

## Evaluation of preprandial and postprandial serum bile acids and plasma ammonia concentrations in healthy dogs, and the effects of frozen storage on plasma ammonia concentrations\*

CORRESPONDENCE TO:  
Márcia Mery Kogika  
Departamento de Cirurgia  
Faculdade de Medicina Veterinária e Zootecnia da USP  
Cidade Universitária Armando de Salles Oliveira.  
Av. Orlando Marques de Paiva, 87  
05508-000 - São Paulo - SP.  
e-mail: mmkogika@usp.br

1-Departamento de Clínica Médica da Faculdade de Medicina Veterinária e Zootecnia da USP-SP

Avaliações dos níveis pré e pós-prandiais de ácidos biliares séricos e de amônia plasmática em cães hígidos e o efeito do tempo de congelamento do plasma nas concentrações de amônia\*

Márcia Mery KOGIKA<sup>1</sup>; Shinobu MATSUURA<sup>1</sup>; Mitika Kuribayashi HAGIWARA<sup>1</sup>; Regina Mieko Sakata MIRANDOLA<sup>1</sup>; Enrico Lippi ORTOLANI<sup>1</sup>

### SUMMARY

Preprandial and postprandial (2 and 4 hours) serum bile acids (SBA) and pre and postprandial (2 hours) plasma ammonia concentrations were evaluated. Additionally, the effects of freezing (for 24 and 48 hours at -20°C) were observed on plasma ammonia concentrations in 22 healthy dogs. The preprandial SBA concentration was  $2.1 \pm 0.3$  mmol/l and  $7.5 \pm 1.2$  mmol/l and  $7.8 \pm 1.4$  mmol/l for samples obtained 2 and 4 hours after feeding, respectively. Fasting and postprandial (2 hours) plasma ammonia concentrations were significantly different when measurement was performed within 30 minutes after blood collection ( $118.2 \pm 13.2$  mg/dl or  $67.3 \pm 7.5$  mmol/l and  $227.9 \pm 59.2$  mg/dl or  $129.9 \pm 33.7$  mmol/l), but the difference between pre and postprandial concentrations was not observed when ammonia was measured in samples stored (-20°) for 24 and 48 hours. Plasma freezing makes ammonia concentrations fall considerably when these levels were initially too high, mainly in postprandial samples. From these results it may be suggested that canine plasma cannot be stored for later ammonia determination by using freezing as the sole stabilizer, and for SBA determinations, blood samples might be collected 2 or 4 hours after feeding. Plasma ammonia values obtained in this study should allow comparisons to data obtained from dogs with hepatic disease or hepatoencephalopathy, so as to confirm the importance of its use as means of diagnosis and prognosis in future.

**UNITERMS:** Bile acids; Ammonia; Dogs.

### INTRODUCTION

**H**epatobiliary diseases, which can develop either as acute or chronic progressive hepatic diseases, are frequently diagnosed in dogs and cats. The most important etiologic agents involved are toxic substances (drugs, mycotoxins, heavy metals - for example hepatitis associated with copper in Bedlington terriers and Dobermans), ischemia (anaemia and congestive right heart

failure), bacteria (leptospirosis), vascular abnormalities (portosystemic shunt, hepatic cysts), virus (infectious hepatitis virus) and metabolic disorders (*diabetes mellitus*, hyperadrenocorticism and hypothyroidism)<sup>7,16</sup>.

Owing to the great storage capacity of the liver and the multiple functions performed by this organ, the early diagnosis of hepatobiliary diseases is still a challenge for clinicians being usually established when clinical signs as ascites and jaundice are noticed, because of extensive loss

\*This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, proc. 95/9417-1 and 95/9415-9)

of hepatic parenchyma. Other symptoms are not specific of hepatic diseases, thus making even more difficult the diagnosis of hepatic lesion or dysfunction<sup>7</sup>.

Laboratorial assays are the most important procedures to detect liver diseases. Nevertheless, as some dogs show minimal or none alterations in routinely used hepatobiliary screening tests, other tests such as serum bile acids (SBA) and plasma ammonia determinations had been suggested to help clinicians to evaluate liver function<sup>13</sup>.

Compared to other more traditional assays, serum bile acids determination can be considered one of the most important tests for the diagnosis of hepatobiliary disease, because of the high sensitivity and specificity. Bile acids are synthesized in liver from cholesterol, being later stored in gallbladder and released into the small intestine postprandially<sup>1,9,10,25</sup>.

Although it has been suggested that only postprandial SBA determination should be considered in the diagnosis of hepatobiliary diseases in dogs<sup>13,25</sup>, some authors recommend their determination in both, fasting and 2-hour postprandial samples<sup>8,9,10,18,19</sup>.

According to Center<sup>7</sup> and Meyer *et al.*<sup>21</sup>, plasma ammonia concentration is as important as SBA determination to detect hepatic dysfunction. Ammonia comes from protein degradation in colon, being considered one of the substances responsible for hepatic encephalopathy (HE) manifestation, a clinical syndrome characterized by abnormal mental status and impaired neurological function in animals that have advanced liver disease (acute or chronic) and/or severe portosystemic shunts<sup>6,7,24</sup>.

Due to great variability of plasma ammonia concentrations in dogs, even in fasting samples, many researchers recommended the ammonia tolerance test in which the plasma concentration of the substance is measured before and after administration of ammonium chloride (NH<sub>4</sub>Cl, 100 mg/kg) via stomach tube<sup>6,11,27</sup>. Slightly increased post-challenge blood ammonia levels are considered normal in healthy dogs, but markedly increased may indicate decreased functional ability owing to the reduction of hepatic parenchyma or lack of integrity of the portal blood supply due to portosystemic shunts.

As ammonia chloride may induce or enhance signs of HE in dogs, even in asymptomatic ones, the test should be taken carefully or, according to Dial<sup>13</sup>, should be replaced by 2-hour postprandial plasma ammonia determination, which is safer.

For evaluation of blood ammonia concentration, the enzymatic method has been routinely used, but now an easy

and feasible method, using a selective ion electrode is available<sup>14</sup>. It allows determining high levels of circulating ammonia (up to 100,000 mg/dl or 1,000 ppm) without the need of diluting the sample, making the method an ideal one for use in clinical routine.

Considering the high instability of ammonia, it is recommended to do its determination in fresh plasma within 30 minutes after blood collection<sup>6,17</sup>; although, according to some researchers<sup>5,22,27</sup>, the plasma could be stored at -20°C for 1 to 2 days without any alteration in ammonia concentration.

In this paper, the authors present the pre and postprandial values of SBA and ammonia, the latter measured by means of ion selective electrode method in healthy dogs. Beside this, the influence of frozen storage on ammonia levels of the sample, as well as the optimum postprandial time for blood collection for SBA analysis were studied.

## MATERIAL AND METHOD

**Dogs** - Twenty-two healthy mixed-breed dogs, both sexes, aged 2 to 6 years, from Experimental Animal Holding Facility of Departamento de Clínica Médica / Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo or housed dogs were used for this study. Health was assessed by history, clinical examination and confirmed by routine biochemical liver functions tests (alanine transaminase, alkaline phosphatase, total protein and albumin).

**Samples** - Blood was collected, via venipuncture, from each dog after a 12-hour fast and 2 hours postprandially\*, another postprandial sample (4 hours after feeding) was obtained for serum bile acids determination. Serum for bile acids determination was separated immediately after blood collection and stored at -20°C.

For ammonia measurement, blood was collected into heparinized glass vacuum tubes, fasting and 2-hour postprandial samples, centrifuged at 2,000g for 10 minutes, and the plasma separated (3 aliquots) and ammonia determination made within 30 minutes; two other aliquots were immediately stored at -20°C.

**Serum bile acids (SBA) determination** - Serum bile acids concentrations were obtained by use of commercial colorimetric enzymatic method - Enzibile® Prod. n°. 1002682/ Standards Prod. n°. 1002657 (NYCOMED PHARMA AS).

**Plasma ammonia determination** - Plasma ammonia concentration was determined in fresh plasma sample (kept

\*Commercial canned food (Pedigree-chicken flavour), 250 gm/15 kg BW (supplied by Éffem Produtos Alimentícios/Waltham).

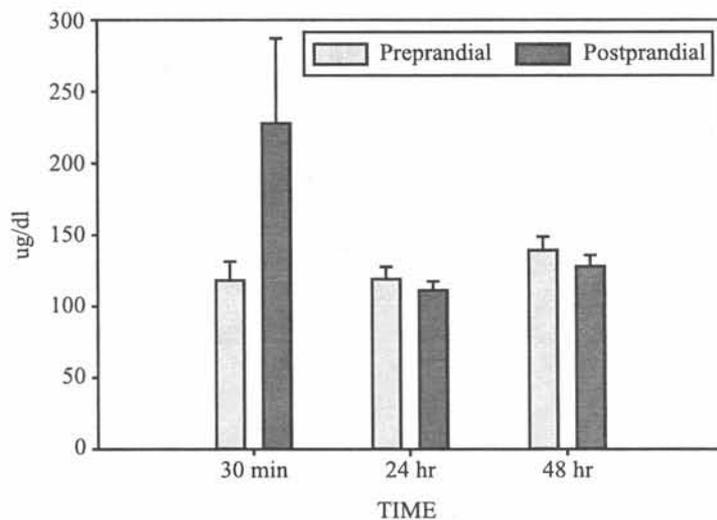
in ice bath) obtained within 30 minutes after blood collection, and in samples stored at  $-20^{\circ}\text{C}$  for 24 and 48 hours, fasting and postprandial blood samples, using a specific ion electrode method, as referred by Attili *et al.* Ammonia was measured by use of Orion<sup>®</sup> electrode - model 95-12; briefly, 1 ml of plasma sample was diluted with 2 ml of pH 12 buffer solution (Titrisol n<sup>o</sup>. 9892 Merck), homogenized with magnetic stirrer, and the mV reading were measured with SA 720 Meter (Procyon). Standard solutions (0.1 to 1,000 ppm of ammonia) were diluted with pH 10.5 buffer solution (8.1 parts of pH 10 buffer Titrisol n<sup>o</sup>. 9890 + 1.9 parts of pH 12 buffer Titrisol n<sup>o</sup>. 9892), processed likewise and the results in mV were plotted in monolog paper. Ammonia concentration was calculated according to standard logarithmic curve referred above.

**Statistical analysis** - To assess whether there is a difference between pre and postprandial SBA concentrations, and also 2 and 4 hours after feeding SBA values, paired Student *t* test was conducted at  $p < 0.05^{**}$ . Analysis of variance (ANOVA) was used to evaluate difference between plasma ammonia concentrations obtained 30 minutes after venipuncture (controls) and aliquots stored at  $-20^{\circ}\text{C}$  for 24 and 48 hours. This test was followed by Dunnett Multiple Test, and significance was considered at a level of  $\alpha = 5\%$ . In order to detect a difference between pre and postprandial plasma ammonia levels, paired Student *t* test was also conducted at  $p < 0.05$ .

## RESULTS

Mean and standard error mean values of SBA concentration were  $2.1 \pm 0.3$  mmol/l (preprandial),  $7.5 \pm 1.2$  mmol/l and  $7.8 \pm 1.4$  mmol/l (postprandial, for samples obtained 2 and 4 hours after food ingestion, respectively) (Tab.1). Analysis of data showed significant difference between mean pre and postprandial SBA concentrations. However, no difference was detected between postprandial SBA values (2 and 4 hours after feeding).

Fasting and postprandial plasma ammonia concentrations that were measured within 30 minutes after venipuncture are presented in Tab. 2. Fasting plasma ammonia concentrations ranged from 74 to 350 mg/dl ( $118 \pm 13.2$  mg/dl) and rose to values ranging 60 to 1,100 mg/dl ( $227.9 \pm 59.2$  mg/dl) for samples collected 2 hours after feeding, with significant difference between pre and postprandial plasma ammonia concentrations.



**Figure 1**

Variations in plasma ammonia concentrations (preprandial and postprandial samples) determined within 30 minutes after collection and in samples stored for 24 and 48 hours ( $-20^{\circ}\text{C}$ ) from healthy dogs.

**Table 1**

Pre and postprandial (2 and 4-hr) serum bile acids concentrations (mmol/l) in healthy dogs. São Paulo, 1997.

	Bile Acids (mmol/l)			
	Preprandial	2-hr Postprandial	4-hr Postprandial	
Values				
mean	2.1*	7.5	7.8	
SD	1.5	5.5	6.5	
SEM	0.3	1.2	1.4	
maximun	6.2	21.2	22.8	
minimun	0.3	0.3	1.9	

SD = standard deviation;

SEM = standard error mean;

\* = significantly different from the postprandial (2 and 4-hr) mean values ( $p < 0.05$ , paired *t* test).

Pre and postprandial plasma ammonia concentrations, after plasma storage at  $-20^{\circ}\text{C}$  for 24 and 48 hours, are also showed in Tab. 2. There was a significant difference between pre and postprandial concentrations when measurement was performed 30 minutes after venipuncture, but no difference was observed when samples were stored for 24 and 48 hours, showing that plasma storage, even at  $-20^{\circ}\text{C}$ , makes ammonia concentrations fall considerably when these levels are high.

**Table 2**

Pre and postprandial plasma ammonia concentrations (mg/dl) in samples determined within 30 minutes after blood collection and in samples stored for 24 and 48 hour at -20°C. São Paulo, 1997.

Storage	Plasma ammonia (µg/dl)					
	Preprandial			Postprandial		
	30 min	24hr	48hr	30 min	24hr	48hr
Mean	118.2 <sup>*</sup>	119.0	139.3	227.9 <sup>**</sup>	111.1	127.9
SD	62.2	40.8	44.0	277.7	30.0	37.8
SEM	13.2	8.7	9.4	59.2	6.4	8.0
Maximum	350	210	240	1100	160	205
Minimum	74	60	62	60	60	66

SD = standard deviation;

SEM = standard error mean;

<sup>\*</sup> Significant difference from the postprandial mean value obtained within 30 minutes after collection (p<0.05, paired t test);

<sup>\*\*</sup> Significantly different from the means observed in samples stored for 24 and 48 h -postprandial (ANOVA - Dunnett Multiple Test,  $\alpha = 5\%$ ).

To convert mg/dl to mmol/l, multiply by 0.57<sup>5</sup>

Fig. 1 shows the mean pre and postprandial plasma ammonia concentrations.

## DISCUSSION

Preprandial SBA concentrations found in this study were significantly different from postprandial SBA values; similar results had been described by Counsell; Lumsden<sup>12</sup> and Jensen<sup>18</sup>. Postprandial SBA concentrations varied more (0.3 mmol/l to 21.2 mmol/l) than preprandial concentrations (0.3 mol/l to 6.2 mmol/l), which could be explained by different bile acids absorption peaks and quantity of food ingested and digestive process in general<sup>3,23,26</sup>. Due to these variations, Beckett *et al.*<sup>3</sup> and Washizu *et al.*<sup>26</sup> recommend that postprandial determination should be made within 8 hours after feeding.

In our study significant difference between values obtained from samples collected 2 and 4 hours after feeding was not observed, allowing to the conclusion that the conventional collecting time (2 hours after feeding) might be considered suitable for routine bile acids determination. Jensen<sup>18</sup> observed similar results with determinations made between 2 and 6 hours after feeding.

Preprandial plasma ammonia concentrations, obtained in fresh samples and determined by specific ion electrode method, were similar to those described by Hardy<sup>15</sup>; Bunch<sup>4</sup> and Willard; Twedt<sup>27</sup>. Postprandial values were significantly different from preprandial ones, but now because of the paucity of information in the literature and the high variability among the samples collected after 2 hours of the meal, the meaning and usefulness of pre and postprandial sampling for ammonia determination are still to be cleared.

Due to plasma ammonia instability, the authors recommended its determination within 30 minutes after venipuncture<sup>13,17,20</sup>, although other researchers did not observe difference in values obtained from plasma stored at -20°C until 48 hours after venipuncture<sup>4,5,22,27</sup>.

Concerning canine species, data obtained in this study demonstrated that plasma storage at -20°C did not modify preprandial low plasma ammonia values measured 30 minutes, 24 and 48 hours after venipuncture. However, as to postprandial ammonia levels from samples stored at -20°C for 24 and 48 hours there was a significant difference among values obtained in the same samples, when they were evaluated at 30 minutes, 24 and 48 hours after venipuncture. Same results had been related by Hitt; Jones<sup>17</sup> and Dial<sup>13</sup>, which explained that ammonia concentrations might decrease during storage due to vaporous loss as equilibrium is established between aqueous and gaseous phases. These results suggest that measurement should be made within 30 minutes after blood collection, at least when plasma levels are elevated.

Therefore, it can be recommended that, for SBA determinations, blood samples should be collected 2 or 4 hours after feeding and, for plasma ammonia determinations, measurement should be made as soon as possible after venipuncture (within 30 minutes).

Regarding to pre and postprandial plasma ammonia determinations, values obtained in this study should be compared to those obtained in animals with hepatic disease or HE, so as to confirm the importance of its use as means of diagnosis in future.

## CONCLUSIONS

- Postprandial SBA evaluation can be performed indifferently 2 or 4 hours after feeding;
- Plasma ammonia determination should be made within 30 minutes after venipuncture.

## RESUMO

Foram avaliadas as concentrações pré e pós-prandiais de ácidos biliares séricos (2 e 4 horas) e de amônia plasmática (2 horas) em vinte e dois cães hígidos. O efeito do tempo de armazenamento (à temperatura de  $-20^{\circ}\text{C}$ ) do plasma sobre as concentrações de amônia também foi estudado. A média e o erro padrão da média em relação aos valores pré-prandiais de ácidos biliares séricos (ABS) foram de  $2,1 \pm 0,3$  mmol/l e de  $7,5 \pm 1,2$  mmol/l e  $7,8 \pm 1,4$  mmol/l para os valores pós-prandiais de 2 e 4 horas, respectivamente. As concentrações plasmáticas de amônia pré e pós-prandiais ( $118,2 \pm 13,2$  mg/dl ou  $67,3 \pm 7,5$  mmol/l e  $227,9 \pm 59,2$  mg/dl ou  $129,9 \pm 33,7$  mmol/l), diferiram ( $p < 0,05$ ) nas amostras mensuradas em até 30 minutos após a colheita de sangue; entretanto, a diferença entre os valores pré e pós-prandiais deixou de existir quando a amônia era mensurada nas amostras que foram congeladas pelo período de 24 e 48 horas. Observou-se que os valores de amônia das amostras pós-prandiais, que foram congeladas, apresentavam-se mais baixos quando comparados aos valores obtidos da mesma amostra mensurada em até 30 minutos após a colheita, e a diminuição da concentração de amônia era mais drástica quando os valores iniciais eram muito elevados. Os resultados obtidos sugerem que o plasma do cão não pode ser estocado para posterior determinação de amônia, utilizando-se apenas do congelamento como forma de estabilizar a amônia. Para a avaliação dos valores séricos pós-prandiais de ácidos biliares, sugere-se que a colheita de sangue possa ser efetuada em 2 ou 4 horas após a alimentação. Os valores de amônia plasmática obtidos no presente estudo podem permitir a comparação com os valores observados em cães com doença hepática ou encefalopatia e assim confirmar a importância da sua utilização no diagnóstico e prognóstico.

**UNITERMOS:** Ácidos biliares; Amônia; Cães.

## REFERENCES

- 1- ANWER, M.S.; MEYER, D.J. Bile acids in the diagnosis, pathology, and therapy of hepatobiliary diseases. **The Veterinary Clinics of North America: Small Animal Practice**, v.25, n.2, p.503-17, 1995.
- 2- ATTILI, A.F.; AUTIZI, D.; CAPACACCIA, L.; CONSTANTINI, S.; COTTA-RAMUSINO, F. Rapid determination of plasma ammonia using an ion specific electrode. **Biochemical Medicine**, v.14, n.1, p.109-16, 1975.
- 3- BECKETT, G.J.; DOUGLAS, J.G.; FINLAYSON, N.D.; PEREY-ROBB, I.W. Differential timing of maximal postprandial concentrations of plasma chenodeoxycholate and cholate: its variability and implications. **Digestion**, v.22, n.5, p.248-54, 1981.
- 4- BUNCH, S.E. Distúrbios hepatobiliares e pancreáticos exócrinos - Manifestações clínicas da doença hepatobiliar. In: NELSON, R.W.; COUTO, C.G. **Fundamentos de medicina interna de pequenos animais**. Rio de Janeiro : Guanabara Koogan, 1994. p.273-303.
- 5- BUSH, B.M. **Interpretation of laboratory results for small animal clinicians**. Oxford : Blackwell, 1991. p.290-3.
- 6- CENTER, S.A. Pathophysiology and laboratory diagnosis of hepatobiliary disorders. In: ETTINGER, S.J.; FELDMAN, E.C. **Textbook of veterinary internal medicine**. Philadelphia : W.B. Saunders, 1995. p.1261-312.
- 7- CENTER, S.A. Pathophysiology and laboratory diagnosis of liver disease. In: ETTINGER, S.J. **Textbook of veterinary internal medicine**. Philadelphia : W.B. Saunders, 1989. p.1421-527.
- 8- CENTER, S.A.; BALDWIN, B.H.; De LaHUNTA, A.; DLETZE, A.E. Evaluation of serum bile acid concentration, for the diagnosis of portosystemic venous anomalies in the dog and cat. **Journal of American Veterinary Medical Association**, v.186, n.10, p.1090-4, 1985.
- 9- CENTER, S.A.; BALDWIN, B.H.; ERB, H.N.; TENNANT, B.C. Bile acid concentrations in the diagnosis of hepatobiliary disease in the dog. **Journal of American Veterinary Medical Association**, v.187, n.9, p.935-40, 1985.
- 10- CENTER, S.A.; MAN WARREN, B.S.; SLATER, M.R.; WILENTZ, E. Evaluation of twelve-hour preprandial and two-hour postprandial serum bile acids concentrations for diagnosis of hepatobiliary disease in dogs. **Journal of American Veterinary Medical Association**, v.199, n.2, p.217-26, 1991.
- 11- CORNELIUS, C.E. Liver function. In: KANEKO, J.J. **Clinical biochemistry of domestic animals**, 4.ed. San Diego : Academic Press, 1989. p.364-91.
- 12- COUNSELL, L.J.; LUMSDEN, J.H. Serum bile acids: reference values in healthy dogs and comparison of two kit methods. **Veterinary Clinical Pathology**, v.17, n.3, p.71-4, 1988.
- 13- DIAL, S.M. Clinicopathologic evaluation of the liver. **The Veterinary Clinics of North America: Small Animal Practice**, v.25, n.2, p.257-74, 1995.
- 14- GAU, N. Ammonia. In: PIECE, A.J.; KAPLAN, L.A. **Methods in clinical chemistry**. St Louis : Mosby, 1987. p.1088-92.
- 15- HARDY, R.M. Pathophysiology of hepatic encephalopathy. **Seminars in Veterinary Medicine and Surgery (Small Animal)**, v.5, n.2, p.100-6, 1990.
- 16- HART, B.D.; STEINBERG, S.A.; VANWINKLE, T.J.; FARNBACH, G.C. Hepatotoxicity of phenobarbital in dogs: 10 cases (1985-1989). **Journal of American Veterinary Medical Association**, v.199, n.8, p.1060-6, 1991.
- 17- HITT, M.E.; JONES, B.D. Effects of storage temperature and time on canine plasma ammonia concentrations. **American Journal of Veterinary Research**, v.47, n.2, p.363-4, 1986.
- 18- JENSEN, A.L. Variations in total bile acid concentration in serum of dogs after a test meal. **Journal of Veterinary Medicine**, v.38, n.4, p.241-6, 1991a.

- 19- JENSEN, A.L. Evaluation of fasting and postprandial total serum bile acid concentration in dogs with hepatobiliary disorders. **Journal of Veterinary Medicine**, v.38, n.4, p.247-54, 1991b.
- 20- JENSEN, D.M. Portal systemic encephalopathy and hepatic coma. **Medical Clinics of North America**, v.70, p.1081-92, 1986.
- 21- MEYER, H.P.; ROTHUIZEN, J.; TIEMESSEN, I.; VAN DEN BROM, W.E.; VAN DEN INGH, T.S.G.A.M. Transient metabolic hyperammonaemia in young Irish Wolfhounds. **Veterinary Record**, v.138, n.3, p.105-7, 1996.
- 22- OGILVIE, G.K.; ENGELKING, L.R.; ANWER, M.S. Effects of plasma sample storage on blood ammonia, bilirubin, and urea nitrogen concentrations: cats and horses. **American Journal of Veterinary Research**, v.46, n.22, p.2619-22, 1985.
- 23- SHAFFER, E.A.; BEARDOIN, M.; SMALL, D.M.; *et al.* Relationship between gallbladder function and the enterohepatic circulation of bile salts during fasting. **Gastroenterology**, v.66, p.775, 1974.
- 24- TABOADA, J.; DIMSKI, D.S. Hepatic encephalopathy: clinical signs, pathogenesis, and treatment. **The Veterinary Clinics of North America: Small Animal Practice**, v.25, n.2, p.337-55, 1995.
- 25- TISDALL, P.L.C.; HUNT, G.B.; TSOKALAS, G.; MALIK, R. Post prandial serum bile acid concentrations and ammonia tolerance in Maltese dogs with and without hepatic vascular anomalies. **Australian Veterinary Journal**, v.72, n.4, p.121-6, 1995.
- 26- WASHIZU, T.; KOIZUMI, I.; KANEKO, J.J. Postprandial changes in serum bile acids concentration and fractionation of individual bile acids by high performance liquid chromatography in normal dogs. **Japan Journal of Veterinary Science**, v.49, n.4, p.593-600, 1987.
- 27- WILLARD, M.D.; TWEDT, D.C. Gastrointestinal, pancreatic and hepatic disorders. *In*: WILLARD, M.D.; TVEDTEN, H.; TURWALD, G.H. **Small animal clinical diagnosis by laboratory methods**. Philadelphia : W.B. Saunders, 1994. p.179-218.

Received: 01/04/1998

Accepted: 22/07/1998