Introduction

In this report, we describe and present the performance characteristics of an immunoassay (ELISA) method to measure the concentration of iohexol in collected samples to obtain a mGFR value. Iohexol has emerged as the non-radioactive GFR probe-of-choice among clinical investigators and ELISA is a readily available and cost-effective analytical platform that can be easily standardized across independent clinical reference laboratories. As part of our validation, we reanalyzed archived human serum and plasma samples using the ELISA wherein the iohexol concentration was previously measured using neutron activation analysis and reported.[1] The results of this study show that the ELISA method provides an accurate measurement of iohexol for the application of obtaining a mGFR value.

Materials and Methods

ELISA Iohexol FIT-GFR Kit Components

The FIT-GFR Iohexol kit (BioPAL, Worcester, MA) contains iohexol standard concentrate, rabbit anti-iohexol, goat anti-rabbit IgG-HRP, HRP substrate reagent, HRP stop reagent, and an iohexol 96-well coated plate. The composition of each component is described in the package insert of the kit.

Sample Interference

Because this diagnostic test has applications in basic research, veterinary medicine and human clinical research, sample interference was evaluated for rat, mouse, feline, canine, and human serum and human urine. Samples were diluted 1:0, 1:10, 1:20, 1:50, 1:100 into sample diluent. Fifty microliters of diluted sera or urine samples were pipetted into sample wells in duplicate and then processed following the standard assay protocol. The resulting optical density values were compared with the mean zero standard value.

Cross Reactivity

The cross reactivity of a number of iodinated compounds with anti-iohexol antiserum were assessed. Among the compounds tested were iopamidol, iothalamate, sodium iodide, L-thyroxine, 3,3',5-triiodo-L-thyronine, 3,5-diiodo-L-tyrosine, and 3-iodo-L-tyrosine. Various common drugs, such as aspirin, Tylenol, and ibuprofen were also tested.

Sensitivity

As previously described by Anderson,[2] the limit of detection (LOD) and the limit of quantitation (LOQ) were determined by measuring 10 blank replicates of human serum containing no iohexol at three dilutions (undiluted, 1:50 and 1:500), 10 blank replicates of human urine containing no iohexol at three dilutions (undiluted, 1:500 and 1:5,000), and for 10 blank diluent samples. The standard curve was run in duplicate. The mean value and standard deviation (SD) for each sample set were determined. The LOD was calculated as the mean + 3 SD and the LOQ calculated from the mean value + 10 SD.

Accuracy and Precision

Intra-assay replicate analysis (n=10) and inter-assay replicate analysis (n=5) at two levels of iohexol (low 0.02 µg/ml; high 1.5 µg/ml) were determined. The inter-assay replicates were collected over five weeks. Accuracy was defined as the range of percentage differences between the mean ± 2 SD of back-calculated concentrations and real standard values. Intra-assay and inter-assay precision was expressed as the percent coefficient of variation of the measured iohexol concentration, i.e. %CV = 100·SD/mean.

Comparison Study

Archived Clinical Samples

Between October 2003 and June 2005, 57 patients were recruited to participate in a renal study to directly compare mGFR values obtained by ^{99m}Tc-DTPA and iohexol.[1] The study was approved by the UMass Institutional Review Board and was performed in adherence to the *Declaration of Helsinki*. Informed consent was obtained from all subjects. Neutron activation analysis (NAA) was used as the read-out system to measure the concentration of iohexol *via* the activation of iodine in serum and urine samples. The results of this published study demonstrated that NAA provides an accurate measure of iohexol in a biological sample and that this analytical tool could be used to obtain an accurate GFR value. Samples from a small cohort of this study (n = 22 patients) were archived at -20 °C to allow for the re-analysis of iohexol. The time of iohexol injection, times for each sample collection and urine volume, as well as the corresponding NAA iohexol measured were also archived. After nearly eight years, these samples were re-assayed using a commercially available ELISA iohexol kit.

Immunoassay Analysis

Using the ELISA kit components, iohexol standards were prepared using BioPAL's diluent (0.01 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µg/ml). Using the same diluent, serum and urine samples were diluted. It was determined that serum required a dilution of 1:500 and urine samples required a dilution of 1:5,000 in order to bring the samples within the active range of the standard curve. Fifty microliters of standard or diluted sample were pipetted into wells of a 96-well coated plate and then 50 µl of rabbit anti-iohexol was added to each well. The plate was incubated for 60 minutes at 25°C on an orbital shaker and then washed with a Tween 20 PBS buffer (Elx50, Biotek Instruments, Inc., Winooski, VT). One hundred microliters of goat anti-rabbit IgG-HRP was then added to each well and again incubated for 30 minutes followed by a second plate wash cycle. Substrate (100 µl) was added to all wells and incubated for 30 minutes. Stop reagent (100 µl) was then added and the optical density at 450 nm for each well was recorded. Using software supplied by the plate reader (Multiskan® Spectrum, Thermo Election Corporation), data from the standards were fit to a four-parameter logistic function. By interpolation, the concentration of iohexol present in each sample was determined. Sample preparation and the analytical procedure required approximately three hours to compete.

GFR Calculation via UV/P

As shown in Figure 1, the protocol used in this study provides three UV/P measurements, as follows:

$$GFR_1 = U2 \cdot V2/[T_{U1\text{-}U2} \cdot (P1+P2)/2],$$
$$GFR_2 = U3 \cdot V3/[T_{U2\text{-}U3} \cdot (P2+P4)/2],$$
$$GFR_3 = U4 \cdot V4/[T_{U3\text{-}U4} \cdot (P4+P6)/2],$$

where U and P is the concentration of Iohexol (µg/ml) in urine or serum, respectively. V is the volume (ml) of urine collected at each time point and T is the time duration between urine collections in minutes. The three GFRs (ml/min) were averaged. Since personal patient data such mass and height was not archived, GFR values for this report could not be normalized per 1.73 m^2 of body surface area (BSA).

GFR Calculation via Blood Clearance Method

In addition to the UV/P measurement, a one-compartment blood clearance method was also used in this study. The concentration of iohexol (µg/ml) in each blood sample was plotted as a function of time. The data was fit to a one exponential decay function, i.e., $Y = B\, e^{-bX}$. The function was integrated over the limits zero to infinity to obtain the area-under-the-curve (AUC), i.e., AUC = B/b (mg·min/ml). The GFR value (ml/min) is then obtained by dividing the administrated dose by the AUC. For comparison, this analysis was preformed based on six samples (P1-P6) and for four samples (P1, P2, P4, P6).

Data Analysis

Because NAA provides a direct measurement of iodine content, NAA served as the analytical standard. For each sample collected (n = 220), the recorded iohexol concentration measured by NAA was directly compared to the concentration measured by ELISA using the analysis of Bland and Altman.[3] ELISA GFR values were compared to the clinical standard by assessing bias of the GFR test (the difference between the GFR test and the clinical standard); precision between the GFR test and the clinical standard (r^2); accuracy expressed as the percent of the GFRs that fell within 20% of the clinical standard. In addition, a Student's *t*-test was also used to evaluate statistical differences between the two analytical methods.

The GFR values measured by NAA were directly compared to GFR values measured by ELISA using the analysis of Bland and Altman.[3] ELISA GFR values were compared to the clinical standard by assessing bias of the GFR test (the difference between the GFR test and the clinical standard); precision between the GFR test and the clinical standard (r^2); accuracy expressed as the percent of the GFRs that fell within 10% of the clinical standard. A one way analysis of variance (ANOVA) was used to evaluate statistical differences, wherein a statistical difference was defined $p<0.05$.

Results

FIT-GFR Kit Performance

The corresponding LOD for the diluent was 0.0074 µg/ml and the corresponding LOD for human serum (undiluted, 1:50 and 1:500) was 0.0453 µg/ml, 0.1678 µg/ml and 1.728 µg/ml, respectively, and the LOD for human urine (undiluted, 1:500 and 1:5,000) was 0.0481 µg/ml, 0.2831 µg/ml and 3.9078 µg/ml, respectively. The LOQ for diluent was 0.0214 µg/ml and human serum (undiluted, 1:50 and 1:500) was 0.0874 µg/ml, 0.2067 µg/ml and 2.3474 µg/ml, respectively, and the LOQ for human urine (undiluted, 1:500 and 1:5,000) was 0.0698 µg/ml, 0.6165 µg/ml and 9.9566 µg/ml, respectively. The presence of human serum or urine did not have a significant effect on the baseline measurement. No significant effect on the serum baseline measurement was also observation for other animal models tested with the exception of felines. The presence of feline serum did have a minor effect on the baseline measurement, but this effect was eliminated by 1:10 dilution.

The optical density readout and the corresponding iohexol concentration for the replicates from the intra-assay analysis of the low and high controls are provided in Table 1. The intra-assay coefficient of variation for the low and high control was 2.8 and 3.9, respectively, and the corresponding measured iohexol concentration was 0.203±0.006 µg/ml and 1.556±0.060 µg/ml, respectively. For the inter-assay coefficient of variation for the low and high control was 3.6 and 3.9, respectively, and the corresponding measured iohexol concentration was 0.204±0.007 µg/ml and 1.559±0.061 µg/ml, respectively.

The cross reactivity of iohexol with the anti-iohexol antiserum was 100%. All other tested iodinated compounds and commonly used drugs had a cross reactivity of less than 0.01%.

Comparison Study

Iohexol concentration range in serum or urine samples as measured by NAA was 39820.6–29.7 µg/ml. The iohexol concentration for each sample measured by ELISA is comparable to the values obtained by NAA demonstrating a high degree of precision and accuracy (Figure 2). The bias between the two methods was 3.28 µg/ml, the precision was 12.41 µg/ml, and the accuracy was such that 90% of the measured values were within 20% of the analytical standard (Figure 2B). There was no statistical difference measured between the two analytical methods.

For the 22 human subjects evaluated, the range of GFR values was 22.7–141.1 ml/min. The ELSIA iohexol test provided comparable results to those previously obtained by NAA, demonstrating a high degree of precision and accuracy (Figures 3 and 4). The comparison of the UV/P method shows that the bias between the two tests was 8.01 ml/min, precision was 8.31 ml/min, and the accuracy was such that 95% of the subjects had ELISA GFRs within 20% of the NAA reported value (Figure 3B). For the blood clearance method based on six time-points, the bias between the two tests was 0.45 ml/min, precision was 7.70 ml/min, and the accuracy was such that 100% of the subjects had ELISA GFRs within 20% of the NAA reported value (Figure 4B). There was no statistical difference measured between ELISA GFR values obtained by either UV/P or the six-point blood clearance method compared to the reported NAA values. When the GFR values were recalculated using a one compartment blood clearance model based on four time points, the results were comparable with no statistical difference in the value as compared to the UV/P value and the six-point blood clearance calculation (Table 2).

Discussion

The results of this study demonstrate that immunoassay is sensitive for the detection of iohexol present in human serum and urine samples and provides an accurate and reproducible measurement with a high degree of precision. The results of this study also demonstrate the feasibility of using immunoassay-based readout system to measure GFR in humans. Although this study is limited in size, the samples population spans a wide range of GFR values. The results of this study show that the ELISA platform accurately obtained the GFR, as compared to the previously reported value.[1] Figure 3 and Figure 4 show the comparison of GFR values obtained by the UV/P method and by the blood clearance method, respectively. In both cases, the data virtually fall on the line of unity (Figure 3A and 4A), wherein the error is evenly distributed across the mean from high to low GFR values (Figure 3B and 4B).

In this study, patients were given a standard iohexol dose volume of 5 ml (647.1 mg iohexol per ml). This dose was required to obtain an accurate NAA measurement and is also the standard dose most widely reported for HPLC-based detection methods. However, the results of this study demonstrate that the required dose for the ELISA platform is significantly less. At the administrated 5 ml dose volume, serum samples required a dilution of 1:500 and the urine samples required a dilution of 1:5,000 to bring the samples within the active range of the standard curve. Because no measurable sample interference is observed with undiluted human serum and urine suggests that the administrated dose could be lowered by as much as two orders of magnitude, which in turn would results in a proportional reduction in the dilution factors for collected serum and urine. In an effort to balance sensitivity, easy-of-use and improving the safe profile, these results suggest that a dose of 0.5 ml (647.1 mg iohexol per ml) for an adult human is achievable. As a result, the expected required dilution would be 1:50 and 1:500 for serum and urine samples, respectively.

Another advantage of the ELISA platform is that it requires only a small amount of sample for processing, i.e., 50 µl of serum or urine. As a result, this diagnostic tool can be more easily used to measure GFR in small animal models, such as rodents. Because only a minute amount of sample is consumed by the analysis and because iohexol is stable in serum and urine samples, samples can be archived and then re-assayed at a later date. Immunoassay is an analyte specific-based detection method. As a result, degradation of other components within the biological sample will not affect the accuracy of the iohexol measurement. However, other non-analyte specific based technologies, such as many HPLC-based procedures, overall sample degradation can be problematic. Because iohexol is a clinically available CT contrast reagent, the FIT-GFR test offers researchers the ability to use the same diagnostic probe throughout the pharmaceutical development pipeline, i.e., pre-clinical through human clinical trials. These are all important advantage for clinical research and drug development.

References

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2. Anderson DJ. Determination of the lower limit of detection. *Clin Chem* 35:2152-2153, 1989.
3. Bland JM and Altman DG. Statistical methods for assessing agreement between two methods of clinical measurements. *Lancet* 1:307-310, 1986.

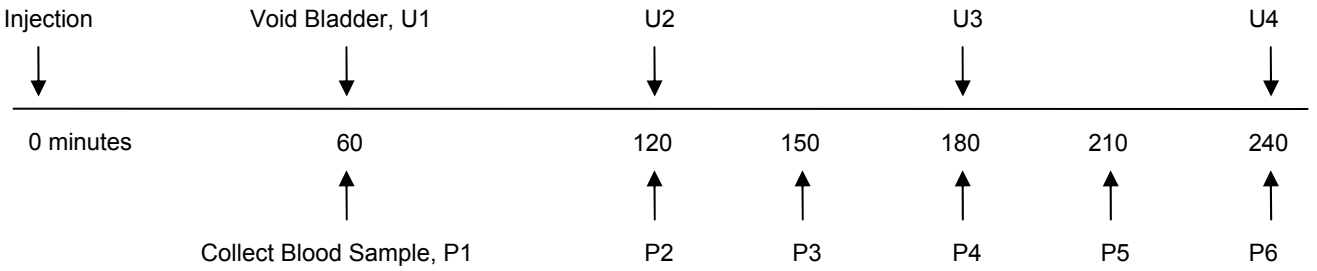

Figure 1: Timeline for the sample collection.

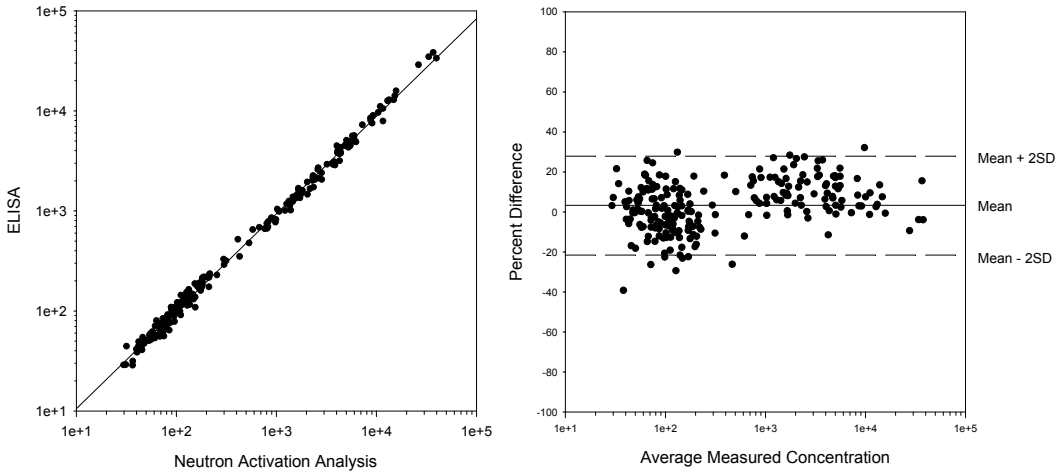

Figure 2: A comparison of two analytical methods of measuring iohexol concentrations (µg/ml) in collected serum samples. **A:** ELISA values are directly compared with NAA values y = 0.987x – 75.818; r = 0.99; P = 0.75. **B:** difference against the mean iohexol value.

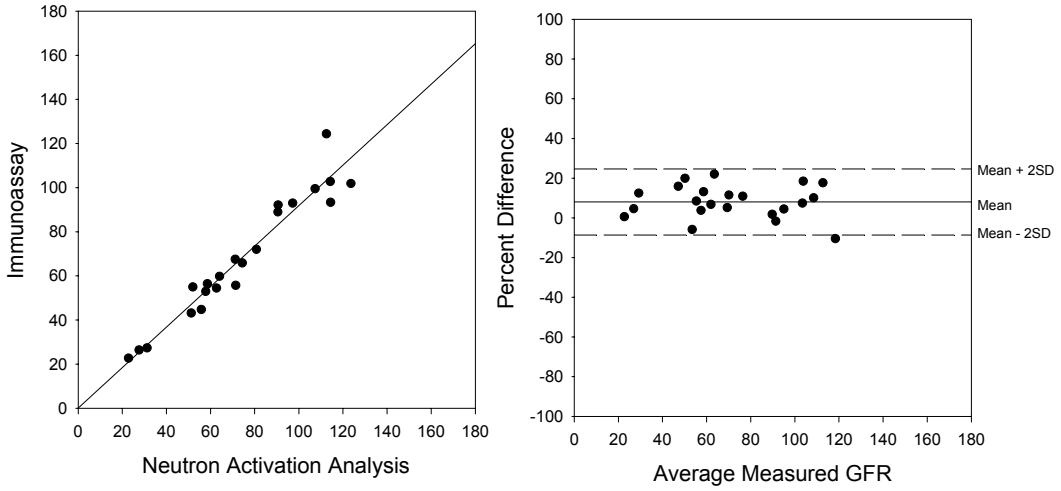

Figure 3: Immunoassay (ELISA) and neutron activation analysis (NAA) glomerular filtration rate (GFR) values (ml/min) calculated by the UV/P method. **A:** ELISA values are directly compared with NAA values y = 0.918x – 0.095; *r* = 0.97; *P* = 0.49. **B:** difference against the mean iohexol value.

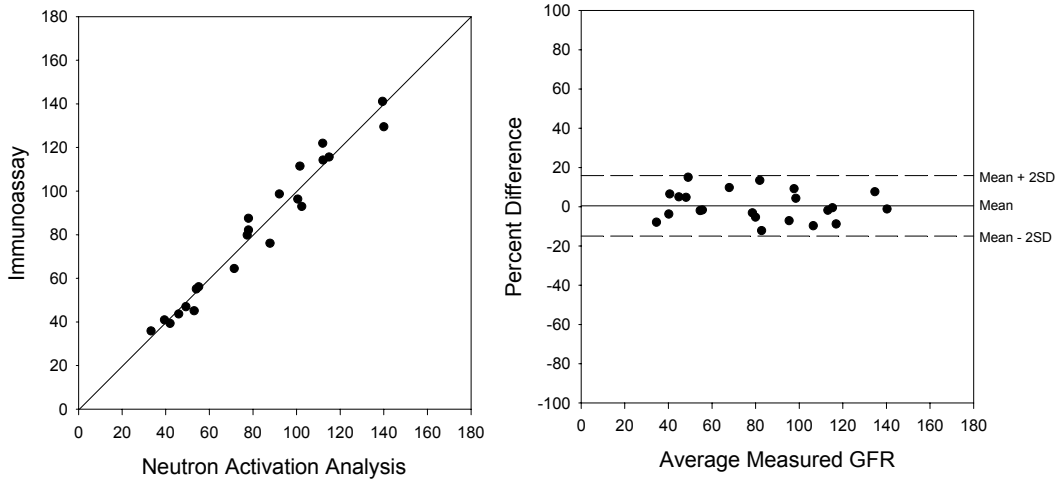

Figure 4: Immunoassay (ELISA) and neutron activation analysis (NAA) glomerular filtration rate (GFR) values (ml/min) calculated by the one compartment blood clearance method using 6 time points. **A:** ELISA values are directly compared with NAA values y = 1.002x – 0.365; *r* = 0.98; *P* = 0.98. **B:** difference against the mean iohexol value.

	Low Control		High Control	
	Optical Density	µg/ml	Optical Density	µg/ml
1	0.593	0.196	0.278	1.510
2	0.590	0.200	0.281	1.469
3	0.590	0.200	0.272	1.581
4	0.593	0.195	0.269	1.623
5	0.583	0.209	0.268	1.640
6	0.578	0.214	0.277	1.525
7	0.588	0.202	0.274	1.555
8	0.584	0.206	0.281	1.474
9	0.586	0.204	0.270	1.608
10	0.588	0.202	0.272	1.579
Mean	0.587	0.203	0.274	1.556
S.D.	0.00467	0.005775	0.00487	0.06030
%CV	0.8	2.8	1.8	3.9

Table 1: The optical density and concentration for iohexol from the replicates from the intra-assay analysis of the low and high controls are presented.

	UV/P		Blood Clearance – 6P		Blood Clearance – 4P	
	ELISA	NAA	ELISA	NAA	ELISA	NAA
Mean	68.16	74.19	80.66	80.90	79.72	79.96
S.D.	27.91	29.38	32.66	31.98	32.14	31.50

Table 2: The average glomerular filtration rate (GFR) and standard deviation values (ml/min) for each calculated method, $P = 0.71$.