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Comparison of storage stability of serum hepatobiliary enzyme activities in murrah buffaloes

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Abstract

The present study was designed and conducted to find out the appropriate physical baseline values for hepatobiliary enzymes such as, Alanine aminotransferase(ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP) and Gamma glutamyltransferase (GGT) in adult healthy Murrah buffaloes of hot humid climatic condition and also to assess the ideal storage condition for buffalo sera samples meant for the assay of hepatobiliary enzymes by storing at room temperature (22 to 27 ºC), 4 ºC and -20 ºC up to 14 days. The normal mean serum activities of ALT, AST, ALP and GGT were 50.0 ± 3.53, 130.0 ± 7.29, 323.6 ± 32.09 and 10.11 ± 1.28 IU/L with 95% confidence interval of 42.02 to 57.98, 113.51 to 146.49, 251.0 to 396.19 and 7.15 to 13.07 IU/L, respectively. Alanine aminotransferase and Gamma glutamyltransferase were found to be sufficiently stable up to the study period of 14 days at both 4 ºC and -20 ºC whereas unstable at room temperature. Aspartate aminotransferase was more stable at 4 ºC up to 11 days and 8 days at room temperature and was stable only 2 days at -20 ^{0}C. Alkaline phosphatase showed great variation upon storage as compared to other hepatobiliary enzymes and it is suggested that its estimation should be performed in fresh serum samples to get a more accurate result. Thus the present study reveals specific reference values for each serum hepatobiliary enzymes in buffaloes of hot humid tropics. From these results it is also advisable to consider stability of each serum hepatobiliary enzymes for different animals separately before preserving sera samples to get a more valid and reliable result.

INTRODUCTION

Enzymes are central to every biochemical process and most of the metabolic pathways in our body are regulated through the coordinated action of enzymes. Most of the enzymes with diagnostic applications function within the cells in which they are synthesized and are present in high concentration in specific tissues. These enzymes leak out into the serum when there is any damage to hepatic tissue. Increased serum activity of these enzymes act as a sensitive marker of cellular damage. So the study of clinically important enzymes has immense practical importance in diagnosis and monitoring the progress of tissue damage. The routinely used enzymes to evaluate hepatic damage in animals includes ALT, AST, ALP, GGT, Sorbitol dehydrogenase (SDH), Lactate dehydrogenase (LDH), Ornithine carbamoyl transferase (OCT) and 5' Nucleotidase (NTP) (Kaneko *et al*., 2008). The enzymes routinely used in human beings for disease diagnosis may not give true indications of hepatic injury in veterinary practice. There is also lack of standard reference values for some species. Each animal species has their own specific hepatobiliary enzyme levels which vary from one species to another (Kaneko *et al*., 2008). The available data on hepatobiliary enzyme levels from literature shows widely divergent values among different species and these data are mainly procured from the animals reared in temperate climate. Even though considerable information is available on normal serum hepatobiliary enzyme levels of domestic animals of exotic breeds, kept under different environment and management conditions, use of these serum enzyme levels for

monitoring health status of indigenous breeds may mislead the diagnosis. So for a more accurate clinical interpretation of hepatic diseases, it is a prerequisite to establish the reference values of these enzymes. When large numbers of blood samples are collected or when many different analyses are required it is inevitable to store the samples. Many investigations have been undertaken on the stability of enzymes, *in vitro*, but the results are widely divergent and most of them showed great instability during preservation. Different treatment of the blood before analysis like conditions of preservation, centrifugation, haemolysis and bacterial growth could account for the variations in the results and partially because of the varying specificities of individual methods of analysis. In veterinary medicine, not much studies have been published on the stability of biochemical markers especially serum enzymes to date. At present, as there is conflicting data regarding the effect of different temperatures and durations of storage on the stability of the activities of hepatobiliary enzymes, which are routinely analyzed for clinical diagnostic use. It is of primary importance to reexamine the storage stability of these enzymes. Besides, data on this line in the hot humid tropical conditions are very meager. Therefore, the present study is on the effects of storage time and temperature on the measured activities of the hepatobiliary enzymes like ALT, AST, ALP and GGT in the sera samples of buffaloes under various storage conditions *viz*, at room temperature (22 to 27 ºC), 4 ºC and -20 ºC for a period of two weeks.

MATERIALS AND METHODS

Ten female Murrah buffaloes between 2 to 3 years of age maintained at University Buffalo Farm, College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, Thrissur were selected randomly for the study. Blood samples were collected by jugular venipuncture using sterile needles (18 gauge) directly into clean dry sterile glass tubes without anticoagulants. Serum was harvested after 30 to 45 minutes following clot formation and by centrifugation for 10 minutes at 2000 g.

The clear serum 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 serum was dispensed into 18 sample tubes, closed tightly and divided into three groups. One of each group was stored upright at room temperature (approximately 25 ºC), 4 ºC and -20 ºC. The stored serum aliquots from all temperature and time points were analyzed 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 condition and time was determined by calculating the percentage change in concentrations from the mean fresh value (day 0) at each time-point for each animal.

The experimental results obtained were analyzed by using analysis of variance (ANOVA) technique followed by Duncan Multiple Range Test and paired t-test as described by Snedecor & Cochran (1994) using a computerized software program, statistical package for social sciences (SPSS) .

RESULT AND DISCUSSION

The results showed that the mean serum activities of ALT, AST, ALP and GGT were 50.0 ± 3.53, 130.0 ± 7.29, 323.6 ± 32.09 and 10.11 ± 1.28 IU/L with 95% confidence interval of 42.02 to 57.98, 113.51 to 146.49, 251.0 to 396.19 and 7.15 to 13.07 IU/L, respectively (Table 1). The mean values of ALT activity observed for female Murrah buffaloes in the present study was found to be 50.00 ± 3.53 IU/L with a reference range of 42.02 to 57.98 IU/L. The results support the findings of Terzano *et al.* (2005) and Grasso *et al.* (2004) who reported mean ALT values of 60 IU/L in buffalo heifers and with a range of 55.35 to 58.49 IU/L in adult female buffaloes kept at intensive and traditional system of management. However, a higher ALT activity of 176 to 219 IU/L and 83 to 116 IU/L was observed for buffaloes at different pre-post partum time intervals and early lactation, respectively (Terzano *et al.,* 2005). A significantly lower ALT level was reported by Mudgal *et al.* (2008) who found a mean ALT level of 37.15 IU/L for 8 to 9 month old buffalo calves. Marked differences were also observed in mean serum ALT activity for adult buffaloes (Pal and Dasgupta, 2006) who reported 28.50 ± 1.32 IU/L which was significantly lower than the present findings.

Table 1. Serum ALT, AST, ALP and GGT activities (IU/L) in buffalo (n=10)

Enzyme	Min.	Max.	Mean± SE	95 % Confidence interval
ALT	30	64	50.00± 3.53	42.02 - 57.98
AST	105	172	130.00± 7.29	113.51 -146.49
ALP	175	479	323.60± 32.09	251.00 – 369.19
GGT	4	15	10.11± 1.28	7.15 - 13.07

Table 2. Activity of ALT and AST in buffalo sera samples preserved at 25 ºC, 4 ºC and -20 ºC for 14 days

Days of storage	ALT			AST		
	25 ºC	4 ºC	-20 ºC	25 ºC	4 ºC	-20 ºC
0 (Base line value)	51.80±3.43 -	51.80±3.43 -	51.8±3.43 -	129.80±8.70 -	129.80±8.70 -	129.80±8.70 -
1	44.50±2.19* (-14.09)	47.30±2.39 (-8.69)	51.30±3.12 (-0.97)	132.80±7.50 (+2.31)	127.00±7.60 (-2.16)	128.30±7.49 (-1.16)
2	44.30±2.46* (-14.47)	47.40±3.10 (-8.49)	50.90±2.29 (-1.74)	130.80±7.90 (+0.77)	125.80±7.05 (-3.08)	121.9±10.09 (-6.09)
5	37.90±2.16* (-26.83)	47.40±2.61 (-8.49)	46.30±1.74 (-10.62)	125.60±8.30 (-3.23)	125.30±7.62 (-3.47)	118.40±10.69* (-8.78)
8	27.10±1.52* (-47.68)	47.60±1.91 (-8.11)	46.60±2.03 (-10.04)	114.30±16.40 (-11.94)	127.60±9.40 (-1.69)	116.30±7.9* (-10.40)
11	20.50±2.09* (-60.42)	46.20±3.22 (-10.80)	45.80±3.80 (-11.58	77.10±12.90* (-40.60)	123.60±7.80 (-4.78)	112.80±6.9* (-13.09)
14	14.8±1.85* (-71.42)	45.10±3.79 (-12.93)	51.10±3.72 (-1.35)	68.0±10.9* (-47.61	121.4±7.8* (-6.47)	109.1±6.9* (-15.95)

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

The 113.51 to 146.49 IU/L of AST reference range observed for adult healthy buffaloes in the present study is in close agreement the reports of Randhawa *et al.* (1997) and Grasso *et al.* (2004) and they reported a mean ALT value of 134.6 ± 4.36 IU/L for adult healthy buffaloes and 146.84 IU/L for buffalo cows maintained under intensive system of management, respectively. In contrast, a slightly increased AST value (164.68 IU/L) was observed for those under traditional system of management (Grasso *et al.,* 2004). The present findings were also comparable with the observations of Terzano *et al.* (2005) who reported 101.2 IU/L of mean AST activity for adult buffaloes even though it is towards the lower margins of the present reference range. Contrary to the results of the present study, significantly lower AST values were reported by Pal and Dasgupta (2006) and Mudgal *et al.* (2008) who reported 54.00 ± 1.22 IU/L for adult healthy buffaloes and 62.47 IU/L for male buffalo calves, respectively.

The reference range of 251.00 to 396.19 IU/L ALP activity obtained in the present study is in close agreement with the studies of Grasso *et al.* (2004) who reported 370.11 IU/L of ALP activity in buffaloes maintained under intensive system of management, whereas a higher ALP values was observed for those maintained under traditional system (443.12 IU/L). A similar study was conducted by Terzano *et al.* (2005) on adult healthy buffaloes and the present findings were within reference range of 200 to 650 IU/L established by them. But Randhawa *et al.* (1997) presented comparatively lower ALP values (113.9 ± 4.25 IU/L) for buffaloes. ALP activity of 76.34 IU/L suggested by Bharti *et al.* (2008) for male Murrah buffalo calves of 6 to 8 months of age was significantly lower than the present findings.

The mean GGT concentration of 10.11 ± 1.28 IU/L obtained for adult healthy buffaloes in the present study is within the range of 4.9 to 25.7 IU/L reported by Hilali *et al.* (2006). The findings of the present study are also comparable to the reports of Randhawa *et al.* (1997) who presented a GGT activity of 16.8 ± 0.82 IU/L for adult healthy buffaloes. However, the results of the present study were significantly lower than the reports of Terzano *et al*. (2005) and Grasso *et al.* (2004) and the reported GGT levels were 21.2 IU/L and 26.95 to 27.43 IU/L, respectively.

The choice of temperature for the assay of enzymes of clinical importance in different species is still under debate. Refrigeration and freezing preserve most of the enzymes but some deteriorate even when frozen. Changes in the temperature of storage alter the rate of reaction and rate of denaturation of enzymes. Stability of an enzyme activity in one species does not mean stability in a second (Kaneko *et al.,* 2008). Species differences in the stability of enzyme activities is also reported in various marmoset and suggested that the enzyme become more concentrated, or that its activity increased at lower temperature (Davy *et al.*, 1984). Intracellularly, enzymes are protected from degradation when bound to their substrates and cofactors. In serum, enzymes, substrates and cofactors are dispersed and binding is uncommon, leaving the enzymes more susceptible to degradation (Kaplan and Pesce, 1989).

Table 3. Activity of ALP and GGT in buffalo sera samples preserved at 25 ºC, 4 ºC and -20 ºC for 14 days

Days of storage	ALP			GGT		
	25 ºC	4 ºC	-20 ºC	25 ºC	4 ºC	-20 ºC
0 (Base line value)	310.20±36.6 -	310.20±36.6 -	310.2±36.6 -	11.30±1.02 -	11.30±1.02 -	11.30±1.02 -
1	254.5±44.10 (-17.96)	261.3±45.17* (-15.76)	274.6±44.7* (-11.48)	13.60±0.95* (+20.35)	12.80±0.58 (+11.27)	12.70±0.57 +12.39
2	229.8±38.31* (-25.91)	279.1±42.38* (-10.03)	275.0±44.62* (-11.35)	15.80±0.87* (+39.82)	13.80±0.43 (+22.12)	12.20±0.039 +7.96
5	165.4±38.59* (-46.68)	274.5±43.75* (-11.51)	311.5±34.19 (+0.42)	17.60±0.80* (+55.75)	12.60±0.56 (+11.50)	12.50±0.41 +10.62
8	110.5±24.59* (-64.38)	272.3±43.32* (-12.21)	319.6±32.3 (+3.03)	20.10±2.48* (+77.88)	13.50±0.38 (+19.47)	13.20±0.40 +16.81
11	77.6±14.99* (-74.98)	270.9±41.95* (-12.67)	284.1±41.22 (-8.41	18.00±0.52* (+59.29)	13.80±0.58 (+22.12)	13.80±0.58 +22.12
14	71.25±11.8* (-77.03)	271.8±43.3* (-12.38)	279.8±42.93 (-9.80)	17.70±1.02* (+56.64)	13.00±0.56 (+15.04)	11.00±0.63 -2.65

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

The stability of ALT activity at room temperature was much less as compared to 4 ^{o}C and -20 ^{o}C (Table 2). The enzyme was highly unstable at room temperature and showed significant decrease (P≤ 0.05) in activity from the very next day of blood collection. At the end of the experimental period only less than 30 % of initial activity was retained in the serum samples. The storage of serum at 4 ^{o}C for two weeks did not result in any significant change in enzyme activity. Insignificant decrease in ALT activity was observed from the first day onwards at 4 ^{o}C. The storage of serum at -20 ^{o}C was also considered to be suitable for ALT assay in buffalo. The activity remained much unaffected up to the study period of two weeks. The observations for serum ALT stability were consistent with the study of Boyanton and Blick (2002) in human plasma. They observed a 20 % decrease in ALT activity at 48 hours and 56 hours at room temperature and the cited reason was due to the increased lactate concentration as a result of bacterial contamination. This study recommends either 4 ºC or -20 ºC for preservation of buffalo sera samples for ALT assay.

Sera samples stored at room temperature maintained the initial AST activity up to 8 days without any significant loss, but thereafter the values decreased to a point of statistical significance on the 11th and 14th day of storage, and more than 40 % decrease in activity was noticed during this period (Table 2). Only negligible changes were found in AST activity when the serum was stored at 4 ^{o}C up to the 11th day and these changes were not statistically significant. After 11 days, a clinically acceptable significant decrease in AST values (P≤ 0.05) was seen. Results obtained for AST stability at -20 ^{o}C revealed a negligible variation on enzyme activity up to 2 days. Beyond then, a statistically significant (P≤ 0.05) decline in activity was observed up to the 14th day. According to Gosling 1986, a class of group-specific enzymes such as proteinases, that attack enzymes, if functional at -20^{0} C, could account for loss of activity of some enzymes stored at freezing temperatures. Another common reason which could be attributed to the loss of activity of enzymes could be the intolerance of thawing from freezing to the analytical temperature. The slower rate of freezing and thawing was more detrimental to the enzyme activity than the faster process (Whittam *et al.*1973). Freezing with slow thawing resulted in more severe damage to proteins due to crystallization on freezing and re-crystallisation on thawing (Cao *et al.*2003). The AST activity under various storage conditions suggested for human serum was 3 days at room temperature, one week at 4 ºC and one month -25 ºC (Kaplan and Pesce, 1989). Due to significant decrease in AST activity at -20 ºC, the present study suggests 4 ºC as the better storage condition for buffalo sera samples.

The ALP activity in the sera samples stored at room temperature did not show any statistically significant change up to the first day, followed thereafter by a significant decline to below baseline values and only less than 23 % of initial activity retained at the end of the experimental period. Results are presented in table 3. At 4 ^{o}C, ALP activities declined markedly beginning within 24 hours of venipuncture, and the changes were statistically significant and the enzyme

was totally unstable at this temperature. However, the percentage change in activity was comparatively lower than that at room temperature. The specimens kept in the frozen state showed great fluctuations in ALP activity over the entire period. Even after 24 hours of storage, significant decline ($P \leq 0.05$) in ALP activity was observed. These results were contradictory to the reports of Kaplan and Pesce (1989) in human sera samples where ALP activity increased with increase in temperature. The present results suggest the instability of buffalo ALP enzyme during preservation of sera samples both at 4 ºC and -20 ºC and the assay should be performed on the day of blood collection. The difference in ALP activity may be due to the occurrence of several isozymes for ALP which differs in their sensitivity to temperature, individual variation, or enzymes and may become more concentrated at lower temperature.

Time of storage had a significant effect on GGT activity in the sera samples kept at room temperature (Table 3). The activities increased significantly over the time of storage with more pronounced degree of change on the 8th day where a 78% increase in activity was observed. In the refrigerator and frozen state, the enzyme showed no appreciable change over a period of two weeks and the percentage change in mean activity was less than 23% in both the conditions. Among these two conditions, the storage of serum at -20 ^{o}C was considered to be more suitable for GGT assay of buffalo serum. The results were in accordance with the study of Donnlley *et al.* (1995) on human serum and they stated that GGT to be highly stable at 4 ºC (14 days) and -20 ºC (4 months). They reported a stability of 48 hours at room temperature, which in the present study was 24 hours. The increase in serum GGT activity at room temperature may be due to bacterial contamination. A similar finding was reported by Lazaroni *et al.* (1958) who stated that bacterial contamination can cause either an increase or a decrease in the enzyme activity in human serum maintained at room temperature. The present study suggests -20 ºC as the most suitable storage condition for GGT assay in buffalo sera samples.

REFERENCES

1. Bharti, V.K., Gupta, M. and Lall, D. 2008. Ameliorative effects of boron on serum profile in buffalo (*Bubalus bubalis*) fed high fluoride ration. *Trop. Anim. Health Prod.* 40: 111–116
2. Boyanton, B.L. and Blick, K.E. 2002. Stability studies of twenty-four analytes in human plasma and serum. *Clin. Chem.* 48: 2242–2247
3. Cao, E., Chen, Y., Cui, Z., and Foster, P. R., 2003. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnology and Bioengineering*. 82 (6): 684-690
4. Davy, C.W., Jackson, M.R. and Walker, J.W. 1984. Stabilities of some constituents of Marmoset (*Callithrix jacchus*) plasma under various conditions of storage. *Clin. Chem.* 30: 101-104
5. Donnelly, J.G., Soldin, S.J., Nealon, D.A. and Hicks, J.M. 1995. Stability of twenty five analytes in human serum at 22 ºC, 4 ºC and -20 ºC. *Pediatr. Pathol. Lab. Med.* 15: 869-874
6. Grasso, F., Terzano, G.M., Rosa, G.D., Tripaldi, C. and Napolitano, F. 2004. Influence of housing conditions and calving distance on blood metabolites in water buffalo cows. *Ital. J. Anim. Sci.* 3: 275-282
7. Gosling P. 1986. Analytical reviews in clinical biochemistry: calcium measurement. *Annals of Clinical Biochemistry.* 23: 146-156
8. Hilali, M., Abdel-Gawad, A., Nassar, A. and Abdel-Wahab, A. 2006. Hematological and biochemical changes in water buffalo calves (*Bubalus bubalis*) infected with *Trypanosoma evansi*. *Vet. Parasitol.* 139: 237–243
9. Kaneko, J.J., Harvey, J.W. and Bruss, M.L. 2008. *Clinical Biochemistry of Domestic Animals.* Fifth edition. Academic Press, California, 932p.
10. Kaplan, L.A. and Pesce, A.J. 1989. *Clinical Chemistry, Theory, Analysis and Correlation.* Second edition. The C.V. Mosby Company, Baltimore, 1149p.
11. Lazaroni, J.A., Maier, E.C. and Gorczyca, L.R. 1958. The stability of Lactic dehydrogenase in serum. *Clin. Chem*. 4: 379-381
12. Mudgal, V., Garg, A.K., Dass, R.M. and Varshney, V.P. 2008. Biological effect of Selenium and Copper supplementation on blood metabolic profile in male buffalo (*Bubalus bubalis*) calves. *Trace Elem. Res.* 121: 31–38
13. Pal, S. and Dasgupta, C.K. 2006. Haemato-biochemical profiles of buffalo in anthelmintics treatment against *Fasciola gigantica* infection. *Buffalo Bulletin*. 25: 25-29
14. Randhawa, C.S., Brar, R.S., Sharma, D.R. and Randhawa, S.S. 1997. Biochemical responses in mixed chronic psoroptic and sarcoptic mange of buffaloes *(Bubalus bubalis)*. *Trop. Anim. Hlth Prod.* 4: 253-254
15. Snedecor, G.W. and Cochran, W.G. 1994. *Statistical Medhods.* Eight edition. Oxford and IBH Publishing Company, Calcutta, 564 p.
16. Terzano, G.P., Allegrini, S., Borghese, A., Roncoroni, C. and Alfieri, L. 2005. Metabolic and hormonal parameters in buffaloes. In: Borghese, A. (eds). *Buffalo Production and Research Paper No: 67.* Food and Agriculture Organization of the United Nations, Rome. pp. 219-248
17. Whittam, J. H., and Rosano, H. L. 1973. Effects of freeze thaw process on alpha amylase. *Cryobiology*. 10 (3): 240-243.