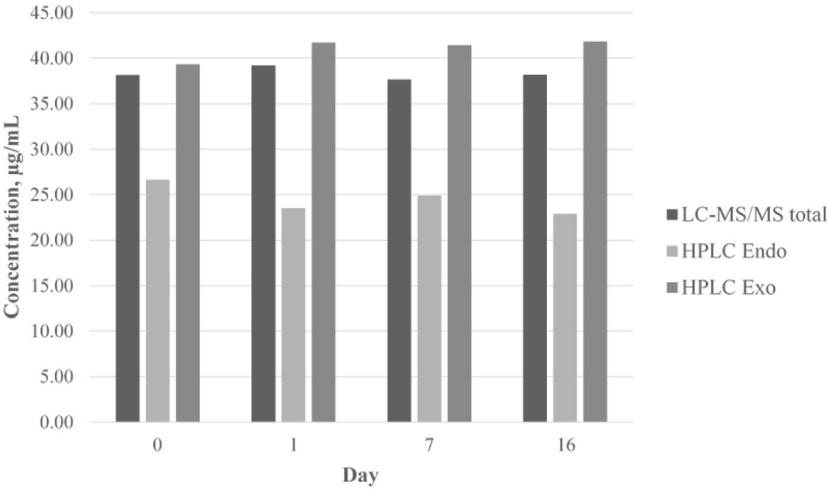


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Fig. 3. A. Iohexol concentration (μg/mL) from a sample derived from crystalline iohexol powder when analyzed by LC-MS/MS Total Iohexol and both HPLC-UV Endo- Exo-Iohexol peaks when stored at room temperature measured at day 0, 1, 4, and 16. B. Iohexol concentration (μg/mL) of sample stored at 4 °C measured at day 0, 1, 4, and 16. C. Iohexol concentration (μg/mL) of sample stored at −20 °C measured at day 0, 1, 4, and 16.

phenomena is also an important consideration should standardization efforts attempt to address iohexol calibration. Laboratories must ensure the calibrator or quality control solutions have equilibrated properly prior to quantitative analysis, as non-equilibrated samples derived from iohexol powder compared to an equilibrated calibration curve could produce erroneous results. Many laboratories use commercially available iohexol solutions (i.e., Omnipaque) for preparation of calibrators and quality control samples. Omnipaque solutions are heat sterilized

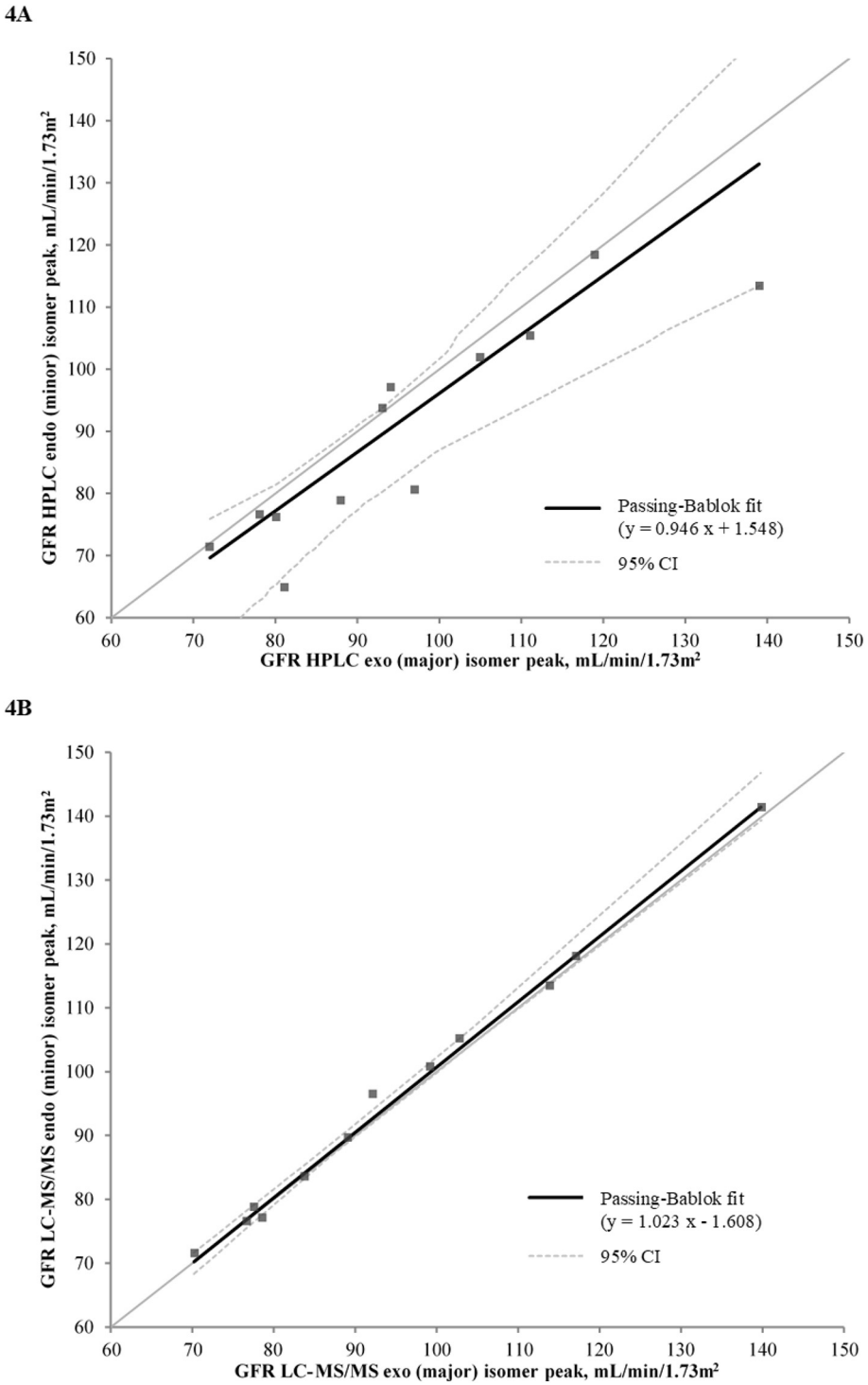

Fig. 4. Passing-Bablok regressions: regression (black), with 95% confidence intervals (dashed), and line of identity (grey). A. Iohexol derived GFR measured by HPLC-UV calculated from the exo isomer (x-axis) or the endo isomer (y-axis). B. Iohexol derived GFR measured by LC-MS/MS *endo- exo-* measurement procedure calculated from the exo isomer (x-axis) or the endo isomer (y-axis).

[20] prior to product release, which is thought to bring the ratio of *exo*- and *endo*-iohexol rapidly into equilibrium [21].

Another distinctive difference between the MPs is the detection method employed by each analytical instrument. The HPLC-UV MP measures iohexol's ultraviolet light absorbance whereas the LC-MS/MS utilizes multiple reaction monitoring to measure iohexol. Result differences in the plasma disappearance mGFR by the HPLC-UV Endo-Exo-Iohxol MP (Fig. 4A) clearly display differences when comparing the two isomers within this same MP, which is attributed to interference in the endo peak using this MP. This scatter was not observed using LC-MS/MS Endo- and Exo-Iohexol MP in Fig. 4B when comparing results between endo and exo isomers suggesting increased analytical specificity using LC-MS/MS. It is important to note that the endo isomer for the HPLC-UV MP is not used in our clinical mGFR determinations for our patient population and was only used to calculate GFR for investigational purposes of this study. It is noted the MPs discussed in this manuscript along with many other HPLC-UV and LC-MS/MS MPs are actively enrolled in an interlaboratory quality control assessments executed by Equalis AB (Uppsala, Sweden).

5. Conclusion

In summary, we found a negligible (< 1%) mean bias between iohexol concentrations and mGFR determinations when using HPLC-UV Exo-Iohexol and LC-MS/MS Total Iohexol from the MP's. These results are in contrast to the findings of Delanaye et al [16] where a ~10% difference in mGFR was observed when using HPLC versus LC-MS/MS MPs. Discrepancies between our two MPs have highlighted differences in the technologies, different calibration approaches, and how potentially analytical interferences in MPs could introduce biases in mGFR. All clinical MP's are slightly different and subtle interferences can vastly differ from MP to MP. Thus, MP biases must be carefully assessed by the testing laboratory. Chromatographic separation is the biggest difference between the MPs investigated here. Analytical interferences from endogenous non-iohexol compounds found in plasma potentially cause mGFR errors. Also, when dissolving crystalline iohexol in solution, sufficient time must be given for equilibration to be reached as the ratio of *endo*- and *exo*-isoforms may change from the initial state. This initial state is likely different from the Omnipaque given to patients for GFR measurements. In any case, GFR measured from the same filtration agent in the same samples should give the same mGFR value across MPs. Differences likely arise from MP analytical error, calibration [15], and possibly analytical interferences in the MPs.

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