STORAGE STABILITY OF HEPATOBILIARY ENZYMES IN DOG SERUM*

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Abstract

Storage stability of hepatobiliary enzymes such as, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP) and Gamma glutamyltransferase (GGT) in the sera of dogs of mixed breeds reared under the hot humid climatic conditions of Kerala was assessed. All the animals were apparently healthy, free of external parasites, dewormed regularly and vaccinated routinely against infectious diseases. Concentrations of ALT, AST, ALP and GGT were determined using an automated blood analyzer. Stabilities of enzymes in sera stored at room temperature (22 to 27°C), 4°C and -20°C were compared with the enzyme activities of fresh sera (day 0) on day 1, 2, 5, 8, 11 and 14. Alanine aminotransferase was found to be sufficiently stable up to 14 days at 4°C, whereas it was stable only for one day at -20°C and was totally unstable at room temperature. Aspartate aminotransferase was stable both at 4 °C and -20°C up to14 days whereas, it was totally unstable at room temperature. Alkaline phosphatase showed great variation upon storage when compared to other hepatobiliary enzymes and it is suggested that its estimation should be performed in fresh sera to get an accurate result even though the study suggested -20°C as better. Gamma glutamyltransferase was found to be highly stable at room temperature, 4°C and -20°C during the entire experimental period. It is therefore advisable to consider stability of each serum hepatobiliary enzyme for different animals separately before preserving sera to get more valid and reliable results.

Keywords: *storage stability, hepatobiliary enzymes, dog serum*

Enzymes may lose activity as a result of proteolysis by proteinase, aggregation and suboptimal buffer condition (Herrmann *et al.,* 2001). The routinely used enzymes to evaluate hepatic damage in animals include ALT, AST, ALP, GGT, Sorbitol dehydrogenase (SDH), Lactate dehydrogenase (LDH), Ornithine carbamoyl transferase (OCT) and 5' Nucleotidase (NTP) (Kaneko *et al*., 2008). Many investigations have been undertaken on the stability of enzymes, *in vitro*, but the results are widely divergent (Kaplan and Pesce, 1989). In veterinary medicine, to date not much studies have been published on the stability of biochemical markers especially serum hepatobiliary enzymes, which are routinely analysed for clinical diagnostic use. Hence, the present study was taken up to evaluate the *in vitro* stability of serum hepatobiliary enzymes like ALT, AST, ALP and GGT in the sera of dogs under various storage conditions *viz*, at room temperature (22 to 27°C), 4 ºC and -20ºC for a period of two weeks.

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Materials and Methods

A total of 13 apparently healthy dogs comprising of 11 male and 2 females below three years of age belonging to different breeds such as Golden Retriever, Great Dane, German Shepherd, Boxer, Labrador Retriever, Rottweiller, Chinese Pug, Cocker Spaniel and Daschund were selected. These dogs were maintained at nearby kennels and were fed on a balanced diet. All dogs were dewormed and immunized against rabies, distemper, parvo and hepatitis virus. They were also free from external parasites.

Blood samples were collected by cephalic / saphenous vein aseptically in sterile disposable syringe, using scalp vein set. Blood was then transferred immediately into clean dry sterile glass tubes without anticoagulants and sera separated.

The clear sera was immediately assayed for the following hepatobiliary enzymes ALT, AST, ALP and GGT within an hour of serum separation to serve as basal fresh values (day 0). The remaining sera were dispensed into 18 sample tubes, closed tightly and divided into three groups. One of each group was stored upright at room temperature (approximately 25 ^{0}C), 4 ^{0}C and - 20 ^{0}C. The stored serum aliquots from all temperature and time points were analysed together in one batch for hepatobiliary enzymes on 1, 2, 5, 8, 11 and 14 days post collection. Prior to analysis, at each designated time, the aliquots of the frozen samples were left to stand at room temperature to thaw and inverted several times to mix. The enzyme assay was performed using Ecoline Merck diagnostic kits (Merck Specialities Pvt. Ltd, Mumbai) on an automated blood analyzer (Microlab 200). The stability of an enzyme activity under each temperature and time was determined by calculating the percentage change in concentrations from the mean fresh values (day 0) at each time-point for each animal.

The results obtained were analyzed by ANOVA followed by Duncan Multiple Range Test and paired t-test as described by Snedecor and Cochran (1994).

Results and Discussion

The time and temperature had significant effects on hepatobiliary enzyme activity in dog sera during their storage (Tables 1 and 2). At room temperature, there was an upward trend in ALT values within 24 h of venipuncture and the values were substantially higher from 1st to 14th day when compared to mean fresh values. Significant increase ($P \leq 0.05$) in activity was found from the first day onwards. During the experimental period, the observed increase in activity was from 48.16 to 84.99 per cent. The increase in serum ALT activity at room temperature might be due to bacterial contamination. A similar finding was reported by Lazaroni *et al.* (1958) who stated that, bacterial contamination could cause either an increase or decrease in the enzyme activity in human serum maintained at room temperature. Activity of ALT in serum stored at 4 ^{0}C remained unchanged for two weeks and retained more than 90 per cent of initial activity and there was no significant difference in mean ALT activities over the entire period of storage. Whilst, ALT activities changed significantly at - 20 ^{0}C with an increase in activity from the second day onwards followed by a decrease in activity on 11th and 14th day and only about 63 per cent of the initial activity remained after two weeks (Fig. 1). These findings were supported by the report of Kaplan and Pesce (1989) who suggested that ALT of human sera was unstable at -20 ^{0}C, stable for five days at 4 ^{0}C and two days at room temperature. According to Gosling 1986, a class of group-specific enzymes such as proteinases, could lead to the loss of activity of some enzymes stored at freezing temperature. Another common reason attributed to the loss of activity of enzymes at -20 ^{0}C could be the intolerance of thawing from freezing to analytical temperature. The slower rate of freezing and thawing was more detrimental to the enzyme activity than the rapid process (Whittam *et al.*, 1973). Freezing with slow thawing resulted in more severe damage to proteins due to crystallisation on freezing and recrystallisation on thawing (Cao *et al.*, 2003). The present study suggested, 4 ^{0}C as the ideal storage condition for preserving dog sera meant for ALT assay as compared to room temperature and -20 ^{0}C.

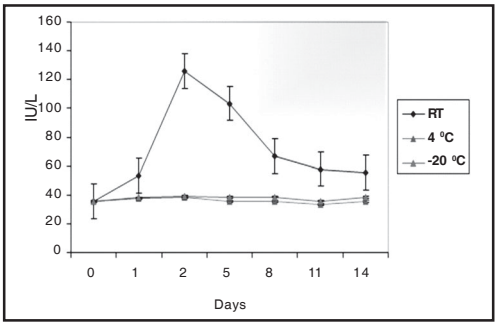

Fig.1. Stability of ALT activity in dog serum

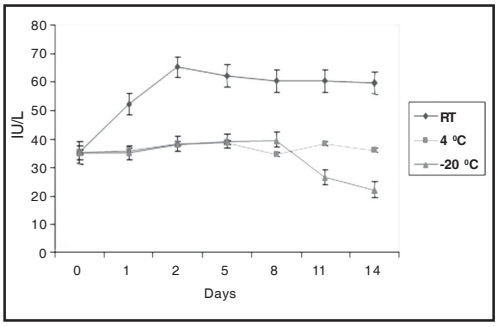

Fig.2. Stability of AST activity in dog serum

Results of the storage stability of AST at room temperature revealed a statistically significant increase ($P \leq 0.05$) in values from the very next day of blood collection up to 14th day. About 50 per cent increase in AST activity was found after 24 h of storage, thereafter the activity increased to 253.08 per cent on second day (Fig. 2). The enzyme was totally unstable at room temperature. Storage at 4 ^{0}C and -20 ^{0}C for two weeks did not result in any change in enzyme activity that would be considered statistically significant. It was found that AST activity changed by about 7 per cent and 10 per cent at 4 ^{0}C and -20 ^{0}C, respectively during their storage. In human sera, Kaplan and Pesce (1989) reported AST stability for a period of one month at 25 ^{0}C. In the present study both refrigerator and freezer could be considered as the suitable storage conditions for AST assay.

Storage of sera at room temperature significantly affected mean ALP activities throughout the study period and the enzyme was found to be unstable. The enzyme showed significant decrease ($P \leq 0.05$) in activity after 24 h of venipuncture at room temperature. At 4°C decrease in activity was seen

Table 1. Activity of ALT and AST in dog sera preserved at 25°C, 4°C and -20°C for 14 days

Days of storage	ALT (IU/L)			AST (IU/L)		
	25°C	4°C	-20°C	25°C	4°C	-20°C
0 (Base line value)	35.30± 4.93 -	35.30± 4.93 -	35.30± 4.93 -	35.60± 2.14 -	35.60± 2.14 -	35.60± 2.14 -
1	52.30± 4.66* (+48.16)	35.80± 4.89 (+1.42)	35.30± 4.40 (0)	53.10± 5.58* (+49.16)	37.90± 2.14 (+6.46)	38.10± 2.26 (+7.02)
2	65.30± 3.94* (+84.99)	38.30± 5.25 (+8.49)	38.20± 4.95* (+8.22)	125.70± 14.00* (+253.08)	38.30± 1.61 (-7.58)	39.00± 2.44 (+9.55)
5	62.30± 4.83* (+76.49)	38.80± 6.51 (+9.92)	39.20± 4.32* (+11.05)	103.40± 9.70* (+190.45)	35.40± 3.15 (-0.56)	38.40± 3.05 (+7.86)
8	60.50± 5.09* (+71.39)	34.50± 6.79 (-2.27)	39.60± 4.87* (+12.18)	67.10± 11.13* (+88.48)	35.60± 2.77 (0)	38.60± 1.79 (+8.43)
11	60.50± 5.09* (+71.39)	46.20±3.22 (-10.80)	26.50± 4.43* (-24.93)	57.90± 10.13 (62.64)	33.50± 2.49 (-5.89)	35.90± 1.79 (+0.84)
14	59.80± 5.69* (+69.41)	45.10±3.79 (-12.93)	22.20± 3.19* (-37.11)	55.40± 9.34 (+55.62)	35.40± 1.69 (-0.56)	38.30± 1.54 (+7.58)

*Percentage change from initial activity in parenthesis, * $P \leq 0.05$*

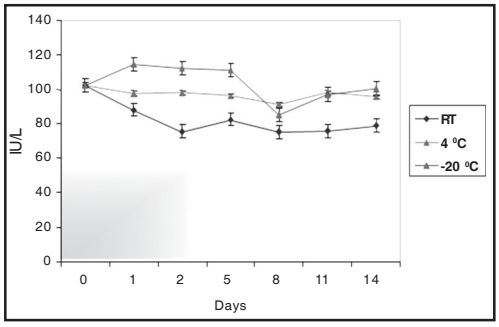

Fig.3. Stability of ALP activity in dog serum

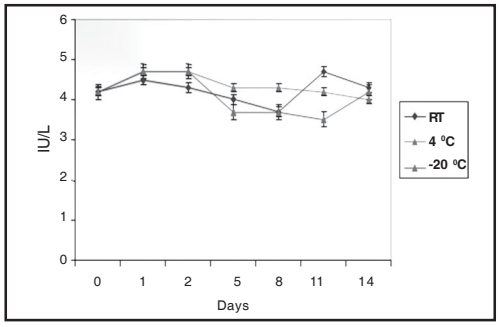

Fig.4. Stability of GGT activity in dog serum

throughout the study period. Statistically significant decrease in activity was observed from second day onwards. There was no significant change in ALP activity in the sera kept overtime, up to 5 days at -20 ^{0}C (Fig.3). Thereafter, significantly lower ALP activity was observed from 8th to 14th day. From the present study, it was found that storing at -20 ^{0}C was more suitable for dog sera for ALP estimation when compared to refrigeration, even though prolonged storage was not recommended. The difference in ALP activity may be due to the occurrence of several isozymes for ALP which differed in their sensitivity to temperature or due to individual variation or high concentration of enzyme at lower temperature (Kaneko *et al.* 2008).

There was virtually no significant change in GGT activity stored at room temperature, refrigerator and freezer. The enzyme was sufficiently stable under all the three storage conditions up to two weeks. This is of much importance as it enables the transportation of samples under sufficiently stable conditions from the farm to a local diagnostic laboratory. During the study period only less than 12 per cent change in activity was ob-

Table 2. Activity of ALP and GGT in dog sera preserved at 25°C, 4°C and -20°C for 14 days

Days of storage	ALP (IU/L)			GGT (IU/L)		
	25°C	4°C	-20°C	25°C	4°C	-20°C
0 (Base line value)	102.30± 7.51 -	102.30± 7.51 -	102.30± 7.51 -	102.30± 7.51 -	102.30± 7.51 -	4.20± 0.17 -
1	88.00± 8.54* (-13.98)	97.70± 6.53 (-4.49)	114.30± 9.61 (+11.73)	88.00± 8.54* (-13.98)	97.70± 6.53 (-4.49)	4.70± 0.21 (+11.90)
2	75.50± 8.66* (-26.19)	98.00± 7.47* (-4.20)	112.30± 14.96 (+9.77)	75.50± 8.66* (-26.19)	98.00± 7.47* (-4.20)	4.70± 0.21 (+11.90)
5	82.30± 8.53* (-19.55)	96.30± 7.56* (-5.86)	111.00± 15.23 (+8.50)	82.30± 8.53* (-19.55)	96.30± 7.56* (-5.86)	3.70± 0.21 (-11.05)
8	75.30± 7.08* (-26.19)	91.20± 6.89* (-10.85)	85.20± 11.50* (-16.72)	75.30± 7.08* (-26.19)	91.20± 6.89* (-10.85)	3.70± 0.33 (-11.05)
11	75.70± 9.94* (-26.00)	97.80± 4.74 (-4.39)	96.80± 7.57* (-5.38)	75.70± 9.94* (-26.00)	97.80± 4.74 (-4.39)	3.50± 0.35 (-11.80)
14	79.00± 9.78* (-22.78)	95.80± 4.25 (-6.35)	100.30± 10.39 (-1.96)	79.00± 9.78* (-22.78)	95.80± 4.25 (-6.35)	4.20± 0.17 (0)

*Percentage change from initial activity in parenthesis, * P≤ 0.05*

served in the samples (Fig.4). Kaplan and Pesce (1989) reported a stability of one week at 4 ^{0}C and one month at -25 ^{0}C for the GGT activity in human sera. The results were also in accordance with the study of Donnlley *et al.* (1995) on human sera who found GGT to be highly stable at 4 ^{0}C for 2 weeks and up to 4 months at -20 ^{0}C.

To summarise, temperatures of -20°C and 4°C were regarded as ideal for storage of dog sera for AST and GGT estimation whereas, storage at 4°C provided better stability for ALT. The study suggested room temperature as an ideal condition for GGT assay in case of dog serum. But ALP estimation should be performed in fresh serum samples to get an accurate result even though the study suggests -20°C as better.

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