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Quantitative Assessment of Hepatic Function by Means of ^{99m}Tc-Mebrofenin in Healthy Horses

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^{99m}Tc-mebrofenin is used in humans and small animals to assess hepatic function. This study was undertaken to measure hepatic clearance of ^{99m}Tc-mebrofenin in healthy horses and to determine whether feed deprivation and increased serum total bilirubin (TBIL) concentration alter ^{99m}Tc-mebrofenin clearance. Plasma clearance of ^{99m}Tc-mebrofenin was determined in 7 healthy horses at 0, 48, and 96 hours of feed withholding. Serum TBIL and nonesterified fatty acid (NEFA) concentrations were measured every 24 hours. ^{99m}Tc-mebrofenin (4.16 ± 0.62 mCi, mean ± SD) was injected into a jugular vein, and blood samples were retrieved from the contralateral jugular vein. A plasma time-activity curve of ^{99m}Tc-mebrofenin was generated, from which the area under the curve (AUC) and the $T_{½}$ of the fast-phase ($T_{½f}$) and slow-phase ($T_{½s}$) were calculated. Mean ± SD AUC was 17,700 ± 4,257, 18,616 ± 8,078, and 16,168 ± 6,031 counts per minute (cpm) at 0, 48, and 96 hours, respectively; mean ± SD $T_{½f}$ was 2.80 ± 0.38 minutes, 3.52 ± 1.46 minutes, and 3.82 ± 1.29 minutes at 0, 48, and 96 hours, respectively; median $T_{½s}$ was 63.9, 49.2, and 45.8 minutes at 0, 48, and 96 hours, respectively. No difference was detected between the values of AUC, $T_{½f}$, and $T_{½s}$ at 0, 48, and 96 hours. There was a significant increase in TBIL with fasting, with a mean ± SD of 6.3 ± 1.3 mg/dL at 96 hours. NEFAs increased, reaching a plateau at 48 hours (650 ± 152 μmol/L). Plasma TBIL concentrations did not correlate with AUC or $T_{½s}$ but correlated weakly with $T_{½f}$ ($r = 0.50$). Plasma NEFA concentrations did not correlate with AUC, $T_{½s}$, or $T_{½f}$ values. This study suggests that ^{99m}Tc-mebrofenin plasma clearance is not affected by feed withholding and that hyperbilirubinemia associated with feed withholding does not affect the hepatic extraction efficiency of this radiopharmaceutical.

Key words: Bilirubin; Hepatobiliary scintigraphy; Iminodiacetic acid; Iminodiacetic acid derivates.

Hepatic failure often remains undetected in horses until >60% of liver function is lost.[1] In one retrospective study, 22 of 44 horses with biopsy-confirmed liver disease had no clinical signs suggestive of liver disease at the time of examination.[1] This finding emphasizes the importance of early and accurate assessment of hepatic function in horses. Serum gamma-glutamyl transferase (GGT) activity is routinely measured in equine blood and can be used to screen horses for subclinical liver disease.[2] Increased serum sorbitol dehydrogenase (SDH), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities may also alert the clinician to the presence of liver disease.[3] Readily available measures of liver function include serum bile acid (SBA) and total bilirubin (TBIL)[3] concentrations. Increased TBIL concentrations are detected in horses suffering from liver disease, but unconjugated bilirubin also accumulates in the blood as a result of hemolysis or feed deprivation.[3] Furthermore, one study showed that serum TBIL concentrations were increased in only 8 of 34 horses with liver disease,[4] which suggests that the sensitivity of this test is low. In contrast, an increase in SBA concentration >20 μmol/L is both a specific and sensitive indicator of hepatic dysfunction in horses with clinical liver disease.[4,5] Bromosulphthalein (BSP) clearance tests have also been performed in the past to measure liver function in horses, but BSP is no longer available for purchase, and >50% of liver function must be lost before the plasma half-life of this substance is prolonged.[3]

Interpretation of blood TBIL concentrations in horses is complicated by hemolysis and anorexia-associated hyperbilirubinemia.[3,6] Hemolysis can be recognized by either the presence of free hemoglobin in the serum or urine or by anemia. Blood unconjugated bilirubin concentrations rise when horses do not eat, and concentrations increase in proportion to the number of days spent without feed.[3,6] Anorexia-associated hyperbilirubinemia has been attributed to a rise in nonesterified fatty acid (NEFA) concentrations[6,7] that occur in response to negative energy balance. Unconjugated bilirubin and NEFA blood concentrations are positively correlated in feed-deprived horses, suggesting that these metabolites compete for a common pathway into the liver.[6,7] Feed deprivation also raises SBA concentrations in ponies and horses.[8,9] SBA concentrations increased by 72% and reached a mean value of 22 μmol/L after healthy ponies were deprived of feed for 3 days.[8] In horses, a 3-fold increase in SBA concentrations was detected after 2–4 days of feed deprivation, but mean concentrations remained <20 μmol/L.[9] Because anorexia alters both TBIL and SBA concentrations, effects of feed deprivation must be assessed as novel methods for evaluating liver function in horses are being developed.

Quantitative hepatic scintigraphy with iminodiacetic acid derivates (IDAs), most commonly ^{99m}Tc-mebrofenin or ^{99m}Tc-disofenin, is routinely used in humans to evaluate liver function[10–13] and has been described in dogs and cats.[14–17] After IV injection, these organic ionic tracers are rapidly bound to plasma proteins (mainly albumin) and circulate to the liver, where they dissociate from their binding proteins and are taken up by the hepatocytes through a carrier-mediated, nonsodium-dependent membrane transport mechanism. This transport mechanism is also shared by bilirubin and, as serum bilirubin rises, competition for a limited num-

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ber of carrier molecules ensues. This competition induces a decrease in radiopharmaceutical uptake by the hepatocytes, which is greater for disofenin than for mebrofenin.[10,18] For this reason, in human medicine, ^{99m}Tc-mebrofenin is the preferred radiopharmaceutical in patients with serum bilirubin concentrations >10 mg/dL.[19]

Normal hepatocytes extract ^{99m}Tc-mebrofenin from the blood rapidly and with high efficiency. The normal hepatic extraction efficiency of ^{99m}Tc-mebrofenin in dogs is 92.2 ± 4.8% (mean ± SD).[9] When hepatic function is diminished, however, the liver cannot completely extract the radiopharmaceutical from the blood, which results in prolonged blood pool activity. This is reflected by a decrease in hepatic extraction, which is proportional to the degree of hepatic damage.[20] Image-based and plasma-based methods have been reported both in humans and small animals.[10,14,16,17,20] Plasma-based methods measure the rate of clearance of the radiopharmaceutical from the blood from sequential venous blood samples. Evaluating the plasma clearance of ^{99m}Tc-mebrofenin after a peripheral injection is a simple, noninvasive, convenient method to measure hepatocellular function that can be carried out without a gamma camera.

This study was undertaken to measure the clearance of ^{99m}Tc-mebrofenin from the blood in healthy, fed horses and to determine whether feed deprivation alters the hepatic clearance of ^{99m}Tc-mebrofenin.

Materials and Methods

Animals

Seven horses donated to the University of Tennessee Veterinary Teaching Hospital for reasons other than hepatic disease were used for both the fed and feed withholding parts of the study. All horses underwent baseline tests, including a physical examination, a serum biochemical analysis, and a CBC. Criteria for inclusion in the study were that each horse had (1) no history of liver disease, (2) normal physical examination parameters, and (3) plasma concentrations of TBIL and GGT that were within reference ranges for our laboratory. Horses were housed in facilities at the University of Tennessee, and a Protocol for the Use of Live Vertebrates was approved by the Institutional Animal Care and Use Committee at the University of Tennessee before undertaking the study.

Plasma Clearance Studies

Fourteen-gauge polypropylene catheters were inserted into the right and left jugular veins, and plasma ^{99m}Tc-mebrofenin clearance studies were performed at 0 hour and then again after 48 and 96 hours of feed deprivation. Each plasma clearance study was performed as follows: ^{99m}Tc-mebrofenin was injected in 1 jugular catheter IV, and blood samples were collected from the controlateral jugular vein at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 minutes after radiopharmaceutical administration. The administered dose of radiopharmaceutical was calculated by subtracting the counts in the syringe after injection from the counts preinjection, and performing decay correction. After completion of the sample collections for the 1st plasma clearance study, horses were denied food for 96 hours. Water was provided at libitum during the period of feed deprivation. Horses were monitored throughout the study for clinical signs consistent with hyperlipemia, including lethargy, weakness, and neurologic signs. Blood was collected every 24 hours throughout the 96-hour fasting period to monitor TBIL and NEFA concentrations.

Analysis of Plasma NEFA and TBIL Concentrations

Plasma NEFA concentrations were measured with an in vitro enzymatic colorimetric test kit[a], with acyl CoA synthetase, acyl CoA oxidase, and ascorbate oxidase reactions and a microtiter plate reader.[b] TBIL was measured in plasma with an in vitro enzymatic colorimetric test kit[c] that used sulfanilic acid and sodium nitrite reactions. An automated discrete analyzer[d] was used for TBIL measurements.

Analysis of the Plasma Disappearance Curve

Plasma was separated within 1 hour of collection, aliquots were obtained, and weight was determined with an analytic balance. One milligram of plasma was considered to have a volume of 1 mL; thus, activity was expressed as counts/mL of plasma. Aliquots were counted in a 10-mm-diameter sodium iodide well detector interfaced with a multichannel analyzer.[e] A window was set over the 140 keV of photopeak of technetium-99m. The samples were decay corrected to the time the 1st sample was counted. A time-activity curve that represented the plasma disappearance of ^{99m}Tc-mebrofenin was created. This time-activity curve was normalized so that the 1-minute sample had a value of 100,000 counts/mL. This normalization has been previously described and is performed to standardize the area under the curve (AUC) and take into account differences in ^{99m}Tc-mebrofenin dose, weight of the animal, and plasma volume of the animal, as well as day-to-day differences in the counting efficiency of the well counter.[16] A biexponential (two-compartment) model was used to fit the plasma disappearance curve by the equation $Y = K_1e^{-K_2t} + K_3e^{-K_4t}$ to obtain the regression coefficients K_1, K_2, K_3, and K_4 by dedicated software.[f] The AUC was calculated by substituting the calculated regression coefficients in the equation AUC = $(K_1/K_2) + (K_3/K_4)$. The $T_{½}$ of the fast (distribution and extraction, $T_{½f}$) and slow (elimination, $T_{½s}$) portions of the curve were calculated. $T_{½f}$ was calculated by the formula $T_{½f} = \ln(2)/K_2$. The $T_{½s}$ was calculated by the formula $T_{½s} = \ln(2)/K_4$.[16]

Statistical Analysis

A one-way analysis of variance was used to compare AUC and $T_{½f}$ at 0, 48, and 96 hours.[g] Comparison between $T_{½s}$ at the 3 time points was carried out by analysis of variance on ranks, because the data were not normally distributed.[g] The All Pairwise Multiple Comparison Procedure was used to compare TBIL and NEFA serum concentrations at 0, 24, 48, 72, and 96 hours.[g] Linear regression analysis was performed to evaluate the correlation between TBIL and NEFA serum concentrations and AUC, $T_{½f}$, and $T_{½s}$, respectively.[g] Level of significance was set at $P < .05$. Values are mean ± SD unless otherwise noted.

Results

Three geldings and 4 mares were used; ages ranged from 3 to 20 years (median, 4 years). No health complications arose as a result of feed deprivation or ^{99m}Tc-mebrofenin injections. Forty-eight–hour intervals between plasma clearance studies permitted clearance of the radiopharmaceutical from the patient.

Injected dose was 4.16 ± 0.62 mCi (154 ± 23 MBq). The time-activity curve illustrating the mean values of plasma disappearance of ^{99m}Tc-mebrofenin at 0 hour is shown in Figure 1. AUC values were 17,700 ± 4,257 counts per minute (cpm) at 0 hour, 18,616 ± 8,078 cpm at 48 hours, and 16,168 ± 6,031 cpm at 96 hours. AUC values did not differ significantly between 0-, 48-, and 96-hour curves ($P = .775$). The shape of the curve at 0 hour did not differ from that of the curves obtained at 48 and 96 hours (data not shown).

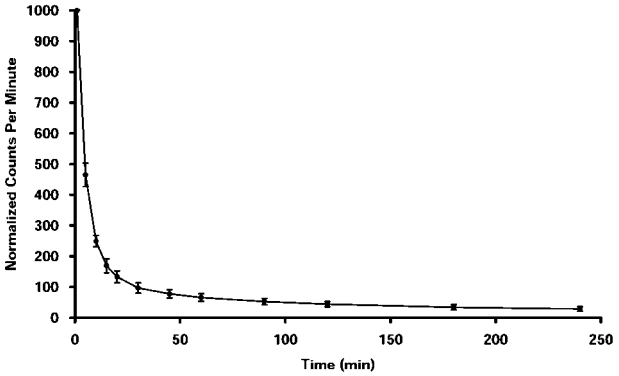

Fig 1. Plasma time-activity curve illustrating the disappearance of ^{99m}Tc-mebrofenin from blood. The curve has the characteristic of a two-compartment model, characterized by an initial rapid decline of the radiopharmaceutical from the blood (fast phase), followed by a slower decline (slow phase). The solid line represents the fitted average curve for the 0-hour samples; the squares represent averages, and the whiskers depict 1 standard deviation above and below the average. At 240 minutes, very little activity remains, indicating near-complete clearance of the radiopharmaceutical from the blood.

$T_{½f}$ values were 2.80 ± 0.38 minutes at 0 hour, 3.52 ± 1.46 minutes at 48 hours, and 3.82 ± 1.29 minutes at 96 hours. Median $T_{½s}$ values were 63.9 minutes (interquartile range, 52.7–69.0 minutes) at 0 hour; 49.2 minutes (interquartile range, 35.0–87.5 minutes) at 48 hours; and 45.8 minutes (interquartile range, 29.6–127.5 minutes) at 96 hours. $T_{½f}$ or $T_{½s}$ values did not differ significantly between 0, 48, and 96 hours ($P = .309$ and $P = .563$, respectively).

Plasma concentrations of TBIL (Fig 2) significantly increased ($P < .001$) as time progressed when horses were deprived of feed, and mean concentrations at 24, 48, 72, and 96 hours differed significantly from baseline values. Mean ± SD TBIL concentrations were 1.3 ± 0.3 mg/dL, 2.9 ± 0.6 mg/dL, 4.2 ± 0.6 mg/dL, 5.5 ± 1.0 mg/dL, and 6.3 ± 1.3 mg/dL at 0, 24, 48, 72, and 96 hours, respectively. Plasma NEFA concentrations increased significantly as time progressed ($P < .001$), reaching a plateau at 48 hours (Fig 3). Mean plasma NEFA concentrations at 48, 72, and 96 hours differed significantly from baseline values. TBIL concentrations detected during the study were positively correlated ($P < .001$) with plasma NEFA concentrations ($r = 0.77$).

Plasma TBIL concentrations were not significantly correlated with calculated AUC values ($r = 0.06$) or ^{99m}Tc-mebrofenin plasma $T_{½s}$ values ($r = 0.28$). However, there was a significant but weak correlation between plasma TBIL concentrations and $T_{½f}$ ($r = 0.50$; $P = .026$). Plasma NEFA concentrations were not significantly correlated with calculated ^{99m}Tc-mebrofenin plasma AUC ($r = 0.02$), $T_{½s}$ ($r = 0.01$), or $T_{½f}$ ($r = 0.30$).

A ^{99m}Tc-mebrofenin curve at 0 hour from 1 horse was excluded from the analysis because blood clotted within the sample collection catheter soon after injection. An attempt was made to use blood samples collected from the same catheter that ^{99m}Tc-mebrofenin was injected through, but residual radiopharmaceuticals interfered with results.

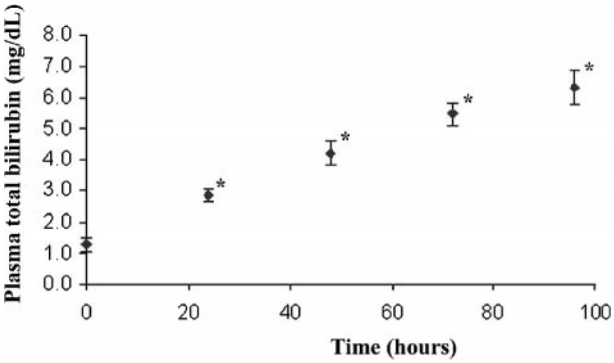

Fig 2. Plasma concentrations of blood total bilirubin (TBIL) as time progresses. Concentrations are expressed as average (squares), with the whiskers depicting 1 standard deviation above and below the average. Asterisks (*) mark the time points that are significantly different from baseline values (0 hour).

Discussion

Our results indicate that ^{99m}Tc-mebrofenin is a radiopharmaceutical that can be repeatedly administered to horses without apparent adverse effects. Techniques used in this study were simple to perform and provided consistent results. Use of this procedure will be limited to institutions that are approved to use radiopharmaceuticals and possess a well detector counter, but results suggest that ^{99m}Tc-mebrofenin plasma clearance studies serve as a useful measure of hepatic function in horses. Our results suggest that this test warrants further evaluation in horses with liver disease.

^{99m}Tc-mebrofenin was selected for this study because hepatic scintigraphy with this radiopharmaceutical is used to evaluate hepatobiliary function in humans with liver disease.[10,11,21] This technique provides information regarding hepatocellular function, as well as information regarding patency of the biliary tree. Hepatic scintigraphy with ^{99m}Tc-mebrofenin has also been described in dogs and cats.[14,15,17,20,22,23] Additionally, there is 1 report describing an image-based technique to evaluate the biliary kinetics of ^{99m}Tc-disofenin in horses.[24] However, the main limitation to the application of the classic, image-based, hepatic scintigraphy technique in horses is that the patient's size makes it

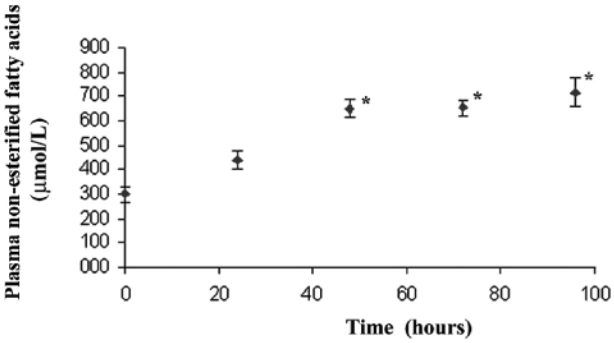

Fig 3. Plasma concentrations of nonesterified fatty acid (NEFA) as time progresses. Concentrations are expressed as average (squares), with the whiskers depicting 1 standard deviation above and below the average. Asterisks (*) mark the time points that are significantly different from baseline values (0 hour).

difficult to obtain adequate images of the liver. A plasma-based method to evaluate hepatic function has been described in normal dogs and in dogs with induced hepatic injury.[16] Rather than measuring ^{99m}Tc-mebrofenin accumulation in the hepatocytes, it measures the amount of radiopharmaceutical remaining in the blood by means of a curve plotting the disappearance of the radiopharmaceutical from the plasma. In the dog, this curve has the kinetics of a two-compartment model and is highly correlated with hepatic extraction efficiency. The plasma clearance technique is simple and noninvasive and can be performed without a gamma camera and without the need for deconvolution analysis.

In this study, we used the same technique described in dogs to evaluate the plasma disappearance of ^{99m}Tc-mebrofenin in normal horses. Effects of feed deprivation were also evaluated in this study because hyperbilirubinemia may be induced by anorexia, and IDA derivates are transported into the hepatocytes through the same membrane transport mechanism as bilirubin. As serum bilirubin concentrations rise, competition for a limited number of carrier molecules ensues, and more radiopharmaceutical remains in the plasma. However, ^{99m}Tc-mebrofenin has a greater resistance to displacement by serum bilirubin than other IDA derivates. In humans, there is normal hepatic extraction of ^{99m}Tc-mebrofenin in patients with serum bilirubin concentrations between 4.8 and 8.2 mg/dL.[20] The critical concentration of serum bilirubin has not been determined in dogs or cats. Results of this study indicate that ^{99m}Tc-mebrofenin clearance values are not affected by hyperbilirubinemia in horses, at least up to 8.5 mg/dL, which was the maximum plasma TBIL concentration recorded.

Feed deprivation raises plasma TBIL concentrations in horses and ponies as unconjugated bilirubin accumulates in the blood.[6,7,25–27] Complete anorexia can increase unconjugated bilirubin concentrations within 12 hours, and concentrations may reach 8 mg/dL as a result of anorexia alone.[3] Mechanisms responsible for these increases have not been determined, but it has been suggested that fatty acids compete with bilirubin for transport into the liver.[6,7] All of the horses of this study developed hyperbilirubinemia by 24 hours, as expected, with TBIL concentrations steadily rising to maximum mean concentrations of 6.3 mg/dL at 96 hours. The highest concentration recorded at 96 hours was 8.5 mg/dL. Plasma NEFA concentrations also increased with time in the 1st 48 hours until a plateau was reached. A similar plateau in plasma NEFA concentrations after 48 hours of feed deprivation was reported by Naylor et al.[7] Plasma concentrations of NEFA and TBIL were also positively correlated, which supports the theory that these metabolites compete for the same clearance pathways. A membrane-bound protein called organic anion transport protein 2 (OATP2) has been identified in human hepatocytes, and this protein facilitates movement of both organic acids and bilirubin into cells.[28,29] If OATP are also present in equine hepatocytes, then NEFA may compete with bilirubin for these transport proteins. Hyperbilirubinemia might also develop independently if feed deprivation triggers events similar to those associated with the inflammatory response. Inflammatory stimuli including lipopolysaccharide and interleukin-1 reduce bilirubin clearance from the blood and induce intrahepatic cholestasis in humans.[30]

This study showed that the plasma clearance curve of ^{99m}Tc-mebrofenin in normal horses resembles that described in dogs. The curve has the characteristics of a two-compartment model, in that there is an initial rapid decline of the radiopharmaceutical from the blood (fast phase), followed by slower decline (slow phase). The initial fast phase is due to the radiopharmaceutical distribution within the blood pool and its high extraction rate by the liver. The slower decline in the 2nd phase of the curve is due to lower concentrations of the radiopharmaceutical within the plasma. The equation representing plasma concentrations of a radiopharmaceutical with 2-compartment kinetics is as follows: $Y = K_1e^{-K_2t} + K_3e^{-K_4t}$, where Y is the plasma concentration of the radiopharmaceutical, K_2 and K_4 are the 1st-order rate constants, and K_1 and K_3 are the intercepts on the y-axis for the 1st and 2nd segments of the curve, respectively. These coefficients are important because they allow the calculation of parameters from the time-activity curve, which can be used to further define the pattern of clearance of the radiopharmaceutical from the blood. The area under the plasma time-activity curve is a measure of the amount of radiopharmaceutical in the systemic circulation.[31] The AUC can be used to predict hepatic extraction efficiency and provide a quantitative measure of hepatocellular function. In this study, there was no difference in the AUC values at the 3 time points during feed deprivation, despite rising TBIL and NEFA concentrations. Therefore, it does not appear that hyperbilirubinemia alone, up to a mean concentration of 6.31 mg/dL, interferes with ^{99m}Tc-mebrofenin hepatic extraction efficiency. The 2 other parameters calculated by the coefficients from the two-compartment kinetics equation, the $T_{½}$ of the fast (distribution and extraction) and slow (elimination) portions of the curve, did not differ at the 3 time points during the study. The AUC and $T_{½}$ values were not affected by variations in TBIL and NEFA concentrations. There was a weak but significant correlation between the $T_{½f}$ and TBIL, which likely reflects some degree of competitive inhibition between the radiopharmaceutical and TBIL. The degree of competitive inhibition from this concentration of hyperbilirubinemia does not appear to be great enough to significantly reduce the extraction efficiency of the radiopharmaceutical by the hepatocytes.

The concentration of hyperbilirubinemia needed to significantly reduce hepatic extraction efficiency in horses remains unknown, but it appears to be higher than that caused by feed deprivation alone. Results of this study validate the use of plasma clearance of ^{99m}Tc-mebrofenin to assess hepatic function in normal horses and indicate that increases in TBIL and NEFA concentrations do not alter the time-activity curve or its parameters. SBA concentrations were not measured in this study because healthy horses were assessed. Concentrations of SBA rise as liver function declines in horses.[9] However, SBA concentrations are not useful for detecting subclinical disease in horses suffering from pyrrolizidine alkaloid toxicosis,[2] and false-negative results have been detected in horses suffering from hepatic lipidosis.[5] Feed deprivation also raises SBA concentrations, but concentrations have remained close to 20 μmol/L in horses

and ponies that have been deprived of feed for 3–4 days.[3,8,9] Future studies should include the measurement of ^{99m}Tc-mebrofenin clearance values in horses with subclinical liver disease and comparisons with SBA concentrations.

Footnotes

[a] Colorimetric test kit, Wako Chemicals USA, Richmond, VA
[b] Universal microtiter plate reader, Bio-Tek Instruments Inc, Winooski, VT
[c] Colorimetric test kit, Roche Diagnostics Corporation, Indianapolis, IN
[d] Cobas Mira discrete analyzer, Roche Diagnostic Systems Inc, Somerville, NJ
[e] MAESTRO multichannel analyzer, Perkin Elmer Instruments, Oak Ridge, TN
[f] IgorPro software, WaveMetrix Inc, Lake Oswego, OR
[g] SigmaStat software, SPSS Inc, Chicago, IL

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