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Development of a simple HPLC-UV method for determination of GFR by serum iohexol clearance

Author:
Diederick J. van der Westhuizen

Student Number:
VWSDIE001

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University of Cape Town

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Declaration page

I hereby declare that this thesis represents my own work which has been done after registration for the degree of M.Med in Chemical Pathology at The University of Cape Town, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications. I have read the University's current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University's Human Research Ethics Committee. I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

Yours faithfully,

Diederick Johannes van der Westhuizen

August 19, 2022

Abstract

The glomerular filtration rate (GFR) is considered the best indicator of kidney function. Iohexol, a contrast agent, is currently considered to be a reference marker since it meets all the requirements of an ideal GFR marker.

The aim of this study was to develop and validate a method for iohexol measurement using high performance liquid chromatography with ultraviolet detection (HPLC-UV).

The method developed includes a mobile phase with water and acetonitrile with a gradient of 5%-30% organic, a C18 analytical column (100 × 4.6 mm, 2.7 μm particle size) at a temperature of 40 °C and a flow rate of 0.5 ml/min. Serum samples were deproteinized by addition of perchloric acid (5%).

The assay met the sensitivity cut-off with a mean signal-to-noise ratio of 17.2 at a level of 10 μg/mL iohexol. Mean recovery was 103.7% (CV=4.4%). The dilution experiment allowed for 5-times dilution up to iohexol levels of 500 μg/mL with an accuracy of 103.1% (CV=1.3%) For selectivity, no interfering endogenous compounds at the retention time of iohexol were observed. The matrix effect experiment showed a clinically acceptable variation at all concentration levels with a CV of the slopes of 2.7%. Stability of the stock solution was proven for at least 9 months at -80°C. Sample post-extraction stability was adequate at 84 hours. Interference testing yielded between -7% and -12% difference at 2% haemolysis and -9% to -13% difference at 10% lipaemia.

With regards to these findings, this method is simple, specific, linear, precise and robust, which allows its application for the measurement of GFR in serum.

Acknowledgements and contributions

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We sincerely thank Mignon McCulloch from Department Internal Medicine at Red Cross Hospital, Anita Brink and Jen Holness from the Nuclear Medicine Departments at Red Cross Children's Hospital and Groote Schuur Hospital for the donation of a vial of iohexol which was used for initial method development and for teaching us the slope-intercept method as well as putting us into contact with the team in the UK, Caroline Booth, Charles Turner and Neil Dalton whose initial advice put us on the right track. We also thank Peter Nourse, pediatric nephrologist at Red Cross Children's Hospital and an anonymous reviewer for the suggestions to the manuscript. This project would not be possible without the support from the staff at the Division of Chemical Pathology at the University of Cape Town. This work was funded by a Research and Development Grant from the University of Cape Town.

Contributions

Method development including standard and working solution preparation was primarily performed by Diederick J. van der Westhuizen (D.J.W.) and Joanne Pillay (J.P.). Sarah Lampert assisted with method optimization and some analytical batches in the method validation. George F. van der Watt (G.F.W.) provided clinical guidance on the protocol, ethics application and with general advice. Data analysis, coordination of the pilot study and draft writing was done by D.J.W.

List of Abbreviations

- $_{dd}H_2O$, double distilled water
- ACN, acetonitrile
- AUC, area under the plasma concentration curve
- BSA, body surface area
- CI, confidence interval
- CKD, chronic kidney disease
- CV, coefficient of variation
- DAD, diode array detection
- DTPA, diethylenetriaminepentaacetic acid
- EDTA, ethylenediaminetetraacetic acid
- ESRD, end-stage renal disease
- GFR, glomerular filtration rate
- HPLC, high performance liquid chromatography
- IS, internal standard
- KDIGO, Kidney Disease: Improving Global Outcomes
- LC-MS, liquid chromatography mass spectrometry
- LLOQ, lower limit of quantification
- MS-MS, tandem mass spectrometry
- MeOH, methanol
- PAR, peak area ratio
- PCA, perchloric acid
- Ref, reference sample
- SD, standard deviation
- UV, ultraviolet
- VAMS, volumetric absorptive micro-sampling
- eGFR, estimated GFR
- mGFR, GFR measurement

Chapter 1
GFR measurement using Iohexol - a literature review

Diederick J van der Westhuizen [a,b],[*], Sarah Lampert[a], George F van der Watt[a,b], Joanne Pillay[a],[*]

[a]Division Chemical Pathology, Department of Pathology, University of Cape Town, Cape Town, South Africa

[b]Groote Schuur Hospital and Red Cross Children's Hospital National Health Laboratory Service, Cape Town, South Africa

[*]Corresponding authors: dietervdwes@gmail.com; joanne.pillay@uct.ac.za

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List of abbreviations

AUC, area under the plasma concentration curve; CKD, chronic kidney disease; ESRD, end-stage renal disease; PCA, perchloric acid;ACN, acetonitrile; MeOH, methanol; KDIGO, Kidney Disease: Improving Global Outcomes; eGFR, estimated GFR; mGFR, measured GFR; HPLC-UV, high performance liquid chromatography with ultraviolet detection; LC-MS, liquid chromatography - mass spectrometry; BSA, body surface area; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid

1 Introduction to GFR

Glomerular Filtration Rate (GFR) is an indicator of renal function[1]. Precise GFR measurement (mGFR) is an invasive clinical procedure necessary in certain clinical scenarios as an alternative to relying on the endogenous markers, serum creatinine and/or cystatin C. The agreed-on most important factor in the staging of chronic kidney disease (CKD) is GFR and it is also the best overall index of kidney function in health and disease[2]. The definitions of all stages of CKD include GFR as parameter. A GFR measurement $\geq$ 90 mL/min/1.73m^2 with other evidence of kidney damage in the KDIGO guidelines indicates CKD category G1 (stage 1) and GFR measurement of $<$ 15 indicates CKD category G5 or end-stage renal disease (ESRD)[3]. Originally inulin clearance was considered the gold standard for GFR measurement. The first researchers employed measuring urine every few minutes together with a urinary catheter and a continuous infusion of inulin - a procedure which is impractical today, especially considering the risks associated with intravenous inulin administration[4]. Subsequently, other exogenous markers have been evaluated for the measurement of GFR. These include the radio-isotopic markers [^{125}I-iothalamate, ^{51}Cr-ethylenediaminetetraacetic acid (EDTA) or ^{99}Tc-diethylenetriaminepentaacetic acid (DTPA)] and 'cold' non-radioisotopic markers (iohexol or iothalamate)[5]. Today, accurate measurement of GFR is performed by measuring the concentration of these markers over time in plasma.[6, 7, 8].

In clinical practice, however, GFR is either estimated, using a number of equations (that have been developed using either creatinine or cystatin C levels, together with biometric data), or it is measured through timed creatinine clearance. Creatinine clearance is usually calculated from a 24 hour urine collection, although in the acute setting a six hour urine collection was also described to outperform an estimated GFR (eGFR) equation[9] and a two hour urine creatinine clearance outperformed an eGFR equation in predicting cessation of continuous renal replacement therapy[10]. Another study in the ICU setting recommended replacing 24-hour urine sampling with 2-hour sampling since the 24 hour samples resulted in a large proportion of non-valid determinations, even under close monitoring[11]. For calculating creatinine clearance, the standard U x V/P clearance equation is used, where U is the urine marker level, V is the urinary flow rate, and P is the plasma marker level[12]. At lower GFR levels, because of tubular creatinine secretion, creatinine clearance systematically overestimates measured GFR[13, 14]. Tubular secretion of creatinine is variable between patients and cannot be directly measured[13, 14]. Creatinine clearance also has high intra-individual variation and is prone to inaccurate urine collection by patients[13, 15, 16, 17].

Estimated GFR only requires measurement of the serum creatinine, which is widely available, convenient and cost-effective. An important caveat of eGFR is its inherent imprecision. Ideally, at least 90% of eGFR's should fall within 30% of mGFR (P30 value)[18, 19]. For a measured GFR of 60 ml/min/1.73m^2, it would require an estimate

that lies between 42 and 78 ml/min/1.73 m^2. A P30 value of $> 90\%$ is in practice only attainable when an eGFR equation based on both creatinine and cystatin C, another endogenous GFR marker, is used[18], but serum creatinine alone is the most widely used marker for estimating GFR. The P30 target is also seldom attainable in population groups other than the population in which the estimating equation was developed, especially outside Europe, North America and Australia[20]. For creatinine-based equations in the African setting, P30 values between 72% and 82% have been found [21, 22, 23, 24]. Considering these limitations, up to 25% of patients have mGFR's that differ more than 30% from the eGFR. Even lower P30 values have been described in a number of African studies[25, 26, 27]. Other co-morbid clinical conditions where eGFR performs poorly are heart failure, liver disease, cancer, morbid obesity and malnutrition[18]. A specific eGFR equation, namely the CamGFR equation has been developed for cancer patients, but this equation also performs poorly. For example, in a 50-year-old man (height 1.7 m, weight 70 kg) with a serum creatinine of 100 µmol/L, the 95% confidence interval on the GFR estimate ranges from 45-100 ml/min/1.73 m^2[28]. This is not of much value to an oncologist making patient management decisions. Despite these limitations, the Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines recommend serial estimations of GFR in individual patients using creatinine-based equations, since they still have value for monitoring individual patients over time[3]. In certain settings mGFR is essential because of the limitations and inaccuracy of eGFR equations. These include: patients receiving nephrotoxic drugs, calculating chemotherapeutic drug dosages, living kidney donors, patients with complex urological disease such as bilateral hydronephrosis and patients with solitary kidneys.

2 Iohexol's application for measuring GFR

2.1 General properties

First used in 1980, iohexol is classified as a non-ionic contrast medium mainly used for computed tomography (CT) and radiographic interventions such as catheter-based angiography[29]. In addition to low toxicity, iohexol is completely cleared by renal excretion at doses up to 500mg iodine (I) per kg. Furthermore, being non-radioactive, iohexol is not subject to the same extensive regulatory requirements of radioactive tracers such as ^{51}Cr-EDTA. Iohexol also meets the characteristics of an ideal GFR marker (low extra-renal clearance, absent tubular secretion and reabsorbtion, simplicity of use, low cost, stability, low protein binding, and a low incidence of allergic reactions or nephrotoxicity) and as a result is increasingly being considered as the gold standard for mGFR[30]. The extra-renal clearance of iohexol appears to be around 0-6 ml/min/1.73m^2. This was determined in anephric patients (2-3 ml/min/1.73m^2) [31, 32] and by studying the difference between plasma and urinary clearances (0-6 ml/min/1.73m^2)[33, 34, 35]. Iothalamate, another non-radioactive contrast medium, differs from iohexol since it is an ionic molecule and has been shown to have higher tubular excretion in a concentration dependent manner[36, 37]. Iothalamate also has higher extra-renal clearance than iohexol (4-10 ml/min/1.73m^2[35] vs. 0-6 ml/min/1.73m^2 for iohexol).

2.2 Safety

The safety of iohexol is well proven[38, 39]. This is supported by the large number of iohexol mGFR's used in Europe, e.g. the roughly 1500 GFR measurements currently performed per year in Sweden and >15 000 iohexol mGFR's performed at the Mario Negri Institute in Italy over the past 25 years with only one adverse event[40]. The reason for the low rate of adverse events, is that very low doses are used for mGFR measurement when compared to radio-contrast investigations. During a typical mGFR, 5 ml of iohexol (300mg I/ml) is administered intravenously vs volumes of up to 300 ml used for coronary angiography. The safety of iohexol has also been verified by measuring urinary N-acetyl-β-glucosaminidase and α1-microglobulin which are sensitive markers of tubular damage[41].

2.3 Measurement method

2.3.1 Method selection

There is less agreement on the best method for measuring iohexol. The best described and validated laboratory methods for measurement of iohexol are high performance liquid chromatography (HPLC) with ultraviolet (UV) detection, HPLC with tandem mass spectrometry (MS-MS) and X-ray fluorescence[30]. Other methods have been described, such as capillary electrophoresis, but these are not well validated and not routinely used[42][43]. In Europe HPLC-UV methods are the most widely used and offer the best compromise between accuracy, cost and sophistication[30]. The aim of the present study was to develop and validate a simple and robust measurement method for serum iohexol utilizing an HPLC-UV system.

2.3.2 Sample preparation

All HPLC-based methods require a degree of sample preparation before analysis of biological samples[44]. To measure iohexol in serum, similar to the majority of analytes on HPLC, a chosen internal standard (IS) is first added to the sample, then proteins are precipitated, followed by chromatographic separation, detection and quantification of iohexol relative to the internal standard (IS) peak area. With urine, due to the relatively low protein concentration, it may be diluted and directly injected onto the HPLC column without protein precipitation, commonly referred to as "dilute-and-shoot"[45]. The internal standard is a substance which has similar properties to the analyte of interest, does not occur endogenously and corrects for extraction or sample preparation inefficiency[46]. Perchloric acid (PCA) protein precipitation appears to be the best described and most successful protein precipitation reagent used by most HPLC-UV based methods[47, 48, 49, 50, 44, 51]. Generally 5-6% PCA in a 1-4:1 ratio with serum/plasma is used. El Assri *et al.* found similar recovery of iohexol with 2, 3 and 4 volumes of 6% PCA to serum[49]. Since PCA is a non-volatile acid, it is however not used in mass spectrometer (MS)-based detection systems, as it may contaminate the detector. For this reason acetonitrile or methanol with volatile acids are usually used for sample preparation with MS detection methods.

2.3.3 Chromatographic conditions

A reverse phase column (C18) is generally employed by HPLC-UV systems and column lengths range between 7.5-25 cm. Both isocratic and gradient elution techniques are described. The mobile phases generally have a low percentage of organic solvent (acetonitrile / methanol). Iohexol is detected photometrically as two isomers, reflecting the endo- and exo-isoforms, both of which (including the total area) can be used for quantification. This is possible since the ratio between the two peaks is constant, even with variations in mobile phase pH[52]. To our knowledge, the best investigation of mobile phase constitution has recently been described by El Assri *et al.* (2020) where various concentrations of the organic components of the mobile phase (acetonitrile and methanol) with water were investigated.

2.4 Stability

Seegmiller *et al.* confirmed that iohexol in whole blood and urine is stable for seven days when stored at ambient temperature (20°C), refrigerated (4°C), or frozen (-20°C) prior to measurement[53]. Another study found iohexol in serum samples to be stable at -17°C for at least the six week testing period[54]. Holleran *et al.* found iohexol to be stable for at least five months at -80°C and autosampler stability for 72 hours was adequate, although sample stability was only tested for four hours[55]. El Assri *et al.* have confirmed similar stability values, however, iohexol recovery was significantly lower in urine after three freeze-thaw cycles (recovery = 92%) and freezing at -80°C for two months (recovery = 90%)[49].

3 Calculations and derivations when measuring GFR

3.1 Correction for the "fast" elimination curve

The clearance of a substance from plasma can be quantified as the volume of plasma from which the substance is entirely removed per unit time. Due to the difficulty of accurately timed urine samples and the high number of urine samples necessary for urine-derived tracer clearance studies, plasma clearance is currently the preferred method for GFR measurement[30]. The most established approach to measuring GFR from plasma sampling is based on the ratio of the tracer administered to the area under the plasma concentration curve[56]. When iohexol is injected into the intravascular space, it distributes throughout the extracellular fluid compartment[30]. This gives rise to the so-called initial "fast" elimination phase. Following redistribution, the second "slow" elimination curve corresponds to renal clearance from plasma, entirely by glomerular filtration. Ideally, both of these curves should be determined and be regressed on a multi-exponential elimination curve to measure GFR most precisely. For the purpose of this discussion, a bi-exponential and mono-exponential elimination model will be discussed.

As illustrated in figure 1, after the administration of iohexol as a single intravenous bolus, GFR is obtained by dividing the injected total quantity of iohexol by the area under the plasma concentration curve (AUC) from injection time to infinity[57]. In patients with good renal function the plasma concentration decreases rapidly, the AUC is low, and the ratio, and thus GFR is high. Determining the initial quantity of tracer administered is easy, but determining the AUC can be achieved using methods of varying practicality and

accuracy.

The AUC is most accurately calculated from multiple plasma sample measurements taken shortly after injection and repeated over several hours. To obtain an accurate AUC representing renal clearance, sampling at 15 minute intervals is required for the first hour followed by half-hourly sampling for up to five hours or more. Due to the impracticality of this approach, this method is limited mainly to research settings. The slope intercept or one-pool method is a more practical approach in clinical practice. This method is based on the assumption that two hours post-injection the plasma clearance curve is nearly mono-exponential. This allows an approximate AUC (AUC_{slow}) to be calculated from as few as two plasma/serum samples, usually taken two and four hours post-injection. The AUC_{slow} is however an underestimate of the true AUC (Figure 2), giving an overestimate of the true GFR.

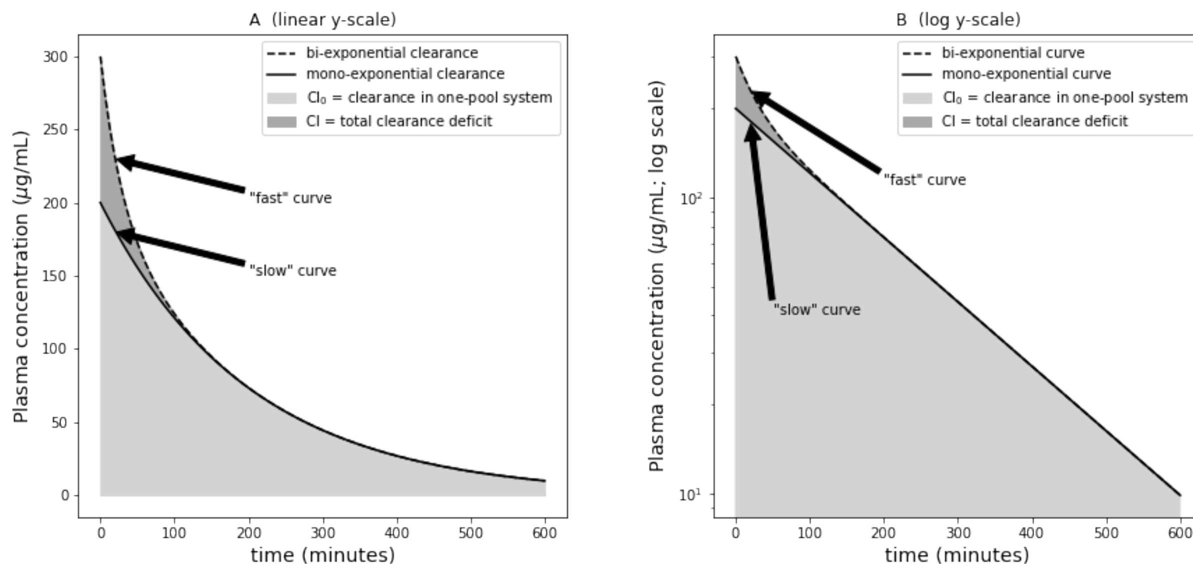

Figure 1: Elimination curves typically seen with an iohexol clearance when 5 mL iohexol is injected intravenously as a bolus in healthy individuals. The same eliminations curves are illustrated in figure A and B, but figure A was plotted on a linear scale y-axis whereas B was plotted on a logarithmic scale y-axis

Fig 1 relates to the work originally described in a well recognised study by Bröchner-Mortensen from Denmark in 1972[56]. Accurate total-AUC based clearance was determined by injecting 74 adults with ^{51}Cr-EDTA. The ^{51}Cr-EDTA was also used to determine clearance from an assumed "one-pool system". Although clearance was originally done with ^{51}Cr-EDTA, iohexol or any other suitable GFR marker follows the same principle. In this study it was shown that clearance from a one-pool system (ml/min) was always higher than the total plasma clearance, according to the formula below, where Cl = total plasma clearance and Cl_1 = plasma clearance with an assumed one-pool system.

$$x = 0.990778y - 0.001218y^2 \tag{1}$$

$$Cl = 0.990778 \times Cl_1 - 0.001218 \times Cl_1^2 \tag{2}$$

Fig 2 illustrates the main findings of Bröchner-Mortensen's original experiment[56]. Although originally performed with ^{51}Cr-EDTA, the relationship between these two variables (total plasma clearance and plasma clearance with an assumed one-pool system)

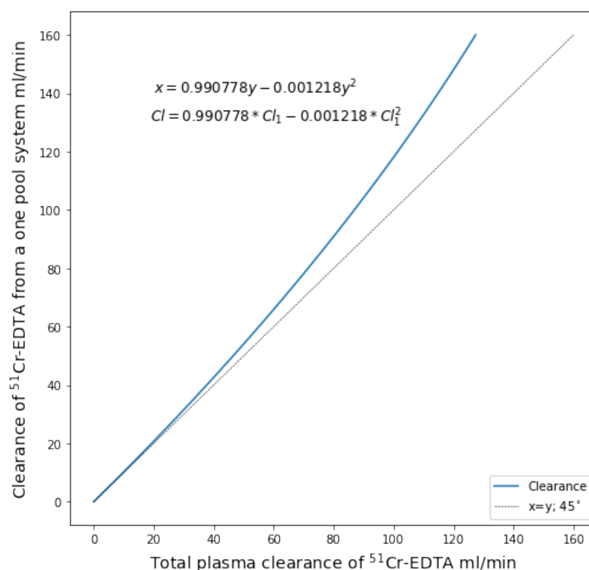

Figure 2: The derivation of the Bröchner-Mörtensen equation illustrated. Comparison of total plasma clearance to when a one-pool system is assumed.

has become accepted practice in determining GFR from two or more plasma sample measurements with tracers, including iohexol[30].

3.2 Timing and number of samples

The timing of the plasma samples is also a subject for discussion[58, 59, 60]. Briefly, in adults and children the recommendation is that serum samples be taken at two and four hours after 5 ml intravenous injection of iohexol (300 mg I/ml)[30, 61]. In suspected or confirmed cases of severe CKD, due to low plasma clearance, at least one of the samples should be taken at 24 hours post-injection to allow a more accurate quantification of clearance[58, 61]. Depending on the degree of CKD, five, six or eight hours post-administration samples have also been recommended[30, 61]. It is recommended that the first sample is not taken before two hours to allow for the distribution ("fast") elimination phase to complete, but there is no consensus on the exact timing, nor on the number of samples used. Multiple sample measurements could still be advantageous in those with ESRD[44].
A single sample method has been described, using the Jacobsson formula[62]. Single plasma time points may produce inaccurate GFR results if there were errors in blood drawing, dilution, contamination of the sample or an analytical error of some kind. The single sample method is however practically advantageous and becoming more commonly used, especially in large epidemiological studies due to lower cost of analysis[63].

3.3 Correction for body surface area

The most accurate method for body surface area (BSA) determination is by coating, surface integration and triangulation methods, described by Boyd[64], but it is impractical and difficult in clinical settings. In stead, formulae that utilize weight and height have been proposed for estimation of BSA. The Haycock formula for calculating BSA is the most accurate over a wide range of ages and populations[65] and is used most often to

estimate BSA in GFR studies.

$$\text{Body surface area}(m^2) = weight(kg)^{0.5378} \times height(cm)^{0.3964} \times 0.024265 \quad (3)$$

Haycock et al. derived this formula from the measured data by multiple regression analysis on 81 subjects, ranging from premature infants to adults. It gave a good fit for all values of BSA from less than 0.2 m^2 to greater than 2.0 m^2 (r = 0.998)[66]. Correcting clearance for BSA is done universally, but also has limitations as it may affect results at the extremes of body sizes[67, 68, 69].

4 Method development and validation

The U.S. Food and Drug Administration (FDA) has developed a set of guidelines called Bioanalytical Method Validation - Guidance for Industry[70]. These guidelines set out the guiding principles for method development, which involve optimizing the procedures and conditions of extraction and detection of the analyte. Method development includes optimizing the following bioanalytical parameters to ensure that the method is suitable for validation: reference standards, critical reagents, the calibration curve, quality control (QC) samples, selectivity and specificity, sensitivity, accuracy, precision, recovery and stability of the analyte in the matrix. In addition, interference testing should be performed for commonly encountered interferents including haemolysis and lipaemia. For clinical application, method development is followed by subsequent method validation, which validates or assesses the above parameters in the FDA guidelines. The European Union's Medicine Agency (EMA) has published similar guidelines for validation of bioanalytical methods[71].

5 Discussion and conclusion

Accurate determination of GFR is essential in several clinical scenarios where eGFR equations are unable to accurately quantify the GFR. Iohexol has been extensively studied and is increasingly considered to be an ideal tracer molecule for mGFR[72]. The most common method used for iohexol determination is HPLC-UV[30]. It does however not have the sensitivity offered by LC-MS, a more complex and costly analyser to run and maintain. The most common sample preparation on HPLC-UV involves PCA as protein precipitant and the chromatography method involves a C18 column as stationary phase with water and acetonitrile or methanol as the respective mobile phases. It appears that iohexol is stable in serum and urine for at least the proposed study duration of roughly six months when frozen at -80°C. Most centers use sampling times of two and four hours post-injection for GFR measurement when the expected value will be in the normal range, and a 24 hour sample when ESRD is suspected. Most centers employ the Bröchner-Mortensen correction factor after correcting for body surface area (BSA) using the Haycock formula[66]. Iohexol is safe[40], stable, and accurate mGFR's can be performed outside of specialized centers when clinicians follow a routine protocol. It can therefore be widely implemented in the South African context, since samples may be collected off-site and sent to a central laboratory for analysis.

6 Aims of the study

The proposed study was initiated to address the need for a reliable and widely available mGFR measurement method in the Southern African context. The objectives are to firstly develop and validate a quantitative method for serum iohexol on HPLC-UV and secondly, to provide proof of principle that the method can be successfully implemented in clinical practice by comparing this iohexol mGFR with creatinine clearance and eGFR equations in a cohort of healthy volunteers. Once successfully implemented, comparison with another available tracer, ^{99}Tc-DTPA will be done in a large cohort of patients in collaboration with the Department of Nuclear Medicine, Groote Schuur Hospital, Cape Town, in a follow-up study.

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Chapter 2
Development of a simple HPLC-UV method for determination of GFR by serum iohexol clearance

Journal of Chromatography B[1]

Diederick J van der Westhuizen[a,b,*], *Sarah Lampert*[a], *George F van der Watt*[a,b], *Joanne Pillay*[a,*]

[a]*Division Chemical Pathology, Department of Pathology, University of Cape Town, Cape Town, South Africa*
[b]*Groote Schuur Hospital and Red Cross Children's Hospital National Health Laboratory Service, Cape Town, South Africa*

Abstract

The glomerular filtration rate (GFR) is considered the best indicator of kidney function. Iohexol, a contrast agent, is currently considered to be a reference marker since it meets all the requirements of an ideal GFR marker. The aim of this study was to develop and validate a method for iohexol measurement using high performance liquid chromatography with ultraviolet detection (HPLC-UV). The method developed includes a mobile phase with water and acetonitrile with a gradient of 5%-30% organic, a C18 analytical column (100 × 4.6 mm, 2.7 μm particle size) at a temperature of 40 °C and a flow rate of 0.5 ml/min. Serum samples were deproteinized by addition of perchloric acid (5%). The assay met the sensitivity cut-off with a mean signal-to-noise ratio of 17.2 at a level of 10 μg/mL iohexol. Mean recovery was 103.7% (CV=4.4%). The dilution experiment allowed for 5-times dilution up to iohexol levels of 500 μg/mL with an accuracy of 103.1% (CV=1.3%) For selectivity, no interfering endogenous compounds at the retention time of iohexol were observed. The matrix effect experiment showed a clinically acceptable variation at all concentration levels with a CV of the slopes of 2.7%. Stability of the stock solution was proven for at least 9 months at -80°C. Sample post-extraction stability was adequate at 84 hours. Interference testing yielded between -7% and -12% difference at 2% haemolysis and -9% to -13% difference at 10% lipaemia. With regards to these findings, this method is simple, specific, linear, precise and robust, which allows its application for the measurement of GFR in serum.

Keywords: Iohexol, Glomerular Filtration Rate(GFR), Lipaemia, Haemolysis, HPLC-UV, Stability

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[*] *Abbreviations:* GFR, glomerular filtration rate; eGFR, estimated GFR; HPLC, high performance liquid chromatography; UV, ultraviolet; LC-MS, liquid chromatography mass spectrometry; CV, coefficient of variation; SD, standard deviation; CI, confidence interval; $_{dd}H_2O$, double distilled water; PCA, perchloric acid; MeOH, methanol; ACN, acetonitrile; mGFR, GFR measurement; MS-MS, tandem mass spectrometry; IS, internal standard; LLOQ, lower limit of quantification; Ref, reference sample; PAR, peak area ratio; VAMS, volumetric absorptive microsampling; HPLC-DAD, high performance liquid chromatography with diode array detection

[*] Corresponding authors
Email addresses: dietervdwes@gmail.com (Diederick J van der Westhuizen), joanne.pillay@uct.ac.za (Joanne Pillay)

[1] journal homepage: www.elsevier.com/locate/jchromb

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1. Introduction

The glomerular filtration rate (GFR) is an indicator of renal function. Precise GFR measurement (mGFR) is an invasive clinical procedure necessary in certain clinical scenarios as an accurate alternative to methods that rely on serum creatinine and/or cystatin C to estimate GFR (eGFR) or formal creatinine clearance based on timed urine collections. Serum iohexol is a good alternative to radioactive labelled tracers for determining mGFR because it is not subject to the extensive regulatory requirements for the measurement, handling and administration of radioactive substances. Iohexol also meets the requirements of an ideal GFR tracer, namely low extra-renal clearance, simplicity of use, low cost, stability, low protein binding, and the absence of allergic reactions or nephrotoxicity at the doses used[1]. There is less agreement regarding the measurement methodology of iohexol. Most validated methods for measurement of iohexol utilise high performance liquid chromatography (HPLC) with ultraviolet (UV) detection, HPLC with tandem mass spectrometry (LC-MS/MS) and X-ray fluorescence[2]. Other methods have also been described, such as capillary electrophoresis, but these are inadequately validated and not routinely available [3][4]. According to Delanaye et al[2], HPLC-UV systems are most commonly employed in Europe and are the best compromise between accuracy, cost and sophistication. The aim of this study was to develop and validate a simple and robust measurement method for serum iohexol measurement utilizing an HPLC system with diode array detection (HPLC-DAD). A DAD detects the absorption in UV to visual (VIS) wavelength range and has multiple (e.g. 1024) photodiode arrays to obtain information over a wide range of wavelengths consecutively[5]. The measurement technique involved precipitation of the serum proteins followed by separation and quantification of iohexol calculated from peak area ratios using an internal standard (IS). Accuracy, precision, reinjection stability, sensitivity, specificity, matrix effects, recovery, process efficiency, interference assessment and dilution verification were evaluated according to the recommendations and criteria published in the FDA and European Medicines Agency Bioanalytical Method Validation Guidelines[6, 7].

2. Materials and Method

2.1. Chemicals and reagents

A 50mL vial of iohexol [Omnipaque™ 300 mg iodine(I)/ml] was sponsored by the Department of Radiology and a vial of iomeprol (Imeron™ 400mg I/ml) by the Department of Cardiology, Groote Schuur Hospital. United States Pharmacopeia (USP) reference standards of iohexol, iothalamate and ioversol, acetic acid, perchloric acid, HPLC grade methanol and acetonitrile were purchased from Merck (St. Louis, USA). Double distilled water (ddH_2O) was produced in the laboratory using an AC-3C-MD Deomed reverse osmosis system (Konstancin-Jeziorna, Poland). Intralipid® was obtained from Groote Schuur Hospital Pharmacy.

2.2. *Equipment*

Chromatography was performed on an Agilent 1260 Infinity instrument (Agilent Technologies, Santa Clara, USA). For the method development we compared two analytical columns: Kinetex C18 column (100 × 3.0 mm; 2.6 μm particle size) from Phenomenex™ (Torrance, USA) and an InfinityLab Poroshell 120 C18 column (100 × 4.6 mm; 2.7 μm particle size) from Agilent Technologies Inc.

2.3. *Sample collections for the pilot study*

Ethical approval was obtained from the University of Cape Town Human Research Ethics Committee and 9 clinically healthy voluntary participants were enrolled for mGFR by iohexol clearance together with collection of timed urine and serum for calculation of creatinine clearance and estimation of GFR. For each participant, after voiding the bladder, a baseline serum sample was taken followed by 5mL Omnipaque™-300 intravenous injection and flushing with 10ml normal saline. The syringes were weighed before and after injection to ascertain and record the precise injected amount. This time point was noted as time T_0. Serum samples were taken at 2 hours (T_{2h}) and 4 hours (T_{4h}) after iohexol injection for measurement of iohexol. After 4 hours the bladder was voided for urine collection and the time noted to complete the urine collection. Urine volume was determined volumetrically. Urine and serum creatinine concentrations were determined on a Cobas 6000 c501 analyzer (Roche Diagnostic, Basel, Switzerland) with the *Creatinine plus ver.2*, an IDMS-traceable enzymatic creatinine assay. Estimated GFR and creatinine clearance for each participant was manually calculated on Microsoft Excel (Office 365). Both the MDRD and CKD-EPI study equations were used for eGFR calculation since these are the two most recent major eGFR equations developed. In current clinical practice, however, mostly the CKD-EPI study equation is used because of its inclusion of a larger cohort and subjects in the normal range.

2.4. *Chromatographic conditions*

The mobile phase consisted of water and acetonitrile (ACN), both adjusted with acetic acid to a final pH of 5.9. An injection volume of 10 μL was used. The gradient elution at 0.5 mL/min flow consisted of 5% ACN at 0 minutes and increased to 30% at 3.0 minutes. The ACN was increased rapidly to 98% at 3.5 minutes and held for one minute until 4.5 minutes. The ACN was then reduced to 5% at 5 minutes and held at 5% ACN until 8.5 minutes to equilibrate the column for the next injection. All exogenous administered contrast agents were detected at an absorbance wavelength of 245 nm. The Agilent MassHunter Workstation software was used for data acquisition (version B .09.00), and quantification (version B .10.1).

Between injections the needle was rinsed with a mixture of water, methanol, acetonitrile and isopropanol in a 1:1:1:1 volume ratio. Column rinsing was carried out after each analytical batch for 10 minutes, at 0.5 ml/min, with a mixture of aqueous: organic mobile phase (20:80, v/v) to eliminate possible non-polar

substances retained in the column. The column was then stored with a 50% methanol or acetonitrile mixture until the next analytical run.

2.5. Sample and standard preparation

Calibration standards and controls were prepared by spiking iohexol into pooled serum known to be devoid of contrast media. To 200 µL of serum samples, standards and controls, 20 µL ioversol internal standard working solution (825 µg/mL) was added in 1.5 ml micro-centrifuge tubes and vortex mixed for 1 minute. For deproteinization, 3.6 volumes (800 µL) of perchloric acid (PCA; 5% v/v) was added to 1 volume (220 µL) of serum, standard or control sample[8][9]. The tubes were then vortex mixed for 1 minute and subsequently centrifuged at 10000 RCF for 5 minutes. The supernatant was then injected onto the HPLC machine. A 5mg/ml stock solution was prepared gravimetrically by dissolving iohexol reference standard into water. Stock solution accuracy was confirmed by two analysts and concentration agreement verified spectrophotometrically. Several aliquots of the stock solution were frozen at -80° C and subsequently used, after thawing in a water bath at room temperature, to make up the working solutions.

2.6. Stability

2.6.1. Stock solution stability

From a USP iohexol analytical reference standard, two fresh stock solutions were prepared by two separate analysts and one standard of the same concentration made up 9 months prior to testing and frozen at -80°C, was thawed. Diluted stock solutions were each analysed 6 times spectrophotometrically at 245nm. The (%) difference was compared to the first analyst's sample preparation (reference sample).

2.6.2. 24- and 84-hour re-injection stability

The post-preparation stability of extracted iohexol samples was assessed at room temperature at 24 and 84 h respectively. Stability was defined by stability ratio according to the equation:

$$\text{Stability Ratio\%} = \frac{\text{Peak area of stored sample}}{\text{Peak area of fresh sample}} \times 100 \qquad (1)$$

2.7. Precision and accuracy

The precision and accuracy of the method was determined on three separate days by the replicate analyses (n = 6) of four quality control (QC) levels. The QC samples were prepared by spiking iohexol into blank human serum at concentrations within the calibration range. The four QC levels were at the lower level of quantification (LLOQ), low (QCL), medium (QCM) and high (QCH) concentrations and corresponded to values which would theoretically be obtained at various time periods after 5mL iohexol injection in vivo. The linearity of each standard curve was confirmed by plotting the peak area ratio of iohexol to ioversol (IS) using weighted least-squares regression. The standard curve samples were prepared on the first day, frozen

at -80° C and a freshly thawed set assayed with the batch. The precision of the method was expressed as the percentage CV and the accuracy was expressed as follows:

$$\%\text{Accuracy} = \frac{\text{mean observed concentration}}{\text{spiked concentration}} \times 100 \tag{2}$$

2.8. Sensitivity and Specificity

To assess sensitivity, six different blank serum samples were spiked at the LLOQ (10 μg/ml) and extracted with IS. The same six serum samples were extracted as blanks (with IS). The signal/noise ratio was observed for each of the six different LLOQ samples.

Specificity was assessed to ensure that the substance quantified was the intended analyte, at the LLOQ. To evaluate specificity, the six LLOQ samples were compared with each corresponding double blank sample for potential endogenous interferences at the retention time of the analyte.

2.9. Matrix effect, Recovery and Process efficiency

The method described by Matuszewski[10] to test for matrix effects, attempts to quantify the effects across the calibration range. For matrix effect, recovery and process efficiency experiments, three sets of samples were prepared, each at low (25 μg/ml), medium (50 μg/ml) and high (120 μg/ml) concentrations of iohexol. Set 1 was a set prepared in pure injection solvent without serum matrix (n=3 at each concentration). Set 2 was prepared by post-extraction spiking at each (n=6 from 6 different patient serums matrix samples) concentration. The different blank serum samples were extracted without IS in triplicate. Each sample was then spiked at low, medium and high concentrations of iohexol and the same concentration of IS after extraction. Set 3 comprised of pre-extraction spiked serum samples that were extracted as per the method described in section 2.5 above (n=6 at each concentration). The peak area ratios of the analyte/IS for each concentration in set 2 was used to generate linear regressions for each matrix lot (n=6).

Total iohexol recovery of the extraction method was assessed by comparing the peak area ratios between Sets 2 and 3. Process efficiency was evaluated by comparing peak area ratios between Set 1 and Set 3, to assess the overall contribution of both the extraction recovery and matrix effect on the analyte quantification.

2.10. Haemolysis and lipaemia

Interference from in vitro haemolysis was tested at 2% haemolysis. Haemolysate was prepared as described by Lovelock, by rapid freeze thawing isolated packed red cells 3 times.[11] To prepare the test samples, 2% haemolysate (by volume) was added to a set of 6 different sources of serum samples known to be devoid of contrast media and without visual evidence of haemolysis. With the use of working solutions of iohexol, to ensure a constant spiking volume of 4%, iohexol was spiked into these haemolysed samples to prepare high, low and LLOQ haemolysed samples. Reference samples were prepared at the same iohexol levels using blank serum from the same six serum sources with double distilled water (ddH_2O) added instead of haemolysate.

Six of each reference and test samples were extracted with IS as in section 2.5.
Interference from lipaemia was tested by adding 10% volume of Intralipid® to six blank serum samples to produce test samples mimicking lipaemia. For the reference samples, ddH_2O was added instead. Working solutions of a constant volume were added to spike samples at the different levels.
Selectivity in the presence of haemolysis and lipaemia was evaluated by extracting replicates of haemolysed and lipaemic serum as double blank samples. The ability of the IS to provide sufficient compensation for the analyte was assessed by comparing the reference and test serum sample response ratios. Mean peak area ratios observed in test samples were compared to those observed in the reference samples.

2.11. Dilution verification

To verify accuracy in diluted samples, a sample was prepared with an iohexol concentration of 500 μg/ml and then diluted 5-fold. The diluent used was an extract from a blank serum sample with internal standard added and prepared similar to the test sample, after the sample preparation as in section 2.5. The rationale for this dilution method, is that the original sample would not need re-extraction for dilution after obtaining a value which is greater than the measuring range of the assay. A dilution factor of 5 was then applied to obtain the final iohexol concentrations. The dilution was performed and analysed six times for a single spiked sample.

2.12. Statistical analysis

Data entry, calculations and figures were performed with Microsoft Excel (Office 365). Generation of graphs and power analysis were done with the Matplotlib (v.3.5.0, 2021) and Statsmodels (v.0.14.0) packages using Python (v.3.9) and regression analysis was performed with Stata SE (v.14.1, StataCorp, USA).

3. Results

3.1. Method optimization

Flow rates between 0.3-0.5 ml/min were evaluated during method optimization. Various isocratic elution techniques were attempted, but these led to increases in the pressure at a constant flow rate. This was ascribed to possible non-polar compounds being retained on the column. Pressure decreased when the column was flushed by retrograde flow of a high organic mobile phase. An injection volume of 10 µL was used in all described experiments as it was found in the initial development to provide the best compromise between peak sensitivity and shape without overloading the columns (results not shown). Iomeprol, iothalamate and ioversol were assessed for suitability as internal standards. These compounds are all iodinated contrast media. Ioversol, iomeprol and iohexol are however non-ionic, whereas iothalamate is ionic. According to

our literature search, we could not find a good reference for an IS in HPLC-UV, where separation of compounds is important as opposed to MS. The sources reviewed, either did not report which internal standard was used or stated iohexol-related compound B or iothalamate as suitable internal standards. Although LC-MS/MS methods discuss suitable internal standards, the chromatography used to achieve good baseline separation between the internal standard and iohexol peak for accurate quantification on a UV detector has not been described. Iohexol-related compound B, used in some HPLC-UV methods is not cost-effective and iothalamate was found to be too unstable during the PCA protein precipitation step (results not shown) in our method. When the pH in the sample is lowered to below 3, the iothalamate peak disappeared from the chromatogram. The reason for this is uncertain, but is likely due to the ionic nature of iothalamate. Iomeprol was tested as an alternative internal standard. Although theoretically an ideal internal standard due to a similar molecular weight and structure to iohexol, we found baseline separation from the iohexol isomer peaks problematic in precipitated serum samples at various mobile phase consistencies with both acetonitrile and methanol. Good baseline separation of iohexol was finally obtained when using ioversol as an internal standard.

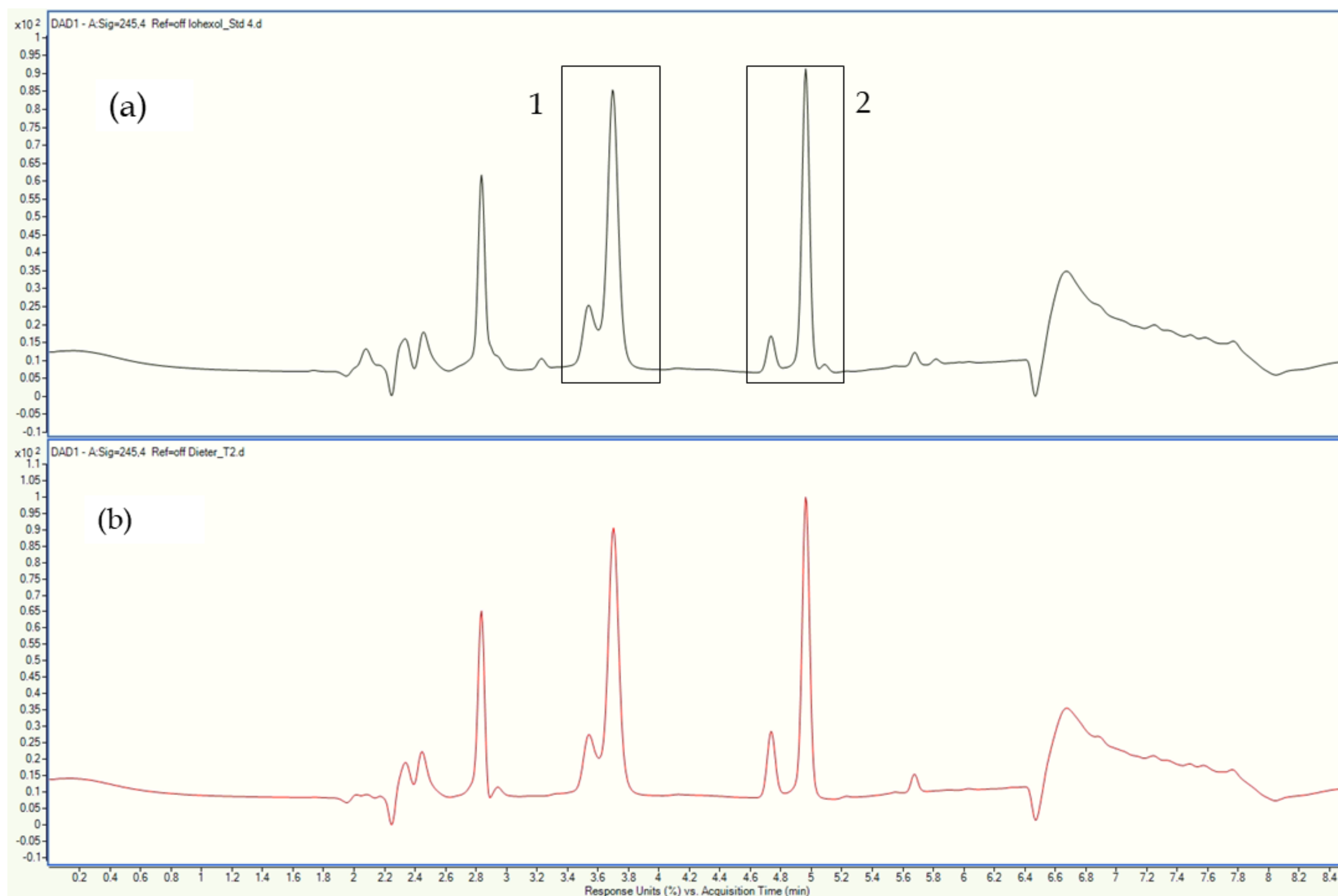

Figure 1: Representative chromatograms of (a) 75.0 μg/mL calibrator and (b) patient serum sample taken 2 hours after 5mL iohexol administration. Box 1, ioversol isomers; Box 2, iohexol isomers.

3.2. Stability

3.2.1. Stock solution stability

The (%) difference between the long term stored sample and the reference sample was 3.2%. The (%) difference between the second analyst's sample and the reference sample was 3.8%. The coefficient of variation of the spectrophotometric procedure on each of the samples measured were 0.2%, 1.9% and 0.9% for analyst 1, analyst 2 and the long term stored reference sample. These were all within acceptable limits[6].

3.2.2. Re-injection stability

After 24 hours, the (%) difference after the validation re-injection was -3.6% and -8.1% at 120 and 50 μg/ml respectively. After 84 hours, the (%) difference was -8.5% and -13.3% at 120 and 50 μg/ml respectively.

3.3. Precision and Accuracy

Table 1: Summary of accuracy and precision data obtained.

		Nominal iohexol concentration (μg/mL)				
		10	**50**	**60**	**120**	**n**
Day 1	Mean	10.6	50.9	61.7	122.2	6
	CV	2.3	4.6	2.8	2.3	
Day 2	Mean	10.4	49.5	58.0	118.5	6
	CV	1.3	2.6	4.1	0.9	
Day 3	Mean	11.0	52.1	57.7	120.2	6
	CV	3.3	3.9	2.2	5.3	
Total	Mean	10.7	50.9	59.1	120.3	18
	Acc	106.9	101.7	98.6	100.3	
	CV	3.3	4.2	4.4	3.4	

CV: % coefficient of variation; Acc: % Accuracy expressed as (obtained concentration)/(nominal concentration)*100

From table 1 it is evident that the inter-run CV of the method ranged from 3.3 - 4.4%. Also evident is the positive bias which is higher at the lower range. Note the %CV at the LLOQ (10 μg/ml) which is better than at all higher concentrations (see section 4 for elaboration on this). Figure 2 illustrates the linearity of a representative calibration curve ($r^2 = 0.9994$) from an accuracy and precision batch. The blue triangles indicate the four QC levels.

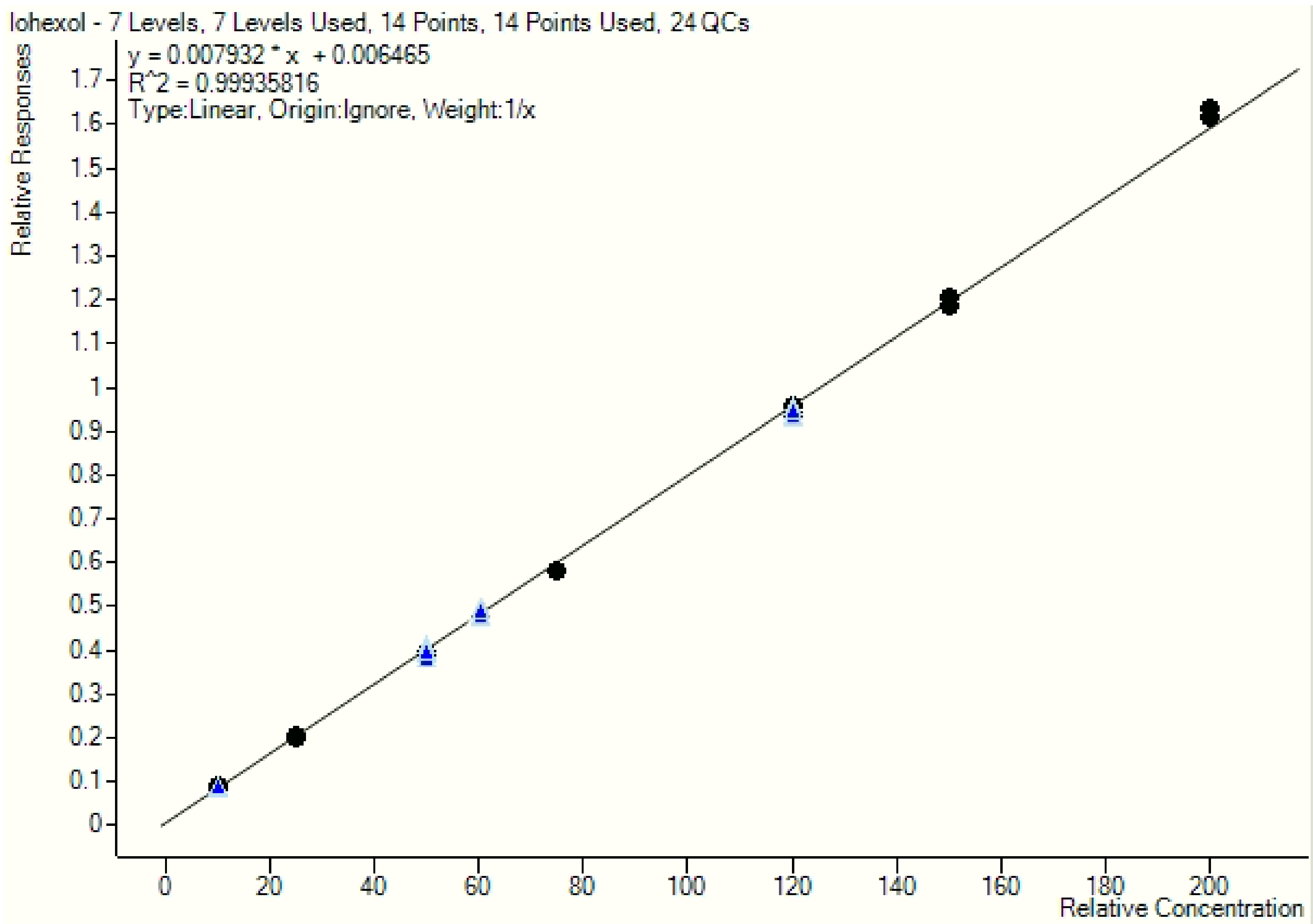

Figure 2: Representative calibration from an accuracy and precision batch. Legend: Black dots, calibrators; Blue triangles, test samples. Relative concentration in μg/ml.

3.4. Sensitivity and Specificity

As evident in table 2, mean signal-to-noise ratio at the tested LLOQ concentration of 10 μg/ml for iohexol was 17.2 (n=6). The average peak area in the blanks and double blanks as a percentage of the average LLOQ peak area were 7.7% and 0.96% respectively, showing that the described method demonstrated acceptable specificity and selectivity for iohexol in serum.

3.5. Matrix effect, Recovery and Process Efficiency

Fig 3 illustrates the findings of the matrix effects experiment. The concentrations that were assayed correspond to the values expected in clinical serum samples between 2-4 hours after IV injection of 5mL iohexol in adults. The individual concentration levels had a CV between 2.9% and 5.9%. The regression slopes for the 6 curves had a CV of 2.7%. Table 3 shows that the recovery was consistent across the iohexol levels. The CV was consistent between 1.7% - 3.4% across these levels.

Process efficiency was 107.9%, 106.2% and 101.0% with %CV's of 6.0, 5.0 and 2.8 at the low, medium and

Table 2: Values obtained during the sensitivity experiment.

Serum sample	Iohexol peak area	Blank peak area	S/N
A	42.7	13.1	3.2
B	46.3	7.8	5.9
C	44.0	1.0	42.9
D	46.6	1.4	33.7
E	47.1	6.1	7.7
F	45.7	4.8	9.5
Average	45.4	5.7	17.2

The different serum samples (n=6) were all tested at 10 μg/mL iohexol and at 0 μg/mL (blank). S/N : signal to noise ratio; Iohexol peak area : peak area of iohexol in the iohexol spiked serum samples; Blank peak area : peak area of iohexol in the blank serum samples

high levels respectively. This corresponded to an average process efficiency of 105.0% and an average CV of 3.4%.

Table 3: Summary of the recovery experiment.

		Set 3	Set 2	Recovery (%)
Low	Peak	0.1734	0.1738	99.7
25 μg/mL(n=6)	CV	6.0	4.5	3.4
Medium	Peak	0.3680	0.3385	108.7
50 μg/mL(n=6)	CV	5.0	5.9	2.4
High	Peak	0.8714	0.8480	102.8
120 μg/mL(n=6)	CV	2.8	2.9	1.7
Collective	Peak			103.7
mean (n=18)	CV			4.4

CV : % coefficient of variation; Peak : mean peak area of all samples for the specific analyte level.

3.6. Dilution

The results obtained from the 500 μg/ml iohexol sample after 5-fold dilution were 511.7, 525.8, 509.6, 520.2, 515.7, 509.3 μg/ml. This yielded a mean iohexol concentration of 515.4 μg/ml (CV: 1.3%) with an

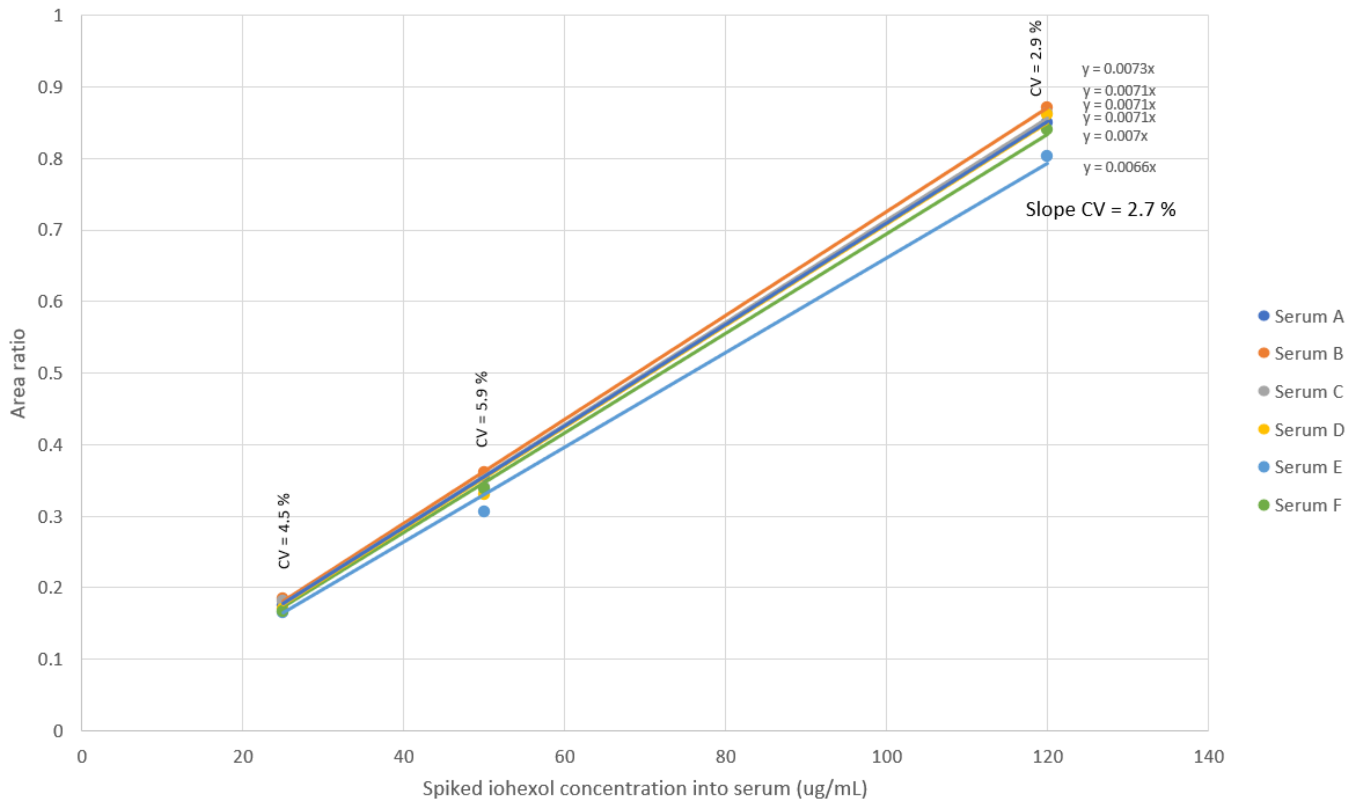

Figure 3: Matrix effects by the Matuszewski method illustrated. The CV of the slopes for the different sources of the matrix was 2.7% (should be <5%) and the CV of the obtained area ratio (precision) at each concentration is indicated as 4.5%, 5.9%, and 2.9% at the low, medium and high concentrations (each should be <15%). All regression lines were forced through (0;0) for slope illustration purposes.

accuracy of 103.1%.

3.7. Haemolysis and Lipaemia

Haemolysis of 2% had a -7.0% and -12.4% difference to the reference samples at the high and low concentrations respectively. Lipaemia of 10% yielded -8.9% and -12.8% difference at the same two concentration values (High: 120 μg/ml and Low: 50 μg/ml). The %CV's for the reference and the test samples are also shown in table 4.

3.8. Pilot study results

The results from the pilot study are shown in table 5. The mean age of the 9 participants was 37.9 years (SD = 11.5 years) with a range from 24 to 61 years. Females represented 44% of participants (not shown). The BMI ranged from 20 to 41 kg/m^2 (mean = 27; SD = 7 kg/m^2) and BSA ranged from 1.57 to 2.34 m^2 (mean = 1.89; SD = 0.23 m^2). The mean Bröchner-Mortensen corrected iohexol clearance in the 9 participants was 85.8 ml/min/1.73m^2 and ranged from 46 to 118 ml/min/1.73m^2. A multiple regression model was constructed to assess if sex, age or BMI was correlated with creatinine clearance. Only BMI

Table 4: Interference testing results.

		Peak area ratios			
		High conc.		Low conc.	
		Ref	Test	Ref	Test
2% Haemolysis	Mean	0.896	0.833	0.392	0.344
	CV	5.2	2.2	3.8	3.9
	%Dif.		-7.0		-12.4
10% Lipaemia	Mean	0.896	0.817	0.392	0.342
	CV	5.2	1.1	3.8	1.5
	%Dif.		-8.9		-12.8

High conc. : 120 μg/mL; Low conc. : 50 μg/mL. Ref : reference sample; Test : Test sample; Mean : mean value for (n = 6) samples tested; CV : % coefficient of variation; %Dif. : % difference from reference sample, where % difference = (ref. sample PAR - test sample PAR) / (ref. sample PAR) $\times 100$, and PAR = peak area ratio.

was found to be significantly correlated with creatinine clearance (p = 0.037), after adjusting for sex and age. This is further discussed in section 4. A power analysis for determining sample size was performed after the pilot study. Effect size was assumed as 0.3, because of the similarity to the P30 values commonly used in eGFR studies and because a difference of 10% between methods in a population with a variability of roughly 30% between patients would need to be detectable. The alpha and power values were assumed as the default 0.05 and 0.8 respectively and analysis was performed with the paired T-test's power module of the Statsmodels package[12]. This showed that the optimum number of participants to compare agreement in GFR methods is 89 patients.

4. Discussion

Iohexol is present in serum and urine as two isomers (endo and exo-iohexol), both of which can be used for quantification[13][14]. A comparative study of the isomers used for quantification showed no significant difference in precision and accuracy if either isomers or total amount was used[15]. Additionally, as explained in section 3.1 we found complete baseline separation of the isomers difficult with chromatography alone (Figure 1), hence decided to integrate both isomers for quantification. The stability results indicate that iohexol stock solution is stable for at least nine months at -80° C, and this will be re-assessed for longer time periods in future analyses. On the HPLC autosampler, it was shown that samples were stable for at least 84 hours after extraction, hence making analysis after three and a half days (a weekend) possible, should a failed

Table 5: Pilot study results.

	Biometric data			mGFR		Creatinine Clearance	eGFR	
ID	Age (y)	BMI	BSA (m^2)	Iohexol Clearance	Bröchner-M. corrected		MDRD	CKD-EPI
1	61	29	1.89	49	46	53	50	49
2	30	21	1.88	90	78	137	98	91
3	34	30	1.71	90	79	129	120	120
4	42	23	1.85	92	80	121	99	93
5	31	41	2.15	96	81	47	79	71
6	50	24	1.91	103	88	136	93	95
7	32	35	2.34	121	96	66	69	64
8	37	24	1.72	126	106	143	114	112
9	24	20	1.57	141	118	143	138	135

ID : participant identification number, BMI : body mass index, BSA : body surface area in m^2 (Haycock formula). Iohexol clearance, Bröchner-Mortensen clearance and creatinine clearance, MDRD eGFR and CKD-EPI eGFR were all corrected for body surface area (units: ml/min/1.73m^2). Ethnicity, weight and sex were omitted from this table to preserve anonymity.

run occur on a Friday afternoon. This is especially relevant in South Africa with frequent loadshedding of electricity[16].

For sensitivity, the assay met the FDA and EMA acceptance criteria which requires a mean analyte signal/noise ratio of >5 times the response of the blank. The signal/noise ratio at our LLOQ of 10 μg/ml iohexol was 17.2.

For specificity, there was no significant response from potential interferent peaks in the blank matrix samples tested at the same retention time as iohexol. Specificity was thus acceptable, since the observed response in the extracted blank matrix samples was less than 20% of the analyte response in the LLOQ sample and the observed response at the retention time of the internal standard was less than 5% of the response of the internal standard in all six serum matrices tested.

Accuracy and precision performance showed that the method performed similarly across the calibration range. A positive bias was however evident at the LLOQ level, but not at higher concentrations.

The matrix is defined as the components of the sample other than the analyte[17]. The matrix can have considerable effect on the way the analysis is conducted, and the quality of the results obtained. Hence, matrix effect is described as the combined effect of all components of the sample other than the analyte on the

measurement of the analyte concentration. If a specific component can be identified as causing an effect, then this is referred to as interference[17]. The matrix effect experiments obtained results which indicated a %CV well below the cut-offs of <15% for the individual levels and <5% for the collective slope variability. This indicated that there were no matrix components present that significantly influenced iohexol quantification.

The recovery of an analyte is the response obtained from an amount of analyte added to and extracted from the matrix, compared to the detector response for the true concentration of the pure standard. It can be understood as the percentage of iohexol originally in the specimen compared to the amount that reaches the end of the extraction procedure - i.e. detection. The extraction recovery refers to the extraction efficiency, which should also be within certain limits of variability. This was determined by comparing the response of six sets of blank serum spiked with iohexol and extracted, to blank serum that was first extracted and then spiked with iohexol, i.e. it assesses the effect of the extraction on the iohexol concentration. When peak area ratios of iohexol were the same for samples spiked before and after extraction, a quantitative recovery and process efficiency of ±100% was established. This confirms that iohexol has negligible protein binding[2] and that almost not iohexol was lost during the extraction process.

Interference testing was performed at 2% haemolysis as described by Lovelock.[11]. Assuming a mean corpuscular haemoglobin concentration of 34 g/dL, this would correspond to 680 mg/dL free haemoglobin in the serum, or roughly 2+ haemolysis index on visual inspection. The 7.0 to 12.4% negative difference across levels is acceptable as a 15% interference is considered significant[6]. Interference testing at 10% lipaemia was also performed using Intralipid®. Although this technique of lipaemia interference is debatable[18], it provides an easy and practical assessment of lipaemia when endogenous lipaemic samples are not available for interference testing. Bornhorst described that the addition of 10 μL of 200mL/L Intralipid® to a 1mL non-lipaemic serum sample yielded a L-index of 225[19]. The finding of 8.9 - 12.8% negative difference during lipaemia in our study with Intralipid®, represents an acceptable degree of interference in samples with lipaemia indices ± 10-fold higher than typical lipaemic samples. This is rarely encountered in the routine laboratory.

There are a few endeavours to which this method may lend itself to further improvement. Given the performance obtained, this method could be further improved for smaller sample volumes such as those obtained from capillary tubes or dried blood spots[20]. Although the specificity results showed no interfering peaks in the six serum samples tested, concomitant drug testing other than the basic interferents of lipaemia and haemolysis was not performed. This is mentioned as it was noted in a subsequent study in healthy participants that one participant's serum showed a peak which eluted near iohexol with a 0.5 min retention time difference. This could not be further explored due to ethical limitations. Chromatograms should thus always be inspected manually to confirm that there are no co-eluting peaks at the retention time of either the IS or the analyte of interest.

In the pilot study we illustrated the measurement of GFR by iohexol plasma clearance and identified one participant with subsequently confirmed stage 3a CKD. The possible reasons for the difference between the creatinine clearance, eGFR equation values and iohexol clearance results are many-fold and beyond the discussion of this article. It should be noted that our sample population was small (n=9), had wide variation in body mass indexes (BMI's), body surface areas (BSA's), ethnicities and sex. Even though sample size estimation should ideally not be performed after the study has been done, it is clear from the power analysis that our sample of nine participants is under-powered. Indexing GFR for BSA is considered by most clinicians including nephrologists as a standard but there are limitations to such an indexation, especially in subjects at the extremes of body sizes[21, 22, 23]. Urine was collected for eight of the nine participants over only four hours. Even though it was well timed and meticulously controlled by the study coordinator, this may not be reliable enough to account for possible residual bladder urine volumes in the participants. Creatinine clearance is usually calculated from a 24-h urine collection, but close monitoring of participants during a 24 hour period was not possible and we attempted to have a simultaneous iohexol and creatinine clearance. This may be better controlled in an ICU setting where patients are catheterized. The fact that the urine collections did not occur over the same daily time period may also be a confounder. Our sample population was all selected from within our Division of Chemical Pathology, and all presumed to have been healthy with no history of renal disease (which was an inclusion criteria). This will not be the case in a hospital or epidemiological setting. Concomitant substance use (caffeine and analgesics) were reported by some participants before urine collection, which are also possible confounders[24, 25]. Concomitant medication interference testing was not performed for the measurement method and will be done in a future study. After a limited regression analysis (n=9), amongst the recorded variables, the most significant variable explaining the discrepancy between the mGFR and creatinine clearance results appears to be the wide variation of BMI among participants, especially the two outlier patients (in table 5 see participant ID's 5 and 7 with low creatinine clearances and high BMI's). There is also considerable debate about whether to use ideal versus real body weight (as used in our study) for GFR estimation. In one study, ideal body weight was shown to provide less bias if used in the BSA calculation[26]. Despite the fact that creatinine clearance is used extensively in intensive and acute care settings as a proxy for GFR, it has previously been shown to perform poorly when compared to reference GFR methods[27, 28, 29], even when adding additional endogenous parameters like beta-2 microglobulin and urea clearance[30]. As a future endeavour, we would like to further validate our method in patients by comparing it with another available reference mGFR method such as ^{99}Tc-DTPA clearance which is available at our institution at the Department of Nuclear Medicine, Groote Schuur Hospital, Cape Town.

5. Conclusion

We described a simple method for the measurement of iohexol in serum which is also robust, precise and selective, ideal for the African context. This method, according to our knowledge, is the first HPLC-UV based method described for serum iohexol measurement in Africa. To address the limitations discussed above, we intend to expand on the work commenced in this study by conducting a direct comparison of the iohexol and ^{99}Tc-DTPA clearance will be simultaneously measured in a larger cohort of renal patients. Iohexol is safe[31], stable, and accurate mGFR's can be performed outside of specialized centers when clinicians follow a routine protocol. After thorough external quality assurance samples have been analyzed, it can be widely implemented in the African context, since samples may be collected at peripheral sites and sent to a central laboratory.

6. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. Acknowledgements

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8. Contributions - CRediT author statement

Diederick J. van der Westhuizen: Methodology, Investigation, Formal analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Software **Joanne Pillay:** Conceptualization, Methodology, Validation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Sarah Lampert:** Investigation, Resources. **George F. van der Watt:** Conceptualization, Methodology, Writing - Review & Editing. All authors have read and agreed to the submitted version of the manuscript.

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Stock Solution Accuracy and Stability (HPLC-UV)

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Preparation Date (SS1 Ref):	**18-Oct-21**
Preparation Date (SS2 Acc):	**18-Oct-21**
Preparation Date (SS3 LTS):	**05-Feb-21**
Stock Solution Concentration (mg/ml):	
Stock Solution Solvent:	
Test Solution Concentration (µg/ml):	
Test Solution Solvent:	
~ Storage Temp. (°C):	
LTS Storage period (days):	**255**

Assay date:		**18Oct2021**
Batch Run ID:		
	Preparation ID:	RS #:
SS 1 (Ref for LTS):	**JP_iohexol_18Oct2021**	
SS 2 (Test Accuracy):	**DJW_iohexol_18Oct2021**	
SS 3 (LTS):	**JP_iohexol_05Feb2021**	
	Accuracy:	LTS:
Reference material in- house Lot#:		

Wavelength λ (nm)	**245**		
Preparation ID:	JP_iohexol_18Oct2021	DJW_iohexol_18Oct2021	JP_iohexol_05Feb2021
Peak Height A	1.721	1.716	1.754
Peak Height B	1.728	1.771	1.778
Peak Height C	1.725	1.787	1.789
Peak Height D	1.725	1.735	1.780
Peak Height E	1.731	1.794	1.787
Peak Height F	1.728	1.794	1.799
Average	1.726	1.766	1.781
STDEV	0.003	0.033	0.015
CV(%)	0.2	1.9	0.9
Accuracy % Difference (vs ref)		2.3	
LTS % Difference (vs ref)			3.2
CV(%) pass/fail	**PASS**	**PASS**	**PASS**
Accuracy % Difference pass/fail		**PASS**	
LTS % Difference pass/fail			**PASS**
Date/Time	**Operator**		

Decision criteria

Max %CV	15.0	according to SOP HP2002/09 V.14
Accuracy Max %Difference	5.0	according to SOP HP2002/09 V.14
LTS Max %Difference	10.0	according to SOP HP2002/09 V.14

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

Stock solution stability

Dilution Validation

Validation ID and version:	IHX v1	Assay date:	2021-10-27
Analyte:	Iohexol	Batch Run ID:	27Oct_Val_day1
Dilution Specification:	Post-extraction (extracted blank matrix)		
Dilution Factor:	5		

	Nominal Conc.	**Observed Conc.**
	µg/ml	**µg/ml**
Sample 1	500.0	511.7
Sample 2		525.8
Sample 3		509.6
Sample 4		520.2
Sample 5		515.7
Sample 6		509.3
Average	500.0	515.4
STDEV		6.572
CV(%)		1.3
% Accuracy		103.1
CV(%) pass/fail	**PASS**	
% Accuracy pass/fail	**PASS**	

Decision criteria

Max CV(%)	15.0	according to SOP HP2002/09 V.14
Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Min %Accuracy	85.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

Sensitivity

Validation ID and version:	**IHX v1**
Assay Method ID and version:	**IHX 5-30 gradient v1**
Analyte:	**Iohexol**
Biological Matrix:	**Serum**
Anticoagulant:	**N/A**

Assay Date:		**2021-10-22**
Batch Run ID:		**22Oct2021_Sensitivity_Selectivity**
Matrix sources:	Lot A	**Patient 1**
	Lot B	**Patient 2**
	Lot C	**Patient 3**
	Lot D	**Patient 4**
	Lot E	**Patient 5**
	Lot F	**Patient 6**

	LLOQ in Matrix Peak Area (with ISTD)	**Blank Matrix Peak Area (with ISTD)**	**LLOQ in Matrix S/N**
Lot A	42.4	13.1	3.2
Lot B	46.3	7.8	5.9
Lot C	44.0	1.0	42.9
Lot D	46.6	1.4	33.7
Lot E	47.1	6.1	7.7
Lot F	45.7	4.8	9.5
Average	45.4	5.7	17.2
		Sensitivity pass/fail	PASS

Decision criteria

Mean analyte response at LLOQ greater than 5.0 **times the response observed in the blank sample.**

according to SOP HP2002/09 V.14

Selectivity and Specificity

Validation ID:	**IHX v1**
Method ID and Version:	**IHX 5-30 gradient v1**
Analyte:	**Iohexol**
Matrix:	**Serum**
Anticoagalant:	**N/A**

Assay Date:		**2021-10-22**
Batch Run ID:		**22Oct2021_Sensitivity_Selectivity**
Matrix sources:	Matrix lot A	**Patient 1**
	Matrix lot B	**Patient 2**
	Matrix lot C	**Patient 3**
	Matrix lot D	**Patient 4**
	Matrix lot E	**Patient 5**
	Matrix lot F	**Patient 6**

	LLOQ in Matrix Peak Area (without ISTD)	**Double Blank Matrix Peak Area (without ISTD)**	**Double Blank peak as % of average LLOQ peak area**	**Blank Matrix Peak Area (with ISTD)**	**Blank peak as % of average LLOQ peak area**
Lot A	47	2	5.8	4	9.3
Lot B	43	0	0.0	4	9.3
Lot C	42	0	0.0	3	7.0
Lot D	45	0	0.0	2	4.6
Lot E	43	0	0.0	4	9.3
Lot F	39	0	0.0	3	7.0
Average	43	0.42	0.96	3.33	7.74
				Selectivity and Specificity pass/fail	PASS

Decision criteria

Observed response from blank < than % of LLOQ 20.0 according to SOP HP2002/09 V.14

Observed response from ISTD < than % of LLOQ 20.0 according to SOP HP2002/09 V.14

These two criteria must be true for all six individual lots of blank matrix. according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

VALIDATION 1: Inter-batch calibration standards and quality control results

Results generated by Analyst®

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Analyst/Technician:	**Dieter**
CL number:	
Analyte peak area lowest STD:	38.3
Analyte peak area lowest STD duplicate:	40.8
Analyte peak area in Blank:	0

Assay Date:	**2021-10-27**
Batch Run ID:	**2021-10-27_AP_day1**

Summary of Calibration Standard Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	CV(%)	%Accuracy	n
S11						
S10						
S9						
S8						
S7	10.0	10.9	0.7	6.8	109.0	2
S6	25.0	26.1	0.0	0.1	104.3	2
S5	50.0	45.3	2.1	4.7	90.5	2
S4	75	70.8	0.0	0.0	94.4	2
S3	120	116.6	1.1	0.9	97.2	2
S2	150	146.8	2.8	1.9	97.9	2
S1	200	213.6	1.4	0.7	106.8	2

Summary of Quality Control Accuracy and Precision

Sample ID	Nominal Conc. µg/ml	Mean Observed Conc. µg/ml	Std Dev	CV(%)	%Accuracy	n
QC LLOQ 2						
QC LLOQ 1	10.00	10.59	0.24	2.3	105.9	6
QC L	50.00	50.92	2.36	4.6	101.8	6
QC M	60.00	61.74	1.73	2.8	102.9	6
QC H	120.00	122.22	2.80	2.3	101.8	6
QC XH						

Note: Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported. - FDA

Results interpretation

Response in Blank as % of LLOQ:	0.0
% of LLOQ STDs meeting criteria:	100.0
% of ULOQ STDs meeting criteria:	100.0
% of total STDs meeting criteria:	100.0
Validation run pass/fail:	PASS
% of total Performance QCs within criteria:	100.0
Do any performance QCs (at one level) have more than 50% of them failing to meet criteria?:	NO
Method development required?:	NO

Decision criteria (for batch pass/fail)

Max Response in Blank as % of LLOQ	20.0	according to SOP HP2002/09 V.14
Lowest STD Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest STD Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining STDs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining STDs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of LLOQ and ULOQ STDs to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total STDs to meet criteria	75.0	according to SOP HP2002/09 V.14

Decision criteria (for method development required)

Lowest Performance QC Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest Performance QC Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of Performance QCs at each level to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total Performance QCs to meet criteria	67.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ____________________ Date : ____________________

Verified by: ____________________ Date : ____________________

VALIDATION 2: Inter-batch calibration standards and quality control results

Results generated by Analyst®

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Analyst/Technician:	**Dieter**
CL number:	
Analyte peak area lowest STD:	43.2
Analyte peak area lowest STD duplicate:	44.7
Analyte peak area in Blank:	1.66

Assay Date:	**2021-11-01**
Batch Run ID:	**01Nov2021_Val_day_2_A&P**

Summary of Calibration Standard Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	CV(%)	%Accuracy	n
S11						
S10						
S9						
S8						
S7	10.0	10.4	0.24	2.3	104.3	2
S6	25.0	24.9	0.30	1.2	99.8	2
S5	50.0	48.9	0.08	0.2	97.8	2
S4	75	72.8	0.00	0.0	97.1	2
S3	120	118.7	1.66	1.4	98.9	2
S2	150	149.9	1.66	1.1	99.9	2
S1	200	204.3	1.58	0.8	102.1	2

Summary of Quality Control Accuracy and Precision

Sample ID	Nominal Conc. μg/ml	Mean Observed Conc. μg/ml	Std Dev	CV(%)	%Accuracy	n
QC LLOQ 2						
QC LLOQ 1	10.00	10.45	0.14	1.3	104.5	6
QC L	50.00	49.51	1.28	2.6	99.0	6
QC M	60.00	58.00	2.38	4.1	96.7	6
QC H	120.00	118.53	1.04	0.9	98.8	6
QC XH						

Note: Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported. - FDA

Results interpretation

Response in Blank as % of LLOQ:	3.8
% of LLOQ STDs meeting criteria:	100.0
% of ULOQ STDs meeting criteria:	
% of total STDs meeting criteria:	100.0
Validation run pass/fail:	PASS
% of total Performance QCs within criteria:	100.0
Do any performance QCs (at one level) have more than 50% of them failing to meet criteria?:	NO
Method development required?:	NO

Decision criteria (for batch pass/fail)

Max Response in Blank as % of LLOQ	20.0	according to SOP HP2002/09 V.14
Lowest STD Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest STD Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining STDs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining STDs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of LLOQ and ULOQ STDs to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total STDs to meet criteria	75.0	according to SOP HP2002/09 V.14

Decision criteria (for method development required)

Lowest Performance QC Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest Performance QC Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of Performance QCs at each level to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total Performance QCs to meet criteria	67.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

VALIDATION 3: Inter-batch calibration standards and quality control results

Results generated by Analyst®

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Analyst/Technician:	**Joanne**
CL number:	
Analyte peak area lowest STD:	42
Analyte peak area lowest STD duplicate:	43.2
Analyte peak area in Blank:	0

Assay Date: **2021-11-05**

Batch Run ID: **05Nov2021_Val_day_3_HM&LP**

Summary of Calibration Standard Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	CV(%)	%Accuracy	n
S11						
S10						
S9						
S8						
S7	10.0	9.8	0.62	6.3	98.2	2
S6	25.0	25.4	0.94	3.7	101.6	2
S5	50.0	50.4	2.03	4.0	100.8	2
S4	75	73.4	0.85	1.2	97.9	2
S3	120	117.3	3.75	3.2	97.7	2
S2	150	155.4	9.39	6.0	103.6	2
S1	200	197.6	7.82	4.0	98.8	2

Summary of Quality Control Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	CV(%)	%Accuracy	n
QC LLOQ 2						
QC LLOQ 1	10.00	11.02	0.36	3.3	110.2	6
QC L	50.00	52.12	2.02	3.9	104.2	6
QC M	60.00	57.68	1.29	2.2	96.1	6
QC H	120.00	120.17	6.31	5.3	100.1	6
QC XH						

Note: Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported. - FDA

Results interpretation

Response in Blank as % of LLOQ:	0.0
% of LLOQ STDs meeting criteria:	
% of ULOQ STDs meeting criteria:	
% of total STDs meeting criteria:	100.0
Validation run pass/fail:	PASS
% of total Performance QCs within criteria:	100.0
Do any performance QCs (at one level) have more than 50% of them failing to meet criteria?:	NO
Method development required?:	NO

Decision criteria (for batch pass/fail)

Max Response in Blank as % of LLOQ	20.0	according to SOP HP2002/09 V.14
Lowest STD Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest STD Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining STDs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining STDs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of LLOQ and ULOQ STDs to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total STDs to meet criteria	75.0	according to SOP HP2002/09 V.14

Decision criteria (for method development required)

Lowest Performance QC Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest Performance QC Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of Performance QCs at each level to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total Performance QCs to meet criteria	67.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

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Summary of calibration standards and quality control results (Validation 1+2+3)
Results generated by Analyst®

Validation ID and version:	IHX v1	
Analyte:	Iohexol	
Validation 1 Assay Date:	2021-10-27	Batch Run ID:
Validation 2 Assay Date:	2021-11-01	Batch Run ID:
Validation 3 Assay Date:	2021-11-05	Batch Run ID:

Summary of Calibration Standard Accuracy and Precision

Validation Batch	Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	Nominal Conc.	200.00	150.00	120.00	75.00	50.00	25.00	10.00				
	Replicates	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.
Validation 1	1	214.66	144.77	117.35	70.750	46.750	26.060	10.3777				
	2	212.62	148.79	115.82	70.780	43.761	26.099	11.421				
Validation 2	1	203.14	148.74	119.91	72.801	48.975	24.735	10.2587				
	2	205.37	151.09	117.56	72.808	48.858	25.158	10.5997				
Validation 3	1	192.08	162.02	114.61	74.001	51.841	26.069	9.3815				
	2											
	n	5	5	5	5	5	5	5				
	Mean	205.57	151.08	117.05	72.23	48.04	25.62	10.41				
	STDEV	8.9465	6.5241	1.9989	1.4221	2.9988	0.6365	0.7314				
	CV(%)	4.352	4.318	1.708	1.969	6.243	2.484	7.028				
	% Accuracy	102.8	100.7	97.5	96.3	96.1	102.5	104.1				

N/A

Summary of Performancy Quality Control Accuracy and Precision

Validation Batch	Sample ID	QC XH	QC H	QC M	QC L	LLOQ 1	QC LLOQ 2
	Nominal Conc.		120.00	60.00	50.00	10.00	
	Replicates	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.
Validation 1	1		127.62	63.14	54.078	10.209	
	2		119.73	58.62	50.618	10.626	
	3		122.39	63.12	49.315	10.560	
	4		121.55	61.06	52.553	10.973	
	5		120.58	62.66	47.434	10.545	
	6		121.43	61.87	51.517	10.618	
Validation 2	1		118.49	54.22	47.522	10.526	
	2		117.77	58.64	49.206	10.346	
	3		119.93	57.66	49.002	10.503	
	4		116.96	56.64	50.190	10.591	
	5		119.02	60.56	49.808	10.500	
	6		119.00	60.28	51.336	10.218	
Validation 3	1		111.23	60.22	51.270	11.700	
	2		128.24	57.64	48.605	10.751	
	3		118.97	57.11	54.290	10.821	
	4		121.00	57.16	52.235	10.768	
	5		115.70	57.32	53.614	11.101	
	6		125.84	56.60	52.724	11.008	
	n		18.000	18.000	18.000	18.000	
	Mean		120.30	59.14	50.85	10.69	
	STDEV		4.0943	2.5784	2.1270	0.3535	
	CV(%)		3.4	4.4	4.2	3.3	
	% Accuracy		100.3	98.6	101.7	106.9	

N/A

Results interpretation

Inter-day (Between-batch) Method Validation pass/fail: PASS

Decision criteria

Criterion	Value	Reference
Lowest STD Max Average %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest STD Min Average %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining STDs Max Average %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining STDs Min Average %Accuracy	85.0	according to SOP HP2002/09 V.14
Lowest Performance QC Max Average %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest Performance QC Min Average %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Max Average %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining Performance QCs Min Average %Accuracy	85.0	according to SOP HP2002/09 V.14
All STDs and QCs Max Average CV(%)	50.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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VALIDATION X : Reinjected after ~X hours

Results generated by Analyst®

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Analyst/Technician:	**Dieter**
CL number:	
Analyte peak area lowest STD:	38.3
Analyte peak area lowest STD duplicate:	40.8
Analyte peak area in Blank:	0

Batch Run ID:	**AP_day1_24h_reinject**
Original Validation injection date:	**2021-10-27**
Original Validation injection start time:	**22h00**
Validation reinjection date:	**2021-10-28**
Validation reinjection start time:	**22h00**
Duration on instrument (hours):	**24**
Other storage conditions if not kept on instrument:	**N/A**

Summary of Calibration Standard Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	% CV	% Accuracy	n
N/A						
N/A						
N/A						
S8						
S7	10.0	10.6	0.4	3.6	105.5	2
S6	25.0	25.6	0.1	0.2	102.3	2
S5	50.0	47.7	0.7	1.4	95.4	2
S4	75	71.6	2.8	3.9	95.5	2
S3	120	119.3	1.5	1.3	99.4	2
S2	150	146.0	4.2	2.9	97.3	2
S1	200	209.3	2.5	1.2	104.7	2

Summary of Quality Control Accuracy and Precision

Sample ID	Nominal Conc.	Mean Observed Conc.	Std Dev	% CV	% Accuracy	n
QC LLOQ 2						
QC LLOQ 1	10.00	11.02	0.36	3.3	110.2	6
QC L	50.00	50.39	2.62	5.2	100.8	6
QC M	60.00	63.32	3.10	4.9	105.5	6
QC H	120.00	124.63	3.07	2.5	103.9	6
QC XH						

Note: Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported. - FDA

Results interpretation

Response in Blank as % of LLOQ:	0.0
% of LLOQ STDs meeting criteria:	100
% of ULOQ STDs meeting criteria:	100
% of total STDs meeting criteria:	100
Do any QCs (at one level) have more than 50% of them failing to meet criteria?:	NO
% of total QCs meeting criteria:	100
Reinjection reproducibility pass/fail:	PASS

Decision criteria (for batch pass/fail)

Max Response in Blank as % of LLOQ	20.0	according to SOP HP2002/09 V.14
Lowest STD and QC Max %Accuracy	120.0	according to SOP HP2002/09 V.14
Lowest STD and QC Min %Accuracy	80.0	according to SOP HP2002/09 V.14
Remaining STDs and QCs Max %Accuracy	115.0	according to SOP HP2002/09 V.14
Remaining STDs and QCs Min %Accuracy	85.0	according to SOP HP2002/09 V.14
Min % of LLOQ and ULOQ STDs to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total STDs to meet criteria	75.0	according to SOP HP2002/09 V.14
Min % of QCs at each level to meet criteria	50.0	according to SOP HP2002/09 V.14
Min % of total QCs to meet criteria	67.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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On-Instrument Stability

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Batch duration:	**24**
Duration on instrument (hours):	**24**
Autosampler temperature (~°C):	**Room temp (25)**
Other storage conditions if not kept on instrument:	**N/A**

Batch Run ID:	**AP_day1_24h_reinject**
Original Validation injection date:	**2021-10-27**
Original Validation injection start time:	**22:00**
Validation reinjection date:	**2021-10-28**
Validation reinjection start time:	**22:00**

Summary of On-Instrument stability results of extracted samples:

Fresh	**High Concentration**		**120 ug/ml**
Validation, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (1st injection)	499.328	391.573	1.28
Injection 2 (1st injection)	498.332	416.48075	1.20
Injection 3 (1st injection)	504.64	412.5915	1.22
Injection 4 (1st injection)	502.98	414.081	1.21
Injection 5 (1st injection)	497.668	413.00525	1.20
Injection 6 (1st injection)	497.668	410.109	1.21
Average	500	410	1.22
STDEV	3	9	0.0279
CV(%)	0.6	2.2	2.3
CV(%) pass/fail			**PASS**
	High Concentration		**120 ug/ml**
Validation, Re-injection End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (Reinjection)	501.486	408.2885	1.23
Injection 2 (Reinjection)	498.996	427.4865	1.17
Injection 3 (Reinjection)	508.458	431.293	1.18
Injection 4 (Reinjection)	505.138	432.7825	1.17
Injection 5 (Reinjection)	501.818	425.1695	1.18
Injection 6 (Reinjection)	487.044	427.652	1.14
Average	500	425	1.18
STDEV	7	9	0.0293
CV(%)	1.5	2.1	2.5
%Diff after Validation Re-injection			-3.6
CV(%) pass/fail			**PASS**
%Difference pass/fail			**PASS**

Summary of On-Instrument stability results of extracted samples:

Fresh	**Low Concentration**		**50 ug/ml**
Validation, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (1st injection)	213.476	393.3935	0.54
Injection 2 (1st injection)	211.65	416.48075	0.51
Injection 3 (1st injection)	202.188	408.2885	0.50
Injection 4 (1st injection)	215.966	409.447	0.53
Injection 5 (1st injection)	199.034	417.722	0.48
Injection 6 (1st injection)	214.14	414.081	0.52
Average	209	410	0.51
STDEV	7	9	0.0235
CV(%)	3.4	2.2	4.6
CV(%) pass/fail			**PASS**
	Low Concentration		**50 ug/ml**
Validation, Re-injection End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (Reinjection)	186.75	408.123	0.46
Injection 2 (Reinjection)	187.414	426.99	0.44
Injection 3 (Reinjection)	195.963	423.349	0.46
Injection 4 (Reinjection)	196.461	427.8175	0.46
Injection 5 (Reinjection)	215.302	434.4375	0.50
Injection 6 (Reinjection)	218.456	433.1135	0.50
Average	200	426	0.47
STDEV	14	10	0.0250
CV(%)	6.8	2.2	5.3
%Diff after Validation Re-injection			-8.1
CV(%) pass/fail			**PASS**
%Difference pass/fail			**PASS**

Decision criteria

Max CV(%)	15.0	according to SOP HP2002/09 V.14
Max % Difference	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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Analyst Signature: ______________________ Date : ______________________

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On-Instrument Stability

Validation ID and version:	**IHX v1**
Analyte:	**Iohexol**
Batch duration:	**84**
Duration on instrument (hours):	**84**
Autosampler temperature (~°C):	**Room temp (25)**
Other storage conditions if not kept on instrument:	**N/A**

Batch Run ID:	**AP_day1_84h_reinject**
Original Validation injection date:	**2021-10-27**
Original Validation injection start time:	**22:00**
Validation reinjection date:	**2021-10-30**
Validation reinjection start time:	**10:00**

Summary of On-Instrument stability results of extracted samples:

Fresh	**High Concentration**		**120 ug/ml**
Validation, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (1st injection)	499.328	391.573	1.28
Injection 2 (1st injection)	498.332	416.48075	1.20
Injection 3 (1st injection)	504.64	412.5915	1.22
Injection 4 (1st injection)	502.98	414.081	1.21
Injection 5 (1st injection)	497.668	413.00525	1.20
Injection 6 (1st injection)	497.668	410.109	1.21
Average	500	410	1.22
STDEV	3	9	0.0279
CV(%)	0.6	2.2	2.3
CV(%) pass/fail			**PASS**
	High Concentration		**120 ug/ml**
Validation, Re-injection End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (Reinjection)	483.06	415.2395	1.16
Injection 2 (Reinjection)	480.072	432.7825	1.11
Injection 3 (Reinjection)	485.882	433.279	1.12
Injection 4 (Reinjection)	486.546	437.7475	1.11
Injection 5 (Reinjection)	468.95	429.969	1.09
Injection 6 (Reinjection)	479.408	431.7895	1.11
Average	481	430	1.12
STDEV	6	8	0.0245
CV(%)	1.3	1.8	2.2
%Diff after Validation Re-injection			-8.5
CV(%) pass/fail			**PASS**
%Difference pass/fail			**PASS**

Summary of On-Instrument stability results of extracted samples:

Fresh	**Low Concentration**		**50 ug/ml**
Validation, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (1st injection)	213.476	393.3935	0.54
Injection 2 (1st injection)	211.65	416.48075	0.51
Injection 3 (1st injection)	202.188	408.2885	0.50
Injection 4 (1st injection)	215.966	409.447	0.53
Injection 5 (1st injection)	199.034	417.722	0.48
Injection 6 (1st injection)	214.14	414.081	0.52
Average	209	410	0.51
STDEV	7	9	0.0235
CV(%)	3.4	2.2	4.6
CV(%) pass/fail			**PASS**
	Low Concentration		**50 ug/ml**
Validation, Re-injection End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1 (Reinjection)	192.062	412.9225	0.47
Injection 2 (Reinjection)	191.066	434.7685	0.44
Injection 3 (Reinjection)	188.659	432.4515	0.44
Injection 4 (Reinjection)	195.05	434.1065	0.45
Injection 5 (Reinjection)	189.24	439.899	0.43
Injection 6 (Reinjection)	191.896	435.7615	0.44
Average	191	432	0.44
STDEV	2	10	0.0123
CV(%)	1.2	2.2	2.8
%Diff after Validation Re-injection			-13.3
CV(%) pass/fail			**PASS**
%Difference pass/fail			**PASS**

Decision criteria

Max CV(%)	15.0	according to SOP HP2002/09 V.14
Max % Difference	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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Matrix Effects - Regression Slope

Validation ID and version:	**IHX v1**	Assay Date:	**2021-11-17**
Analyte:	**IHX 5-30 gradient v1**	Batch Run ID:	**17Nov2021_ME+PE+RE**
Biological Matrix:	**Iohexol**	Matrix sources: Matrix lot A	**Patient serum A**
Anticoagulant:	**Serum**	Matrix lot B	**Patient serum B**
Preparation Date:	17-Nov-21	Matrix lot C	**Patient serum C**
ISTD used:	YES	Matrix lot D	**Patient serum D**
Concentration units:	µg/ml	Matrix lot E	**Patient serum E**
		Matrix lot F	**Patient serum F**

Sample Concentrations:	**High conc.**	**Medium conc.**	**Low conc.**
	120	50	25

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix lot A	484	569	199	558	99	568
Matrix lot B	498	572	201	555	108	582
Matrix lot C	498	577	193	568	101	556
Matrix lot D	482	560	189	573	97	570
Matrix lot E	480	598	179	584	102	618
Matrix lot F	483	576	189	559	96	575

	High Conc. Peak Area Ratio	**Medium Conc.** Peak Area Ratio	**Low Conc.** Peak Area Ratio	Area Ratio vs. Conc Regression Slope
Matrix lot A	0.85	0.36	0.1742	0.0071
Matrix lot B	0.87	0.36	0.1851	0.0072
Matrix lot C	0.86	0.34	0.1809	0.0072
Matrix lot D	0.86	0.33	0.1705	0.0073
Matrix lot E	0.80	0.31	0.1652	0.0068
Matrix lot F	0.84	0.34	0.1669	0.0071
Average	0.85	0.34	0.1738	0.0071
STDEV	0.0246	0.0200	0.007883	0.000190
CV(%)	2.9	5.9	4.5	2.7
CV(%) pass/fail	**PASS**	**PASS**	**PASS**	
Slope variability CV(%) pass/fail				**PASS**

Decision criteria		
Max CV(%) for each concentration	15.0	according to SOP HP2002/09 V.14
Max slope variability CV(%)	5.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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Absolute Recovery

Validation ID and version:	**IHX v1**	Assay Date:	**2021-11-17**
Assay Method ID and version:	**IHX 5-30 gradient v1**	Batch Run ID:	**17Nov2021_ME+PE+RE**
Analyte:	**Iohexol**	Matrix sources: Matrix lot A	**Patient serum A**
Biological Matrix:	**Serum**	Matrix lot B	**Patient serum B**
Anticoagulant:	**N/A**	Matrix lot C	**Patient serum C**
Extration type:	Serum extraction with 5% PCA 4:1	Matrix lot D	**Patient serum D**
Preparation Date:	2021-11-17	Matrix lot E	**Patient serum E**
		Matrix lot F	**Patient serum F**
ISTD used:	Yes		
Concentration units:	µg/ml		

Sample Concentrations:	**High conc.**	**Medium conc.**	**Low conc.**
	120	50	25

Pre-extraction spiked QC's
Set 3

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix lot A	533	626	231	602	108	587
Matrix lot B	536	601	233	602	111	608
Matrix lot C	527	596	223	593	104	585
Matrix lot D	534	603	215	587	103	601
Matrix lot E	522	629	213	627	98	623
Matrix lot F	546	616	216	611	102	612

Post-extraction spiked blank plasma
Set 2

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix lot A	484	569	199	558	99	568
Matrix lot B	498	572	201	555	108	582
Matrix lot C	498	577	193	568	101	556
Matrix lot D	482	560	189	573	97	570
Matrix lot E	480	598	179	584	102	618
Matrix lot F	483	576	189	559	96	575

Peak area ratios of Set 3 compared to Set 2 samples

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Set 3 Peak Area Ratio	**Set 2 Peak Area Ratio**	**Set 3 Peak Area Ratio**	**Set 2 Peak Area Ratio**	**Set 3 Peak Area Ratio**	**Set 2 Peak Area Ratio**
Sample 1	0.85	0.85	0.38	0.36	0.1839	0.1742
Sample 2	0.89	0.87	0.39	0.36	0.1834	0.1851
Sample 3	0.88	0.86	0.38	0.34	0.1781	0.1809
Sample 4	0.89	0.86	0.37	0.33	0.1713	0.1705
Sample 5	0.83	0.80	0.34	0.31	0.1573	0.1652
Sample 6	0.89	0.84	0.35	0.34	0.1663	0.1669
Average	0.87	0.85	0.37	0.34	0.1734	0.1738
STDEV	0.0245	0.0246	0.0183	0.0200	0.010469	0.007883
CV(%)	2.8	2.9	5.0	5.9	6.0	4.5
% Recovery		102.8		108.7		99.7
Recovery CV(%)		1.0		0.8		1.3
CV(%) each conc. pass/fail		**PASS**		**PASS**		**PASS**

Average % RE	103.7
Average CV(%)	4.4
CV(%) across the mean of each conc. pass/fail	**PASS**

Decision criteria

Max CV(%) for each concentration	15.0	according to SOP HP2002/09 V.14
Max CV(%) across the mean of each concentration	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

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Process Efficiency

Validation ID and version:	**IHX v1**	Assay Date:	**2021-11-17**
Assay Method ID and version:	**IHX 5-30 gradient v1**	Batch Run ID:	**17Nov2021_ME+PE+RE**
Analyte:	**Iohexol**	Matrix sources: Matrix lot A	**Patient serum A**
Biological Matrix:	**Serum**	Matrix lot B	**Patient serum B**
Anticoagulant:	**N/A**	Matrix lot C	**Patient serum C**
Extration type:	Serum extraction with 5% PCA 4:1	Matrix lot D	**Patient serum D**
Preparation Date:	2021-11-17	Matrix lot E	**Patient serum E**
		Matrix lot F	**Patient serum F**

ISTD used:	Yes
Concentration units:	µg/ml

Sample Concentrations:	**High conc.**	**Medium conc.**	**Low conc.**
	120	50	25

Pre-extraction spiked QC's

Set 3	**High Concentration**		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Matrix lot A	533	626	231	602	108	587
Matrix lot B	536	601	233	602	111	608
Matrix lot C	527	596	223	593	104	585
Matrix lot D	534	603	215	587	103	601
Matrix lot E	522	629	213	627	98	623
Matrix lot F	546	616	216	611	102	612

Neat unextracted samples

Set 1	**High Concentration**		**Medium Concentration**		**Low Concentration**	
	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Injection solvent 1	478	553	203	571	90	566
Injection solvent 2	479	560	195	561	93	567
Injection solvent 3	479	552	196	583	92	575

Peak area ratios of Set 3 compared to Set 1 samples

	High Concentration		**Medium Concentration**		**Low Concentration**	
	Set 3 Peak Area Ratio	**Set 1 Peak Area Ratio**	**Set 3 Peak Area Ratio**	**Set 1 Peak Area Ratio**	**Set 3 Peak Area Ratio**	**Set 1 Peak Area Ratio**
Sample 1	0.85	0.87	0.38	0.36	0.1839	0.1583
Sample 2	0.89	0.86	0.39	0.35	0.1834	0.1635
Sample 3	0.88	0.87	0.38	0.34	0.1781	0.1605
Sample 4	0.89		0.37		0.1713	
Sample 5	0.83		0.34		0.1573	
Sample 6	0.89		0.35		0.1663	
Average	0.87	0.86	0.37	0.35	0.1734	0.16
STDEV	0.024	0.0062	0.0183	0.0099	0.010469	0.00260
CV(%)	2.8	0.7	5.0	2.9	6.0	1.6
% Process Efficiency		101.0		106.2		107.9
Process Efficiency CV(%)		**3.9**		**1.7**		**3.7**
CV(%) each conc. pass/fail		**PASS**		**PASS**		**PASS**

Average % PE	105.0
Average CV(%)	3.4
CV(%) across the mean of each conc. pass/fail	**PASS**

Decision criteria

Max CV(%) for each concentration	15.0	according to SOP HP2002/09 V.14
Max CV(%) across the mean of each concentration	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

Recovery, Process Efficiency and Matrix Effects
(CPQA Requirements)

Validation ID and version:	**IHX v1**
Assay Method ID and version:	**IHX 5-30 gradient v1**
Analyte:	**Iohexol**
Biological Matrix:	**Serum**
Anticoagulant:	**N/A**
Extration type:	Serum extraction with 5% PCA 4:1
Preparation Date:	2021-11-17
ISTD used:	Yes
Concentration units:	µg/ml

Assay Date:		**2021-11-17**
Batch Run ID:		**17Nov2021_ME+PE+RE**
Matrix sources:	Matrix lot A	**Patient serum A**
	Matrix lot B	**Patient serum B**
	Matrix lot C	**Patient serum C**
	Matrix lot D	**Patient serum D**
	Matrix lot E	**Patient serum E**
	Matrix lot F	**Patient serum F**

Notes:	
Set 1	Neat, Unextracted samples prepared in injection matrix
Set 2	Post extraction spiked samples
Set 3	Spiked, extracted samples, pre-extraction spike of analyte(s) into matrix and IS into then extract
ME= (Set 2 PAR/Set 1 PAR) x 100	
RE = (Set 3 PAR/Set 2 PAR) x 100	
PE = (Set 3 PAR/Set 1 PAR) x 100	

Sample Concentrations:	High conc.	Medium conc.	Low conc.
	120	50	25.0

Concentration	Matrix Source	Analyte Peak Area			Internal standard (IS) Peak Area			Peak Area Ratio (Analyte/IS)			Recovery (%)	Process Efficiency (%)	Matrix Effects (%)
		Set 1 (Injection Solution)	**Set 2 (Post-extraction)**	**Set 3 (Pre-extraction)**	**Set 1 (Injection Solution)**	**Set 2 (Post-extraction)**	**Set 3 (Pre-extraction)**	**Set 1 (Injection Solution)**	**Set 2 (Post-extraction)**	**Set 3 (Pre-extraction)**			
High	Lot A	478	484	533	553	569	626	0.865	0.851	0.852			
	Lot B	479	498	536	560	572	601	0.855	0.870	0.891			
	Lot C	479	498	527	552	577	596	0.867	0.863	0.883			
	Lot D		482	534		560	603		0.862	0.885			
	Lot E		480	522		598	629		0.803	0.830			
	Lot F		483	546		576	616		0.840	0.887			
	Average	479	488	533	555	575	612	0.863	0.848	0.871	102.8	101.0	98.3
	STDEV	0	8	8	4	13	14	0.0062	0.0246	0.0245			
	CV(%)	0.1	1.6	1.5	0.8	2.2	2.2	0.7	2.9	2.8			
							CV(%) each conc. pass/fail	**PASS**	**PASS**	**PASS**			
Medium	Lot A	203	199	231	571	558	602	0.36	0.36	0.38			
	Lot B	195	201	233	561	555	602	0.35	0.36	0.39			
	Lot C	196	193	223	583	568	593	0.34	0.34	0.38			
	Lot D		189	215		573	587		0.33	0.37			
	Lot E		179	213		584	627		0.31	0.34			
	Lot F		189	216		559	611		0.34	0.35			
	Average	198	192	222	572	566	604	0.346	0.338	0.368	108.7	106.2	97.7
	STDEV	4	8	9	11	11	14	0.01	0.020	0.02			
	CV(%)	2.2	4.2	3.9	1.9	2.0	2.3	2.9	5.9	5.0			
							CV(%) each conc. pass/fail	**PASS**	**PASS**	**PASS**			
Low	Lot A	90	99	108	566	568	587	0.2	0.2	0.2			
	Lot B	93	108	111	567	582	608	0.2	0.2	0.2			
	Lot C	92	101	104	575	556	585	0.2	0.2	0.2			
	Lot D		97	103		570	601		0.2	0.2			
	Lot E		102	98		618	623		0.2	0.2			
	Lot F		96	102		575	612		0.2	0.2			
	Average	92	100	104	569	578	603	0.161	0.174	0.173	99.7	107.9	108.1
	STDEV	2	4	5	5	21	15	0.00	0.01	0.01			
	CV(%)	1.8	4.2	4.5	0.9	3.7	2.5	1.6	4.5	6.0			
							CV(%) each conc. pass/fail	**PASS**	**PASS**	**PASS**			
										Average	104	105	101
										STDEV	4.56	3.6	5.84
										CV(%)	4.4	3.4	5.8
										Result	**PASS**	**PASS**	**PASS**
												ME Interpretation	**Ion Enhancement**

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ______________________

Verified by: ______________________ Date : ______________________

Decision criteria

Max CV(%) for each concentration	15.0	according to SOP HP2002/09 V.14
Max CV(%) across the mean of each conc.	15.0	according to SOP HP2002/09 V.14
Matrix Effects show Ion supression below	100.0	according to SOP HP2002/09 V.14
Matrix Effects show Ion enhancement above	100.0	according to SOP HP2002/09 V.14

Haemolysis

Validation ID and version:	**IHX v1**	Preparation Date:	**05-Nov-21**
Assay Method ID and version:	**IHX 5-30 gradient v1**	Assay Date:	**05-Nov-21**
Analyte:	**Iohexol**	Batch Run ID:	**05Nov2021_Val_day-3_HM&LP**
Biological Matrix:	**Serum**	ISTD used:	**YES**
Anticoagulant:	**N/A**		

Reference Samples	**High Concentration**		**Low Concentration**	
Non-haemolysed	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Sample 1	474	571	200	517
Sample 2	488	510	200	545
Sample 3	484	546	216	530
Sample 4	482	534	208	528
Sample 5	474	550	210	520
Sample 6	485	517	209	526
Test Samples	**High Concentration**		**Low Concentration**	
2% haemolysed	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Sample 1	510	630	211	627
Sample 2	516	636	210	630
Sample 3	519	621	209	621
Sample 4	512	610	228	623
Sample 5	519	608	212	632
Sample 6	515	608	213	602

Peak Area Ratio	**High Concentration**		**Low Concentration**	
	Reference Peak Area Ratio	2% haemolysed Peak Area Ratio	Reference Peak Area Ratio	2% haemolysed Peak Area Ratio
Sample 1	0.83	0.81	0.3861	0.3362
Sample 2	0.96	0.81	0.3664	0.3336
Sample 3	0.89	0.84	0.4085	0.3363
Sample 4	0.90	0.84	0.3933	0.3667
Sample 5	0.86	0.85	0.4035	0.3358
Sample 6	0.94	0.85	0.3969	0.3532
Average	0.90	0.83	0.39	0.34
STDEV	0.0467	0.0181	0.014952	0.013387
CV(%)	5.2	2.2	3.8	3.9
%Difference		-7.0		-12.4
CV(%) pass/fail	PASS	PASS	PASS	PASS
%Difference pass/fail		PASS		PASS

Decision criteria

Max CV(%)	15.0	according to SOP HP2002/09 V.14
Max %Difference	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Enter/Save

Analyst Signature: ______________________ Date : ____________

Verified by: ______________________ Date : ____________

Lipemia

Validation ID and version:	IHX v1	Preparation Date:	05-Nov-21
Assay Method ID and version:	HX 5-30 gradient v	Assay Date:	05-Nov-21
Analyte:	Iohexol	Batch Run ID:	05Nov2021_Val_day-3_HM&LP
Biological Matrix:	Serum	ISTD used:	YES
Anticoagulant:	N/A		

Reference Samples	**High Concentration**		**Low Concentration**	
Non-lipemic	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Sample 1	474	571	200	517
Sample 2	488	510	200	545
Sample 3	484	546	216	530
Sample 4	482	534	208	528
Sample 5	474	550	210	520
Sample 6	485	517	209	526
Test Samples	**High Concentration**		**Low Concentration**	
10% Lipemia	Analyte Peak Area	ISTD Peak Area	Analyte Peak Area	ISTD Peak Area
Sample 1	503	630	208	623
Sample 2	512	628	214	629
Sample 3	507	620	210	612
Sample 4	509	619	211	619
Sample 5	507	616	204	591
Sample 6	502	610	214	614

Peak Area Ratio	**High Concentration**		**Low Concentration**	
	Reference Peak Area Ratio	10% Lipemia Peak Area Ratio	Reference Peak Area Ratio	10% Lipemia Peak Area Ratio
Sample 1	0.83	0.80	0.3861	0.3344
Sample 2	0.96	0.82	0.3664	0.3398
Sample 3	0.89	0.82	0.4085	0.3431
Sample 4	0.90	0.82	0.3933	0.3414
Sample 5	0.86	0.82	0.4035	0.3460
Sample 6	0.94	0.82	0.3969	0.3492
Average	0.90	0.82	0.39	0.34
STDEV	0.0467	0.0092	0.014952	0.005111
CV(%)	5.2	1.1	3.8	1.5
%Difference		-8.9		-12.8
CV(%) pass/fail	PASS	PASS	PASS	PASS
%Difference pass/fail		PASS		PASS

Decision criteria

Max CV(%)	15.0	according to SOP HP2002/09 V.14
Max %Difference	15.0	according to SOP HP2002/09 V.14

Date/Time	**Operator**

Analyst Signature: ______________________ Date : __________

Verified by: ______________________ Date : __________

INDEX

No	Age	Weight (kg)	Height (cm)	BMI	BSA m^2 (Haycock et al)	BSA m^2 (Du Bois)	Weight (g) syringe before	Weight (g) syringe after	Grams iohexol injected	ml injected	mg Iohexol injected (647 mg/ml) - Omnipaque PI
1	24	54	164.5	20.0	1.56729	1.58254	10.8128	4.0221	6.7907	5.166	3342
2	32	108	176	34.9	2.33710	2.23135	11.6647	5.0078	6.6569	5.064	3276
3	31	101	157	41.0	2.15456	1.99632	11.5876	4.9123	6.6753	5.078	3285
4	37	64	164	23.8	1.71517	1.69728	11.5934	4.962	6.6314	5.045	3264
5	42	70	175	22.9	1.84678	1.84814	11.6873	4.9866	6.7007	5.097	3298
6	34	68	150	30.2	1.71043	1.63248	11.6925	4.9752	6.7173	5.110	3306
7	61	77	163	29.0	1.88993	1.82794	11.6548	4.9235	6.7313	5.121	3313
8	30	70	184	20.7	1.88386	1.91658	10.7632	4.9956	5.7676	4.387	2839
9	50	74	177	23.6	1.91139	1.90797	11.6069	5.1223	6.4846	4.933	3192
Mean:		76.222222	167.8333	27.3276	1.890724		11.45146667	4.8786	6.572866667	5.000	3235

No	Time injected	Time T2h	delta Minutes T0-T2	Time T4h	delta Minutes T4-T2	Time T7h	Bottle weight before discard	Bottle weight after discard	Urine volume t0-4h	Urine volume t4-7h	Serum Creat [T0] uM	Urine Creat [4hcol] uM	Urine Creat 4-7h
1	9:31	11:31	120.00	13:35	124.00		754.59	131.93	622.66		56	2861	
2	10:08	12:22	134.00	14:08	106.00		504.93	130.48	374.45		93	5044	
3	10:19	12:25	126.00	14:19	114.00		426.28	129.26	297.02		71	3138	
4	10:36	12:36	120.00	14:36	120.00		748.13	130.91	617.22		73	3995	
5	10:54	12:54	120.00	14:54	120.00		341.96	130.6	211.36		79	11599	
6	11:12	13:12	120.00	15:12	120.00		609.69	129.91	479.78		54	3290	
7	11:25	13:30	125.00	15:40	130.00		545.43	130.87	414.56		97	3358	
8	11:56	13:56	120.00	15:56	120.00	19h10 (urine a	668.47	48.11	620.36	460.5	84	4948	5304
9	12:00	14:00	120.00	16:00	120.00				334		73	7848	
Mean:									441	460			

No	Creatinine Clearance	Creatinine Clearance /1.73m2	Serum iohexol [T2] ug/ml	Serum iohexol [T4] ug/ml	Kd (min^-1)	T0.5 (min)	Theoretical initial plasma concentration (Cp0) (ug/ml)	Volume of distribution (Vd) (L)	Vd / kg (L/kg)	Iohexol Clearance (ml/min)	Clearance per $1.73m^2$ (ml/min)
1	130	143	79.89	31.54	0.0075	92	196.4	17.0	0.315	128	141
2	85	66	54.8	27	0.0067	104	134.1	24.4	0.226	163	121
3	55	47	74.59	40.59	0.0053	130	146.1	22.5	0.223	120	96
4	141	143	78.61	34.94	0.0068	103	176.9	18.5	0.288	125	126
5	129	121	99.59	46.17	0.0064	108	214.8	15.4	0.219	98	92
6	122	129	111.46	32.86	0.0102	68	378.1	8.7	0.129	89	90
7	56	53	137.62	87.78	0.0035	200	212.1	15.6	0.203	54	49
8	152	137	87.3	38.79	0.0068	103	196.5	14.4	0.206	98	90
9	150	136	79.7	41.13	0.0055	126	154.4	20.7	0.279	114	103
Mean:		108	89	42	0.00651	115	201.036995	17.46906	0.23	109.82	100.90

No	Brochner-Mortensen (original)	Brochner-Mortensen and Jodal	Fleming	MACS and CKiD equation	eGFR CKD-EPI	eGFR CKD-EPI BSA adjusted	eGFR MDRD Race-adjusted	eGFR MDRD Not Race-adjusted	eGFR MDRD Not Race-adjusted BSA adjusted
1	118	115	114	122	125	138	123	123	135
2	96	103	100	101	93	69	105	87	64
3	81	84	83	84	98	79	107	89	71
4	106	105	104	109	113	114	112	112	112
5	80	81	80	82	105	99	99	99	93
6	79	79	78	81	118	120	119	119	120
7	46	46	46	46	54	50	54	54	49
8	78	79	78	80	107	98	99	99	91
9	88	89	88	91	103	93	105	105	95
Mean:	86	86.73	85.46	88.66					

Mean weight injected: 6.572866667
Mean SG 1.314573333

GFR calculated by different slope-intercept equations

Iohexol individual elimination curves

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

Development on an HPLC based method for determination of glomerular filtration rate by iohexol clearance

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR: Prof George van der Watt

ADDRESS: NHLS Chemical Pathology
Red Cross Children's Hospital
Klipfontein Road, Rondebosch

CONTACT NUMBER: 021 658 5220

CO-INVESTIGATOR (1): Dr Dieter van der Westhuizen

ADDRESS: Chemical Pathology Registrar Office
C20, NHLS
Groote Schuur Hospital
Anzio Road, Observatory

CONTACT NUMBER: 021 404 4135

CO-INVESTIGATOR (2): Ms Joanne Pillay

ADDRESS: Division of Chemical Pathology
6th Floor, Falmouth Building
Faculty of Health Sciences, University of Cape Town
Anzio Road, Observatory

CONTACT NUMBER: 021 406 6104

CONTACT NUMBER OF HEALTH RESEARCH ETHICS COMMITTEE: 021 938 9677

You are invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline participation. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Human Research Ethics Committee of the University of Cape Town** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

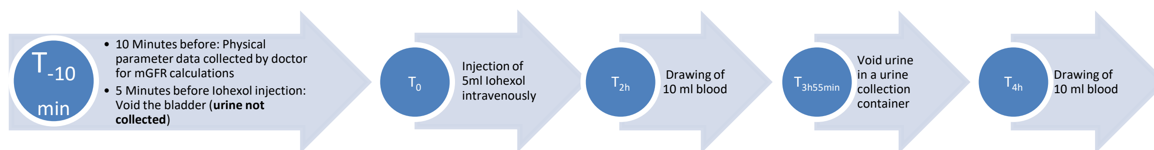
What is this research study all about?

This study will be conducted at Groote Schuur Hospital. Approximately 5 participants will be recruited from the Division of Chemical Pathology.

Tests to see how well the kidneys are working are performed every day on hundreds of patients worldwide. It is important that these tests are as accurate and reliable as possible, because often, important decisions are made on the basis of the results. For example, the results may be used to diagnose kidney failure, or to decide when dialysis is necessary, or to monitor kidney function when patients are treated with medication that can be harmful to the kidneys. The most accurate test to see how well the kidneys are working is called the glomerular filtration rate, or GFR test. The GFR test is very accurate. The aim of this research is to develop a method of testing for GFR in South Africa using Iohexol injection into a vein.

Research that has been done previously has shown that this test works well and is reliable. In Europe, there have been around 25 000 tests done on patients and study participants with no known negative effects (side-effects).

If you agree to participate in this study, the following is a representation of what will be done:



Why have you been invited to participate?

You have been invited to participate because you are working in the Division of Chemical Pathology. You should not have known renal disease. You should not have a known allergy to contrast mediums. We are developing a method to measure GFR.

What will your responsibilities be?

You will have no responsibilities, other than to allow the investigators to use your information in this study.

Will you benefit from taking part in this research?

This research will likely not benefit you directly, except that your measured GFR can be made known to you personally via email by accepting the voluntary disclosure at the bottom of this consent form. This research will benefit the South African patients in the future who need a test of their kidney function.

Are there any risks involved in your taking part in this research?

There are no major risks to you if you take part in this research. There is a small risk of minor discomfort in the upper arm from the tourniquet while the blood sample is being taken, but this will not take longer than 30 seconds.

None of the risks as described below, has been described in more than roughly 25 000 mGFR's done in Europe (using 5ml iohexol intravenously), estimating the risk of any of the described adverse events as < 1 / 25 000.

The risks associated with the intravenous administration of iohexol, albeit at the dose for CT scan (80 - 180 ml), are:

Common adverse effects:

- Reversible contrast-induced nephropathy
- Hypersensitivity reactions such as skin reactions, e.g. itching, urticaria
- Nausea and vomiting

Uncommon adverse effects:

- Severe adverse reactions such as anaphylaxis, bronchospasm, angioneurotic oedema, hypotension, arrhythmias and renal failure

To our knowledge, mGFR does not pose any significant risk when using Iohexol clearance as the marker.

If you do not agree to take part, what alternatives do you have?

Whether you agree to take part or not, will not change the outcome for you. The only benefit to you for participation in this study, is the fact that you will get a GFR measurement done.

Who will have access to your medical records?

If you agree to participating, the following information will be recorded: your age, sex, ethnicity, weight, height, any medical condition you have, the medication you are taking, the result of the blood and urine creatinine tests, and the result of the GFR test. All information will be treated as confidential and protected on a cloud storage using a password. Only the investigators will have access to your information. When the results of this research are published, the identity of all participants will remain anonymous.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

It is extremely unlikely that any form of injury will occur as a direct result of taking part in this research study.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study and there will be no costs involved for you, if you do not take part.

Is there anything else that you should know or do?

- You can contact Prof. George van der Watt at **021 658 5220** or Dr. Dieter van der Westhuizen at **021 404 4135** if you have any further queries or encounter any problems.
- You can contact the Human Research Ethics Committee of the University of Cape Town at 021 650 1236 or hrec-enquiries@uct.ac.za if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ______________________________ agree to take part in a research study entitled "Development on an HPLC based method for determination of glomerular filtration rate by iohexol clearance"

I declare that:

- I have read this information and consent form and it is written in a language (English) with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I **do not have renal disease** and I **do not have a known allergy to any contrast mediums.**
- Applicable to females: I am not pregnant.

Circle whichever option is applicable (Voluntary Disclosure):

- I (do / do not) want to receive my own mGFR results by email after the study.
  - Email:____

Signed at (*place*)_____________________________ on (*date*)__________________________________

____ ____

Name of witness **Signature of witness**

Declaration by investigator

I *(name)* _____________________________________ declare that:

- I explained the information in this document to ______________________________
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did not use an interpreter.

Signed at (*place*) ______________________________ on (*date*) ________________________________

____ ____

Signature of investigator **Signature of witness**

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences

Human Research Ethics Committee

Room G50- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-enquiries@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

22 September 2020

HREC REF: 556/2020

Prof G van der Walt
Division of Chemical Pathology
Email: george.vanderwalt@uct.ac.za

Dear Prof van der Walt

PROJECT TITLE: DEVELOPMENT OF AN HPLC BASED METHOD FOR THE DETERMINATION OF GLOMERULAR FILTRATION RATE BY IOHEXOL CLEARANCE-MMED CANDIDATE-DR. DIETER VAN DER WESTHUIZEN

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 September 2021.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Dr Dieter van der Westhuizen will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

HREC/REF:556/2020sa

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HUMAN RESEARCH
ETHICS COMMITTEE
2 5 MAR 2022
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

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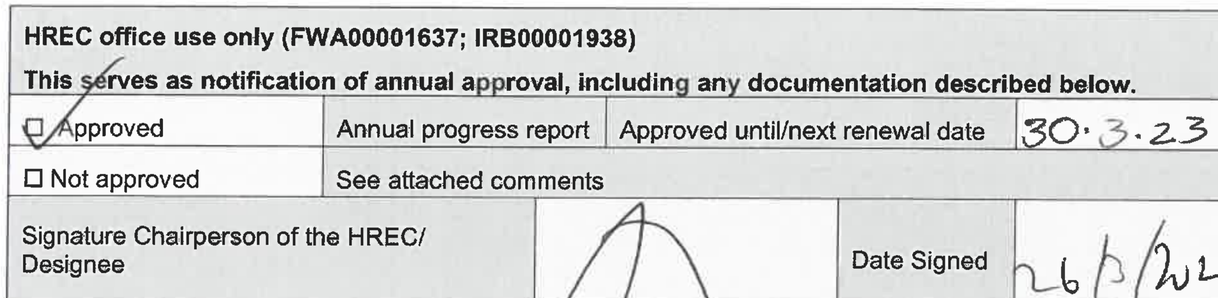
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

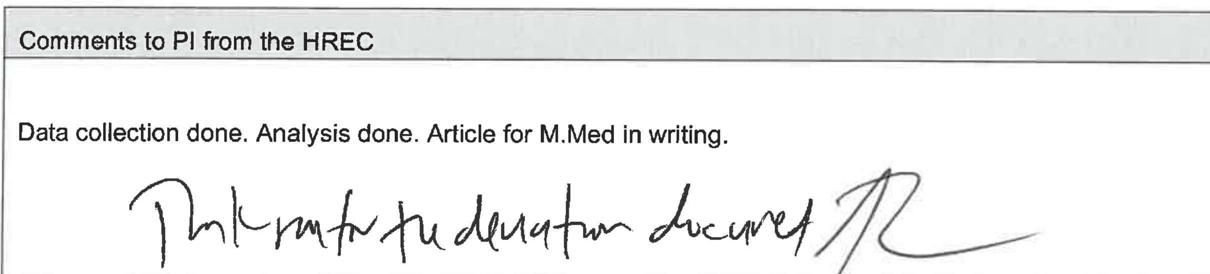
FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal



HREC office use only (FWA00001637; IRB00001938) **This serves as notification of annual approval, including any documentation described below.**			
☑ Approved	Annual progress report	Approved until/next renewal date	30.3.23
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee	[signature]	Date Signed	26/3/2022

Note: Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.
Please clarify your plan for research-related activities during COVID-19 lockdown.
Please use the latest form found on our website:
http://www.health.uct.ac.za/fhs/research/humanethics/forms



Comments to PI from the HREC
Data collection done. Analysis done. Article for M.Med in writing. Thank you for the detailed document [signature]

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	2022-03-18		
HREC REF Number	556-2020	Current Ethics Approval was granted until	2021-09-30
Protocol title	Development on an HPLC based method for the determination of glomerular filtration rate by iohexol clearance – M.med candidate Dr. Dieter vd Westhuizen		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	☐ Yes	☑ No	
If yes, could you please provide the HREC Reference number for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study.			

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

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FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Principal Investigator	Ass. Prof. George van der Watt
Department / Office Internal Mail Address	Division of Chemical pathology - carmen.stout@uct.ac.za george.vanderwatt@uct.ac.za / dieter.vdwesthuizen@nhls.ac.za / joanne.pillay@uct.ac.za

1.1 Does this protocol receive US Federal funding?	☐ Yes	☑ No
1.2 If the study receives US Federal Funding, does the annual report require full committee approval? **Note:** Any annual approvals for **Full Committee** review MUST be submitted on the monthly HREC submission dates. (Please send electronic copy for full committee review to hrec-submission@uct.ac.za)	☐ Yes	☐ No

If yes in 1.2 please complete section 1.3 below for invoicing purposes

1.3 Ethics Renewal Fee

Please **(tick ✓)**appropriate box for billing purposes:

Submission Type	***Description***	***New fee (Vat Incl.)***	***tick ✓***
Research funded solely from UCT departmental/ divisional/group budget	Annual evaluation of research progress report for re-certification	**R0,00**	☑
Non-sponsored student research for degree purposes at UCT/Other Universities & Colleges	Annual evaluation of research progress report for re-certification	**R0,00**	☐
Annual re-certification / Progress report (FHS016 Form)	Clinical Trial & International Grant Funded Research - Annual evaluation of research progress report for re-certification for Full Committee Approval	**R7000,00**	☐
Annual re-certification / Progress report (FHS016 Form)	Clinical Trial & International Grant Funded Research - Annual evaluation of research progress report for re-certification for Expedited review	**R3 710.00**	☐
Annual re-certification / Progress report (FHS016 Form)	National grant funded research - Annual evaluation of research progress report for re-certification for Full Committee Approval	**R6000.00**	☐
Annual re-certification / Progress report (FHS016 Form)	National Grant funded research for Annual evaluation of research progress report for re-certification for Expedited review	**R1 500,00**	☐

NB: Protocols funded by UCT (e.g. departmental funding / student research) and by certain grant funding organizations (e.g. MRC, NRF, CANSA,) are exempt from these charges.

Please provide details for Invoicing, either complete section 1 or 2 :

1. Invoice billing – Directly to Sponsor

Sponsor's name	

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

Billing Address of Sponsor:	
Vat Number:	
Contact person	
Telephone number	
Email Address	
2. **Internal Journal Billing:**	
Fund Number:	
Cost Centre Number:	
Account Holder Name:	
Division of Account Holder:	

2. List of documentation for approval

Progress report

3. Protocol status (tick ✓)

☐	Open Enrolment
☑	Closed to enrolment (tick ✓)
☐	Research-related activities are ongoing
☐	Research-related activities are complete, long-term follow-up only
☑	Research-related activities are complete, data analysis only
☐	Main study is complete but sub-study research-related activities are ongoing
☐	Study is closed → Please submit a Study Closure Form (FHS010)

4. Enrolment

Number of participants enrolled to date	9
Number of participants enrolled, since last HREC Progress report (continuing review)	As above - 9.
Additional number of participants still required	0

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

5. Refusals

Total number of refusals (participants invited to join the study, but refused to take part)	0

6. Cumulative summary of participants

Total number of participants who provided consent	9
Number of participants determined to be ineligible (i.e. after screening)	0
Number of participants currently active on the study	0
Number of participants completed study (without events leading to withdrawal)	9
Number of participants withdrawn at participants' request (i.e. changed their mind)	0
Number of participants withdrawn by PI due to toxicity or adverse events	0
Number of participants withdrawn by PI for other reasons (e.g. pregnancy, poor compliance)	0
Number of participants lost to follow-up. Please comment below on reasons for loss of follow-up.	0
Number of participants no longer taking part for reasons not listed above. Please provide reasons below:	

7. Progress of study

Please provide a brief summary of the research to date including the overall progress and the progress since the last annual report as well as any relevant comments/issues you would like to report to the HREC:
GFR measured successfully in 9 healthy candidates. No adverse events. Data analysis complete. Article in writing currently.

8. Protocol violations and exceptions (tick ✓ all that apply)

☑	No prior violations or exceptions have occurred since the original approval
☐	Prior violations or exceptions have been reported since the last review and have already been acknowledged or approved
☐	Unreported minor violations that have occurred since the last review, as well as significant deviations not yet reported, are attached for review

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

9. Amendments (tick ✓ all that apply)

☑	No Prior amendments have been made since the original approval
☐	Prior amendments have been reported since the last review and have already been approved
☐	New protocol changes/ amendments are requested as part of this continuing review (See note below)

Note: If new protocol changes are being requested in this review, please complete an amendment form (FHS006).
Specific changes in the amended protocol and consent/assent forms must be **bolded**, *italicised* or tracked and all changes must include a rationale.

10. Adverse events

10.1 Please provide below or attach a narrative summary of serious adverse events and/ or unanticipated problems since the last progress report. Please indicate changes made to the protocol and informed consent document(s) as a result (if not already reported to the HREC). Please comment on whether causality to any study procedure or intervention could be established.
N/A

10.2 Have participants received appropriate treatment/ follow-up/ referral when indicated (e.g. in the case of abnormal or incidental clinical findings, distress or anxiety)?		
☐ Yes	☐ No	☑ Not applicable
If yes, please describe:		

11. Summary of Monitoring and Audit Activities (tick ✓)

11.1 Was this study monitored or audited by an external agency (e.g. SAHPRA, FDA)?		
☐ Yes	☐ No	☑ Not applicable

11.2 Did a Data and Safety Monitoring Board publish a report?		
☐ Yes	☐ No	☑ Not applicable

11.3 If yes, please identify the agency and attach a summary of the findings.					
Agency Name		Report attached	☐ Yes	☐ No	☐ Not applicable
		DSMB report attached	☐ Yes	☐ No	☐ Not applicable

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

11.4 Has there been any agency, institutional or other inquiry into non-compliance in this study, or any finding of non-compliance concerning a member of the research team?	
☐ Yes	☑ No
If yes, please explain:	

12. Level of risk (tick ✓)

12.1 In light of your experience of this research, please indicate whether the level of risk to participants has:	
☐	Increased
☐	Decreased
☑	Shown no change
If there has been a change, please explain:	

12.2 Please provide a narrative summary of recent relevant literature that may have a bearing on the level of risk.
Iohexol injection at the volume used for GFR measurement (5mL) is still considered to have minimal risk of adverse events.

13. Insurance

Please confirm that valid no fault insurance is still in place? **(tick ✓)**			
☐ Yes	☐ No		☑ Not Applicable – N/A
If yes, please complete the following:			
Insurer's name:			
Policy no.		*Coverage Period:	
For UCT sponsored studies please liaise the Insurance office via fhs.sponsorship@uct.ac.za regarding the required documentation and information required obtain a renewed UCT No-fault Insurance Certificate.			

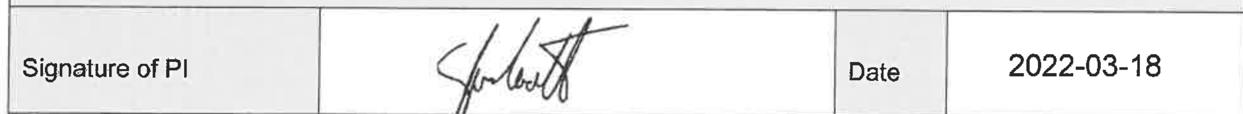
14. Statement of conflict of interest

Has there been any change in the conflict of interest status of this protocol since the original approval? (tick ✓)	
☐ Yes	☑ No

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

If yes, please explain and if necessary, attach a revised conflict of interest statement (Section #7 in the New Protocol Application Form FHS013):

15. Signature

My signature certifies that the above is complete and correct.			
Signature of PI		Date	2022-03-18

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

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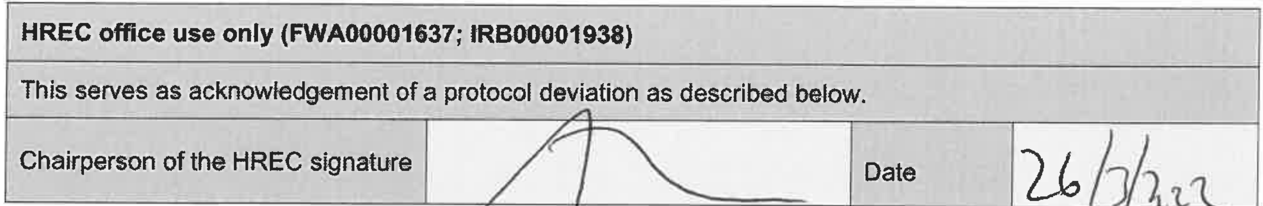
HUMAN RESEARCH ETHICS COMMITTEE
25 MAR 2022
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES

Human Research Ethics Committee

Form FHS011: Study deviation



HREC office use only (FWA00001637; IRB00001938)			
This serves as acknowledgement of a protocol deviation as described below.			
Chairperson of the HREC signature	[signature]	Date	26/3/22

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	2022-03-24
HREC REF Number	556-2020
Project Title	Development on an HPLC based method for the determination of glomerular filtration rate by iohexol clearance – M.med candidate Dr. Dieter vd Westhuizen
Protocol number (if applicable)	
Principal Investigator	A.Prof George van der Watt
Department / Office Internal Mail Address	Division of Chemical pathology secretary: Carmen.stout@uct.ac.za Investigators: George.vanderwatt@uct.ac.za / dieter.vdwesthuizen@nhls.ac.za / joanne.pillay@uct.ac.za

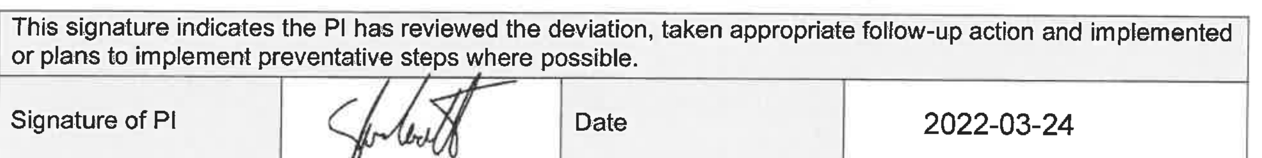
2. Protocol deviation description

Please describe the deviation below, including the reason why the deviation occurred.
Progress report not submitted in time due to the following reasons: candidate wrote final exam during that time period, we thought we would be done writing the article before 30 Sept. 2021. We are done with data collection and currently analyzing it.

3. Follow-up actions

3.1 Please describe any follow-up action(s) taken or planned as a result of this deviation e.g. DSMB reporting, report to sponsor, informing participants.
None needed.
3.2 Please describe what action(s) have or will be taken to prevent similar deviations in future.
Registrars informed to send progress reports in due time.

4. Principal Investigator's acknowledgement of responsibility



This signature indicates the PI has reviewed the deviation, taken appropriate follow-up action and implemented or plans to implement preventative steps where possible.			
Signature of PI	[signature]	Date	2022-03-24