Clinica Chimica Acta 396 (2008) 80–85

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Performance of iohexol determination in serum and urine by HPLC: Validation, risk and uncertainty assessment

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ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 1 July 2008
Accepted 13 July 2008
Available online 18 July 2008

Keywords:
Glomerular filtration rate
Analytical validation
Measurement uncertainty
Iohexol
Reference method

ABSTRACT

Background: Determination of glomerular filtration rate plays an important role in nephrological practice. Iohexol is a reference marker for glomerular filtration rate determination. It is available and safe. The aim of this study was to develop a simple, efficient and easy to use analytical method for the quantification of iohexol in serum and urine by high performance liquid chromatography and to thoroughly validate this method.
Methods: The HPLC method was inspired from the method published by Krutzen. The e.noval software V2.0 (Arlenda, Liège, Belgium) was used to compute all validation results.
Results: The validation results indicate that the method will give accurate and reliable results for serum values ranging from 12.95 to 1295 µg/ml and for urine values ranging from 86.0 to 4144 µg/ml. In routine practice, iohexol concentrations found in plasma after injection range from 40 to 600 µg/ml. The expected urinary values are much wider. One should not hesitate to dilute urine samples to fit with the validated range over 5000 µg/ml.
Conclusion: This is the first time that a reference method for the determination of GFR is validated with such a rigorous and thorough protocol. Contrary to other GFR markers, iohexol is now strongly validated from an analytical point of view.

1. Introduction

Determination of glomerular filtration rate (GFR) plays an important role in daily nephrological practice. Indeed, its determination is crucial to diagnose chronic kidney diseases (CKD) or evaluate risks of developing CKD.

Several steps are required in order to achieve a reliable GFR. The first point is the selection of the GFR marker, the substance that will be monitored in order to determine the filtration rate. The ideal features of the substance to be monitored in order to evaluate the GFR are to appear endogenously in the plasma at constant rate, be freely filtered by the glomerulus without renal tubule absorption or secretion and without extra-renal elimination [1]. This ideal substance does exist but is exogenous: inulin (a fructose polymer). The renal clearance of inulin is thus the "gold standard" for the GFR measurement. However, its application is expensive and tedious [2]. Moreover, inulin is not always easily available on the market. The most used marker in clinical practice is endogenous and is serum creatinine. However, it does not comply to all of these last features [3] and it is indeed a poor marker for GFR, especially in specific population like obese, anorectic and transplanted patients [4,5]. In these specific patients and in clinical nephrological studies, a precise GFR measurement is needed. As inulin clearance measurement is not easy, other methods have been developed as isotopic methods such as ^{51}Cr-ethylenediaminetetra-acetic acid (EDTA) (although not available in the United States) or ^{99}Tc-diethylenetriaminepentaacetic acid (DTPA) [6,7]. However these markers require special handling, radiation exposure and are costly. Contrast agents like iothalamate or iohexol (*N*, *N*'-bis (2,3-dihydroxypropyl)-5-(*N*-2,3-dihydroxypropyl)acetamido)-2,4,6-triiodoisophtalamide) are also available and have the features of the ideal marker together with being easily available and safe. Thus iohexol clearance is replacing inulin clearance as a marker of choice for GFR [2,8–10].

Once the ideal marker has been chosen, the next step is the development of an accurate quantitative analytical method to obtain reliable concentration values of this marker in the serum. The objective of any quantitative analytical method is to obtain results that are close enough to the unknown true value of analyte under investigation in the samples analyzed. One way to demonstrate that the analytical method will provide reliable results is to perform method validation. The objective of method validation is thus to give guarantees that most of the results that will be generated during routine use of this method

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doi:10.1016/j.cca.2008.07.011

will be close enough to unknown true value of analyte. If the validation fails to give this guarantee then decision based on the results obtained by this method will be unreliable and may cause errors in diagnostics: declaring a healthy patient as ill and *vice versa*.

Various chromatographic methods have been described for iohexol determination, mainly in serum [11–13]. However, one should notice that these methods have been poorly analytically validated. Even more, validation for the determination in urine matrix with these methods is clearly lacking.

Thus, the aim of this paper is first to develop a simple, efficient and easy to use routinely analytical method for the quantification of iohexol in serum and urine by high performance liquid chromatography (HPLC) inspired from the method published by Krutzen et al. [12]. Secondly, a thorough validation of this method is performed, which will provide guarantees about the accuracy and thus the reliability of the results that will be obtained by this method during future day to day analysis. In order to attain maximum guarantees risk to obtain results outside pre-specified acceptance limits is provided as well as the measurement uncertainty.

2. Materials and methods

2.1. Terminology

Before going on with the main aims of this paper it is important to define several crucial terms, which are sources of confusion depending on the environment in, which one works or, which are not usually well known.

- Accuracy, as defined in ISO documents or in documents ICH Q2R1 section terminology, is "the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found." [14–23]. It therefore refers to total measurement error.
  Trueness refers to "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value" [15,16,19]. This concept is therefore related to the systematic error of a measurement process.
- Precision refers to "the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions." [14–23].This is related to the random error of a measurement process.

As can be understood from these definitions, the main point is that accuracy is the simultaneous combination of both systematic and random errors, *i.e.* total error [15,16,19].

- Uncertainty of measurement is defined as "a parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand" [19–21].
- β-expectation tolerance intervals are intervals that contain a proportion β of the individual values, such as results, of the population under investigation (e.g. 0.95). These intervals allow to describe the entire population and are less known than confidence intervals [22]. These intervals are called "β-expectation tolerance intervals" (also known as "mean coverage tolerance intervals" or "prediction intervals"). If β=0.95, this means that on average, 95% of the future individual values (results) of the population are included in the interval [L; U] [23].

2.2. Chemicals

Iohexol (Omnipaque ™) was purchased from Amersham Health (South Plainfield, NJ, USA). Perchloric and orthophosphoric acid were both reagent grade and were purchased from Merck (Darmstadt, Germany) and HPLC grade acetonitrile from Lab-Scan (Dublin, Ireland).

Ultrapure distilled and deionized water was prepared in-house and filtered prior to use.

2.3. HPLC equipment and mobile phase

The HPLC equipment consisted of a Hewlett–Packard (HP) 1100 Model (Agilent Technologies, Palo Alto, CA, USA). The Lichrospher analytical column (Merck; 250×4 mm I.D.; particle size: 5 µM) was packed with C18 material.

The mobile phase consisted of a mixture of distilled water and acetonitrile (95:5: v/v) adjusted to pH 3.0 with orthophosphoric acid. The separation was performed isocratically with a flow rate of 1.0 ml/min. Typical HPLC operating pressure was approximately 70 bars at a thermostatised column oven temperature of 40 °C.

An injection volume of 20 µl for serum and urine samples was accomplished using the HP 1100 autosampler. Detection was achieved with the HP 1100 DAD detector at an absorbance wavelength of 254 nm.

2.4. Standard and control preparation

Standards stock solutions of iohexol (520 mg/ml in deionized water) were prepared and stored at 4 °C. Working serum and urine standards as well as control samples were prepared by dilution of the stock solutions with adequate volumes of blank human serum and urine.

2.5. Calculations

The e.noval software V2.0 (Arlenda, Liège, Belgium) was used to compute all validation results and build the accuracy profiles.

2.6. Serum and urine samples preparation

To deproteinize the serum matrix, 100 µl of serum were treated with 100 µl of 5% perchloric acid. The tubes were then spun at 11,000 g at 4 °C during 10 min. The supernatant was directly transferred to glass HPLC vials.

Fifty microlitres of urine were directly diluted in the glass autosampler vial with 1 ml of deionised water and vortex mixed for 10 s.

3. Results

3.1. Method optimization

The method previously published by Krutzen et al. [12], was modified by first, adjusting the pH of the mobile phase from 2.5 to 3.0, then the volume of perchloric acid necessary to precipitate the serum proteins was reduced from 5 volumes to 1 volume and finally the flow rate reduced from 1.5 to 1 ml/min. These conditions provided good peak shape. The iohexol isomers eluted at 4.3 and 4.7 min (Fig. 1).

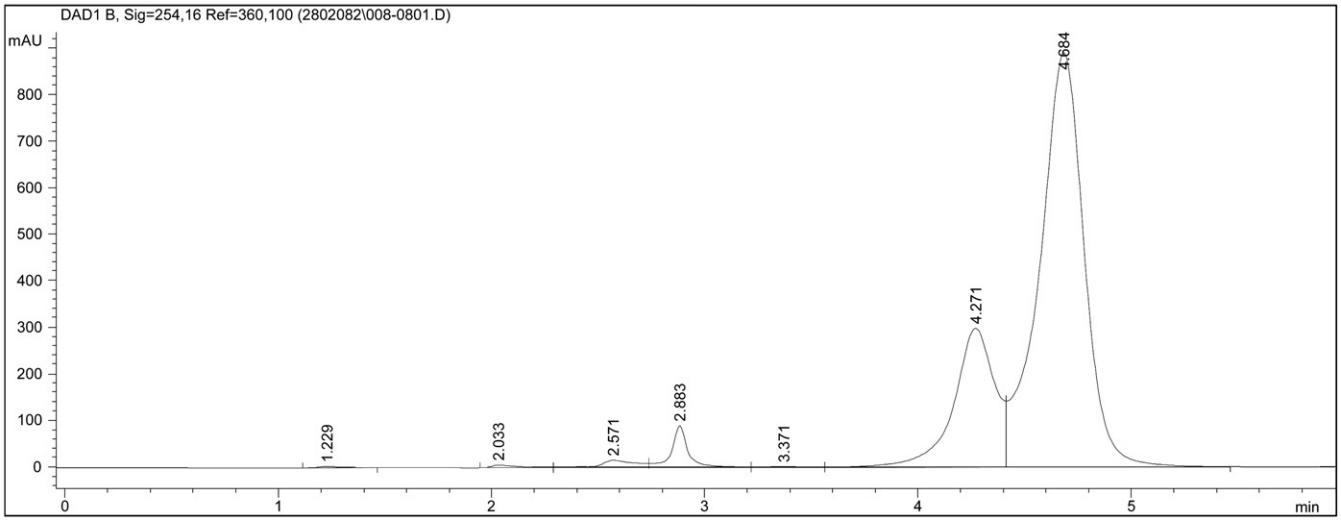

Fig. 1. Chromatogram of a serum 130 µg/ml iohexol calibrator.

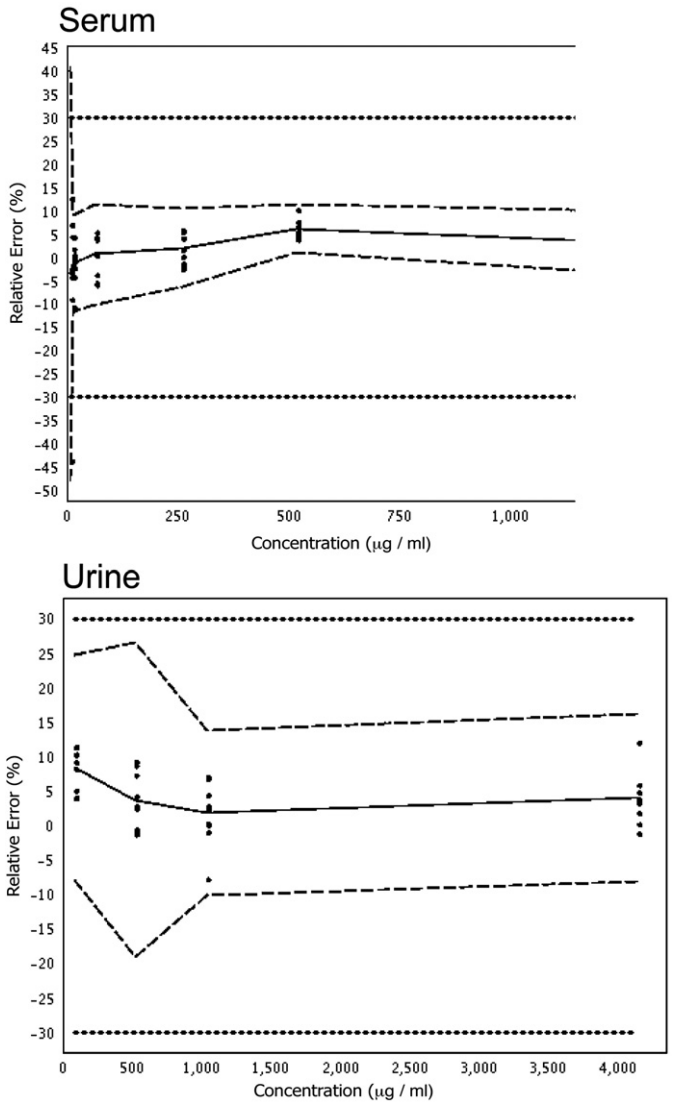

Fig. 2. Accuracy profiles, using a for calibration curve a weighted (1/*X*) linear regression model for serum and urine. Acceptance limits (….), β-expectations tolerance limits (----) and relative bias (___).

3.2. Method validation

3.2.1. Selectivity

The absence of interfering endogenous components of urine and serum at the retention time of iohexol was demonstrated after analysis of serum and urine spiked with iohexol and blank samples from six different sources of the same matrices (data not shown).

3.2.2. Response function

The response function is, within a certain range, the relationship between the response observed and the concentration of the analyte in the sample [24]. Thus, the selection of the calibration curve for the quantification of iohexol in the two matrices should be the fit for purpose response function [25]. To determine the most adequate one for our goal, different calibration models have been evaluated. As iohexol is not naturally present in serum or urine, the calibration curves were prepared by spiking a pool of serum and of urine in order to reach 7 concentration levels ranging from 6.47 to 1295 µg/ml for serum and from 52.0 to 4144 µg/ml for urine, respectively. Each calibration point was analyzed in duplicate on three consecutive days.

Independent validation standards were also prepared by proceeding in the same way: for serum, 6 validation standards ranging from 8.63 to 1295 µg/ml were analyzed in triplicate on three consecutive days and for urine, 4 validation standards ranging from 86.0 to 4144 µg/ml were analyzed in triplicate on three consecutive days.

From the results obtained, the concentrations of the validation standards were back-calculated to determine the mean relative bias, the standard deviation for intermediate precision and finally the upper and lower β-expectation tolerance limits at 95% level [16,26].

Different accuracy profile were thus plotted to select the most convenient regression model for the analytical method. The acceptance limits were set at ±30% as recommended [26]. As shown in Fig. 2, the best response function for serum as well as for urine was achieved with the weighted (1/*X*) linear regression. The response functions obtained by applying these regression models are presented in Table 1.

3.2.3. Trueness

Trueness, expressed in terms of relative bias (%), was calculated from the validation standards [16,24]. As can been seen in Table 1, trueness was acceptable (relative bias smaller than 10%) for both matrices serum and urine.

3.2.4. Precision

The precision of the method was determined by computing the Relative Standard Deviations (RSDs) for repeatability and time-different intermediate precision at each concentration level of the validation standards [16,24,26]. For serum and urine, the precision at each concentration level of the validation standards did not exceed 15% (Table 1).

3.2.5. Accuracy, LOQ and LOD

Accuracy takes into account the total error (sum of the systematic and random errors) of the test results [16,24,26]. As shown in Table 1, the relative upper and lower β-expectation tolerance limits (%) did not exceed the acceptance limits (±30%) for each concentration level and for both matrices.

Table 1
Method validation for iohexol determination in serum and urine.

Validation criterion	Serum		Urine	
Response function	Weighted 1/*X* Linear regression Calibration range (7 points): 6.47–1295 µg/ml		Weighted 1/*X* Linear regression Calibration range (7 points): 52.0–4144 µg/ml	
Trueness	Concentration	Relative bias	Concentration	Relative bias
Level 1	8.63 µg/ml	−3.4%	86 µg/ml	8.4%
Level 2	12.95 µg/ml	−1.3%	518 µg/ml	3.7%
Level 3	64.75 µg/ml	0.7%	1036 µg/ml	1.9%
Level 4	259 µg/ml	2.0%	4144 µg/ml	4.0%
Level 5	518 µg/ml	6.2%		
Level 6	1295 µg/ml	3.3%		
Precision	Repeatability/intermediate precision (RSD%)		Repeatability/intermediate precision (RSD%)	
Level 1	15.0/17.0		0.8/3.5	
Level 2	4.2/4.2		0.8/4.8	
Level 3	4.4/4.4		4.2/4.7	
Level 4	3.1/3.3		2.9/4.1	
Level 5	2.1/2.1			
Level 6	2.4/2.7			
Accuracy	β-expectation lower and upper tolerance limits of the relative error (%)		β-expectation lower and upper tolerance limits in relative error (%)	
Level 1	−47.8; 41.1		−8.0; 24.7	
Level 2	−11.6; 9.1		−19.0; 26.4	
Level 3	−10.1; 11.6		−10.1; 13.8	
Level 4	−6.4; 10.5		−8.2; 16.1	
Level 5	1.1; 11.4			
Level 6	−3.6; 10.1			
Linearity				
Slope	1.035		1.040	
Intercept	0.5372		−5.441	
r^2	0.9989		0.9977	

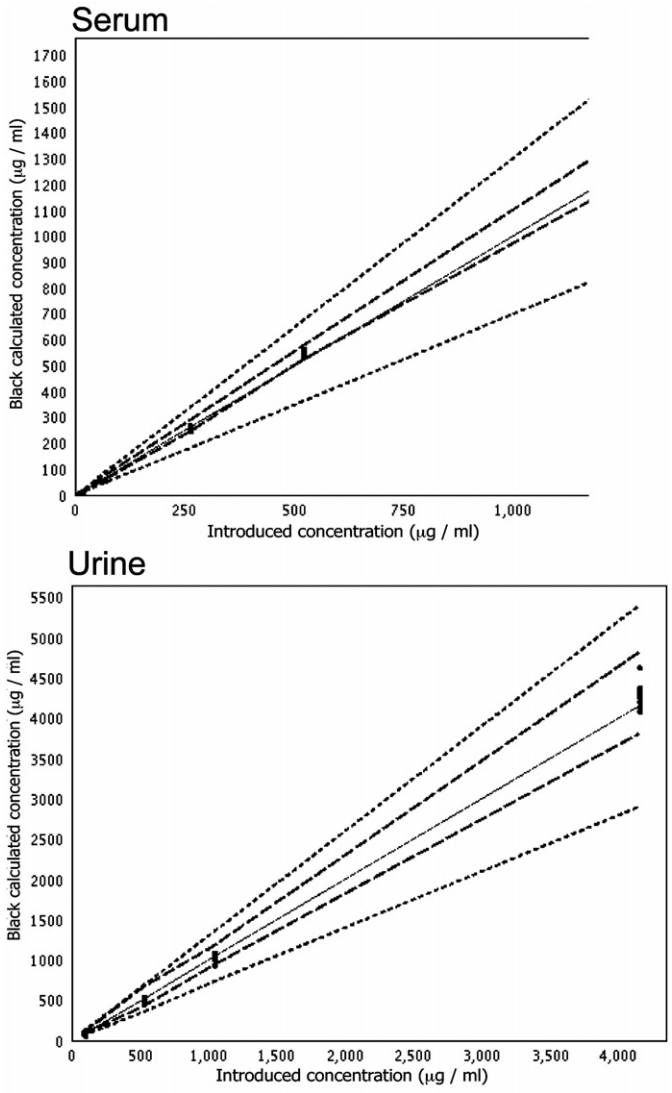

Fig. 3. Linearity profiles for serum and urine. Identity line (___) when $Y=X$, upper and lower acceptance limits in absolute values (….), and upper and lower β-expectation tolerance limits (----).

Thus the method can be considered as giving accurate results between 12.95 and 1295 µg/ml for serum samples, and between 86.0 and 4144 µg/ml for urine.

The limit of detection (smallest quantity of the analyte than can be detected in the sample, but not quantified) was evaluated at 3.08 µg/ml for serum and 26.06 µg/ml for urine. The limits of quantification, obtained by calculating the smallest and the highest concentration beyond, which the β-expectation limits go outside the acceptance limits were 10.76 and 1295 µg/ml for the lowest and highest limits for serum, respectively. For urine, they were at 86.0 and 4144 µg/ml.

3.2.6. Linearity of the results

To evaluate the linearity of the method results, a linear regression line was fitted on the back-calculated concentrations of the validation standards [16,24,26]. The equations obtained and the coefficients of determination are shown in Table 1. The linearity was demonstrated using the β-expectation tolerance interval approach, as the absolute upper and lower β-expectation tolerance limits were included inside the absolute acceptance limits, irrespective of the concentrations studied (Fig. 3).

3.3. Risk assessment

The effective risk of having a future measurement falling outside the specified acceptance limits of ±30% total error by using the β-expectation tolerance intervals obtained with the selected regression models was also evaluated. This risk is calculated as the sum of the proportion of results lying above the upper and under the lower acceptance limits for each concentration level tested [27]. The maximum risk tolerated was set at 5%, it means that in routine practice, we do not accept no more than 5% of our samples to fall outside the ±30% acceptance limits, which is acceptable in clinical biology. Fig. 4 shows the risk profile associated at each level studied for both matrices. None of the levels included in the validated range presented a risk higher than 5% for serum. However for the lowest concentration tested (8.63 µg/ml) where the method is not anymore valid the effective risk to obtain a result out of the acceptance limits is of about 22%. For urine samples, the risk associated to all the concentration levels tested is well under the maximum risk of 5% reaching its maximum value 3.7% for the 518 µg/ml level. The integration of the risk profile in the validation process allows us to determine how far our method will be reliable for its routine utilisation by knowing the effective risk of obtaining out of specification results during the future routine use of the method.

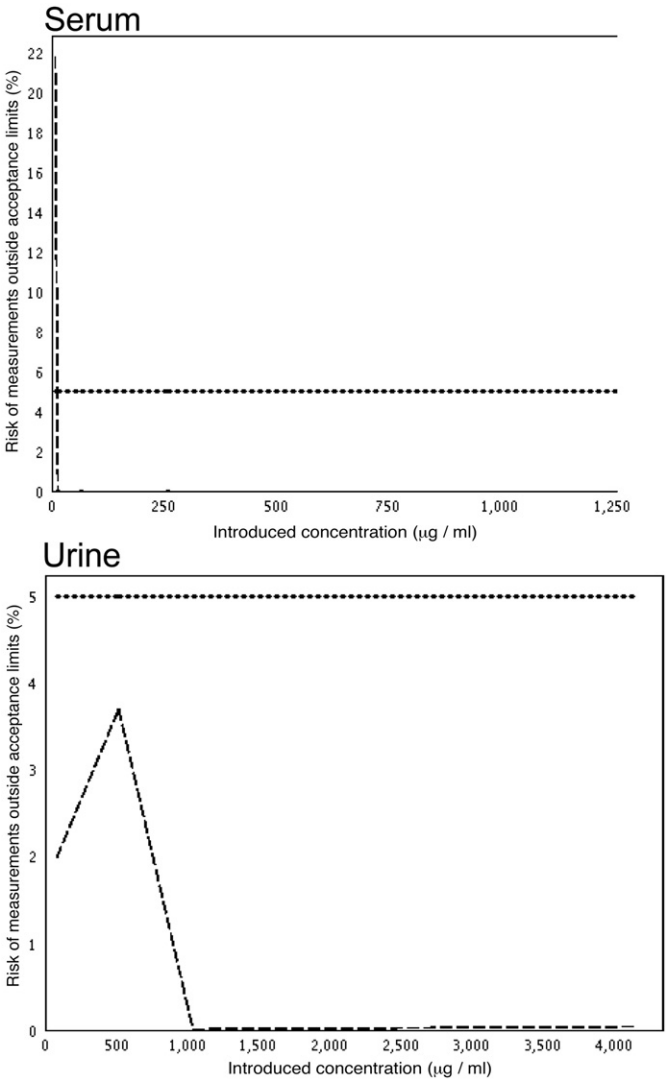

Fig. 4. Risk profiles for serum and urine. The maximum tolerated risk is set at 5%.

Table 2
Estimates of measurement uncertainties related to iohexol measurement in serum and urine, at each concentration level investigated.

SERUM	Concentration (µg/ml)	Uncertainty of the bias (µg/ml)	Uncertainty (µg/ml)	Expanded uncertainty (µg/ml)	Relative uncertainty (%)
Level 1	8.63	0.59	1.58	3.16	36.7
Level 2	12.95	0.18	0.58	1.16	8.9
Level 3	64.75	0.96	3.02	6.05	9.3
Level 4	259.0	3.19	9.22	18.44	7.1
Level 5	518.0	3.62	11.44	22.89	4.4
Level 6	1295	13.33	37.10	74.20	5.7
URINE	Concentration (µg/ml)	Uncertainty of the bias (µg/ml)	Uncertainty (µg/ml)	Expanded uncertainty (µg/ml)	Relative uncertainty (%)
Level 1	86.00	1.71	3.47	6.94	8.1
Level 2	518.0	14.07	28.35	56.70	10.9
Level 3	1036	18.65	51.72	103.4	9.9
Level 4	4144	78.55	185.6	371.1	8.9

3.4. Uncertainty assessment

The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand [28]. Several uncertainty results were generated and are presented in Table 2: the uncertainty of bias of the method at each concentration level of the validation standard, the uncertainty, which combines the uncertainty of the bias with the uncertainty of the method obtained during the validation step, *i.e.* the intermediate precision standard deviation, and the expanded uncertainty, which equals to the uncertainty multiplied by a coverage factor $k=2$, representing an interval around the results where the unknown true value can be observed with a confidence level of 95% [28]. In addition, the relative expanded uncertainties (%) sor serum and urine obtained by dividing the corresponding expanded uncertainties with the corresponding introduced concentrations (Table 2) are not higher than 10%, which means that with a confidence level of 95%, the unknown true value is located at a maximum of ±10% around the measured result. Only for the first concentration level of iohexol in serum (8.63 µg/ml) is the relative expended uncertainty extremely high, about 36%.

4. Discussion

A simple HPLC/UV method for iohexol determination in serum and urine was successfully analytically validated. This is the first time that a reference method for the determination of GFR is validated with such a rigorous and thorough protocol. Contrary to other GFR markers, iohexol is now strongly validated from an analytical point of view and the method is now ready for its clinical application. Moreover, iohexol determination in urine has been poorly studied. Now, on particular conditions and especially in patients with ascite or interstitial oedema, the accurate measure of the urinary clearance is of importance and better than the plasma clearance [29]. Moreover, the reproducibility of GFR measurement is relatively high (around 10%). This reproducibility could certainly be improved if the analytical performance of the marker used for determination (inulin, isotopic or contrast agent) was improved [30,31].

The validation results indicate that the method will give accurate and reliable results for serum values ranging from 12.95 to 1295 µg/ml and for urine values ranging from 86.0 to 4144 µg/ml. In routine practice, iohexol concentrations found in plasma after injection of 5 ml of Omnipaque® (520 µg/ml) usually range from 40 to 600 µg/ml, related to the renal status of the patient and the delay between injection and sampling. The expected urinary values are much wider, depending on the hydratation and the renal function of the patients. As the response of the detector is no linear any more over 4144 µg/ml, one should not hesitate to dilute urine samples to fit with the validated range over 4144 µg/ml. Indeed, urine values usually found in our experience can range from 700 µg/ml to more than 12000 µg/ml. These values are much higher than what had previously been validated for urinary iohexol (10–50 µg/ml) [32].

The original validation approach applied, using accuracy profiles based on β-expectation tolerance intervals for the total measurement error, allowed to evaluate the capability of the method to give fit for purpose results. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the limit of quantification (LOQ) and the range over, which the method can be considered as valid.

Moreover, the risk with respect to the future use of the validated method was estimated and it was demonstrated to be lower than 5% over the whole validated range of concentration for both matrices. Finally, the measurement uncertainties were estimated, allowing correct interpretation and comparison of the results.

From an analytical point of view, we have shown that iohexol determination by HPLC/UV is accurate to meet the clinical goals in GFR measurement.

Acknowledgment

Thanks are due to the Fonds Leon Fredericq and the University of Liège for a research grant to Eric Rozet.

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