Evaluation of a Single Sampling Method for Estimation of Plasma Iohexol Clearance in Dogs and Cats with Various Kidney Functions

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ABSTRACT. Plasma iohexol clearance (PCio) is a practical method for measuring the glomerular filtration rate (GFR) in clinical settings. However, it is too time-consuming for routine application and requires hospitalization for at least half a day. Therefore, the development of a simpler procedure for plasma clearance is necessary to allow the frequent measurement of GFR in clinical settings. The purpose of the present study was to evaluate a single sampling method for estimation of PCio in dogs and cats with various kidney functions. The PCio determined by the 1-compartment model using 3 samples (PCio 3samples) was used as a reference for the evaluation of the single sampling method (PCio single). Plasma iohexol concentration was determined by a cerium arsenite colorimetric method. PCio single was significantly correlated with PCio 3samples in both dogs and cats (dogs: R²=0.985, P<0.001, cats: R²=0.986, P<0.001). In a receiver operating characteristics analysis, the area under the curve, sensitivity, and specificity for detecting decreased GFR were 0.995 [SE, 0.003], 98%, and 93% for dogs and 0.993 [SE, 0.003], 98%, and 93% for cats, respectively. These results demonstrate that PCio single may be very useful for the detection of decreasing GFR in dogs and cats.

KEY WORDS: chronic kidney disease, iohexol clearance, single sampling method.

In veterinary clinical practice, chronic kidney disease (CKD) is diagnosed by detection of decreases in the glomerular filtration rate (GFR) or the presence of renal damage [24]. Plasma urea and creatinine concentrations are widely used as endogenous markers for evaluating renal function in dogs and cats because their measurement is easy and inexpensive. However, they usually increase after a severe reduction in GFR [4]. Extrarenal factors that can alter the plasma urea concentration include high protein intake, fever, intestinal hemorrhaging, and catabolic state. In dogs, it should be noted that the plasma creatinine concentration is influenced by the ratio of body weight to muscle mass [4], and small quantities of creatinine are secreted by the renal tubules in male dogs [11]. Thus, plasma urea and creatinine concentrations do not accurately reflect GFR in dogs and cats.

The GFR is an adequate marker of renal function in humans and animals. The methods for measuring GFR in dogs and cats include plasma or urinary clearance of markers such as inulin, creatinine, and iohexol [6, 8, 11, 14, 18, 21, 20]. The urinary clearance of inulin and creatinine are considered as the gold standards for measuring GFR in humans and animals. However, these methods are impractical in clinical settings because they require insertion of a bladder catheter, which is associated with a risk of developing a urinary tract infection or urethral injury, complete collection of a timed urine sample, and continuous infusion of the marker. Alternative methods for determination of GFR include nuclear imaging of the kidney after intravenous injection of radio labeled substances [2, 32]. However, these methods require radioisotopes and specialized equipment that are not commonly available in clinical settings.

The single injection technique can be used to determine plasma clearance from the elimination curve of the marker in the plasma after a bolus injection. Plasma clearance can be determined by dividing the dosage of the administered marker by the area under the plasma concentration versus time curve (AUC). This method is easier to perform than the urinary clearance method because it does not involve the collection of urine samples and continuous infusion of the marker. Recently, plasma clearance tests for inulin, creatinine, iohexol, ⁹⁹ᵐTc-diethylenetriaminepetaacetic (⁹⁹ᵐTc-DTPA) acid, and ⁵¹Chromium-ethylene diaminic tetraacetic acid (⁵¹Cr-EDTA) have been developed to estimate GFR in dogs and cats [1, 2, 6, 8, 14, 15, 18, 20, 21, 34]. Iohexol, a nonradioactive iodinated contrast media, is easy to use in the clinic and laboratory. Iohexol has been evaluated as a filtration marker in dogs, cats, pigs, foals, and sheep [6, 10, 12, 14, 16, 21, 23]. Limited sampling methods for plasma clearance procedures are used for clinical expediency to estimate GFR in dogs and cats [1, 6, 10, 14, 21, 34]. In this method, AUC is generally determined by the modified 1-compartment model (CM) using the Brosner-Mortensen (BM) formula, which was derived from human data using a radio labeled index [5]. Bexfield et al. showed that this formula was inaccurate in small dogs weighing 2 to 6 kg [3]. Although several studies have used this formula in both dogs and cats, it has not been properly validated in cats [6,
MATERIALS AND METHODS

Study 1

The purposes of study 1 were to evaluate the assay quality of ceric arsenite colorimetric assays for measuring plasma iodine concentration and the utility of PCio using a combination of the modified 1CM and ceric arsenite colorimetric assays.

Animals: Thirty-seven dogs were included in study 1. These dogs were clinically healthy (body weight: 9.4 ± 2.2 [range: 6.7 to 14.1] kg, age: 3.3 ± 3.2 [range: 0.8 to 10.0] years). All dogs were raised as laboratory animals in the Department of Veterinary Internal Medicine at NVLU. All dogs were housed in individual cages and fed a commercial maintenance diet until the measurement of PCio. Water was given ad libitum.

Plasma iohexol clearance (PCio): PCio was performed for all dogs as previously described to determine GFR [21]. The animals were fasted for half a day before the PCio study, and water was given ad libitum during the procedure. The dose of iohexol (Ominiparque 300, DAIICHI SAN-KYO Co., Ltd., Tokyo, Japan) was 90 mg of iodine/kg. A half-milliliter of heparinized blood was collected from the jugular vein before iohexol injection. Iohexol was administered via the cephalic vein (time 0), and then heparinized blood was sampled again at 5, 10, 15, 30, 60, 90, 120, 180, and 240 min.

The plasma iodine concentration was determined by the cerium arsenite colorimetric method [2]. This assay involved the use of 50 µl of plasma, as well as the use of 5 ml of 1 mol sodium hydroxide, 2 ml of working solution, and 100 µl of ceric ammonium sulfate for the colorimetric assay. The working solution was made by mixing five parts of 1 mol sulfuric acid with one part of the bromide/bromade solution and four parts of arsenite reagent. Then, absorbance at 410 nm of samples was read using an absorptiometer.

PCio was calculated using the 2CM (PCio samples) and 1CM (PCio samples) corrected with the BM formula [5]. The 2CM corresponds to a curve that can be resolved into 2 straight lines:

\[
C(t) = A \times e^{-\alpha t} + B \times e^{-\beta t}
\]
\[
AUC = \frac{A}{\alpha} + \frac{B}{\beta}
\]

where A is the concentration at the intercept of the line of slope(-\(\alpha\)), and B is the concentration at the intercept of the line of slope(-\(\beta\)). The plasma concentrations of the initial steep part of the curve were determined by the distribution in body tissues; whereas, the last part of the curve was primarily influenced by elimination from the body.

In the 1CM, PCio was estimated from the slope (-\(\alpha\)) and intercept (A) of the elimination phases of the curves, as determined by linear regression analysis of the final three plasma samples (120, 180, and 240 min). Clearance values (CI) were calculated as CI = dose of iohexol/AUC (AUC = \(A/\alpha\)). The PCio was then calculated as PCio = 0.990778 \times CI - 0.001218 \times CF [5].

The CI (ml/min) were standardized to body surface area (BSA, ml/min/m²). BSA (m²) was calculated from BW (g) using the general formula: BSA = \(K \times (BW)^{0.75}\times10^{2}\), where K is a shape constant (10.1 for dogs, 10.0 for cats), and \(\alpha\) is the mass exponent (0.71 for dogs, 0.66 for cats) [25].

Assay validation: To validate the precision of this procedure, standard serum ranging from 7.5 to 120 µg iodine/ml was analyzed five times. The standard serum was prepared from iohexol solution by serial dilution. The maximum and minimum limits of detection of the cerium arsenite colorimetric method for measuring plasma iodine concentration were determined by iohexol solution with iodine concentrations ranging from 1.87 to 240 µg iodine/ml. Intra-assay variability was assessed in samples collected from 5 dogs. The analysis was repeated 10 times. The coefficient of variation (CV [%] = SD/mean \times 100) for each sample was calculated.

Inter-assay variability was evaluated by 10 measurers analyzing the same samples from 1 healthy dog. The CV was also calculated.

Study 2

The purposes of study 2 were to determine a reference range for PCio using 1CM in clinical healthy dogs and cats and to evaluate a single sampling method for the estimation of PCio in dogs and cats with various kidney functions.

Animals: Seven hundred and seventy-nine dogs and 339 cats were included in study 2. One hundred of the 779 dogs and 36 of the 339 cats were raised as laboratory animals in the Department of Veterinary Internal Medicine at NVLU. These animals were clinically healthy and were assigned to the control group to determine the reference range of PCio. The 679 dogs and 303 cats were client-owned animals and were presented to the Nephrology Service of the Veterinary Medical Teaching Hospital at NVLU or 99 veterinary hospitals in Japan for measurement of PCio. Of these, 480 dogs and 211 cats were diagnosed with chronic kidney dis-
Plasma iohexol clearance (PCio)

**Three sampling method (PCio 3samples):** The detailed procedure for measuring PCio was described above. Plasma samples with iodine concentrations > 120 \( \mu g/ml \) were diluted with appropriate amounts of plasma obtained from the same animal before iohexol injection. The dosages of iohexol in the non-azotemic animals and azotemic animals were 90 mg of iodine/kg and 45 mg of iodine/kg, respectively. Iohexol was administered via the cephalic vein (time 0), and then heparinized blood was sampled again at 120, 180, and 240 min for the non-azotemic dogs and 120, 240, and 360 min for the azotemic dogs. AUC was calculated by 1CM. The Cl (ml/min) was standardized to BSA (ml/min/m²) [25].

Reference ranges were determined by PCio 3samples from control dogs (n=100) and cats (n=36) under the same conditions including fasting, environment, and hydration state. These animals were well hydrated and fasted for half a day before PCio study. All PCio procedures were performed around the same time.

**Single sampling method (PCio single):** The volume of distribution (Vd) of the marker at 120 min was calculated as the injected dose (Dose) divided by the plasma concentration at 120 min (C120); i.e., Vd = Dose/C120. A nonlinear regression analysis was performed using the unstandardized PCio (ml/min) and Vd with a linear quadratic model in the form of Cl = aVd² + bVd + c. Cl was estimated from the linear quadratic equation and standardized to BSA.

**Statistics:** Statistical analysis was performed using commercial computer software (Dr. SPSS for Windows [SPSS Japan Inc.]). Descriptive statistics were calculated for the assay validation and were used to determine the reference range of PCio in dogs and cats. Linear regression analysis was used to assess the PCio value and the correlation between PCio 3samples and PCio 3samples in study 1 and between PCio 3samples and PCio single in study 2. Receiver operating characteristics (ROC) analysis was performed to assess the sensitivity and specificity of the single sampling methods for detection of decreasing GFR in dogs and cats. The values are presented as means ± SD. A \( P \) value of <0.05 was considered statistically significant.

### RESULTS

**Study 1**

**Assay validation:** The relationship between standard serum concentrations (ranging from 7.5 to 120 \( \mu g/ml \)) and theoretical measurement values was linear (\( R^2=0.999 \)). Dilutional linearity was not observed between 120 to 240 \( \mu g/ml \) and 1.87 to 3.75 \( \mu g/ml \) (Table 1). The intra-assay validation value for measurement of 3.75 \( \mu g/ml \) [the CV: 13.8%] was low compared to that for measurement of 7.5 \( \mu g/ml \) [the CV: 4.4%]. The maximum and minimum detection limits of this method for measuring plasma iodine concentration were considered to be 120 and 7.5 \( \mu g/ml \), respectively. Thus, plasma samples with iohexol iodine concentrations > 120 \( \mu g/ml \) were diluted with appropriate amounts of plasma obtained from the same animal before iohexol injection.

In the intra-assay validation, the CV was 3.28 % for PCio values from 5 control dogs (61.61 ± 1.94 ml/min/m²). In the inter-assay validation, the CV of the 10 measurers was 3.17% for PCio values from 1 healthy dog (54.22 ± 1.72 ml/min/m²).

**Comparison of 2CM and 1CM:** The PCio values determined from PCio 9samples and PCio 3samples were 51.39 ± 8.70 (range, 33.19 to 68.99) and 56.19 ± 10.42 (range, 36.09 to 79.71) ml/min/m², respectively. PCio 3samples was significantly higher than PCio 9samples (P<0.001). The \( R^2 \) value for the relationship between PCio 9samples and PCio 3samples was 0.918 (P<0.001) (Fig. 1). Mean difference values and limits of agreement were calculated to represent the difference between the 2 methods. Mean difference values were 4.80 ± 3.68 ml/min/m² and the limits of agreement were –0.97–11.05 ml/min/m².

To assess the adequacy of the BM formula for dogs, a nonlinear regression analysis was performed between the unstandardized PCio 9samples (ml/min) and PCio 3samples (ml/min). The equation for the regression line was \( y = 0.0087x^2 + 0.5966x + 8.9646 \), where \( x \) is the unstandardized PCio 9samples (ml/min) and \( y \) is the unstandardized PCio 3samples (ml/min). PCio 3samples (ml/min/m²) calculated using this original formula were significantly correlated with that

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<th>Dilution (%)</th>
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The unit of data: \( \mu g/ml \).
using the BM formula ($R^2=0.960$, $P<0.001$). In all 37 dogs, PCio\textsubscript{3} samples by the BM formula were higher than that by the original formula, especially at high PCio values (Fig. 2).

**Study 2**

**Reference range of PCio\textsubscript{3} samples:** In 100 control dogs, the PCio value was $74.09 \pm 16.85$ (range, 46.62 to 109.17) ml/min/m$^2$, and in 36 control cats, the PCio value was $51.66 \pm 8.50$ (range, 37.19 to 96.72) ml/min/m$^2$. As these data showed a log-normal distribution, the reference ranges were calculated by the logarithmic transformation of the data (mean $\pm$ 2SD). The reference ranges for dogs and cats were 40.59 to 106.54 and 36.14 to 71.12 ml/min/m$^2$, respectively. Therefore, PCio values < 40 for dogs and < 35 ml/min/m$^2$ for cats were considered as decreased GFR in this present study.

**Comparison of PCio\textsubscript{3} samples and PCio\textsubscript{single}:** In all 779 dogs, the PCio value determined by the limited sampling method was $39.13 \pm 23.75$ (range, 3.78 to 142.88) ml/min/m$^2$. The linear quadratic regression analysis between Vd and the unstandardized PCio determined by the limited sampling method was obtained from the following quadratic equation:

$$PCio\textsubscript{single} = -0.0012Vd^2 + 2.5964Vd - 0.1057$$

In all 339 cats, the PCio value determined by limited sampling method was $32.13 \pm 17.34$ (range, 3.60 to 118.22) ml/min/m$^2$. The linear quadratic regression analysis between Vd and the unstandardized PCio determined by the limited sampling method was obtained from the following quadratic equation:

$$PCio\textsubscript{single} = -0.0844Vd^2 + 3.1788Vd - 0.6042$$

PCio\textsubscript{single} was significantly correlated with PCio\textsubscript{3} samples in dogs and cats (dogs: $R^2=0.985$, $P<0.001$, cats: $R^2=0.986$, $P<0.001$) (Fig. 3), respectively.

The mean difference between PCio\textsubscript{3} samples and PCio\textsubscript{single} in all dogs was $0.144 \pm 3.057$ ml/min/m$^2$, and the limits of agreement given by $\pm 2SD$ were $-5.969$ and $6.258$ ml/min/m$^2$. 

![Fig. 1. The correlation between PCio\textsubscript{3} samples and PCio\textsubscript{3} samples in 37 dogs.](image1)

![Fig. 2. The relationship between PCio\textsubscript{3} samples by the use of the original equation and that by the use of the BM formula.](image2)

![Fig. 3. The correlation between PCio\textsubscript{3} samples and PCio\textsubscript{single} in dogs (A) and cats (B).](image3)
In 455 CKD dogs with decreased PC\text{io}_3\text{samples} (< 40 ml/min/m\text{^2}), the mean difference between PC\text{io}_3\text{samples} and PC\text{io} single was 0.288 ± 2.562 ml/min/m\text{^2}, and the limits of agreement given by ±2SD were −4.835 and 5.412 ml/min/m\text{^2}. In 324 dogs with normal PC\text{io}_3\text{samples} (> 40 ml/min/m\text{^2}), the mean difference between PC\text{io}_3\text{samples} and PC\text{io} single was −0.059 ± 3.635 ml/min/m\text{^2}, and the limits of agreement given by ±2SD were −7.329 and 7.211 ml/min/m\text{^2}.

The mean difference between PC\text{io}_3\text{samples} and PC\text{io} single in all cats was 0.053 ± 2.032 ml/min/m\text{^2}, and the limits of agreement given by ±2SD were −4.011 and 4.118 ml/min/m\text{^2} (Fig. 4-B). In 211 CKD cats with decreased PC\text{io}_3\text{samples} (< 35 ml/min/m\text{^2}), the mean difference between PC\text{io}_3\text{samples} and PC\text{io} single was 0.085 ± 1.753 ml/min/m\text{^2}, and the limits of agreement given by ±2SD were −3.421 and 3.590 ml/min/m\text{^2}. In 128 cats with normal PC\text{io}_3\text{samples} (> 35 ml/min/m\text{^2}), the mean difference between PC\text{io}_3\text{samples} and PC\text{io} single was 0.002 ± 2.430 ml/min/m\text{^2}, and the limits of agreement given by ±2SD were −4.859 to 4.862 ml/min/m\text{^2}.

In order to evaluate the diagnostic accuracy of PC\text{io} single by ROC analysis, all dog and cats were classified as normal GFR or decreased GFR. PC\text{io} single was normal (> 40 ml/min/m\text{^2}) in 8 (1.8%) of 455 dogs with decreased PC\text{io}_3\text{samples}, and was decreased (<40 ml/min/m\text{^2}) in 24 (7%) of 324 dogs with normal PC\text{io}_3\text{samples}. PC\text{io} single was normal (> 35 ml/min/m\text{^2}) in 4 (1.9%) of 211 cats with decreased PC\text{io}_3\text{samples}, and was decreased (< 35 ml/min/m\text{^2}) in 9 (7%) of 128 cats with normal PC\text{io}_3\text{samples}. The sensitivity and specificity of the ROC analysis in dogs were 98 and 93%, respectively, and the AUC was 0.995 (standard error [SE], 0.003). In cats, the sensitivity and specificity were 98 and 93%, respectively, and the AUC was 0.993 (SE, 0.003) (Fig. 5).

DISCUSSION

Iohexol distribution in the body is the extracellular volume, and can be described with a 2CM, including the plasma as the 1st compartment with the interstitial space as
the 2nd compartment [26]. The 2CM is the standard method for calculating the AUC, but collecting a lot of plasma samples may lay a burden on the patients. To apply PCio to more patients, a limited sampling method would be preferable in a clinical setting. In study 1, PCio 3samples by the BM were significantly higher than PCio 9samples (P<0.001). This mean difference might indicate that the 1CM showed a poor correlation with 2CM. However, PCio 3samples by the BM was significantly correlated with PCio 9samples (R²=0.918, P<0.001). These results have showed that PCio 3samples could be available as an alternative method to 2CM, but that reference ranges are necessary to define each method individually. In study 2, the reference ranges of PCio 3samples were obtained from dogs and cats under the same conditions. The mean PCio 3samples in control dogs in study 1 and 2 showed significantly different results (P<0.05). Many studies also showed that the range of GFR values is quite wide in healthy dogs. This may be caused by the variability of GFR in healthy animals which have the large functional reserve of the kidneys. In addition, GFR may be affected by several nonrenal factors such as diet, environment, hydration state, glomerular marker measurement method, and the type of a pharmacokinetic model using to calculate the AUC. Therefore, the reference ranges for the CI should be established in each laboratory. Actually, determination of the reference range may need to consider age, body weight, and breed, because these factors may also affect GFR [3, 19].

In limited sampling method, AUC is generally determined by the modified 1CM using the BM formula. In this study, the differences between PCio 3samples by the BM formula and the original formula were presented, as in other study [3]. PCio 3samples by the BM formula may overestimate GFR in dogs, especially in the individuals with higher PCio value. However, as the equation was obtained from only the healthy dogs, this study could not show whether this equation is appropriate in the dogs with decreased GFR. Further study is needed to evaluate the correction formula for limited sampling method in both dogs and cats.

Iohexol is a nonionic radiographic contrast agent and has been demonstrated to be an ideal GFR marker. Its protein binding is < 1%, and almost 100% is eliminated unmetabolized in the urine 24 hr after an intravenous administration [26]. Plasma iodine content can be measured by the X-ray fluorescence method [6, 7], high performance liquid chromatography [13], the ceric arsenite method [10, 21], and capillary electrophoresis [29]. The X-ray fluorescence method is a simple and accurate method, but is requires a higher iohexol dosage (300 mg iodine/kg) and larger sample volume (3 mℓ of plasma) [6]. The ceric arsenite method used in the present study is labor intensive, but allows lower dosage and only requires a small sample volume. In this study, the errors caused by intra or inter-assay validations in the ceric arsenite method for plasma iodine measurement were considered to be acceptable. However, plasma samples with iohexol iodine concentrations > 120 µg/ml were required to be diluted with appropriate amounts of plasma obtained from the same animal before iohexol injection because of the measurement range of 7.5 to 120 µg/ml. Therefore, the risk of error from dilution of the samples was present, especially in dogs and cats with severely decreased GFR. Finco et al. [10] considered the accuracy of PCio method using a ceric arsenite method and modified 1CM in dogs. In our study, the accuracy of the PCio method using same procedures might need to be confirmed in dogs, because the dosage in our study was different from that in their study [10]. In cats, Miyamoto [21] considered the accuracy of the PCio method using the same procedures including the same dosage and sampling times. For this reason, the accuracy of the PCio method in cats was not evaluated in the present study.

PCio is a practical method in a clinical setting because the clearance value can be determined using the 1CM, and the number of samples collecting from the animal can be reduced [6, 10, 14, 18, 21, 34]. The owners of the patients have discretion as to whether the test is performed. Therefore, a simpler, but accurate method is needed for clinical settings. The single sampling method is the simplest way of measuring plasma clearance. In the present study, PCio estimated by a single sample was made available for detection of decreased GFR in dogs and cats. The utility of the single sampling method has been reported in dogs, cats, and humans [1, 17, 28, 31, 33]. Goy-Thollot et al. [17] showed that PCio determined by a single sample was significantly correlated with PCio determined by the 2 CM (10 samples) in dogs and cats. The single sampling method was calculated using the regression equation obtained from nonlinear regression analysis. Therefore, many samples are necessary to improve the accuracy of the single sampling method. In the present study, the numerous animals (779 dogs and 339 cats) used may provide the requisite accuracy for the regression equation to calculate PCio single. Some studies using iohexol and 99mTc-DTPA showed that optimal sampling times were similar in dogs (120 min) and cats (80 or 90 min) [1, 17]. In another study using 51Cr-EDTA in cats [33], the optimal sampling time was 48 min. In the present study, the assessment of optimal sampling time for determination of PCio single could not be performed, because a limited sampling method was used to collect a higher number of cases. The accuracy of this method in dogs with normal GFR was lower than that in dogs with decreased GFR. The dogs with higher PCio values showed a greater difference between PCio 3samples and PCio single. This difference could be caused by measurement error, because plasma iohexol concentrations in the dogs with higher PCio have very low. Alternatively, this might be the result of the overestimation of PCio by the use the BM formula. However, this did not become a major problem for the diagnosability of PCio single for detection of decreased GFR. The accuracy of this method in cats made little difference between the normal GFR and decreased GFR groups.

Finally, iohexol has a potential nephrotoxic effect. Iodine containing contrast agents have been reported to cause adverse reactions, and potential causes have been investigated in dogs [30]. Acute nephrotoxicity of water soluble
iodinated contrast media has been reported in humans [22]. A nonionic low osmolar contrast media including iohexol has few adverse reactions in human, and most adverse effects were immediate or delayed allergic reaction [27]. No adverse reaction was observed in other studies using iohexol for GFR estimation in dogs and cats [3, 6, 10, 12–14, 16, 17, 19, 21, 34]. In the present study, immediate or delayed allergic reactions were not observed for 12 hr after the iohexol injection. In addition, no animals showed the signs of acute renal failure, such as hypouria, rapid worsening of azotemia for 12 hr after the iohexol injection. No temporary adverse effects were clinically evident in dogs and cats in this study. The measuring reagent waste should be disposed of according to the Wastes Disposal and Public Cleansing Act, because the working solution contains arsenite.

In conclusion, this study presented the regression equation for estimation from the single sample using many dogs and cats. PCiO single obtained by this equation should be useful to detect decreased GFR in dogs and cats. The variation of the Cl values in healthy animals is large because of several nonrenal factors. To assess precisely GFR by the use of the Cl methods, further study is necessary to establish the reference ranges based on age, body weight, or breed.

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