Changes in ALT, AST and ALP Values of Plasma and Serum Samples Stored at Refrigerator (4 ^{0}C) and Room Temperature (32 ^{0}C) for up to Five Days

[1]Nwosu, O. K., [2]Aloh, G. S. and [3]Ihedioha, J. I.

[1] Department of Chemical Pathology, Faculty of Clinical Medicine, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria.

[2]Department of Biochemistry and Biotechnology, Faculty of Applied and Natural Sciences, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria.

[3]Clinical Pathology Unit, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nsukka, Enugu State, Nigeria.

Corresponding author: Ihedioha, J. I. Clinical Pathology Unit, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, POBox 3236, Nsukka, Enugu State, Nigeria. Email: jiiferh@yahoo.com

Abstract

This study investigated the time-related changes in the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) values of serum and plasma samples stored at refrigerator (2 - 8^{o}C, average of 4^{0}C) and room temperature (30-34^{0}C, average of 32^{0}C) for a period of 120 hours (5 days). Blood samples were obtained from a total of 20 patients that presented with cases of liver malfunction at the Ebonyi State University Teaching Hospital, Abakaliki, Nigeria. The enzyme assays were carried out immediately upon sample collection and separation to obtain the baseline value (BV), and thereafter at specified time intervals across the 120 hours. Results showed that values not significantly different (p > 0.05) from the BV can be obtained from serum and plasma samples within the specified storage durations at the different temperatures as follows: For serum ALT, 4^{0}C – 48 hours, 32^{0}C – 16 hours; plasma ALT, 4^{0}C – 36 hours, 32^{0}C –10 hours; serum AST, 4^{0}C – 36 hours, 32^{0}C – 10hours; plasma AST, 4^{0}C – 36 hours, 32^{0}C – 8 hours; serum and plasma ALP, 4^{0}C – 30 hours, 32^{o}C – 10 hours. All the enzymes were found to be more stable in refrigerated samples than in those kept at room temperature; also the enzymes were more stable in serum than in plasma for all the storage temperatures. It was concluded that in general, reliable values for serum and plasma ALT, AST and ALT (values not significantly different from the BV) can be obtained from refrigerated samples if analysed within 30 hours of blood collection and separation, while for samples kept at room temperatures (32^{0}C) analysis should be carried out within 8 hours. Beyond these time points (30 hours for refrigerated samples and 8 hours for samples kept at room temperature) values obtained for the serum and plasma enzymes studied were significantly different (P<0.05) from the BV and therefore not reliable for diagnosis.

Keywords: Liver enzymes, Liver malfunction, Blood collection, Stability, Temperature

Introduction

The laboratory measurement of the serum/plasma activity of some liver specific enzymes has become an invaluable tool for the assessment of the functional status of the liver, the degree of damage to the organ and its response to therapy (Mayne, 1994; Tolman and Rej, 1999; Dufour *et al*., 2000). Commonly, serum/plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity are measured as indicators of liver damage, while serum/plasma alkaline phosphatase (ALP) activity is used to assess for cholestatic disorders (Mayne, 1994; Kenneth, 1996; Dufour *et al*., 2000; Sotil and Jensen, 2004). Currently in medical practice, a diagnosis of liver dysfunction cannot be considered definitive if information obtained from the patients' history and clinical examination is not combined with laboratory test results (including those of enzyme determinations) in arriving at it (Dufour *et al*.,2000).

A general challenge faced by all clinical laboratories worldwide is to ensure the integrity and reliability of their laboratory test results. In addition to pathological processes, laboratory test results are affected by certain pre-analytical, analytical and normal biological variations (Mayne, 1994; Navayanan, 1995; Young and Bermes, 1999). As analytical variations are being reasonably minimized by the development of new technologies and test kits, the relative contribution of pre-analytical variations has become a more dominant element in overall test variability (Navayanan, 1995) Specifically, the results of enzyme activity determinations can be strongly influenced by pre-analytical factors such as method of keeping, storage or handling of the blood, plasma or serum samples between the time of collection of blood and the time of analysis, and improperly handled/stored samples could generate results that may lead to wrong diagnosis or prognosis (Young and Bermes,1999; Clark *et al.,* 2003).

It is generally recommended that ALT, AST and ALP activity determination in serum and plasma samples be carried out immediately upon sample collection and separation of serum/plasma from clot/red cells and if not possible the sample should be refrigerated and analysed within 24 hours (Young and Bermes, 1999). There are however reports suggesting that these enzymes activities may be stable for several hours when

serum/plasma samples are kept at room/ambient temperature conditions (Ono *et al.,* 1981; Heins *et al.,* 1995). The available reports regarding the stability of these enzymes activities at room/ambient temperature are however conflicting, most probably because of the variations in room/ambient temperatures in different parts of the world, where the different studies were carried out. At present, there are no reports (known to the authors) on the stability of these enzyme activities when serum/plasma samples are kept under tropical temperature conditions. In these tropical countries, especially the developing ones, laboratory personnel are constantly faced with the problems of inconsistent power supply and non-functional refrigerators, such that serum/plasma samples often have to be left on the laboratory bench at room temperature for several hours or even days before analysis/determinations can be carried out. Hence the study, which investigated the changes in ALT, AST and ALP activities of serum and plasma samples kept at refrigerator (4^0C) and room temperature (30 – 34^0C, average of 32^0C) for a total of 120 hours (5 days).

Material and Methods

Blood samples for the study were collected from a total of 20 patients who presented with cases of liver diseases at the Ebonyi State University Teaching Hospital, Abakaliki, Nigeria. Ten milliliters (10 mls) of blood was collected by venopuncture from each of the patients after his/her oral consent was obtained. The blood samples were divided into two parts: five milliliters (5 mls) was added to a sample bottle containing appropriate quantity of lithium heparin, and another 5ml was put into a clean dry plain bottle. The sample put into the lithium heparin-treated bottle was centrifuge immediately at 3000 rpm for 5 minutes to separate plasma from the red blood cells. The sample put in the plain bottle was allowed to stay at room temperature for 30 minutes to clot; thereafter the serum was separated from the clot by centrifugation at 3000 rpm for 5 minutes.

The serum and plasma samples from each patient was analysed in duplicates immediately upon separation from clot/blood cells to obtain the baseline value (BV). This represents the value obtained at zero hour. Thereafter the plasma and serum samples were divided into two equal parts: one part was kept in a refrigerator at 4^0C while the other was kept on the laboratory bench at room temperature range of 30 – 34^0C (average of 32^0C). Further determinations on the samples were carried out in duplicates at times 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 42, 48, 72, 96 and 120 hours.

ALT and AST activity in the serum and plasma samples were determined by the method of Reitman and Frankel (1957). Working AST/ALT substrate (200 mmol L – aspartic acid, 2 mmol $\propto$ - oxoglutarate for AST; 200mmol L – alanine, 2 mmol $\propto$ - oxoglutarate for ALT) solution (0.5ml) was placed in a test tube and warmed in a water bath (37^oC) for 5 minutes. Then 0.1ml of serum/plasma was added. The mixture was incubated for exactly 60 minutes in the water bath at 37^oC. After which 0.5ml of 2,4 – dinitrophenylhydrazine (1 mmol) was added. The mixture was then allowed to stand for exactly 20 minutes at between 20 – 25^oC. Five milliliters (5mls) of sodium hydroxide (0.4N) was then added. The activity of AST/ALT was then determined spectrophotometrically (Beckman Model 24) at 505nm after 10 minutes. The ALP activity was determined by the method of King and King (1954). This was done by pipetting 2.0ml of buffered substrate (equal volumes of 0.01M sodium carbonate – sodium bicarbonate buffer at pH 10.0 and 0.01M disodium phenyl phosphate substrate) into a test tube. It was left in a water bath (37^oC) to warm for 5 minutes. Then, 0.1ml of serum/plasma was added. The mixture was then incubated at 37^oC for exactly 15 minutes. After which 0.8ml of 0.5N sodium hydroxide was added followed by 1.2ml of 0.5M sodium bicarbonate. Then 1.0ml of 0.6 % 4 – aminoantipyrene was added. Lastly 1.0ml of 2.4 % potassium ferricyanide was added. The activity of ALP was determined immediately spectrophotometrically (Beckman Model 24) at 520nm. All determinations were done on duplicate samples each time and the average of the two results was recorded. Results obtained from subsequent determinations (after the hour 0 baseline value) were compared with the BV obtained at hour 0 using repeat measure analysis of variance (ANOVA). The results obtained from the samples kept at 4^0C were also compared with that obtained from samples kept at 32^0C at each point of determination using t-test. Also the serum and plasma results per unit time were compared with each other using the t-test. In this paper the results are presented as mean percentage changes from the BV. Percentage increases above the BV were indicated with a positive sign (increased enzyme activity from the BV) while percentage decreases were indicated with a negative sign (decreased enzyme activity from the BV).

Results

The ALT determinations on the serum samples showed a decrease in activity across the period of storage (Table 1) from the baseline value (BV) for both storage temperatures. The mean serum ALT was found to be significantly different ($P < 0.05$) from the BV from the 72^{nd} hour of storage onwards till the 120^{th} hour for the samples storage at 4^0C, while for the serum samples stored at 32^0C a significant different ($P< 0.05$) from the BV was recorded from the 20^{th} hour of storage onwards till the 120^{th} hour. The mean serum ALT activity of the samples stored at 4^0 C was found to be significantly different ($P < 0.05$) from that of samples stored at 32^0 C from the 30^{th} hour of keeping. For the plasma ALT, enzyme activity also decreased across the period of storage from the BV (Table1) for both storage temperatures. Significant differences ($P < 0.05$) from the BV were recorded as from the 42^{nd} hour of storage for the plasma samples stored at 4^0 C and 12^{th} hour of storage for the samples stored at 32^0 C. The mean plasma ALT activity of the samples stored at 4^0C was found to be significantly different from those stored at 32^0C as from the 96^{th} hour of storage.

Table 1: The percentage change in alanine aminotransferase in serum and plasma samples stored at varied temperatures

Duration of storage (hrs)	Mean percentage change ± SD			
	Serum (4^0C)	Serum (32^0C)	Plasma (4^0C)	Plasma (32^0C)
2	± 0.00	- 0.3 ± 0.06	- 0.5 ± 0.10	- 1.0 ± 0.32
4	-0.3 ± 0.09	- 0.9 ± 0.13	- 0.5 ± 0.14	- 1.8 ± 0.25
6	- 0.5 ± 0.14	- 1.5 ± 0.21	- 0.7 ± 0.08	- 2.3 ± 0.39
8	- 0.9 ± 0.08	- 2.2 ± 0.42	- 1.1 ± 0.16	- 3.5 ± 0.54
10	- 1.5 ± 0.15	- 2.9 ± 0.51	-1.7 ± 0.23	- 3.9 ± 0.68
12	- 1.7 ± 0..30	- 3.7 ± 0.64	- 2.5 ± 0.38	- 4.61 ± 1.01
16	- 2.2 ± 0.40	- 4.4 ± 1.15	- 2.8 ± 0.30	- 5.1 ± 1.21
20	- 2.6 ± 0.51	- 5.0 ± 1.37	- 3.1 ± 0.34	- 5.9 ± 1.51
24	- 2.9 ± 0.33	- 5.5 ± 1.51	- 3.6 ± 0.65	- 6.5 ± 2.13
30	- 3.3 ± 0.29	- 7.1 ± 1.45	-4.2 ± 1.07	- 7.4 ± 2.26
36	- 3.8 ± 0.47	- 7.8 ± 1.58	- 4.9 ± 1.09	- 8.6 ± 2.37
42	- 4.5 ± 0.71	- 8.5 ± 1.70	- 5.9 ± 1.58	- 9.7 ± 2.65
48	- 4.8 ± 0.82	- 9.3 ± 1.58	- 6.8 ± 2.00	- 10.8 ± 1.97
72	- 6.0 ± 1.54	-11.2 ± 2.14	- 8.2 ± 1.89	- 14.5 ± 2.45
96	- 6.7 ± 1.71	-15.0 ± 2.41	- 9.5 ± 2.57	- 19.9 ± 2.61
120	- 7.5 ± 2.10	-18.7 ± 2.89	- 11.2 ± 3.05	- 23. 4 ± 3.41

Table 2: The percentage change in aspartate aminnotransferase in serum and plasma samples stored at varied temperatures

Duration of storage (hrs)	Mean percentage change ± SD			
	Serum (4^0C)	Serum (32^0C)	Plasma (4^0C)	Plasma (32^0C)
2	- 0.5 ± 0.08	- 0.8 ± 0.10	- 0.8 ± 0.08	- 1.2 ± 0.21
4	- 1.0 ± 0.30	- 1.6 ± 0.16	- 1.3 ± 0.16	- 1.9 ± 0.34
6	- 1.3 ± 0. 20	- 2.4 ± 0.20	- 1.9 ± 0.15	- 2.8 ± 0.54
8	- 1.6 ± 0.27	- 3.0 ± 0.23	- 2.3 ± 0.20	- 3.9 ± 0.67
10	- 2.1 ± 0..43	-3.8 ± 0.18	- 2.7 ± 0.27	- 4.7 ± 0.92
12	- 2.6 ± 0.41	- 4.5 ± 1.21	-3.0 ± 0.35	- 5.4 ± 1.64
16	-3.0 ± 0.47	- 5.3 ± 1.50	-3.7 ± 0.54	- 6.3 ± 2.07
20	-3.6 ± 0.54	-7.4 ± 1.56	- 4.2 ± 0.80	-7.9 ± 2.21
24	-4.0 ± 0.80	- 8.1 ± 1.72	- 4.3 ± 0.75	- 9.6 ± 2.58
30	-4.2 ± 0.98	-8.6 ± 1.65	- 4.8 ± 0.84	- 11.4 ± 3.01
36	-4.8 ± 1.47	- 10.5 ± 1.71	-5.6 ± 1.09	- 12.8 ± 2.94
42	-5.7 ± 1.56	-11.3 ± 1.94	-6.4 ± 1.94	- 15.7 ± 3.20
48	-6.4 ± 2.05	- 11.9 ± 2.08	- 7.3 ± 2.10	- 19.3 ± 3.38
72	-7.5 ± 2.20	-15.2 ± 2.41	- 9.2 ± 2.64	- 38.1 ± 5.09
96	-7.2 ± 2.25	- 30.0 ± 3.57	- 8.8 ± 2.24	- 45.2 ± 6.54
120	-6.8 ± 2.09	-37.5 ± 4.09	- 7.1 ± 2.19	- 40.4 ± 7.94

Table 3: The percentage change in alkaline phosphatase in serum and plasma samples stored at varied temperatures

Duration of storage (hrs)	Mean percentage change ± SD			
	Serum (4^0C)	Serum (32^0C)	Plasma (4^0)	Plasma (32^0C)
2	+ 0.6 ± 0.05	+ 1.0 ± 0.22	+ 0.8 ± 0.07	+ 1.7 ± 0.15
4	+ 1.3 ± 0.15	+ 1.8 ± 0.17	+ 1.6 ± 0.14	+ 2.3 ± 0.21
6	+1.7 ± 0.27	+ 2.2 ± 0.24	+ 2.1 ± 0.21	+ 3.1 ± 0.35
8	+ 1.4 ± 0.22	+ 1.5 ± 0.19	+ 3.0 ± 0.34	+ 2.0 ± 0.40
10	+ 0.7 ± 0.12	- 2.7 ± 0.55	+1.9 ± 0.15	- 3.0 ± 1.05
12	- 1.5 ± 0.19	- 5.4 ± 1.67	- 0.4 ± 0.09	- 5.9 ± 1.26
16	- 1.9 ± 0.26	- 5.9 ± 1.59	-1.7 ± 0.33	- 6.5 ± 1.57
20	- 2.6 ± 0.25	- 6.5 ± 1.97	- 3.2 ± 1.59	- 6.8 ± 2.07
24	- 4.1 ± 1.11	- 7.3 ± 2.17	-4.1 ± 1.45	- 7.7 ± 2.21
30	-4.9 ± 1.29	- 8.0 ± 2.30	- 4.8 ± 1.50	-8.2 ± 2.24
36	- 5.7 ± 1.46	- 8.5 ± 2.25	- 6.5 ± 1.74	- 9.5 ± 2.51
42	-6.4 ± 1.92	- 9.4 ± 2.50	-7.6 ± 1.96	- 11.1 ± 3. 01
48	-6.9 ± 2.03	-10.2 ± 2.79	- 8.9 ± 2.30	- 13.7 ± 3.21
72	-7.3 ± 2.27	-11.6 ± 3.05	- 9.9 ± 2.60	- 17.3 ± 3.38
96	-7.8 ± 2.40	-14.7 ± 3.42	- 11.6 ± 3.21	- 23.6 ±3.67
120	- 8.3 ± 2.53	- 20.2 ± 3.83	- 13.5 ± 3.09	- 30.4 ± 3.59

There were no significant differences ($P > 0.05$) between the serum ALT and plasma ALT values of samples stored at both 4^oC and 32^oC.

The AST values of the serum samples decreased in activity (Table 2) from their BV across the period of storage for samples stored at both 4^oC and 32^oC. Statistical analysis showed that a significant change from the BV occurred as from the 42^{nd} hour of storage for serum samples stored at 4^0C and as from the 12^{th} hour of storage for the serum samples stored at 32^0C. Significant differences between the mean AST activity of the

serum samples stored at 4^0C and 32^0C was recorded as from the 72^{nd} hour of storage. The results of the plasma AST determinations also showed that there was a decrease in enzyme activity (Table 2) from the BV across the storage period for samples stored at both 4^0C and 32^0C. Significant differences ($p < 0.05$) from the plasma AST BV was recorded as from the 42^{nd} hour of storage for the samples stored at 4^0C and as from the 10^{th} hour of storage for the plasma samples stored at 32^0C. The AST activity of the plasma samples stored at 4^0C and 32^0C were found to significantly differ as from the 8^{th} hour of storage. Again, there were no significant differences ($P > 0.05$) between the serum AST and plasma AST activities of samples stored at both 4^oC and 32^oC.

Results of the ALP determinations on the serum samples showed that for both 4^0C and 32^0C storage temperatures, the ALP activities of the serum samples at first increased from its BV during the first 8 hours of storage but much later started decreasing across the storage period (Table 3). Significant differences ($p < 0.05$) from the BV were recorded as from the 36^{th} hour of storage for the serum samples stored at 4^0C and as from the 10^{th} hour of storage for the serum samples stored at 32^0C. The ALP activities of the samples stored at 4^0C were found to significantly differ ($p < 0.05$) from that of the samples stored at 32^0C as from the 10^{th} hour of storage. The ALP activities of the plasma samples also increased from their BV during the first 6 - 8 hours of storage at both 4^0C and 32^0C, and later decreased progressively across the storage period (Table 3). Significant differences ($p < 0.05$) from the BV was recorded as from the 36^{th} hour of storage for the samples stored at 4^0C and as from the 10^{th} hour of storage for the samples stored at 32^0C. The ALP activities for the plasma samples stored at 4^0C and 32^0C were found to significantly differ from each other as from the 10^{th} hour of storage. A comparison of the serum and plasma ALP activities obtained at the two temperatures of storage showed that the ALP activities of the serum samples stored at 4^0C significantly differed ($p < 0.05$) from that of the plasma samples stored at 4^0C as from the 48^{th} hour of storage, while for the samples stored at 32^0C, the ALP activities of the serum samples significantly differed ($p < 0.05$) from that of the plasma samples as from the 42^{nd} hour of storage.

Discussion

The results of the determinations of ALT on the samples indicate that refrigerated serum and plasma samples can give reliable ALT values (values not significantly different from the BV) if analysis is done within 48 hours and 36 hours of collection of sample respectively, while serum and plasma samples stored at 32^0C can give reliable results within 16 hours and 10 hours of collection respectively. With respect to AST, the results of the study indicate that reliable AST values can be obtained for serum and plasma stored 4^0C if analysis is carried out within 36 hours of sample collection, while for serum and plasma samples stored at 32^0C reliable AST values can be obtained if analysis is carried out within 10 hours and 8 hours of collection respectively. For the ALP, our study indicates that both serum and plasma samples stored at 4^0C can give reliable results within 30 hours of storage, while serum and plasma stored at 32^0C can give reliable ALP values within 10 hours of storage.

In general, the outcome of this study had shown that reliable results (values not significantly different from the BV) can be obtained from both serum and plasma samples stored at both 4^0C and 32^0C for definite storage durations, with the refrigerated samples being more stable than the samples stored at 32^0C for the enzymes assayed. The greater stability of these enzymes in refrigerated samples when compared to those kept at room temperature is in agreement with earlier reports on related studies (Rehak and Chiang, 1988; Heins *et al*., 1995), though the room temperatures of storage in these studies were not 32^0C. This could be due to the denaturation of enzyme proteins at higher temperature leading to greater loss of activity at 32^oC (Anfinsen, 1973).

Only few previous studies have investigated the stability of clinical chemistry analytes in blood kept unseparated beyond 24 hours. Ono *et al* (1981) drew blood from 10 volunteers into plain serum tubes and left aliquots of whole blood to stand at 4^oC, 23^oC, and 30^oC for up to 48 hours. While they found little change in ALT and AST at 4^oC throughout the storage duration, the activities of these enzymes increased significantly after 8 hours at 23^oC. Greater increase in enzyme activity was observed for samples stored at 30^oC.

In another study, Clark *et al* (2003) investigated the stability of plasma analytes in whole blood samples drawn from 12 volunteers and stored at 21^oC and 4^oC for up to 7 days before plasma separation. The aliquoted plasma samples were stored at -80^oC and subsequently analysed in one analytical run for a range of chemistries. They found ALT to change by only 2% up to 48 hours at 4^oC and by 4% up to 48 hours at 21^oC.. They also reported that AST changed by 5% at 4^oC and by 35% at 21^oC after 48 hours. The study designs of Ono *et al* (1981) and Clark *et al* (2003) were similar to the present work except that whole blood samples were stored under each temperature conditions up to the appropriate time-point, then centrifuged and the plasma/serum aliquoted while in the present work, whole blood samples were centrifuged and the serum/plasma aliquoted and stored at the temperature conditions up to the appropriate time point.

Ono *et al* (1981) and Clark *et al* (2003) reported that the activity of AST and ALT increased over time. By contrast, we, however, found the activities of these enzymes to decrease over time. The discrepancy between these results may reflect differences in the study design. Since serum/plasma specimens were left in contact with clot/red blood cells in the previous studies, changes in cell membrane integrity leading to movement of water into cells (haemoconcentration) and haemolysis could contribute to the increase in AST and ALT activities because there is higher activity of

these enzymes in erythrocytes compared with serum/plasma (Sonntag, 1986).

Fadiglo *et al*, (2004) investigated the thermal and carbon dioxide inactivation of ALP in buffer and milk. The thermal stability of the enzyme was examined by measuring the residual activity after heat treatment for various periods. They observed that the inactivation of ALP in glycine/NaOH buffer, pasteurized milk and raw milk was found to be higher as the temperature increased from 20 to 50^{o}C. Similar effect of temperature on the stability of ALP was observed in the present study. At both storage temperatures (4^{o}C and 32^{o}C), there was an initial increase in the activity of ALP followed by a decline in enzyme activity. This initial increase in the activity of ALP may be due to the formation of a phosphate-lipoprotein complex or a multimeric form of the enzyme during storage (Kim and Wyckoff, 1989).

For the ALT and AST results, there was a time-related progressive decline in enzyme activity, but for ALP there was an initial increase followed later by a decline in enzyme activity. The progressive decline with time in ALT and AST values and the initial increase followed by decline in ALP is consistent with the earlier reports of some investigators (Cuccherini *et al.,* 1983; Murphy *et al.,* 2000).

It should be noted that for both storage temperatures the enzyme activity in serum samples was more stable than that recorded for plasma samples. Other investigators (Young and Bermes, 1999; Dufour *et al.,* 2000; Boyanton and Blick, 2002) have also reported the greater stability of enzymes in serum when compared to plasma, and this makes serum a preferred sample for the assay of these enzyme activities. The greater instability of these enzymes in plasma samples could be attributed to the interference by the anticoagulant used in sample collection and in addition to increase in lactate concentration in the case of plasma ALT (Boyanton and Blick, 2002).

It can thus be generally advised based on the results of this present study that in order to obtain reliable results for serum and plasma ALT, AST and ALP, samples stored at 4^{0}C should be analysed within 30 hours of collection while for samples stored at 32^{0}C, analysis should be carried out within 8 hours.

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