Aus der Klinik für Nephrologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Comparison of separate measurement procedures for determination of GFR in chronic kidney disease: iohexol quantification by HPLC and UPLC

Vergleich separater Methoden zur Bestimmung der GFR bei chronischer Nierenerkrankung: iohexol-Quantifizierung durch HPLC und UPLC

zur Erlangung des akademischen Grades

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List of abbreviations

GFR	Glomerular filtration rate
CKD	Chronic kidney disease
GBD	Global Burden of Disease
KDIGO	The kidney disease improving global outcomes guideline
eGFR	Estimated glomerular filtration rate
mGFR	Measured glomerular filtration rate
Scr	Serum creatinine
Scys	Serum cystatin C
MDRD	Modification of Diet in Renal Diseases
CG	Cockcroft-Gault
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
BIS	Berlin Initiative Study
Rev LM	Revised Lund-Malmö
^{51}Cr-EDTA	^{51}Cr- ethylene diamine tetraacetic acid
^{99}Tc-DTPA	^{99}Tc-diethylene triamine pentaacetic acid
CT	Computed tomography
XRF	X-ray fluorescence
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
HPLC	High-performance liquid chromatography
UPLC	Ultra-High-Performance Liquid Chromatography
CCC	Lin’s concordance correlation coefficient
LC	Liquid chromatography

BEH	Bridged ethane hybrid technology
BSA	Body Surface Area
SD	Standard deviation
FDA	Food and Drug Administration
CV	Coefficient of variation
LOA	Limit of agreement
QC	Quality control
ISTD	Internal standard
NPC	Normal phase chromatography
RPC	Reversed-phase chromatography
UV	Ultraviolet
AUC	Area under the curve
ULOA	Upper limit of agreement
LLOA	Lower limit of agreement
CI	Confidence interval

Zusammenfassung

Hintergrund: Eine genaue und präzise Messung der glomeruläre Filtrationsrate (GFR) ist wichtig für die Diagnostik der chronischen Nierenerkrankung (CKD). Iohexol ist ein weithin akzeptierter exogener Marker für die Bestimmung der GFR bei CKD-Patienten. Die Messung der Iohexolkonzentration durch Hochleistungs-Flüssigkeitschromatographie (HPLC) und Ultra-Hochleistungs-Flüssigkeitschromatographie (UPLC) sind die derzeit am häufigsten verwendeten Methoden. Bislang sind Vergleichsstudien zu HPLC- und UPLC-basierten Iohexolquantifizierung limitiert. Das Ziel dieser Studie ist daher, die HPLC- und UPLC-basierte Iohexolquantifizierung in Proben innerhalb einer Studie, der Berliner Initiative Studie (BIS), direkt zu vergleichen. Des Weiteren soll anhand der Messung die Langzeitstabilität der Iohexolproben analysiert werden.

Methode: Die Quantifizierung von Iohexol erfolgt in 386 Serumproben von 56 Patienten der BIS Studie mittels UPLC und HPLC. Es erfolgt ein Vergleich der Iohexolkonzentration und einhergehender GFR.

Ergebnis: Der Vergleich der HPLC- und UPLC-Messverfahren für die Iohexol-Konzentration mittels Passing-Bablok-Regression ergibt: y = −1.02 x - 1.11 (Steigung 95% KI: 1.01, 1.04; Y-Achsenabschnitt 95% KI: −2.50, 0.04 µg/ml). Der Lins Konkordanz-Korrelationskoeffizient (CCC) zeigt eine nahezu vollständige Übereinstimmung (ρ>0.99). Die Bland-Altman-Plot zeigt eine mittlere Abweichung von 1.70%. Für die von Iohexol abgeleiteten GFR zeigt die Passing-Bablok-Regression einen Steigungswert von 1.00 (95%-KI: 0.94, 1.04) und einen Schnittpunktwert von 0.28 ml/min/1.73m^2 (95% KI: −3.05, 3.96 ml/min/1.73m^2). Der CCC ergibt eine Übereinstimmung von ρ=0.97. Der Bland-Altman-Plot zeigt eine mittlere Abweichung von 1.66%.

Der Vergleich von HPLC- und HPLC_BIS-Messungen für die Iohexol-Konzentration ergibt die Passing-Bablok-Regression y = 0.95 x -1.10 (Steigung 95% KI: 0.94, 0.97; Schnittpunkt 95% KI: −2.32, 0.22 µg/ml). Der CCC ergibt eine Übereinstimmung von ρ=0.97. Der Bland-Altman-Plot zeigt eine mittlere Abweichung von 5.93%. Für die GFR zeigt die Passing-Bablok-Regression keine signifikante Abweichung der Linearität. Der CCC ergibt eine

Übereinstimmung mit ρ=0.97. Die Bland-Altman-Analyse zeigt eine mittlere Abweichung von 5.62%.

Schlussfolgerung: Diese Studie fand keinen signifikanten Unterschied oder Bias in der Quantifizierung der Iohexol-Konzentration und der von Iohexol abgeleiteten GFR zwischen der HPLC-Methode und der UPLC-Methode oder zwischen lang- und kurzfristig gelagerten Proben.

Abstract

Background: Accurate and precise measurement of glomerular filtration rate (GFR) is important for the diagnosis and stratification of chronic kidney disease (CKD). Iohexol is a widely accepted exogenous marker to measure GFR. High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UPLC) are the most commonly used methods for measuring iohexol concentration. However, there is limited research on the HPLC and UPLC methods and studies comparing the two. Therefore, this study fills this gap by comparing the two above-mentioned methods for measuring iohexol concentration to identify the differences, if any, in the results produced by the two. In addition, the samples were measured by Berlin Initiative Study (BIS) ten years ago; the same samples were re-measured to investigate if there are differences between long-term and short-term stored samples.

Method: Serum sample iohexol concentrations were measured using both HPLC and UPLC. Iohexol serum concentrations and iohexol-derived mGFR of 56 patients (386 serum samples) were compared. Since the data was measured ten years ago as part of the BIS study (HPLC_BIS measurement), the same samples were re-measured using HPLC for this study (HPLC measurement), and the differences between the results produced by the two were compared.

Result: When comparing HPLC and UPLC, Passing-Bablok regression revealed y = −1.02 x - 1.11 (slope 95% CI: 1.01, 1.04; Y-intercept 95% CI: −2.50, 0.04 µg/ml). Lin's concordance correlation coefficient (CCC) showed almost perfect agreement (ρ>0.99). Bland-Altman analysis illustrated a mean bias of 1.70%. For iohexol-derived GFR results, Passing-Bablok regression illustrated a slope value of 1.00 (95% CI: 0.94, 1.04), and an intercept value of 0.28 ml/min/1.73m^2 (95% CI: −3.05 - 3.96 ml/min/1.73m^2). CCC indicated that the agreement was substantial (ρ = 0.97). The Bland-Altman analysis demonstrated a mean bias of 1.66%.

When comparing HPLC and HPLC_BIS, Passing-Bablok regression line demonstrated y = 0.95 x -1.10 (slope 95% CI: 0.94, 0.97; intercept 95% CI: −2.32, 0.22 µg/ml). CCC indicated substantial agreement (ρ = 0.97). The Bland-

Altman plot showed a mean bias of 5.93%. For iohexol-derived GFR results, Passing-Bablok regression showed an intercept value of 2.07 ml/min/1.73m^2 (95% CI: −1.32, 6.20 ml/min/1.73m^2) and a slope value of 1.03 (95% CI: 0.97, 1.09). CCC revealed substantial agreement (ρ = 0.97). The Bland-Altman analysis showed a mean bias of 5.62%.

Conclusion: This study found no significant difference or bias in the quantification of iohexol concentration and iohexol-derived GFR between either the HPLC method and the UPLC method or between long-term and short-term stored samples.

1. Introduction

Chronic kidney disease (CKD) has been recognized as a heavy burden on global public health. Approximately 10–15% of the global population suffers from CKD [1]. In 2015, the Global Burden of Disease (GBD) study reported that approximately 1.2 million people died due to CKD [2]. Accurate measurement of glomerular filtration rate (GFR), a key indicator of renal function, is important for CKD classification and management.

1.1. Kidney function and glomerular filtration rate

The kidney is an important organ that regulates the water, electrolyte, and acid-base balance while clearing metabolic waste from the body. The basic functional unit of the kidneys is the nephron [3]. Each human kidney contains approximately 1.2 million nephrons. Each nephron consists of a renal corpuscle and a tubule system, as shown in Figure 1. The renal corpuscle consists of glomerular capillaries and the Bowman's capsule. The tubule system consists of the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct system. The pathway of fluids flowing within a nephron begins in the Bowman's capsule, descends into the descending limb of the loop of Henle, returns to the thick ascending limb of the loop of Henle, and passes down into the collecting tubule, and ends up in a renal calyx. Each renal calyx is connected with the ureter, which empties urine into the urinary bladder, where urine is temporarily stored, and from which it is intermittently eliminated. Approximately 1–2 L of urine is excreted each day [4, 5].

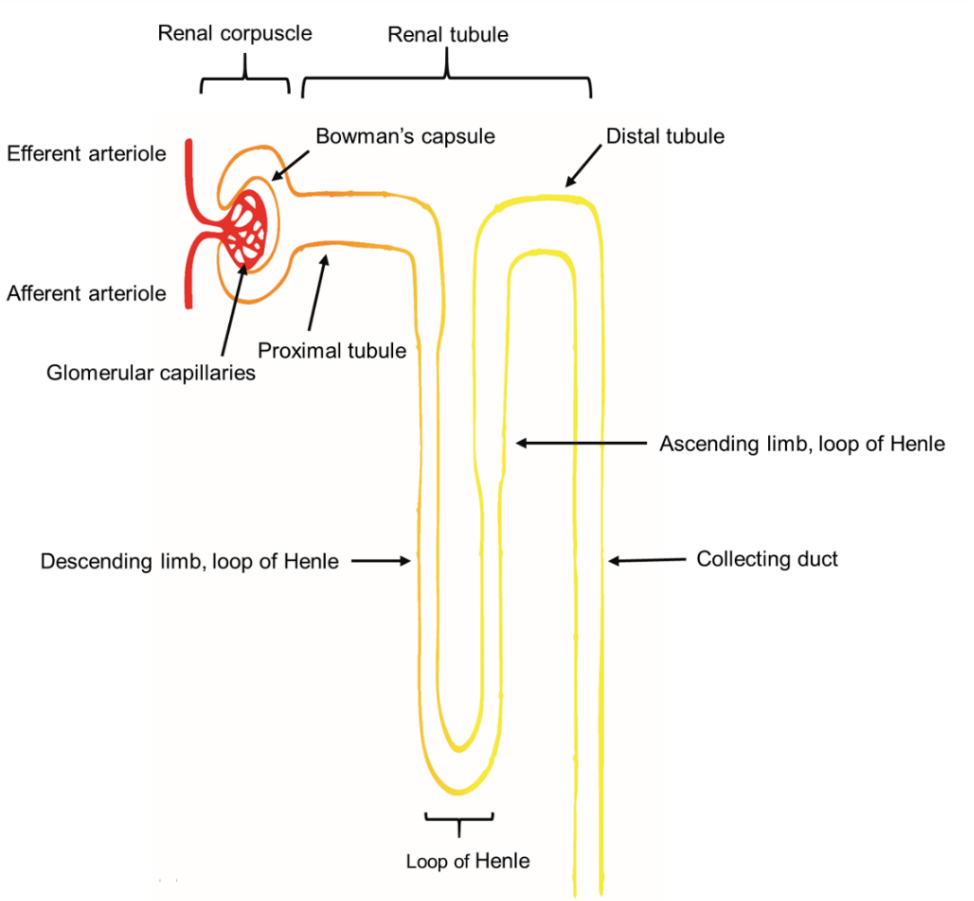

Figure 1. Structure of a nephron

Filtration is the process of water and solutes in the blood leaving the vascular system through the filtration barrier and entering the Bowman's space. The GFR of the kidney is equal to the sum of the filtration rates of all functioning nephrons, which is the amount of fluid that filters into the Bowman space per unit of time. Normal GFR is >90 ml/min/1.73 m^2, which is important for normal kidney function [3-5].

1.2. GFR categories in CKD

CKD is characterized by a decrease in the GFR to less than 60 ml/min/1.73 m^2 or signs of renal damage for at least three consecutive months. GFR assessment (whether estimated or measured) is required for the classification, diagnosis, and treatment of CKD. The Kidney Disease Improving Global Outcomes (KDIGO) guideline proposed a classification scheme based on the GFR [6] (see Table 1). A decrease in the GFR usually indicates disease progression, whereas an improvement in the GFR means

recovery [4]. Therefore, evaluating the patient's GFR is essential for assessing the severity and course of CKD.

Table 1. Changes in GFR seen in different stages of CKD (according to KDIGO, clinical practice guidelines for evaluation and management of CKD, 2012)

GFR category	GFR (ml/min/1.73 m^2)	Terms
G1	≥ 90	Normal or high
G2	60 - 89	Mild decrease
G3a	45 - 59	Mild-to-moderate decrease
G3b	30 - 44	Moderate-to-severe decrease
G4	15 - 29	Severe decrease
G5	< 15	Kidney failure

1.3. Determination of kidney function

Glomerular filtration rate (GFR) is regarded as an important sensitive indicator to evaluate renal function [6]. GFR can not be measured directly; however, it can be evaluated from serum levels of endogenous filtration markers (that is, estimated GFR, eGFR), or the measured clearance of exogenous filtration markers (that is, measured GFR, mGFR).

1.3.1. Determination of kidney function using eGFR

Estimated GFR (eGFR) is derived from the serum concentration of endogenous filtration markers, including serum creatinine (Scr) and serum cystatin C (Scys), without requiring clearance measurement. Over the past decades, researchers have been working on the development of eGFR equations, such as the Modification of Diet in Renal Diseases (MDRD) [7], Cockcroft-Gault (CG) [8], Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [9], Berlin Initiative Study (BIS) [10], Revised Lund-Malmö (Rev LM) [11], some of which have been validated and suggested by regulatory agencies and clinical practice guidelines [12-15]. However, in specific clinical practice, the precision of the eGFR equations can be relatively poor, because serum creatinine is significantly dependent on muscle mass [16] cystatin C is less

affected by muscle mass but is more affected by smoking, obesity, levels of thyroid, inflammation, and cardiovascular risk factors [17-25]. Therefore, mGFR is required and recommended as a confirmatory test in special situations, including the evaluation of living kidney donor candidates, patients with muscle wasting, or if highly accurate GFR evaluation is required for clinical decision-making [6, 26, 27].

1.3.2. Determination of kidney function using mGFR

Measured GFR (mGFR) can be determined based on the serum clearance of exogenous filtration markers. An ideal exogenous filtration marker should have the following characteristics: freely and instantaneously distributed across the extracellular space, free filtration at the glomerulus, not subject to plasma protein binding, no secretion or reabsorption at the tubules, eliminated by the kidneys completely, no synthesis, no degradation, non-toxic, easy and inexpensive to measure [5]. Exogenous filtration markers that meet the criteria include inulin, isotopic (^{51}Cr-EDTA, ^{99}Tc-DTPA) and non-isotopic (iothalamate, iohexol). Currently, iohexol is widely used in Europe since it fulfils all the requirements of an ideal marker.

1.3.2.1. Inulin

Inulin is considered the gold standard for the assessment of kidney function. Although inulin is an ideal marker with few side effects, there are some drawbacks. On one hand, the disadvantage of inulin is that it requires constant intravenous injection and continuous urine collection, which is very time-consuming. On the other hand, urine collection over 24 hours easily causes errors. Overall, the measurement procedure is complicated to carry out in clinical practice. Thus, this measurement method is often restricted to research purposes and can't be carried out for routine clinical diagnosis [28, 29].

1.3.2.2. Radioactive markers ^{51}Cr-EDTA and ^{99}Tc-DTPA

Given all the drawbacks of inulin, two radioactive substances have been proposed as alternatives: ^{51}Cr- ethylene diamine tetraacetic acid (^{51}Cr-EDTA) and ^{99}Tc-diethylene triamine pentaacetic acid (^{99}Tc-DTPA). These agents need to be given only once, by a single injection, then the GFR can be calculated based on the total area under the disappearance curve versus time. Previous studies have demonstrated that radioactive markers ^{51}Cr-EDTA and ^{99}Tc-DTPA are comparable with standard renal

inulin clearance for accurate measurement of GFR [30, 31]. However, there are disadvantages with each of these compounds: ^{51}Cr-EDTA is not available in America, and the kidney's ability to handle ^{99}Tc-DTPA can differ due to different commercial sources [32]. Moreover, due to their radioactivity, these markers require special licensing for complicated handling, storage, and disposal of waste, which complicates the procedure. Further, it cannot be used in all patients, such as pregnant women [33].

1.3.2.3. Iothalamate

Iothalamate is an ionic contrast media, commonly used for urography. Sigman from the New York University used iothalamate as a GFR marker for the first time in 1965 [34]. Following that, most, but not all, studies comparing iothalamate urinary clearance to inulin clearance reported iothalamate clearance to be slightly higher than inulin clearance, probably due to tubular secretion of iothalamate [35, 36]. Moreover, extra-renal clearance is not so negligible, especially in patients with severe CKD. In a recent study, Visser estimated the extra-renal excretion of iothalamate to be 14 ± 12% by calculating its urinary clearance for 24 h [37]. The patients who are allergic to contrast media are considered a clinical limitation [38].

1.3.2.4. Iohexol

Iohexol is also a common exogenous marker used for the accurate determination of the GFR in CKD patients. It is a tri-iodinated benzene-derivative, with a molecular weight of 821.1Da, non-ionic, low osmolality, non-radioactive X-ray contrast medium, widely used for computed tomography (CT), catheter-based angiography, and interventions in clinical practice [39]. Figure 2 shows the chemical structure of iohexol [40].

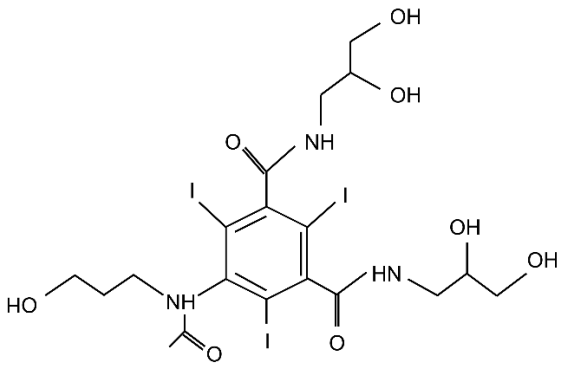

Figure 2. Iohexol chemical structure: C19H26I3N3O9

It was developed in the early 1980s. The first human study found that iohexol was eliminated unchanged in the urine, and had almost the same renal clearance as ^{51}Cr-EDTA [41]. A few years later, Krutzén et al proved the reliability of iohexol clearance for GFR evaluation [42]. Then, several researchers studied the value of iohexol as a marker for GFR estimation.

In a recent review, authors gathered data from seven studies comparing iohexol clearance with inulin clearance, which revealed an excellent correlation and agreement between them [26]. Several researchers have investigated the correlation and agreement between iohexol plasma clearance and ^{51}Cr-EDTA plasma clearance. All relevant studies have found the good correlation and agreement by comparing plasma clearances of both markers [42-48]. Houlihan C et al demonstrated a good correlation between plasma clearance of iohexol and ^{99}Tc-DTPA in 21 diabetic patients [49]. Comparing the clearances of iohexol and iothalamate is important because iothalamate is the most commonly used GFR marker in the US. Since iothalamate is not commercially available in Europe, it is now rarely used. A recent study compared iothalamate and plasma clearance of iohexol in 102 patients for GFR measurement. They found a good concordance between iohexol and iothalamate plasma clearance measured by HPLC, while iothalamate overestimated iohexol results measured by mass spectrometry [50]. The interpretation was probably due to tubular secretion of iothalamate [51].

There appears to be relatively little iohexol-protein bonding. The initial investigation found that the binding rate with protein was just 1.5% [52], which was validated by further other studies [42, 53, 54]. As a marker, extra-renal clearance is important. When the difference between urine clearance and plasma clearance was studied in healthy subjects, the extra-renal clearance of iohexol was found to be between 0 and 6 ml/min/1.73 m^2, [39, 42, 53, 55] or 5% [56]. Extra-renal clearance of iohexol in anephric individuals ranged from 2 to 3 ml/min/1.73 m^2 [57-60]. The absorption and tubular secretion of iohexol have not been demonstrated yet.

Like all other drugs and filtration agents, the safety of iohexol is a problem. Iohexol dosage for coronary interventions ranges from 130 to 300 ml in clinical practice, and from 80 to 180 ml for computed tomography (CT) scans. Nevertheless, the dosage currently used for GFR measurement is usually much lower (5 – 10 ml or less).

Therefore, the risk of immediate adverse events in the evaluation of renal function is minimal or negligible [61]. It has a lower allergic potential than ionic contrast media, which is probably due to a lower histamine release and/or a lower activation of the complement cascade [62]. Recently, a systematic report on the safety of 5 ml iohexol administration for GFR measurement was published. In a total of 15,147 GFR measurements, 2,891 patients were reviewed for the immediate adverse reactions associated with iohexol administration. A single case of moderate-intensity (itching and flushing) reaction was reported in a diabetic renal disease patient a few minutes after iohexol administration, and the patient then recovered after intravenous corticosteroid injection. Overall, the total rate of iohexol-related incidents was as low as 0.0066% regardless of illness circumstances and renal function level [63]. These findings rule out any concerns associated with iohexol's safety as an ideal exogenous filtration marker for GFR measurement [61].

An external quality assurance program for iohexol provided by Equalis AB in Uppsala, Sweden. For other GFR markers, there is no such external quality program, which is another advantage of iohexol [64].

Overall, little amount of iohexol was subjected to protein binding, almost all of it got eliminated through the kidney, and it was neither secreted nor reabsorbed. Therefore, iohexol is a commonly used marker to measure GFR in Europe.

1.4. Quantification of iohexol concentration

To calculate GFR, the concentration of iohexol must be quantified accurately. Iohexol can be quantified by different methods, including X-ray fluorescence (XRF), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and High-performance liquid chromatography (HPLC). Iohexol measurement by HPLC is specific, sensitive, and reproducible [42, 53, 64-72]. Compared with XRF, HPLC has higher sensitivity, especially at low serum concentrations [43, 73]. Compared with LC-MS/MS, HPLC is not expensive and easy to implement [74-77]. Therefore, HPLC is the most commonly used method in Europe [41, 42]. However, the main limitation of HPLC is that it is time-consuming and technically cumbersome [78]. For many decades, researchers have tried to overcome these barriers. A more advanced and innovative technique called Ultra-High-Performance Liquid Chromatography (UPLC) has been developed, which evolved from HPLC. Quantification by the UPLC system can improve analysis

efficiency by using the smaller size of packing particles, and higher flow rates under high pressure.

1.4.1. High-performance liquid chromatography (HPLC)

At the beginning of the twentieth century, Russian botanist Mikhail Tswett came up with the concept of chromatography. He passed the solution mixture through a glass column containing fine particles of calcium carbonate to separate the plant pigments. The separation of these pigments appears as colored bands on the chromatographic column. Tswett gave a very detailed description of the newly discovered phenomena and named his separation method by combining the two Greek words “chroma” and “graphein,” which mean “color” and “to write,” respectively [79, 80].

Originally, HPLC stood for "High-Pressure Liquid Chromatography," an abbreviation created by Professor Csaba Horvath [81] in a Pittcon paper in 1970, indicating the fact that high pressure is applied to generate the flow needed for liquid chromatography in a packed column. Pumps initially had a pressure capacity of 500 psi. Technology took a huge leap in the early 1970s. These new HPLC devices could generate pressures of up to 6,000 psi of pressure. Small particles with a particle size of 3–10 micrometers were used as stationary phase packed in a steel tube, which was subjected to high pressure. It can dramatically improve the analytical ability of column chromatography. In the late 1970s, with the continuous improvement of performance (smaller particles, even higher pressure), the resolution was improved and the analysis time shortened. The acronym HPLC kept unchanged, however, the name changed to high-performance liquid chromatography to distinguish it from the earlier approaches [82].

HPLC is a kind of liquid chromatography (LC) that separates substances using a mobile phase (solvent) and a stationary phase (packing material of the column). When the sample passes through the stationary phase, sample constituents get separated due to the interaction between the sample and the stationary phase. There are four different separation modes depending on the property of the stationary phase, which includes adsorption chromatography, partition chromatography, ion-exchange chromatography, and size exclusion chromatography. Adsorption chromatography is the most widely used, in which the separation is based on adsorption–desorption steps. Depending on the polarity of the two phases, there are normal phase chromatography (NPC) and reversed-phase chromatography (RPC) [83]. Currently,

RPC eclipses NPC due to its ease of use, fast equilibration, reproducible retention time, and the fact that the basic principles of the retention mechanism can be understood easily [84]. The separation mechanism of reversed-phase chromatography is based on the hydrophobic binding interaction of solute molecules between the mobile phase and stationary phase. The mobile phase of reversed-phase chromatography is polar (such as water, methanol, or acetonitrile), and the stationary phase is non-polar (such as hydrocarbon). The separation of RPC relies on the reversible adsorption/desorption of solute molecules with different degrees of hydrophobicity in the hydrophobic stationary phase [85]. Non-polar compounds are retained longer on the column [86]. In practice, special experimental conditions are designed to promote the adsorption of solutes from the mobile phase to the stationary phase. Then, the mobile phase composition is adjusted to promote the desorption of solutes from the stationary phase back into the mobile phase. Finally, the separation of species occurs in the form of bands or regions created by various retentions. Therefore, qualitative and/or quantitative analysis of chemical information can be performed based on these separate areas [87, 88].

HPLC system [89]:

The components of a basic HPLC system are an HPLC solvent, pump, injector, column, detector, computer data station, and waste. Figure 3 is a simplified schematic representation of an HPLC system.

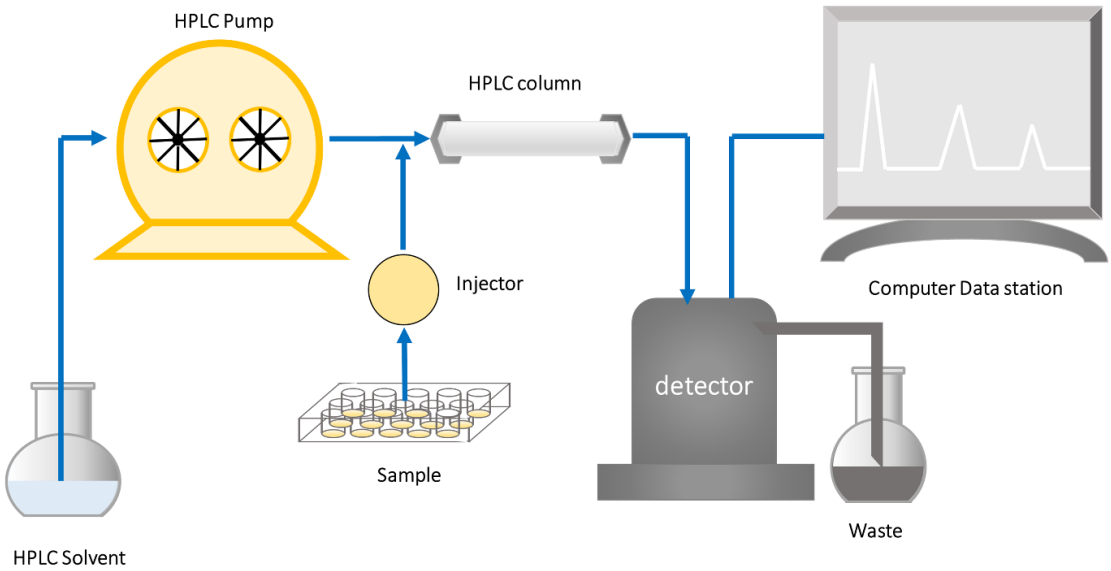

Figure 3. High-performance liquid chromatography system

The mobile phase reservoir holds the solvent. A constant pressure pump is used to drive the mobile phase through the column and generate the specified mobile phase flow rate. The injector can insert the samples into the constantly running mobile phase stream that transports the sample to the column.

HPLC columns are normally made of tubing in stainless steel, containing the packing materials required for separation. Because it is held in place by the column hardware, this packing material is referred to as the stationary phase. Guard columns are often used in front of the column. This short column helps to protect the analytical column and extend its lifetime by removing large particles and impurities before they enter the column.

A detector is required in order to see the separate compound bands from the HPLC column. The detector is linked to a computer data station, which captures the electrical signal necessary for displaying the chromatogram and calculating and quantifying sample concentrations. Different types of detectors have been developed since the compounds can have different characteristic features. For example, an ultraviolet (UV) absorption detector is used if the compound can absorb UV light. A fluorescence detector is used whenever the compound fluoresces.

1.4.2. Ultra-performance liquid chromatography (UPLC)

Although HPLC has many advantages as compared with previously used liquid chromatographic techniques, its efficiency is questionable, which is a major limitation [78]. Chromatographic researchers tried to develop a rapid analytical separation without sacrificing the ability to perform high-quality analyses. As a result, a new system design with smaller high-quality packing materials, the capability to resist very high pressures, advanced technology in the pump, autosampler, and detector, was developed, called ultra-high-performance liquid chromatography (UPLC), which has evolved from HPLC [90].

UPLC mainly benefits from the ‘‘power of small particles’’. From HPLC to UPLC, one of the primary drivers has been the evolution of packing materials used for the separation. The fundamental principles of evolution are governed by the Van Deemter equation [91], according to which, particle size is one of the variables. If the particle size decreases to less than 2 µm, there is a significant gain in efficiency, analysis time, and peak capacity [92]. UPLC columns are usually packed with particles smaller than

2 µm, while HPLC columns are usually packed with particles of size 3–5 µm. The use of smaller particles allows for better resolution, higher efficiency, and higher sensitivity due to sharper and higher peaks, as well as shorter chromatographic analysis time [93]. For example, due to less band spreading occurring during migration through a column with smaller particles (peak width is less and peak height greater), efficiency with 1.7 µm particles is three times greater than with 5 µm particles and two times greater than with 3.5 µm particles. The resolution obtained with 1.7 µm particles is 70% higher compared to 5 µm particles and 40% higher compared to 3.5 µm particles. The column length with 1.7 µm particles can be shortened by one-third compared to 5 µm particles of the same efficiency, and the flow rate can be increased by three times. This means that separation can be completed in $1/9^{th}$ of the time while maintaining resolution [94-96].

Using smaller particles for packing UPLC columns requires a greater pressure range to obtain high peak capacity separations than in HPLC columns. Therefore, to take full advantage of the small particle technology, the system is designed with increased resistance to high pressures; UPLC systems can tolerate up to 1,200 bar, whereas HPLC columns can tolerate a maximum pressure of 400 bar.

In UPLC columns, the particle material provides enhanced mechanical stability and an extended pH range. The current UPLC system employs a second-generation bridged ethane hybrid (BEH) technology [97-99]. This not only enhances the mechanical stability of 1.7 µm particles by bridging the methyl groups in the silica matrix but also extends the pH application range and helps withstand high pressure.

The technology used in the instrument's design must keep up in order to take advantage of the higher resolution, sensitivity, and faster speed provided by smaller particles. The conventional HPLC system is incapable of handling particles smaller than 2 mm. In early 2004, the first commercially available UPLC system to meet these requirements called the ACQUITY UPLC system was suggested for the separation of various drug-related organic small molecules, peptides, and proteins. The system is fully designed to significantly improve resolution, sample throughput, and sensitivity [100, 101].

1.5. Aim of the study

CKD is considered a heavy burden on global public health. Therefore, an accurate assessment of kidney function is necessary for the classification, diagnosis, and treatment of CKD. GFR is recognized as a sensitive indicator for the assessment of kidney function, which includes eGFR and mGFR. mGFR can be measured by different exogenous markers. Iohexol is a widely used exogenous marker used to measure GFR. To calculate GFR, iohexol concentration needs to be quantified accurately. HPLC is the most commonly used method for measuring iohexol. However, the major disadvantage of HPLC is that it is time-consuming. An advanced technology called UPLC has thus been developed to speed up the analysis.

However, currently, there is limited comparative research on HPLC and UPLC for iohexol quantification. A key question is whether different measurement methodologies affect iohexol results, thereby affecting the iohexol-derived GFR results.

This study aims to compare iohexol concentration measured by UPLC and HPLC to see whether different measurement methods affect the results, thereby affecting the iohexol-derived GFR results. In addition, given the samples were obtained and measured ten years ago by the Berlin Initiative Study (BIS), the same samples were remeasured in this study to investigate whether there are differences between long-term and short-term stored samples.

2. Materials and methods

2.1. Manufacturer information of devices, reagents and materials, and software

Table 2. Devices

Devices	Manufacturer
HPLC	Waters, USA
HPLC pump	VWR, Germany
HPLC diode array detector	Hitachi, Mannheim
HPLC column RP-18e 100*4.6 mm	Merck, Germany
HPLC guard column RP-18e 5*4.6 mm	Merck, Germany
UPLC	Waters, USA
UPLC pump	Waters, USA
UPLC diode array detector	Waters, USA
UPLC column BEH peptide 50*2.1 mm	Waters, Ireland
UPLC guard column Acquity BEH C18 2.1*5 mm	Waters, Ireland

Table 3. Reagent and materials

Medium and materials	Manufacturer
Accupaque™ 300, 647 mg/mL Iohexol	GE Healthcare Buchler GmbH &Co.KG, Germany
Acetonitrile	VWR, France
O-phosphoric acid	Carl Roth, Germany
Methanol	VWR, EC
Perchloric acid	Segama, USA
Formic acid	Fisher chemical, Czech Republic
Iopromid	Merck, Germany
UPLC grade water	VWR, France
UPLC 96-well multi-well plates	Pall Corporation, Puerto Rico
Adhesive film for microplates	VWR, USA
HPLC micro insert	VWR, Germany
HPLC vial	VWR, Germany

Table 4. Software

Software	Manufacturer
HPLC software EZCHrom Elite	Waters, USA
UPLC software ChromasterUltra Rs	Waters, USA
Graphpad Prism 5.0 software	San Diego, USA
MedCalc statistical software version 18.2.2	MedCalc software bvba, Ostend, Belgium

2.2. Samples

All the patients were selected from the Berlin Initiative Study (BIS) ten years ago and were approved by the Charité Ethics Committee. Each patient was administered 5 ml iohexol (containing 3235 mg iohexol) intravenously into a forearm, antecubital, or hand vein and flushed with 10 ml saline. Blood samples of each patient were collected at 7-time points (30, 60, 90, 120, 150, 180, 240, and 300 minutes) from the contralateral arm after injection. The samples were centrifuged and transported on dry ice to be stored at -80°C until further analysis at the Charité University Hospital [10, 102, 103]. For my study, 56 patients (386 serum samples) from the BIS study were selected. Since CKD is divided into five stages, we selected patients from each of the different stages according to the eGFR level (CKD-EPI equation).

2.3. Determination of iohexol concentration using HPLC

2.3.1. HPLC calibration standard preparation

Iohexol calibration standard series were made by diluting Accupaque™ 300, (647mg/ml iohexol) to designated concentrations (291.15 µg/ml, 218.36 µg/ml, 145.58 µg/ml, 87.35 µg/ml, 43.67 µg/ml, 23.29 µg/ml) with distilled water. Healthy serum samples were taken from the -80°C refrigerator, thawed at room temperature, and centrifuged at 161,000 rpm for 15 min. 10 µl of the standard series samples were added to 90 µl of healthy serum, and the standard was prepared in doubled rows. Perchloric acid (400 µl 5%) was added to each sample, vortexed quickly and immediately, and centrifuged at 161000 rpm (table centrifuge) at 15°C for 60 min. The vials were prepared, and inserts were put into the vials. 100 µl of the supernatant was transferred into the inserts and put into the HPLC system for measurement.

2.3.2. HPLC quality control preparation

Quality control series were prepared by diluting Accupaque™ 300, (647 mg/ml iohexol) to different concentrations (140.86 µg/ml, 60.69 µg/ml, 34.36 µg/ml, 24.64 µg/ml) with distilled water. Healthy serum samples were taken from the -80°C refrigerator, thawed, and centrifuged at 161,000 rpm for 15 min. 10 µl of the quality control series samples were added to 90 µl of healthy serum. 400 µl 5% of perchloric acid was added to each serum sample, vortexed immediately and quickly, and centrifuged at 161,000 rpm at 15°C for 60 min. The vials and inserts were prepared for samples. 100 µl of the

supernatant was transferred into the inserts and put into the HPLC system for measurement.

2.3.3. HPLC sample preparation

Patient serum samples were taken from the -80°C refrigerator, thawed at room temperature, and centrifuged at 2,500 rpm for 15 min. Samples measuring 100 µl were pipetted into tubes and prepared in doubled rows with an automatic pipette. Perchloric acid (400 µl, 5%) was added to each sample, vortexed quickly and immediately, and centrifuged at 161,000 rpm (table centrifuge) at 15°C for 60 min. The vials were prepared, and inserts were put into the vials. 100 µl of the supernatant was transferred into the inserts and put into the HPLC system for measurement.

2.3.4. HPLC chromatographic condition

The patient samples and standards were prepared in duplicates and run in duplicates. The quality standards were run in triplicates. The analysis of the iohexol concentration was carried out on the HPLC system with a diode array detector, HPLC column (RP-18e 100 x 4.6 mm, Merck, Darmstadt), and a guard column (RP-18e 5 x 4.6 mm, Merck). Iohexol was eluted with acetonitrile/water (4:96%) at a flow rate of 1.5 ml/min and kept at 30°C for a total run time of 4 min. The UV absorbance was monitored at 254 nm. For analysis, the main iohexol peak was used. The basic components of an HPLC system are shown in Figure 4.

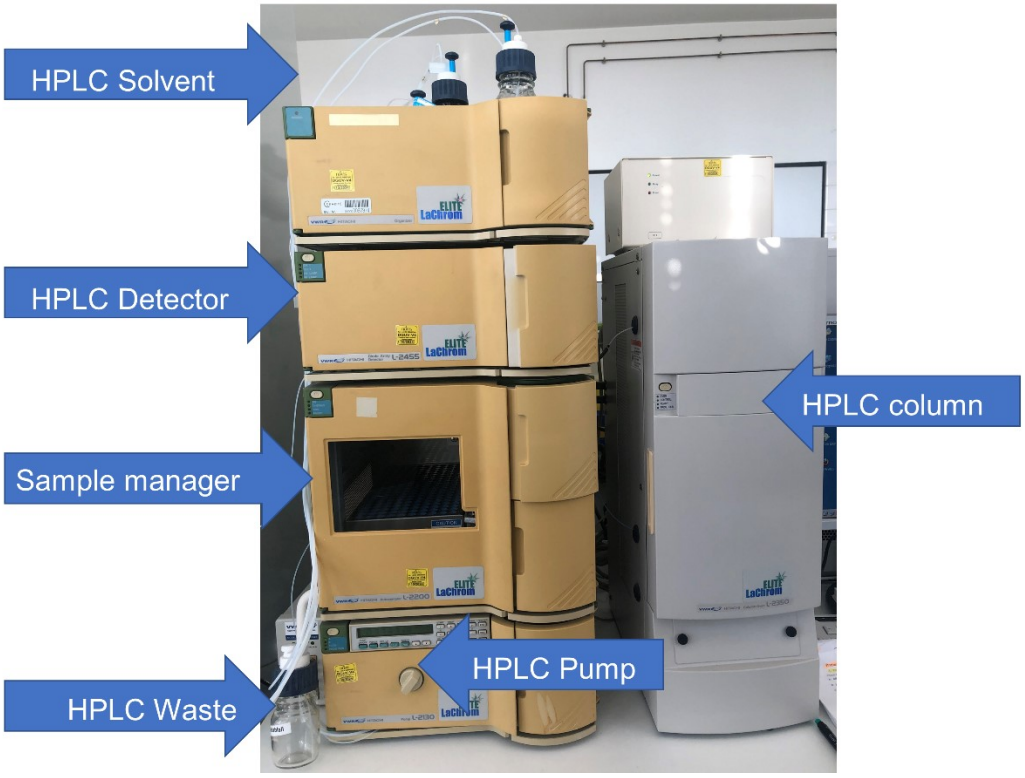

Figure 4. Basic components of an HPLC system

2.3.5. Quantitation of iohexol concentration using external standard calibration

The iohexol standard series was diluted with distilled water to designated concentrations separately, which has been introduced in detail above: C1 = 23.29 μg/ml, C2 = 43.67 μg/ml, C3 = 87.35 μg/ml, C4 = 145.58 μg/ml, C5 = 218.36 μg/ml, and C6 = 291.15 μg/ml. The standards were prepared in duplicates and run in duplicates. The system calculated the mean of four values and recorded the corresponding peak height automatically. The linearity calibration curve between the instrument response (peak height) and analyte concentration (iohexol concentration) was established, as shown in Figure 5. After evaluating the calibration curve (if $R^2 \geqslant 0.999$), the calibration curve was used to calculate the concentration of samples.

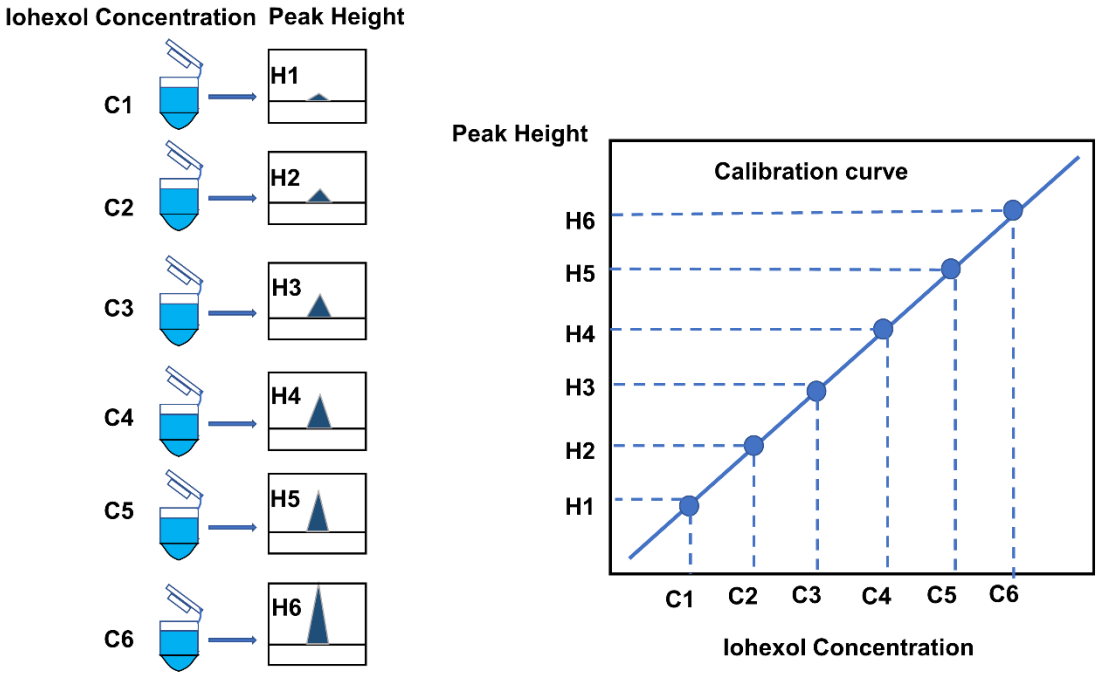

Figure 5. Quantitation of iohexol concentration by external standard calibration

2.4. Determination of iohexol concentration using UPLC

Iohexol calibration standard series was made by diluting Accupaque™ 300 (647 mg/mL iohexol) to 500 µg/ml, 222.22 µg/ml, 98.76 µg/ml, 43.89 µg/ml, 19.50 µg/ml, and 8.67 µg/ml separately with healthy serum. Quality control was achieved by diluting iohexol to 500 µg/ml, 333.33 µg/ml, 148.14 µg/ml, 65.84 µg/ml, 29.26 µg/ml, 13.00 µg/ml, and 8.67 µg/ml separately with healthy serum. Internal standard iopromide (10 µg/ml) was added to the iohexol standard series, quality control, and samples separately. Analysis of the iohexol serum concentration was carried out on a UPLC system with a UPLC column (BEH Peptide 50*2.1 mm, 1.7 µm particles, Waters), a guard column (Acquity BEH C18 2.1*5 mm, Waters), and a diode array detector. For each run, the samples, quality control standards, and calibrators were prepared in duplicates and run as a single analysis. Chromatography was performed at 50°C at a flow rate of 1.0 ml/min with a run time of 1.5 min. Mobile phases were composed of Solvent A (water, 0.1% formic acid) and Solvent B (acetonitrile, 0.1% formic acid). The basic components of a UPLC system are shown in Figure 6.

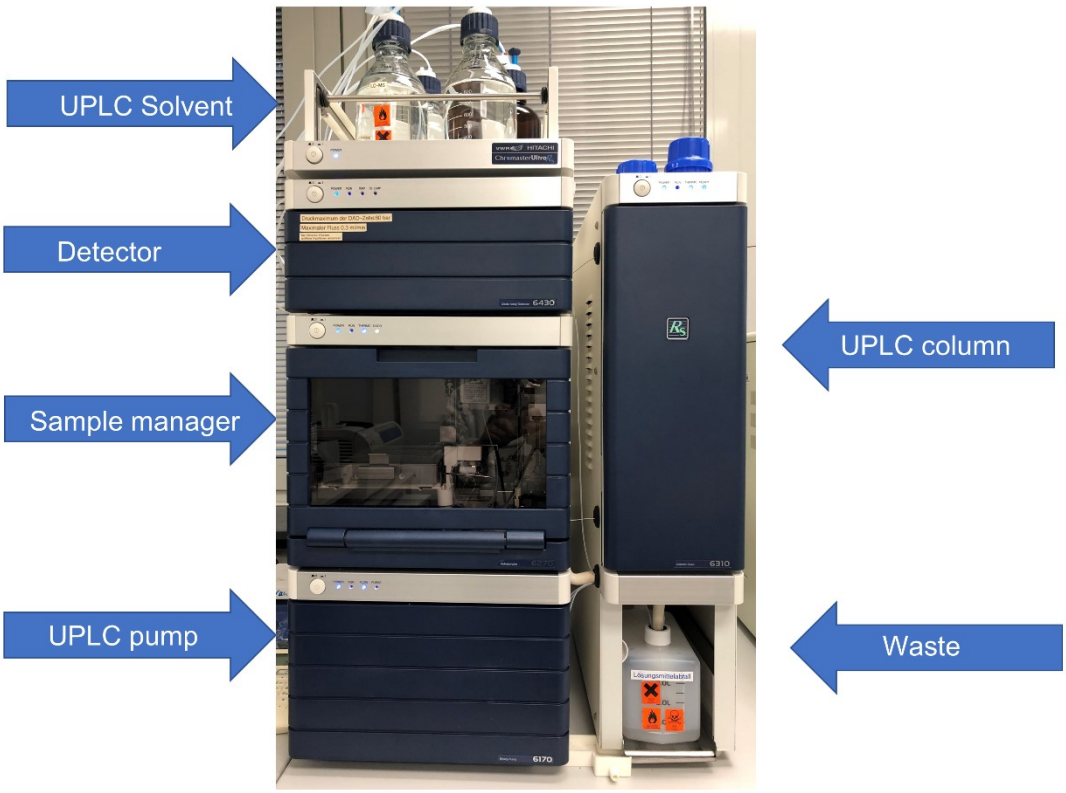

Figure 6. Basic components of a UPLC system

2.4.1. Quantitation of iohexol concentration using internal standard calibration

Iohexol standard series was diluted with distilled water to achieve designated concentrations separately, which has been introduced in detail above: C1 = 8.67 μg/ml, C2 = 19.50 μg/ml, C3 = 43.89 μg/ml, C4 = 98.76 μg/ml, C5 = 222.22 μg/ml, C6 = 500 μg/ml. The same amount (10 μg/ml) of internal standard iopromide was added to the iohexol standard series separately. The calibration standards, including iohexol standard series and internal standard iopromide, were put into the UPLC system for analysis. The standards were prepared in duplicates and run as a single analysis. The system recorded the corresponding iohexol peak area (A1–A6) and internal standard iopromide (A_{ISTD}) automatically. The linearity calibration curve between the instrument response (peak area ratio) and analyte concentration (iohexol concentration) was established as shown in Figure 7. After evaluating the calibration curve (if $R^2 \geqslant 0.999$), the calibration curve was used to calculate the iohexol concentration in samples.

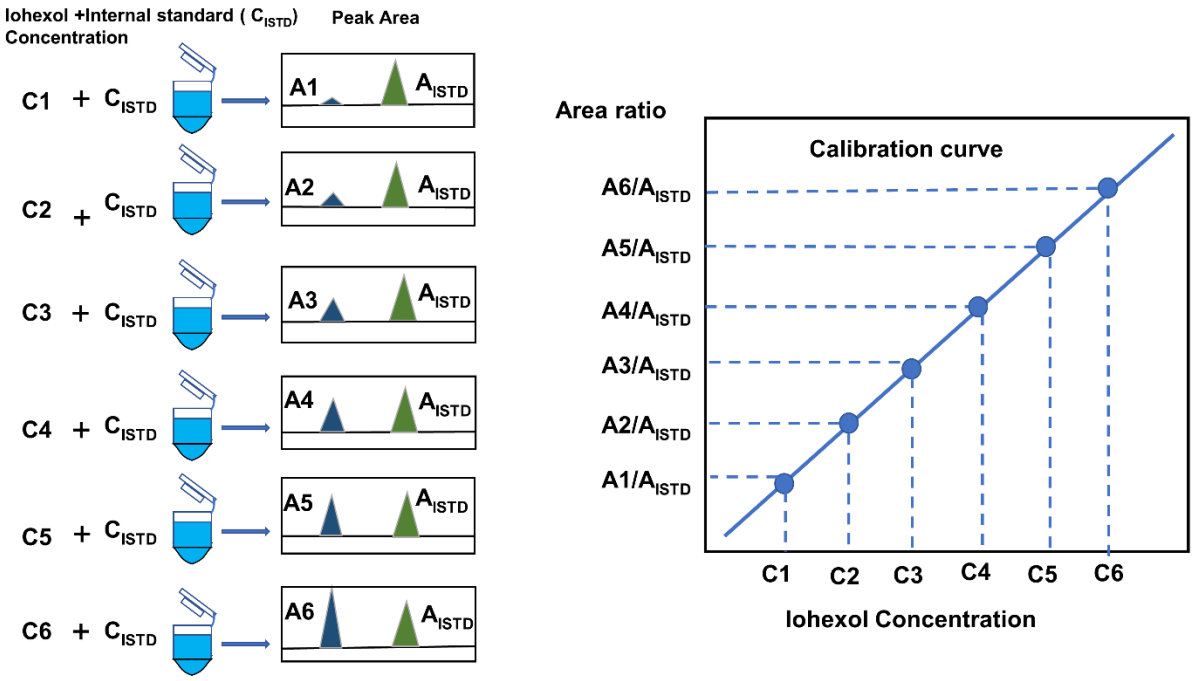

Figure 7. Quantitation of iohexol concentration by internal standard calibration

2.5. Calculation of GFR

After obtaining the concentration of iohexol, the GFR (i.e., the iohexol clearance) was calculated from the given iohexol dose divided by the area under the curve (AUC) see Equation 1.

$$\text{Iohexol clearance} = \frac{\text{Dose}}{\text{AUC}}$$

Equation 1. Iohexol clearance. Dose: Single injection dose of iohexol; AUC: Area under the curve.

After administration of iohexol, it took several hours to collect blood samples and then quantify the concentration of the iohexol in the serum [104]. Excellent and comparable results can be obtained using the one-compartment model as described by Brochner-Mortensen see (Equation 2) [105]. Therefore, in this study, the one-compartment model was employed.

$$GFR = C1 \times GFR(A) + C2 \times [GFR(A)]^2 (\text{ml/min per } 1.73\ m^2)$$

Equation 2. GFR. GFR (A) = Dose/[exp (A)/α]; α is the slope of the curve in the linear elimination phase; A is the Y-intercept, which is the point of intersection with the Y-axis of the linear elimination phase; C1 = 0.9908; C2 = - 0.001218

Body Surface Area (BSA)

The unit of primarily determined GFR is ml/min. To achieve comparability between the individual values of the test persons, these absolute values are corrected to a nominal body surface area (BSA) (see Equation 3). For this, 1.73 m^2 is the standard used [106].

$$\mathrm{BSA} = \mathrm{W}^{0,425} \times \mathrm{H}^{0,725} \times 0,007184$$

Equation 3. BSA. W is the weight in kg; H is the height in centimeters

2.6. Data analysis

All statistical analyses were performed using GraphPad Prism 5.0 and the Medcalc software version 18.2.2. To assess the normality of all measured iohexol concentrations and GFR results, the D'Agostino-Pearson normality test was performed. Passing-Bablok regression, Lin's Concordance Correlation Coefficient, and the Bland-Altman method were used to assess the correlation and agreement between the two methods. Passing - Bablok linear regression was used to calculate the intercept and slope of the linear regression. Lin's concordance correlation coefficient was used to measure deviations from identity between two measurements, where ρ expressed precision and C_b expressed accuracy. Strength of agreement was described according to the values of the concordance correlation coefficient: ρ < 0.90 (poor); ρ = 0.90–0.95 (moderate); ρ = 0.95–0.99 (substantial); ρ > 0.99 (almost perfect). The mean bias was calculated using Bland-Altman analysis from the percentage difference. Limits of agreement were expressed as the mean ± 1.96 times the standard deviation (SD) of the differences. 95% confidence intervals for bias were also estimated. $p < 0.05$ was considered statistical significance.

3. Results

To investigate whether different measurement methods affect the iohexol results and then affect the iohexol-derived GFR results, the study compared the HPLC and UPLC methods. A total of 56 patients with 7-time points each, including a total of 386 serum samples, were measured using the HPLC and UPLC methods. The study also compared the remeasured HPLC measurements with the original results measured using HPLC by the BIS study ten years ago to investigate whether there are differences between long-term and short-term stored samples.

3.1. Representative chromatograms of HPLC and UPLC methods

Using the HPLC method, iohexol was eluted from the chromatographic column as two peaks, including one major peak and a small peak (endo- and exo-iohexol forms), which were not separated from each other. A representative iohexol chromatogram from a 291 µg/ml calibration was obtained, as shown in Figure 8A, and a representative patient chromatogram is shown in Figure 8B. The retention time of iohexol was 2.5 min, which drifted from 2.2 to 3.2 min during the whole experiment. The eluting time was 4 min.

Using the UPLC method, iohexol was eluted from the chromatographic column as one peak and the internal standard iopromide eluted as two peaks. A representative iohexol chromatogram from a calibration obtained is shown in Figure 8C, and a representative patient's chromatogram is shown in Figure 8D. The retention time of the iohexol calibrator at the 43.89 µg/ml level was 0.9 min. The retention time of iopromide (internal standard) was 1.1 min. The eluting time was 1.5 min. There was no retention time drift during the whole experiment.

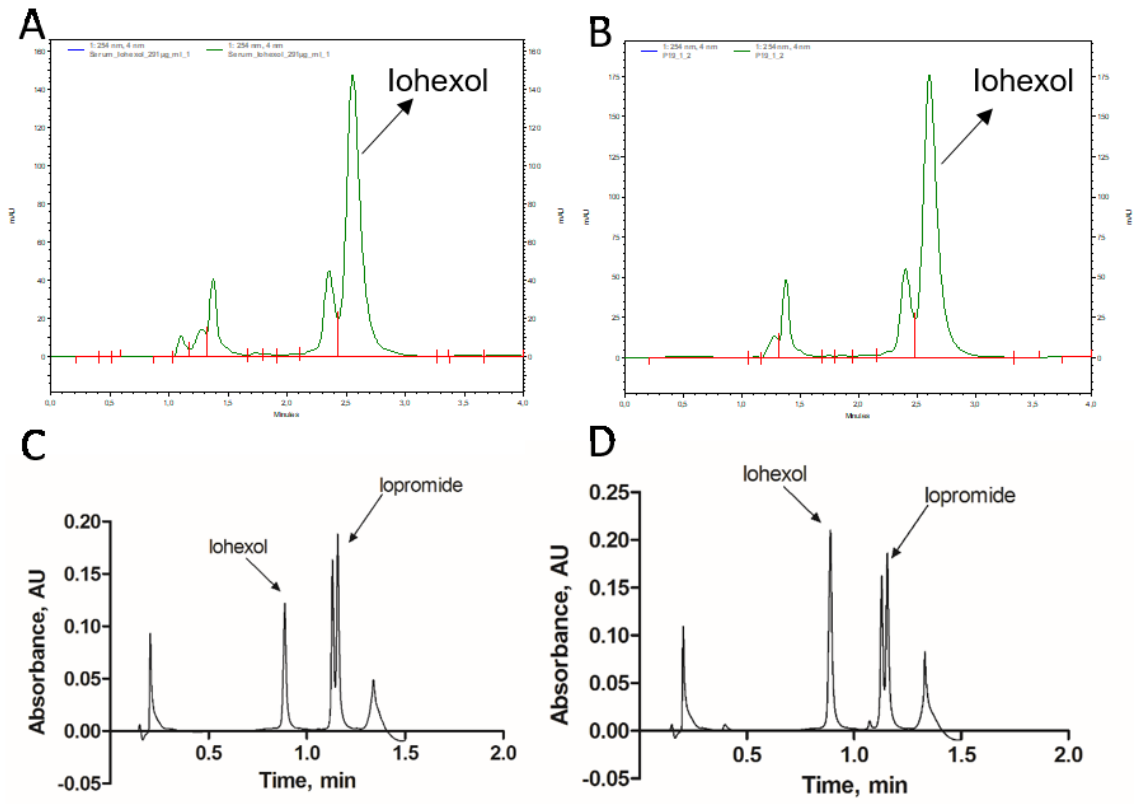

Figure 8. Representative chromatograms: A. Representative chromatogram of an iohexol-standard solution at 291 µg/ml separated by HPLC; B. Representative patient chromatogram separated by HPLC; C. Representative chromatogram of an iohexol-standard solution at 43.89 µg/ml separated by UPLC; D. Representative patient chromatogram separated by UPLC.

3.2. Representative calibration curves of HPLC and UPLC methods

The calibration curve of the HPLC method was constructed in the range of 23.29–291.15 µg/ml. Iohexol was quantified by external standard calibration. The calibration curve was calculated by plotting the peak height of extracted iohexol chromatogram versus. the corresponding concentration of iohexol. A representative iohexol linear calibration curve of one measurement was calculated as y = 1997.62 x + 3438.89 (Figure 9A). The calibration curve showed a coefficient of determination (R^2) of 0.9996. There were no outliers.

However, the measurement of iohexol by UPLC was quantified by internal standard calibration. The calibration curve was constructed by plotting the peak area ratio of extracted iohexol chromatogram and iopromide chromatogram versus. the corresponding concentration of iohexol, which ranges from 8.67 to 500 µg/ml. The

representative linear calibration curve of one particular measurement, as shown in Figure 9B, was calculated as y = 0.0057 x + 0.0058 (R^2 = 0.9998). There were no outliers.

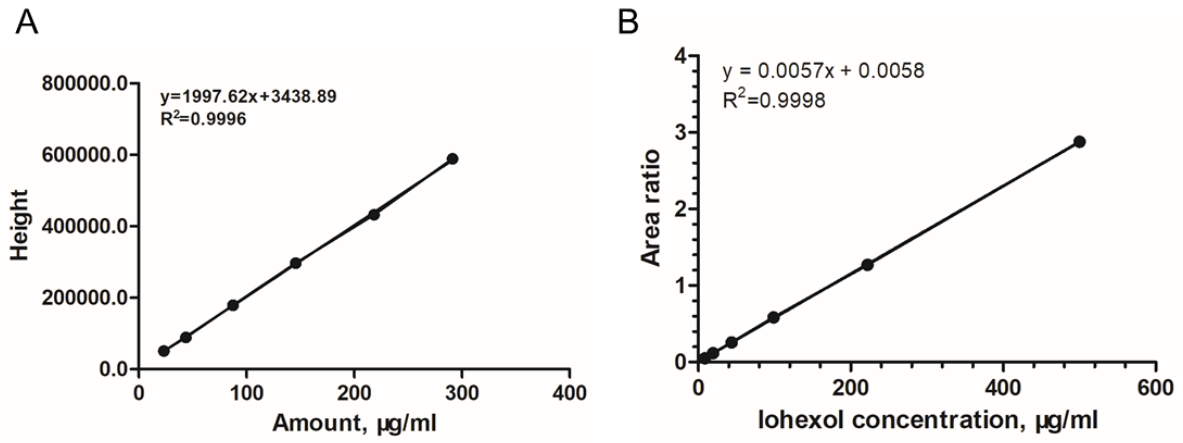

Figure 9. Representative calibration curves: A. Representative calibration curve of one measurement using the HPLC method; B. Representative calibration curve of one measurement using the UPLC method.

3.3. Quality assessment of iohexol measured by HPLC and UPLC

The study aimed to compare UPLC and HPLC in terms of their ability to measure iohexol concentrations in order to understand whether the results differ when different measurement methods are used, thereby affecting the derived GFR. Therefore, a reliable quantitation method was needed to quantify iohexol concentrations. In our study, we performed the internal and external quality assessment of the iohexol measurements using HPLC and UPLC to confirm the reliability of the methods.

3.3.1. Internal quality assessment

For internal quality assessment, we used accuracy and precision. Accuracy is defined as the closeness of agreement between the test value and the accepted reference value. The precision is determined by calculating the coefficient of variation (%CV) of intra- and inter-day at each concentration level of the quality control (QC) standards [107-109]. In this study, the intra-day accuracy of quantification of iohexol using the HPLC method was 90.6–108.8%. The inter-day accuracy was 96.93–98.25%. The CV of intra-day was 0.1–5.1%. The CV of inter-day was 2.27–4.86 % (Table 5). For the

HPLC method, the coefficient of variation never surpassed 5%, and the accuracy was within 10% in this study.

Table 5. Accuracy and precision of quantification of iohexol concentration by HPLC

	QC (µg/ml)	Accuracy[a] (%)	Precision[b] (%)
Intra-day	140.86	94.0-106.1	0.1-1.0
	60.69	91.7-100.8	0.1-1.4
	34.36	91.9-105.8	0.2-2.5
	24.64	90.6-108.8	0.1-5.1
Inter-day	140.86	98.25	2.27
	60.69	97.14	2.33
	34.36	97.65	3.61
	24.64	96.93	4.86

[a] Calculated as (mean determined value/nominal value×100)

[b] Calculated as % CV. (SD/mean value) ×100

Using the UPLC method, the intra-day accuracy was 93.5–103.0%. The inter-day accuracy was 98.7–100.6%. The CV of intra-day for iohexol analyzed using UPLC was 0–7.8%. The CV of inter-day was 0.8–2.5%. The detailed analysis of the accuracy and precision is shown in Table 6. The coefficient of variation and accuracy was within 8% at all concentrations.

Table 6. Accuracy and precision of quantification of iohexol concentration by UPLC

	QC (µg/ml)	Accuracy[a] (%)	Precision[b] (%)
Intra-day	500	96.4-100.5	0-4.9
	333.33	97.7-101.3	0.3-4.4
	148.14	98.5-102.9	0.1-4.3
	65.84	98.1-101.9	0.4-4.8
	29.26	98.2-102.7	0.8-5.7
	13.00	97.2-102.9	0.4-5.8
	8.67	93.5-103.0	0.3-7.8
Inter-day	500	98.7	0.8
	333.33	99.8	1.1
	148.14	100.1	1.1
	65.84	100.4	1.1
	29.26	100.6	1.1
	13.00	99.4	1.6
	8.67	99.0	2.5

[a] Calculated as (mean determined value / nominal value × 100)

[b] Calculated as % CV (SD / mean value) × 100

According to the Food and Drug Administration (FDA) bioanalytical method validation document, the recommendations and acceptance criteria for the accuracy and CV in-study should be within 15% [110]. Therefore, for internal quality assessment, the variation in the results is acceptable.

3.3.2. External quality assessment

An external quality assurance program was conducted by Equalis AB, Uppsala, Sweden, in which more than 30 laboratories participated. Serum or plasma samples with spiked iohexol were distributed to participants four times a year. The spiked concentrations measured in our laboratory were then compared with the overall mean as the index of external quality control. The inter-laboratory CV has a quality goal of 8% [111]. About 62.5% of HPLC results were within ± 8%, and 100% of UPLC results were within ± 8%, as shown in Table 7.

Table 7. Interlaboratory CV of Equalis samples

	1	2	3	4	5	6	7	8
Interlaboratory CV of HPLC[a], %	4.7	4.3	7.5	20.4	13.5	10.9	5.3	7.9
Interlaboratory CV of UPLC[b], %	2.47	-2.9	3.7	4.2	3.9	-2.8	1.0	-1.5

[a] Calculated as (value determined by HPLC - overall mean value / overall mean value) ×100

[b] Calculated as (value determined by UPLC - overall mean value / overall mean value) ×100

3.4. Serum iohexol elimination curves of UPLC, HPLC, HPLC_BIS

The patients' serum samples from the BIS study were measured using HPLC ten years ago, and the process is referred to as HPLC_BIS measurement. The same samples from the BIS study were remeasured using HPLC in my study, referred to as HPLC measurement. The iohexol concentration in the serum samples was measured using UPLC in my study, referred to as UPLC measurement. Representative iohexol elimination curves of one particular patient are shown in Figure 10.

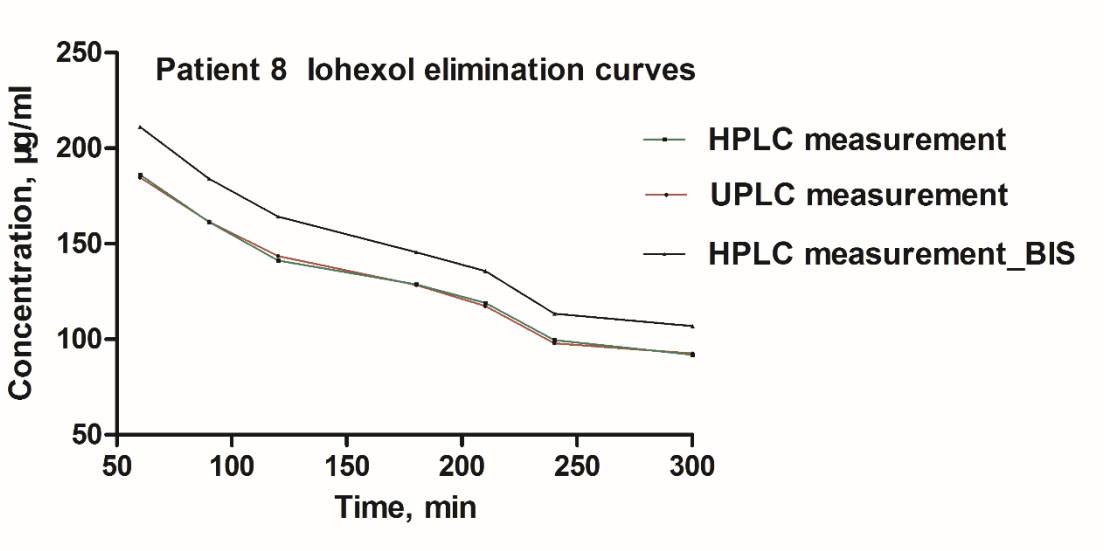

Figure 10. Iohexol elimination curves after intravenous injection in one representative patient. UPLC measurement refers to samples measured by UPLC in this study. HPLC_BIS measurement refers to the samples measured by HPLC ten years ago. HPLC measurement refers to the samples remeasured by HPLC in this study

3.5. Comparison of iohexol concentration measured by UPLC and HPLC

To investigate the correlation and agreement between UPLC and HPLC, I used Passing-Bablok regression, Lin's concordance correlation coefficient, and Bland-Altman plots on 386 serum samples, as shown in Table 8.

For UPLC and HPLC, the Passing-Bablok regression line was y = -1.02 x -1.11 (Y-intercept 95% CI: -2.50, 0.04 µg/ml; slope 95% CI: 1.01, 1.04), as shown in Figure 11. The Cusum test for linearity showed no significant deviation from linearity (P > 0.05). Lin's concordance correlation coefficient ρ was >0.99 and C_b was >0.99 (Table 8). The accuracy statistic C_b measures how far the best-fit line deviates from the 45° line through the origin [112]. According to McBride's proposed criteria for ρ, this level of agreement is characterized as almost perfect [113]. To better understand the agreement, I also performed the Bland-Altman plot analysis, as shown in Figure 12. A mean bias of 1.70% was observed with the upper limit of agreement (ULOA) of 15.26% and lower limit of agreement (LLOA) of -11.86%. About 4.6% of the total points fell outside of the 95% limit of agreement.

Table 8. Comparison of iohexol concentration measured by UPLC and HPLC

Passing-Bablok Regression	**N**	**Intercept (95% CI)**	**Slope (95% CI)**	**Cusum linearity test**
HPLC vs UPLC	386	-1.11(-2.50, 0.04)	1.02(1.01, 1.04)	P = 0.68(p > 0.05)
Lin's Concordance Correlation Coefficient [a]	**N**	**ρ**	**C_b**	
HPLC vs UPLC	386	> 0.99	> 0.99	
Bland-Altman Analyses [b]	**N**	**Bias (95% CI)**	**LLOA (95% CI)**	**ULOA (95% CI)**
HPLC vs UPLC	386	1.70%(1.01%,2.39%)	-11.86%(-13.05%,-10.68%)	15.26%(14.08%,16.45%)

[a] Lin's concordance correlation coefficient measures the deviations from identity between two methods, where ρ expresses precision and C_b expresses accuracy. Strength of agreement was described according to the values of the concordance correlation coefficient: ρ < 0.90 (poor); ρ = 0.90–0.95 (moderate); ρ = 0.95–0.99 (substantial); ρ > 0.99 (almost perfect).

[b] Bland-Altman Analyses: Bias is expressed as the percentage difference between two methods.

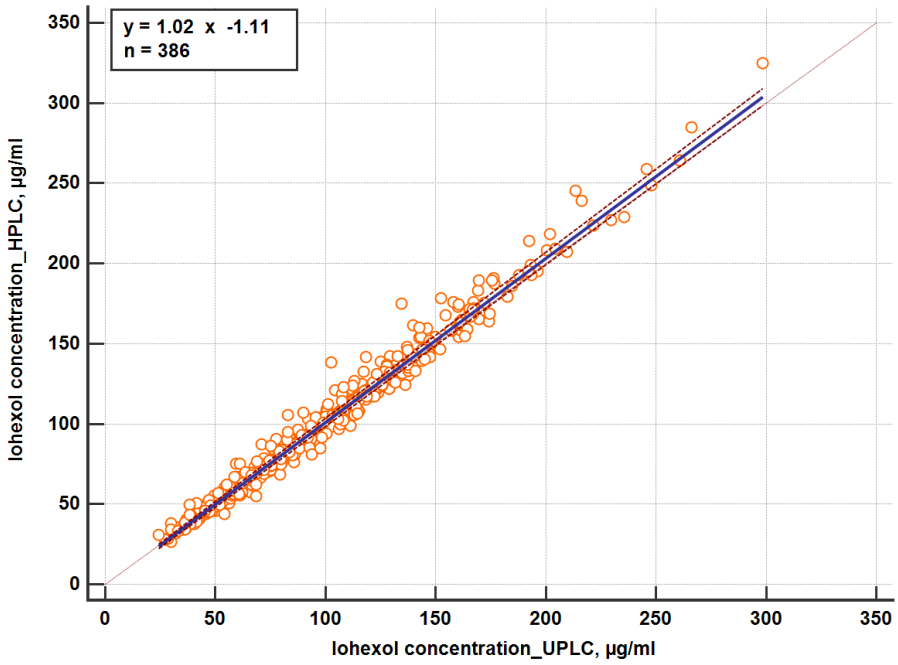

Figure 11. Passing-Bablok regression: Intercept: -1.11 µg/ml (95% CI: -2.50, 0.04 ug/ml); slope: 1.02 (95% CI: 1.01, 1.04). Yellow circles represent measured samples. The solid blue line represents the regression line, the dashed yellow line represents the line of identity, and the dashed red lines represent 95% confidence intervals. The Cusum test indicates no significant deviation from linearity (P > 0.05).

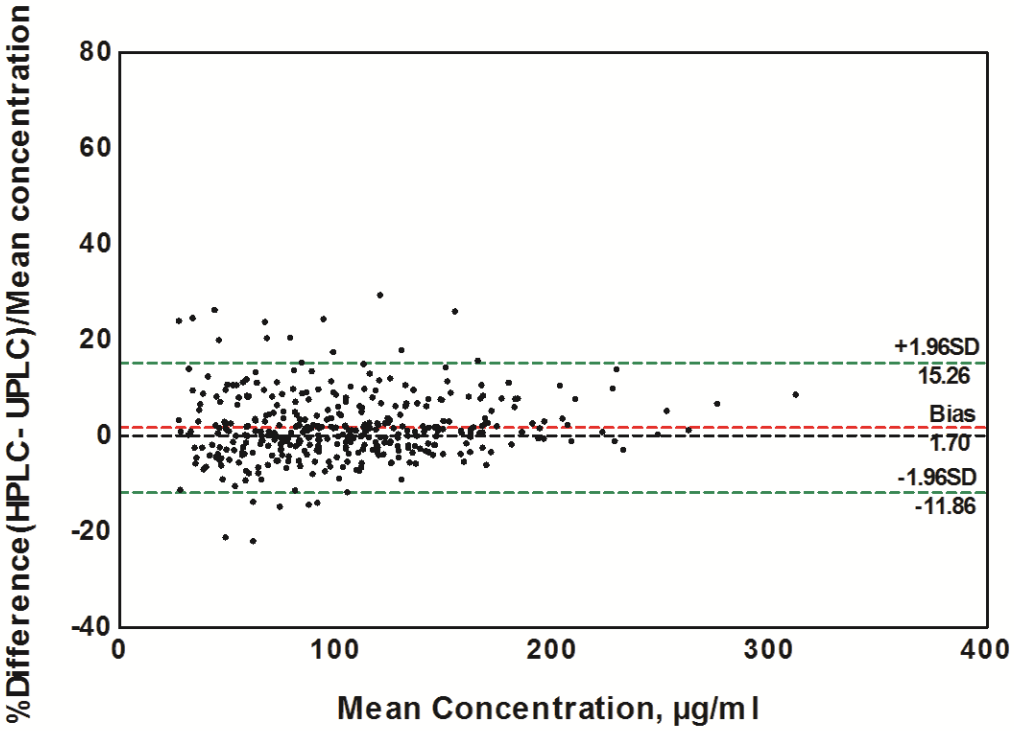

Figure 12. Bland-Altman plot of % differences between the two methods vs. the mean of the two measurements. The dashed red line represents mean bias, the dashed black line represents the identity line, and the dashed green lines represent the 95% limit of agreement (LOA).

3.6. Comparison of iohexol-derived GFR results between UPLC and HPLC

I further compared the UPLC and HPLC methods in terms of their ability to measure iohexol-derived GFR as in the case of the 56 selected patients, as shown in Table 9. Passing-Bablok regression analysis showed a slope value of 1.00 with 95% CI of 0.94–1.04 and an intercept value of 0.28 ml/min/1.73m^2 with 95% CI of -3.05–3.96 ml/min/1.73m^2 (Figure 13). The Cusum test showed no significant deviation from linearity (P = 0.74). Lin's concordance correlation coefficient showed that the concordance was substantial (ρ = 0.97). The Bland-Altman analysis comparing the mGFR results derived from the HPLC and UPLC measurements showed a mean bias of 1.66%, with the limits of agreement (LOA) ranging from -12.65% to 15.98%. About 96.43% of the total points fell within the 95% limit of the agreement (Figure 14).

Table 9. Comparison of iohexol-derived GFR results between UPLC and HPLC

Passing-Bablok Regression	**N**	**Intercept (95% CI)**	**Slope (95% CI)**	**Cusum linearity test**
HPLC vs UPLC	56	0.28 (-3.05, 3.96)	1.00 (0.94,1.04)	P = 0.74 (p > 0.05)
Lin's Concordance Correlation Coefficient [a]	**N**	**ρ**	**C_b**	
HPLC vs UPLC	56	0.97	1.00	
Bland-Altman Analyses [b]	**N**	**Bias (95% CI)**	**LLOA (95% CI)**	**ULOA (95% CI)**
HPLC vs UPLC	56	1.66% (-0.29%,3.62%)	-12.65% (-16.01%,-9.29%)	15.98% (12.62%,19.34%)

[a] Lin's concordance correlation coefficient measures the deviations from identity between two methods, where ρ expresses precision and C_b expresses accuracy. Strength of agreement was described according to the values of the concordance correlation coefficient: ρ < 0.90 (poor); ρ = 0.90–0.95 (moderate); ρ = 0.95–0.99 (substantial); ρ > 0.99 (almost perfect).

[b] Bland-Altman analyses: Bias is expressed as the percentage difference between two methods.

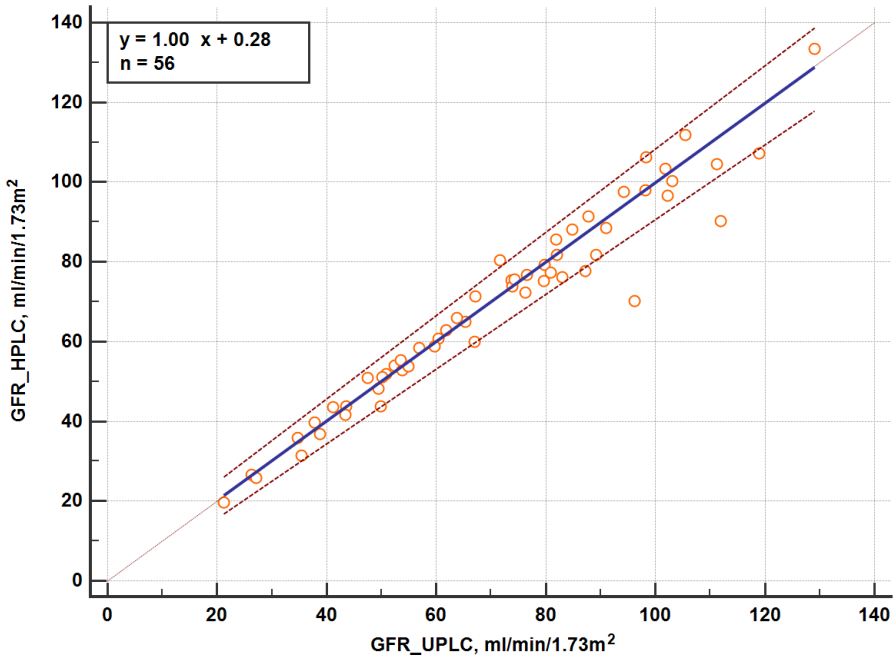

Figure 13. Passing-Bablok regression: Intercept: 0.28 ml/min/1.73m² (95% CI: -3.05, 3.96 ml/min/1.73 m²); slope: 1.00 (95% CI: 0.94, 1.04). Yellow circles represent measured GFR results. The solid blue line represents the regression line, the dashed yellow line represents the line of identity, and the dashed red lines represent 95% confidence intervals. The Cusum test for linearity indicates no significant deviation from linearity (P > 0.05).

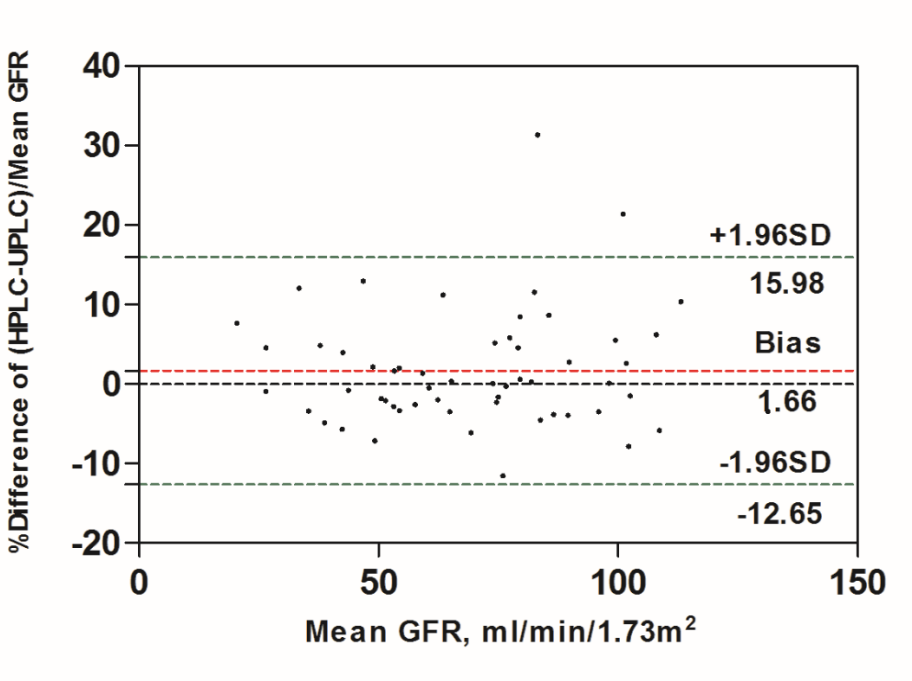

Figure 14. Bland-Altman analysis: Comparison of HPLC and UPLC for measuring iohexol-derived GFR. The dashed red line represents mean bias, the dashed black line represents the identity line, and the dashed green lines represent the 95% limit of agreement (LOA).

3.7. Comparison of iohexol concentration measured by HPLC and HPLC_BIS

Since the HPLC_BIS data is from ten years ago, I remeasured the same samples using HPLC. I compared the results produced by HPLC with that by HPLC_BIS to observe whether there existed any differences between long-term and short-term stored samples, as shown in Table 10.

For the HPLC_BIS measurement and the HPLC measurement, the Passing-Bablok regression line was y = 0.95 x -1.10 (intercept 95% CI: -2.32, 0.22 μg/ml; slope 95% CI: 0.94, 0.97), as shown in Figure 15. No significant deviation from linearity was observed (P = 0.51). Lin's concordance correlation coefficients indicated substantial agreement between HPLC and HPLC_BIS measurement ability, as shown in Table 10. The Bland-Altman plot analysis showed a mean bias of 5.93%. (Figure 16). About 5.6% of the total points fell outside of the 95% limit of agreement (upper limit: 23.10%; lower limit: -11.34%).

Table 10. Comparison Iohexol concentration measured by HPLC and HPLC_BIS

Passing-Bablok Regression	N	Intercept (95% CI)	Slope (95% CI)	Cusum linearity test
HPLC vs HPLC_BIS	386	-1.10(−2.32, 0.22)	0.95(0.94, 0.97)	0.51
Lin's Concordance Correlation Coefficient [a]	**N**	**ρ**	**C_b**	
HPLC vs HPLC_BIS	386	0.97	0.99	
Bland-Altman Analyses [b]	**N**	**Bias (95% CI)**	**LLOA (95% CI)**	**ULOA (95% CI)**
HPLC vs HPLC_BIS	386	5.93%(5.03%,6.83%)	-11.63%(-13.16%,-10.09%)	23.49%(21.96%,25.02%)

[a] Lin's concordance correlation coefficient measures the deviations from identity between two methods, where ρ expresses precision and C_b expresses accuracy. Strength of agreement was described according to the values of the concordance correlation coefficient: ρ < 0.90 (poor); ρ = 0.90–0.95 (moderate); ρ = 0.95–0.99 (substantial); ρ > 0.99 (almost perfect).

[b] Bland-Altman Analyses: Bias is expressed as the percentage difference between two methods.

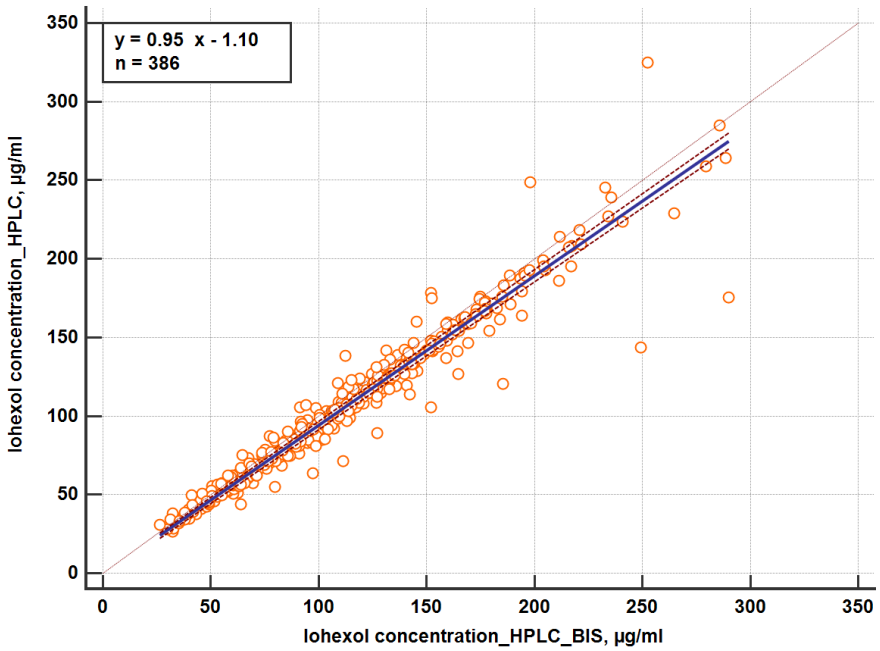

Figure 15. Passing-Bablok regression. Intercept: -1.10 µg/ml (95% CI: 2.32, 0.22 µg/ml); slope: 0.95 (95% CI: 0.94, 0.97). Yellow circles represent measured samples. The solid blue line represents the regression line, the dashed yellow line represents the line of identity, and the dashed red lines represent 95% confidence intervals. The Cusum test indicates no significant deviation from linearity (P > 0.05).

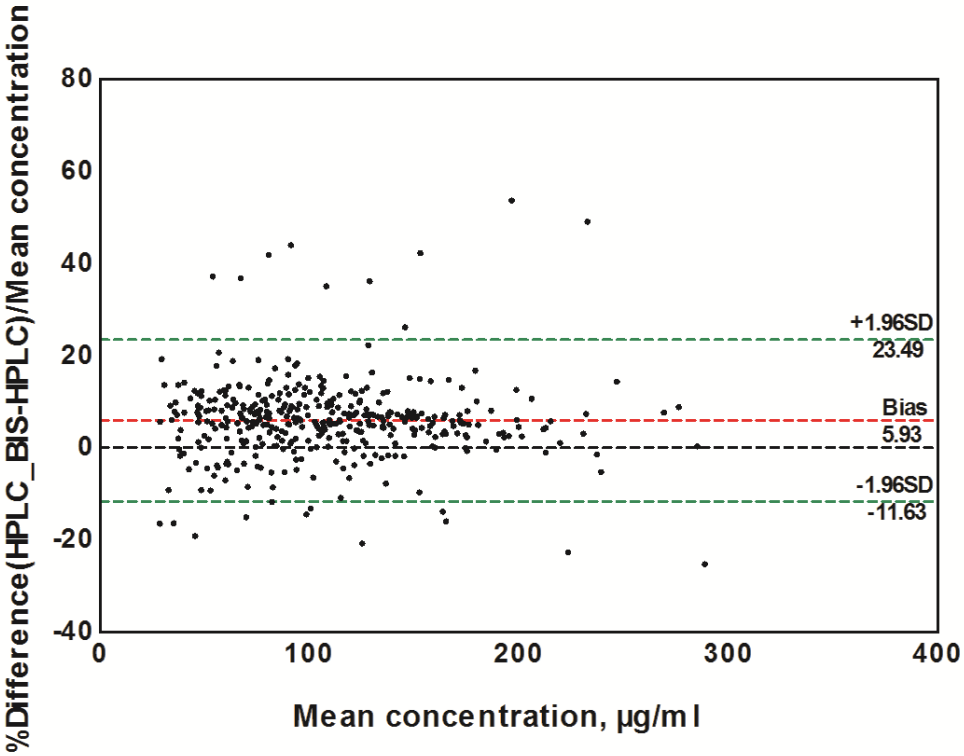

Figure 16. Bland-Altman plot of % differences between two methods vs. the mean of the two measurements. The dashed red line represents mean bias, the dashed black line represents the identity line, and the dashed green lines represent the 95% limit of agreement (LOA).

3.8. Comparison of HPLC and HPLC_BIS for measuring iohexol-derived GFR

When comparing the results produced by HPLC_BIS and HPLC in terms of iohexol-derived GFR of 56 patients (Table 11), the Passing-Bablok regression analysis showed an intercept value of 2.07 ml/min/1.73m^2 (Y-intercept 95% CI: -1.32, 6.20 ml/min/1.73m^2) and a slope value of 1.03 (slope 95% CI: 0.97, 1.09). It showed no significant deviation from linearity (Cusum test: $P > 0.05$), as shown in Figure 17. Lin's concordance correlation coefficient showed that the agreement was substantial ($\rho = 0.97$). The Bland-Altman analysis showed a mean bias of 5.62%, with the limits of agreement ranging from -9.91% to 21.15% between the HPLC_BIS and HPLC measurements. About 3.5% of the total points fell outside of the 95% limit of agreement, as shown in Figure 18.

Table 11. Comparison of HPLC and HPLC_BIS for measuring iohexol-derived GFR

Passing-Bablok Regression	**N**	**Intercept (95% CI)**	**Slope (95% CI)**	**Cusum linearity test**
HPLC vs HPLC_BIS	56	2.07(-1.32, 6.20)	1.03(0.97, 1.09)	P = 0.93(p > 0.05)
Lin's Concordance Correlation Coefficient [a]	**N**	**ρ**	**C_b**	
HPLC vs HPLC_BIS	56	0.97	0.99	
Bland-Altman Analyses [b]	**N**	**Bias (95% CI)**	**LLOA (95% CI)**	**ULOA (95% CI)**
HPLC vs HPLC_BIS	56	5.62%(3.50%,7.74%)	-9.91%(-13.55%,-6.26%)	21.15%(17.50%,24.80%)

[a] Lin's concordance correlation coefficient measures the deviations from identity between two methods, where ρ expresses precision and C_b expresses accuracy. Strength of agreement was described according to the values of the concordance correlation coefficient: ρ < 0.90 (poor); ρ = 0.90–0.95 (moderate); ρ = 0.95–0.99 (substantial); ρ > 0.99 (almost perfect).

[b] Bland-Altman Analyses: Bias is expressed as the percentage difference between two methods.

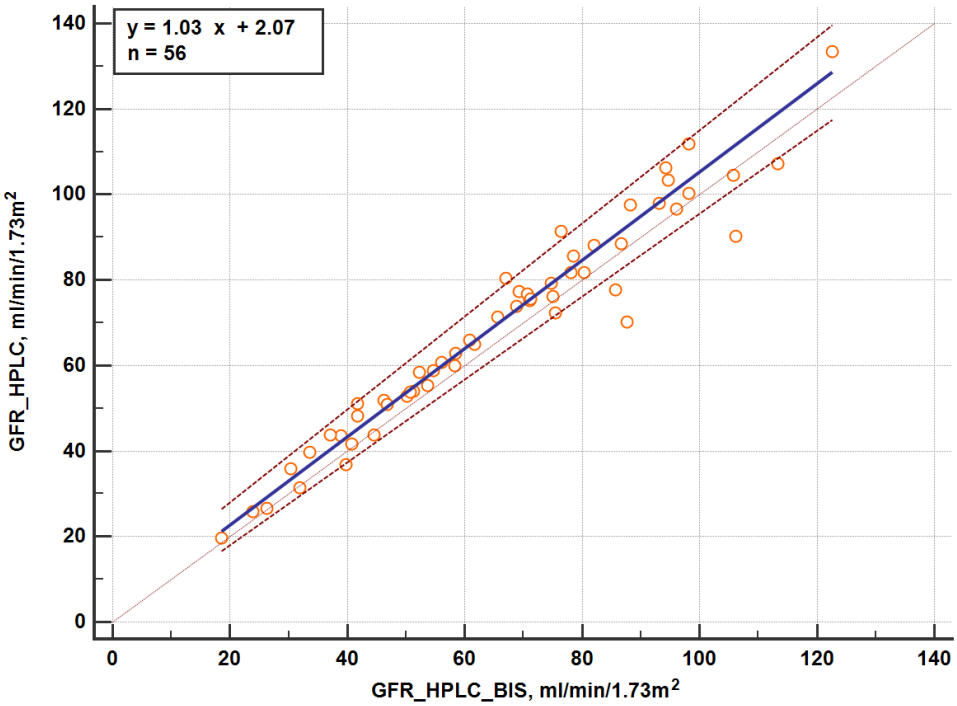

Figure 17. Passing-Bablok regression: Intercept: 2.07 ml/min/1.73m² (95% CI:-1.32, 6.20 ml/min/1.73m²), slope: 1.03 (95% CI: 0.97, 1.09). Yellow circles represent measured samples. The solid blue line represents the regression line, the dashed yellow line represents the line of identity, and the dashed red lines represent 95% confidence intervals. The Cusum test indicates no significant deviation from linearity (P > 0.05).

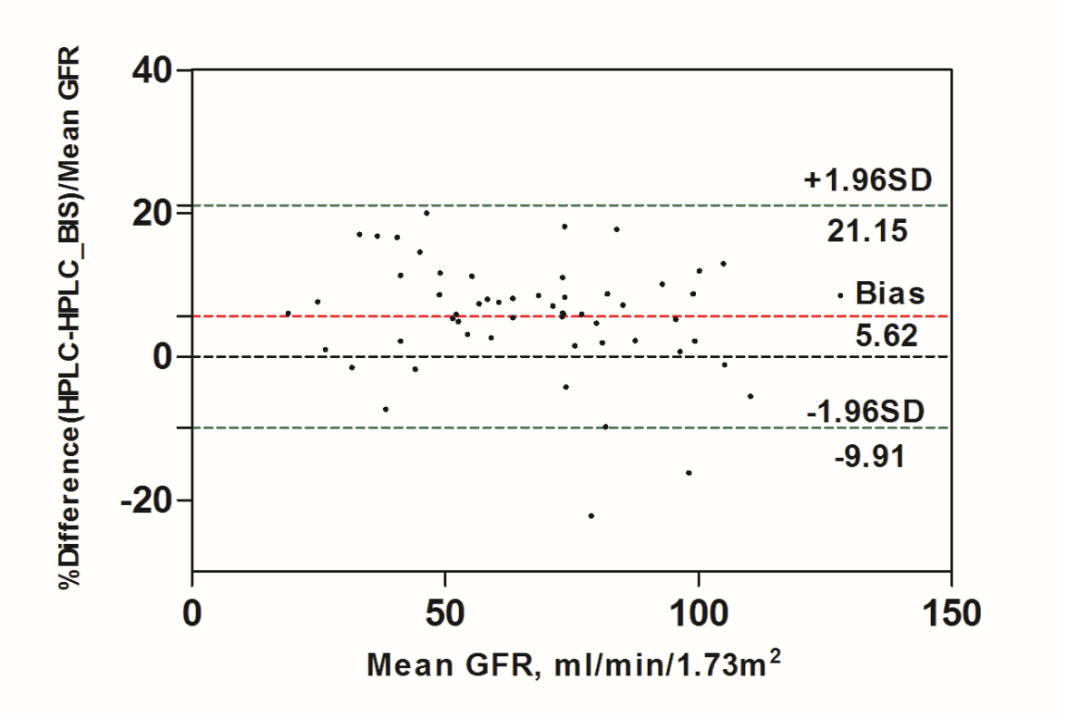

Figure 18. Bland-Altman analysis: Comparison of iohexol-derived GFR measured using the HPLC and HPLC_BIS methods. The dashed red line represents mean bias, the dashed black line represents the identity line, and the dashed green lines represent the 95% limit of agreement (LOA).

4. Discussion

GFR is regarded as an important sensitive indicator for the assessment of kidney function. mGFR values most closely represent true kidney function. Iohexol is widely used in Europe as an exogenous marker to measure GFR. HPLC and UPLC are commonly used methods for measuring iohexol concentrations. However, currently, there is limited comparative research on HPLC and UPLC for iohexol quantification. An important question thus arises as to whether different measurement methodologies affect iohexol results and then affect the iohexol-derived GFR results. My results suggest that no significant difference or bias exists between the two methods in terms of iohexol concentration and iohexol-derived GFR. In addition, I also compared the original iohexol concentrations and iohexol-derived GFR measured in the BIS study ten years ago with remeasured results obtained in my study, both using the HPLC method; no significant bias was observed between the long-term and short-term stored samples.

4.1. Comparison of iohexol concentrations and iohexol-derived GFR using HPLC and UPLC

The HPLC and UPLC are popular methods for quantifying serum iohexol concentration and can be used to determine the GFR. Our study results showed that both the HPLC and UPLC chromatographic methods provided good accuracy and precision, as shown in Table 5 and Table 6. When the results produced by the two methods for measuring the iohexol concentration and the iohexol-derived mGFR were compared, the Passing-Bablok showed no significant deviation from linearity. The Lin's concordance correlation showed almost a perfect level of agreement for iohexol concentration with the coefficient ρ (precision) >0.99 and C_b (accuracy) >0.99 using both methods (Table 8). The data derived from the UPLC and HPLC method had a minimal mean bias (< 2%), and more than 95% of iohexol concentration and the derived mGFR fell within the 95% LOA (Figure 12 and Figure 14). It suggests that the iohexol concentration and iohexol-derived GFR measurement capability of the two methods were comparable. This finding is in agreement with previous studies. Other researchers also posited that HPLC and UPLC can be used interchangeably when measuring different compounds [114-118]. However, UPLC has obvious advantages, the main advantage being a significant reduction in analysis time, which means a reduction in solvent consumption

[116-119]. Analysis duration, solvent consumption, and consequent analysis cost are important factors to be considered in analytical laboratories. UPLC is more eco-friendly than HPLC because a large number of analyses can be performed per unit time and consumption of eluent is much lower.

In addition, inter-laboratory exchange of blinded participant serum samples for method comparison with a well-established analytical method is a valuable tool for assessing the performance of the iohexol determination procedure. Iohexol was included in an external quality assurance program carried out by Equalis AB, Uppsala, Sweden. The program's goal was to assess the performance of the European laboratories that participated in this project. Each laboratory could compare its results with the other participants. The inter-laboratory CV has a quality goal of 8% [63, 111]. We compared the iohexol concentration measured in our lab with the overall mean of iohexol measured by other participants, using the UPLC method; the inter-laboratory CV was calculated to be below 5%. Using the HPLC method, the inter-laboratory CV was found to be 4.3–20.4%. It suggests that the established UPLC method in our lab provided probably more stable and reliable results than the HPLC method. Therefore, changing the measurement method from HPLC to UPLC might allow for more consistency among the laboratories.

Besides, for the UPLC method used in this study, the calibration curve of serum iohexol was linear over the entire concentration range of 8.67–500 µg/ml, with a high positive correlation ($R^2 \geqslant 0.999$). Using the HPLC method, the linear correlation of the iohexol amount and signal intensity was in the range of 23.29–291.15 µg/ml ($R^2 \geqslant 0.999$). The linear range of the UPLC standard curve was wider than HPLC. After injection of 5 ml iohexol, the serum concentration was between 11.42 and 325.14 µg/ml in our study. Some values were outside the range for the HPLC method. It might increase the analytical error when quantifying the lower or higher concentration of iohexol. This would be an aspect to focus on in order to improve the HPLC method in the future.

Finally, the retention time using the UPLC method was relatively stable during the whole experiment, while the retention time using the HPLC method varied with time. Several common reasons could contribute to HPLC retention time drift, including HPLC column temperature fluctuations, inadequate mobile phase mixing, inadequate mobile phase degassing, system leaks or flow rate instability, column fouling, sample

overloading, sample injection volume variation, and changes in the pH of the mobile phase [120]. In our experiment, the samples collected were from patients' serum which contains many compounds, and in the HPLC method, the sample preparation only included one-step perchloric acid removal of proteins; therefore, some complex compounds may have remained in the serum. These compounds can be retained on the column and might not get eluted during each run. They increase over time, thereby changing the retention time. Other studies have demonstrated that the acetonitrile–ethanol-water precipitating reagent could be more effective than perchloric acid in precipitating proteins [121-123]. This would be another aspect to focus on in order to improve the HPLC protocol in the future.

4.2. Comparison of iohexol concentrations and iohexol-derived GFR using HPLC and HPLC_BIS

During biochemical analysis, a major challenge is to minimize the potential introduction of bias during the pre-analytical, analytical, and post-analytical phases. As shown by Binita et al. and Szecsi et al., the frequency of pre-analytical errors can reach up to 80% [124, 125]. Ellervik and Vaught identified sample collection, processing, transport, and storage as the most important technical factors influencing analytical outcomes during the pre-analytical phase [126]. The difference in analytical results might be attributed to storage time. In biological samples, iohexol is a relatively stable chemical. It is stable at room temperature, -80°C, or -20°C [63, 127, 128]. Because of the high stability of iohexol, GFR measurements can be carried out in nearly all clinical settings; collected samples can be delivered to a central laboratory for analysis without any special precautions. Previous studies have shown that the iohexol concentration in freeze-thaw and short-term stability evaluation does not differ significantly from the fresh calibrators [74, 122, 129]. However, there is limited research on whether the iohexol concentration remains stable after long-term storage.

In this study, I compared the remeasured HPLC measurement with the original HPLC_BIS measurement that was performed ten years ago. The results showed substantial agreement and no significant bias in terms of measurement of iohexol concentration and iohexol-derived GFR between the two methods. This guarantees that samples can be re-analyzed safely after long-term storage, whenever needed, without any effect on iohexol concentrations and the derived mGFR.

4.3. Limitation of the present study

Despite the relevance of the study, it does have certain limitations. Firstly, in general, measuring instead of estimating GFR is invasive and time-consuming. Performing mGFR in patients with CKD is an even more complicated task, as it requires multiple-sample serums to assess the mGFR. The practical issues of multiple sampling become more challenging in older patients and outpatients. This study employed the multiple-sample serum clearance method to measure GFR. In the future, we will investigate whether the performance of the multiple-sample serum clearance method is similar to the simpler single-sample serum clearance method to simplify the measurement procedure and reduce the patients' pain. Another limitation of the present study is the lack of comparison with the independent gold-standard inulin clearance method. Although previous studies have shown iohexol clearance to have acceptable concordance with inulin clearance [67, 130-132], we cannot exclude the possibility that, compared with inulin clearance, the iohexol clearance measured using the HPLC and UPLC methods in this study is biased. Finally, iohexol exists as a mixture of conformational exo- and endo-isomers [133]. Most published HPLC measurement procedures separate these isomers and quantify iohexol using only the major (exo-iohexol) isomer [67, 111, 134]. However, for this study, for the HPLC method, iohexol eluted from the chromatographic column as two peaks, including one major peak and a small peak, and complete baseline separation could not be achieved. This shows that the system might have a problem identifying the peak of the major (exo-iohexol) isomer, especially when quantifying low concentrations of iohexol. This aspect needs further improvement in the future.

5. Conclusion

This study's findings suggest that no significant difference or bias exists in the quantification of iohexol concentration between the HPLC method and the UPLC method. Further, iohexol-derived GFR measurement by the two methods was also comparable.

In addition, there was substantial agreement and no significant bias observed in the measurement of iohexol concentration and iohexol-derived GFR between long-term and short-term stored samples. The iohexol serum samples can safely be re-analyzed after long-term storage in the refrigerator at -80°C.

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7. Statutory Declaration

“I, Nana Hu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic: “Comparison of separate measurement procedures for determination of GFR in chronic kidney disease: Iohexol quantification by HPLC and UPLC; Vergleich separater Methoden zur Bestimmung der GFR bei chronischer Nierenerkrankung: iohexol-Quantifizierung durch HPLC und UPLC ”, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

8. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

9. Acknowledgements

During my study in Germany, I received a lot of help from many people, and I would like to take this opportunity to express my gratitude to them. Everyone I've met here has helped me through such a wonderful and unforgettable time.

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10. Confirmation by a statistician

The statistical method was confirmed by a statistician from Institut für Biometrie und klinische Epidemiologie (iBikE) attached below.

The project had adopted the following essential advice regarding a meaningful evaluation and interpretation of the data were given during the consultation:

Bland–Altman plot

Passing-Bablok regression

Lin's Concordance Correlation Coefficient

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Bescheinigung

Hiermit bescheinige ich, dass Frau *Nana Hu* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *01.06.2022*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Bland–Altman plot
- Passing-Bablok regression
- Lin's Concordance Correlation Coefficient

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 02.06.2022

Name des Beraters/der Beraterin: Pimrapat Gebert

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