Evaluation of Iohexol Clearance to Estimate Glomerular Filtration Rate in Normal Horses

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Abstract

In adult horses and foals, renal dysfunction can occur as a secondary complication to gastrointestinal disorders, dehydration, septicemia, endotoxemia and nephrotoxic drug administration. Measurement of renal function is an important feature not only in the diagnosis, but also in the prognosis and management of renal disease. Commonly used drugs such as phenylbutazone and gentamicin can be highly nephrotoxic under certain conditions.

Estimation of the glomerular filtration rate (GFR), accepted as one of the earliest and most sensitive assessments of renal function, can be determined in horses using standard techniques such as endogenous or exogenous renal creatinine clearance. These techniques can be time consuming, dangerous to perform on fractious patients, require trained personnel and are subject to errors most often associated with improper or incomplete urine collection. Recently, tests using iohexol, a radiographic contrast agent, have been developed to estimate the GFR in human beings, pigs, sheep, dogs, cats and horse foals with results that have been validated by traditional standards.
Serum clearance of a substance that is freely filtered by the kidneys without tubular secretion or reabsorption, that is not protein bound, and that is not metabolized, is a measurement of glomerular filtration rate. Iohexol meets all of these requirements and thus its clearance from serum should accurately estimate GFR. Utilization of serum clearance studies for estimation of GFR provides a clinically feasible and reproducible method in order to measure GFR in horses. Other commonly used methods to assess renal function in horses are fraught with inherent and operator error. Serum clearance of iohexol does not necessitate collection of urine and has been shown to be a safe, reproducible method using collection of timed blood samples to assess renal function in humans and animals. The objectives of this project were 1) to determine a method of estimation of GFR based on serum clearance of a substance that meets the requirements of a marker for GFR, and 2) to make the method clinically applicable by developing a method using two blood samples to derive clearance and thus GFR in normal adult horses.

Results of this study showed good agreement between GFR derived by exogenous creatinine clearance and serum clearance of iohexol. In addition, GFR values for all horses using either method were within published reference ranges for this species. The results of this study indicate that a single intravenous injection of iohexol at a dose of 150 mg/kg, followed by collection of 2 serum samples at 3 and 4 hours post injection can be used to estimate the GFR in healthy horses.
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TABLE OF CONTENTS

ABREVIATIONS ................................................................................................. vi

LIST OF TABLES .............................................................................................. vii

LIST OF FIGURES .............................................................................................viii

CHAPTER 1: LITERATURE REVIEW .................................................................1
  1) Renal Physiology .....................................................................................1
  2) Glomerular Filtration .............................................................................2
  3) Renal Function Testing .........................................................................8
  4) Iohexol ..................................................................................................14

CHAPTER 2: MATERIALS AND METHODS .....................................................17
  1) Horses ...................................................................................................17
  2) Subject preparation ..............................................................................17
  3) Iohexol clearance ................................................................................18
  4) Exogenous creatinine clearance ..........................................................19
  5) Iohexol pharmacokinetic calculations .................................................20
  6) Statistical analysis ...............................................................................21

CHAPTER 3: RESULTS ....................................................................................23
  1) Urinary clearance of creatinine ............................................................23
  2) Serum clearance of iohexol .................................................................23
  3) Predicted plasma clearance of iohexol ..................................................23
  4) Comparison of iohexol clearance and creatinine clearance ...............24

CHAPTER 4: DISCUSSION .............................................................................30

REFERENCES ..................................................................................................35

VITA ..................................................................................................................39
ABBREVIATIONS

GFR  glomerular filtration rate

NSAIDS  non-steroidal anti-inflammatory drugs

ARF  acute renal failure

CRF  chronic renal failure

\([\text{Cr}_{\text{serum}}]\)  creatinine concentration in serum

SUN  serum urea nitrogen

\(\text{CL}_{\text{creatinine}}\)  creatinine clearance

\(\text{CL}_{\text{renal}}\)  urinary clearance

\(\text{CL}_{\text{serum}}\)  serum clearance

\(^{99m}\text{Tc-DTPA}\)  \(^{99m}\text{Tc}\)-labeled diethylenetriaminepentaacetic acid

\(^{51}\text{Cr-EDTA}\)  Chromium-51-Ethylenediaminetetraacetic Acid

\(\text{CL}_{\text{iohexol}}\)  3-compartment serum clearance of iohexol

HPLC  high performance liquid chromatography

AUC  area under the curve

\(\text{AUC}_{4\text{ and }6}\)  area under the curve at 4 and 6 hours post iohexol injection

\(\text{CL}_{3\text{-}4\text{ hour}}\)  predicted \(\text{CL}_{\text{plasmaC-3}}\) from \(\text{AUC}_{3\text{ and }4}\)
List of Tables

Table 1: Estimated GFR values (ml/min/kg) for
CL\textsubscript{creatinine}, CL\textsubscript{iohexol} and CL\textsubscript{3-4hour} in 10 horses……………………………………………….25

Table 2: Pharmacokinetic values describing the
disposition of iohexol in horses after IV administration
of a single 150 mg/kg dose……………………………………………………………….26
List of Figures

Figure 1: Semi-logarithmic serum concentration
vs. time plot for iohexol in horses (n=10) after IV
administration of a single 150 mg/kg dose. .....................................................27

Figure 2: The relationship between AUC_{iohexol}
and AUC_{3-4hour}. ..........................................................................................28

Figure 3: Limits of agreement plot depicting the mean
difference between CL_{creatinine} and CL_{iohexol} in 10
healthy horses....................................................................................................29
Chapter 1: Literature Review

1) Renal physiology

Mammalian kidneys are paired, retroperitoneal organs that are located in the caudal half of the abdomen, lateral to the spinal column. They have many essential functions including excretion of the waste products of metabolism and exogenous chemicals, regulation of water and electrolyte balances, regulation of fluid osmolality, regulation of acid-base balance, regulation of arterial blood pressure, production, metabolism and excretion of hormones, and gluconeogenesis. These functions are integral to maintenance of homeostasis such that after total loss of renal function death of most mammals results within one week.

Grossly the kidney is divided into two major regions: the outer cortex and inner medulla. Blood flow to the kidneys comprises approximately 15-20% of the cardiac output. Blood enters the kidney through the hilus as the renal artery which then branches into interlobar arteries, arcuate arteries and afferent arterioles and thus into the capillary beds of the glomeruli. Efferent arterioles exit the glomeruli and lead to secondary capillary beds, the peritubular capillaries which drain into the interlobular veins, arcuate veins, interlobar veins and finally the renal vein, which exits the kidney at the hilus.

The functional unit of the kidney is the nephron. The number of nephrons is species dependent, ranging from 190,000 in the cat, to approximately 4 million in the horse and cow. The kidney cannot regenerate nephrons, thus this number decreases with disease and age. Each nephron is composed of a renal corpuscle, a proximal tubule,
a loop of Henle, a distal convoluted tubule, a connecting tubule and collecting ducts. The renal corpuscle is composed of the glomerulus surrounded by Bowman’s capsule. Blood flows into the glomerular capillaries where fluid is filtered under high hydrostatic pressures into Bowman’s space. As the filtrate flows through the sequence of tubules it is modified through electrolyte, urea and water secretion and reabsorption in order to regulate total body electrolyte, water and acid base balances. The resultant solution is urine that flows from the collecting ducts of the kidney into the renal pelvis, ureters and excreted from the body.

2) Glomerular filtration

Urine is formed through the combination of glomerular filtration, reabsorption of substances from the renal tubules and secretion of substances into the tubules. The composition of glomerular filtrate is almost exactly that of blood with the exception of cells and most proteins. Fluid filtered through the glomerulus passes through three anatomic barriers: the capillary endothelium, the basement membrane and the capillary epithelium. The endothelium of the glomerular capillaries is fenestrated with pores that are larger than those of most other capillary beds in the body. Glomerular endothelial cells also possess fixed negative charges which prevent passage of large negatively charged molecules, particularly proteins. The basement membrane is a relatively thick layer of collagen and proteoglycan fibrils that allows passage of water and small solutes. Proteoglycans also carry a strong fixed negative charge, preventing filtration of proteins. Glomerular capillary epithelial cells (podocytes) possess long foot-like processes that
interdigitate to form a filtration barrier. The foot processes are separated by areas called slit pores that allow passage of the glomerular filtrate. Glomerular epithelial cells also possess strong negative charges and hinder the filtration of like-charged particles. Thus whether particles are filtered through the glomerulus is dependent both on their molecular weight and charge. In humans the capillary endothelium accounts for approximately 2% of the resistance to filtration, with the basement membrane and podocytes contributing approximately 50% and 48% respectively \(6\).

Forces affecting glomerular filtration rate (GFR) are those that control capillary fluid dynamics elsewhere in the body. GFR is determined by 1) glomerular plasma hydrostatic pressure \(P_G\), 2) glomerular plasma colloid oncotic pressure \(\pi_G\), 3) hydrostatic pressure within Bowman’s capsule \(P_B\), 4) colloid oncotic pressures within Bowman’s capsule \(\pi_B\), and 5) the glomerular capillary filtration barrier (filtration coefficient, \(K_f\)) \(^1\). As most proteins are prohibited from filtration due to their size and charge, the oncotic pressure of Bowman’s capsule is negligible and favors filtration of fluid and solutes. Thus GFR can be expressed as:

\[
GFR = K_f \times (P_G - P_B - \pi_G + \pi_B)^1
\]

The glomerular filtration coefficient \((K_f)\) is the product of the permeability of the filtration barrier \((k)\) and the surface area for filtration \((S)\) \(^6\). The surface area of the glomerular capillary is approximately 400 times as high as that of capillaries elsewhere in
the body\(^1\) thus contributing to the large amount of fluid filtered by the kidney. \(K_f\) can be decreased (and thus GFR decreased) by a decrease in the number of functional nephrons (decreased filtration surface area) or through an increase in glomerular filtration barrier thickness as occurs in chronic hypertension or diabetes mellitus\(^1\).

Increases in the hydrostatic pressure in Bowman’s capsule (\(P_B\)) occur rarely but may serve to decrease GFR. In humans \(P_B\) averages 18 mmHg, and increases in this pressure rarely are significant enough to exceed that of \(P_G\). Significant increases in \(P_B\) may occur with urinary tract obstruction and may cause corresponding decreases in GFR.

As blood passes through the glomerular capillaries, the glomerular capillary plasma oncotic pressure (\(\pi_G\)) increases due to a loss of fluid by filtration and an increase in capillary protein concentration. Therefore \(\pi_G\) is determined by 1) arterial plasma colloid oncotic pressure and 2) the fraction of plasma filtered through the glomerulus (filtration fraction)\(^1\). Filtration fraction is defined as:

\[
\text{Filtration fraction} = \frac{\text{GFR}}{\text{renal plasma flow}}
\]

Thus as renal plasma flow increases and GFR is maintained, the filtration fraction decreases, \(\pi_G\) decreases and GFR increases. Decreases in renal plasma flow have the opposite effect of decreasing GFR through an increase in \(\pi_G\). Therefore, independent of changes in \(P_G\), changes in renal plasma flow may have significant effects on GFR.

In many species the glomerular capillary plasma hydrostatic pressure (\(P_G\)) averages 60 mm Hg\(^1,6\). GFR is directly dependent on \(P_G\) and changes in \(P_G\) account for
the majority of physiologic regulation of GFR. $P_G$ is determined by 1) arterial pressure, 2) afferent arteriolar resistance and 3) efferent arteriolar resistance$^1$. Increases in arterial blood pressure increase glomerular hydrostatic pressure and GFR and this effect is closely regulated by feedback processes in the normal animal. Constriction of the afferent arterioles increases afferent resistance, decreases renal blood flow and decreases GFR whereas dilation of the afferent arterioles increases $P_G$ and GFR. Constriction of the efferent arterioles serves to increase $P_G$ but may also decrease renal blood flow. A decrease in renal blood flow causes an increase in glomerular capillary plasma oncotic pressure ($\pi_G$) which, if severe, may oppose the increase in $P_G$ enough to cause a decrease in GFR. An increase of efferent arteriolar resistance of approximately 300$^1$ is necessary for this to occur, thus with mild to moderate increase in efferent arteriolar resistance, GFR increases and with severe resistance, GFR decreases.

In normal animals GFR is maintained primarily through physiologic control of glomerular hydrostatic pressure and glomerular capillary colloid oncotic pressure. This regulation is mediated by the sympathetic nervous system, hormones, locally acting vasoactive substances and other local feedback mechanisms. Autoregulation of GFR and renal blood flow by the kidneys serve to maintain these variables at constant rates in the face of changes in arterial blood pressure.

The sympathetic nervous system innervates all blood vessels in the kidney. Moderate activation of the sympathetic nervous system as occurs during pressure decreases at the carotid sinus or cardiopulmonary baroreceptors does not appear to affect GFR. However, strong stimulation of the sympathetic nervous system such as during
fight or flight responses, brain ischemia, or severe hypotension causes constriction of the renal arterioles. This serves to decrease both renal blood flow and GFR. In the normal animal, sympathetic tone to the kidney appears to have minimal effect on GFR.

In concordance with increased sympathetic tone, epinephrine and norepinephrine are released from the adrenal medulla. These circulating hormones also have the effect of constricting both efferent and afferent renal arterioles and thus causing a decrease in GFR. Like the sympathetic nervous system, these hormones appear to play a minimal role in the normal regulation of GFR.

Autocoids that affect GFR include endothelial-derived nitric oxide, prostaglandins and bradykinin. Endothelial-derived nitric oxide is released by vascular endothelium and a constant basal level is important for preventing vasoconstriction in the kidneys. Prostaglandins (PGE$_2$ and PGI$_2$) and bradykinin actively vasodilate the renal arterioles and serve to oppose vasoconstriction caused by the sympathetic nervous system$^1$. Administration of pharmaceuticals that inhibit synthesis or action of prostaglandins, bradykinin and nitic-oxide may contribute to significant reductions in GFR. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly administered therapeutic agents in horses and may have significant toxic effects on the kidneys due to their vasoconstrictive effects$^{57}$.

Autoregulation of renal blood flow and GFR is maintained primarily through feedback mechanisms specific to the kidney. The major role of autoregulation is to maintain a relatively constant GFR in the face of large fluctuations in systemic blood pressure in order to allow precise control of water and electrolyte excretion.
Tubuloglomerular feedback detects changes in sodium and chloride concentrations in the distal tubule and serves to maintain these concentrations in order to prevent changes in net electrolyte excretion\(^1\).

The tubuloglomerular feedback mechanism is controlled by the juxtaglomerular complex. This complex consists of specialized cells in the distal convoluted tubule termed the macula densa and corresponding juxtaglomerular cells in the walls of the afferent and efferent arterioles. Macula densa cells sense changes in volume in the distal tubule that reflect fluctuations in delivery of sodium and chloride molecules. In response to a decrease in sodium and chloride concentrations in the distal tubule, the macula densa 1) signals the afferent arterioles to dilate and 2) increases renin release from the juxtaglomerular cells. Both of these effects serve to increase GFR and thus delivery of sodium and chloride to the distal tubules\(^1\).

Renin functions to increase release of angiotensin I which is then converted to angiotensin II as it passes through the lungs. Angiotensin II preferentially constricts renal efferent arterioles and as a result increases glomerular hydrostatic pressure. As the tubuloglomerular feedback mechanism also serves to dilate the afferent arterioles, renal blood flow is increased and this in addition to the increase in glomerular hydrostatic pressure serves to increase GFR. These mechanisms maintain GFR within a narrow range during large fluctuations in systemic blood pressure.
3) Renal function testing

Renal dysfunction in horses may occur secondary to changes in hemodynamics, intrinsic renal disease or post-renal disease. Acute and chronic renal disease have occurred in horses associated with systemic disease, renal hypoplasia, polycystic kidney disease, bacterial pyelonephritis, obstructive uropathy, and nephrotoxins. An accurate determination of renal function would be useful when potentially nephrotoxic drugs such as NSAIDs and aminoglycosides are administered in order to select appropriate dosages.

Serum urea nitrogen (SUN) and serum creatinine (Cr) concentrations are the most commonly utilized indices of renal function as measurements of renal retention of nitrogenous wastes. Due to extensive renal reserve capacity, changes in SUN and Cr do not occur until approximately 75% of GFR has been affected. Thus changes in SUN or Cr are insensitive indicators of early or minor changes in renal function. However, once elevated, small changes in SUN or Cr reflect corresponding changes in nephron function. Urea nitrogen is produced by the liver after ammonia uptake and metabolism. The amount of ammonia taken up by the liver is dependent upon 1) dietary protein and amino acid intake, 2) the amount of amino acids and proteins that are broken down to ammonia, and 3) the rate of catabolism of lean body tissue. Thus an increase in SUN may reflect increased protein catabolism rather than decreased urinary excretion. In humans, processes that increase protein catabolism and cause non-renal increases in SUN include hemorrhage into the gastrointestinal tract with digestion and absorption of amino acids, fever, burns, corticosteroid administration, and starvation/cachexia. Urea nitrogen is freely filtered across the glomerulus, and as much as 60% is reabsorbed in the
renal tubules. The rate of tubular reabsorption is dependent upon the rate of tubular fluid flow; the higher the flow rate, the less reabsorption of urea. In summary, SUN is a measure of renal function only after 75% of the GFR has been diminished and is affected by non-renal factors, making it an insensitive indicator of renal dysfunction.

Creatinine is a byproduct of hydrolysis of creatine phosphate in muscle. Creatinine is produced at a constant rate in the normal animal and freely filtered by the glomerulus without tubular reabsorption. Serum creatinine concentrations are affected by muscle damage, with elevated concentrations occurring during rhabdomyolysis. In addition loss of muscle mass as in severe emaciation may cause low serum creatinine concentrations and elevations above reference intervals may not occur with renal dysfunction and result in falsely low values. Like SUN, serum creatinine concentrations do not elevate until greater than 75% of GFR has been affected and is a relatively insensitive method of assessing early or minor renal dysfunction.

Creatinine can be assayed in several ways but the most frequently utilized is the Jaffé method, a colorimetric assay based on the formation of a complex between creatinine and alkaline picrate. Non-creatinine chromagens (glucose, pyruvate, acetoacetate, fructose, uric acid, and ascorbic acid) in the serum which are also measured by the Jaffé assay may contribute to up to 20% of the measured serum creatinine concentration causing overestimation of the true creatinine concentration. As serum creatinine concentrations elevate, the proportion of non-creatinine chromagens decreases and the total value becomes more accurate. Thus elevations in Cr are accurate and sensitive for patients with severe renal dysfunction but not in early or mild cases.
Another frequently used and simple measurement of renal function is urine specific gravity (USG). Urine specific gravity may be used to categorize urine concentration as 1) urine that is more dilute than serum (hyposthenuric = USG < 1.008); 2) urine that is of similar concentration as that of serum (isosthenuric = 1.008 ≤ USG ≤ 1.014); and 3) urine that is more concentrated than serum (hypersthenuric = USG > 1.014). A urine specific gravity outside of the isosthenuric range represents the ability of the kidneys to actively concentrate or dilute the urine. Animals with chronic renal failure lose the ability to concentrate or dilute their urine and a USG within the isosthenuric range corresponds to a degree of renal dysfunction. Isosthenuria occurs when at least two thirds of the nephrons become dysfunctional.

Fractional excretion (FE) of electrolytes in the urine can also be used as estimates of renal function. Fractional excretions reflect renal tubular reabsorptive and secretory capacities and are defined as the ratio of clearance of an endogenous electrolyte to clearance of endogenous creatinine. FE of an electrolyte can be calculated as a percentage of endogenous creatinine clearance as follows:

\[
FE = \left(\frac{U_x}{S_x}\right) / \left(\frac{U_{Cr}}{S_{Cr}}\right) \times 100^d
\]

Where \(U_x\) = urine concentration of substance, \(x\); \(S_x\) = serum concentration of substance, \(x\); \(U_{Cr}\) = urine concentration of creatinine; and \(S_{Cr}\) = serum concentration of creatinine. This calculation requires only simultaneous measurements of serum and urine concentrations of the substance and creatinine, and thus is easy and convenient to derive.
Fractional excretion of Na, K, Cl, and P are used most commonly to assess renal function in horses\textsuperscript{4,24}. The normal equine kidney conserves more than 99% of all filtered sodium and chloride through reabsorption in the renal tubules\textsuperscript{4}. Thus normal fractional excretion of sodium and chloride is less than 1%. Increases in FE of Na or Cl may represent tubular dysfunction through inability to reabsorb these electrolytes. These results must be interpreted in light of the animal’s hydration status, fluid therapy, medication history or recent exercise as all of these may significantly vary the FE of Na and Cl.

Although the above described techniques are convenient and easy to use in clinical practice, they are not sensitive to early or small changes in renal function and are plagued by inaccuracies. Accurate estimation of glomerular filtration rate would allow more appropriate monitoring of renal function. Quantitative measures of renal function can be categorized as plasma disappearance curves or clearance studies involving timed urine collections. The two techniques involve measurement of an endogenous or exogenous substance and either its disappearance from the plasma and/or appearance in the urine. In order to reflect GFR the substance must meet the following requirements of a filtration marker: 1) freely filtered by the glomerulus, 2) no significant renal tubular secretion or reabsorption, 3) no significant binding to plasma protein, 4) non-toxic and 5) not significantly metabolized by the body. For urine clearance studies, GFR is calculated as:

\[
\text{GFR} = \frac{\text{Urine} [x]}{\text{Plasma} [x]} \times \text{urine flow}^4
\]
Urine flow is calculated as the total volume of urine produced throughout the collection time period, usually over 24 hours. Thus, all urine must be collected during the time period making these techniques difficult and lengthy to perform, and not practical clinically. Plasma clearance studies involve measurement of the disappearance of an exogenous marker (as described above) from the plasma. These studies do not require urine collection and are thus clinically easier to perform.

The traditional standard for measurement of GFR is inulin clearance. Inulin clearance has determined GFR values in horses of 1.86 +/- 0.14\textsuperscript{22}, 1.66 +/- 0.38\textsuperscript{19}, 1.63 +/- 0.33\textsuperscript{16}, 1.88 +/- 0.67\textsuperscript{23}, 1.83 +/- 0.21\textsuperscript{7} and 1.55 +/- 0.04\textsuperscript{13}. Creatinine meets the requirements for a marker of glomerular filtration; it is neither secreted nor reabsorbed by the renal tubules in horses\textsuperscript{19}. However, endogenous creatinine clearance frequently underestimates GFR in horses\textsuperscript{19,21}. This is due to the presence of non-creatinine chromagens which are measured as creatinine using the Jaffé reaction\textsuperscript{3}. A falsely high serum creatinine concentration in the denominator of the clearance calculation results in an underestimation of GFR. Exogenous creatinine clearance circumvents this problem by minimizing the contribution of non-creatinine chromagens to the measured serum creatinine concentration by dilution. Exogenous creatinine clearance has been shown to approximate values for GFR that correlate with those of other methods\textsuperscript{12,19}. Creatinine may be injected as an intravenous bolus or as a constant rate infusion in order to achieve steady state conditions. Steady state conditions may be mimicked through subcutaneous injection of creatinine and its subsequent absorption. Exogenous creatinine clearance has been safely performed using subcutaneous injection in dogs\textsuperscript{30}. Although theoretically
accurate, creatinine clearance methods in horses are time-consuming (requiring 24-hour urine collection) and difficult to perform. Urine clearance studies necessitate collection of the total amount of urine produced by the animal in a given time period. Total urine collection should ideally be performed by catheterization of the ureters to prevent omission of part of the urine volume in the urinary bladder. This technique is not practical for clinical purposes. Catheterization of the urinary bladder of the adult horse is a simple procedure but long term catheter maintenance is problematic and collection of total urine volume is not assured. Catheters may become dislodged from the bladder and are a risk for induction of urinary tract infection.

Other markers have been used to estimate GFR, including radio-labeled pharmaceuticals such as $^{125}$I-iodothalamate, $^{99m}$Tc-diethylenepentaaacetic acid ($^{99m}$Tc-DTPA) and Chromium-51-Ethylenediaminetetraacetic Acid ($^{51}$Cr-EDTA). Studies in humans, dogs, pigs and horses have shown good correlation between these methods and those based on inulin or creatinine. However, these methods require specialized and careful handling of the compounds, animals and are expensive to perform, limiting their use to facilities with the necessary equipment in clinical practice.

Iohexol clearance has been used to estimate GFR in humans,\textsuperscript{5,35-38} dogs,\textsuperscript{8,9,11,30,31,39-40} cats,\textsuperscript{31,42} pigs,\textsuperscript{36} sheep\textsuperscript{43}, and recently, horse foals\textsuperscript{28}. It has been shown to be a safe and easy method which has given reproducible estimates of GFR when compared to inulin and creatinine clearance techniques. In normal horse foals iohexol clearance agreed with GFR determined by exogenous creatinine clearance\textsuperscript{28}. The results of this study indicated that a single intravenous injection of iohexol followed by
collection of 2 serum samples at 4 and 6 hours post injection can be used to estimate the GFR in healthy horse foals.

4) Iohexol

Iohexol, commercially available as Omnipaque®, is a non-ionic compound of low osmolality. It is used most commonly in humans and animals as a radiographic contrast agent for urography, contrast enhanced computed tomography and angiography. Nephrotoxic effects have been reported with use of non-ionic compounds with low osmolality such as iohexol. Intravenous injection of iohexol is not associated with adverse side effects, even in humans and animals with renal insufficiency. Once injected, iohexol is not metabolized by the body, bound to plasma proteins, secreted or absorbed by the renal tubules, and is freely filtered at the glomerulus, making it a useful marker for GFR studies.

Iohexol has been used to estimate the GFR in humans and has been shown to be accurate in healthy patients and those with evidence of renal dysfunction. Recent studies completed in dogs, and horse foals indicate that it is also safe in these species following intravenous administration. A wide variety of doses have been administered to animals with normal and impaired renal function and range from 45mg/kg in nephrectomized cats to 600 mg/kg in normal dogs. Iohexol has been administered to healthy horse foals at a dose of 150 mg/kg IV and no adverse effects were seen. Iohexol has been shown to cause osmotic diuresis in dogs at high doses and renal vasoconstriction in humans after intravenous injection, but these changes are rapid and transient, and did not effect GFR in those animals. Both Effersoe et al and
Olsson et al.\textsuperscript{50} incorporated simultaneous GFR measurements comparing iohexol with $^{99m}$Tc-DTPA and $^{51}$Cr-EDTA respectively, and found no change in renal function associated with iohexol during, or after its administration. Simultaneous clearance studies using iohexol with other markers have demonstrated that such protocols are safe, with no interference between injected markers and no adverse reactions.\textsuperscript{11}

Iohexol clearance studies have been performed in pigs, sheep, dogs, cats and horse foals. Finco et al.\textsuperscript{9} and Brown et al.\textsuperscript{30} evaluated the plasma clearance of iohexol as compared to the renal clearance of exogenous creatinine in dogs considered to have normal renal function and those with experimentally reduced renal mass. Gleadhill et al.\textsuperscript{39} and Moe et al.\textsuperscript{8} compared the plasma clearance of iohexol and $^{99m}$Tc-DTPA to determine GFR in healthy dogs and those with confirmed renal disease. Gonda et al.\textsuperscript{28} examined serum clearance of iohexol as compared to exogenous creatinine clearance as an estimation of GFR in normal horse foals. Results of these studies are similar, with GFR values obtained using iohexol showing good agreement when compared to the standard markers selected for each respective study.

Serum clearance of a substance that is freely filtered by the kidneys without tubular secretion or reabsorption, that is not protein bound, and that is not metabolized, is a measurement of glomerular filtration rate. Iohexol meets all of these requirements and thus its clearance from serum should accurately estimate GFR. Utilization of serum clearance studies for estimation of GFR provides a clinically feasible and reproducible method in order to measure GFR in horses. Other commonly used methods to assess renal function in horses are fraught with inherent and operator error. Serum clearance of
iohexol does not necessitate collection of urine and has been shown to be a safe, reproducible method using collection of timed blood samples to assess renal function in humans and animals. The goals of this project were 1) to determine a method of estimation of GFR based on serum clearance of a substance that meets the requirements of a marker for GFR, and 2) to make the method clinically applicable by developing a method using two blood samples to derive clearance and thus GFR in normal adult horses.
CHAPTER 2: MATERIALS AND METHODS

1) Horses: Ten adult horses (6 mares and 4 geldings) were obtained from the university teaching herd or as donations to the Veterinary Teaching Hospital for use in this study. They ranged in age from 6 to 21 years of age and weighed between 436 and 682 kilograms. Breeds included were thoroughbred (4/10), American Quarter Horse (3/10), warmblood cross (1/10), Arabian (1/10) and Morgan (1/10). A complete physical exam, complete blood count, serum biochemistry profile including electrolytes and urinalysis were performed on each horse by a veterinarian at least two days prior to their use in the study. All horses appeared healthy and the results of all laboratory data were within reference intervals. Simultaneous exogenous creatinine clearance and iohexol clearance methods were performed on two horses per day for a total of 5 days. Each pair of horses was brought to the Veterinary Teaching Hospital 24 hours prior to their use and allowed to acclimatize to their surroundings. Horses’ physical parameters were monitored every 6 hours for at least 24 hours following completion of the procedures. All procedures were approved by the Virginia Tech Animal Care and Use Committee.

2) Subject Preparation- Sterile IV catheters\(^a\) (16 gauge 5.5 inch) were placed aseptically in both the left and right jugular veins of each horse. Geldings were sedated with 0.5 mg/kg xylazine\(^b\) intravenously to facilitate aseptic placement of a 100 cm 28-french Foley catheter in the urinary bladder. Mares were not sedated for placement of a 30 cm

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\(^a\) Abbocath-T® Abbott Laboratories, Abbott Park, IL USA  
\(^b\) Rompun® Bayer Corporation, Shawnee Mission, KS USA
24-french Foley catheter in the urinary bladder. Thirty mls of sterile saline were placed in the balloon of each Foley catheter to ensure maintenance of the catheters within the urinary bladder during the study period. Geldings were allowed at least three hours for elimination of xylazine prior to initiation of the study. All horses were fed free choice grass hay and water during the study period.

3) Iohexol Clearance: Iohexol® 150 mg/kg was injected as an intravenous bolus through the right jugular catheter. Time “0” corresponded to the time of completion of the bolus. After injection, the right jugular catheter was flushed with heparinized saline and removed. Blood samples were taken from the left jugular catheter at 5, 20, 40, 60, 120, 240 and 360 minutes after iohexol injection. Blood collection was performed as follows: the catheter was flushed with 6 mls of heparinized saline, 10 mls of blood were drawn from the catheter and discarded, 10 mls of blood were drawn from the catheter and immediately placed into a serum tube, and the catheter was flushed again with 6 mls of heparinized saline. The left jugular catheter was removed following collection of the 360 minute sample. Each serum tube was labeled with the time of blood collection and the horse’s name and allowed to sit at room temperature (22º C) for at least 2 hours in order to clot. Blood tubes were then centrifuged (1000 x g) at room temperature at for 5 minutes and approximately 3 mls of serum were collected from each tube. Serum was then divided into two aliquots and placed in plastic vials which were frozen at -70ºC. Frozen samples were sent to the Animal Health Diagnostic Laboratory at Michigan State

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University\textsuperscript{d} for analysis. Iohexol concentration in serum samples was determined by HPLC using the method of Shihabi et al\textsuperscript{44} as modified in Finco et al\textsuperscript{9}. Equipment included a Waters Corporation (Milford, MA) Alliance system 2695 Separations Module with a Waters Corporation 2487 Dual Absorbance Detector at 254 nm, and 125 x 4.6 mm Phenomenex (Torrance, CA) Prodigy 5μ ODS column. The detection limit was 5 mg iohexol iodine/ml, and the limit of quantification in serum was 15 mg iohexol/L.

4) Exogenous Creatinine Clearance: Creatinine solution\textsuperscript{e} was prepared aseptically by dissolving 1 gram of creatinine per 12 mls of lactated Ringer’s solution. The final concentration of creatinine solution was 80 mg/ml. Creatinine solution was individually prepared for each horse at a dose of 60 mg/kg body weight and stored in a sterile glass container for no more than 18 hours prior to injection. Simultaneously to iohexol injection, 65 % of the horse’s total creatinine dose was injected subcutaneously in the axillary, pectoral and caudal cervical areas. In order to minimize the number of injections sites needed as much volume as possible was injected into each area until the horse demonstrated signs of discomfort. Twenty-five minutes later the remaining 35% of the total creatinine dose was injected subcutaneously. Immediately following the second creatinine injection the bladder was emptied and washed with sterile 0.9% saline in order to ensure removal of all urine. The urinary catheter was then clamped in order to retain all urine produced during the collection period. Blood collection was performed as for

\textsuperscript{d}Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI USA
\textsuperscript{e}Creatinine (C-4255), Sigma Chemical Co., St.Louis, MO USA
Iohexol except that 6 mls were collected and placed in heparinized blood tubes. Forty-five minutes after iohexol and creatinine injection all urine was collected from the bladder. The bladder was washed three times with 500 mls of saline and all fluid recovered was added to the previously collected urine. The total volume was noted and 2 mls of urine/wash mixture was placed in a sterile tube in order to measure urine creatinine concentration. Concurrently, 6 mls of blood were obtained from the jugular catheter for serum creatinine concentration. After the collection period, the urinary catheter was again clamped. The urine collection procedure was repeated sixty-five and eighty-five minutes after iohexol/creatinine injection and blood and urine samples were collected for serum and urine creatinine concentrations. After the eighty-five minute collection the urinary catheter was removed. Serum and urine creatinine concentrations were obtained using automated analysis by an Olympus AU400 through a kinetic modification of the Jaffe method. Creatinine clearance (Cl$_{\text{creatinine}}$) was calculated for each time interval by Cl$_{\text{creatinine}}$ = Urine Volume $\times$ [Creatinine$_{\text{urine}}$] / [Creatinine$_{\text{plasma}}$] / (kg, body weight). Comparisons to iohexol clearance were made using the mean of the three time points for each horse.

5) Iohexol pharmacokinetic calculations: Monoexponential, biexponential and triexponential equations were calculated to describe the data. Data were analyzed by nonlinear least squares regression analysis with equal weighting of the data, using

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1 Olympus AU400, Dallas, TX USA
commercial software. The triexponential equation $C_s(t) = C_1 e^{-\lambda_1 t} + C_2 e^{-\lambda_2 t} + C_3 e^{-\lambda_3 t}$ where $C_s(t)$ is the serum concentration at any time $t$, described the data for each horse. Pharmacokinetic variables were then calculated using the intercepts ($C_1$, $C_2$, and $C_3$) and absolute values of the slopes ($\lambda_1$, $\lambda_2$, and $\lambda_3$) for each horse. The area under the serum concentration versus time curve (AUC) was calculated from the intercepts and slopes of the triexponential equations for each individual animal according to $AUC = C_1/\lambda_1 + C_2/\lambda_2 + C_3/\lambda_3$. The total serum clearance ($Cl_t$) was calculated from $Cl_t = dose/AUC$.

6) Statistical Analysis: Clearance values are expressed as milliliters per minute per kilogram and values are reported as mean. Analysis of serum concentration, verses time profiles were performed for each individual horse in the study. Analysis was performed using WinNonlin (version 1.5) running on a pentium-based personal computer. The $CL_{iohexol}$ and $CL_{creatinine}$ were compared to assess agreement between the two methods. A paired t-test was used to test for mean bias between methods and proportional bias was evaluated using a plot of the differences between mean values of both methods as suggested by Bland and Altman. Standard deviation of the difference was calculated and limits of agreement were set and declared significant at $p \leq 0.05$. ANOVA was performed to compare the AUC of the 3-compartment model to those of the two-timepoint samples after calculating the terminal slopes extracted from the model at 3 and

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9 WinNonlin, ver. 1.5, Pharsight Corporation, Mountainview, California USA
h WinNonlin, ver. 1.5, Pharsight Corporation, Mountainview, California USA
4 hours; 4 and 6 hours; and 3 and 6 hours. The correction factor used to predict a
CL_{iohexol} from CL_{3-4\text{hours}} was derived by errors in variables regression.
CHAPTER 3: RESULTS:

1) **Urinary clearance of creatinine:** Baseline serum creatinine for all horses ranged from 0.9 to 1.3 mg/dl. Forty-five minutes after the total subcutaneous dose of creatinine had been injected, serum creatinine concentrations ranged from 3.8 to 6.8 mg/dl. Values obtained for exogenous creatinine clearance ranged from 1.68 to 2.69 mg/min/ kg body weight with a mean of 2.11 ml/min/kg (Table 1).

2) **Serum clearance of iohexol:** After intravenous injection of iohexol, mean serum iohexol concentrations ranged from 961.18 mg/ml (5 minutes) to 17.77 mg/ml (360 minutes). By using the Akaike information criteria it was determined that a three-compartment model best described the data for 8/10 horses and a two-compartment model best described the data for 2/10 horses. Because the majority of the data was best described with a 3-compartment model, this model was used for all horses. A semi-logarithmic plot of mean iohexol concentration vs. time is shown in Figure 1. Pharmacokinetic variables for each model are represented in Table 2. The mean clearance of iohexol was 2.38 ml/min/kg with a range of 1.95 to 3.33 ml/min/kg (Table 1).

3) **Two timepoint estimates of iohexol serum clearance:** Terminal slopes of the elimination curve were calculated from each combination of the 3 and 4 hour, 4 and 6 hour and 3 and 6 hour time points. ANOVA comparing the AUCs for each of the curves generated from each of the 2-sample estimates revealed no statistical difference among
them. The 3 and 4 hour sampling model was chosen as it was not significantly different from the other models, and it was the most convenient clinically. Errors in variables regression for the AUCs of the 3 and 4 hour model and the 3-compartment model were performed (Figure 2). The following equation was generated to predict $\text{AUC}_{\text{corrected}}$ from $\text{AUC}_{3-4\text{hour}}$:

$$\text{AUC}_{\text{corrected}} = 1.107716 \times \text{AUC}_{3-4\text{hour}} + 15731$$

Predicted clearance was calculated for each horse by:

$$\text{CL}_{\text{predicted}} = \frac{\text{Dose}}{\text{AUC}_{\text{corrected}}}$$

4) Comparison of iohexol clearance and creatinine clearance: Clearance values for creatinine, the 3-compartment model for iohexol and the corrected 3-4 hour sampling times were compared. For $\text{CL}_{\text{creat}}$ vs. $\text{CL}_{\text{iohexol}}$, $\text{CL}_{\text{creatinine}}$ vs. $\text{CL}_{3-4\text{hour}}$ and $\text{CL}_{\text{iohexol}}$ vs. $\text{CL}_{3-4\text{hour}}$ the mean of the two methods was plotted against the difference between the two methods as described by Bland and Altman$^{56}$ (Figure 3). A paired t-test was performed between the means of each method. $\text{CL}_{\text{creatinine}}$ was statistically different from $\text{CL}_{\text{iohexol}}$ and $\text{CL}_{3-4\text{hour}}$ with $p=0.01$ and $p=0.02$ respectively. There was no difference between $\text{CL}_{\text{iohexol}}$ and $\text{CL}_{3-4\text{hour}}$ ($p=0.12$).
<table>
<thead>
<tr>
<th>Horse</th>
<th>CL$_{\text{creatinine}}$</th>
<th>CL$_{\text{iohexol}}$</th>
<th>CL$_{3\text{-4hour}}$</th>
</tr>
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<tr>
<td>01</td>
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<td>2.47</td>
<td>2.31</td>
</tr>
<tr>
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<td>2.44</td>
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<td>2.15</td>
<td>2.30</td>
</tr>
<tr>
<td>Mean</td>
<td>2.11</td>
<td>2.38</td>
<td>2.32</td>
</tr>
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</table>

Table 1: Estimated GFR values (ml/min/kg) for CL$_{\text{creatinine}}$, CL$_{\text{iohexol}}$ and CL$_{3\text{-4hour}}$ in 10 horses.
### Table 2: Pharmacokinetic values describing the disposition of iohexol in horses after IV administration of a single 150 mg/kg dose.

Equation describing the 3-compartment model $C_{st} = C_1 \times e^{-\lambda_1 t} + C_2 \times e^{-\lambda_2 t} + C_z \times e^{-\lambda_z t}$

- $C_{st} =$ Serum iohexol concentration at any time “t”
- $C_1, C_2 =$ concentration intercept for distribution phase; $C_z =$ concentration intercept for post distribution phase; $\lambda_1, \lambda_2 =$ slope of distribution phase curve; $\lambda_z =$ slope of post-distribution phase curve; AUC = area under the curve; $CL_t =$ Total clearance; Vc = volume of distribution of the central compartment; $V_{d_{area}} =$ volume of distribution during the terminal phase.

<table>
<thead>
<tr>
<th>Horse</th>
<th>$C_1$ (ug/ml)</th>
<th>$\lambda_1$ (min$^{-1}$)</th>
<th>$C_2$ (ug/ml)</th>
<th>$\lambda_2$ (min$^{-1}$)</th>
<th>$C_z$ (ug/ml)</th>
<th>$\lambda_z$ (min$^{-1}$)</th>
<th>AUC (ug/ml*hr)</th>
<th>$CL_t$ (ml/min/kg)</th>
<th>Vc (L/kg)</th>
<th>$V_{d_{area}}$ (L/kg)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.127</td>
<td>515.5</td>
<td>0.019</td>
<td>229.4</td>
<td>0.0076</td>
<td>60651.2</td>
<td>2.47</td>
<td>0.1238</td>
<td>0.3250</td>
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<td>0.1548</td>
<td>0.2936</td>
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<td>543.3</td>
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<td>45071.1</td>
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<tr>
<td>5</td>
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<td>159.6</td>
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<td>8</td>
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<td>0.1277</td>
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<tr>
<td>9</td>
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<td>0.154</td>
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<td>88.7</td>
<td>0.0050</td>
<td>62792.3</td>
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<tr>
<td>10</td>
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<td>515.4</td>
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<td>229.5</td>
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<td>Median</td>
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<td>136.5</td>
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<td>45071.1</td>
<td>1.95</td>
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<tr>
<td>Max</td>
<td>543.2</td>
<td>0.236</td>
<td>754.4</td>
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<td>0.0115</td>
<td>77039.9</td>
<td>3.33</td>
<td>0.1548</td>
<td>0.5197</td>
</tr>
</tbody>
</table>
Figure 1: Semi-logarithmic serum concentration (mean +/- SD) vs. time profile for iohexol in horses (n=10) after IV administration of a single 150 mg/kg dose.
Figure 2: Errors in variables regression for AUC of the 3-compartment model for serum clearance of iohexol vs. AUC of corrected 3 and 4 hours sampling points.
Figure 3: Method comparison plot for clearance of creatinine vs. clearance of iohexol in 10 horses. Clearance values were calculated as the mean of the two methods and plotted against the difference between the two methods. Positive differences indicate that clearance of iohexol exceeded clearance of creatinine.
CHAPTER 5: DISCUSSION

Renal dysfunction in horses may occur due to primary renal disease or secondary to systemic disease, toxins or drug administration. Many commonly used equine pharmaceuticals, including non-steroidal anti-inflammatory drugs and aminoglycoside antibiotics, may cause significant renal damage. Evaluation and monitoring of renal function should be a standard of practice in order to prevent, treat and monitor renal damage. Commonly used methods of assessing renal function, such as SUN, Cr and urine specific gravity are simple to perform and readily available to practitioners, but are insensitive at determining early or mild renal dysfunction. Using Cr and SUN to estimate GFR is unsatisfactory and may lead to delays in diagnosis and treatment of renal disease. Fractional excretion of electrolytes are also easy to determine but values are significantly affected by non-renal factors complicating the interpretation of results. Clearance methods are accurate and precise but are time consuming, require specialized equipment and trained personnel, involve costly substances and assays, and leave much room for technical error. However, GFR is the best overall measurement of kidney function and the measurement most easily understood by clinicians.

Inulin clearance is considered the traditional standard of measurement of GFR. However, neither inulin nor its assay is readily available commercially making this method impractical clinically or for research purposes. Another reproducible method of estimating GFR in horses is clearance of 99mTc-DTPA. Results of this method have shown good correlation with inulin clearance in horses\textsuperscript{13,14}. Unfortunately, use of this
method requires specialized equipment and personnel, limiting its use to referral facilities capable of performing nuclear scintigraphy.

Creatinine also meets the requirements of a filtration marker and its clearance from serum has been used to estimate GFR in the horse. Endogenous creatinine clearance routinely underestimates GFR in most species including the horse\textsuperscript{19,21}. Inclusion of non-creatinine chromagens in the measurement of serum creatinine causes the measured value to be higher than the true concentration of creatinine in the serum. This in turn results in a lower calculated clearance value and an underestimation of GFR. Utilization of a bolus injection of exogenous creatinine circumvents this error by essentially diluting out the contribution of non-creatinine chromagens such that their effect on clearance is negligible. Exogenous creatinine clearance is the most clinically feasible method available for estimation of GFR in horses and has been shown to produce reproducible estimates of GFR\textsuperscript{24}. Despite its potential value, exogenous creatinine clearance is seldom used in clinical practice in order to determine GFR due to the time required and the technical challenge of 24-hour urine collection in the adult horse.

Comparison of iohexol clearance to exogenous creatinine clearance should determine the usefulness of the former as an assessment of renal function. Clearance values determined in this study by both iohexol clearance and exogenous creatinine clearance are within published reference intervals for GFR in adult horses determined by a variety of methods\textsuperscript{12,13,16,19,21-23}. Serum clearance of iohexol has been shown to be a safe and reliable assessment of GFR in other species and in normal horse foals\textsuperscript{8}. 
The technique involves use of a commercially available product and assay, and avoids the time-consuming and error-fraught necessity of collecting urine.

Most studies comparing methods of assessment of GFR in humans and horses have used correlation analysis to determine the strength of the relationship between the two methods. Bland and Altman have described a method to measure the agreement between two methods where the differences between methods are plotted against their mean. Limits of agreement are calculated as the 95% confidence interval of the mean difference. The limits should be interpreted with respect to the clinical range of that which is being measured. Results of this study showed narrow limits of agreement that are within the reference ranges for previous estimates of GFR in adult horses, and thus good agreement between the methods.

A full 3 compartment pharmacokinetic analysis is not practical in clinical patients because it necessitates multiple, frequent timed collection of blood and multiple costly assays. In order to determine a more clinically useful method for estimation of GFR, limited sampling times were chosen and clearance was calculated based upon them. All of the models created by the elimination curve formed by two terminal time-points agreed statistically with the 3-compartment model for iohexol clearance. As the 3 and 4 hour time sampling point was not significantly different from the other methods, and would be clinically the easiest to perform, this method of iohexol clearance may be an accurate and accessible technique to measure GFR in horses.

Iohexol clearance has been used in numerous species to determine GFR. In humans and animals, intravenous iohexol administration appears to be safe and fulfills
all of the requirements of a marker for GFR\textsuperscript{39}. The non-ionic composition of iohexol and its low osmolality cause it to be a stable and safe compound, even in patients with renal insufficiency\textsuperscript{52}. No adverse effects of iohexol administration were seen in the horses in this study.

Iohexol clearance is a technically simple procedure and has a number of advantages as compared to other clinical methods of measuring GFR in horses. First, it avoids the necessity of timed urine samples. In two geldings used during this study, problems were encountered in the maintenance and patency of the urinary catheter and urine could not be collected. These horses were excluded from the study. Urine collection techniques necessitate collection of total urine produced over a period of time, usually 24 hours. The time and personnel required for such procedures makes such techniques impractical for clinical use. The large size of the equine bladder and its ventral location in mares makes collection of total urine volume difficult, if not impossible to ensure. Serum clearance of iohexol avoids all of these difficulties.

Exogenous creatinine clearance was chosen as a clinical standard of measurement of GFR in order to compare iohexol clearance to for this study. Although exogenous creatinine clearance has been shown to be an accurate and reliable method of measurement of GFR in horses, the technique is fraught with potential error, as discussed previously. In order to better assess the accuracy of iohexol clearance as a measure of GFR, the technique may be compared to more accurate, but more clinically impractical methods such as inulin or \textsuperscript{99m}Tc-DTPA clearance. Further studies are also necessary in
order to determine estimation of GFR by serum clearance of iohexol in horses with
evidence of established renal dysfunction or failure.

Accurate assessment of renal function in horses is challenging and fraught with
numerous sources of error. The most commonly used methods are insensitive at
measuring early or minor renal dysfunction and may be affected by non-renal factors.
Traditional clearance methods necessitate collection of total urine volumes produced
during a period of time, a feat difficult if not impossible in most clinical settings. The
development of a method of estimation of GFR through serum clearance of iohexol and
timed blood collection provides the practitioner with a safe, non-invasive, simple, and
reproducible method for assessing renal function in horses. Use of this diagnostic test
would provide more precise estimates of renal function and allow earlier diagnosis and
treatment of renal dysfunction in horses.
REFERENCES


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