ISSN 2394–806X (Print), ISSN 2454-5139 (Electronic)
IJHRMLP, Vol: 04 No: 01 January, 2018
Printed in India




**ORIGINAL PAPER**

# A study of effect of storage condition on blood alcohol concentration in living subjects

***Kishorkumar DG[1], Anand P Rayamane[2], Kumar MP[3]***

***Received on July 20, 2017; editorial approval on November 17, 2017***

**ABSTRACT**

***Introduction****: Many studies have demonstrated that both generation and loss of alcohol in stored blood samples. Studies have concluded that both high temperature and an insufficient enzyme inhibitor concentration can result in alcohol generation, presumably as a result of bacterial fermentation. Factors most affecting the stored blood samples, to be used for ethanol determination were the duration and temperature of storage and concentration of preservative.* ***Objectives****: To determine whether alcohol is generated or lost in blood samples stored at different periods and with the presence or absence of preservative and refrigeration.* ***Methods****: 40 adult males of drunkenness were selected as subjects for estimation of blood alcohol. Then after taking written informed consent 30ml of blood is collected from the each individuals. 5 samples were preserved in sodium fluoride vacutainer and refrigerated at 4°C. Another 5 samples were preserved in plain vacutainer and kept at room temperature. Then these were subjected to estimation of blood alcohol concentration (BAC) by Gas Chromatography-Flame Ionization Detector (GC-FID) at various interval of time i.e., on 2nd, 7th, 14th, 30th and 60th day.* ***Conclusion****: BAC in samples without preservative and without refrigeration has fallen significantly as storage period increases at each point of time compared to BAC with preservative NaF (Sodium Fluoride) and with refrigeration at 4°C. NaF and refrigeration of samples at 4°C significantly prevents loss of BAC in stored samples.*

***Keywords****: Blood Alcohol Concentration; Sodium Fluoride*

## INTRODUCTON

The accurate determination of alcohol concentration levels in human blood samples is important for valid results in research studies and often has critical medical and legal ramifications in forensic and toxicological reports.[1] Many studies have demonstrated that both generation and loss of alcohol in stored blood samples. Studies have concluded that both high temperature and an insufficient enzyme inhibitor concentration can result in alcohol generation, presumably as a result of bacterial fermentation.[2] Factors most affecting the stored blood samples, to be used for ethanol determination were the duration and temperature of storage and concentration of preservative.[3] Ethanol losses in samples are positively correlated with the length of storage and the original ethanol concentration in the blood.[4] Antemortem blood samples stored at room temperature or higher will cause a decrease in BAC, not an increase.[5]

Post-mortem production of ethanol up to 70 mg% till 7th day and in few cases even up to 14th day. After 14th day there is loss of ethanol that further decreased on 28th day to become alcohol free.[6] Majority of cases showed higher number of BAC till 20 days thereafter from 21 days to 30 days they found subsequent decrease in BAC.[7] The possibility of investor synthesis of ethanol in samples has been raised, as well as loss due to evaporation or adsorption of the ethanol onto rubber stopper.[8] Increase in post-mortem ethanol production is due to the presence of bacteria. More than 50 species of bacteria, yeast and fungus were capable of producing post-mortem ethanol.[9] However in some studies freshly collected blood samples have shown that concentrations do not change in preserved samples stored in room temperature for up to two months or refrigerates samples stores up to 10 months.[10]

## OBJECTIVE

To determine whether alcohol is generated or lost in blood samples stored at different periods and with the presence or absence of preservative and refrigeration.

## METHODOLOGY

40 adult males who were brought by the police to the emergency

**Address for correspondence**:

[1]Asst Prof of Forensic Medicine, Karwar Institute of Medical Sciences, Karwar

[2]Asst Professor **(Corresponding Author)**

**Email**: anandprayamane@gmail.com

**Mobile**: +917259114490

[3]Prof and HOD of Forensic Medicine, Mysore Medical College and Research Institute Mysore

department for drunkenness examination were selected as subjects for estimation of blood alcohol. Then after taking written informed consent 30ml of blood is collected from the individuals who have consumed alcohol 2 hours prior. Then the collected 30ml blood sample was equally divided into 10 parts of 3ml each, out of which 5 samples were preserved in sodium fluoride vacutainer which contained 3mg NaF per ml of blood. Samples were well mixed and refrigerated at 4°C. Another 5 parts are preserved in plain vacutainer and kept at room temperature. Then these were subjected to estimation of blood alcohol concentration (BAC) by Gas Chromatography-Flame Ionization Detector (GC-FID) at various interval of time i.e., on 2nd, 7th, 14th, 30th and 60th day. Institutional Ethical committee clearance was obtained prior to conducting this study.

**RESULTS**

Tables 1 depicts BAC with preservative and with refrigeration and BAC without preservative and without refrigeration (comparison of mean differences of BAC in two groups at individual points of time) shows significant changes i.e., BAC in samples without preservative and without refrigeration had fallen significantly as storage period increases at each point of time compared to BAC with preservative and with refrigeration.

**Table 1** Comparison of mean differences of BAC in two groups at individual points of time

| BAC at end of | Group | Number of samples | Mean BAC | P value |
|---|---|---|---|---|
| **2nd Day** | With Preservative and with Refrigeration | 40 | 57.21 | 0.001 |
| | Without Preservative and Without Refrigeration | 40 | 47.09 | |
| | Total | 80 | | |
| **7th Day** | With Preservative and with Refrigeration | 40 | 51.45 | 0.0003 |
| | Without Preservative and Without Refrigeration | 40 | 42.50 | |
| | Total | 80 | | |
| **14th Day** | With Preservative and with Refrigeration | 40 | 55.84 | 0.0089 |
| | Without Preservative and Without Refrigeration | 40 | 49.20 | |
| | Total | 80 | | |
| **30th Day** | With Preservative and with Refrigeration | 40 | 49.20 | 0.0168 |
| | Without Preservative and Without Refrigeration | 40 | 44.40 | |
| | Total | 80 | | |
| **60th Day** | With Preservative and with Refrigeration | 40 | 41.92 | 0.0177 |
| | Without Preservative and Without Refrigeration | 40 | 34.68 | |
| | Total | 80 | | |

Mann-Whitney Test the two-tailed P value is considered significant.

**Table 2** depicts, when BAC samples were analysed within the groups i.e., samples with preservative and with refrigeration, we found that BAC had fallen significantly as storage time increased in this group.

**Table 2** BAC (in mg %) level with Preservative and with Refrigeration at 2nd, 7th, 14th, 30th and 60th day

| BAC at end of | Mean Rank |
|---|---|
| 2nd at end of | 4.08 |
| 7th Day | 2.78 |
| 14th Day | 3.95 |
| 30th Day | 2.80 |
| 60th Day | 1.40 |

*Chi Square 75.34, Degree Freedom 4*

***Friedman Test**: The P value is < 0.0001, considered extremely significant.*

**Table 4** depicts, Mean BAC in samples with preservative and with refrigeration, in which Mean BAC had fallen gradually as storage period increased except on the 14th day where Mean BAC increased and then fell gradually.

**Table 3** depicts, when BAC samples were analysed within the groups i.e. samples without preservative and without refrigeration, we found that BAC had fallen significantly as storage time increased in this group.

**Table 3** BAC (in mg %) level without Preservative & without Refrigeration at 2nd, 7th, 14th, 30th and 60th day

| BAC at end of | Mean Rank |
|---|---|
| 2nd at end of | 3.50 |
| 7th Day | 2.70 |
| 14th Day | 4.00 |
| 30th Day | 3.08 |
| 60th Day | 1.73 |

*Chi Square 47.54, Degree Freedom 4*

***Friedman Test**: The P value is < 0.0001, considered extremely significant.*

**And** Mean BAC in samples without preservative and without refrigeration in which Mean BAC has fallen gradually as storage period increased except on 14th day where Mean BAC had increased and then fell gradually.

**Table 4** Mean BAC (in mg %) level with Preservative & with Refrigeration *v/s* Mean BAC (in mg %) level without Preservative & without Refrigeration

| Days | Mean BAC in Sample with Preservative & with Refrigeration | SD in Sampe with Preservative & with Refrigeration | Mean BAC in Sample without Preservative & without Refrigeration | SD in Sampe without Preservative & with Refrigeration |
|---|---|---|---|---|
| **2nd Day** | 57.21 | 11.47 | 47.09 | 11.08 |
| **7th Day** | 51.45 | 12.13 | 42.50 | 6.35 |
| **14th Day** | 55.84 | 9.26 | 49.20 | 11.03 |
| **30th Day** | 49.20 | 13.08 | 44.40 | 10.74 |
| **60th Day** | 41.92 | 10.72 | 34.68 | 14.75 |

## DISCUSSION

**BAC at 2nd, 7th, 14 4h, 30th and 60th day in Samples with preservative and with refrigeration *v/s* Samples without preservative and without refrigeration**

BAC with preservative and with refrigeration and BAC without preservative and without refrigeration which shows significant changes i.e., BAC in samples without preservative and without refrigeration have fallen significantly as storage period increases at each point of time compared to BAC with preservative and with refrigeration.

The highlight of our study is use preservative NaF & refrigeration of samples at 4°C for analysing BAC in which fall of BAC is significantly less than those samples without preservative and without refrigeration.

Lewis RJ et al.[11] concluded that NaF and refrigeration is preferred way of storage for estimation of blood alcohol concentration of the blood samples.

In the study conducted by dubowskietal.[12] the samples which were analysed without preservative and without refrigeration showed a decrease in the BAC and when they studied the samples with preservative (NaF and biocide sodium aside) and with refrigeration there was no significant change in BAC. So also Brown et al.[3] in his study concluded that factors most affecting the stored blood samples were duration, temperature of storage and concentration of preservative. Similarly Wigmore JG[5] in his study concluded that the most accurate determination of BAC is from the blood samples which are refrigerated and Ma Dong[13] concluded the best condition for keeping ethanol stable in blood is refrigeration with preservative and with 50% of air chamber in container. Also the results of Slavka et al.[14] showed that alcohol concentrations were significantly reduced with the increase of temperature and prolongation of storage. Room temperature storage of samples is the least suitable way of keeping them, independent of the duration of storage. The temperature of storage, duration of storage, selection of preservatives and air quantity above the sample are said to be the most common causes of changes in the value of ethanol in whole blood samples. There is the synergism of these influences and it is hard to discuss the conditions separately. Wichai Wong chanapai[15] concluded that the concentrations of ethanol in bloods with 1% sodium fluoride as preservative stored at 4°C were more stable than at -20°C and room temperature.

**BAC at 2nd, 7th, 14 4h, 30th and 60th day in Samples with preservative and with refrigeration**

When BAC samples analysed within groups i.e., samples with preservative and with refrigeration, we found that BAC has fallen significantly as storage time increased in this group.

**AND BAC at 2nd, 7th, 14 4h, 30th and 60th day in Samples without preservative and without refrigeration**

When BAC samples analysed within groups i.e. samples without preservative and without refrigeration, we found that BAC has fallen significantly as storage time increased in this group.

BAC in above mentioned both groups i.e. samples with preservative NaF and with refrigeration at 4°C and samples without preservative and without refrigeration has significantly fallen gradually as storage period increased.

Reason for loss of BAC in above mentioned both groups can be attributed to the chemical oxidation of the stored samples as well as due to the evaporation and adsorption.

Jones AW[16] showed that ethanol losses in samples are positively correlated with the length of storage and the original ethanol concentration in the blood. Moynham et al.[17] found that in blood taken from living subjects, there was no alcohol generation regardless of varying storage temperatures, times and the presence or absence of an enzyme inhibitor, but there was some alcohol depletion after longer storage times. Shan X et al.[18] found that alcohol positive cases showed various changes in BAC ranging from no significant change to a 47% decrease and concluded long term storage either under refrigeration, at or above room temperature decreased BAC. Tracey Winek[19] inferred that whole blood samples stored for 35 days at 26.7°C to 37.8°C lost alcohol and the percentage loss of BAC averaged between l0-19%. And important mechanism with regard to stability of alcohol in stored blood was a strongly temperature dependent alcohol oxidation reaction which was not inhibited by sodium fluoride. Avbel AJ.[20] showed blood samples without preservative stored under refrigeration (3°C) for 18 months to 2 years, showed decrease in ethanol content. The decrease were attributed to oxidation and (or) evaporation. Slavka Mandic-Radic[14] showed that alcohol concentrations were significantly reduced with the increase of temperature and prolongation of storage. Wichai Wong chanapai[15] concluded that the loss of ethanol in stored whole blood sample was due to the chemical oxidation rather than the physical loss. Anderson SG et al.[2] found that consistently

higher rates of alcohol depletion in the preserved samples might reflect salting-out effect and/or some reaction alcohol and sodium fluoride. Dubowski et al.[12] showed that ethanol levels in whole blood samples stored up to 1 year (refrigerated at 4°C) without preservative declined slightly (less than 5%), but this decrease was not statistically significant. Samples stored with the preservative and biocide sodium aside did not show any ethanol degradation over the 12-month storage period. Glendenning BL and Waugh TC[10] concluded freshly collected blood samples have shown that concentrations do not change in preserved samples stored in room temperature for up to two months or refrigerated samples stores up to 10 months. Charies L et al.[8] concluded in their study that Alcohol analyses of blood obtained aseptically from living humans can be delayed for as long as 14 days without a significant change in alcohol content. This hold true whether the blood sample is refrigerated or not, or whether a preservative is added to sample or not.

**Mean BAC in with preservative and with refrigerated samples *v/s* Mean BAC in without preservative and without refrigeration samples**

Mean BAC in samples with preservative and with refrigeration, in which Mean BAC has fallen gradually as storage period increased except on the 14th day where Mean BAC is increased and then fell gradually.

Mean BAC in samples without preservative and without refrigeration in which Mean BAC has fallen gradually as storage period increased except on 14th day where Mean BAC has increased and then fell gradually.

In above mentioned both groups mean increase in BAC at 14th day could be attributed to microbial fermentation due to contamination.

C B Jani et al.[7] concluded that majority of cases showed higher number of BAC till 20 days thereafter from 21 days to 30 days they found subsequent decrease in BAC in antemortem sample. Avbel AJ[20] studied post-mortem human blood samples without preservative stored under refrigeration (3°C) for 18 months to 2 years, observing increase and decrease in ethanol content. The decrease were attributed to oxidation and (or) evaporation, the increases to post-mortem synthesis of ethanol by microbial fermentation of glucose. Anderson SG et al.[2] concluded that both high temperature and an insufficient enzyme inhibitor concentration can result in alcohol generation, presumably as a result of bacterial fermentation. Stojan Pet kovic et al.[21] confirmed that the absences of preservative and prolonged storage at higher temperatures are not necessarily sufficient for alcohol production in antemortem blood samples. Singh and Chandra[6] have reported that on 14th day of analysis there is post-mortem loss of ethanol that further decreased on 28day to that maximum to become alcohol free. However they also reported that maximum production of ethanol occur as 70mg % mostly within 7th day.

Usually the samples for BAC estimation which were stored without preservative at room temperature were analysed after longer duration (approximately 1 to 2 months), which lead to significant loss of BAC as the storage period increased. We recommend that samples for BAC estimation should be ideally preserved in sodium fluoride vacationer with refrigeration at 4°C.

## CONCLUSION

Present study concludes BAC in samples without preservative and without refrigeration has fallen significantly as storage period increases at each point of time compared to BAC with preservative NaF and with refrigeration at 4°C. NaF and refrigeration of samples at 4°C significantly prevents loss of BAC in stored samples.

**Source of funding**: Nil.

**Acknowledgements**: RFSL Mysore.

**Conflict of interest**: Nil.

## REFERENCES

1. Penetar D M, McNeil J F, Ryan E T, Lukas S E. Comparison among plasma, serum, and whole blood ethanol concentrations: impact of storage conditions and collection tubes. J of Analytical Toxicology 2008 September;32:505-10.
2. Anderson SG. The effects of storage on the accuracy of blood alcohol readings. [cited 2017 November 20]; Available from: URL:www.jiscmail.ac.uk
3. Brown G A, Neylan D, Reynolds W J, Smalldon KW. The stability of ethanol in stored blood. Part 1: Important variables and interpretations of results. AnlChimActa 1973;66:271-83.
4. Justice Kannan K, Mathiharan K, editors. A textbook of medical jurisprudence and toxicology. 24th ed. Nagpur: Lexis Nexis Butterworths Wadhwa; 2011.p. 177-85.
5. Wigmore, JG. Do Blood Samples Need to be Refrigerated.[cited 2017 November 20]; Available from: URL:http://www.wigmoreonalcohol.com/do-blood-samples-need-to-be-refrigerated
6. Singh R K and Chandra H. Estimation of post-mortem production of and loss of ethanol in blood with respect of its duration of storage at room temperature. Int J Med Tocicol Leg Med 1999;2(1):1-4.
7. CB Jani, Sanjay Gupta, Hitendra Barot, Jaydeep Gadhavi. Retrospective study of cases of drunkenness with emphasis on procedure and interpretation of results. J Indian Acad Forensic Med 30(3):128-35.
8. Charles L, Winek and Loutte, J Paul. Effects of short term storage conditions on alcohol concentrations in blood from living human objects. Cli Chem 1983;29(11):1959-60.
9. CO'Neal, Poklis A. Post-mortem production of ethanol and factors that influence interpretation a critical review. Am J Forensic Med Pathol 1996;17:8–20.
10. Glendening BL, Waugh TC. The study of ordinary blood alcohol samples held various periods of time under different conditions. J Forensic Sci 1965;10:192-200.
11. Lewis R J, Johnson R D, Angier M K, Vu T N. Ethanol formation in unadulterated post-mortem tissues. Forensic Science International 2004 May;146:17-24.
12. Dubowski K M, Gadsden R H, Poklis A. The stability of ethanol in human whole blood controls: an interlaboratory evaluation. J of Analytical Toxicology 1997 Oct;21:486-91.
13. Ma D, Zhuo XY, Bu J, Xiang, Shen BH. Research of on the stability of ethanol in preservation of ethanol in blood. Fa Yi Xue Za Zhi 2007 Apr; 23(2):117-9.
14. Slavka Mandic-Radic, Gordana Dzingalasevic, Nevena Lukovic. Stability of ethanol in blood and urine samples. JMB 2007;26(3):241-4.
15. Wichai Wongchanapai, Dech Dokpuang, Siriwan Sasithonrojanachai, Somboon Tamtakerngkit. Stability of post-mortem blood ethanol under experimental conditions. Siriraj Med J 2008 Mar-Apr;60(2):62-5.
16. A W Jones. Are changes in blood ethanol concentration during storage analytically significant importance of method imprecision. Am J Clin Patho 2007;74:1299-304.
17. Moynham AF, Beverstock R, Perl J, Starmer GA. The effects of storage on the blood alcohol readings. Paper presented at: proceedings of the ninth international conference on alcohol, drugs and traffic safety.1983; San Juan, Puerto Rico.
18. Xiaoqin Shan, Nicholas B Tiscione, Ilene Alford, Dustin Tate Yeatman. A study of blood alcohol stability in forensic antemortem blood samples. Forensic Science International 2011;211:47–50.
19. Tracey Winek, Charles L Winek, Wagdy W Wahba. The effect of storage at various temperatures on blood alcohol concentration. Forensic Science International 1996;78:179-85.
20. Avbel AJ. Some factors affecting the analytical determination of ethanol in human blood and tissues. [Masters Thesis]. Duquense University Graduate school, Pittsburgh, PA:1972.
21. Stojan Petkovic, Slobodan Savic, Dragana Zgonjanin, Isidora Samojlik. Ethanol concentrations in antemortem blood samples under controlled conditions. Alcohol and Alcoholism 2008;43(6):658–60.