Routine Determination of GFR in Renal Transplant Recipients by HPLC Quantification of Plasma Iohexol Concentrations and Comparison With Estimated GFR

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Estimated glomerular filtration rate (eGFR) methods are not sufficiently reliable in renal transplant recipients (RTR) and should be replaced by iohexol plasma clearance measurement. However, this method has poor availability in health centers. The aim of our study was to develop a high-performance liquid chromatography (HPLC) method for plasma iohexol measurement in routine practice and to evaluate its plasma clearance as a reference of GFR. We developed an HPLC method using UV detection. We evaluated sample storage conditions to provide recommendations for routine practice. Then, we compared GFR based on plasma iohexol clearance (GFR-iohexol) to eGFR using modification of diet in renal

disease, Cockcroft and Gault, and CDK-EPI equations in 40 RTR. The method was validated over a concentration range of 15–300 µg/l. Excellent linearity ($r > 0.998$), inter- and intraday precision (CV < 3.3%), and accuracy (>96.8%) were complied with ICH guidelines. We also demonstrated excellent samples stability (9 days). Although eGFR methods are not references in RTR, we found a correct concordance between eGFR and GFR-iohexol in our population. To conclude, our method is simple, rapid, accurate, and reliable for routine clinical and research use especially in RTR. *J. Clin. Lab. Anal.* 26:376–383, 2012.

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INTRODUCTION

The accurate and precise evaluation of renal clearance is a real challenge. The glomerular filtration rate (GFR) is generally considered the best measure of overall kidney function (1). Among the methods for GFR evaluation, inulin clearance is the reference but its dosage is costly, time expensive, and not widely available in routine practice. Several GFR estimation methods based on creatinine concentration, such as modification of diet in renal disease (MDRD) or Cockcroft and Gault are used in routine practice but they are not highly correlated to inulin clearance in most patients (2–7). Other markers of GFR such as cystatin C or beta-trace proteins have also been eval-

uated, especially in renal transplant recipients (RTR) but they still require validation (3, 8–10).

It remains particularly important to have an accurate method to evaluate GFR in RTR for the standard post-transplantation follow-up (11). Some authors showed

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that many predictive formulas (12 published prediction equations) estimating creatinine clearance over- or underestimate the real value, particularly in the low range and in posttransplant kidney patients (12). Thus, they suggested that graft function should be preferably monitored using a reference method of GFR measurement, such as iohexol plasma clearance (12). Moreover, Mariat et al. (13) compared performances of six GFR estimation methods to inulin clearance in 284 renal transplant patients. They demonstrated that no formula among Cockcroft–Gault, Walser, Jelliffe, Nankivell, and Levey formulas correlated enough to inulin clearance to be reliable in these patients. A consensus from the Lisbon Conference (14) on the care of the kidney transplant recipients reported that estimation of GFR is frequently used to check kidney function and to make decision, even though it is far from the gold standard. Direct measurement of GFR is recommended in some circumstances such as estimation of kidney disease severity (classification K/DOQI) (14). White et al. (15) showed that the new Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation better estimated GFR in RTR with a correction for low creatinine levels, but its precision remained insufficient.

Many studies showed a high correlation between plasma clearance of iohexol, a nonionic molecule commonly used as X-ray contrast media agent, and other exogenous GFR markers such as DTPA (diethylenetriaminepentaacetic acid), radiolabeled EDTA (ethylenediaminetetraacetic acid), and inulin, in adult patients with normal or impaired renal function (16–21). Several analytical methods have been described to measure plasma iohexol concentrations such as X-ray fluorescence (22,23), mass spectrometry (24, 25), and chromatographic methods (16, 21, 26–31).

The aims of our study were (i) to develop a fast and reliable HPLC method to measure iohexol concentration in plasma samples, (ii) to evaluate its direct application in daily practice in RTR, (iii) to assess discrepancies between different eGFR equations and the measured iohexol clearance. Although eGFR methods are not gold standards in RTR, they are usually applied in routine practice. Thus, we aimed at evaluating the bias of renal function evaluation when using these inappropriate but currently used formulas.

MATERIAL AND METHODS

Chemicals

All chemicals used were HPLC-grade. Perchloric acid at 65% and methanol 99.9% were purchased from Carlo-Erba (Val de Reuil, France). Para-aminohippuric acid (PAH), trifluoroacetic acid, acetonitrile were obtained by Sigma (Darmstadt, Germany). The solution

of PAH (Sigma) at 30 µg/ml was prepared in ultra-pure water. The solution of iohexol was prepared by dilution of omnipaque® (300 mg/ml) in ultra-pure water.

Equipment

The HPLC-UV system used to measure iohexol concentrations consisted of a pump (Ultimate 3000 Pump) connected to a thermostated Ultimate® 3000 autosampler (Dionex, Sunnyvale, CA). The ultimate 3000 VWD detector 414 (Dionex, Voisins Le Bretonneux, France) was set at 254 nm. Chromatographic separation was achieved on a reversed-phase column Xterra C18 3.5 µm (4.8 × 100 mm, Waters, St. Quentin en Yvelines, France). The mobile phase was a mixture of 0.1% trifluoroacetic acid and methanol (5:95, v/v) adjusted to pH 2.2 with sodium hydroxide. The mobile phase was degassed, and then a flow rate of 0.4 ml/min was applied. The standardized Jaffe's method on AU640 analyzer (Beckman Coulter, Villepinte, France) was used to measure creatinine concentrations.

Calibration Curve and Quality Control Samples

We used calibrators and quality controls from our own design. We chose PAH as an internal standard. PAH was added in a constant amount to plasma samples, blank sample, and calibration standards. Calibration was performed by plotting the ratio of the calibration standards signals to the internal standard signal. The area of the iohexol peak was systematically divided by that of the internal standard. Calibration points were chosen according to concentrations values expected after iohexol injection. They were obtained by dilution of a commercial solution of omnipaque® (i.e., iohexol) in blank human serum to yield concentrations of 15–300 µg/ml (15, 75, 100, 150, 200, and 300 µg/ml). We chose two levels of quality controls: 50 µg/ml (CQ1) and 200 µg/ml (CQ2).

Preparation of the Samples

A minimum of 1 ml of blood was collected on heparin tube, immediately protected from light, and then centrifuged for 15 min at 800 × g. The plasma was frozen at –20°C until analysis. After thawing, plasma sample was mixed thoroughly by inversion, and then centrifuged for 10 min at 800 × g. A total of 160 µl of calibrator, quality control, or patient plasma sample was added to 20 µl of internal standard and 800 µl of perchloric acid (1 M). Then, the solution was vortexed for 1 min. After a centrifugation at 10,000 × g for 10 min, 20 µl of the supernatant was injected in the HPLC system.

Fig. 1. Chromatogram showing peaks of iohexol isomers and peak corresponding to internal standard (PAH).

ASSAY VALIDATION

Precision and Accuracy

Validation was conducted according to the ICH guidelines (32). The assay was considered satisfactory if precision, expressed as relative standard deviation (RSD) or coefficient of variation (%CV), was less than 15% for intra- and interday variation. RSD for lower limit of quantification (LLOQ) was set at <20%. The accuracy compared to the nominal value (%deviation) should be within 15% for intra- and interday comparison (%deviation for LLOQ was set at <20%). The calibration curve should have a correlation coefficient r^2 of 0.995 or more. The back-calculated values for quality controls should be within 15% of target (20% at LLOQ). At least six nonzero concentration points must be used to derive the standard curve, and >75% of points must be within 15% of the targeted value (LLOQ must be within 20%). We evaluated possible interference with other plasma metabolites by analyzing ten plasma samples from patients not having iohexol administration. The LLOQ was estimated for the first calibration curve's point. The accuracy should

be within 20% of the nominal concentration and precision should be <20%. Within-day precision and accuracy of iohexol concentrations measurements were determined by analysis of six replicates of each quality-control sample ($n = 6$) at low (50 $\mu\text{g/ml}$) and high (200 $\mu\text{g/ml}$) concentration. The interassay reproducibility was assessed by repeated analysis of the two samples for 20 days, not necessarily consecutive. Precision was reported as CV (%) and accuracy as percent of the nominal value (% deviation).

Stability of iohexol in Different Storage Conditions

We analyzed the stability of iohexol in plasma under different conditions to propose recommendations for sample storage in routine practice. The basal value considered as reference was determined on fresh plasma quality controls (50, 100 and 150 $\mu\text{g/ml}$) and the concentrations were measured under different storage conditions. Plasma samples were stored at room temperature, 4°C, and -20°C for 9 days and the measurements were repeated at day 2 (D2), day 7 (D7), and day 9 (D9). We also assessed the

Fig. 2. Calibration curve of iohexol measured by HPLC method.

stability over three freeze-thaw cycles with concentrations measurements after 2, 7, and 9 days of freezing at -20°C . Bland and Altman plot with the intraclass correlation coefficient determination were used to compare iohexol concentrations between different storage and time conditions.

Application to Clinical Samples

Sample collection

Blood samples were collected from 40 Caucasian patients 3 months after kidney transplantation. An evaluation of GFR by MDRD was performed for each patient to adapt the protocol of iohexol clearance measurement. Patient with GFR estimated by MDRD formula $>40\text{ ml/min/1.73 m}^2$ or $<40\text{ ml/min/1.73 m}^2$ required four or six blood samples, respectively. Iohexol injection was contraindicated in any patient with a confirmed allergy to an iodinated product. For each patient, 5 ml of Omnipaque[®] 300 mg/ml was administered by intravenous injection. Blood samples were collected at 120, 150, 180, and 210 min after injection for patients having $\text{GFR} > 40\text{ ml/min/1.73 m}^2$ and supplementary times at 240 and 270 min for $\text{GFR} < 40\text{ ml/min/1.73 m}^2$. Immediately after sampling, the tubes were protected from light and stored at 4°C until centrifugation.

Calculation of iohexol clearance

Although some authors have described equations to calculate GFR from iohexol blood samples (33), we used Bröchner–Mortensen (34) method. This formula was established from pharmacokinetic data of Cr-51-EDTA (and not iohexol) in nonkidney transplant recipients, but we kept this method considered as a reference, currently used in routine practice of biochemical laboratories. The clearance of iohexol (Clr) was estimated using the concentration values from all time points according to a one-compartment model by the formula: Clr

TABLE 1. Intra- and Interday Reproducibility in six and 20 Replicates

		Quality controls	
		50 µg/ml	200 µg/ml
Intraday reproducibility	<i>n</i>	6	6
	CV (%)	0.7	2.7
	RE (%)	1.9	3.2
Interday reproducibility	<i>n</i>	20	20
	CV (%)	3.3	2.9
	RE (%)	1.1	1.1

$= \text{Dose/AUC}$ (with AUC: area under the plasma concentration–time curve). Then, plasma clearance of iohexol was modified for the early distribution phase by the Bröchner–Mortensen formula: $\text{Clr}_{\text{BM}} = (0.990778 \times \text{Clr}) - (0.001218 \times \text{Clr}^2)$.

GFR Estimations (eGFR)

We estimated GFR for each patient by using three validated formulas based on creatinine concentrations. We used Cockcroft and Gault formula defined as $\text{Cl creatinine (ml/min)} = [(140 - \text{age (years)}) \times \text{weight (kg)} \times A] / \text{creatinine (µmol/l)}$; $A = 1.23$ in men and 1.04 in women (35). We used the following MDRD equation: $186.3 \times (\text{creatinine (µmol/l)}/88.4) - 1.154 \times \text{age} - 0.203 \times K \times k$; $K = 1.00$ (man) or 0.742 (woman), $k = 1.180$ (African) (36). The new CKD-EPI equation was $\text{GFR} = 170 \times [\text{serum creatinine concentration (mg/dl)}]^{-0.999} \times [\text{Age}]^{-0.176} \times [0.762 \text{ if patient is female}] \times [1.180 \text{ if patient is African}] \times [\text{serum urea nitrogen concentration}]^{-0.170} \times [\text{Alb}]^{0.318}$ (36).

We compared GFR estimated by Cockcroft and Gault, MDRD, CKD-EPI, and iohexol clearance using Bland and Altman plot with the intraclass correlation coefficient. We considered iohexol clearance as the reference.

Statistical Analysis

Bland and Altman plot with intraclass correlation coefficient was performed with R version 2.8.0, an open-source program developed by the R Foundation for Statistical Computing (Vienna, Austria).

RESULTS

Chromatography, Linearity, and LLOQ

Retention times for internal standard and iohexol were 6.25 and 9.5 min, respectively (Fig. 1). Presence of iohexol on the chromatogram was characterized by two peaks (9.2–9.5) corresponding to both stereoisomers (30). We observed good linearity ($r^2 = 0.9987$) over the studied

Fig. 3. [Color online.] Bland and Altman plots representing different storage and time conditions of plasma samples (i.e., quality controls: 50–100–150 $\mu\text{g/mL}$). Blue, green, black, red points and lines represent the median and the first and third quartiles for the following storage conditions: freeze-thaw cycles (blue), storage at -20°C (green), storage at 4°C (black), storage at room temperature (red), respectively. The reference was the measure of iohexol concentrations on fresh plasma.

iohexol concentration ranges of 15–300 $\mu\text{g/mL}$. The LLOQ was 15 $\mu\text{g/mL}$. Calibrations curves are shown in Figure 2.

Precision, Accuracy, and Specificity

Repeated measurements of iohexol concentrations on quality control specimens (50 and 200 $\mu\text{g/mL}$) gave excellent intra- and interassay CV less than or equal to 2.7% and 3.3% for both controls, respectively (Table 1). Moreover, intra- and interday reproducibility assays revealed an RE value inferior to 3.2% for both controls (Table 1). We detected no peak after analysis of blank plasma, thus showing no analytical interference between iohexol and plasma metabolites.

Stability

The measurement of quality controls concentrations after different storage conditions revealed intraclass correlation coefficient at 0.81 [0.32–0.96], 0.97 [0.91–0.99], 0.97 [0.90–0.99], and 0.97 [0.89–0.99] for storage between 2 and 9 days at room temperature, 4°C , -20°C , and

after freeze-thaw cycles (Fig. 3). The stability of iohexol in plasma is thus excellent, until 9 days before analysis.

Iohexol Clearance in RTR and Comparison With eGFR

We evaluated iohexol clearance in 40 RTR. Patients' characteristics are described in Table 2; the mean level of serum creatinine was superior to normal value:

TABLE 2. Patients Characteristics

Variables	Mean $\pm$ SD	Minimum	Maximum
Age (years)	52.0 $\pm$ 13.5	21.0	73.0
Height (cm)	168.0 $\pm$ 9.6	148.0	182.0
Weight (kg)	70.2 $\pm$ 11.0	49.0	90.0
Body Mass Index (kg/m^2)	24.9 $\pm$ 3.28	19.5	32.4
Serum creatinine ($\mu\text{mol/L}$)	144 $\pm$ 41	71	227
GFR Cockcroft–Gault	50.6 $\pm$ 15.1	29.6	81.5
GFR iohexol	53.7 $\pm$ 15.2	21.7	84.1
GFR MDRD	48.0 $\pm$ 15.0	21.0	75.0
GFR CDK-EPI equation	48.0 $\pm$ 15.8	21.7	77.4

GFR, glomerular filtration rate (mL/min/1.73 m^2); SD, standard deviation.

Fig. 4. Bland and Altman plots representing different GFR evaluation methods: (A) MDRD equation vs. iothexol clearance; (B) Cockcroft and Gault equation vs. iothexol clearance; (C) CKD-EPI equation vs. iothexol clearance. Gray lines represent the median and the first and third quartile.

144 $\mu\text{mol/l}$ (± 41). Bland and Altman plots with MDRD equation, Cockcroft and Gault formula, and CKD-EPI equation versus iothexol clearance are shown in Figure 4. Intraclass correlation coefficients were evaluated at 0.68 [0.53–0.80], 0.81 [0.70–0.88], and 0.71 [0.56–0.81], respectively.

DISCUSSION

GFR estimation (eGFR) methods using creatinine concentration have been described as unreliable in some subgroups of the general population, especially in RTR (15, 37). Thus, graft function should preferably be evaluated

using a reference method of GFR measurement, based on the clearance of an exogenous marker such as iothexol. As iothexol has the main characteristics of a convenient marker of GFR, its plasma clearance seems to be the best choice to determine GFR in routine practice. Contrary to determination of renal clearance of inulin, iothexol plasma clearance does not require urine collection and continuous intravenous infusion. However, iothexol plasma clearance is not largely used in clinical practice and its determination remains limited to some health centers.

Few HPLC methods have been described for iothexol quantification (16, 21, 28–31). We developed a rapid (run time <10 min) HPLC method validated according to the recommendations of ICH guidelines, compatible with those described in the literature. As this method requires an acid precipitation of the proteins extraction steps, we used PAH as an internal standard. Although Cavalier et al. did not use any internal standard in their method (30), Klenner et al. (28) chose the sodium diatrizoate dehydrate. We suggest that it is important to correct for a possible loss of analyte during sample preparation or sample inlet.

The LLOQ, evaluated at 15 µg/ml is quite similar to that described by other authors evaluating iothexol concentrations in plasma samples (10.8 µg/ml) (30). This value is low enough to evaluate concentrations until 270 min after injection of 1,500 mg of Omnipaque®. Moreover, accuracy and precision are also in the same ranges as previously described (28–31).

Moreover, we showed excellent stability of iothexol in blood samples at 4°C. This condition, easy to obtain in clinical practice, could contribute to a larger use of this GFR determination by nephrology units.

Estimated GFR based on MDRD, Cockcroft and Gault, and CDK-EPI equations are not gold standards in RTR but are currently used in routine practice in most of biochemical laboratories. We compared eGFR and GFR-iothexol to highlight bias when using eGFR methods and to assess eGFR performance in RTR. We observed an acceptable overall concordance between different eGFR methods and GFR-iothexol [Intraclass correlation (ICC) 0.6]. We suggest that this concordance could be due to the characteristics of our cohort, containing few overweight patients (BMI: 24.9 ± 3.28 ; [19.5–32.4] kg/m²) and no patients with deleterious kidney function (serum creatinine 144 ± 41 ; [71–227] µmol/l). However, it appeared that for some patients, the eGFR showed estimation error of about 50%, which is meaningless and of no clinical utility. Interestingly, Goerdt et al. compared four mathematical models used to estimate GFR (the Cockcroft–Gault equation, the Jelliffe equation, the Walser equation, and the Mawer equation) to iothexol plasma clearance. They concluded that the Jelliffe and Walser equations gave the least biased and most precise estimations of GFR in RTR (38). Moreover, Gaspari et al. showed that 12 prediction equa-

tions do not allow a rigorous assessment of renal function in RTR compared to plasma iothexol clearance (12). Despite a tendency toward GFR overestimation, Walser and MDRD equations gave the best performance. Thus, results from published studies are heterogeneous but suggest that eGFR methods are not sufficiently reliable in RTR.

CONCLUSION

Estimated GFR equations have poor performance to evaluate renal function in RTR and should be replaced by iothexol assay. We developed a fast, sensitive, and accurate HPLC-UV method for the quantification of iothexol in plasma. This method has been successfully used in daily practice to measure GFR in RTR. The performance of this method makes it suitable for routine and research in RTR. Further experiments are necessary to determine whether this method could also be of interest in other patients such as obese, older, or malnourished patients.

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