การตรวจวัดระดับไอโอเฮกซอลในพลาสมาด้วยวิธีโครมาโทกราฟีของเหลวสมรรถนะสูงพิเศษอย่างง่ายและรวดเร็ว

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บทคัดย่อ

การวัดค่าไอโอเฮกซอลในพลาสมาอย่างแม่นยำถือเป็นสิ่งสำคัญในการตรวจวัดค่าอัตราการกรองของไต (GFR) ในการศึกษานี้ เราได้พัฒนาและตรวจสอบความใช้ได้ของวิธีวิเคราะห์ใหม่ในการหาปริมาณไอโอเฮกซอลโดยใช้โครมาโทกราฟีของเหลวประสิทธิภาพสูงพิเศษพร้อมการตรวจจับอัลตราไวโอเลต (UPLC-UV) การศึกษานี้เกี่ยวข้องกับการเตรียมตัวอย่างและการแยกทางโครมาโทกราฟีสำหรับไอโอเฮกซอล ค่าพารามิเตอร์ต่าง ๆ ประกอบด้วยประเภทของคอลัมน์ องค์ประกอบและพีเอชของเฟสเคลื่อนที่ ได้รับการปรับให้เหมาะสมเพื่อให้ได้การแยกที่เหมาะสมที่สุด สภาวะโครมาโทกราฟีที่เหมาะสมที่สุดเป็นดังนี้ เฟสเคลื่อนที่คือร้อยละ 3.5 (ปริมาตรต่อปริมาตร) อะซีโตไนไตรล์ต่อน้ำ โดยไม่ต้องปรับความเป็นกรด-ด่าง คอลัมน์คือ Poroshell 120 EC-C18 โดยควบคุมอุณหภูมิที่ 30 องศาเซลเซียส และอัตราการไหล 0.2 มิลลิลิตรต่อนาที วิธีการที่พัฒนาขึ้นมีความเป็นเส้นตรงที่ดีเยี่ยมในช่วงความเข้มข้น 0 ถึง 640 ไมโครกรัมต่อมิลลิลิตร (R^2 = 0.9999) การทดสอบความเที่ยงของการวิเคราะห์แสดงด้วยค่าร้อยละ RSD ซึ่งเป็นไปตามเกณฑ์ยอมรับ (<ร้อยละ 2) ทั้งสำหรับการวิเคราะห์ with-in run และ between run การทดสอบความแม่นยำมีค่าการคืนกลับอยู่ระหว่างร้อยละ 97.70 ± 2.11 ถึงร้อยละ 102.91 ± 1.47 วิธีนี้มีขีดจำกัดในการตรวจพบ และขีดจำกัดในการวัดเชิงปริมาณเท่ากับ 0.97 และ 3.26 ไมโครกรัมต่อมิลลิลิตรตามลำดับ โดยใช้เวลาการวิเคราะห์ต่อตัวอย่างที่สั้น (6 นาที) โดยรวมแล้วการทดสอบ UPLC-UV ที่พัฒนาขึ้นนี้ให้วิธีการที่ง่าย รวดเร็ว และเชื่อถือได้สำหรับการตรวจวัดไอโอเฮกซอลในพลาสมา ทำให้เหมาะสำหรับการใช้งานทางคลินิกในการปฏิบัติงานด้านไตวิทยาและการวิจัยเพื่อการประเมิน GFR โดยการวัดตรง

คำสำคัญ: อัตราการกรองผ่านไต ไอโอเฮกซอล เครื่องโครมาโทกราฟีของเหลวสมรรถนะสูงพิเศษ การหาค่าที่เหมาะสมของวิธีทดสอบ การตรวจสอบความใช้ได้ของวิธีทดสอบ

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A Simple and Rapid Ultra-performance Liquid Chromatography Method for Plasma Iohexol Determination

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Abstract

Accurate measurement of iohexol in plasma is essential for determining glomerular filtration rate (GFR). In this study, we developed and validated a novel method for quantifying iohexol using ultra-performance liquid chromatography with ultraviolet detection (UPLC-UV). The method involved sample preparation and chromatographic separation for iohexol. Various parameters including the type of stationary phase, mobile phase composition, and pH of mobile phase were optimized to achieve optimal separation. The optimal chromatographic conditions are shown as follow; the mobile phase was 3.5% (v/v) of acetonitrile/water without adjusting pH, a flow rate was set at 0.2 mL/min, the analytical column was Poroshell 120 EC-C18 with a column temperature of 30 °C. The developed method exhibited excellent linearity over a concentration range of 0 to 640 μg/mL (R^2 = 0.9999). Precision analyses demonstrated %RSD values within acceptable limits (<2%) for within-run and between-run analyses. Recovery experiments yielded accurate results ranging from 97.70 ± 2.11% to 102.91 ± 1.47%. The method also achieved low limits of detection and quantitation (0.97 and 3.26 μg/mL, respectively) with a short analysis time per sample (6 minutes). Overall, the developed UPLC-UV assay provides a simple, rapid, and reliable method for iohexol determination in plasma, making it suitable for clinical applications in nephrology practice and research for direct GFR assessment.

Keywords: Glomerular filtration rate, Iohexol, Ultra-high-performance liquid chromatography, Method optimization, Method validation

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Introduction

The glomerular filtration rate (GFR) is the amount of plasma filtered by the renal glomerular capillaries into the Bowman's capsule per unit of time. It plays a key role in the clinical assessment of kidney function, the diagnosis of chronic kidney disease, the evaluation of living kidney donation before nephrectomy, and the assessment of the progression of renal function decrease in kidney disease. Understanding GFR is essential for dosing potentially toxic drugs with a narrow therapeutic index and monitoring treatment's impact on renal function.[1, 2]

There are two methods for measuring GFR: direct measure GFR (measurement GFR; mGFR), which involves injecting exogenous substances like inulin, iothalamate, ^{51}Cr-ethylenediaminetetraacetic acid (^{51}Cr-EDTA), or iohexol that are almost completely excreted through the kidneys[3, 4], and then calculating the GFR through the excretion rate of that exogenous substance by measuring its level in the blood and urine. Another method involves estimating GFR (estimated GFR; eGFR) by calculating the GFR using an accurate formula after assessing the amounts of endogenous substances produced by the body and cleared in the urine, such as creatinine and cystatin C.[5] These include the CKD-EPI equation (CKD-epidemiology cooperation), the MDRD equation (modification of diet in renal disease), and the Cockcroft-Gault formula.[6, 7]

Direct measurement of GFR for obtaining a precise value is required in particular circumstances because it affects the choice of treatment. For instance, when a drug has a narrow therapeutic range and is toxic to the kidneys, or when assessing a potential living kidney donor.[8] In addition, it is advised to assess GFR using the direct measurement approach in chronic conditions such as liver cirrhosis because GFR estimations based on endogenous substances like creatinine in patients with liver cirrhosis may be invalid under certain conditions, such as reduced muscle mass, malnutrition, or excessive bilirubin.[9, 10] Moreover, the obese patient is encouraged to employ direct measurement since, in individuals with a BMI >30 kg/m^2, a variety of body composition variables, like reduced muscle mass, may cause the use of creatinine-based equations to overstate GFR.[9, 11]

In daily practice, estimating GFR-based equations are most frequently utilized. They have the advantage of being inexpensive and providing results immediately.[9] Their disadvantage is that they rely on endogenous biomarkers, which can be affected by factors including age, sex, muscle mass, medications, some chronic illnesses, food, and probably many more.[12] Therefore, in situations where more precise GFR is required, a technique for evaluating exogenous compounds qualified as an ideal marker for measurement of GFR properties was used instead, and iohexol is

another widely used exogenous marker.[13]

Iohexol is a safe and effective nonionic contrast medium, structure as shown in Fig. 1, that has various advantages in clinical practice and could be utilized as a reliable GFR marker in patients with severe renal failure. As a result, it is becoming a more widely acknowledged marker for determining GFR.[14-17] Iohexol clearance is recommended as a reference method for finding the glomerular filtration rate, which is important in nephrology practice and research.[17, 18]

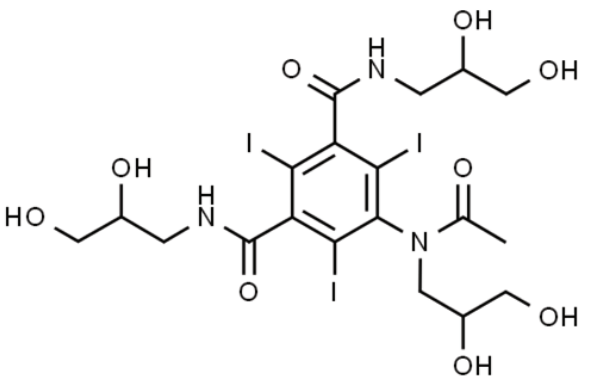

Fig. 1 The chemical structure of iohexol (N,N´-Bis (2,3-dihydroxypropyl)-5-[N-(2,3-dihydroxypropyl) acetamido] triiodoisophthalamide).[26]

Currently, several studies have published methods for the determination of iohexol using high-performance liquid chromatography (HPLC)[14, 15, 19] methods, and these methods keep fulfilling the requirements of clinicians and researchers. Using the HPLC-UV method, the two isomers have different retention times.[20] Since the endo-isomer is proportionally less than the exo-isomer (ratio about 20:80), most investigators measured only the major compound and generally used this isomer for the calculations of iohexol clearance and consequently GFR determination.[16, 21] The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is another highly accurate and precise method of determining iohexol in plasma.[22] However, because of the analyzer's limitations and high cost, routine laboratories are unable to perform analysis using this method. That is why this method is still not widely used. However, the advantages of ultra-high performance liquid chromatography (UPLC), which include speed, good separation, and sensitivity and could be considered to be a new direction for chromatography method because of the analysis time and

sensitivity in clinical laboratories, are very important. As a result, UPLC is becoming increasingly important. UPLC differs from traditional HPLC in the size of particles of the stationary phase and the liquid handling system can be used to operate at much higher pressure. The knowledge from the Van Deemter equation, the efficiency of the chromatographic technique is proportional to particle size decrease. The small particle diameter can reduce height equivalent to a theoretical plate (HETP) which results in higher efficiency.[23]

Due to the superior advantages of the UPLC method in terms of speed, separation, and sensitivity than HPLC, the development of measurement work has increasingly focused on this technique. This study aimed to develop a sensitive, fast, and reliable UPLC-UV method for iohexol determination. Validation results were conducted following the International Conference on Harmonization (ICH) criteria[24] and acceptance criteria following the study of Shabir G *et al.*[25]

Material and method

1. Samples

The 20 left-over EDTA plasma samples from the Division of Clinical Chemistry Department of Pathology, Faculty of Medicine Ramathibodi Hospital were collected in June 2020 and kept at -80 ˚C until use. These samples were used for method development, optimization, and validation. This study received a proper institutional review and broad approval from the Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. The ethics number for this study is COA. MURA2020/915.

2. Materials and reagents

Iohexol standard was purchased from US Pharmacopeia (Rockville, USA). The iohexol-related compound B (5-amino-N, N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide) was purchased from Toronto Research Chemicals (Toronto, Canada). HPLC-grade acetonitrile was purchased from RCI Labscan Limited (Bangkok, Thailand). AR-grade perchloric acid was purchased from Ajax Finechem (Taten Point NSW, Australia). AR-grade orthophosphoric acid was purchased from RCI Labscan Limited (Bangkok, Thailand). Purified water was prepared with a Millipore (Molsheim, France) Milli-direct Q3 water-purification system.

3. UPLC-UV: condition and equipment

The study was carried out using the Agilent 1200 series UPLC system (Santa Clara, USA), consisting of a pump, an online vacuum degasser, an autosampler, a column oven, and a UV/VIS detector. The whole UPLC system was controlled by Agilent ChemStation Software, by which data were

acquired and quantified. The autosampler loop volume was kept at 10 μL. Detector wavelengths were set at 254 nm. The iohexol-related compound B was used as an internal standard (IS). For the optimized test, we used three analytical columns: Poroshell 120 EC-C18 (50 × 3.0 mm; 2.7 μm), Zorbax Eclipse plus C18 (50 × 2.1 mm; 1.8 μm), and Zorbax SB-C18 (50 × 2.1 mm; 1.8 μm) and the three analytical columns were purchased from Agilent Technologies (Santa Clara, USA).

4. Preparation of standards

The stock solution of iohexol standard (10 mg/mL) was prepared in acetonitrile. Stock solutions were kept at -80 ˚C. A total of six concentrations of iohexol standards (5, 20, 80, 160, 320, and 640 μg/mL) were prepared in EDTA plasma and used as calibrators. The iohexol-related compound B at a concentration of 10 μg/mL was prepared in 5% (v/v) perchloric acid and used as the protein precipitant solution containing IS.

5. Sample and sample preparation

The previous study by El Assri *et al.*[26] was adopted and optimized for the current UPLC-UV study. For sample preparation, the ratio of the volume of sample and protein precipitate solution was tried to determine the optimal ratio based on % extraction recovery, and it was calculated by using a formula as shown below. In short, aliquots of the calibrator, control material, or EDTA plasma samples (50 μL) were added to 1.5 mL microcentrifuge tubes added with a protein precipitation solvent containing 10 μg/mL IS in the volume 250 μL, 500 μL, 750 μL, and 1,000 μL representing the ratio of 1:5, 1:10, 1:15, and 1:20 respectively. Then, mixed well and centrifuged at 10,000 rpm for 10 minutes. The clear supernatant was then taken into the vial for further injection with a UPLC-UV analyzer.

$$\%\text{Extraction recovery} = \frac{\text{peak area of spike standard in plasma sample}}{\text{peak area of neat standard}} \times 100$$

6. Method optimization

The optimization of the UPLC-UV method in this study was experimentally adjusted for several parameters such as the type of column, the composition of the mobile phase, and the pH of the mobile phase. The resolution (Rs) between endo and exo-iohexol, retention time (RT), asymmetry factor (As), and theoretical plate (N) were all calculated using Agilent Technologies' ChemStation software in order to determine whether the optimization was adequate.

6.1 Stationary phase

Initially, we were inspired by the study of Niculescu *et al.* [27], who developed an HPLC method to measure iohexol, so we adopted the same mobile phase composition (3.5% acetonitrile/water, pH 2.5) to select appropriate column in our study. The three candidate columns consisting of Poroshell 120 EC-C18, Zorbax Eclipse plus C18, and Zorbax SB-C18 were enrolled to test for iohexol separation. The column properties for each candidate column are shown in Table 1. The Poroshell 120 EC-18 was selected because it is a superficial porous silica column that is a new trend of support materials used in the production of columns. The Eclipse plus C18 was selected because it is the endcap bonded phase column, and Zorbax SB-C18 was selected because it is the non-endcap bonded phase column.

Table 1 The specification of three candidate columns for iohexol measurement by UPLC-UV technique.

Specification	Poroshell 120 EC	Zorbax Eclipse Plus	Zorbax SB
Silane phase	C18	C18	C18
Porous silica	Superficial	Total	Total
Column length	3.0 x 50	2.1 x 50	2.1 x 50
Particle size (μm)	2.7	1.8	1.8
Pore size (°A)	120	95	80
Column endcapping	Yes	Yes	No
Max.Pres.	600	600	1200
Carbon load (%)	10	9	10

6.2 Mobile phase

After obtaining an appropriate column for the determination of iohexol from trial 2.6.1, the composition of the mobile phase was studied. For the separation of iohexol, acetonitrile in water was optimized. Iohexol separation was investigated for the four different concentrations of acetonitrile in the mobile phase (2%, 3.5%, 5%, and 7% (v/v)).

6.3 pH of mobile phase

After an appropriate column and the composition of the mobile phase were obtained by trials 2.6.1 and 2.6.2, the impact of the pH of the mobile phase on the determination of iohexol was examined. Orthophosphoric acid was added to the mobile phase to change the pH from no modification to 4 and 2.5 to assess the

impact of pH. The pH values were selected based on previous studies of Niculescu *et al.*[27] at pH 2.5 and Nilsson-Ehle *et al.*[28] at pH 4. No adjustments were selected because it was easy to prepare.

7. Validation of the method

7.1 Selectivity

Comparing the chromatograms of the six blank EDTA plasma samples with those plasma samples spiked with iohexol and iohexol-related compound B allowed researchers to evaluate the method's selectivity. Selectivity was guaranteed that each blank sample had its interference levels checked. It was acknowledged that no interfering peaks existed.

7.2 Linearity of range

To assess the linearity of the calibration range, stock standard solutions containing 10 mg/mL of iohexol were added to the plasma and made in triplicate at seven different concentrations including a sample blank that covered the analytical range. The calibration curve was constructed by plotting the peak area ratios of the exo-iohexol to the IS (y) against the analyte concentration (expressed as μg/mL). For each concentration, the operation was done three times. The applied criteria for a valid calibration model were the coefficient of determination higher than 0.999 (R^2), and the relative standard deviation (%RSD) to be within 3% for all concentrations of calibrators.

7.3 Precision

Twenty samples per level injected into the UPLC-UV system were used to assess the intra-day precision. Free iohexol human plasma was spiked with standards to create three final concentrations of 40, 150, and 300 μg/mL. On five successive days, the inter-day precision was evaluated similarly. After determining the iohexol concentrations, %RSD was estimated. The acceptable criteria for precision are %RSD lower than 2%.

7.4 Accuracy

By using the recovery approach, the method's accuracy was evaluated. The % recovery was estimated at three nominal concentrations by spiking the fresh plasma with iohexol standards. The final concentrations were comparable to those attained using the previously disclosed precision evaluation approach. The percentage of recovery was calculated using the formula below and the acceptable criteria for accuracy is %recovery range between 90-110%.

$$\%\text{Recovery} = \frac{\text{observed conc.} \times 100}{\text{expected conc.}}$$

7.5 Lower limit of detection and quantitation

The detection and quantitation limits were calculated using the relationship between the standard deviation (SD) of the iohexol response at the lowest point on the calibration curve and the slope of the calibration curves. The quantitation limit was to be 10 SD,

whereas the detection limit was to be a relation of 3 SD.

Results

1. Sample extraction

The extraction of iohexol during sample preparation is essential for accuracy quantification. The data in Table 2 represent the mean of the %recovery and peak area values of endo-iohexol and exo-iohexol obtained by triplicate run, it shows the percentage recovery of iohexol obtained from the protein precipitation with a ratio of sample to precipitant solution at 1:5, 1:10, 1:15, and 1:20. The extraction efficiency range between 90.53 and 103.36% and 95.13 and 99.55% for endo-iohexol and exo-iohexol, respectively. The ratio of 1:10 shows a satisfied %recovery of 97.82 ± 1.27% and a high peak area in both isomers (shown in Fig. 5), Moreover, it has been shown to precipitate proteins by several previous studies.

Table 2 Recovery (%) of endo-iohexol and exo-iohexol with various ratios of sample to protein precipitation solution.

The ratio of sample to protein precipitant solution	Endo-iohexol			Exo-iohexol		
	Recovery (%)	Peak area (mAu*s)	SD (mAu*s)	Recovery (%)	Peak area (mAu*s)	SD (mAu*s)
1:5	103.36	367.89	0.85	99.55	1462.83	1.29
1:10	100.50	199.33	0.23	97.82	787.48	1.27
1:15	100.55	139.35	0.16	98.61	545.29	0.79
1:20	90.53	103.56	0.63	95.13	401.57	0.41

In this table, the recovery (%) and peak area (mAu*s) for both endo-iohexol and exo-iohexol are provided at various ratios of sample-to-protein precipitation solution. The standard deviation (SD) of the peak area is also included. This data helps assess the efficiency of protein precipitation and the recovery of the analytes under different conditions.

2. Method optimization

2.1 Stationary phase

Three columns were investigated for iohexol determination by the UPLC-UV system, including Poroshell 120 EC-C18, Zorbax Eclipse plus C18, and Zorbax SB-C18 column. Iohexol could be separated into endo- and exo-iohexol peaks by all three columns, and the IS was the final peak, as seen in Fig. 2. Table 3 displays the results of the column performance evaluation against this iohexol separation, and the result is the mean of the triplicate run. The RT, As, N, peak area, Rs, and isomer ratios mentioned are ordered by using the Poroshell 120 EC-C18, Zorbax Eclipse plus C18, and Zorbax SB-C18 column, respectively. The RT of endo-iohexol were 3.56, 2.19, and 2.17 minutes, and for exo-iohexol were 3.92, 2.51, and 2.48 minutes and for IS were 4.76, 3.11, and 3.09 minutes. The As of endo-iohexol were 0.80, 0.76, and 0.88, and for exo-iohexol were 0.87, 0.76, and 0.90, and for IS were 0.67, 0.58, and 0.73. The N of endo-iohexol were 5,507, 3,009, and 3,710, and for exo-iohexol were 4,543, 2,520, and 2,810, and for IS were 7,357, 4,567, and 5,973. The peak area of endo-iohexol were 606, 624, and 598 mAu*s, and for exo-iohexol were 2,137, 2,192, and 2,140 mAu*s, and for IS were 536, 553 and 558 mAu*s. The Rs between endo-iohexol and exo-iohexol were 1.71, 1.78, and 1.88. The peak area between the isomer of endo-iohexol and exo-iohexol were 0.283, 0.285, and 0.279. The Poroshell 120 EC-C18 column performs that it fulfilled the acceptance criteria with values of Rs between the iohexol isomers with >1.5, asymmetry factor <2, N >2,000, high peak area, and lower back pressure with 65 bar.

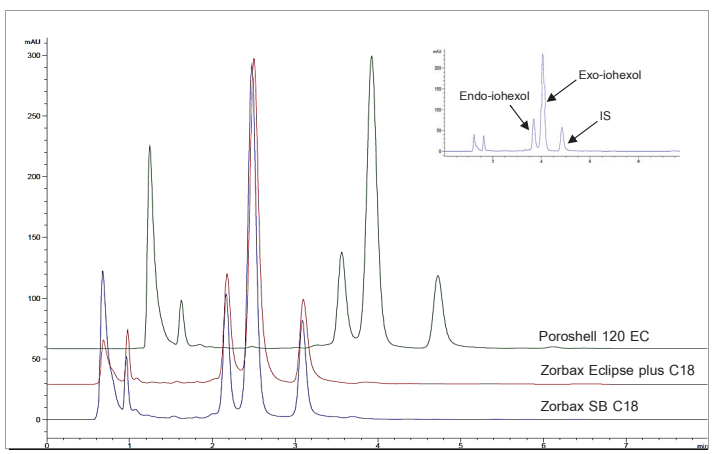

Fig. 2 Chromatograms obtained from plasma sample adding iohexol concentration of 600 μg/mL and internal standard (IS) by using the three columns with the mobile phase composition suggested by Niculescu *et al.*[27] (3.5% acetonitrile: water at pH 2.5).

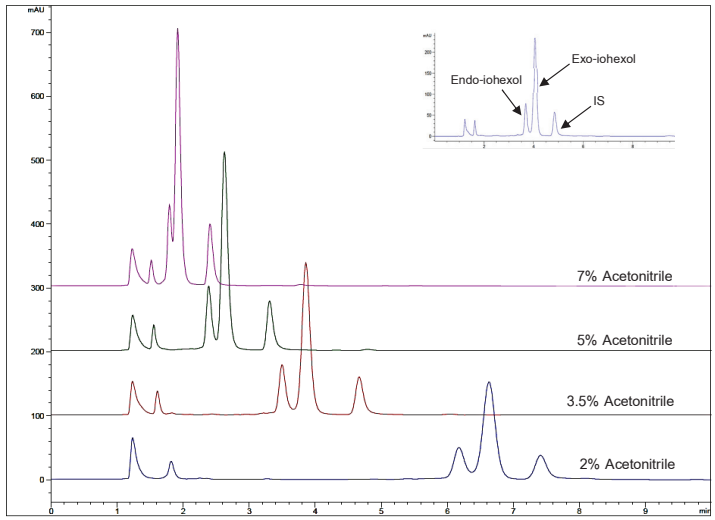

Fig. 3 Chromatograms obtained from plasma sample adding iohexol concentration of 600 μg/mL and internal standard (IS) by using various acetonitrile/water (v/v) in the mobile phases by using the Poroshell EC120 column.

2.2 Mobile phase

The mobile phase composition was investigated to optimize the UPLC-UV assay parameters. Table 4 shows the value of peak parameters including RT, As, N, peak area, Rs, and isomer ratio obtained by usage of various acetonitrile/water as the mobile phase, and the result is the mean of the triplicate run. All studied parameters are ordered by using the mobile phase at concentrations of 2%, 3.5%, 5%, and 7% (v/v) acetonitrile/water, respectively. The RT of endo-iohexol were 6.40, 3.56, 2.39, and 1.80 minutes, and for exo-iohexol were 6.86, 3.92, 2.63, and 1.93 minutes and for IS were 7.60, 4.76, 3.32 and 2.42 minutes. The As of endo-iohexol were 0.92, 0.80, 0.81, and 1.02, and for exo-iohexol were 0.96, 0.87, 0.80, and 0.77, and for IS were 0.81, 0.67, 0.62 and 0.65. The N of endo-iohexol were 6,471, 5,507, 3,964, and 2,638, and for exo-iohexol were 5,911, 4,543, 3,456, and 2,844, and for IS were 8,560, 7,357, 5,794 and 4,321. The peak areas of endo-iohexol were 602, 606, 613, and 551 mAu*s, and for exo-iohexol were 2,053, 2,137, 2,180, and 2,244 mAu*s, and for IS were 463, 536, 553 and 542 mAu*s. The Rs between endo-iohexol and exo-iohexol were 1.34, 1.71. 1.43, and 0.87. The peak area ratio between the isomer of endo-iohexol and exo-iohexol were 0.293, 0.283, 0.281, and 0.246. The typical chromatograms for the iohexol and IS by using different acetonitrile/water as the

mobile phase are given in Fig. 3. The 3.5% acetonitrile/water as mobile phase fulfill the acceptable criteria with good Rs between the iohexol isomer with > 1.5, asymmetry factor <2, N > 2,000, high peak area, and a short total run time of 6 minutes.

Table 3 The optimization of the stationary phase by using three columns with the mobile phase composition suggested by Niculescu *et al.* [27] (3.5% acetonitrile: water at pH 2.5).

Column Type	Analytes	RT (min)	As	N	Peak area (mAu*s)	Rs*	Ratio**	Back pressure (bar)
Poroshell EC120	Endo-iohexol	3.56	0.80	5,507	606	1.71	0.283	65
	Exo-iohexol	3.92	0.87	4,543	2,137			
	IS	4.72	0.67	7,357	536			
Zorbax SB	Endo-iohexol	2.19	0.76	3,009	624	1.78	0.285	125
	Exo-iohexol	2.51	0.76	2,520	2,192			
	IS	3.11	0.58	4,567	553			
Eclipse plus	Endo-iohexol	2.17	0.88	3,710	598	1.88	0.279	130
	Exo-iohexol	2.48	0.90	2,810	2,140			
	IS	3.09	0.73	5,973	558			

In this table, the retention time (RT), asymmetric factor (As), theoretical plate number (N), peak area, resolution (Rs), ratio values, and back pressure are provided for each column type (Poroshell EC120, Zorbax SB, and Eclipse Plus) and each analyte (Endo-iohexol, Exo-iohexol, and IS). * The Rs and Ratio values are provided for endo-iohexol and exo-iohexol. The data allows for the evaluation of the performance of different stationary phases under the specified mobile phase conditions, aiding in the selection of the optimal stationary phase for the analysis.

Table 4 The optimization of acetonitrile concentration in the mobile phase using the Poroshell EC120 column.

Acetonitrile/ water	Analytes	RT (min)	As	N	Peak area (mAu*s)	Rs*	Ratio**
2%	Endo-iohexol	6.40	0.92	6,471	602	1.34	0.293
	Exo-iohexol	6.86	0.96	5,911	2,053		
	IS	7.60	0.81	8,560	463		
3.5%	Endo-iohexol	3.56	0.80	5,507	606	1.71	0.283
	Exo-iohexol	3.92	0.87	4,543	2,137		
	IS	4.72	0.67	7,357	536		
5%	Endo-iohexol	2.39	0.81	3,964	613	1.43	0.281
	Exo-iohexol	2.63	0.80	3,456	2,180		
	IS	3.32	0.62	5,794	553		
7%	Endo-iohexol	1.80	1.02	2,638	551	0.87	0.246
	Exo-iohexol	1.93	0.77	2,844	2,244		
	IS	2.42	0.65	4,321	542		

In this table, the retention time (RT), asymmetric factor (As), theoretical plate number (N), peak area, resolution (Rs), and ratio values of endo-iohexol, exo-iohexol, and IS. * The Rs and Ratio values are provided for endo-iohexol and exo-iohexol at different acetonitrile concentrations in the mobile phase. The data allows for the evaluation of the chromatographic performance under varying solvent compositions, aiding in the selection of the optimal acetonitrile concentration for the analysis.

2.3 pH

The pH is a parameter that has been carefully chosen for iohexol measurement. Orthophosphoric acid changes the mobile phase's pH from no adjustment to 4 and 2.5. Table 5 displays the outcomes of a three-variable variation. The RT, As, N, peak area, Rs, and isomer ratios mentioned are ordered by using the mobile phase's pH of no adjustment, 4 and 2.5, respectively. The RT of endo-iohexol were 3.56, 3.67, and 3.69 minutes, and for exo-iohexol were 3.92, 4.04, and 4.06 minutes, and for IS were 4.72, 4.83, and 4.85 minutes. The As of endo-iohexol were 0.80, 0.83, and 0.82, and for exo-iohexol were 0.87, 0.89, and 0.90, and for IS were 0.67, 0.69, and 0.68. The N of endo-iohexol were 5,507, 5,706, and 5,575, and for exo-iohexol were 4,543, 4,710, and 4,611, and for IS were 7,357, 7,479, and 7,215. The peak areas of endo-iohexol were 606, 620, and 619 mAu*s, and for exo-iohexol were 2,137, 2,188, and 2,182 mAu*s, and for IS were 536, 535, and 534 mAu*s. The Rs between endo-iohexol and exo-iohexol were 1.71, 1.71, and 1.71. The peak area between the isomer of endo-iohexol and exo-iohexol were 0.283, 0.283, and 0.284. The mobile phase with no pH adjustment produces a good chromatogram as same as pH of 2.5 and 4, however, it is easy to prepare (Fig. 4).

Table 5 The optimization of the pH of the mobile phase using the Poroshell EC120 column with 3.5% acetonitrile: water as mobile phase.

pH of mobile phase	Analytes	RT (min)	As	N	Peak area	Rs*	Ratio*
Not adjust pH	Endo-iohexol	3.56	0.80	5,507	606	1.71	0.283
	Exo-iohexol	3.92	0.87	4,543	2,137		
	IS	4.72	0.67	7,357	536		
pH 4	Endo-iohexol	3.67	0.83	5,706	620	1.71	0.283
	Exo-iohexol	4.04	0.89	4,710	2,188		
	IS	4.83	0.69	7,479	535		
pH 2.5	Endo-iohexol	3.69	0.82	5,575	619	1.71	0.284
	Exo-iohexol	4.06	0.90	4,611	2,182		
	IS	4.85	0.68	7,215	534		

In this table, the retention time (RT), asymmetric factor (As), theoretical plate number (N), peak area, resolution (Rs), and ratio values of endo-iohexol, exo-iohexol, and IS. * The Rs and Ratio values are provided for endo-iohexol and exo-iohexol at different pH values of the mobile phase. The data indicates the performance of the chromatographic system under various pH conditions, allowing for the selection of the optimal pH for the analysis.

Table 6 The optimized parameters for developing iohexol assay with UPLC.

Parameters	Value
Analyzer	UPLC
Stationary phase	Poroshell EC120 column
Mobile phase	3.5% ACN: water with no pH adjustment
UV Detector wavelength	254 nm
Injection volume	10 µL
Column oven	30 °C
Sample (µL): Precipitant solvent (µL)	50:500 (1:10)

3. Method validation

According to method-optimized data, the best chromatographic conditions are 3.5% (v/v) acetonitrile in water as the mobile phase and the Poroshell 120 EC-C18 column as the stationary phase. These conditions were selected for iohexol separation because they provide a clear resolution between endo- and exo-iohexol isomer peaks, have a total run time of less than 6 min per analysis, and have good peak characteristics for all analytes.

3.1 Selectivity

Six different blank plasma samples were extracted to test the selectivity. The method's selectivity was inferred from the lack of competing peaks at the retention times for iohexol and IS. Fig. 5 displays the chromatogram of a blank plasma sample spiked with iohexol and IS at a concentration of 600 µg/mL. With a resolution of 1.71 for endo- and exo-iohexol and 4.55 for exo-iohexol and IS, this result showed 3 peaks with retention times of 3.56, 3.92, and 4.72 minutes for endo-iohexol, exo-iohexol, and IS respectively. A blank plasma chromatogram (Fig. 5A) revealed no interference peaks at the iohexol and IS retention times. The outcome demonstrated that none of the endogenous compounds from the six distinct sources' blank plasma interfered.

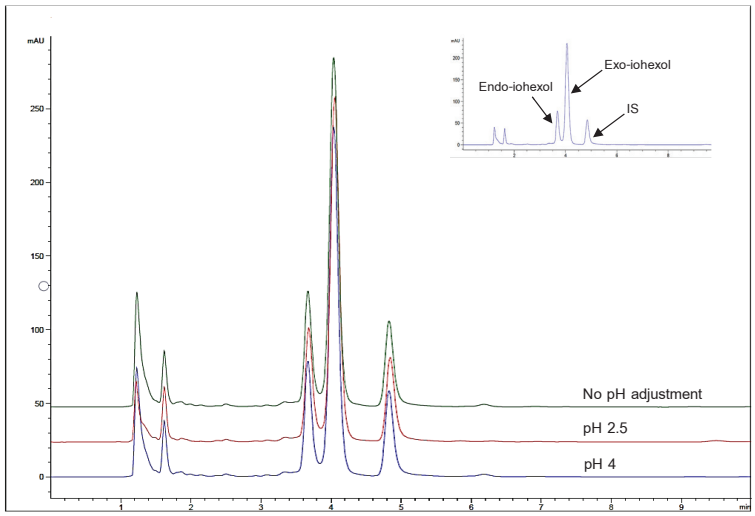

Fig. 4 Chromatograms obtained from plasma sample adding iohexol concentration of 600 μg/mL and internal standard (IS) by using various pH of mobile phases using the Poroshell EC120 column with 3.5% acetonitrile: water as mobile phase.

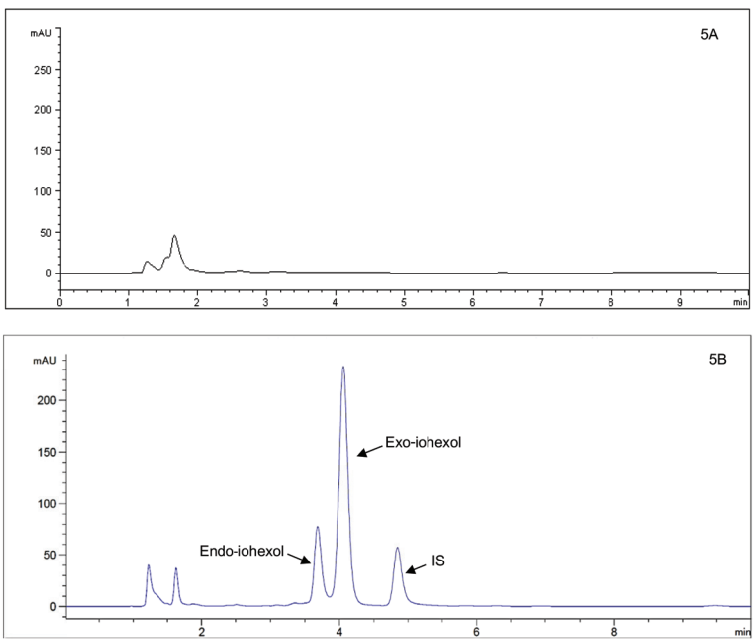

Fig. 5 The chromatogram of blank samples (5A) and blank sample adding iohexol concentration of 600 μg/mL and internal standard (IS) (5B) by using the Poroshell EC120 column with 3.5% acetonitrile: water as mobile phase no pH adjustment.

3.2 Linearity of range

The peak area ratio of exo-iohexol to IS (y) versus the iohexol concentration was used to obtain the calibration curves for iohexol in EDTA plasma, which showed a strong linear relationship over the range of 0-640 μg/mL as shown in Fig. 6. The equation for a typical calibration curve was y=0.0119x-0.0424 (R^2 = 0.9999). The iohexol concentrations fell within the 0-640 μg/mL assayed range. The %RSD at all levels of the calibration was < 3% and that is falling in acceptable criteria in the test range.

3.3 Limit of detection and quantitation

The detection limits and quantitation limits for the developed UPLC technique were 0.97 and 3.26 μg/mL, respectively. The low detection and quantitation limit values showed that iohexol had appropriate analytical sensitivity.

3.4 Precision and accuracy

As indicated in Table 7, precision and accuracy were determined using the within-run and between-run variation of the spiked sample at three concentrations. Within-run and between-run analyses had precision (%RSD) values of 1.43 to 2.16% and 1.38 to 1.53%, respectively. The recovery (%) range was between 97.70 (2.11) to 102.91 (1.47)%. These results showed that this method fulfilled the acceptance criteria with values of %RSD <2% for precision and within 90-110% for accuracy.

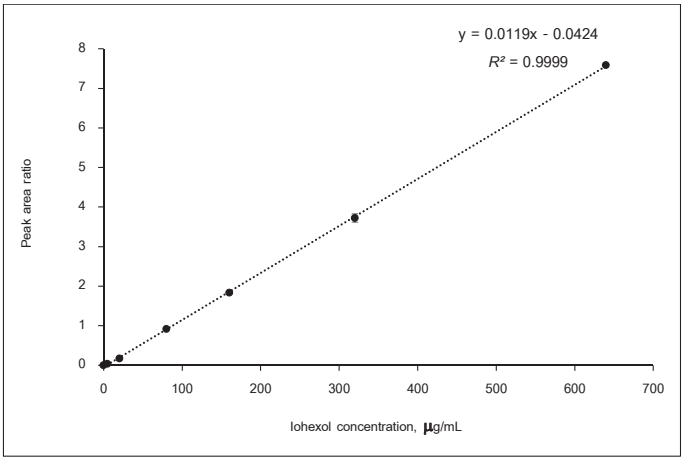

Fig. 6 Linear responses in exo-iohexol/internal standard (IS) peak area ratio versus iohexol concentrations.

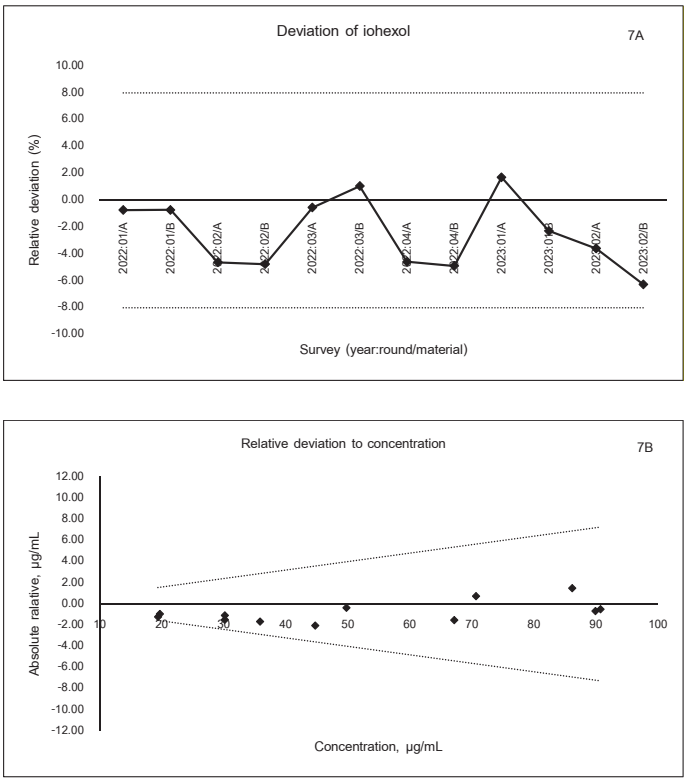

Fig. 7 The performance of the new UPLC-UV method was evaluated by measuring EQA samples (Equalis (Uppsala, Sweden). The % relative deviation between the new UPLC-UV method and Equalis mean (7A), and the relative deviation to concentration (7B) are determined. The dashed line indicates the maximum acceptable of relative deviation and absolute relative value. This indicates that the new UPLC-UV method staying within the acceptable limits defined by the evaluation criteria.

4. Method application

Following the optimization process detailed in Table 6, the UPLC-UV conditions deemed optimal for iohexol determination were implemented in our laboratory. These conditions were applied to analyze External Quality Assessment (EQA) samples obtained from Equalis (Uppsala, Sweden). In the time period 2022 to 2023, a total of 6 EQA rounds were conducted, each comprising two samples. Before analysis, the EQA samples were stored at -80°C. The results of the EQA sample analysis are presented in Table 8.

In Table 8, "True concentration" refers to the concentration obtained from the weight standard added to the pooled plasma produced by EQUALIS. "Equalis mean" represents the average concentration determined by

all participating laboratories globally, with more than 50 laboratories involved. "UPLC-UV" denotes the concentration of iohexol determined using the newly developed UPLC-UV method, ranging from 19.4 to 90.8 μg/mL. "Trueness" indicates the percentage closeness of agreement between the values obtained from the new UPLC-UV method and the true values, with a range of 99.40% to 102.07%. The "% relative deviation" represents the difference between the Equalis mean and the values obtained from the new UPLC-UV method, ranging from -5.92% to 1.75%. The deviation to concentration varies from -2.05 to 1.48 μg/mL.

The performance of the new UPLC-UV method is further depicted in Fig. 7. Fig. 7A illustrates the percentage relative deviation of EQA samples over different periods, while Fig. 7B shows the deviation to concentration. All EQA sample results fell within the allowed range specified by the Equalis quality goal, which dictates that the relative deviation for iohexol should be less than 8%.

Table 7 Analytical performance of the developed UPLC-UV method for iohexol determination.

Analytical characteristics		Analytical performance	
LLOD (μg/mL)		0.97	
LLOQ (μg/mL)		3.26	
Precision		within-run	between-run
	Low, 40 μg/mL	2.16	1.53
	Medium, 300 μg/mL	1.43	1.38
	High, 600 μg/mL	1.83	1.49
Accuracy			
	Low, 40 μg/mL, %	97.70 ± 2.11	
	Medium, 300 μg/mL, %	102.91 ± 1.47	
	High, 600 μg/mL, %	101.72 ± 1.86	

LLOD, lower limit of detection; LLOQ, lower limit of quantification

Table 8 The performance of the new UPLC-UV method is assessed by EQA samples.

Survey	True concentration, μg/mL	Equalis mean group value, μg/mL (n)	New UPLC-UV value, μg/mL	Trueness (%)	Relative Dev. (%)*
2022:01/A	90.0	90.65 (53)	90.0	100.00	-0.72
2022:01/B	50.1	50.15 (52)	49.8	99.40	-0.70
2022:02/A	35.4	37.56 (56)	35.9	101.41	-4.42
2022:02/B	19.3	20.64 (56)	19.7	102.07	-4.55
2022:03/A	90.0	91.28 (48)	90.8	100.89	-0.53
2022:03/B	70.7	69.95 (48)	70.7	100.00	1.07
2022:04/A	44.9	46.85 (50)	44.8	99.78	-4.38
2022:04/B	30.1	31.67 (51)	30.2	100.33	-4.64
2023:01/A	86.5	84.72 (56)	86.2	99.65	1.75
2023:01/B	67.3	68.73 (56)	67.2	99.85	-2.23
2023:02/A	302.0	31.28 (52)	30.2	100.00	-3.45
2023:02/B	19.2	20.62 (52)	19.4	101.04	-5.92

In this table, EQA survey, true concentration (μg/mL), Equalis mean group value (μg/mL) with sample size (n), new UPLC-UV value (μg/mL), trueness (%), and relative deviation (%) are provided for each EQA sample. The relative deviation is calculated to assess the accuracy of the new UPLC-UV method, and it's noted that the relative deviation for iohexol is less than 8% for all EAQ samples which is acceptable.

Discussions

Serum clearance of iohexol is a safe and reliable method for GFR determination. It has shown that there is a good correlation between iohexol clearance and inulin clearance, the gold standard method. HPLC-UV technique remains a method of choice for serum iohexol measurement since this method is reliable, precise, and accurate. The HPLC-UV technique is nevertheless less efficient than the UPLC-UV technique in terms of speed, separation, and sensitivity. Additionally, there is currently no research that provides a method for measuring iohexol utilizing the UPLC-UV method.

In this study, we established a novel method for determining iohexol clearance from serum with UPLC-UV, which is simple, reliable, precise, and accurate and can be used to determine iohexol in clinical settings. Based on the results, the condition of 3.5% acetonitrile with no pH adjustment as the mobile phase, Poroshell 120 EC-C18 column as the stationary phase, UV wavelength set at

254 nm, oven temperature set at 30˚C, and injection volume set at 10 μL was identified as excellent chromatography condition for iohexol determination. We selected the Poroshell 120 EC-C18 because it provides good resolution (>1.5) between the analytes (endo-iohexol and exo-iohexol) and any interfering peaks. This is crucial for accurate quantification, especially in complex sample matrices, the theoretical plate number (N) for the Poroshell EC120 column is higher compared to other columns, indicating better peak shape and separation efficiency, this can result in sharper, better-defined peaks, facilitating accurate quantification. Moreover, the back pressure of the Poroshell EC120 column is lower compared to other columns, this is likely due to its superficially porous silica property, indicating good column performance without excessive pressure buildup in the chromatographic system. Overall, the Poroshell EC120 column appears to offer favorable characteristics for iohexol determination, including high resolution, efficiency, and manageable back pressure, making it a suitable choice for this application.

By this condition, it showed a better peak shape that is easy to integrate, and capable of clearly distinguishing interfering peaks leading to good accuracy and precision. This method provides a high speed, given that it can completely elute iohexol in less than 6 minutes as opposed to other methods' common elution periods of more than 10 minutes, it offers an advantage over other published HPLC methods that have been described in the past.[14, 15, 19, 29] The ability to separate endo-/exo-iohexol is another advantage of using this method. Based on the evaluation of resolution between isomers, it was found that this isomer can be clearly separated, and it is better than the HPLC-UV method previously published by Cavalier *et al.*[30], Castagnet *et al.*[19], and Slack *et al.*[15], resulting in integration peaks being easy and get a good precision.

An efficient sample preparation method is very important for accurate and reliable bioanalysis by UPLC-UV. A simple and rapid protein precipitation using 5% (v/v) perchloric acid was employed in this work. The previous publications used the volume of plasma sample to precipitation solvent range from 1:5 to 1:15. In trials to find the appropriate amount of precipitant solution, the ratio of 1:10 was chosen since it provided a satisfactory amount of %recovery and a high peak area of iohexol that resulted in a good sensitivity, assuming that the sample was not overly diluted with protein precipitation solution. Based on observations of more than 20 blank samples, this amount can also remove the majority of interfering substances; no interfering peaks were seen at the RT of iohexol and IS.

Method validation results showed a larger linearity range (0-640 μg/mL) than the previous study.[19, 29] In routine practice, after injecting 5 mL of Omnipaque® usually found the concentration of iohexol ranged from 40-600 μg/mL related to the renal function of the patient.[30] The advantages of larger linearity are to serve the patients who reduce the dose of iohexol administrated to avoid risk and get accurate results from a small iohexol concentration remaining in the blood circulation at the last of sampling time after administering iohexol to patients 300 min. By the routine analytical work that we have done in Ramathibodi Hospital, at 300 mins of the patient's sample after iohexol administration, the lowest concentration that could be found was 6.59 μg/mL. However, our developed method can cover such low concentrations, which is an advantage over the traditional HPLC-UV method. The method has a low within-run and between-run %RSD and high %recovery and no substance coelute was found in any patient samples. Our method was able to measure a minimum value (LLOQ) of 3.23 μg/mL, which was significantly better than that of Soman *et al.*[14], Castagnet *et al.*[19], and Slack A *et al.*[15], which measured a minimum value of 10, 15, 15 and μg/mL respectively.

The studies by Assri *et al.*[26] and Cavalier *et al.*[30] both developed HPLC methods for measuring iohexol in plasma and urine matrices. These studies concluded that the optimal chromatographic conditions were similar to those investigated in our study. Assri *et al.* addressed chromatographic factors affecting iohexol separation and provided satisfactory HPLC conditions for iohexol determination in serum and urine. However, UPLC conditions were not explored in their study, prompting our investigation into UPLC parameters for iohexol analysis to fill this gap. On the other hand, Cavalier *et al.*'s study aimed to fully validate iohexol determination using risk assessment and uncertainty analysis. Their findings confirmed that the chromatography-based method for iohexol measurement is highly accurate and reliable, suggesting its potential as a reference method for direct glomerular filtration rate (GFR) measurement.

A very important issue that affects the results of the analysis of iohexol in plasma is the ratio of isomers (endo-/exo-iohexol). If the ratio is very different between the calibrator used to create the calibration curve and the patient sample it will affect the analysis results, causing errors. According to the researcher's routine work, the difference in this ratio shouldn't be more than 10%; however, if the difference is more than the specified threshold, the extracted samples should stand at room temperature for about 2 hours before being injected into the UPLC-UV system. If it measures, it will

be observed that the ratio returns to being less different (the data are shown in Table 9). This might be a consequence of the two isomers achieving equilibrium. The factors determining the ratio have not, however, been clearly studied in relation to this topic. Therefore, further research into this topic is necessary.

Table 9 The characteristics of isomerization are expressed by the ratio of exo/endo iohexol.

	Immediate injection			Equilibration at ambient temperature for 2 hours before injection		
	% Area		Ratio	% Area		Ratio
	endo-iohexol	exo-iohexol	**exo/endo iohexol**	endo-iohexol	exo-iohexol	**exo/endo iohexol**
Calibrator (n=6)	18.2 ± 0.67	81.8 ± 0.67	4.50	20.7 ± 0.92	79.3 ± 0.92	3.84
Sample (n = 10)	21.0 ± 0.22	79.0 ± 0.22	3.76	20.7 ± 0.23	79.3 ± 0.23	3.82
%difference			**-16.53**			**-0.35**

In this table, the percentage area and the ratio of exo/endo iohexol are provided for both calibrator and sample. The immediate injection results are compared to those obtained after allowing the extraction solution to stand at room temperature for 2 hours before injection. The percentage difference between the two conditions is also calculated.

Conclusion

We have developed a simple, rapid, sensitive, and reliable UPLC-UV method for measuring iohexol concentration in plasma. A large number of samples can be taken each day because the sample extraction is very straightforward and just requires one step of protein precipitation using 5% perchloric acid, along with the UPLC run time of 6 minutes which allows a large number of samples for each day. The technique was successfully used to figure out a patient's GFR by calculating the plasma clearance of iohexol. This is crucial for usage in both clinical practice and research. However, an ongoing challenge with the UPLC-UV method is the variation in the ratios of the two iohexol stereoisomers between calibrators and samples. To address this issue, it is important to establish an appropriate acceptable threshold for these ratios. Further research and optimization may be necessary to refine the method and ensure accurate and reliable results in clinical and research settings.

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