Evaluation of Iohexol Clearance to Estimate Glomerular Filtration Rate In Normal Horse Foals

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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. Of particular concern are those drugs, including the aminoglycoside antibiotics, that are eliminated almost exclusively by the kidney. Knowledge of a patients renal status prior to treatment would direct efforts at; 1) restoring kidney function prior to protracted therapy with potentially damaging drugs, 2) adjusting the dose of a life-saving drug based on the magnitude of dysfunction, or 3) selecting a drug that is not dependant on renal function for elimination.

Estimation of the glomerular filtration rate (GFR), accepted as one of the earliest and most sensitive indicators of renal dysfunction, can be determined in horses using standard techniques

such as endogenous or exogenous renal creatinine clearance. Unfortunately, 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, dogs and cats with results that have been validated by traditional standards. Most testing protocols require a single bolus injection of iohexol, followed by 2 or 3 blood samples obtained over a few hours. Compared to traditional testing methods, samples are easily and rapidly obtained making the testing procedure less stressful for the patient. A simple method to measure GFR in horses that does not require urine collection, would allow veterinarians in a clinical setting the ability to determine a patient's renal status easily and safely.

The objectives of this study were; 1) model the pharmacokinetic profile of iohexol in horse foals, 2) compare creatinine clearance, an accepted standard for GFR determination in patients, with iohexol clearance, and 3) develop sampling parameters and calculation methods for a practical test, based on iohexol clearance, that compares favorably with creatinine clearance in horse foals.

Iohexol concentration time data were best described using a 3-compartment open model. Mean creatinine clearance (2.17 ml/min/kg) and mean iohexol clearance (2.15 ml/min/kg) showed good agreement. In addition, GFR values for all foals 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 4 and 6 hours post injection can be used to estimate the GFR in healthy horse foals. Mean corrected GFR value ($CL_{predicted}$) for 10 foals in this study was 2.15 ml/min/kg.

DEDICATION

This work is dedicated to my husband Les, for

his love and enduring support.

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ABBREVIATIONS

GFR	glomerular filtration rate
ARF	acute renal failure
CRF	chronic renal failure
$[Cr_{serum}]$	creatinine concentration in serum
SUN	serum urea nitrogen
$CL_{creatinine}$	creatinine clearance
CL_{renal}	urinary clearance
CL_{plasma}	plasma clearance
^{99m}Tc-DTPA	^{99m}Tc-labeled diethylenetriaminepentaacetic acid
^{51}Cr-EDTA	Chromium-51-Ethylenediaminetetraacetic acid
$CL_{plasmaC\text{-}1}$	clearance – 1-compartment model
$CL_{plasmC\text{-}2}$	clearance – 2-compartment model
$CL_{plasmaC\text{-}3}$	clearance – 3-compartment model
$CL_{trapezoid}$	clearance calculated by trapezoidal method
HPLC	high performance liquid chromatography
ICP-AES	inductively coupled plasma-atomic emission spectroscopy
CE	capillary electrophoresis
AUC	area under the curve
$AUC_{4\ and\ 6}$	area under the curve at 4 and 6 hours post iohexol injection
$CL_{predicted}$	predicted $CL_{plasmaC\text{-}3}$ from $AUC_{4\ and\ 6}$

CHAPTER 1: LITERATURE REVIEW

1) Kidney Function

Renal physiology**—**The kidneys play host to a myriad of functions concerned with water and electrolyte balance, glucose metabolism, hormone production, and removal of waste products from the body.[1] In addition, many drugs such as aminoglycoside antibiotics are eliminated from the body by renal excretion.[2] Mechanisms central to these renal processes include selective glomerular filtration, tubular re-absorption and tubular secretion of various substances. Alterations in renal function occur in association with changes in blood flow, and damage associated with therapeutic agents or disease. These alterations can be mild, or in the extreme, life-threatening.[3]

The architecture and physiology of the mammalian kidney has been well described in many texts.[1,3,4] In mammals, both kidneys are identical and composed of hundreds of thousands to millions of functional units termed nephrons. In human beings there are approximately two million nephrons [1] (one million in each kidney). Taken individually, each nephron is composed of the glomerulus and a complex tubule that courses variously through the renal parenchyma. The kidneys receive approximately 20% of the total cardiac output[4] and depend on this blood flow for adequate perfusion, to filter the plasma of metabolic waste products[3] and maintain body water and solute homeostasis.[1] Blood from the renal arteries reaches the glomerulus through an afferent arteriole and is selectively filtered as it passes through a nest of capillaries that terminate in an efferent arteriole.

The filtrate that accumulates within the area surrounding the capillaries (Bowman's space) contains approximately 25-30% of the total plasma water, cations and solutes that are less than 5200 molecular weight in size. Filtrate enters the tubules where the gross product or "primary urine" continues to be selectively modified[5] as it passes throughout the length of the tubule. The tubules converge to form collecting ducts that deliver the processed urine to the renal pelvices, ureters and bladder, sequentially. The rate of glomerular filtration is closely maintained within a narrow range. It is a function of the hydrostatic pressure produced by the cardiovascular system, the oncotic pressure of the plasma and complex auto-regulatory elements that prevent abrupt changes in renal blood flow.[1,4] Adequate glomerular filtration relies on a minimum number of functionally normal nephrons between both kidneys and a reduction in this number is directly proportional to the rate of filtration.[6]

Glomerular filtration rate—The glomerular filtration rate (GFR) is considered one of the earliest and most sensitive indicators of renal dysfunction.[7-11] It has been used clinically in man and animals [11] to assess the magnitude of damage or reduced function due to various diseases. It is used to monitor the progression of renal disease or a patient's response to potentially nephrotoxic drug administration.[11] As an example, use of non-steroidal anti-inflammatory drugs (NSAIDS) in dehydrated or diseased patients can directly affect the GFR by depressing the production of prostanoids[12] essential for renal autoregulation. Early detection of renal dysfunction is particularly important in patients with acute renal failure, as it is potentially reversible.[3] Unfortunately, traditional methods used to estimate the GFR are rarely performed outside of research institutions. They are cumbersome and time-consuming, necessitating constant IV infusions of test molecules, urinary catheterization, and timed urine collections. Lack of reproducibility is a major limitation,[13]

and these methods are subject to inaccuracies associated with incomplete bladder rinsing and failure to retrieve all urine and cleared marker.[9,14] A particular problem in adult horses, is the difficulty in obtaining a self-retaining urinary catheter that is long enough to accommodate the male urethra. Using the simplest definition, the GFR estimates the volume of plasma filtered by all glomeruli collectively, over a certain interval of time. Unlike many mammalian neonates, foals have mature kidneys at birth with GFR values that equal or exceed those of adults.[15] Although there have been a wide range of values described for horses, the average GFR approximates 2ml/min/kg [14] In a study with horse and pony foals Brewer *et al*.[16] used single injection inulin clearance, serum and urine inulin clearance (using continuous infusion), and 12 hour endogenous creatinine clearance to obtain mean GFR values (±1 SEM) of 2.30 ± 0.34, 2.56 ± 0.30, 2.82 ± 0.32 and 2.81 ± 0.55 ml/kg/min, respectively. In another study by Holstock *et al*.,[17] the GFR was determined in pony foals (n=13) by single injection serum clearance of inulin and urinary clearance of endogenous creatinine. Mean values for GFR (±1 SEM) were 3.21 ± 0.36 for inulin and 1.92 ± 0.14 for creatinine. Additional studies in adult horses have also obtained a wide range of GFR values using a variety of methods.[7,14,18-22] Examples from a few of these studies estimated GFRs (±1 SEM) at 1.46 ± 0.24[18], 1.83 ± 0.20[7], 1.92 ± 0.51[21] and 2.24 ± 0.07 [22] ml/min/kg.

In the presence of acute renal failure (ARF) or chronic renal failure (CRF), alterations in the GFR occur. The magnitude of the alteration is dependent on the etiology of the dysfunction (glomerular vs. tubular disease) and the intrinsic ability of the kidneys to compensate. Any change in the mechanisms that govern renal perfusion and renal plasma flow or changes in glomerular hydraulic and oncotic pressures will lead to a reduction in GFR.[1] A reduction in GFR results in the build-up of several substances and metabolites in the blood, which under normal conditions, the kidneys would rapidly remove.

In clinical practice, the two common metabolites measured when renal compromise is suspected are serum creatinine ([Cr_{serum}]) and serum urea nitrogen (SUN), also referred to as blood urea nitrogen (BUN). The tests for these metabolites are economical and widely available to most practitioners. Renal and non-renal factors can result in elevations of one or both of these substances. Dehydration, which leads to a reduction in cardiac output and renal perfusion can cause significant elevations in SUN and [Cr_{serum}].[6,23] Though both metabolites are considered crude indicators of renal function, [Cr_{serum}] is considered to be somewhat more sensitive.[1] During periods of reduced flow, urea is passively reabsorbed with water and sodium in the proximal tubule, the magnitude of which varies inversely with GFR. In addition, non-renal factors may falsely elevate [Cr_{serum}] and make it an even less sensitive tool with which to detect existing renal dysfunction. In horses [Cr_{serum}] may be falsely elevated due to acute rhabdomyolysis or cachexia that results in the release of preformed creatinine from damaged muscle cells.[24] Serum creatinine values as high as 27 mg/dl have been reported in normal neonatal foals <72 hours old (normal reference interval: 0.4 – 2.1 mg/dl).[25] With severe-acute injury, such as that associated with ethylene glycol poisoning in dogs or mild damage to a small percentage of renal tissue, elevations of [Cr_{serum}] and SUN are often not present.[26] In response to injury, neighboring healthy nephrons hypertrophy and increase their activity to compensate for the reduced function.[2,3] This increase in individual, or single nephron GFR (SNGFR),[23,27]occurs early in renal failure. It maintains filtration and urine production within normal limits for a period of time, depending on the magnitude of damage, but does not continue indefinitely. This compensation, relative to creatinine, results in its continued excretion and maintenance of normal serum concentrations, even in the presence of advanced renal disease. The GFR must be less than 25% of normal [9] or greater than 75% of renal function must be lost or abnormal before it is reflected clinically by an increase in serum creatinine concentration.[6,24]

2) Renal Function Tests

Various methods have been used to assess renal function in animals and man and several diagnostic tests have been developed to estimate the GFR. Most measure the disappearance or appearance of an intravenously injected substance referred to as a "marker" in the blood or urine, respectively. Any substance used as a marker should fulfill certain criteria;[28] it should be "freely" filtered by the glomerulus and not metabolized, bound to plasma proteins, secreted into or reabsorbed by the renal tubules. Ideally, the amount of the substance should appear unchanged in urine and be equal to the amount of injected dose. In addition, the marker itself should have no effect on GFR.[5] Inulin, a natural fructose polymer long considered the "standard" exogenous marker[28] and creatinine are close to fulfilling such criteria in most mammals, including horses and can be used to estimate GFR in this species.[19,21,29] Inulin and exogenous creatinine may be injected intravenously as a bolus dose, or as a constant infusion that results in maintaining steady state concentrations. Exogenous creatinine has been safely administered subcutaneously in dogs [29] and cats [30] during clearance studies to mimic steady state concentrations achieved by constant infusions. Assuming that the injected marker observes the previously described criteria, and is excreted completely and unchanged by the kidney, the rate of its clearance from the plasma (CL_{plasma}) should equal the rate of filtration or renal clearance (CL_{renal}) at the glomerulus, or the GFR as follows:

$$CL_{renal} = U \cdot [X]_u / [X]_p \quad (1)$$

$$CL_{renal} = GFR = CL_{plasma}$$

where $[X]_u$ and $[X]_p$ are the concentrations of substance "X" in urine and plasma and "U" is the urine flow in ml/min. Calculations used to obtain estimates of GFR using renal clearance techniques require careful and complete urine collection to prevent errors.[9] Failure to adequately

rinse and retrieve all urine present in the bladder will lead to an underestimation of GFR.[3,31] The analytical methods used to determine the concentration of marker may also be a source for error. In the case of endogenous creatinine, most laboratories use automated instrumentation that relies on the Jaffe method to determine [Cr_{serum}].[32] The presence of non-creatinine chromogens in the serum interfere with analysis by falsely elevating the true value by as much as 50% in dogs,[3,6,31] because they can not be differentiated from creatinine. In dogs with experimentally reduced renal mass,[3] goats[3] and humans,[28] overestimation of GFR using endogenous creatinine clearance has been reported to occur, but does not appear to be the case in the pony.[19] Tubular secretion of creatinine that occurs in dogs, approximates the magnitude if non-creatinine chromogens present in serum. Since non-creatinine chromogens are only present in serum, urine creatinine concentrations are not effected by the analysis.[6] The use of exogenous creatinine circumvents the problems associated with Jaffe analysis by diluting, or significantly reducing the proportion of non-creatinine chromogens present in the serum, thereby diminishing their impact on GFR estimation.[31] Evidence exists for validation of exogenous creatinine as a reference marker for the GFR in dogs[11] where a close correlation between renal clearance of exogenous creatinine and ^{14}C-labled inulin has been shown.[29] Clearance studies using endogenous and exogenous creatinine have been used in horses to estimate GFR. In the few, variously designed studies completed thus far, results agree favorably with GFRs obtained using inulin.[15,19,22,33] Other markers that are used to determine the GFR include radio labeled pharmaceuticals such as ^{99m}Tc-labeled diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA) and Chromium-51-Ethylenediaminetetraacetic Acid (^{51}Cr-EDTA). Both ^{99m}Tc-DTPA and ^{51}Cr-EDTA used in human,[34] dog,[8] pig[35] and horse[7,14] studies, have shown excellent agreement when compared with the GFRs obtained using traditional markers, such as inulin. Unfortunately, problems associated with handling radioactive materials and the risks of exposure to humans have

limited their use in all but a few clinical and research facilities. Recently, protocols have been developed in humans,[5,34-37] dogs,[8,9,11,30,38-40] cats,[30,41] pigs [35] and sheep[42] using iohexol as a marker to estimate GFR. Iohexol fulfills all of the characteristics necessary of a clearance marker used to determine GFR [43]

3) Iohexol

Introduction

Iohexol, known commercially as Omnipaque®, is a non-ionic, low osmolality radiographic contrast agent. In humans, common uses include urography, contrast enhanced computed tomography and angiography. Although some ionic contrast media such as diatrizoate have been shown to have nephrotoxic potential,[2] this does not appear to be the case with iohexol.[44] It has a low prevalence of adverse drug reactions after intravenous injection, even when given to young or elderly human patients.[45] Once injected, iohexol is not metabolized by the body,[38] bound to plasma proteins, secreted or absorbed by the renal tubules, and is freely filtered at the glomerulus,[43,46] making it a useful marker for GFR studies. Iohexol is commonly used to estimate the GFR in humans [5,34-37,40,46-49] and yields good precision in healthy patients and those with renal disease or varying degrees of renal dysfunction.[36] Recent studies completed in dogs, indicate that it is also safe in this species following intravenous administration.[9,30,38] A wide variety of doses have been administered to animals with normal and impaired renal function[8-10,35,41] and range from 45mg/kg in nephrectomized cats[41] to 600 mg/kg in normal dogs.[35] In a study by Heine and Moe,[39] iohexol (600 mg/kg) administered by IV injection to 50 dogs suffering from pyometra was well tolerated. Iohexol has been shown to cause osmotic diuresis in dogs[50] at high doses and renal vasoconstriction in humans after intravenous injection, but these changes are rapid and transient.[2] Both Effersoe *et*

al.[47] and Olsson *et al.*[49] 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.[10] Various doses, combinations and routes of administration have been used with bolus IV injections of iohexol, including SQ exogenous creatinine,[9 30] IV bolus injection followed by constant infusion of exogenous creatinine,[41] IV bolus injection of ^{99m}Tc-DTPA[8] and ^{51}Cr-EDTA.[35]

Success in obtaining accurate, reproducible estimations of the GFR in humans using iohexol has lead to similar studies in animals. Methods to estimate the GFR in dogs, using iohexol, have been investigated by several researchers.[8,9,30] Finco *et al.*[9] and Brown *et al.*[30]evaluated the plasma clearance of iohexol against the renal clearance of exogenous creatinine in dogs considered to have normal renal function and those with experimentally reduced renal mass. Gleadhill *et al.*[38] and Moe *et al.*[8]compared the plasma clearance of iohexol and ^{99m}Tc-DTPA to determine GFR in healthy dogs and those with confirmed renal disease. 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. However, direct comparison of data generated by these studies is difficult due to variations in methodology and in the choice of mathematical models used to determine the plasma clearance values.

In horses, the use of iohexol has been limited to contrast enhancing procedures, such as myelography, in which iohexol is injected directly into the sub-arachnoid space of the spinal cord.[51]In most cases, horses are anesthetized and placed in recumbency during such procedures. To our knowledge, the pharmacokinetic behavior of intravenously injected iohexol has not been

evaluated in the horse. In fact, the author did not find reference to its IV administration in this species.

In preparation for this project, the author completed a pilot study in 2001 that included 4 horses less than 1 year of age. Three of these horses were healthy and determined to have normal renal function. The fourth had been diagnosed with chronic renal failure secondary to NSAID toxicity prior to inclusion in the study. The ante-mortem diagnosis was based on serum biochemical profiles and renal biopsies. Ante-mortem diagnosis was verified by histopathology of renal tissue taken from both kidneys at necropsy. Iohexol, at a dose of 150 mg/kg (Finco, personal communication) was administered intravenously, as a bolus to each horse. Blood samples were obtained at 120, 180, 240 and 300 minutes after injection of iohexol. The pharmacokinetic behavior of iohexol was similar and the distribution phase was complete by approximately 120 minutes in the three horses with normal renal function. The mean iohexol concentration in these horses ranged from 195.4 mg/L (120 min) to 48.2 mg/L (300 min) and the mean GFR, based on a 1-compartment pharmacokinetic model using the 2,3 and 4 hour samples, was 2.42 ml/min/kg. In the horse with confirmed renal failure, the iohexol concentration after injection was 397.7 mg/L (120 min) and 300.0 mg/L (240 min) and the GFR was 0.689 ml/min/kg. The dose of injected iohexol was well tolerated by all 4 horses during and after administration.

Pharmacokinetics of iohexol

Distribution and elimination of iohexol following a bolus intravenous injection is assumed to follow first order kinetics. Equilibration of the injected dose between the vascular and extravascular compartments is followed by an exponential decline during the elimination phase. In humans with normal renal function, final distribution is complete within approximately 2 hours.[5]

Iohexol clearance studies[30] in dogs and cats indicate that the marker behaves similarly in these species. In dogs with normal to moderately reduced renal function, the distribution phase was completed within 2 hours.[39] In rats and dogs iohexol is rapidly distributed and found almost exclusively in the extracellular space .[42] In a study using 3 dogs, 98% of the iohexol dose was excreted unchanged in the urine[11] supporting its utility as a marker for use in GFR studies. Clearance studies using iohexol have agreed closely with traditional methods used to estimate GFR among a variety of species, therefore, it is logical to believe that it will behave similarly in horses as well.

Iohexol clearance has been calculated using either compartmental models or model-independent methods. Significant variations in clearance values may be obtained depending on the pharmacokinetic methods used, the behavior of the injected substance, the number of samples obtained and the time intervals chosen.[9,39] The clearance (CL_{plasma}) of an injected filtration marker, such as iohexol, can be calculated by measuring the concentration of the marker in the plasma versus time as shown by the formula:

$$CL_{plasma} = Dose/AUC \qquad (2)$$

Where Dose is the injected dose of iohexol and AUC is the calculated area under the concentration versus time curve. The AUC is calculated using mono, bi- or tri-exponential formulas[39,52] that describe 1-compartment ($CL_{plasmaC-1}$), 2-compartment ($CL_{plasmaC-2}$) and 3-compartment ($CL_{plasmaC-3}$) models, depending on the available data. Model independent methods calculate the AUC by use of the trapezoidal rule to obtain clearance ($CL_{trapezoid}$). Accurate assessment of iohexol clearance using either compartmental models or the model-independent method requires collection and analysis of plasma samples starting “x” minutes after injection and continuing at frequent intervals. Analyses

of this kind almost always demonstrate one or more distribution and elimination phases [52] if sampling begins after the distribution phase or phases have been completed, a $CL_{plasmaC-1}$ model provides an accurate description of the data. Clinically, use of the $CL_{plasmaC-1}$ is attractive because only two or three samples are required for analysis and the procedure can be completed in 3 or 4 hours with a minimum of discomfort to the patient. However, there are important considerations associated with the use of the $CL_{plasmaC-1}$ model. The $CL_{plasmaC-1}$ assumes an immediate distribution of the injected marker, and in patients with normal renal function, the $CL_{plasmaC-1}$ may overestimate clearance by up to 30%[53] because it ignores the amount of injected substance present that corresponds to the AUC during the distribution phase.[11] To alleviate this discrepancy, and allow clinical use of the $CL_{plasmaC-1,}$ Brochner-Mortensen[53] developed a formula for use in human GFR studies to correct for such error as expressed by the regression equation:

$$CL = 0.990778\ CL_1 - 0.001218\ CL_1^2 \qquad (3)$$

Where CL is the corrected clearance and CL_1 is the $CL_{plasmaC-1.}$

Using ^{51}Cr-EDTA, Brochner-Mortensen compared the corrected $CL_{plasmaC-1}$ with that of the total plasma clearance in 74 patients with varying degrees of renal dysfunction and found that clearance values could be accurately predicted using the $CL_{plasmaC-1}$ value corrected with his regression equation. This correction equation was based on the disappearance curve derived from the 5 terminal points 3 to 5 hours after injection of the marker. Clearance studies completed in humans[5,34,37] and animals[30,38,41] have applied this correction formula in an attempt to simplify estimation of GFR for use in the clinical setting. Brown et al[30] found that results of the corrected $CL_{plasmaC-1}$ compared well with those obtained using more complex methods in a study using renal intact and partially nephrectomized cats (n=10) and dogs (n=7). Gleadhill and Michell[38] calculated

the clearance of iohexol and ^{99m}Tc-DTPA and compared the corrected $CL_{plasmaC\text{-}1}$ with a $CL_{plasmaC\text{-}2\ model}$ in a study that included 24 dogs with known or suspected renal impairment to validate the use of the corrected $CL_{plasmaC\text{-}1}$. They determined that the additional early sampling required for the $CL_{plasmaC\text{-}2}$ model did not make a significant difference in clearance values when compared to the corrected $CL_{plasmaC\text{-}1\ model}$. Miyamoto[41] found adequate correspondence between renal creatinine clearance and plasma iohexol clearance using the only the Brochner-Mortensen corrected $CL_{plasmaC\text{-}1}$ model. Though varied in approach, all investigators found a close correlation between GFR values obtained using traditional estimations of GFR and those using Brochner-Mortensen's single compartment correction equation. Caution must be used when interpreting the data from animal studies that attempt to use a correction formula developed for use in human studies. Animal study groups are often limited in size and rarely include a true cross section of the normal or diseased population. Unknown effects may occur due to age, species differences, concurrent non-renal disease or variation between individual patients. In addition, the reduction in GFR that attends renal impairment results in a longer elimination phase for the marker. This could effect use of a correction formula that requires calculation of the decay curve based on early sampling 2-5 hours after injection of the marker if the terminal phase has not been reached. In those patients with profoundly reduced renal function, this could lead to inaccuracies when attempting to estimate GFR.[36,37] In such cases, it has been recommended that an additional sample beyond 4 or 5 hours be obtained to determine a more precise measurement of clearance.

Analysis of iohexol

Iohexol is very stable.[5] Samples can be chilled for up to two-weeks[11] or frozen indefinitely without affecting analysis.[54] Several methods have been developed to determine the concentration of

iohexol in urine, serum and plasma samples. These include; (1) high performance liquid chromatography (HPLC),[43] (2) inductively coupled plasma-atomic emission spectroscopy (ICP-AES),[54] (3) capillary electrophoresis (CE),[55] (4) ceric-arsenite ("simple chemical method"),[56] (5) X-ray fluorescence (Renalyzer PRX90)[5] and (6) scintillation (gamma) counters. The Renalyzer is no longer available and scintillation counters are primarily available at institutions permitted to handle radioactive materials. Capillary electrophoresis provides a safe, inexpensive and accurate determination of iohexol concentration,[55] but unless carefully initiated, problems with evaluation of serial samples at very low or very high concentrations may occur.[54] The ceric-arsenite method is accurate, but necessitates handling of restricted chemicals and requires a bench chemist. Additionally, it is purported to have poor repeatability in method comparison studies (Finco, personal communication). Analysis of iohexol using either HPLC or ICP-AES is commercially available (Animal Health Diagnostic Lab, Michigan State University, East Lansing Michigan) and both have been used to develop pharmacokinetic profiles of iohexol in cats and dogs (Braselton-personal communication). Method comparison studies examining HPLC and IPC-AES have demonstrated that these two methods can be used interchangeably without risk of error[9] (Braselton, personal communication). Because of its sensitivity,[10] HPLC analysis offers several advantages over some of the other methods; 1) size of serial blood samples can be reduced, especially important in studies using rodents, very small animals (cats) or neotates,[54] 2) in the presence of severe renal impairment, a reduced dose of iohexol could be administered without risk of nephrotoxicity, yet remain detectable.

CHAPTER 2: MATERIALS AND METHODS

Horses—Ten horse foals, 5 colts and 5 fillies were obtained as orphans from a nurse mare herd in northern Kentucky. They ranged from 6 to 12-weeks of age and weighed between 82 and 106 kilograms. Breeds represented included 1 American Quarter Horse, 3 Tennessee Walkers, 5 American Saddlebreds and 1 draft/thoroughbred cross. None of the foals had a history of failure of passive transfer or previous illness. All foals received a physical examination by a veterinarian two-weeks prior to arrival at the Virginia-Maryland Regional College of Veterinary Medicine research facility and were deemed healthy and free of infectious diseases prior to shipping. In addition, as a requirement for interstate transport, all foals tested negative for the presence of antibodies to the Equine Infectious Anemia Virus. Physical examinations were performed on each foal upon arrival and once each day during the one-week acclimation period. Foals were de-wormed with 10% fenbendazole[a] at a dose of 10mg/kg orally and suffered no ill effects. All foals remained healthy during the 7 days prior to the experiment.

Subject preparation—On the day of the study all foals were lightly sedated with Xylazine HCl[b]at a dose of 0.50 mg/kg, IV to facilitate intravenous catheter and urinary catheter placement.

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[a] Panacure® paste, Hoechst-Roussel Agri-Vet Co., Somerville, NJ USA

[b]Rompun® Bayer Corporation, Shawnee Mission, KS USA

[c]Angiocath™ Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT USA

Following sedation, a 16 gauge, 83 mm long, I.V. catheter[c] was placed aseptically in both right and left jugular veins of each foal. Blood samples were obtained for CBC and chemistry profile analysis. A 30 cm long, 12-french Foley urinary catheter was placed aseptically in each filly and a 55 cm long, 12-french Foley urinary catheter was placed aseptically in each colt. Thirty-milliliters of sterile saline was injected into the balloon tip to retain the catheter in place during the collection procedure. Once prepared, foals were offered free-choice hay and water and held for a minimum of 3 hours prior to initiating the study. Foals were monitored for straining against the urinary catheter and urine was removed from the bladder when necessary to keep them comfortable and prevent passage of urine around the catheter.

Iohexol clearance—Just prior to iohexol injection, 6ml of blood was taken from the right jugular catheter and discarded, followed by removal of a 10ml blood sample which was immediately placed in a serum tube. The catheter was flushed with 2 ml of heparinized saline followed by a slow bolus injection of the calculated dose of iohexol[d] (150mg/kg) through the right jugular catheter with completion noted as time "0". Immediately following the injection, the catheter was flushed as above with heparinized saline and removed. Sequential blood samples were taken from the left jugular catheter at 5, 20, 40, 60, 120, 180, 240 and 360 minutes post iohexol injection and precise withdrawal times (minutes, seconds) noted. Samples were obtained after flushing the catheter with 2 ml of sterile saline, removing and discarding 6 ml of blood followed by removal of 10 ml of blood that was immediately placed in a serum tube and held for 2 hours to allow primary clot formation. The catheter was then flushed with 2 ml of sterile saline as previously described and sealed. The catheter was removed immediately after the 360 minute sample was

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[d] Omnipaque® 350 Nycomed Amersham, Princeton, NJ USA

obtained. Serum was separated further by centrifugation at ambient temperature (1000 X g for 10 minutes). Two milliliters of serum was removed from clotted serum tubes, transferred to labeled plastic vials, chilled to 5 C and shipped to AHDL[e] for analysis. Iohexol concentration in serum samples was determined by HPLC using the method of Shihabi et al[43] as modified in Finco et al.[9]. Equipment included a Waters Corporation (Milford, MA) 600 E gradient HPLC system with Waters tunable absorbance detector at 254nm, Waters 712 WISP autosampler, 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.

Creatinine clearance—The exogenous creatinine solution was prepared aseptically by dissolution of 1 gram of creatinine[f] in 12 mls lactated Ringers solution resulting in a final concentration of 80 mg of creatinine per milliliter. Individual doses were calculated at 100 mg creatinine per liter of total body water for each foal as follows:

Body Weight (kgs) x 0.60 = Total Body Water

One hour after iohexol was injected, 65% of the foal's total calculated dose of exogenous creatinine was injected subcutaneously in 3 approximately equal amounts on the left side of the foal under the skin of the axilla, pectoral area and caudo-lateral to the withers.

At 1 hour and 25 minutes post iohexol injection, the remaining 35% was injected in a similar manner on the right side of the foal. At 1 hour and 45 minutes the urinary bladder was emptied and a sample of this urine retained for urinalysis. The bladder was then washed with sterile isotonic saline. Care was taken to insure that the entire wash volume was recovered. The catheter was

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[e] Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI USA

[f] Creatinine (C-4255), Sigma Chemical Co., St.Louis, MO USA

clamped to prevent any urine loss during the collection periods. Concurrently, a blood sample was obtained from the jugular catheter in a manner previously described and placed in 7.5 ml evacuated tubes containing 100 U of heparin to determine serum creatinine concentration ($[Cr_{serum}]$). Time was noted and the clock started at time "0". At approximately twenty-minute intervals the bladder was emptied of all urine and washed with saline several times to assure complete retrieval of creatinine. The entire urine collection, including the wash was saved, and its volume determined followed by removal from this volume of a 2 ml aliquot sample to determine urine creatinine concentration ($[Cr_{urine}]$). Midway through each urine collection period, a blood sample was obtained to determine $[Cr_{serum}]$. This procedure was repeated 3 times and the urinary catheter removed immediately after the final urine collection. Creatinine concentrations in urine and serum samples were determined within 3 hours of collection, by the clinical laboratory at Virginia Tech via automated analysis[g] using the Jaffe reaction.[32] Creatinine clearance ($CL_{creatinine}$) was calculated for each time interval using $CL_{creatinine}$ = Urine Volume x $[Cr_{urine}]$ / $[Cr_{serum}]$ / (body weight in kilograms). Comparisons to iohexol clearance were made using the mean of the three time points for each foal.

Pharmacokinetic calculations (model dependent and model independent)— Monexponential, biexponential, and triexponential equations were fit to the plasma iohexol concentration versus time profiles. The data were analyzed by nonlinear least squares regression analysis with equal weighting of the data, using commercial software. The number of exponential terms required to describe the data for each foal was determined by application of Akaike's information criterion.[57] Standard pharmacokinetic equations were used to describe the disposition of

[g] Olympus AV 400, Dallas, TX USA

iohexol. The triexponential equation $Cpt=C_1 \times e^{-\lambda 1t} + C_2 \times e^{-\lambda 2t} + C_z \times e^{-\lambda zt}$ where Cpt is the plasma concentration at any time t, described the data for each foal. Pharmacokinetic variables were then calculated using the intercepts (C_1, C_2, and C_z) and absolute values of the slopes (λ_1, λ_2, and λ_z) of the best fit equation for each foal. The area under the plasma concentration versus time curve (AUC) was calculated from the intercepts and slopes of the triexponential equations for each individual animal according to $AUC = C_1/\lambda_1 + C_2/\lambda_2 + C_z/\lambda_z$. The total plasma clearance (Clt) was calculated from Clt = dose/AUC. The volume of distribution of the central compartment (Vc) was calculated, using $Vc=dose/ (C_1+C_2+C_z)$. The volume of distribution was calculated as Vd(area) = dose $*\lambda_z$/AUC.

Model independent pharmacokinetics— Area under the plasma versus time profile was determined using the trapezoidal rule. Model independent iohexol clearance was calculated using $Clt_{(trap)} = dose / AUC_{trapezoid}$

Statistical Analysis—Clearance values are expressed as milliliters per minute per kilogram and values are reported as mean. Analysis of serum concentration versus time profiles were performed for each individual foal in the study. Analysis was performed using WinNonlin[g] (version 1.5) running on a Pentium-based personal computer. The CL_{plasma} 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.[58] Standard deviation of the difference was calculated and limits of agreement were set and declared significant at $p \leq 0.05$. The

correction factor used to predict a $CL_{plasmaC\text{-}3}$ was derived by geometric mean regression[59] after calculating the terminal slopes extracted from the model at; 3 and 4 hours; 4 and 6 hours; 3 and 6 hours; and 3,4 and 6 hours. A paired t-test was used to assess agreement between the model and extracted slopes.

CHAPTER 3: RESULTS

Urinary clearance of creatinine—Baseline serum creatinine concentration for all foals prior to subcutaneous injection of exogenous creatinine ranged from 0.9 to 1.7 mg/dl. At forty-five minutes after the second injection, serum creatinine concentrations increased a minimum of 6 times the pre injection values in each foal with a range 5.6 to 11.9 mg/dl. Values for exogenous creatinine clearance ranged from 1.34 to 3.20 ml/min/kg body weight with a mean of 2.17 ml/min/kg (Table 1).

Plasma clearance of iohexol—After IV administration, iohexol concentration ranged from 915.68 (5 minutes) to 18.32 mg/L (360 minutes). Visual inspection of individual arithmetic and semi-logarithmic plots suggested first order elimination of iohexol (Fig 1). A 3-compartment open model was used to describe disposition. Individual pharmacokinetic constants are presented in (Table 2). The mean $CL_{plasmaC\text{-}3}$ of iohexol was 2.15 ml/min/kg with a range of 1.68 to 2.69 ml/min/kg (Table 1). The $CL_{plasmaC\text{-}3}$ model resulted in the best fit of the data when compared to the $CL_{plasmaC\text{-}1}$, $CL_{plasmaC\text{-}2}$ and model-independent methods ($CL_{trapezoid}$).

Predicted plasma clearance of iohexol— $CL_{predicted}$ from the $CL_{plasmaC\text{-}3}$ using the correction factor (formula 5) was 2.15 ml/min/kg with a range of 1.60 to 2.67 ml/min/kg (Table1). Terminal slopes were calculated for each combination of the; 3 and 4 hour; 4 and 6 hour; 3 and 6 hour; and 3,4 and 6-hour time points. These slopes were compared to the terminal slope of the 3-compartment model using a paired t-test. The rate constant derived from $AUC_{4\ and\ 6\ was}$ not significantly different from

the terminal slope described by the study model ($P = 0.20$). Once the combination producing the best estimate of the terminal slope was selected, those two points were used to calculate an area under the curve according to the equation:

$$AUC = \text{Intercept} / [\text{slope}] \qquad (4)$$

The area under the curve for the 2-sample method ($AUC_{4\ \text{and}\ 6}$) was then plotted versus the AUC derived from the $CL_{plasmaC\text{-}3}$ analysis. Geometric mean regression was used to develop an equation (formula 5) that describes the relationship between these two areas [59] (Figure 2) and applied to the $AUC_{4\ \text{and}\ 6}$ to obtain the $AUC_{predicted}$ as shown:

$$AUC_{predicted} = (AUC_{4\ \text{and}\ 6}) \times 0.9603154 + 18529.162 \qquad (5)$$

Predicted clearance ($CL_{predicted}$) was determined for each foal as follows:

$$CL_{predicted} = \text{Dose} / AUC_{predicted} \qquad (6)$$

CHAPTER 4: DISCUSSION

Glomerular filtration rate is considered the most important parameter used to assess renal function. Traditional function tests, such as creatinine and inulin clearance, are accurate at determining renal competence,[16,19,21] but are impractical and rarely used. Error[9] and the inherent difficulties associated with performing these tests properly, even under ideal conditions, are compounded by the large size and unpredictable nature of horses. As a result, renal function tests are rarely used, even at referral institutions or sophisticated private practices where invasive diagnostic tests, such as renal biopsy, predominate. Measurement of serum urea (SUN) and serum creatinine ($[Cr_{serum}]$) as determinants of renal function, and as an index of GFR are easily and safely obtained, but have proven to be crude indicators of renal dysfunction until GFR is severely compromised. In addition, $[Cr_{serum}]$ may be falsely elevated in adult horses and neonatal foals by non-renal factors, such as acute rhabdomyolysis and placental incompetence, respectively.[15]

Plasma clearance techniques offer distinct advantages over those that require urine collection. They are minimally invasive, require less time and are easy to perform. As a result, protocols using plasma clearance of iohexol are rapidly replacing the use of traditional techniques used to estimate the GFR in humans,[40] dogs[9,11,30,38,39] and cats.[30,41] The iohexol molecule satisfies the criteria of an "ideal" filtration marker,[43] and has proven to be safe, and most importantly, accurate at estimating GFR among patients with a wide range of renal dysfunction.[36,37,40] Simultaneous clearance studies using iohexol in conjunction with other markers, including exogenous creatinine[9]have demonstrated no interference[10] or adverse reactions.

To determine the utility of a new diagnostic method, comparisons are typically made against an established standard. Exogenous creatinine clearance, regarded as an accurate indicator of GFR in foals and adult horses, determined in this study, had a range of 1.34 to 3.20 ml/min/kg, which corresponds to published reference ranges.[16,18,19] In addition, the range of GFR values obtained in our foals using exogenous creatinine clearance are also in agreement with the values obtained by researchers using a variety of other methods and markers.[7,14,17] This suggests that plasma clearance of iohexol could be validated by direct and simultaneous comparison to exogenous creatinine clearance. The aims of this study were; to determine the pharmacokinetic behavior of iohexol in normal foals and compare the GFR values obtained with those of exogenous creatinine clearance. And, develop a simplified method to predict the plasma clearance of iohexol defined by two or three blood samples.

The disposition of iohexol after IV bolus injection was best described by the 3-compartment open model, and gave the best estimate of GFR in all foals when compared to $CL_{creatinine}$ (Table 1). Previous studies have used straight linear regression to assess agreement between two methods and correlation coefficients determined in method comparison studies of iohexol and exogenous creatinine clearance by Finco *et al*.[9,30] demonstrated results that approached unity. In our study, mean GFR values for $CL_{plasmaC\text{-}3}$ (2.15 ml/min/kg) and $CL_{creatinine}$ (2.17 ml/min/kg) were not significantly different and highly correlated as well. Although it is attractive to assume that the two methods could be used interchangeably, a high correlation coefficient implies only the strength of the relationship between two variables, not how closely they agree. Bland and Altman[58] suggest that it is more accurate to plot the differences between methods against the average of the two measurements and set limits of agreement. To substantiate our correlation as more than just a close association, the mean differences between $CL_{creatinine}$ and $CL_{plasmaC\text{-}3}$ for each foal in this study were

determined and compared against the mean of the two methods (Figure 3). The mean difference between $CL_{creatinine}$ and $CL_{plasmaC-3}$ in the foals was 0.02003 with limits of agreement set at –0.56732 and 0.60738.

The simplified clearance method presented in this study is an example of how a more complex model could be modified for use clinically. The 1-compartment model, which is described by a monoexponential plasma disappearance curve, is often applied to clearance studies used to estimate GFR. Protocols requiring only 2 or 3 blood samples have been used in human patients to eliminate the inconvenience of serial sampling and extended clinic visits. But, use of the 1-compartment model may underestimate the area under the curve, because the area ignored during the distribution phase is large, resulting in an overestimation of the GFR. An overestimation of as much as 30% can occur when using the 1-compartment model[8,53] in patients with normal GFR values. To account for this discrepancy, a modified 1-compartment model and correction factor to describe the AUC and calculate the GFR has been developed[53] and applied to GFR studies in humans and several animal species.[30,38,41,48] Although this is a 1-compartment analysis of the data gathered, in reality it is a model of a single compartment (either 2nd or 3rd) extracted from a multi-compartment model. Two-sample methods for determining GFR using iohexol pharmacokinetics depend on obtaining an accurate estimate of the terminal elimination phase of the elimination profile and a correction for the area or areas under the curve(s) for existing distribution phases. From a clinical point of view, preference should be given to the shortest test duration. Visual inspection of the plasma versus time data in this study suggested that any or all of the 3, 4 and 6-hour time points might produce an accurate estimate of the terminal slope and AUC. Clearance was calculated using samples from different time points (3 and 4 hour; 3 and 6 hour; 4 and 6 hour; 3, 4 and 6 hour) fitted to a 1-compartment model. As long as the distribution phase or phases have been completed, a $CL_{plasmaC-1}$

provides an accurate description of the data. The rate constant, that was derived from the 4 and 6-hour time points and used to determine the AUC, was highly correlated (R^2 =0.95) and not statistically different from the AUC obtained from the study model. Therefore, this value was used to predict the $CL_{plasmaC-3}$ and determine the $CL_{predicted.}$ Application of our correction formula to $AUC_{4 \text{ and } 6}$ to obtain $AUC_{predicted}$ resulted in a mean $CL_{predicted}$ GFR value (2.15 ml/min/kg) that was not statistically different from the study model ($CL_{plasmaC-3}$) which required eight samples.

The use of iohexol clearance to determine the GFR in humans has, for the most part, replaced the use of traditional markers, and in addition has proven safe and reliable in recently completed animal studies.[9,11,39,41] When developing a renal function test for use in horses, certain features are necessary if it is to be used in clinical practice. It should be easy to perform, safe for the staff and patient, and produce results that can be used interchangeably with those obtained by a currently accepted standard. The mean GFR values obtained in this study using urinary clearance of exogenous creatinine were not different from those obtained using plasma clearance of iohexol. This indicates that the use of plasma clearance of iohexol may obviate the use of traditional methods of estimating GFR in horses. Iohexol, injected intravenously at a dose of 150 mg/kg, was well tolerated by all foals and the clearance values obtained suggest that plasma clearance of iohexol, using a 3-compartment model, can be used to estimate the GFR in healthy foals. In addition, the correction formula introduced here allowed us to predict GFR accurately without the use of a more complex method.

Additional studies are necessary to verify these findings in healthy adult horses, and foals and adults with reduced renal function or concurrent disease. Age related factors might be particularly important in very young foals who have a much larger volume of distribution than adult horses.[60] This may have an effect on the distribution and elimination of iohexol in this age group. Use of the

$CL_{predicted}$ method developed to estimate GFR in this study, requiring only two blood samples obtained 4 and 6 hours after injection of iohexol, could easily be utilized in the clinical setting. Iohexol maintains its stability in chilled or frozen in serum samples and analysis is inexpensive and commercially available.

	$CL_{creatinine}$	$CL_{plasmaC-3}$	$CL_{predicted}$
Foal 01	2.84	2.66	2.55
Foal 02	1.65	1.76	1.89
Foal 03	1.34	1.68	1.75
Foal 04	2.57	2.69	2.54
Foal 05	1.37	1.84	1.83
Foal 06	3.20	2.60	2.67
Foal 07	1.80	1.71	1.61
Foal 08	2.48	2.36	2.25
Foal 09	2.37	2.25	2.52
Foal 10	2.08	1.96	1.88
Mean	2.17	2.15	2.15

Table 1. Estimated GFR values (ml/min/kg) for $CL_{creatinine}$, $CL_{plasmaC-3}$ and $CL_{predicted}$ in 10 foals.

Foal	C_1	L_1	C_2	L_2	C_z	L_z	AUC	Clt	Vc	Vdarea
	(µg/ml)	(min^{-1})	(µg/ml)	(min^{-1})	(µg/ml)	(min^{-1})	(µg/ml*hr)	(ml/min/kg)	(l/kg)	(l/kg)
1	367.5	0.0695	351.3	0.0117	107.4	0.0051	56377.1	2.66	0.1816	0.5208
2	441.1	0.1045	349.3	0.0151	263.8	0.0046	85116.7	1.76	0.1423	0.3855
3	569.0	0.3030	349.7	0.0333	428.6	0.0056	89265.8	1.68	0.1113	0.3014
4	320.8	0.1497	321.7	0.0334	321.7	0.0073	55858.9	2.69	0.1556	0.3679
5	726.5	0.4255	412.7	0.0304	359.4	0.0054	81418.6	1.84	0.1001	0.3390
6	504.6	0.1093	389.3	0.0238	274.0	0.0075	57707.1	2.60	0.1284	0.3486
7	280.0	0.1087	230.8	0.0198	356.8	0.0048	87807.1	1.71	0.1729	0.3523
8	521.2	0.2159	327.6	0.0219	272.7	0.0059	63607.9	2.36	0.1337	0.3996
9	523.7	0.0798	451.7	0.0150	149.0	0.0050	66584.4	2.25	0.1334	0.4527
10	217.0	0.1499	354.8	0.0308	373.7	0.0059	76731.5	1.96	0.1587	0.3337
Median	472.8	0.1295	350.5	0.0228	297.9	0.0055	71657.9	2.10	0.1380	0.3803
Mean	447.1	0.1716	353.9	0.0235	290.7	0.0057	72047.5	2.15	0.1418	0.3651
Min	217.0	0.0695	230.8	0.0117	107.4	0.0046	55858.9	1.68	0.1001	0.5875
Max	726.5	0.4255	451.7	0.0334	428.6	0.0075	89265.8	2.69	0.1816	0.2253

Table 2. Pharmacokinetic constants describing the disposition of iohexol in foals after IV administration of a single 150 mg/kg dose.

Equation Describing the Three Compartment Open Model $C_{pt} = C_1 \text{ x } e^{-\lambda 1t} + C_2 \text{ x } e^{-\lambda 2t} + C_z \text{ x } e^{-\lambda zt}$

C_{pt} = plasma concentration at any time “t”

C_1, C_2 = concentration intercept for distribution phase; C_z = concentration intercept for post distribution phase; L_1, L_2 = slope of distribution phase curve; L_z = slope of post distribution phase curve; AUC = area under the curve; Clt =Total clearance; Vc = volume of distribution of the central compartment; Vd area = volume of distribution during the terminal phase.

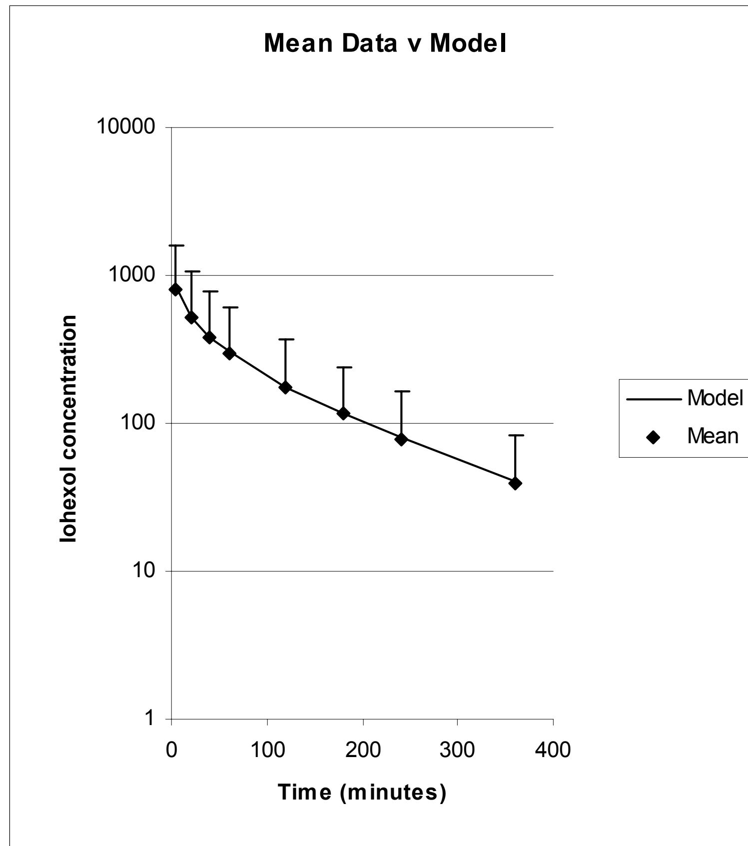

Figure 1. Semi-logarithmic plasma concentration verses time plot for iohexol in foals (n=10) after IV administration of a single 150 mg/kg dose

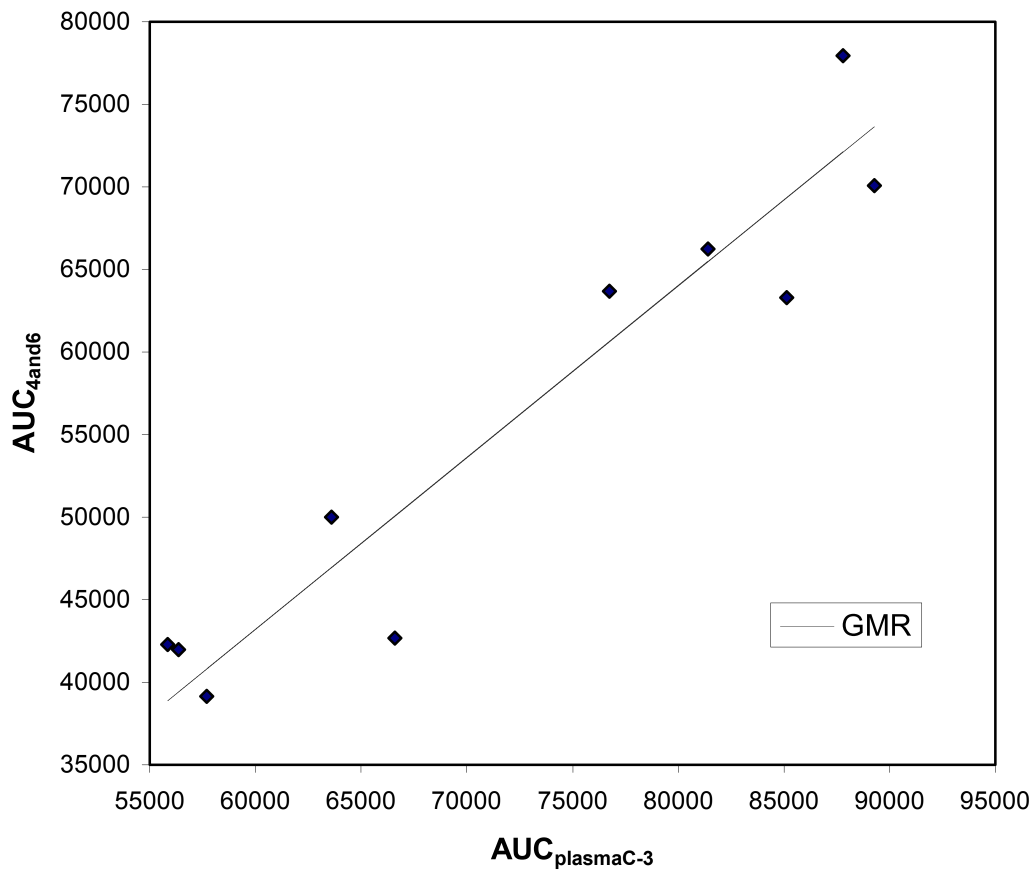

Figure 2. The relationship between $AUC_{plasmaC-3}$ and $AUC_{4\ and\ 6}$. $R^2 = 0.95$

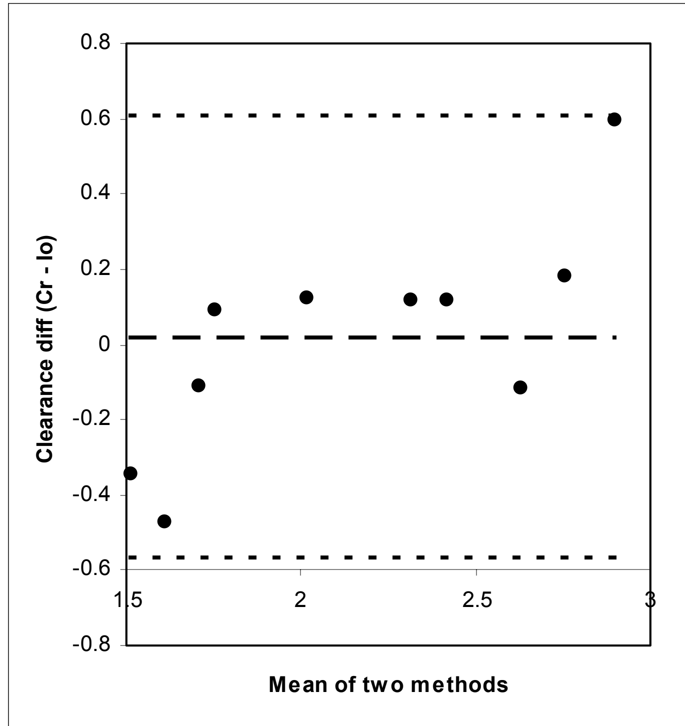

Figure 3. Limits of agreement plot depicting the mean difference between $CL_{creatinine}$ and $CL_{plasmaC-3}$ in 10 healthy foals. (-----);Limits of agreement (2SD)

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