Introduction

The direct measurement of glomerular filtration rate (mGFR) remains the standard of excellence for the evaluation in renal performance (1). There are numerous procedural methods available to obtain an accurate mGFR value and, if properly administrated, each method will provide a comparable result. Despite superior accuracy and reproducibility, this diagnostic measurement is underutilized both in research and in clinical medicine due to cumbersome analytical procedures that add excessive cost to the analysis, such as iohexol HPLC, or present regulatory barriers, such as radioactivity. As a result, only highly specialized laboratories run mGFR studies on a routine basis.

The medical community now recognizes renal disease as a significant world-wide healthcare problem, which in turn has renewed focus on effective and reliable renal diagnostics (2). The necessity of mGFR testing for many clinical situations, such as transplantation and as a confirmatory test for biomarker-based screening programs, has become apparent (3). Likewise, researchers and regulatory agencies now recognize the importance of mGFR testing to ensure the reliability of pre-clinical and clinical investigations (4).

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. As part of our initial validation we also present the results for a feline study to demonstrate the accuracy of the ELISA detection method to measure iohexol clearance to obtain a mGFR value, as compared to neutron activation analysis (NAA) and to HPLC (5-7). The results of this study show that the ELISA method provides an accurate measurement of iohexol for the application of obtaining a measured GFR value in felines.

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.

Iohexol FIT-GFR Kit Validation

Sample Interference

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 (8), the limit of detection (LOD) and the limit of quantitation (LOQ) were determined by measuring 10 blank replicates of feline serum containing no iohexol at three dilutions (undiluted, 1:10 and 1:100) 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

Experimental Procedure

Three male adult cats and two female spayed adult cats were used for this study. All animals were between 7-9 years of age. Each animal was implanted with a venous access port (VAP) which allowed intravenous access to the jugular vein. All experimental procedures were approved by the Tufts University Institutional Animal Care and Use Committee.

Food was withheld from each animal for at least 12 hours prior to the experiment. At the start of the study, animals were weighed. Iohexol (Omnipaque® 300, 300 mg/kg, 1.0 mL/kg) was injected into the VAP and the exact time of injection was recorded. The iohexol was given as a bolus followed by a saline flush. Blood samples (~3 mL) were collected from the jugular vein at baseline (pre-injection) and at 2, 3, and 4 hours after injection. Blood was placed into serum collection tubes and allowed to clot for 15 minutes. The tubes were then centrifuged and the serum was harvested and divided into two portions. A portion of the sample was shipped to Michigan State University (MSU) for iohexol analysis by HPLC for the determination of GFR. The remaining portion was analyzed by ELISA and by NAA.

Neutron Activation Analysis

As previously described (5,6), each serum sample was centrifuged and 100 µl of serum was transferred to a vial designed for neutron activation analysis (BioPAL, Inc., Worcester, MA). Each vial contains a known amount of a metallic monitor to account for potential neutron-flux variations during the neutron activation process (9). All samples were dried in a warming oven at 70°C overnight. In addition, iohexol standards and NIST traceable iodine standards were also prepared in the same manner. Prepared samples from each subject were then analyzed by neutron activation analysis to quantify the amount of iohexol (BioPAL, Inc., Worcester, MA). Because the iodine component of iohexol can be correctly verified using a NIST standard, NAA is the analytical standard for this study.

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 samples were diluted 1:300 in order to bring the samples within the active range of the standard curve. 50 µl 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). 100 µl of goat anti-rabbit IgG-HRP was 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

A one-compartment blood clearance method was used in this study (10). 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. The GFR was further adjusted on the basis of the feline's body weight, and these adjusted GFR value (ml/min/kg) were used for statistical analyses.

Data Analysis

Because NAA provides a direct measurement of iodine content, NAA served as the analytical standard. For each sample collected (15 total), the iohexol concentration measured by NAA was directly compared to the concentration measured by ELISA using the analysis of Bland and Altman (11). 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. In addition, a student pair *t*-test was also used to evaluate statistical differences between the two analytical methods. The comparable analysis for HPLC could not be preformed because MSU does not report individual sample concentration. MUS only reports the GFR value for each feline subject.

The GFR values measured by NAA and HPLC were directly compared to GFR values measured by ELISA using the analysis of Bland and Altman (11). ELISA GFR values were compared to both clinical standards 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. In addition, a student *t*-test was also used to evaluate statistical differences between analytical methods.

<u>Intra-assay and Inter-assay Variability</u>

To evaluate intra-assay variability of obtaining a GFR value, samples from one feline subject were evaluated in duplicate eight times on one plate. Intra-assay precision was expressed as the coefficient of variation (%CV = 100·SD/mean) of the measured optical density value, the corresponding iohexol concentration and the resulting GFR value. To evaluate inter-assay variability, the same feline subject's GFR value was measured every other month for one year for a total of six measurements. During the one-year period, serum samples were stored at 4 °C.

Results

FIT-GFR Kit Performance

The corresponding LOD for the diluent was 0.0006 µg/ml and the corresponding LOD for feline serum (undiluted, 1:10 and 1:100) was 0.0261 µg/ml, 0.0098 µg/ml and 0.0002 µg/ml, respectively. The LOQ for diluent was 0.0018 µg/ml and feline serum (undiluted, 1:10 and 1:100) was 0.1173 µg/ml, 0.0449 µg/ml and 0.0015 µg/ml, respectively. The presence of feline serum did have a minor effect on the baseline measurement, but this effect was eliminated by 1:10 dilution. In contrast, for additional species tested there was no measurable sample interference observed with serum at any dilution level, including undiluted human serum and urine.

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 controls 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 controls 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

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 1). The bias between the two methods was -3.34 µg/ml, the precision was 5.06 µg/ml, and the accuracy was such that 100% of the measured values were within 10% of the analytical standard (Figure 1B). There was no statistical difference measured between the two analytical methods.

Table 2 lists the GFR values obtained for each method. The ELISA test provides comparable results to both NAA and HPLC tests. For the ELISA to NAA comparison, the bias between the two tests was 0.098 ml/min/kg, precision was 0.205 ml/min/1.73m^2, and the accuracy was such that 90% of the subjects had ELISA GFR values within 10% of the NAA GFR value. There was no statistical difference measured between GFR values obtained by ELISA and NAA.

A bias was observed for the HPLC test. For the ELISA to HPLC comparison despite a good correlation, the bias between the two tests was 0.252 ml/min/1.73m^2, precision was 0.185 ml/min/1.73m^2, and the accuracy was such that none of the subjects had ELISA GFR value within 10% of the HPLC value. Comparable results were also obtained when NAA values are compared directly to HPLC values. The bias between these two tests was 0.350 ml/min/1.73m^2, precision was 0.037 ml/min/1.73m^2. Due to the bias, there was a statistical difference measured when NAA or ELISA GFR values are compared to HPLC values, see Table 2.

Intra-assay and Inter-assay Variability

The intra-assay coefficients of variation for the optical density readout, the corresponding iohexol concentration and measured GFR value are provides in Table 3. The inter-assay coefficient of variation for the measured GFR value is provided in Table 4. The intra-assay and inter-assay coefficient of variation for the measured GFR value was 5.7 and 3.1, respectively.

Discussion

This report presents for the first time a technological approach using immunoassay to measure the GFR probe iohexol and thereby obtain a measured GFR value. Two immediate benefits of this advance are apparent. First, since the analytic arm of this test relies on immunoassay, many analytic barriers for high throughput processing iohexol samples are eliminated. Second, because this ELISA test is provided in kit format, all laboratories using this method perform the same analytical procedure with the same reagents in the same way. Therefore, identical results and better standardization will be found among independent investigators and core laboratories.

Iohexol is an iodinated contrast dye designed to enhance X-ray computed tomography (CT). Iohexol does not bind to serum proteins and is freely filtered through the glomerulus with no identifiable reabsorption or tubular secretion making it an ideal marker for the measurement of GFR. Unlike iothalamate, iohexol has been reported to have a low allergenic potential (12). Due to its clinical availability, iohexol has emerged as the GFR probe-of-choice and the most widely reported analytical method for its measurement is HPLC.

The results of this study demonstrate that immunoassay is sensitive for the detection of iohexol and provides an accurate and reproducible measurement with a high degree of precision. Undiluted pooled feline serum showed minor cross reactively, but this effect was eliminated by 1:10 dilution. The results of this study also demonstrate the feasibility of using this immunoassay-based readout system to measure the clearance of injected iohexol to obtain a measured GFR value in cats. Although this study is limited in size, individual samples for each subject span a wide range of iohexol concentrations. Figure 1 shows the comparison of iohexol values obtained from the immunoassay method to values obtained from NAA, the analytical standard. This direct comparison show a strong linear correlation (Figure 1A), wherein the error is evenly distributed across the mean from high to low iohexol values (Figure 2A). As a result, the GFR values generated from the data also are in statistical agreement (Table 2). When ELISA or NAA GFR values are directly compared to HPLC values the results demonstrate good correlation but with a systematic bias that prevented statistical agreement. Because the reference laboratory that conducted the HPLC analysis did not report iohexol concentration for individual samples, it cannot be determine if the bias is due to an analytical component of the HPLC measurement or due to a systematic mathematical factor introduced in the GFR calculation.

At the iohexol dose used in this study, feline serum required a dilution of 1:300 in order to bring the samples within the active range of the standard curve. Because potential feline serum sample interference could be effectively eliminated by a 1:10 dilution suggests that the administrated dose could be lowered by a factor of ten. The lower dose would result in a proportional reduction in the dilution factors and a resulting theoretical 1:30 dilution would be sufficient to ensure an accurate ELISA measurement. The lower dose would also provide an additional level of safety for veterinary applications. In addition, the lower dose volume combined with the small sample size requirements makes the ELISA method attractive for GFR determination in small research models, such as rodents. Because iohexol is a clinically available reagent and the ELISA reagents are commercially available and provided in kit format, this approach may offer a low-dose method to measured GFR in human clinical investigations that will allow for better standardized across different core facilities. The ELISA iohexol platform provides researchers the ability to use the same diagnostic probe throughout the pharmaceutical development pipeline, i.e., pre-clinical through human clinical trails and therefore warrants additional investigation.

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	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.

	Analytical Method		
	HPLC	NAA	ELISA
Subject 1	1.778	2.090	2.065
Subject 2	1.653	1.972	1.995
Subject 3	2.282	2.677	2.593
Subject 4	3.120	3.464	3.510
Subject 5	2.510	2.891	2.437
Average	2.269	2.619	2.520
S.D.	0.592	0.610	0.607

Table 2: Glomerular filtration rate (min/min/kg) values obtained by each analytical method. NAA vs. ELISA, r = 0.95, *P* = 0.341; HPLC vs. ELISA, r = 0.96, *P* = 0.039; NAA vs. HPLC, r = 1.00, *P* = 0.00002.

	2hr Serum		3hr Serum		4hr Serum		
Sample	O.D.	Act.	O.D.	Act.	O.D.	Act.	GFR
1	0.2477	1145.0	0.3137	686.3	0.3852	424.3	1.891
2	0.2447	1173.8	0.3070	719.8	0.3817	433.8	1.825
3	0.2498	1123.8	0.3159	675.0	0.3909	409.3	1.906
4	0.2575	1054.3	0.3197	657.0	0.3982	391.8	2.035
5	0.2596	1036.3	0.3200	656.5	0.4050	375.3	2.041
6	0.2554	1072.5	0.3164	672.5	0.3861	422.0	2.019
7	0.2433	1189.0	0.3086	711.5	0.3799	438.8	1.813
8	0.2398	1224.0	0.3013	750.0	0.3668	477.5	1.764
Average	0.2494	1127.3	0.3128	691.1	0.3867	421.6	1.912
S.D.	0.006789	67.9	0.006619	33.2	0.0117	31.1	0.1089
%CV	2.7	6.0	2.1	4.8	3.0	7.4	5.7

Table 3: The intra-assay analysis obtained from one feline subject. The optical density readout (O.D.), the corresponding iohexol concentration (Act.) and measured GFR value are listed.

Month	GFR
1	1.995
3	1.998
5	1.911
7	1.912
9	1.892
11	2.041
Average	1.958
S.D.	0.0609
%CV	3.1

Table 4: The inter-assay analysis obtained from one feline subject.

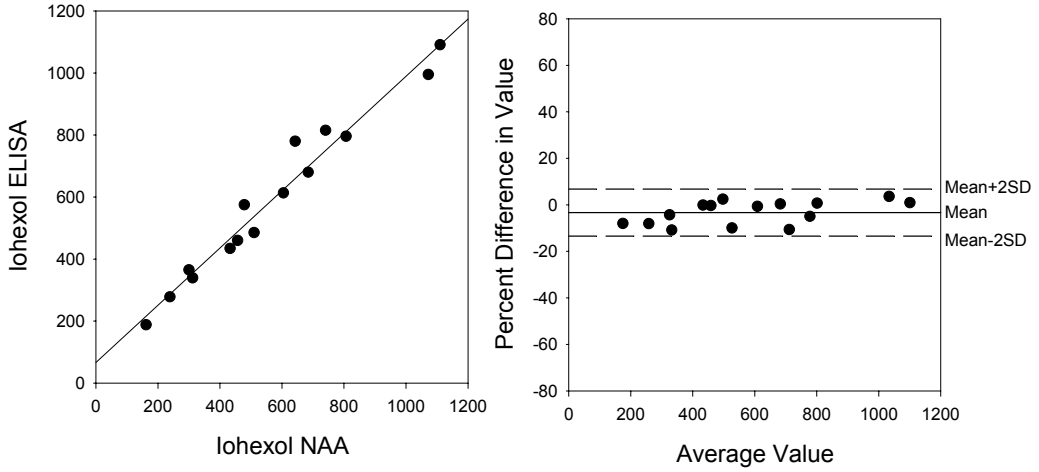

Figure 1: A comparison of two analytical methods of measuring iohexol concentrations (µg/ml) in collected serum samples. **A:** ELISA values are directly compared with NAA values y = 0.923x + 66.4; *r* = 0.98; *P* = 0.124. **B:** difference against the mean iohexol value.