DOCTORAL THESIS

University of La Laguna

PLASMA CLEARANCE OF IOHEXOL SIMPLIFIED BY DRIED BLOOD SPOT TESTING

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Directed by Dr. Esteban Porrini

Co-directed by Dr. Flavio Gaspari

2017

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D. **Esteban Porrini**, doctor en Medicina y Cirugía por la Universidad de La Laguna, D. **Flavio Gaspari**, doctor en Química por la Universidad de Milán,

CERTIFICAN

Que **Sergio Luis Lima**, licenciado en Biología, ha realizado bajo su dirección los trabajos de investigación correspondientes a la Tesis Doctoral con Mención Internacional titulada: **"Plasma clearance of iohexol simplified by dried blood spot testing",** que presenta para optar al grado de Doctor Europeo por la Universidad de La Laguna.

Revisada la presente Memoria, la consideramos apta para ser presentada y defendida, y autorizamos su presentación para ser juzgada por el Tribunal que sea designado al efecto.

Y para que conste y surta los efectos oportunos, en cumplimiento de las disposiciones vigentes, firmamos el presente certificado en San Cristóbal de La Laguna, a X de Julio de 2017.

Dr. Esteban Porrini   Dr. Flavio Gaspari

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To my family

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“Genius is 1% talent and 99% hard work”

(Albert Einstein)

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ABBREVIATIONS

CKD: chronic kidney disease

DBS: dried blood spot

GFR: glomerular filtration rate

24h-CrCl: twenty four hour creatinine clearance

LA: limits of agreement

MCG: modified Cockcroft-Gault

TDI: total deviation index

CCC: concordance correlation coefficient

Cp: coverage probability

HPLC-UV: ultra violet high performance liquid chromatography

IS: internal standard

QC: quality control

CL_1: one-compartment model

EMA: European Medicine Agency

LLOQ: Lower limit of quantification

ULOQ: upper limit of quantification

CV: coefficient of variation

SD: standard deviation

DMU: 1,3-dimethyluric acid

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INTRODUCTION

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A reliable determination of renal function is fundamental in many clinical situations such as the evaluation of patients with renal disease, staging of chronic kidney disease (CKD), risk prediction for disease progression, assessment of renal function over time, indication for dialysis therapy, screening of kidney living donors and adjustment of toxic drugs or procedures (contrast media) in patients with renal impairment, among others. Also, in clinical research, in studies in which renal function is the main outcome measure, an accurate and precise evaluation of renal function is essential to assess the evolution of renal disease and the efficacy of new therapies designed to prevent renal function loss.

The most widely used methods to evaluate renal function entail measurement of serum creatinine, twenty four hour creatinine clearance (24h-CrCl), or formulas based on creatinine and/or cystatin-C. During the last decades, more than 60 formulas to estimate renal function have been published. Some of them have become a very popular method to estimate renal function. However, there is abundant evidence in the literature showing that formulas do not reflect real renal function properly. The average error of any formula is about ±30% of measured renal function. This has been observed in diverse populations like: chronic kidney disease of diverse causes, type 2 diabetes, renal transplantation, polycystic kidney disease, cancer, heart failure, cirrhosis, liver transplantation, healthy living kidney donors, among others. On the other hand, gold standard methods are cumbersome and time-consuming, and so their use is mostly restricted to research centres. Thus, an alternative method to evaluate renal function is urgently needed in clinical practice and research.

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In this doctoral thesis we propose a simplification of a gold standard method to measure renal function: the plasma clearance of iohexol. This approach consists in the measurement of iohexol by dried blood spot (DBS) testing. This technique represents a huge reduction of the pre-analytical phase of the method. Also, DBS testing is a safe and cheap procedure. Firstly, we will discuss the limitations of current methods to estimate renal function. Secondly, we will describe the advantages of DBS testing.

SERUM CREATININE

Creatinine is an organic compound derived from creatine, a nitrogenous organic acid (methyl guanidine-acetic acid) synthesized in the liver and located mainly (~98%) in the muscles, where non-enzymatic dehydration converts creatine to creatinine (1) (Figure 1).

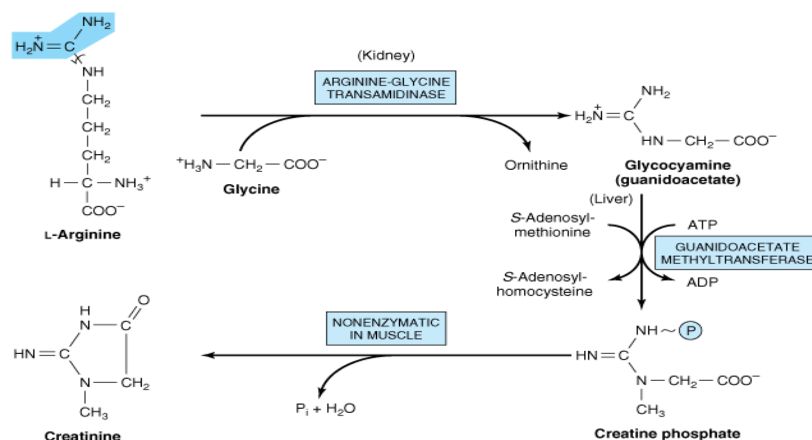

Figure 1: Schematic representation of creatine and creatinine metabolism, adapted from http://www.namrata.co/creatinine-a-marker-of-renal-function/

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SERUM CREATININE AS AN INDEX OF RENAL FUNCTION

Serum creatinine is the most widely used marker of renal function in routine clinical practice. In 1926, Rehberg first described the use of creatinine as a marker of glomerular filtration rate (GFR) (2). The technique to measure creatinine became available in 1938 (3). Since then, serum creatinine and 24h-CrCl have been used as reliable indicators of renal function.

An ideal marker of GFR must fulfil the following characteristics (4)**:**

1.- its production and plasma concentration must be constant
2.- it must be free in plasma (not binding to protein), freely and fully filtrated through the glomerulus.
3.- it must be neither secreted nor absorbed by renal tubules.
4.- it must be inert and non-toxic.
5.- it must be exclusively excreted by kidneys.
6.- it must be easily measured in both plasma and urine.

Moreover, the relationship between an ideal marker of GFR and real renal function (i.e. measured) must be rectilinear and inverse, as shown in Figure 2.

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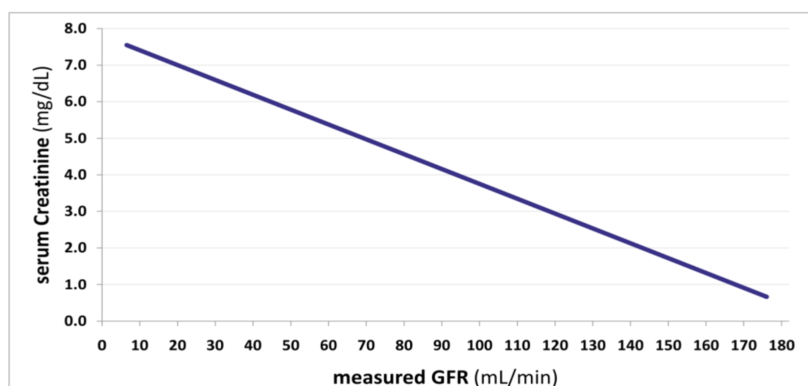

Figure 2: Relationship between a perfect marker of GFR and real renal function.

This means that a one-unit change in the marker i.e. creatinine, from 1 to 2 or from 2 to 3, correlates with a similar change in GFR. However, creatinine has a curvilinear relationship with real renal function, as depicted in Figure 3.

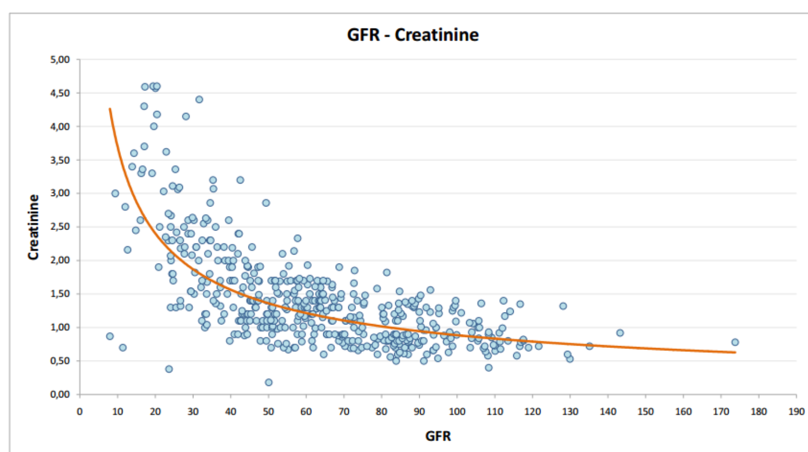

Figure 3: Relationship between serum creatinine and measured glomerular filtration rate (plasma clearance of iohexol), in 650 subjects with diverse causes of renal disease i.e. renal transplantation, chronic kidney disease, type 2 diabetes, living donors, heart failure, cancer, cirrhosis, liver transplantation and autosomal dominant polycystic kidney disease.

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LIMITATIONS OF SERUM CREATININE

The limitations of creatinine to reflect GFR are explained by the following causes: a.- tubular handling; b.- extra-renal clearance; c.- changes in muscular mass; d.- alterations in dietary intake; and e.- differences in the methods to measure creatinine.

a.- Tubular handling of creatinine: an ideal marker of renal function should not be metabolized, reabsorbed or secreted by tubular cells. More than eighty years ago, in 1935, Shannon demonstrated ***tubular secretion*** of creatinine for the first time (5). Later studies supported this finding (6). Increased tubular secretion to urine decreases serum creatinine, giving rise to a false overestimation of real renal function. Under normal conditions, tubular cells account for about 10% of total creatinine secretion, and this increases proportionally to the decrease in GFR (6-8), reaching 80-100% in advanced CKD (stages 3-4). Thus, tubular secretion of creatinine masks the reduction of renal function in patients with renal disease. Little evidence is available regarding ***the reabsorption of creatinine by tubular cells***. It has been reported in patients with uncontrolled diabetes (9,10) or even in healthy individuals (11). Increased tubular reabsorption of creatinine decreases urinary excretion and may lead to underestimation of real renal function.

b.- Extra-renal clearance of creatinine: In 1974, Jones et al demonstrated that up to 66% of creatinine can be metabolized or excreted via extra-renal routes, particularly in the gastrointestinal tract by gut flora, similarly to urea or uric acid. (12,13). This may

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contribute to the overestimation of GFR by creatinine and creatinine-based formulas in patients with renal disease.

c.- Changes in muscle mass: greater or lesser muscle mass may influence creatinine. In cases of reduced muscle mass as occurs in anorexia, advanced cirrhosis, children or in elderly subjects with age-related muscle mass loss, (14) the reduced production of creatinine may lead to overestimation of renal function. On the other hand, increased muscular mass or muscle consumption may determine a rise in serum creatinine and therefore an underestimation of GFR. These can be observed in patients on chronic glucocorticoid therapy (15), hyperthyroidism (16) or muscular atrophy. Additionally, vigorous exercise increases muscle mass and creatinine production from its precursor creatine, as well as changes in renal handling of creatinine (17,18). Finally, men generally have more muscle mass than women and this accounts for their greater creatinine production than in women.

d- Alterations in dietary intake: Meat is the major source of creatine and meat intake influences the total creatine pool and urinary creatinine excretion (*1*). Dietary protein deficiency leads to negative nitrogen balance and loss of muscle mass due to a reduced availability of the creatine precursors arginine and glycine (19,20). This is the case of malnourished individuals, and in vegetarians and vegans in whom dietary protein intake from meat is abolished.

e.- Methods to measure creatinine: Creatinine was named in 1847 for the first time by Liebig (21) and synthesized in 1885 by Horbaczewski (22). Jaffe described in 1886 its reaction with picrate under alkaline conditions to form a complex whose red-orange colour is easily detected and quantified (23). Then, Folin (24)

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demonstrated the utility of this reaction for the assay of creatinine in urine in 1904, and later in blood (25). Since then, this method has remained dominant in clinical laboratories. Currently, there are two principal methods for measuring serum creatinine in clinical laboratories: Jaffe and enzymatic. The method proposed by Jaffe is less expensive than the enzymatic method, 0.30€ versus 2.00€ per determination. However, the former is also more susceptible to interferences since a significant percentage (~20%) of the colour reaction comes from substances other than creatinine (non-creatinine chromogens) that are present in plasma. These positive interferences results in a creatinine value 20% higher than the true value and therefore an overestimation of creatinine levels in serum. Also, the Jaffe reaction has interference with high concentrations of bilirubin or other compounds present in the serum of jaundiced patients. So, the Jaffé method is gradually being replaced by the enzymatic method which is also more sensitive and specific.

IS CREATININE A GOOD MARKER OF RENAL FUNCTION?

Considering the above, serum creatinine is not a perfect marker of renal function. The correlation between creatinine and measured GFR is weak. A single value of creatinine is associated with a wide spectrum of GFR with a curvilinear pattern, as shown in Figure 3. Moreover, this indicates that a single value of creatinine, 1.50 mg/dl for example, is associated to a wide range of GFR from 30 to 90 ml/min (Figure 4).

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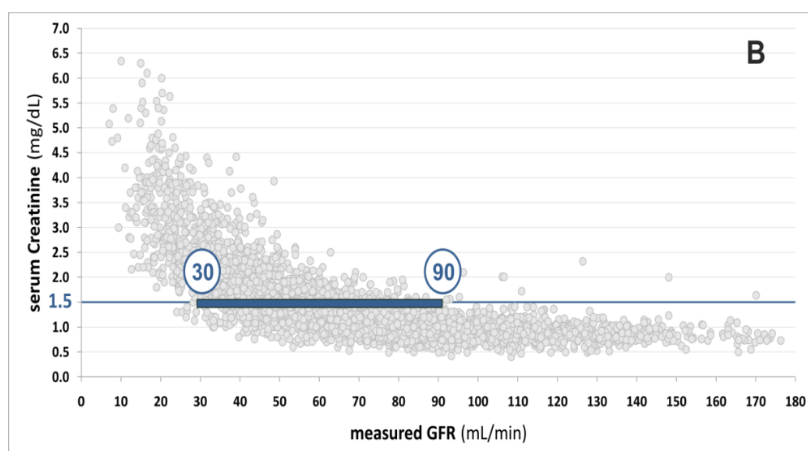

Figure 4: Relationship between serum creatinine and measured glomerular filtration rate (plasma clearance of iohexol) as in Figure 3. Serum creatinine of 1.5 mg/dl is associated with measured GFR ranging from 30 to 90 ml/min.

Notably, the curvilinear relationship between creatinine and was actually observed more than 30 years ago, in 1985, when Shemesh et al described the limitations of creatinine as a filtration marker in patients with CKD (6) (figure 5).

Importantly, the curvilinear correlation between creatinine and GFR was similar when creatinine was determined with the Jaffé method (Figure 5) or with the enzymatic method (Figure 3). Thus, the method used to measure creatinine seems not to have a major impact on the relationship between the marker and renal function.

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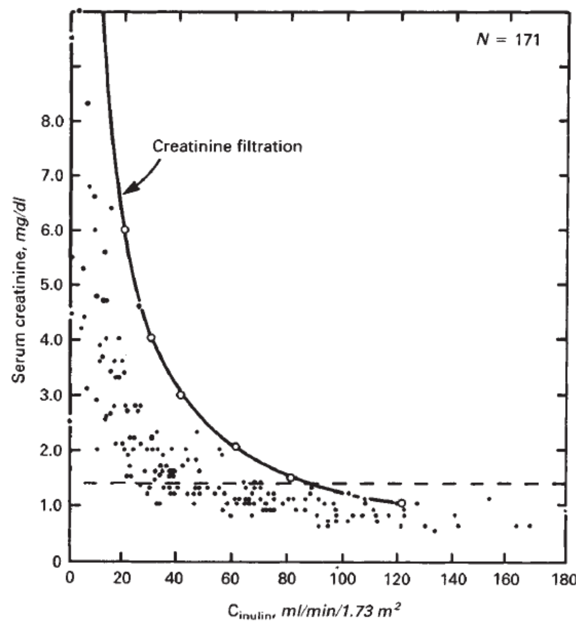

Figure 5: Correlation between creatinine and measured GFR in 171 patients with glomerular disease, adapted from Shemesh et al in Kidney International. 1985; 28 (5):830-838.

URINARY CREATININE CLEARANCE

Twenty four hour creatinine clearance (24h-CrCl) is considered a surrogate marker of GFR. It is calculated as the urinary concentration of creatinine multiplied by the volume of the timed urine sample (usually urine output during 24 hours), and divided by the average of creatinine serum concentration during the same time period:

$$\text{Creatinine Clearance} = \frac{\text{Creatinine}_{urine} \times \text{Volume}_{urine}}{1{,}440 \times \text{Creatinine}_{serum}}$$

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The limitations of serum creatinine as a marker of renal function are also applicable to urinary clearance of creatinine. Moreover, an additional limitation is that the collection of urine can be burdensome in some patients and timed collections are subject to errors caused by incomplete urine collection.

All the above evidence indicates poor agreement between creatinine and real renal function. This is possibly the major cause of the error of estimated GFR by creatinine-based formulas. However, we are not claiming that creatinine should not be used in clinical practice.

In spite of the lack of agreement between serum creatinine and measured GFR, this marker could be considered useful to evaluate renal function in daily clinical practice for those patients who do not require an accurate assessment of renal function. On the other hand, it is necessary to investigate new markers of renal function that can replace creatinine as a marker of GFR.

CYSTATIN-C

New biomarkers of renal function have been studied to obviate the error of creatinine in reflecting real GFR. In 1985, Grubb et al proposed cystatin-C as an accurate and precise biomarker of GFR (*26*).

CYSTATIN-C AS AN INDEX OF RENAL FUNCTION

Cystatin-C is a low-molecular weight (13kD) protein of the super-family of cysteine protease inhibitors. It is produced at a constant rate by a “housekeeping” gene that is present in all nucleated cells. Cystatin-C is freely filtered across the glomerulus.

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However, it is **reabsorbed and metabolized** by tubular epithelial cells (27,28), which precludes the use of 24h urine collection for clearance analysis. Also, high levels of cystatin-C are associated with age, male gender, black race, weight, height, cigarette smoking and subclinical inflammation, central adiposity, diabetes and metabolic syndrome (29-33). In fact, high cystatin-C predicts the appearance of diabetes. Thus, cystatin-C levels can increase as a consequence of the clinical situations listed above but that does not necessarily indicate reduced renal function. Finally, like serum creatinine, the relationship between cystatin-C and measured GFR is not rectilinear but curved as indicated in Figure 5.

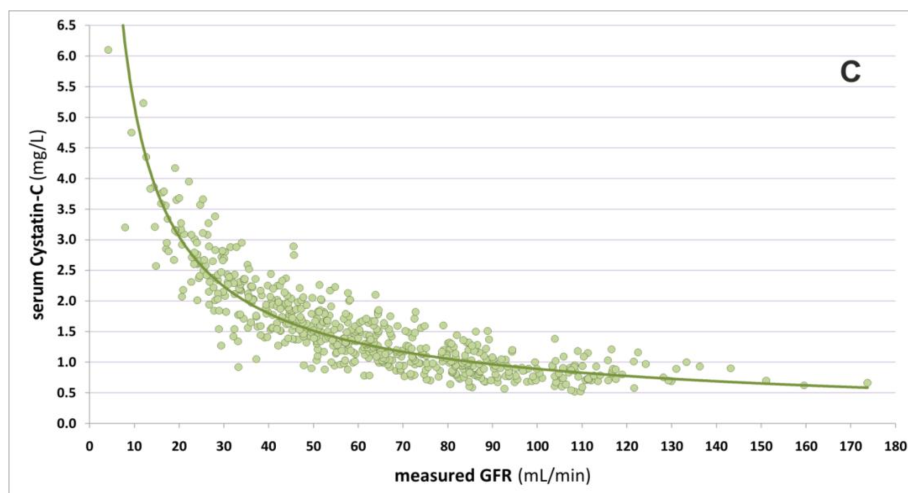

Figure 6: Relationship between serum cystatin-C with measured glomerular filtration rate (plasma clearance of iohexol), in 650 subjects with diverse causes of renal disease i.e. renal transplantation, chronic kidney disease of diverse causes, type 2 diabetes, living donors, heart failure, cancer, cirrhosis, liver transplantation and autosomal dominant polycystic kidney disease.

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The correlation between cystatin-C and measured GFR is low. Cystatin-C can remain stable while GFR decreases. For example, for a value of cystatin-C of 1.50 mg/dl, GFR value may range from 30 to 90 ml/min, as indicated in Figure 7.

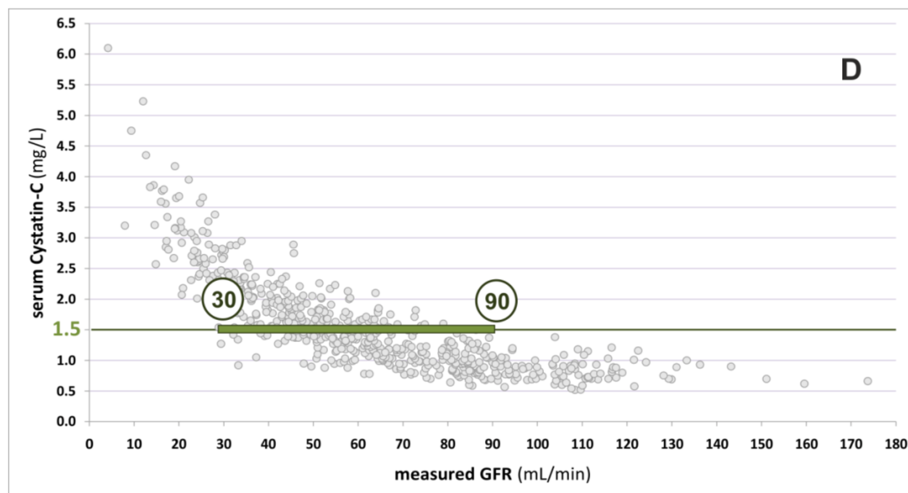

Figure 7: Relationship between serum cystatin-C and measured GFR (plasma clearance of iohexol) as in Figure 3.

GFR ESTIMATED BY FORMULAS

To overcome the limitations of creatinine and cystatin-C as markers of renal function, more than 60 formulas have been developed to estimate GFR. Formulas combine creatinine and/or cystatin-C with other variables like age, height, weight or gender in algorithms that can be very simple or extremely complex. Equations represent a simple and practical approach to the determination of renal function and accordingly have been extensively used in clinical practice and research. However, there is no consensus on the reliability of GFR estimated by formulas. Some

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authors support the use of equations (34-38), while others claim that the error of formulas precludes their use (39-44).

In 1957, Effersoe published the first formula based on serum creatinine to facilitate dose adjustments of toxic drugs in cancer patients (*45*). Few creatinine-based equations were reported in the following years (46-48). Then, in 1976 Cockcroft and Gault published a simple formula which became very popular among physicians (49). Many other formulas were reported during the eighties and nineties, until 2000, when Levey developed the MDRD equation using data from the Modification of Diet in Renal Disease study. In 2009, Levey proposed the Chronic Kidney Disease Epidemiology Collaboration formula (38). In 2000, Le Bricon published the first formula using cystatin-C (50). During the next few years more than 15 formulas based on cystatin-C were reported. The last approach has been the combination of creatinine and cystatin-C in the same equation (51-54). In 2012, the CKD-EPI Collaboration reported a formula using these two markers. (26).

However, no formula has proved to have acceptable agreement with measured GFR. The error of eGFR by any formula is at least ± 30% compared with measured GFR, and this error has been observed in the whole spectrum of GFR, from glomerular hyperfiltration, normal renal function, and moderate to advanced CKD and in diverse clinical situations, such as chronic kidney disease, diabetes, renal transplantation, polycystic kidney disease, cancer, heart failure, cirrhosis, liver transplantation, children with CKD, among others (Table 1).

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Author (year) Disease Type of study	N Ethnicity Method	Formulas	Statistics and error	Comments
Inker (55) (2016) CKD *Cross-sectional*	3551* Multiracial Iothalamate	**Cr:** CKD-EPIcr; **Cy:** CKD-EPIcy **CrCy:** CKD-EPIcc	**P30**:CKD-EPIcr: 84;CKD EPIcy: 83;CKD EPIcc:	error > ± 30% of mGFR: 10-25%
Padala (53) (2012) CKD *Longitudinal*	3531* Multiracial iothalamate	**Cr:** CKD-EPI	Difference between eGFR and mGFR decline greater than ± 5% in 11 to 33% of the subjects (average 22%).	Poor agreement between eGFR and mGFR decline.
Iliadis (56) (2011) Diabetes *Cross-sectional*	448 Caucasian ^{51}Cr-EDTA	**Cr:** MDRD, CKD-EPI **Cy**: 9 formulas **Cr-Cy**: Stevens	**P30:** from 21 (Perkins) to 81 (CKD-EPI)	error > ± 30% of mGFR: 20- 80% similar bias between creatinine-based and cystatin-C-based formulas
Gaspari (57) (2013) Diabetes *Longitudinal*	600* Caucasian Iohexol	**Cr:** 17 formulas	**GFR Baseline:** TDI: from 32% (Rule) to 93% (Jelliffe-2) CCC: from 0.21 (Jelliffe-2) to 0.52 (Rule) **GFR decline***:* CCC: from -0.21 (Effersoe) to 0.36 (Hull)	eGFR slower than mGFR decline
Luis-Lima (40) (2015) Renal Transplantation Cross-sectional	193 Caucasian Iohexol	***Cr***: 34 formulas **Cy:** 14 formulas **Cr-Cy***:* 3 formulas	***Cr***: CCC: from 0.04 (Rowe) to 0.84 (Matsuo); TDI (%): greater than 49 (Matsuo) **Cy:** CCC: from 0.67 (Perkins) to 0.90 (Rule,Hoek); TDI (%): from 34 (Hoek) to 83 (Perkins) **Cr-Cy:** CCC:Ma:0.85;Stevens:0.90;CKD-EPI:0.87; TDI (%): Ma: 49; Stevens: 37;CKD-EPI:47	eGFR showed a poor agreement with mGFR for all formulas 30 to 60% error in the classification of CKD
Bosma (58) (2005) Renal Transplantation Longitudinal	798 Caucasian ^{125}I-iothalamate	**Cr:** 9 formulas	**GFR baseline:** **P30**: from 72 (Rule) to 88 (MDRD, Jelliffe2) **GFR decline (ml/min/y):** mGFR: –1.9 ± 15 mL/min; eGFR: from 0.5 ± 12 (Jeliffe-1) to –2.3 ± 12 (Hull)	error > ± 30% of mGFR: 10-30% Poor agreement between eGFR and mGFR decline over time

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Author (year) Disease Type of study	N Ethnicity Method	Formulas	Statistics and error	Comments
Ruggenenti (59) (2012) ADPKD Longitudinal	111 Caucasian iohexol	Cr: MDRD, CKD-EPI	**GFR baseline:** **LA:** MDRD: -30.6 to 19.5; CKD-EPI: -21.3 to 27. **GFR decline (ml/min/y):** mGFR: - 8 ±10; CKD-EPI: -5 ± 9, MDRD: -4.5 ± 10. **LA:** MDRD: -22 to 29; CKD-EPI: -21 to 28.	Poor agreement between eGFR and mGFR decline. mGFR decline faster than eGFR decline
Craig (60) (2012) Cancer Cross-sectional	288 Caucasian ^{51}Cr-EDTA	**Cr:** CG, MDRD, CKD-EPI	**CD30:** CG: 86, MDRD: 86, CKD-EPI: 82 **CD10:** CG: 43, MDRD: 32, CKD-EPI: 25 **P30:** CG: 75, MDRD: 66, CKD-EPI: 65	Error of eGFR in carboplatin dose > ± 30%: 15-20% ~30% of cases had an overestimation dose based on eGFR > 20%
Gonwa (61) (2004) Cirrhosis Cross-sectional	1447 Caucasian Iothalamate	**Cr:** CG, MDRD, Nankivell	**P30**: CG: 61, MDRD: 67, Nankivell: 64	error > ± 30% of mGFR: 30-40%
Francoz (62) (2014) Liver transplantation Cross-sectional	300 Caucasian iohexol	**Cr:** MDRD, CKD-EPI	**LA:** MDRD: -67 to 37; CKD-EPI: -54 to 35	Wide limits of agreement
Schwartz (63) (2009) Pediatrics, Cross-sectional	349 Caucasian (16% black/other) iohexol	**Cr:** Counahan, Leger **Cy:** Filler, Grubb **Cr-cy:** Bouvet, Zappitelli, Schwartz	P30: Bouvet: 80, Zappitelli: 82, Schwartz: 83, Filler: 72, Grubb: 72, Counahan: 73, Leger: 71	error > ± 30% of mGFR: 20-30%

Table 1: Studies that evaluated the performance of estimated GFR in several clinical conditions. P30, P10: percentage of estimations included within an error of ±30% or ±10% of measured GFR, respectively; Cr: creatinine; Cy: Cystatin-c; CG: Cockcroft-Gault; LA: limits of agreement, GFR in ml/minutes/1.73m^2; CKD: chronic kidney disease, MCG: modified Cockcroft-Gault; TDI: total deviation index, CCC: concordance correlation coefficient.

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CONSEQUENCES OF THE ERROR OF FORMULAS

The low accuracy and precision of estimated GFR has important **consequences in clinical practice:**

1.- in the evaluation of patients with chronic kidney disease such as diabetes, renal transplantation, polycystic kidney disease, glomerulonephritis, children with renal diseases, etc. As the average error of any formula is about ±30% of measured GFR, in patients with real GFR of 60 ml/min, formulas may range from 42 (-30%) to 78 (+30%) ml/min. This makes the evaluation of renal function difficult in clinical practice. (40, 55, 56, 63)

2.- to detect changes of GFR decline during follow-up. Several studies have shown the lack of reliability of equations to assess the evolution of GFR over time. Formulas may estimate either a faster or a slower decline compared with real GFR decline. (53, 57-59)

3.- in the risk assessment of renal disease progression and the determination of CKD stages. Diverse studies show that 30 to 60% of patients were incorrectly classified in lower or higher CKD stages.

4.- in the selection of living donors for kidney transplantation. Overestimation of GFR may lead to the selection of living donors with real renal function below the cut-off for donation.

5.- in clinical conditions associated with a high risk for renal disease: non-renal transplant patients, chronic liver disease, chronic heart failure, etc. (61, 62, 64)

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6.- in drug dosing or procedures that involve potentially toxic drugs like carboplatin or contrast media in patients with CKD. Several studies have demonstrated over-dosing of carboplatin when formulas are used to calculate the dose. (60)

The error of formulas also has consequences in **clinical research**. Several studies have shown that GFR decline estimated with formulas was either faster or slower than that of measured GFR. Therefore, these formulas do not allow a rigorous assessment of renal function and their use may generate misleading information on the effectiveness of new drugs to slow renal disease progression. A recent review indicated that the use of estimated GFR by formulas (and not gold standard methods) is responsible for the failure of many studies in nephrology (65).

An alternative to estimated GFR is the use of gold standard techniques to measure renal function. However, they are cumbersome due to technical and logistic limitations, as mentioned below.

MEASUREMENT OF RENAL FUNCTION BY GOLD STANDARD METHODS

Direct measurements of GFR that evaluate the disappearance of exogenous substances eliminated only by glomerular filtration have been available since 1951 (66-68). These include inulin which is considered the gold standard to measure GFR. However, it requires continuous intravenous infusion of the marker and urine collections at timed intervals by bladder catheterization. Therefore, simpler methods have been developed,

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using the plasma disappearance of radioactive exogenous markers such as ^{51}Cr-EDTA (Ethylenediaminetetra-acetic acid), $^{Tc\text{-}99m}$DTPA (diethylenetriaminepentaacetic acid), and non-radioactive substances like iothalamate and iohexol. All these methods showed good agreement with inulin clearance (67). However, they require specialized procedures and are time consuming, which limit their use in daily clinical practice. ^{51}Cr-EDTA and $^{Tc\text{-}99m}$DTPA have the specific limitations of using radio-labeled substances, which require radioactive licensing and compliance with regulations governing the storage and disposal of radioactive material.

Non-radioactive markers like iothalamate (mainly used in the United States) and iohexol (mainly used in Europe) have been proposed as more practical approaches to evaluate GFR. The choice of a method mostly depends on previous experience and tradition of each centre. In our laboratory, we have used the plasma clearance of iohexol since 2012 (69). This method, from our point of view, is an excellent choice to measure GFR, since it is simple, reproducible, reliable, inexpensive and safe. The method is simple because it only requires the intravenous injection of a small volume of the marker (5 mL), followed by repeated blood extraction. Additionally, neither extra-renal clearance nor renal metabolism of iohexol have been reported, which makes plasma and urinary clearances interchangeable and urine collection unnecessary (67). Moreover, iohexol clearance is reproducible and reliable since it is measured by a standard chromatographic technique, ultra violet high performance liquid chromatography (HPLC-UV) which enables accurate quantification of low plasma concentrations. Notably, the inter-laboratory reproducibility of iohexol

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measurement is about 5%, according to an external quality programme developed by Equalis AB (Uppsala, Sweden) in which 35 laboratories compared their results every 3 months. Furthermore, the cost of iohexol plasma clearance is low (about 200 euros). Finally, its safety profile is excellent, with only minor side effects reported (70, Flavio Gaspari and Sergio Luis Lima, personal communications). An extensive review on the advantages of the plasma clearance of iohexol in clinical practice and research has recently been published (71,72).

However, gold standard methods to measure GFR are still considered cumbersome and impractical by the medical community, and are therefore underused. They require multiple venous blood sample extractions which make the nursing protocol costly and time consuming. The pre-analytical phase is cumbersome because the liquid blood samples must subsequently be centrifuged, aliquoted to separate plasma from red blood cells, stored in freezer, thawed and re-aliquoted for analysis. All this requires the availability of pre-analytical infrastructures that make the whole procedure burdensome.

In our view, a simplification of the plasma clearance of iohexol may help to disseminate its use in clinical practice and research.

HOW TO SIMPLIFY THE MEASUREMENT OF GFR?

The plasma clearance of iohexol requires several blood extractions during 4 to 8 hours. Some authors have proposed simplifying the method by **reducing the number of blood**

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extractions to one sample (73). However, this approach has shown a ± 40% error of real renal function (74).

Another way to simplify the measurement of GFR is to use **dried blood spots (DBS) on filter paper.** DBS sampling offers the following *practical, clinical and financial* advantages in comparison with the conventional blood testing:

(a) Improvement of patient comfort: capillary blood samples are extracted after painless finger-prick with a lancet and so DBS sampling is less invasive than standard venipuncture. The procedure also minimizes the number of phlebotomies. Also, it can be performed at health centers, so the patient does not need to travel to a third level hospital.

(b) Simplification of sample storage because DBS samples are collected on filter paper where the capillary blood is placed and dried. So, no tubes are needed, easing the storage in clinical laboratories and biobanks as well as reduces cost.

(c) Reduction of shipment costs since the samples can be sent by regular mail at room temperature. This is especially useful for sampling in remote communities located far away from a testing laboratory and in multi-centre clinical trials;

(d) Increased safety because there is neither virus nor bacterial-related biohazard due to the loss of infectivity as a consequence of disruption of their envelope on drying.

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(e) Significant overall cost reduction due to fewer requirements in trained staff and processing since catheterization and tubes sampling are not necessary.

DBS TO MEASURE IOHEXOL AND RENAL FUNCTION

The use of a dry matrix of blood as a method of sampling was first described by Ivar Bang in 1913 (75). In 1963, Robert Guthrie used DBS analysis for the detection of phenylalanine for the diagnosis of phenylketonuria in neonates. Since then, many uses of DBS have been reported for collection, storage, and analysis of human blood in the screening of metabolic disorders (76), therapeutic drug monitoring (77), and epidemiological studies.

The general advantages of DBS for the measurement of iohexol in comparison with conventional blood sampling are those listed above. Moreover, the molecule of iohexol has several advantages *per se* which make it particularly suitable for the DBS technique: (a) it is very stable at room temperature which greatly simplifies the pre-analytical phase; (b) it is a non-isotopic cold marker, which makes it measurable by standard techniques like HPLV-UV; (c) there is no red blood cell partitioning for iohexol, allowing the use of whole blood and therefore DBS.

In DBS sampling, blood is obtained via finger-prick with an automatic lancet. Next, the drop of blood is applied to sampling paper and allowed to dry. Finally a circle of filter paper containing the DBS is punched out to extract and analyse the molecule of interest. Two approaches for DBS sampling have been described:

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(a) **NON-VOLUMETRIC SAMPLING** (partial spot methods) in which a blood drop is placed directly on the paper and a non-fixed volume of the sample is taken for analysis. This approach does not consider the effect of hematocrit on blood viscosity, which affects the flux properties of blood. Thus, the diffusion of blood on filter paper is lower for cases with high hematocrit, and higher for cases with low hematocrit. This issue is important, since the diffusion of blood will determine the volume of plasma contained in the punched out DBS sample.

(b) **VOLUMETRIC SAMPLING** (whole spot methods) which involves an intermediate step for sampling using a volumetric device (i.e. heparinized capillary pipette) and a paper circle containing the whole blood sample is punched out, and hence a fixed volume of the sample is taken for analysis. The same volume must be taken and placed on the filter paper in all cases. For example, a capillary pipette of 10 µl must be completely filled with blood and the whole volume must be placed on the filter paper. Finally, a spot is punched out and the circle of paper will contain the whole volume of blood.

To our knowledge, only one study has evaluated the measurement of iohexol using the DBS technique. In 2007, Niculescu-Duvaz assessed the performance of DBS analysis to determine GFR in comparison with the gold standard method (plasma analysis) (78). The authors demostrated low accuracy and precision and wide limits of agreement between the gold standard and the DBS-based methods: ranging from -14 to 16 ml/min. This error is unacceptable which indicates that the DBS cannot replace

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the standard method. The authors used the non-volumetric approach for DBS sampling, and so the cause of the error may be related to the non-reproducible blood volume absorbed on the fixed portion of the filter paper used for the analysis of iohexol.

Thus, a reliable, accurate and precise DBS technique to measure iohexol and GFR has not yet been completely developed. Also, there is no rigorous validation of DBS (volumetric and non-volumetric) against the plasma method.

The results of our study may represent a huge simplification of the measurement of renal function in clinical practice, without sacrificing accuracy and precision. This may offer the medical community a more reliable and practical tool to assess renal function. Also, simplification of the method will facilitate the plasma clearance of iohexol to be used in clinical trials.

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OBJECTIVES

(a) To simplify the iohexol plasma clearance method to measure GFR by DBS analysis in order to disseminate its use in clinical practice and research.

(b) To evaluate the agreement between the simplified and the reference method to measure GFR.

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HYPOTHESIS

The DBS method to measure GFR can be used without loss of accuracy and precision with respect to the gold standard technique.

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METHODS

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The implementation of a new method requires validation with a reference method. For this study, the reference method used to determine iohexol and glomerular filtration rate (GFR) is based on the analysis of plasma samples. The new method to determine iohexol and GFR is based on the analysis of DBS samples on filter paper.

REFERENCE METHOD BASED ON PLASMA SAMPLES

Sampling

The sampling procedure to determine the clearance of iohexol by the reference plasma analysis method consists in the following steps: a) injection of iohexol: 5 ml of the iohexol solution (Omnipaque 300, GE Healthcare) are intravenously injected during 2 minutes; b) sample extraction: 3 mL of blood are taken at different time intervals according to a long protocol: 120, 180, 240, 300, 360, 420, and 480 min after iohexol injection, for individuals with expected mGFR ≤ 40 mL/min (estimated using the MDRD formula); and a short protocol: 120, 150, 180, 210, and 240 min for those with expected mGFR >40 mL/min; c) blood samples are centrifuged, aliquoted to separate plasma from red blood cells, and stored at -20ºC; d) the plasma samples are thawed and re-aliquoted for analysis.

Sample preparation

Two-hundred microliters of the plasma samples are added to 50 µl of the internal standard, that is 1,3-dimethyluric acid, DMU (500 µg/ml in phosphate buffer, pH 7.4), and deproteinized by

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adding 750 µl of perchloric acid (5%), which is then subject to vortex mixing and centrifuging.

Chromatographic conditions

Five microliters of the supernatant are analyzed with a HPLC system (Agilent Series 1260, Spain) equipped with a diode array detector set at 254 nm, and chromatographed by a C18 reverse phase column (5 µm, 150 × 4.6 mm, Advanced Chromatography Technologies LTD, Scotland). Iohexol and internal standard (IS) DMU are eluted in a mobile phase of deionized water (96%, adjusted to pH 2.5 with phosphoric acid) and acetonitrile (4%), pumped at a flow rate of 1.0 mL/min.

Quality assurance

A working solution of iohexol (647 µg/ml) is prepared in deionized water and used for the calibration curve and quality control samples. A total of five concentrations of iohexol: 32.35; 64,7; 97,05; 129,4 and 161,75 µg/ml, in iohexol-free plasma (healthy individuals), are used as calibrators. Two in-house quality control standards (QCs), containing iohexol at low (64,7 µg/ml) and high (129,4 µg/ml) concentrations are also prepared and used for assay validation (table 2). Aliquots of the calibrators, quality control samples and reference standard solutions are stored at −20ºC until use and thawed for analysis. Calibration curves and quality controls are prepared for each set of samples. The chromatographic method is based on the study reported by Krutzen et al in 1984 (79), and has been previously validated and published by other authors (80,81).

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	Plasma (µL)	IS (µL)	Iohexol (µL)	$HClO_4$ (µL)	Added iohexol (µg)	Iohexol conc (µg/mL)
Calibrator 1	200	50	10	740	6,5	32,4
Calibrator 2	200	50	20	730	12,9	64,7
Calibrator 3	200	50	30	720	19,4	97,1
Calibrator 4	200	50	40	710	25,9	129,4
Calibrator 5	200	50	50	700	32,4	161,8
Blank	200	0	0	800	-	-
QC 1	200	50	20	730	12,9	64,7
QC 2	200	50	40	710	25,9	129,4

Table 2: Quality assurance for the reference method (plasma analysis). IS: DMU (500 µg/ml); Iohexol (working solution, 647 µg/mL); $HClO_4$: perchloric acid.

Iohexol and GFR determination

Iohexol levels are determined by measuring the height of the second iohexol peak (iohexol eluted from the chromatographic column as two peaks, at 5.8 and 6.8 minutes, reflecting the endo- and exo- isomers present in the pharmacologic preparation) and the internal standard peak. The peak height ratio is calculated and its values by means of the calibration curve return the iohexol concentration.

GFR determined by the plasma clearance of iohexol is calculated according to a one-compartment model (CL_1) by the formula: CL_1 = Dose/AUC, where AUC is the area under the plasma concentration-time curve. In accordance with the procedure described by Bröchner-Mortensen (82), the plasma clearance of iohexol is finally corrected using the formula $CL = (0.990778 \times CL_1) - (0.001218 \times CL_1^2)$.

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METHOD BASED ON DRIED BLOOD SPOT

In order to select the best DBS sampling approach (*non-volumetric* or *volumetric)* to determine iohexol and therefore GFR, the agreement between plasma and both DBS analyses was evaluated.

Sample preparation

The procedure to extract iohexol from DBS samples is based on that described by Niculescu-Duvaz with minor modifications (84). The DBS sample is subject to the following procedure: 1. the filter paper is punched out to obtain a partial (non-volumetric) or the whole blood sample (volumetric) (figure 8); 2. the sample is placed in a tube with 250 µl of 5% perchloric acid with the internal standard (DMU, 100 µg/mL) and subjected to vortex mixing for 3 minutes to deproteinize the sample; 3. The tube is ultrasonicated for 15 minutes, incubated at room temperature for 30 minutes and then centrifuged at 12.500 rpm for 10 minutes; finally 25 µl of supernatant is analyzed in the HPLC-UV system.

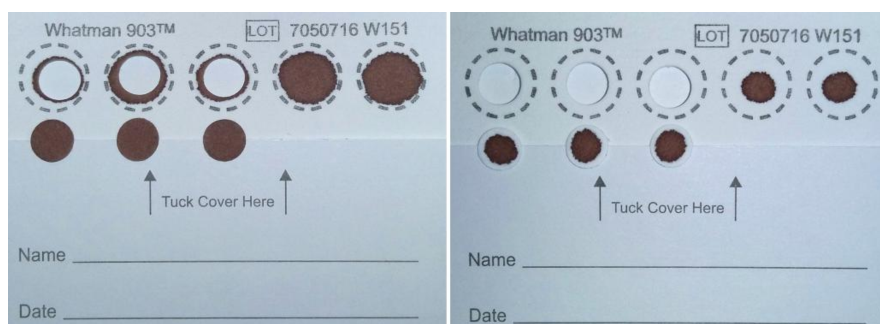

Figure 8: Punch out of DBS samples for the non-volumetric (left panel) and volumetric (right panel) sampling to obtain a partial blood sample.

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Chromatographic conditions

The chromatographic conditions are the same as those described for the analysis in plasma.

DBS TESTING: STUDIES “IN VITRO”

PRELIMINARY ANALYSIS

Five samples of total blood from healthy individuals were spiked with the following concentrations of iohexol: 32,35; 64,7; 97,05; 129,4 and 161,75 μg/mL to prepare the standards for analysis. Each standard was created in triplicate. Then, a fixed volume of blood (10 μL) from these samples was taken from the tube using a heparinized capillary pipette, placed on filter paper and allowed to dry at room temperature.

Analysis of the results

Precision (expressed as the coefficient of variation of the three replicas) of the height of the peaks for iohexol and the internal standard (DMU) is determined.

ANALYSIS WITH PHARMACOKINETIC CURVES

For this analysis, we took advantage of 50 consecutive patients undergoing the plasma clearance of iohexol procedure at our centre. After blood extraction, both plasma and blood samples were taken from the tubes to determine iohexol concentrations based on the volumetric and non-volumetric methods. Then, GFR was determined using iohexol concentrations obtained by plasma analysis, and both DBS methods. Finally, the agreement between these methods was evaluated using the statistics described below.

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Non-volumetric sampling

This procedure consists in the following steps: a) injection of iohexol as previously described; b) sample extraction: 3 mL of blood are taken at different time intervals according with the long or the short protocols; c) a variable volume of blood (from 20 to 40 µL) to simulate different sizes of real drops is taken from the tube with and automatic pipette, placed onto a filter paper and allowed to dry at room temperature.

Volumetric sampling

The procedure consists in the following steps: a) injection of iohexol as previously described; b) sample extraction: 3 mL of blood are taken at different time intervals according to the long or the short protocols; c) a fixed volume of blood (10 µL) is taken from the tube using a heparinized capillary pipette, placed onto a filter paper and allowed to dry at room temperature

Sample preparation

The sample preparation is the same as that described for the preliminary analysis in DBS testing: studies "in vitro".

Chromatographic conditions

The chromatographic conditions are the same as those described for the analysis in plasma.

Quality assurance

A working solution of iohexol (647 µg/ml) is prepared in deionized water and used for the calibration curve and quality control samples. A total of five concentrations of iohexol: 32.35;

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64,7; 97,05; 129,4 and 161,75 µg/ml iohexol-free blood (healthy individuals) are used as calibrators. Two in-house quality control standards (QCs), containing iohexol at low (64,7 µg/ml) and high (129,4 µg/ml) concentrations are also prepared and used for assay validation (table 3). Aliquots of the calibrators, quality control samples and reference standard solutions are stored at −20ºC until use.

	Blood calibrators preparation			DBS calibrators preparation		
	Blood (µL)	Iohexol (µL)	Added iohexol (µg)	DBS (µL)	$HClO_4$+ IS (µL)	Iohexol conc (µg/mL)
C1	190	10	6,5	10	250	32,4
C2	180	20	12,9	10	250	64,7
C3	170	30	19,4	10	250	97,1
C4	160	40	25,9	10	250	129,4
C5	150	50	32,4	10	250	161,8
Blank	200	0	-	10	250	-
QC 1	180	20	12,9	10	250	64,7
QC 2	160	40	25,9	10	250	129,4

Table 3: Quality assurance for the DBS method. IS: iopamidol (3 µg/ml), Iohexol (working solution, 647 µg/mL); $HClO_4$: perchloric acid.

Iohexol and GFR determination

To estimate the real plasma levels of iohexol using DBS samples for both non-volumetic and volumetric approaches, the measured concentrations of iohexol are corrected according to hematocrit using the following formula: iox_pl = iox_dbs / (1- hto), where "iox_pl" is the estimated plasma concentration of iohexol; "iox_dbs" is the measured level of iohexol in DBS samples; and "hto" is the patient's hematocrit value. The hematocrit value is determined using an automated Beckman Coulter instrument at the

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Central Laboratory, Hospital Universitario de Canarias. GFR was determined in the same way as described for the analysis in plasma.

DBS TESTING: STUDIES "IN VIVO"

Renal function of 200 patients with diverse clinical conditions was determined using both the reference (plasma analysis) and the DBS methods. **Based on the results of this thesis (please see results), only the volumetric approach was used "*in vivo*".** GFR values were compared using the statistics of agreement proposed by Lin et al (86)

Sampling

The volumetric DBS used in humans consists in the following steps: a) injection of iohexol as previously described; b) sample extraction: capillary blood is obtained via finger-prick with an automatic lancet, and a fixed volume of 10 μL is taken and applied to filter paper and allowed to dry; c) this procedure is repeated at each interval according to the long and short protocols; d) the DBS samples are stored at room temperature until analysis.

Simultaneously, the plasma analysis is performed in the same patient (as described in point 1) for the comparison of the two methods.

Sample preparation

The sample preparation is the same as that described in the section DBS testing: Studies "in vitro".

Chromatographic conditions

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The chromatographic conditions are the same as those described in the section DBS testing: Studies "in vitro".

Quality assurance

Quality assurance is the same as that described in the section DBS testing: Studies "in vitro".

GFR determination

GFR determination is the same as that described for the analysis in plasma.

STABILITY OF GFR MEASUREMENT BY DBS TESTING

Storage conditions

To demonstrate the stability of iohexol in DBS samples at room temperature, 20 samples corresponding to time points of pharmacokinetic curves were stored during 2, 4, 8 and 12 weeks and then analyzed and compared to the plasma analysis. In the same way, 15 samples corresponding to time points on pharmacokinetic curves were frozen at -20ºC during 4, 12 and 24 weeks, and then thawed, analyzed and compared to the plasma analysis. Acceptable differences between GFR values should be within ±10%.

Shipment conditions

Eighteen samples corresponding to different time points on pharmacokinetic curves, at room temperature, were shipped by regular mail to different European centers, which returned the DBS samples to our laboratory. The samples were analyzed and GFR

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values were compared to the results of plasma analysis. Acceptable differences between GFR values should be within ±10%.

STATISTICS OF AGREEMENT

Validation is one of the most important challenges before implementing a new method in the laboratory. To assess the validity of DBS testing, it is necessary to define *a priori* meaningful margins of error and the proportion of observations that should fall within these margins. In other words, one needs to establish how much disagreement between the new and the reference methods is tolerable. Although a margin of error of 15% is acceptable in chromatography, it seems reasonable for the case of measured GFR to propose that the vast majority of the observations by DBS testing, i.e. 90%, should fall within a ±10% error of those values obtained with the reference method. The ±10% criterion derives from the fact that it reflects the overall reproducibility of a reference method to measure GFR (71).

The agreement between the reference (plasma analysis) and the new method (DBS testing) was assessed by the statistics of agreement proposed by Lin et al (85). These tests include the limits of agreement described in 1986 by Bland and Altman (88), total deviation index (TDI), concordance correlation coefficient (CCC) and coverage probability (CP). The limits of agreement are a simple graphic tool which describes the limits that include the majority of the differences between two measurements. The narrower these limits are, the better the agreement. CCC combines elements of accuracy and precision, with scores ranging from 0 to 1, and a value greater than 0.90 reflects optimal concordance between

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measurements. TDI is a measure that captures a large proportion of data within a boundary for allowed differences between two measurements (87). CP ranges from 0 to 1; it is a statistic that estimates whether a given TDI is less than a pre-specified percentage (87). The ideal situation is to have a TDI <10%, meaning that 90% of the estimations fall within an error of ± 10% of mGFR. Finally, these statistics provide confidence intervals which allow generalization of the results.

VALIDATION OF DBS TESTING IN COMPLIANCE WITH EMA GUIDELINES

The final method was tested and validated "in vitro" before implementation in vivo, in accord with EMA guidelines. Validation of the DBS method was performed in compliance with the European Medicine Agency (EMA) guidelines (83). The main objective was to demonstrate the reliability of DBS testing for the determination of iohexol concentrations in a specific biological matrix like dried blood, using the same anticoagulant as for the study samples. We evaluated the following aspects: selectivity, lower limit of quantification, response function and calibration range (calibration curve performance), accuracy, precision, matrix effects, stability of the analyte in the biological matrix, and in stock and working solutions, as well as the stability of the internal standard.

To prepare calibration standards, quality control and stability samples, as well as internal standard, we spiked samples of the biological matrix (blood) with iohexol from stock solution.

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Selectivity: the analytical method should be able to differentiate the analyte of interest (iohexol) and the internal standard from different interferences: endogenous components of the matrix, metabolites, degradation products formed during sample preparation or possible co-administered medications. Selectivity was proved using 20 different blank matrixes from patients with several clinical conditions such as (CKD, type 2 diabetes, renal transplantation, polycystic kidney disease, cancer, heart failure, *cirrhosis,* liver transplantation, healthy living kidney donors). Absence of interfering components was accepted with a response < 20% of the lower limit of quantification for iohexol and < 5% for the internal standard.

Carry-over: was assessed by injecting blank blood samples after a high concentration of iohexol at the upper limit of quantification (161,75 µg/mL). Carry-over in the blank sample following the high concentration of iohexol should not be greater than 20% of the lower limit of quantification (16,17 µg/mL) for iohexol and 5% for the internal standard.

Lower limit of quantification (LLOQ): is the lowest concentration that can be quantified with acceptable accuracy and precision. The LLOQ is considered the lowest calibration standard. The analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample.

Calibration curve: the response of the instrument should be evaluated over a specified concentration range of iohexol. The calibration standards were prepared by spiking the blank matrix (whole blood) with known concentrations of iohexol. The calibration curve should range from the lowest calibration

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standard (lower limit of quantification, LLOQ) to the highest calibration standard (upper limit of quantification, ULOQ). A minimum of six concentration levels were used (16,17; 32,35; 64,7; 97,05; 129,4; 161,75 μg/mL) to calculate the calibration curve parameters. Additionally, a blank sample (without iohexol and internal standard) and a zero sample (with internal standard) were analyzed. The experimental back-calculated concentrations of the calibration standards for iohexol were compared with the theoretical levels, which should be within ±15% of the nominal value, except for the LLOQ that should be within ±20%. The slope and intercept were calculated. At least 75% of the calibration standards must fulfill this criterion. At least three acceptable pharmacokinetics curves obtained during validation were recorded.

Accuracy was assessed using quality control (QC) samples spiked with known amounts of iohexol, independently from the calibration standards, and using separately prepared stock solutions. The QC samples were analyzed against the calibration curve, and the concentrations obtained were compared with the nominal value. Accuracy was evaluated for the values of the QC samples obtained within a single run (within-run accuracy) and in different runs (between-run accuracy).

Within-run accuracy was determined in a single run using 5 samples (replicas) for the following concentrations of iohexol: 16,17 μg/mL (LLOQ) 32,35 μg/mL (low QC), 64,7 μg/mL (medium QC), 161,75 μg/mL (high QC), which cover the whole range of the calibration curve. The mean concentration of the five replicas should be within 15% of the nominal values for the QC samples,

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except for the LLOQ which should be within 20% of the nominal value.

Between-run accuracy was evaluated using LLOQ, low, medium and high QC samples analyzed in three runs on at least two different days. The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Precision: describes the closeness of repeated individual measures of iohexol, and is expressed as the coefficient of variation (CV). Precision was demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs.

Within-run precision: was determined by the analysis of five samples (replicas) for each level of iohexol: 16,17 μg/mL (LLOQ) 32,35 μg/mL (low QC), 64,7 μg/mL (medium QC), 161,75 μg/mL (high QC), which cover the whole range of the calibration curve. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Between-run precision: was evaluated using LLOQ, low, medium and high QC samples analyzed in three runs on at least two different days. The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%. Accuracy was expressed as the ratio between the experimental back-calculated concentrations and the nominal values, multiplied by 100. Precision was expressed as the ratio between the standard deviation (SD) and the mean, multiplied by 100.

Dilution integrity: dilution of samples should not affect accuracy and precision. Dilution integrity was tested by spiking the

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matrix with an iohexol concentration above the ULOQ (323,5 μg/mL) and diluting this sample with blank matrix. Accuracy and precision should be within ±15%.

Stability of iohexol in DBS samples was evaluated using QC samples at a concentration of 32,35 μg/mL (low QC) and 161,75 μg/mL (high QC) and analyzed after different storage conditions (room temperature, -20ºC and -80ºC). The calibration curve for this analysis was obtained from freshly spiked calibration standards, and the concentrations of QC samples were compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration.

The following stability tests were evaluated:

1. **Freeze and thaw stability**: low QC and high QC were frozen at -20 and thereafter thawed at room temperature. After complete thawing, samples were frozen for at least 12 hours and then thawed again. These freeze-thaw cycles were repeated three times.

2. **Short-term stability**: five replicas of each QC samples, at 32,35 μg/mL (low QC) and 161,75 μg/mL (high QC) were stored at room temperature and analyzed after 2 and 4 weeks. Also, five replicas of low and high QC samples were frozen at -20 and -80ºC and thereafter thawed at room temperature and analyzed after 4 weeks. The mean, standard deviation (SD) and coefficient of variation (CV) of the 5 replicas were calculated and compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration.

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3. **Long-term stability**: five replicas of each QC sample, at 32,35 µg/mL (low QC) and 161,75 µg/mL (high QC) were stored at room temperature and analyzed after 8 weeks. Also, five replicas of low and high QC samples were frozen at -20 and -80ºC and thereafter thawed at room temperature and analyzed after 12 and 24 weeks. The mean, SD and CV% of the 5 replicas were calculated and compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration.

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RESULTS

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DBS TESTING: STUDIES "IN VITRO"

PRELIMINARY ANALYSIS

Analysis of iohexol for DBS testing

The variability in the height of the peaks of iohexol for the three replicas of each sample was extremely low (CV% < 3%) for all the QC samples (table 4). *This indicates that the procedure to extract iohexol from filter paper is highly reproducible.*

Preliminary analysis of iohexol by DBS					
QC/replica	1	2	3	4	5
1	17,9	35,4	55	72,9	91,8
2	18,6	35,9	54,0	72,9	91,0
3	17,5	36,1	54,4	72,6	90,7
mean ± SD	18,0 ± 0,5	35,8 ± 0,3	54,5 ± 0,5	72,8 ± 0,2	91,2 ± 0,6
precision %	2,89	0,96	0,99	0,24	0,63

Table 4: Analysis of iohexol by DBS testing. Values represent the heights for the peak of iohexol.

Analysis of the internal standard for DBS testing

The variability in the height of the peaks of DMU for the fifteen analyzed samples was high (CV = 9%) (Table 5). This may indicate red blood cell partitioning for DMU, which disqualifies its use as an internal standard. Thus, based on the previous results, we performed the analysis in DBS without an internal standard, using external calibration.

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Preliminary analysis of DMU by DBS	
QC/replica	Height
1	33,4
2	31,6
3	26,4
4	25,4
5	27,6
6	26,5
7	31,2
8	31,0
9	30,4
10	32,2
11	32,5
12	33,0
13	32,5
14	33,5
15	29,7
mean ± SD	30,5 ± 2,7
precision %	9,0

Table 5: Analysis of 1,3-dimethyluric acid (DMU) by DBS testing. Values represent the heights for the peak of DMU.

ANALYSIS WITH PHARMACOKINETIC CURVES

Non-volumetric sampling

A variable volume of blood (from 30 to 40 µL) was taken with a manual pipette to simulate different sizes of real drops. The drops were placed on filter paper and allowed to dry at room temperature for at least 24 hours (Figure 9).

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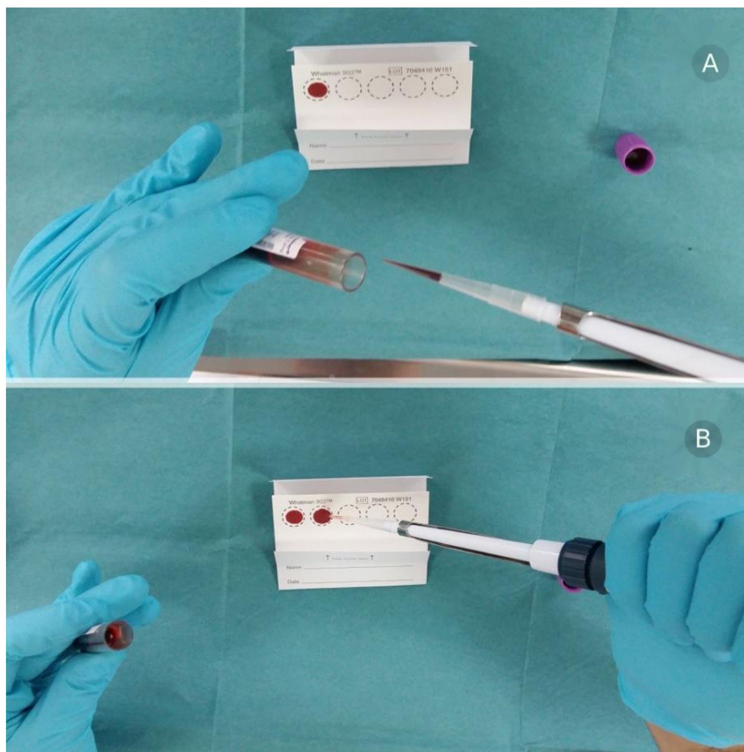

Figure 9: DBS “in vitro” sampling for non-volumetric DBS analysis.

Volumetric sampling

A fixed volume of blood (10 µL) was taken with a heparinized capillary pipette. The drops were placed on filter paper and allowed to dry at room temperature for at least 24 hours (figure 10)

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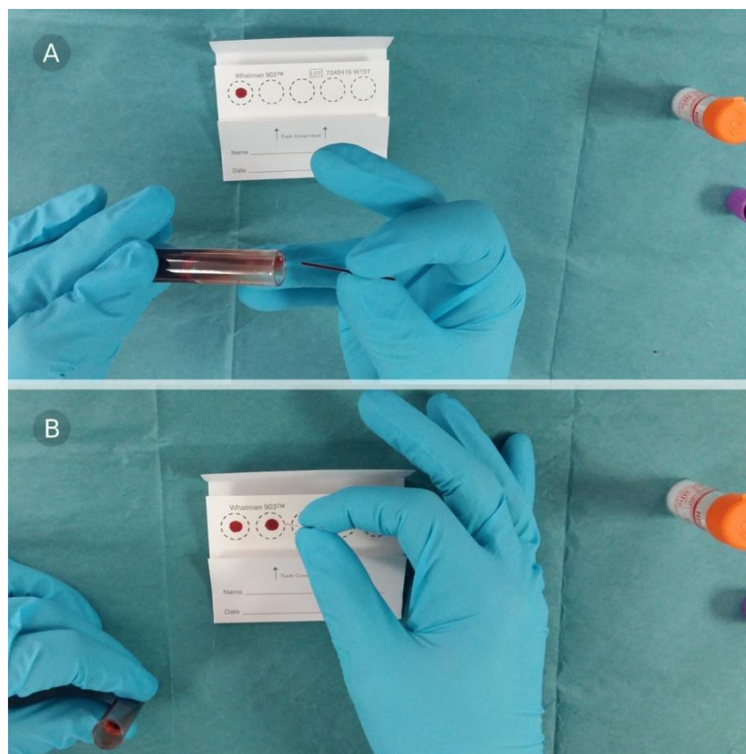

Figure 10: DBS "in vitro" sampling for non-volumetric DBS analysis.

GFR determination. Agreement analyses "in vitro".

Reference method vs non-volumetric DBS:

Compared with the reference method in plasma, the non-volumetric DBS had a CCC of 0.87 (0.74, upper CI), reflecting moderate precision and accuracy. Also, TDI was 26.03 (35.24, upper CI), which means that 90% of the GFR values showed an error ranging from -26 to +26% when compared with the reference method. Finally, cp was 45 (30, upper CI), which indicates that more than 55% of the GFR values had an error range greater than ±10% of the measurements using the plasma method (table 6).

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Bland and Altman plots (figure 11) showed wide limits of agreement (from -11.0 to 12.3 ml/min) and a mean difference of 0.7 ml/min between values measured with the DBS and the reference method, indicating excellent agreement.

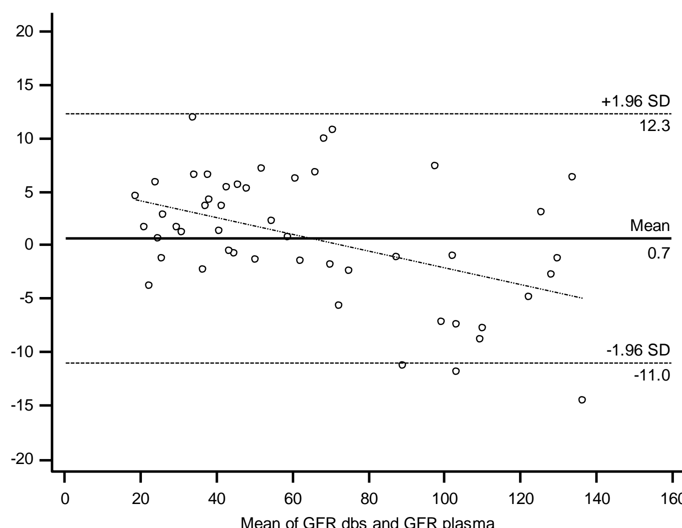

Figure 11: Bland-Altman plots of the difference between the GFR values (mL/min) measured by the reference and non-volumetric DBS method versus the mean of both. The straight and the dashed lines indicate mean difference and 95% limits of agreement, respectively.

GFR determination. Agreement analyses "in vitro".

Reference method vs volumetric DBS:

Compared with the method in plasma, the volumetric DBS had a CCC of 0.97 (0.95, upper CI), reflecting high precision and accuracy. Also, TDI was 13.01 (15.56, upper CI), which means that 90% of the GFR values showed an error ranging from -13 to +13% when compared with the reference method. Finally, cp was 79 (70,

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upper CI), which indicated that > 21% of the GFR values had an error range greater than ±10% of the method in plasma (Table 6)

DBS "in vitro"	Non-volumetric	Volumetric
TDI (%)	26,03 (35,24)	13,0 (15,56)
CCC	0,87 (0,74)	0,97 (0,95)
cp	45,1 (30,3)	79,10 (70,30)

Table 6: Agreement between the reference method (plasma analysis) and DBS "in vitro" (non-volumetric and volumetric). TDI: total deviation index, TDI (total deviation index); CCC (concordance correlation coefficient); CP (coverage probability). n=50.

Bland and Altman plots (figure 12) showed narrow limits of agreement (from -7.2 to 5.9 ml/min) and a mean difference of -0.7 ml/min between values measured with the DBS and the reference method, indicating excellent agreement.

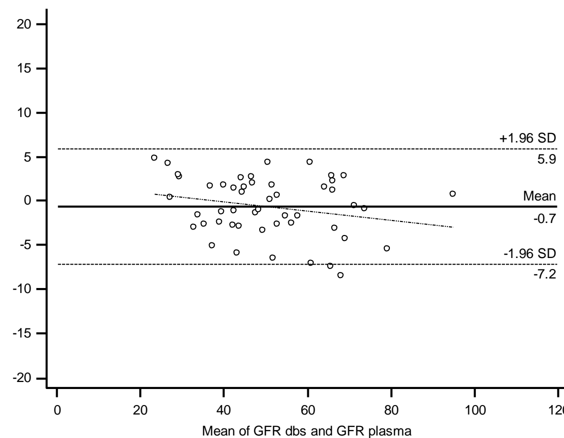

Figure 12: Bland-Altman plots of the difference between the GFR values (mL/min) measured by the reference and DBS volumetric method "in vitro" versus the mean of both. The straight and the dashed lines indicate mean difference and 95% limits of agreement, respectively.

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According to the results shown in table 5, the non-volumetric method had low precision and accuracy and was therefore rejected. **On the other hand, volumetric DBS sampling showed acceptable agreement with the reference method.** However, the TDI of 13% was greater than the ±10% limit of agreement that we defined *a priori* to accept a new method i.e. TDI < 10%, CCC > 0.99 and cp > 90. Thus, we decided to improve the volumetric method to increase the agreement with the reference method (plasma analysis).

OPTIMIZATION OF THE VOLUMETRIC DBS

In the previous analysis, solutions were added to DBS samples using a classic manual pipette. Also, iohexol was measured based on external calibration, without the use of an internal standard. Then, we developed two consecutives steps to improve the performance of the volumetric DBS:

1) Use of a mechanical repetitive pipette which improves the precision and accuracy of the volumes and solutions added to DBS samples.

2) Testing different molecules as internal standards: iodixanol, iopromide and iopamidol.

Selection of an internal standard for DBS samples

We tested three different iodine contrast media: iodixanol, iopromide and iopamidol as possible internal standards. These compounds were chromatographed with iohexol to evaluate retention time and resolution of the chromatographic peak. Iodixanol was not detected in the analysis. Iopromide had a long

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retention time, eluting at minute 12, and also had three isomers. **Iopamidol** had short retention time (minute 4) and excellent resolution *and was therefore selected as the new internal standard for DBS analysis* (Figure 13)

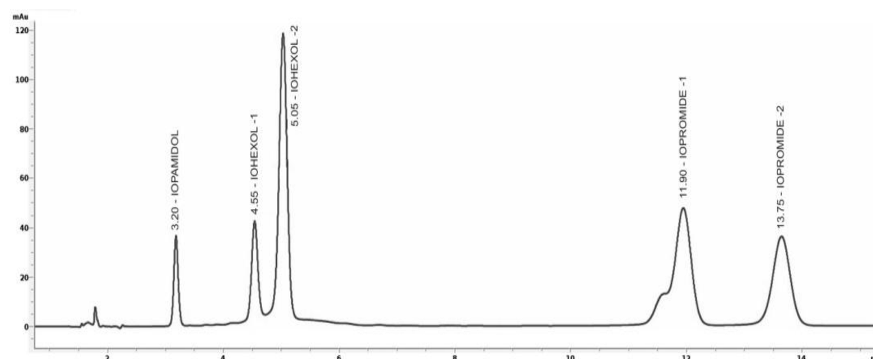

Figure 13: Chromatogram of iohexol and iodixanol, iopromide and iopamidol, detected at 254 nm.

Then, we took advantage of the presence of 50 new patients who underwent plasma clearance of iohexol at our centre. After blood extraction, samples were taken from the tubes to determine iohexol using the mechanical pipette. Then, samples were analyzed with or without the internal standard (iopamidol). The agreement between both "in vitro" approaches and the reference method was evaluated.

The use of a mechanical repetitive pipette did not substantially improve the performance of the DBS method as reflected by only minor changes in TDI and cp, compared with the manual pipette method (Table 6).

However, the introduction of an internal standard improved the agreement between the method in plasma and the DBS

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approach:CCC was 0.993 (0.990, upper CI), reflecting high precision and accuracy; TDI was 9.03 (10.77, upper CI), which means that 90% of the GFR values showed an error ranging from -9 to +9% compared with the reference method. Finally, cp was 92 (86, upper CI), which indicates that only 8% of the GFR values had an error range greater than ±10% of the method in plasma (table 7)

Volumetric DBS	Manual pipette	Mechanical pipette	Mechanical pipette + IS
TDI	13,01 (15,56)	12,27 (14,70)	9,03 (10,77)
CCC	0,97 (0,95)	0,99 (0,99)	0,99 (0,99)
cp	79,1 (70,3)	81,5 (72,8)	92,4 (85,7)

Table 7: Agreement between the reference method (plasma analysis) and DBS “in vitro”. TDI: total deviation index, TDI (total deviation index); CCC: concordance correlation coefficient); CP: coverage probability.

Bland and Altman plots (Figure 14) showed narrow limits of agreement (from -6.8 to 6.1 ml/min) and a mean difference of -0.3 ml/min between values measured with the DBS using and internal standard and the reference method, indicating excellent agreement.

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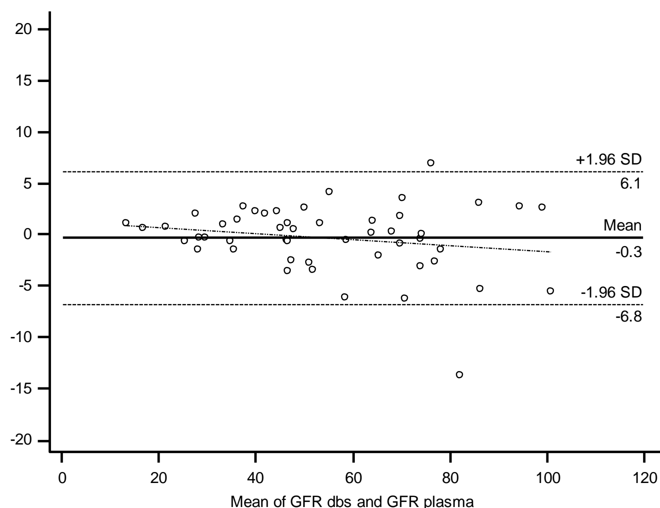

Figure 14: Bland-Altman plots of the difference between the GFR values (mL/min) measured by the reference and DBS volumetric method "in vitro" (internal standard) versus the mean of both. The straight and the dashed lines indicate mean difference and 95% limits of agreement, respectively.

DBS TESTING: STUDIES "IN VIVO"

Patients

The plasma clearance of iohexol was performed in 203 patients attended by the Department of Nephrology, Hospital Universitario de Canarias (Tenerife, Spain), between July 2015 and March 2017. The final DBS volumetric method (using a mechanical pipette and iopamidol as internal standard) was performed simultaneously with the plasma analysis (reference method) to evaluate the agreement between both techniques. Clinical characteristics of the patients are shown in table 8.

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N	203
Age (y) (mean ± SD)	57.3 ± 15.3
Gender (male-%)	138 (67.9)
Renal Disease (n - %)	
Renal transplantation	52 (26.0)
Predialysis	36 (18.0)
Type 2 diabetes	30 (15.0)
Chronic kidney disease	24 (12.0)
Living kidney donors	21 (10.0)
Chronic liver disease	18 (9.0)
Liver transplantation	9 (4.5)
Children with renal disease	5 (2.5)
Cancer patients	3 (1.5)
Others	7 (3.5)
Weight (kg) (mean ± SD)	79.0 ± 17.3
BMI (mean ± SD) (kg/m^2)	28.3 ± 5.2
Measured GFR	
mean ± SD (ml/min)	63.6 ± 34.8
range (ml/min)	9.1 -159.7

Table 8: Clinical characteristics of the patients included in the study

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Chromatography

In a typica chromatogram of human plasma, the peak of the first isomer of iohexol appears at 4.034 minutes, the second at 4.478 minutes and the internal standard DMU at 6.105 minutes. All peaks were detected at 254 nm (Figure 15)

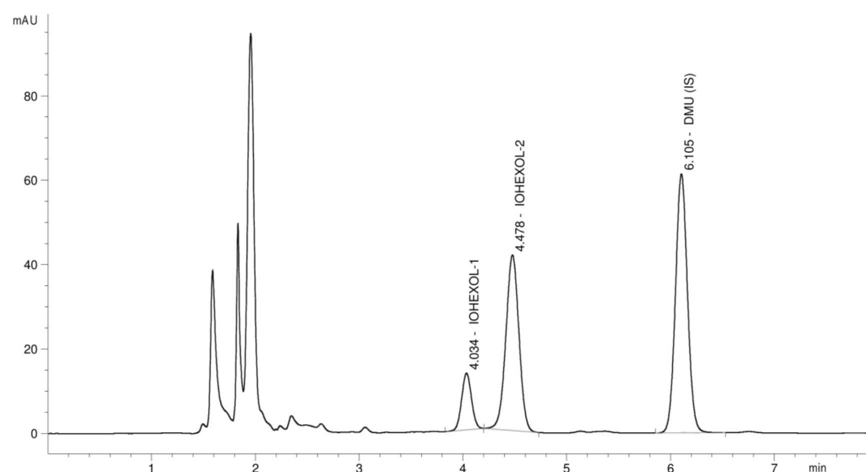

Figure 15. Representative chromatogram of human plasma after iohexol (3.235 g) injection. Iohexol isomers and DMU were detected at 254 nm.

GFR determination

GFR values averaged 63.8±35.0 ml/min, ranging from 9.1 to 159.7 ml/min for the reference method. For the short procedure, the pharmacokinetic curve of iohexol consists in five points measured at 120, 150, 180, 210, 240 minutes after injection (Figure 16).

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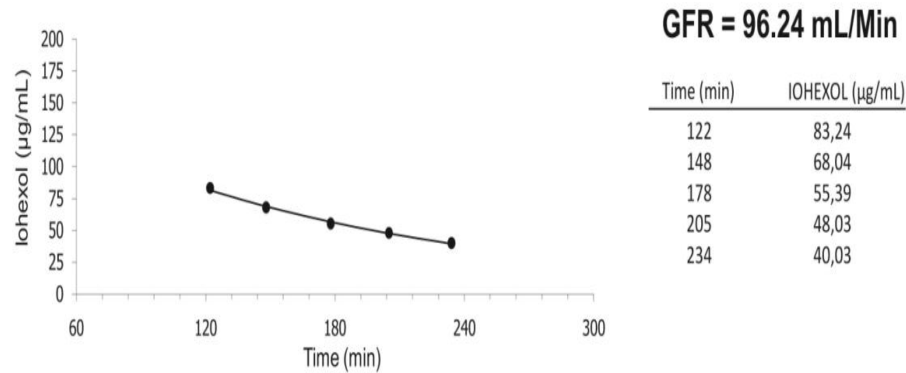
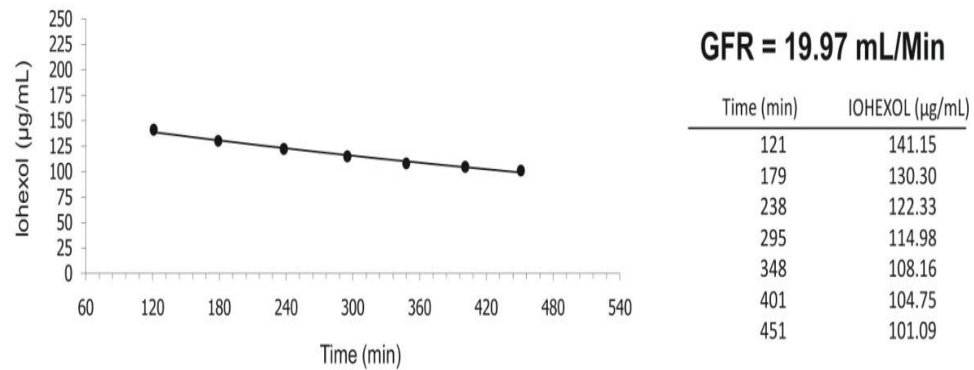


Time (min)	IOHEXOL (µg/mL)
122	83,24
148	68,04
178	55,39
205	48,03
234	40,03

Figure 16: Plasma clearance of iohexol for the short protocol.

For the long procedure, the pharmacokinetic curve of iohexol consists in seven points measured at 120, 180, 240, 300, 360, 420, and 480 min after injection (figure 17). Iohexol concentrations are expressed in µg/mL.



Time (min)	IOHEXOL (µg/mL)
121	141.15
179	130.30
238	122.33
295	114.98
348	108.16
401	104.75
451	101.09

Figure 17: Plasma clearance of iohexol for the long protocol.

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DBS sampling "in vivo": Finger prick

Figure 18 shows the sampling procedure to extract capillary blood via finger-prick with an automatic lancet for the DBS volumetric sampling approach "in vivo". A fixed volume of blood (10 μL) is taken, using a volumetric device (heparinized capillary pipette), and placed on filter paper to make the DBS samples and allowed to dry at room temperature.

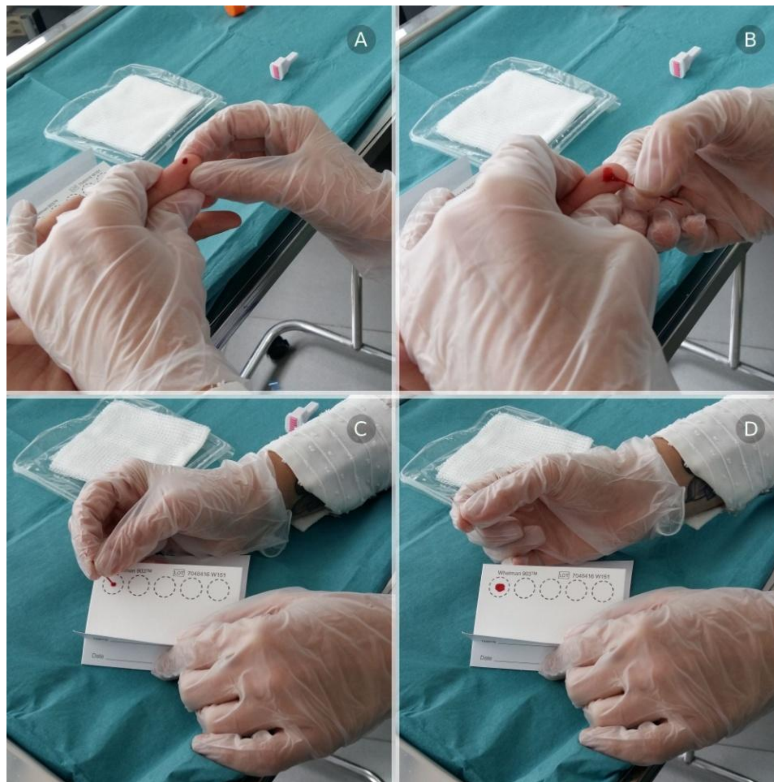

Figure 18: DBS "in vivo" sampling for volumetric DBS analysis.

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Agreement between the reference method and DBS volumetric: Studies “in vivo”

The analysis of the reference method (plasma analysis) and the volumetric DBS approach using the internal standard showed excellent agreement between the two methods. CCC was 0.997 (0.996, upper CI), reflecting an excellent level of precision and accuracy. TDI was 9.53 (10.39, upper CI), and therefore 90% of the GFR values showed an error ranging from -9.5 to +9.5% when compared with the reference method (plasma analysis), and cp was 91 (88, upper CI). This indicates that less than 10% of the GFR values had an error range greater than ±10%. (table 9)

DBS studies “in vivo” (n = 203)	**DBS Volumetric method**
TDI	9,63 (10,49)
CCC	0,99 (0,99)
cp	91,0 (88,0)

Table 9. Agreement between the reference method (plasma analysis) and DBS “in vivo” volumetric sampling approach. TDI: total deviation index, TDI (total deviation index); CCC (concordance correlation coefficient); CP (coverage probability).

Bland and Altman plots (figure 19) showed narrow limits of agreement (from -6.9 to 9.3 ml/min) and a mean difference of 1.6 ml/min between GFR measured with the DBS and the reference method, indicating excellent agreement.

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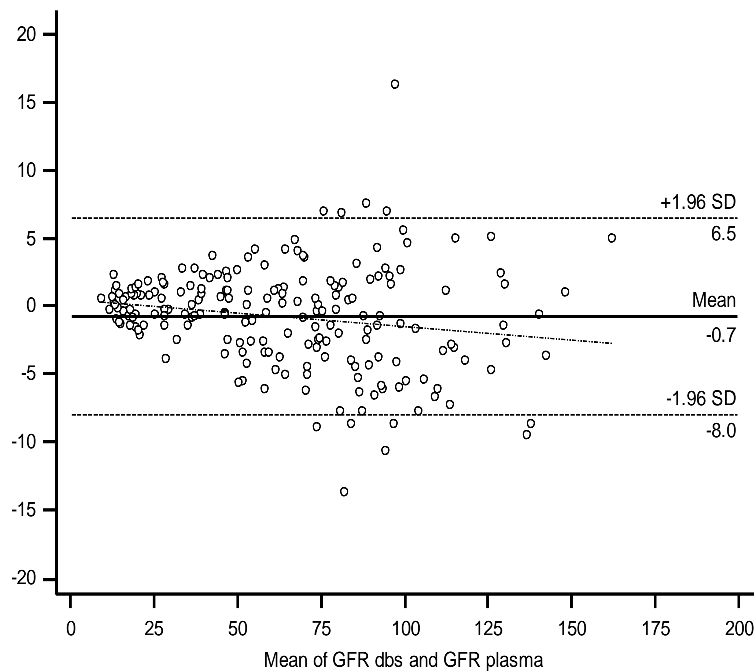

Figure 19. Bland-Altman plots of the difference between the GFR values (mL/min) measured by the reference and DBS method versus the mean of both. The straight and the dashed lines indicate mean difference and 95% limits of agreement, respectively.

To support the previous result we performed a Bland and Altman analysis comparing iohexol concentrations measured with the DBS and the reference method (Figure 20). The plot showed narrow limits of agreement (from -13,2 to 10,1 µg/mL) and a mean difference of -1,5 µg/mL between both methods, indicating excellent agreement.

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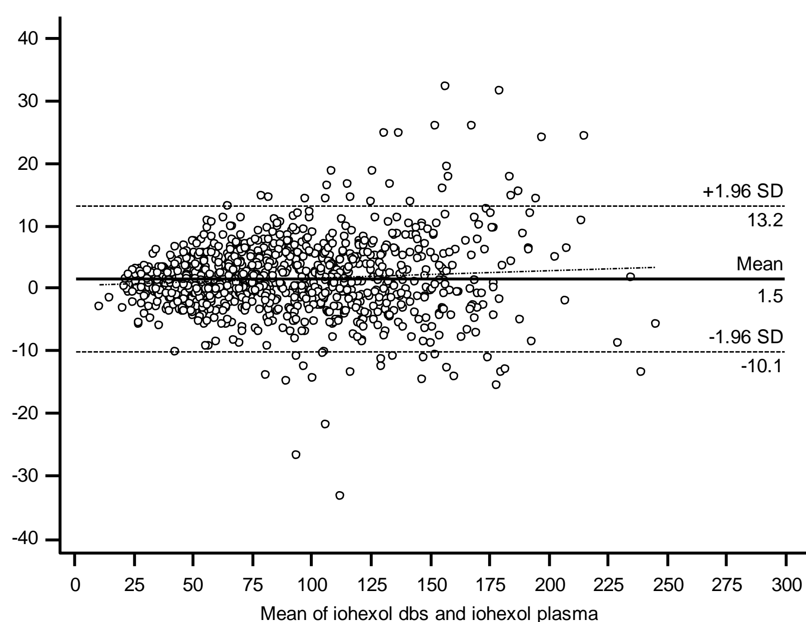

Figure 20. Bland-Altman plots of the difference between the iohexol concentrations (μg/mL) determined by the reference and DBS method versus the mean of both. The straight and the dashed lines indicate mean difference and 95% limits of agreement, respectively.

Stability of GFR measurement by DBS testing

Storage conditions

Stability was evaluated using twenty real pharmacokinetic curves and analyzed after storing at room temperature for 2, 4, 8, and 12 weeks, analyzed and compared to the plasma analysis.

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Room Temperature: 2 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	64,8	69,4	7,2
2,0	32,1	32,2	0,3
3,0	19,5	20,6	5,9
4,0	94,1	89,2	-5,2
5,0	147,8	145,3	-1,7

Room Temperature: 4 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	13,5	14,1	3,8
2,0	18,4	18,9	2,4
3,0	55,5	59,5	7,2
4,0	73,6	66,3	-10,0
5,0	113,2	102,0	-9,9

Room Temperature: 8 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	27,6	29,4	6,9
2,0	21,3	19,9	-6,7
3,0	78,5	73,9	-5,8
4,0	77,0	82,2	6,7
5,0	72,9	74,2	1,7

Room Temperature: 12 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	90,0	91,0	1,1
2,0	84,4	85,3	1,1
3,0	19,7	20,8	5,4
4,0	22,6	19,8	-12,4
5,0	19,9	21,4	7,4

Table 10. Stability of GFR measurement (ml/min) by DBS testing after storing at room temperature for 2, 4, 8, and 12 weeks.

In the same way, fifteen real pharmacokinetic curves were stored at -20ºC during 4, 8, 12 and 24 weeks, then thawed, analyzed and compared to the plasma analysis.

Freezer (-20ºC): 4 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	88,5	84,2	-4,8
2,0	55,0	51,3	-6,7
3,0	55,3	54,6	-1,2
4,0	59,4	58,5	-1,6
5,0	27,2	28,0	3,0

Freezer (-20ºC): 8 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	36,9	35,7	-3,2
2,0	91,3	99,9	9,5
3,0	39,3	39,5	0,6
4,0	33,3	29,9	-10,4
5,0	53,0	52,9	-0,3

Freezer (-20ºC): 12 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	36,9	35,7	-3,2
2,0	91,3	99,9	9,5
3,0	39,3	39,5	0,6
4,0	33,3	29,9	-10,4
5,0	53,0	52,9	-0,3

Freezer (-20ºC): 24 weeks			
Case	GFR plasma	GFR dbs	Diff %
1,0	77,0	76,8	-0,3
2,0	86,2	79,3	-8,0
3,0	61,7	61,8	0,1
4,0	53,1	51,9	-2,2
5,0	74,4	73,0	-1,8

Table 11. Stability of GFR measurement by DBS testing after storing at -20ºC for 4, 8, 12 and 24 weeks. Values represent GFR in mL/min.

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Shipment conditions

Stability was evaluated using eighteen real pharmacokinetic curves and analyzed after shipping by regular mail to different European centers, which returned the DBS samples to our laboratory that were analyzed and compared to the plasma analysis.

Case	GFR plasma	GFR dbs	Diff %	Destination	Days
1	126,9	118,0	-7,0	Bergamo (IT)	9
2	99,6	92,3	-7,4	Bergamo (IT)	9
3	18,7	19,3	3,1	Bergamo (IT)	9
4	15,9	17,4	9,0	Madrid (ES)	7
5	15,4	14,1	-8,1	Madrid (ES)	7
6	127,6	124,6	-2,4	Madrid (ES)	7
7	12,4	11,6	-6,8	Barcelona (ES)	4
8	92,9	92,5	-0,4	Barcelona (ES)	4
9	79,4	78,0	-1,7	Barcelona (ES)	4
10	98,8	102,8	4,1	Lisbon (PT)	33
11	93,0	88,4	-5,0	Lisbon (PT)	33
12	38,8	40,8	5,2	Lisbon (PT)	33
13	84,7	86,4	1,9	Copenhague (DK)	13
14	18,2	18,9	3,8	Copenhague (DK)	13
15	40,9	40,4	-1,3	Copenhague (DK)	13
16	75,7	72,7	-4,0	Madrid (ES)	27
17	51,6	56,1	8,7	Madrid (ES)	27
18	112,8	115,5	2,4	Madrid (ES)	27

Table 12. Stability of GFR measurement by DBS testing after shipping by regular mail to different European centers. Values represent GFR in mL/min.

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Validation of volumetric DBS in compliance with the EMA guidelines

Selectivity

Figure 21 represents a typical chromatogram of a blank DBS sample. We analyzed 15 blank DBS samples from patients with several clinical conditions such as renal transplantation, predialysis, type 2 diabetes, chronic kidney disease, living kidney donors, chronic liver disease, liver transplantation, children with renal disease, cancer patients. No interfering chromatographic peaks were observed at the retention time of iohexol and iopamidol.

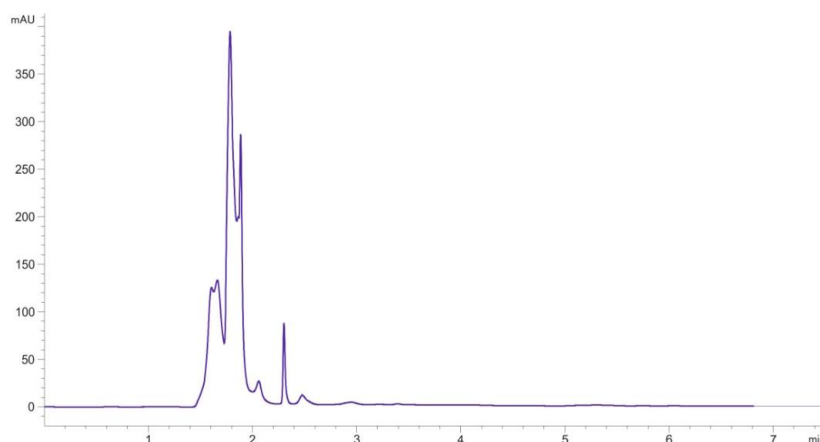

Figure 21: Representative chromatogram of DBS blank sample.

Figure 22 represents a chromatogram of iohexol and iopamidol (internal standard) in an extracted DBS sample from a representative patient represents iohexol eluted as two isomers at 4.83 and 5.36 minutes, whereas iopamidol eluted at 3.31 minutes.

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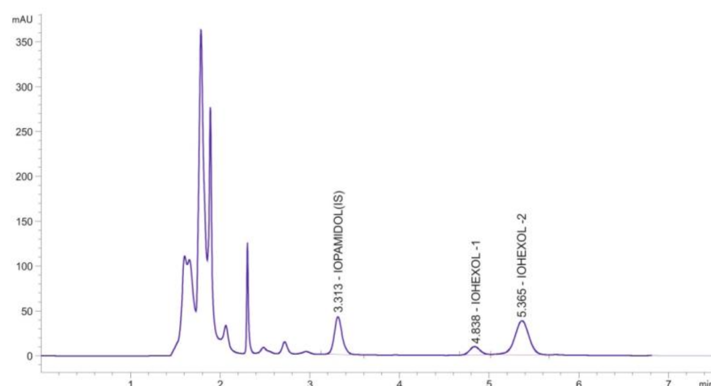

Figure 22: Chromatogram of DBS sample after iohexol (3.235 g) injection. Iohexol isomers and iopamidol (IS) were detected at 254 nm.

Carryover

We injected a high amount of iohexol at the upper limit of quantification (161,75 µg/mL). Carry over in the blank sample following this high concentration of iohexol was not observed for iohexol or for the internal standard (figure 23)

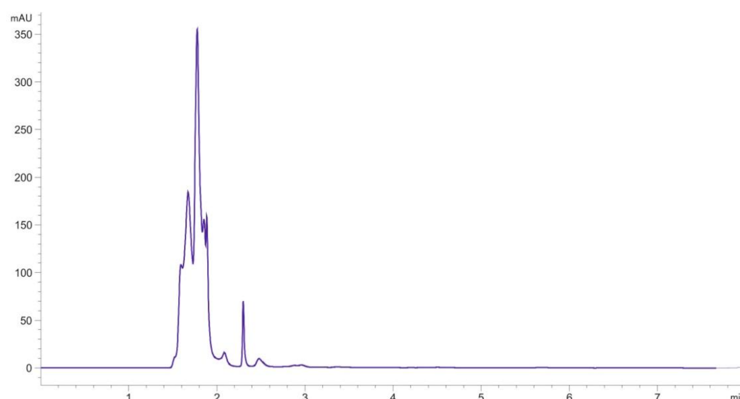

Figure 23. Representative chromatogram of DBS blank sample after analyzing a previous sample corresponding to high amount of iohexol (161,75 µg/mL)

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Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was the lowest concentration of iohexol that could be quantified with acceptable accuracy and precision. Within-run accuracy was 105.66% for 5 replicas in the same run of analysis. Within-run precision was 1.69% (CV%) for 5 replicas in the same run of analysis (table 13). Between-run accuracy was 100.46% for 3 replicas in three runs on at least two different days. Between-run precision was 2.52% (CV%) for 3 replicas in three runs on at least two different days (table 14). The iohexol signal was at least 5 times the signal of the blank sample.

Within-run accuracy and precision									
replica	1	2	3	4	5	mean ± SD	nominal	accuracy	Precision
LLOQ	16.6	17.3	17.1	16.9	17.4	17.0 ± 0.29	16.17	105.6	1.69

Table 13: Within-run accuracy and precision for the lower limit of quantification (LLOQ). Values represent iohexol concentrations in μg/mL.

Between-run accuracy and precision							
replica	1	2	3	mean ± SD	nominal	Accuracy	Precision
LLOQ	16.6	15.8	16.2	16.2 ± 0.41	16.17	100.4	2.52

Table 14: Between-run accuracy and precision for the lower limit of quantification (LLOQ). Values represent iohexol concentrations in μg/mL.

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Calibration curves

Three acceptable curves are shown in tables 15-17. The differences between the experimental back-calculated concentrations of the calibration standards and the theoretical levels were within ±5%, except for the LLOQ of the first curve which was 7.7%.

Calibrator	Theorethical concentration	Observed amount	Diff %	Iohexol height	IS height	Ratio
1	16,2	14,9	-7,8	9,1	53,6	0,2
2	32,4	32,6	0,9	20,2	53,9	0,4
3	64,7	64,1	-1,0	39,4	53,5	0,7
4	97,1	99,4	2,4	61,7	54,0	1,1
5	129,4	131,1	1,3	81,5	54,0	1,5
6	161,8	159,3	-1,5	97,4	53,1	1,8

Table 15

Calibrator	Theorethical concentration	Observed amount	Diff %	Iohexol height	IS height	Ratio
1	16,2	16,6	2,8	9,5	53,9	0,2
2	32,4	32,2	-0,5	18,9	53,1	0,4
3	64,7	64,7	0,1	38,8	52,7	0,7
4	97,1	98,5	1,6	60,7	53,7	1,1
5	129,4	124,8	-3,6	72,4	50,5	1,4
6	161,8	164,4	1,7	101,7	53,6	1,9

Table 16

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Calibrator	Theorethical concentration	Observed amount	Diff %	Iohexol height	IS height	Ratio
1	16,2	15,5	-4,2	8,8	53,9	0,2
2	32,4	33,0	1,9	19,8	54,0	0,4
3	64,7	65,0	0,5	39,9	54,0	0,7
4	97,1	94,5	-2,6	56,5	52,2	1,1
5	129,4	130,0	0,5	80,8	54,0	1,5
6	161,8	162,1	0,2	100,9	54,0	1,9

Table 17

Tables 15-17: Performance of three representative clearance curves for iohexol analysis by DBS testing. IS: internal standard. Values represent iohexol concentrations in µg/mL.

The slope and intercept are based on the ratio between the heights of the second peak of iohexol and the internal standard, and were calculated for each calibration curves (figure 24-26)

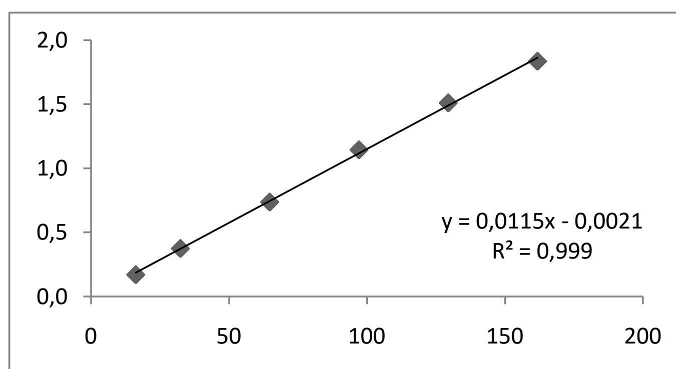

Figure 24

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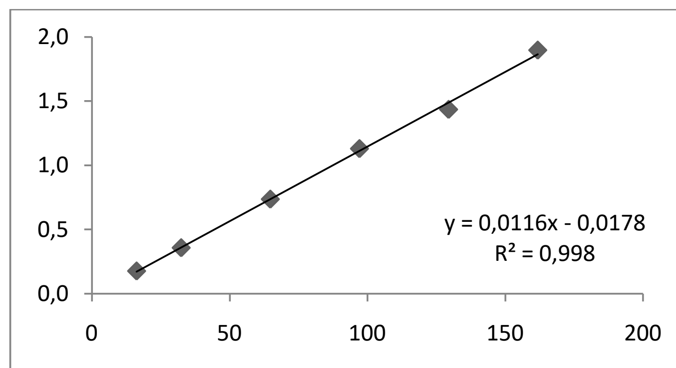

Figure 25

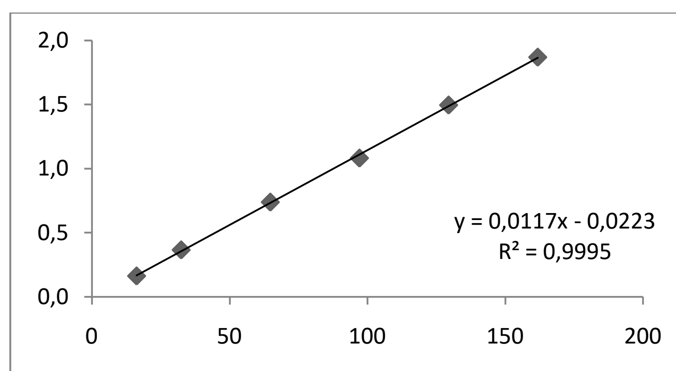

Figure 26

Figure 24-26: Calibration parameters for the three calibrations curves represented in tables 15-17.

Accuracy and precision

Within-run accuracy and precision

Five replicas were analyzed for each QC sample in a single run. The mean and standard deviation (SD) of the iohexol concentrations were calculated. Within-run accuracy was higher than 97.5% for 32,35 µg/mL (low QC), 64,7 µg/mL (medium QC), 161,75 µg/mL (high QC), and 105,6% for 16,17 µg/mL (LLOQ).

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CV% for within-run precision was lower than 2% for all the QC samples, including LLOQ (table 18).

Within-run accuracy and precision				
QC/replica	LLOQ	low	medium	high
1	16,6	32,4	62,6	157,7
2	17,3	32,5	64,3	157,8
3	17,1	32,2	63,3	161,2
4	16,9	32,2	63,0	154,7
5	17,4	32,2	63,3	157,6
mean ± SD	17.0 ± 0.3	32.3 ± 0.1	63.3 ± 0.6	157.8 ± 2.2
nominal value	16,7	32,35	64,7	161,75
accuracy %	105,6	99,9	97,8	97,5
precision %	1,69	0,43	0,98	1,45

Table 18: Within-run accuracy and precision for the lower limit of quantification (LLOQ), and low, medium and high quality controls. Values represent iohexol concentrations in μg/mL.

Between-run accuracy and precision

Five replicas were analyzed for each QC sample in five runs on different days. The mean and standard deviation (SD) of the iohexol concentrations were calculated.

Between-run accuracy was higher than 96.0% for all the QC samples, including LLOQ (table XXX). CV% for within-run precision was lower than 3.2% for all the QC samples, including LLOQ (table 19)

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Between-run accuracy and precision				
QC/replica	**LLOQ**	**low**	**medium**	**high**
1	16,6	32,8	63,3	153,6
2	15,8	31,3	64,3	158,3
3	16,2	31,2	62,3	156,2
4	15,5	35,2	62,7	154,7
5	15,4	31,8	62,5	155,3
mean ± SD	15.95 ± 0.5	31.0 ± 0.9	63.0 ± 0.8	155.6 ± 1.7
nominal value	16,7	32,35	64,7	161,75
accuracy %	98,5	98,5	97,4	96,2
precision %	3,16	3,12	1,30	1,13

Table 19: Between-run accuracy and precision for the lower limit of quantification (LLOQ), and low, medium and high quality controls. Values represent iohexol concentrations in µg/mL.

Dilution integrity

Five replicas were analyzed for each QC sample in a single run. The mean and standard deviation (SD) of the iohexol concentrations were calculated. Between-run accuracy was higher than 98.0% for all the QC samples (table 20). CV% was lower than 1.7% for all the QC samples, including LLOQ

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QC/Dilution	Dilution integrity 1	1:02	1:04	1:06	1:08	1:10
1	321,4	163,1	79,3	53,0	40,0	32,3
2	326,2	164,4	79,2	52,7	41,3	32,2
3	325,5	166,3	80,1	53,3	41,1	32,2
4	323,5	163,1	78,4	53,0	40,7	32,4
5	325,5	164,1	79,4	53,3	41,8	32,1
mean±SD	324,4	164,2	79,3	53,1	41,0	32,2
nominal value	323,5	161,7	80,9	53,9	40,4	32,3
accuracy %	100,3	101,5	98,0	98,4	101,4	99,8
precision %	0,6	0,8	0,7	0,5	1,7	0,4

Table 20: Analysis of dilution integrity. Values represent iohexol concentrations in µg/mL.

Stability

Stability was evaluated using QC samples at a concentration of 32,35 µg/mL (low QC) and 161,75 µg/mL (high QC) and analyzed after different storage conditions (room temperature, -20ºC and -80ºC). The calibration curve for this analysis was obtained from freshly spiked calibration standards, and the concentrations of QC samples were compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration. The following stability tests were performed:

Freeze and thaw stability

Five replicas were analyzed for each QC sample, 32,35 µg/mL (low QC) and 161,75 µg/mL (high QC), in three runs on different days after the freeze-thaw cycle. The mean and standard deviation (SD) of the iohexol concentrations were calculated.

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Accuracy was expressed as the ratio between the experimental back calculated concentrations and the nominal values, multiplied by 100. Precision was expressed as the ratio between the standard deviation (SD) and the mean, multiplied by 100.

Accuracy was higher than 95% for both low and high QC samples for the three freeze-thaw cycles, except for the low QC of the third cycle which was 94.8% (table 21). CV% precision was lower than 4.0 % for both low and high QC samples.

Freeze/thaw	Cycle 1		Cycle 2		Cycle 3	
Replica	Low QC	High QC	Low QC	High QC	Low QC	High QC
1	32.6	154.9	33	159.7	30.7	159.4
2	32.1	154.9	35.4	154.8	30.9	152.5
3	32.6	152.6	35.6	158.5	30.0	155.9
4	32.5	155.2	33.1	160.9	31.1	160.2
5	32.8	156.2	35.7	159.3	30.6	157.9
mean ± SD	32.5 ± 0.2	154.8 ± 1.3	34.5 ± 1.4	158.6 ±2.3	30.7 ± 0.4	157.2 ±3.1
nominal value	32.3	161.7	32.3	161.7	32.3	161.7
accuracy %	100.6	95.7	106.8	98.1	94.8	97.2
precision %	0.73	0.86	3.96	1.47	1.38	1.96

Table 21: Freeze and thaw stability. Values represent iohexol concentrations in µg/mL.

Short-term stability

Accuracy was higher than 95% for both low and high QC samples after storing the DBS samples at room temperature for 2 weeks. The same was observed at 4 weeks, except for the accuracy of the high QC which was 94.8% (table 22). CV% precision was lower than 2.2 % for both low and high QC samples.

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Storage conditions	Fresh sample value		2 weeks at RT		4 weeks at RT	
Replica	Low QC	High QC	Low QC	High QC	Low QC	High QC
1	32.1	154.8	31.8	156.3	33.5	154.6
2	32.6	154.9	32.5	160.0	33.0	154.4
3	31.9	152.6	33.5	160.3	33.1	152.6
4	32.4	155.2	32.0	158.8	33.0	153.7
5	32.8	156.1	33.0	158.9	33.1	152.6
mean ± SD	32.4 ± 0.3	159.7 ± 2.4	32.6 ± 0.7	158.9 ± 1.5	33.2 ± 0.2	153.6 ± 0.9
nominal value	32.3	161.7	32.3	161.7	32.3	161.7
accuracy %	100.1	98.9	100.8	98.2	102.6	94.9
precision %	1.03	1.52	2.14	1.0	0.67	0.62

Table 22: Short-term stability after storing DBS samples at Room Temperature for 2 and 4 weeks. Values represent iohexol concentrations in µg/mL.

Accuracy was higher than 95.5 % for both low and high QC samples after storing the DBS samples at -20ºc and -80ºC for 4 weeks. (table 23). CV% precision was lower than 1.6 % for both low and high QC samples.

Storage conditions	Fresh sample value		4 weeks at -20ºC		4 weeks at -80ºC	
Replica	Low QC	High QC	Low QC	High QC	Low QC	High QC
1	32.1	154.8	33.4	156.2	32.1	156.1
2	32.6	154.9	34.0	152.2	32.7	153.5
3	31.9	152.6	34.7	156.6	32.9	154.3
4	32.4	155.2	34.4	152.1	32.5	154.2
5	32.8	156.1	34.7	156.2	32.4	158.0
mean ± SD	32.4 ± 0.3	159.7 ± 2.4	34.3 ± 0.5	154.7 ± 2.3	32.5 ± 0.3	155.2 ±1.8
nominal value	32.3	161.7	32.3	161.7	32.3	161.7
accuracy%	100.1	98.9	106.0	95.6	100.6	95.9
precision%	1.03	1.52	1.60	1.49	0.88	1.18

Table 23: Short-term stability after storing DBS samples at -20ºC and -80ºC for 4 weeks. Values represent iohexol concentrations in µg/mL.

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Long-term stability

Accuracy was higher than 98% for both low and high QC samples after storing the DBS samples at room temperature for 8 weeks. (table 24). CV% precision was lower than 2.0 % for both low and high QC samples.

Storage conditions	Fresh sample value		8 weeks at RT	
Replica	**Low QC**	**High QC**	**Low QC**	**High QC**
1	32.1	154.8	32.2	168.5
2	32.6	154.9	31.4	164.3
3	31.9	152.6	31.2	160.1
4	32.4	155.2	31.9	162.0
5	32.8	156.1	31.7	165.5
mean ± SD	32.4 ± 0.3	159.7 ± 2.4	31.7 ± 0.4	164.1 ± 3.2
nominal value	32.3	161.7	32.3	161.7
accuracy %	100.1	98.9	98.0	101.4
precision %	1.03	1.52	1.29	1.95

Table 24: Long-term stability after storing DBS samples at Room Temperature for 8 weeks. Values represent iohexol concentrations in µg/mL.

Accuracy was higher than 93% for both low and high QC samples after storing the DBS samples at -20ºC and -80ºC for 12 weeks. (table 25). CV% precision was lower than 1.6 % for both low and high QC samples.

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Storage conditions	Fresh sample value		12 weeks at -20ºC		12 weeks at -80ºC	
Replica	Low QC	High QC	Low QC	High QC	Low QC	High QC
1	32,2	154,9	29,8	152,3	29,9	149,8
2	32,6	154,9	30,1	151,8	30,4	150,8
3	31,9	152,6	29,9	153,7	30,5	153,4
4	32,4	155,2	30,4	149,6	30,4	148,5
5	32,8	156,2	30,6	153,7	30,1	151,3
mean ± SD	32.4 ± 0.3	159.7 ± 2.4	30.2 ± 0.35	154.7 ± 2.3	30.3 ± 0.24	150.8 ± 1.81
nominal value	32,35	161,75	32,35	161,75	32,35	161,75
accuracy %	100,2	98,9	93,3	94,1	93,5	93,2
precision %	1,03	1,52	1,16	1,10	0,80	1,20

Table 25: Long-term stability for storing DBS samples at -20ºC and -80ºC for 12 weeks. Values represent iohexol concentrations in µg/mL.

Accuracy was higher than 94.8 % for both low and high QC samples after storing the DBS samples at -20ºC and -80ºC for 24 weeks. (table 26). CV% precision was lower than 5.9 % for both low and high QC samples.

Storage conditions	Fresh sample value		24 weeks at -20ºC		24 weeks at -80ºC	
Replica	Low QC	High QC	Low QC	High QC	Low QC	High QC
1	32,2	154,9	33,5	152,8	30,5	153,9
2	32,6	155,0	31,8	154,8	30,7	154,9
3	32,0	152,6	33,5	155,3	32,7	156,9
4	32,4	155,2	36,9	153,1	31,0	154,3
5	32,8	156,2	35,6	151,5	31,4	153,2
mean ± SD	32.4 ± 0.3	159.7 ± 2.4	34,2	153,5	31,3	154,6
nominal value	32,35	161,75	32,35	161,75	32,35	161,75
accuracy %	100,2	98,9	105,9	94,9	96,7	95,6
precision %	1,03	1,52	5,82	1,01	2,77	0,92

Table 26: Long-term stability for storing DBS samples at -20ºC and -80ºC for 24 weeks. Values represent iohexol concentrations in µg/mL.

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DISCUSSION

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We described a simplification of the plasma clearance of iohexol, a gold standard method to measure renal function. We proposed to replace plasma samples by capillary blood deposited in filter paper using DBS. The agreement between both methods was excellent, showing that GFR can be measured with DBS without losing accuracy and precision. This represents a major simplification of the measurement of renal function.

THE ERROR OF ESTIMATED GFR

Renal function can be determined by two different methods: the estimation by formulas or the measurement by gold standard methods. The estimation of GFR by formulas is a simple and popular tool in clinical medicine. Formulas are algorithms based on few variables: creatinine and/or cystatin-c, age, weight and gender. However, formulas estimate real GFR leaving a large margin of error, as indicated by several studies in the literature (40, 53, 55-63). It is optimistic to suppose that the complex physiology of renal function can be evaluated by equations based on so few variables. Moreover, some of these variables are related between them. In example, gender and age are correlated with muscle mass, the source of creatinine. Also, obesity and inflammation influence the levels of cystatin-C, whereas creatinine can be secreted or reabsorbed by renal tubular cells (5-11). Thus, the relationship between GFR and these markers can be altered by several situations unrelated with renal function.

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The error of estimated GFR has major consequences:

I.- in day-to-day clinical practice:

(a) the bias of estimated GFR limits the correct diagnosis of renal function in patients with renal disease. The average error of eGFR is ±30% of mGFR, meaning that in a patient with mGFR of 60 ml/min, a value given by a formula may range from 42 ml/min to 78 ml/min. This is clinically unacceptable. Formulas overestimate or underestimate real GFR at random and the percentage of estimations with an acceptable error i.e. ± 10 %, are the exception rather than the norm. Thus, the magnitude of renal dysfunction and the stage of CKD are very difficult to evaluate. Also, formulas fail to detect early stages of renal disease like hyperfiltration (57).

(b) formulas do not reflect properly GFR changes over time. Estimated GFR decline can be slower or faster than measured GFR decline as shown by several studies in patients with a renal transplantation (58), type 2 diabetes (57) and polycystic renal disease (59). Thus, the evolution of renal function over time can not be analyzed with estimated GFR.

II.- in clinical research:

The error of formulas in assessing GFR decline limits its use in clinical trials or prospective studies that evaluate risk factors for renal disease progression or new therapies designed to slow renal function loss. Moreover, the unreliability of estimated GFR has been associated with the lack of success of clinical trials in nephrology (65). Accordingly, the European Medical Association recommended the use of mGFR in clinical research when renal function is the main outcome of the study (83).

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Renal function can be measured by gold standard methods such as renal or plasma clearances of exogenous substances, eliminated only by glomerular filtration like inulin, ^{51}Cr-EDTA, $^{Tc\text{-}99m}$DTPA, ^{125}I-iothalamate, iothalamate or iohexol. Although these methods are more accurate and precise, they are also cumbersome and time consuming. Urinary clearance of inulin requires continuous intravenous infusion and urine collections at timed intervals by bladder catheterization. Also, inulin has become expensive. ^{51}Cr-EDTA, $^{Tc\text{-}99m}$DTPA and ^{125}I-iothalamate have the specific limitations of using radio-labeled substances, such as licensing and compliance with regulations governing the storage and disposal of radioactive material. Then, the use of these methods is restricted to specialized centres for research studies.

Hence, Clinical Medicine stands at a crossroads: on the one hand, algorithms based on creatinine and cystatin-C are simple but inaccurate estimators of renal function; on the other hand, gold standard methods are reliable but unpractical.

The low reliability of estimated GFR by formulas is in contrast with the accuracy and precision of several techniques that evaluate outcomes in clinical medicine. In cardiology, myocardial function is evaluated by echocardiography. In neumology, spirometry is the most common of the pulmonary function tests and is helpful in assessing breathing patterns that identify conditions such as asthma, pulmonary fibrosis, cystic fibrosis, and chronic obstructive pulmonary disease. In gastroenterology, endoscopy is indispensable for the diagnosis of severe diseases like

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gastric, esophageal or colorectal cancers as well as inflammatory bowel diseases. Also, CT-scan and nuclear magnetic resonance are established tools in clinical oncology, neurology, surgery, neumology, vascular diseases, among others. **All these procedures are costly and time consuming but their utility and relevance in clinical medicine is undisputed.** Finally, the need of reliable tools in medicine has been remarked by an initiative of the White House of the United States of America, named "The Precision Medicine Initiative" that promotes research advances to enable better assessment of disease risk, understanding of disease mechanisms, and prediction of optimal therapy for diseases (89).

However, in Nephrology, renal function is still evaluated with a high level of uncertainty.

SIMPLIFICATION OF A GOLD STANDARD BY DBS TESTING

In this doctoral thesis, we applied the DBS method to the plasma clearance of iohexol in order to simplify the measurement of GFR and facilitate its use. We chose to work with the plasma clearance of iohexol because this method is simple and reproducible (71). The use of DBS determined an important simplification of the pre-analytical phase of the procedure, from the collection of blood samples to the chromatographic analysis in the laboratory, including:

a.- **avoiding venopuntures** to extract venous blood, which is replaced by finger-prick with a painless lancet to collect capillary blood samples. This improves the comfort of the patients and is particularly important in cases with difficult venous access and in very young children with renal disease.

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b.- **avoiding tubes for blood collection and plasma storage,** since capillary blood is deposited on paper and left to dry (figure 27-28)

c.- **avoiding cold-storage facilities (fridges/freezers),** since DBS can be safely stored at room temperature preserving the characteristics of the molecules included on paper.

d.- **increased safety,** due to the loss of infectivity of agents like virus or bacteria as a consequence of disruption of the envelope during drying.

e.- **avoiding special shipment and transport regulations,** because iohexol is stable at room temperature in DBS samples and so it can be sent by regular mail without the need for sending the samples on dry ice and special containers. Also, no special regulations for the shipment are required since DBS samples do not represent a biohazard. Based on the above, the DBS method implies a huge reduction in the cost of the plasma clearance of iohexol.

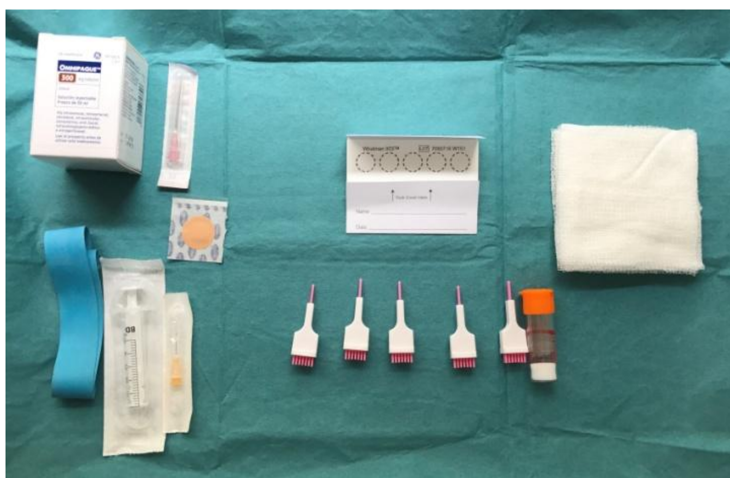

Figure 27: Material required for the DBS technique.

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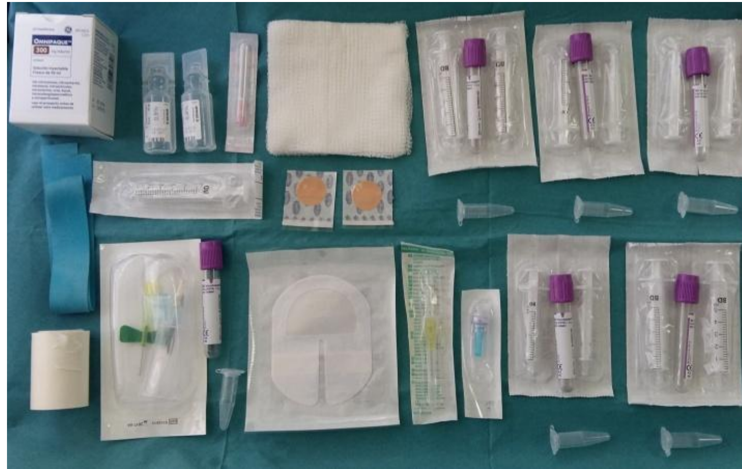

Figure 28: Material required for the standard technique in plasma.

Finally, the DBS testing represents an important cost reduction related with the measurement of renal function. Recent publications showed that the cost of the plasma clearance of iohexol in diverse EU countries is about 100 to 200 € (72). The simplification of the technique using DBS sampling may reduce the costs to 50-100 €. The most important advantage in cost reduction is possibly the elimination of sample storing in freezer. It is noteworthy that, this is not different from the cost of many tests described above: spirometry (50 €), CT-scan (200-400€), magnetic resonance (400 €), echocardiography (120 €).

Thus, the DBS method applied to the clearance of iohexol may be especially useful to evaluate renal function in clinical practice and research.

The advantages of DBS testing in comparison with the conventional plasma analysis are summarized in table 27.

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	Plasma	**DBS**	***Comment***
Sampling	Venous puncture	Finger-prick	Finger-prick is painless. *Increases patient comfort.*
Blood volume	Venous (18-24 mL)	Capillary (0.18-0.24 mL)	Reduced blood samples. *Increases patient comfort.*
Centers	Specialized centers	Specialized and Healthy centers	The procedure can be done in peripheral centres and the samples can be sent to a reference laboratory. *Increases patient comfort.*
Storage	Fridge/Freez er	Room temperature	*Reduced costs*
Shipments	Special mail	Regular mail	No need for shipment in dried ice or special containers. *Reduced costs*
Phlebotomy material	Required	Not required	*Reduced costs*
Aliquoting/ Centrifuging	Required	Not required	*Reduced costs*
Biohazard	Yes	No	*Reduced costs*

Table 27: Practical, clinical and financial advantages of DBS testing in comparison with the conventional blood analysis.

TECHNICAL ASPECTS OF DBS TESTING

The DBS method was used for the first time in 1963 by Robert Guthrie for the diagnosis of phenylketonuria in neonates. Since then, DBS has been used for the screening of several

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metabolic disorders (76), therapeutic drug monitoring (77), and epidemiological studies. Two approaches for DBS sampling have been described: (a) non-volumetric sampling (partial spot methods) that uses a non-fixed volume of the sample for analysis (b) volumetric sampling (whole spot methods) that uses a fixed volume of the sample. (Figure 8)

In our knowledge, this is the first study that compares these two DBS approaches for measuring renal function. We observed that the non-volumetric DBS approach was inaccurate and imprecise whereas the volumetric method had acceptable agreement with the reference method (plasma analysis).

The error of the non-volumetric method may be explained by a non-homogeneous diffusion of blood and analytes on filter paper which is directly related with the hematocrit. Low hematocrit reduces blood viscosity which facilitates high diffusion of blood on paper, whereas high hematocrit increases blood viscosity which determines low diffusion of blood in paper. Thus, the quantity of analytes in the sample will vary according to the hematocrit. The major limitation of the non-volumetric method is that the sample of paper does not contain the whole drop of blood (FIGURE 8 in Methods). Thus, the quantity of the analyte in the sample will be highly variable depending on the diffusion of blood and the hematocrit.

The better performance of the volumetric approach may be related with the collection of a fixed volume of blood which is completelly included in the punched-out sample for analysis.

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There are other special technical considerations that must be taken into account for the correct use of the volumetric approach: (a) the capillary pipette of 10 µl should be completely filled with blood, since incomplete filling or even small bubbles inside the capillar invalidate the procedure (figure 30) and (b) the whole volume of blood must be placed on the filter paper

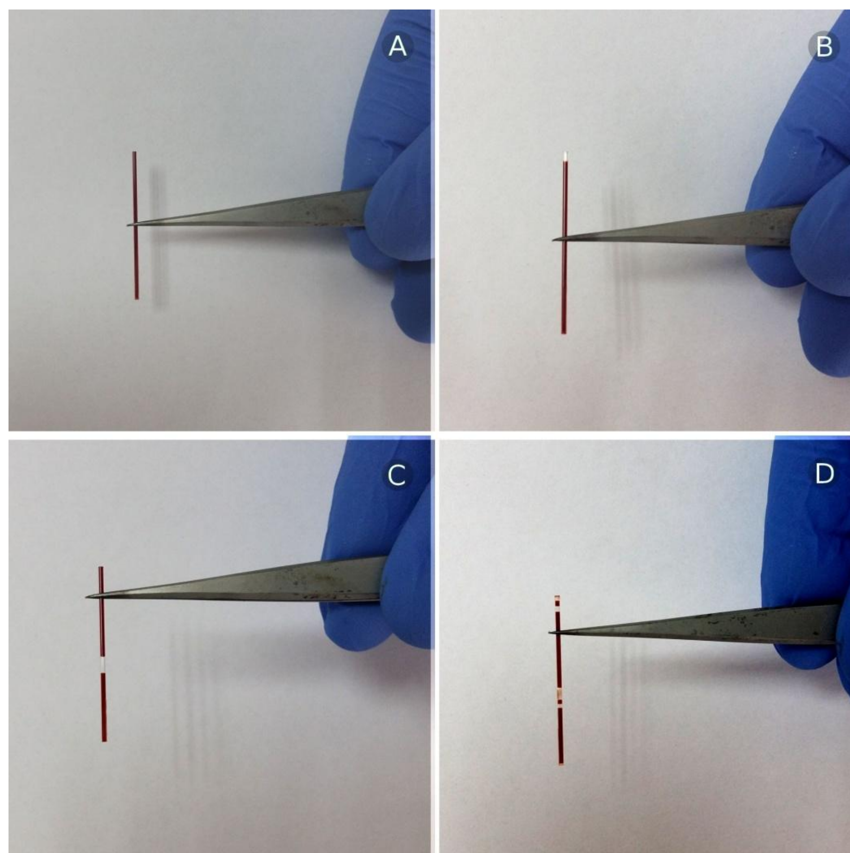

Figure 30: Errors for DBS volumetric samplin using a capillar pipette. Incomplete filling of the capillar (panel A) and an air buble inside the capillar (panel B)

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Finally, to improve even more the accuracy and precision of the volumetric approach, we tested several potential internal standards such as iodixanol, iopromide and iopamidol. The use of and internal standard is important in chromatography to correct and normalize possible errors during the analytical process. **We described for the first time the use of iopamidol as internal standard for the analysis of iohexol in whole blood by HPLC.** This improved the agreement between the volumetric sampling approach and reference technique: as indicated by a TDI of 9.53 and a CCC of 0.997 and a cp of 91.3. **This makes the DBS and the plasma method interchangeable.**

To evaluate the reliability of GFR measured with DBS sampling, we used specific statistical tests for agreement like the limits of agreement, total deviation index (TDI), coverage provability (cp) and concordance correlation coefficient (CCC). Also, we defined *a priori* meaningful margins of error and the proportion of the values that fall within these margins. It seems reasonable to propose that the vast majority of the values calculated by the new method i.e. 90% should fall within a ±10% error of the values determined for the reference method. The ±10% criterion derives from the fact that it considers the overall reproducibility of a gold standard method to measure GFR that is 7% (71). **Thus, we used specific methods of agreement that allowed us a thorough evaluation of the new DBS method.**

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APPLICABILITY OF GFR MEASREMENT WITH DBS SAMPLING

DBS represents a huge simplification of the plasma clearance of iohexol and this may facilitate the use of a reliable tool to measure GFR in clinical practice and research.

In clinical practice, there are special clinical conditions where direct measurement of GFR could be relevant, such as:

1.- Adjustment of potentially toxic drugs: such as cancer patients who will start chemotherapy i.e. carboplatin; patients with systemic lupus erythematosus or other glomerulonephritis who will receive cyclophosphamide or calcineurin inhibitors; patients with CKD who will be treated with other toxic drugs, etc.

2.- **Patients with renal disease who will undergo a potentially nephrotoxic procedure**: such as the use of contrast media (coronarography, percutaneous angioplasty of the renal artery, tomography) in order to evaluate the risk of contrast-induced nephropathy for renal function.

3.- Evaluation of renal function in living donors: to ensure that renal function before nephrectomy is above the cut-off for donation and to evaluate the evolution of renal function after donation.

4.- To assess the evolution of renal function in patients with CKD or at risk of renal disease: like patients with a renal transplantation or a liver transplantation.

5.- Children with renal disease, in whom formulas are particularly unreliable.

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6.- Patients in which it is not possible to assess renal function due to high discordance between 24-hour creatinine clearance and formulas.

At the Hospital Universitario de Canarias, in collaboration with the University of La Laguna, we started a clinical protocol to measure renal function with the plasma clearance of iohexol using DBS in the conditions described above. The preliminary results are promising: the DBS sampling proved to be simple and reproducible, and the results of measured GFR helped in clinical evaluations and decision making (Esteban Porrini and Armando Torres, personal communication). For example, an accurate determination of GFR before kidney donation is crucial to reduce the risk of renal impairment after living kidney donation and to provide acceptable renal mass to the donor. The cut-off level for living kidney donation in our center is 80 ml/min. In about 10% of the potential living donors (8 of 80 cases), mGFR measured by the plasma clearance of iohexol was <80 ml/min (~70 ml/min) while GFR estimated by MDRD or other formulas and creatinine clearance showed eGFR >90 ml/min. These cases were therefore excluded for donation according to the measured GFR (Ana González Rinne, personal communication).

DBS testing is especially useful in cases with difficult venous access which according to our experience may represent 10-15% of the patients scheduled for the plasma clearance of iohexol. Finally, this method is crucial to measure renal funciton in very young children with renal disease.

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Another important applicability of DBS testing is clinical research. Several studies have shown that estimated GFR decline with formulas persistently failed to detect the evolution of renal function overtime. The use of estimated GFR by formulas (and not gold standard methods) may be responsible for the failure of clinical studies in nephrology (65). Hence, the European Medicines Agency (EMA) has recently recommended that clinical trials in which renal function is the main outcome measure of the study, should use a gold-standard method like the plasma clearance of iohexol to assess the effect of new drugs on the progression of kidney disease (83). DBS testing offers a simple, reliable and relatively inexpensive method measurement of renal function based on the plasma clearance of iohexol. **Also, DBS offers two important advantages: neither cold-storage facilities (freezer) nor dried ice for sample transportation are required.** This implies a huge cost reduction for clinical studies in nephrology.

Finally, DBS testing can be suitable for studies in small animals (rats and mice) in which the volume of blood is limited, or in large animals (swine, sheep) to improve the animal-welfare during the procedures, in accordance with Russell and Burch's 3Rs model for animal research (refinement, reduction and replacement; Russell, 1995) (91)

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LIMITATIONS

There are two possible limitations of DBS testing: firstly, the method requires knowing the value of hematocrit of the same day renal function is measured. However, the determination of hematocrit is a standarized analysis with high reproducibility. Secondly, the collection of blood with a pipette has to be accurate, avoiding incomplete filling or the presence of air bubbles inside the capillar. Likewise, the whole volume of blood must be placed on the filter paper. Nevertheless, we think that after a brief and appropiate training, the collection of blood for DBS sampling can be properly achieved.

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CONCLUSIONS

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1.- We simplified the plasma clearance of iohexol to measure renal function using dried blood spot (DBS) testing.

2.- The agreement between DBS and the reference method (plasma analysis) was excellent, making both techniques interchangeable.

3.- The DBS method improves the comfort of patients since blood is drawn by finger-pricking with a painless lancet.

4.- We described that the volumetric method is the best approach for DBS sampling.

5.- The DBS method reduces the amount of the material used for the procedure: syringes, tubes, needles, etc.

6.- DBS samples can be stored at room temperature, so, freezers and dried ice are not required for storage and shipment of samples.

7.- The DBS testing may help to disseminate the measurement of renal function in clinical practice and research.

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