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Linear Regression Analysis and Correlation Findings in Hemoglobin Oxidation Studies in Diabetics Blood and Normal Blood: The Effects of Amyl Nitrite and Butyl Nitrite

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Abstract

Enhanced susceptibility of amyl nitrite or butyl nitrite induced oxidation of oxyhemoglobin into methemoglobin in the Type 2 diabetics blood compared to normal blood in independent studies was confirmed ($P<0.05$). For amyl nitrite induced oxidation of hemoglobin a linear regression line was calculated from 40 donors, 20 of whom were diabetics and 20 of whom were normal or non diabetics, using all their HbA1C (%) values and the measured hemoglobin oxidation times. The resultant equation was that of a straight line which is most accurately described by the equation $Y = 4.43 - 0.25X$ where X = percent of HbA1C and Y = hemoglobin oxidation time in minutes. The standard deviation of points around the fitted line was 0.449 and the standard errors of the slope and intercept were 0.0230 and 0.207, respectively. The product moment correlation coefficient (r) was found to be - 0.87. For butyl nitrite induced oxidation of hemoglobin another linear regression line was calculated from all donor HbA1C values and all of the measured hemoglobin oxidation times from another 40 donors, 20 of whom were diabetics and 20 of whom were normal. The resultant equation was that of a straight line which is most accurately described by the equation $Y = 7.17 - 0.53X$ where X = percent of HbA1C and Y = hemoglobin oxidation time in minutes. The standard deviation of points around the fitted line was 1.00 and the standard errors of the slope and intercept were 0.0586 and 0.472, respectively. The product moment correlation coefficient (r) was found to be - 0.83. Thus, based on the fact that both correlation coefficients are less than -0.75 this study demonstrates that there is a very good to excellent inverse relationship between HbA1C percentage and the hemoglobin oxidation time for both amyl nitrite and butyl nitrite. This similar finding could be attributed to the fact that these alkyl nitrites differ only in one methylene molecule. It then follows that both of the aforementioned equations can be used to predict the rate of hemoglobin induced oxidation by amyl nitrite or butyl nitrite based solely upon the HbA1C (%) value. Such equations could then be of good predictive value as to the susceptibility of hemoglobin induced oxidation by either of these alkyl nitrites.

Keywords: Amyl Nitrite; Butyl Nitrite; Diabetes; HbA1C; Hemoglobin Oxidation; Methemoglobin

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Introduction

Amyl nitrite and butyl nitrite are chemical compounds that cause the oxyhemoglobin to undergo oxidation, i.e. the iron (II) in the hemoglobin loses an electron to become iron (III). When this occurs the ruby red oxyhemoglobin changes in color to a chocolate brown which signifies it has become methemoglobin or iron (III) hemoglobin. While iron (II) hemoglobin can carry oxygen to the tissues as oxyhemoglobin, iron (III) hemoglobin or methemoglobin cannot carry oxygen to the tissues and is therefore useless in oxygen transport to the tissues [1]. With the usage of amyl nitrite in the treatment of coronary heart disease and its effect to cause methemoglobinization and possible side effects therefrom, a study of diabetics blood's susceptibility to enhanced methemoglobin formation by this nitrite appears warranted [2, 3]. With wide usage of butyl nitrites for both medicinal and recreational uses and possible side effects therefrom a study of diabetics blood due to their increased susceptibility by butyl nitrite also appears warranted. In addition both amyl nitrite and

butyl nitrite are used as inhalants which are inexpensive and easy to obtain but can cause the heart to beat quickly and irregularly and then suddenly stop (cardiac arrest) [4, 5]. Furthermore methemoglobinization is an important indicator for oxidative stress in diabetes [6]. As is well known people with diabetes mellitus also have hemoglobin that differs from ordinary adult blood in that it is glycosylated to a level of 6.5% or greater by the abnormally high level of glucose in the untreated diabetic's blood [7]. As a result of the high glucose the hemoglobin becomes hemoglobin A1C by the Amadori rearrangement to form a 1-deoxy-1-amino-D- fructopyranose. Specifically the sugar has reacted with the N-terminal valine of the hemoglobin's beta globin chain in an irreversible manner to form the HbA1C molecule. Moussa reported that methemoglobinization, that is the oxidation of iron (II) of oxyhemoglobin into iron (III) to form methemoglobin, is an important indication of oxidative stress in certain diabetic patients. Specifically, those afflicted with Type 1 diabetes mellitus have a higher hemoglobin auto-oxidation rate than those with Type 2 diabetes mellitus or non diabetics [8]. Increased susceptibility to the oxidation of diabetes blood by amyl nitrite would be worthwhile information to establish as it would be a possible contraindication for the use of this drug to treat certain diabetics with heart disease (angina pectoris). In addition these studies may establish that certain diabetics blood (e.g., those with type 2 diabetes mellitus) is less stable than those of a normal adult which could put them at even greater risk of all heart disease (both angina pectoris and myocardial infarction) due to hemoglobin with a higher auto-oxidation rate. Increased susceptibility of diabetics blood due to enhanced methemoglobin formation by butyl nitrite would be good evidence to establish that diabetics' blood in general are more susceptible to enhanced oxidation by alkyl nitrite. This could then become good reason to indicate that all alkyl nitrite use in any form should be an absolute contraindication for diabetics. In addition this could pose a greater threat to those with certain hemoglobinopathies or modified hemoglobins such as with diabetics with their elevated HbA1C levels.

Materials

Amyl nitrite and butyl nitrite were purchased from Fisher Scientific. Other required chemicals were obtained from the Sigma and Aldrich Chemical Company. Blood products such as normal adult blood and diabetics' blood were purchased from Physicians Plasma Alliance (PPA). All blood was tested and certified to be non-viral by PPA.

The data for the amyl nitrite was obtained from 40 donors 20 of whom had type 2 diabetes mellitus and 20 of whom were non diabetics. In Table 1 the HbA1C (%) values and hemoglobin oxidation times are given for all the diabetics samples (1D-10D) and the normal samples (1N-10N). The data for the butyl nitrite was obtained from 40 donors 20 of whom had type 2 diabetes mellitus and 20 of whom were non diabetics. In Table 2 the HbA1C (%) values and hemoglobin oxidation times are given for all the diabetics samples (1D'-10D') and the normal samples (1N'-10N'). The amyl nitrite and butyl nitrite studies were performed independently and with mainly different blood samples. The samples were provided as matched sets of diabetics and non diabetics blood wherein the two groups were matched with respect to age, gender, number of obese and number of cigarette smokers as evenly as was possible. Also these donors took similar vitamins and medications according to their medical histories. In an earlier publication the selected characteristics of the patients used in these studies have already been presented, i.e., age, gender, weight and smoker status [9]. All blood was drawn into Acid-Citrate-Dextrose (ACD) tubes and stored at 2-4 C prior to use.

Methods

The Hemoglobin A1C (HbA1C) percentages were determined using a Bayer DCA-2000 test kit. Diabetes was assessed as a HbA1C percentage greater than 6.5% [7]. A laboratory spectrophotometer equipped with a strip chart recorder was employed to monitor the formation of methemoglobin at 436 nm. A small table top centrifuge was used to separate plasma from the red blood cells. To determine the oxidation times blood samples were centrifuged for 2000g for 20 min to remove any remaining plasma. The remaining packed Red Blood Cells (RBCs) were aerated and washed in 20 mM Phosphate Buffer Saline (PBS) at pH 7.2 followed by another centrifugation to remove the saline. This procedure of centrifugation, aeration and washing was repeated. The RBCs were then resuspended in 20 mM PBS (pH 7.2) for a maximum of 60 min prior to testing.

Table 1: HbA1C and Oxidation Times of Patients for Amyl Nitrite

Samples	HbA1C, %	Oxidation Time, min
1D	9.3	1.4
2D	10.2	1.6
3D	11	2
4D	9.7	1.8
5D	12.4	1.8
6D	10.9	1.8
7D	12.4	1.6
8D	13.3	1.3
9D	11	1.7
10D	12.4	1.6
11D	11.8	1.6
12D	9.9	1.3
13D	12.4	1.2
14D	11	1.6
15D	10	1.4
16D	11.4	1.8
17D	12.2	1.3
18D	12	1.5
19D	13.3	1.1
20D	11.6	1.3
1N	5.5	3
2N	5.3	3.8
3N	5.7	2.4
4N	5.1	2.6
5N	5.7	3
6N	5.8	3
7N	5.5	3.8
8N	5.4	3.8
9N	5.4	2.7
10N	5.5	4
11N	5.4	2.7
12N	5.3	2.7
13N	5.4	4.2
14N	5.7	2.8
15N	5.3	3
16N	5.4	2.7
17N	5.4	2.7
18N	5.3	2.7
19N	5.5	3
20N	5.7	3.6
Mean±SEM	8.4±1.3	2.3±0.4

Table 2: HbA1C and Oxidation Times of Patients for Butyl Nitrite

Samples	HbA1C, %	Oxidation Time, min
1D'	11.7	1.4
2D'	11	1.7
3D'	10	1.7
4D'	13.7	1.5
5D'	11.4	1.5
6D'	11.6	1.2
7D'	12.2	1.3
8D'	11.6	1.3
9D'	8	1.3
10D'	12	1.3
11D'	7.1	1.3
12D'	7.6	1.2
13D'	8.1	1.5
14D'	11.6	1.5
15D'	7	1.5
16D'	9.5	1.7
17D'	8.3	2
18D'	8.7	1.3
19D'	7.5	1.4
20D'	9.3	1.5
1N'	5.2	4.3
2N'	5.7	5.2
3N'	4.9	6.4
4N'	5.5	5.1
5N'	5.4	4
6N'	5.7	4
7N'	5	5.3
8N'	5.1	5.7
9N'	5	5.1
10N'	5.2	4.5
11N'	5.3	5.5
12N'	5.4	4.9
13N'	5.2	4.4
14N'	5.4	4
15N'	5.3	4.9
16N'	5.4	4.9
17N'	4.6	4
18N'	5.5	4.1
19N'	5.5	4.7
20N'	5.7	4.9
Mean±SEM	7.6±0.4	3.1±0.3

A 0.01 mL portion of resuspended RBCs was hemolyzed by the addition of 1.0 mL of distilled water and adjusted to a final volume of 2.6 mL by the addition of 20 mM PBS (pH 7.2). The hemoglobin solutions were then adjusted to a standard absorbance (e.g., A = 1.0 ± 0.2) at a wavelength of 436nm with more 20 mM PBS (pH 7.2). The 2.6 mL aliquot of this hemoglobin solution was then added to a 0.05 mL aliquot of a 0.1% amyl nitrite (or butyl nitrite) in ethanol solution. A final concentration of 140 micromoles per liter (μmol/L) of alkyl nitrite was obtained after its addition to the hemoglobin solution. The above gave a final hemoglobin concentration between 6 and 9 μmol/L [10].

All of the above solutions were then placed in cuvettes and the reaction measured in a spectrophotometer equipped with a chart recorder set at a wavelength of 436 nm. This is a suitable wavelength for measuring and distinguishing oxyhemoglobin and methemoglobin. The spectrophotometer chart recorder then generated graphic representations of the conversion of oxyhemoglobin into methemoglobin as a function of time. The terminal period or asymptotic phase corresponds to essentially 100% methemoglobin formation. The final absorbance was found to be approximately A = 0.5 ± 0.1. All hemoglobin oxidation times (in min) obtained in these studies along with HbA1C values have been included in Tables 1 and Table 2 for these samples. As seen in Table 1 for the amyl nitrite studies the overall mean ± SEM obtained for HbA1C was 8.4 ± 1.3% and the mean ± SEM oxidation time was 2.3 ± 0.4 min. As seen in Table 2 for the butyl nitrite studies the overall mean ± SEM obtained for HbA1C was 7.6 ± 0.4% and the mean ± SEM oxidation time was 3.1 ± 0.3 min.

According to Colton [11] the appropriate tests to use for these data are linear regression analysis and correlation determinations for the samples. The data was analyzed using an Excel spreadsheet on a Microsoft computer.

Results and Discussion

As revealed in an earlier study an independent Student's t-test has already revealed that the time taken for diabetics erythrocytes to undergo oxidation was significantly shorter ($P<0.05$) than the non diabetic controls [9]. This increased sensitivity of diabetics erythrocytes to oxidation thus gives impetus to both correlation and linear regression studies of this phenomenon in an attempt to better quantify the overall relationship between the observed hemoglobin oxidation times as a function of HbA1C values obtained from the patient group studied. For the amyl nitrite studies, to obtain this end first a scatter diagram of the reaction time to form methemoglobin as a function of percent HbA1C was made as shown in Figure 1 from the patient's HbA1C values and respective hemoglobin oxidation times. The raw data used to generate the scatter diagram can be found in Table 1. Next the method of least squares was employed to generate a linear regression line which was calculated from the percent of all donor HbA1C values and their hemoglobin oxidation times as shown in Figure 2. The resultant equation of the straight line was Y = 4.43 - 0.25X where X = HbA1C percentage and Y = hemoglobin oxidation time in minutes as illustrated in this figure. The standard deviation of points around the fitted line was 0.449 and the standard errors of the slope and intercept were 0.0230 and 0.207, respectively. In a likewise fashion for the butyl nitrite studies first a scatter diagram of the reaction time to form methemoglobin as a function of percent HbA1C was made as shown in Figure 3 from the patient's HbA1C values and respective hemoglobin oxidation times. The raw data used to generate the scatter diagram can be found in Table 2 below. From this a linear regression line was then calculated from the percent of all donor HbA1C values and their hemoglobin oxidation times as shown in Figure 4. The resultant equation of the straight line was Y = 7.17 - 0.53X where X = HbA1C percentage and Y = hemoglobin oxidation time in minutes as illustrated in this figure. The standard deviation of points around the fitted line was 1.00 and the standard errors of the slope and intercept were 0.0586 and 0.472, respectively.

Figure 1: Scatter diagram of the reaction time (in min) to form methemoglobin as a function of HbA1C percentage for amyl nitrite

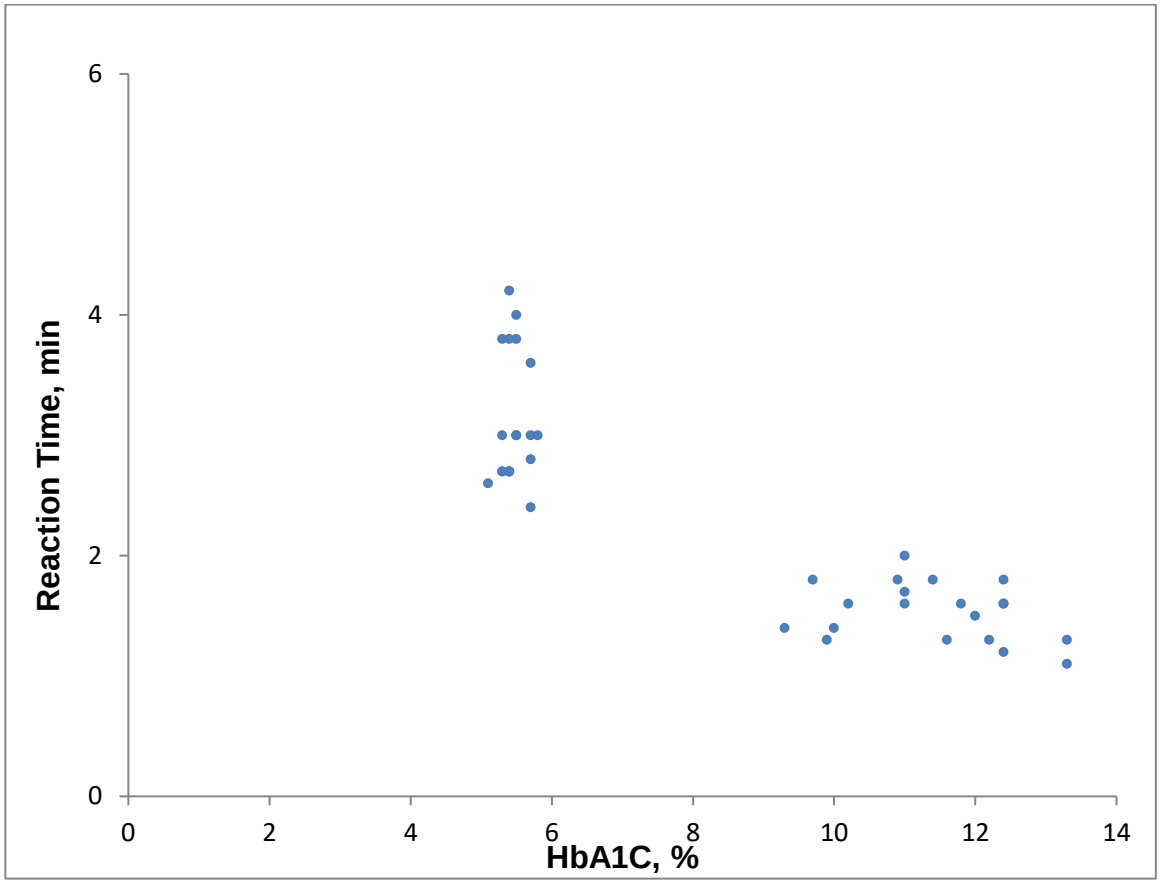


Figure 2: Linear regression line y = 4.43-0.25x of hemoglobin oxidation times (in min) with 95% confidence interval error bars for amyl nitrite

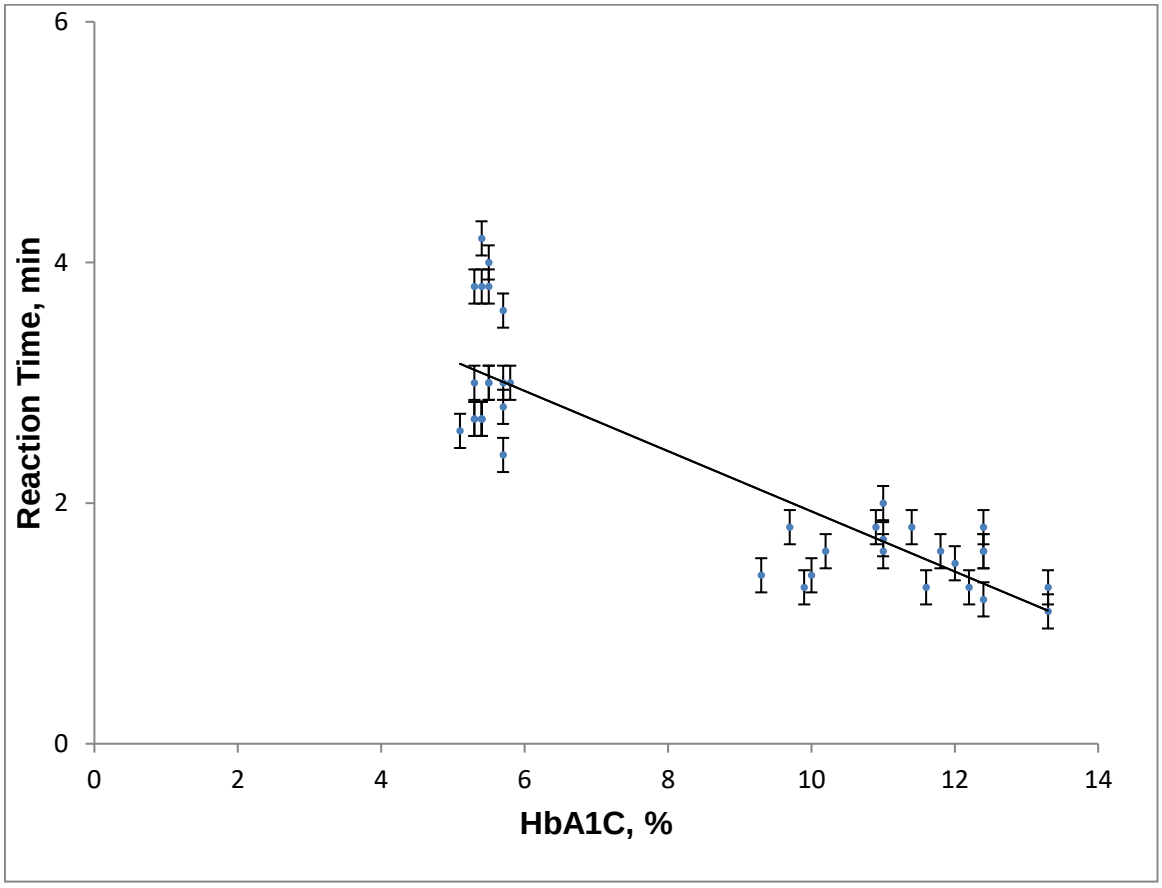


Figure 3: Scatter diagram of the reaction time (in min) to form methemoglobin as a function of HbA1C percentage for butyl nitrite

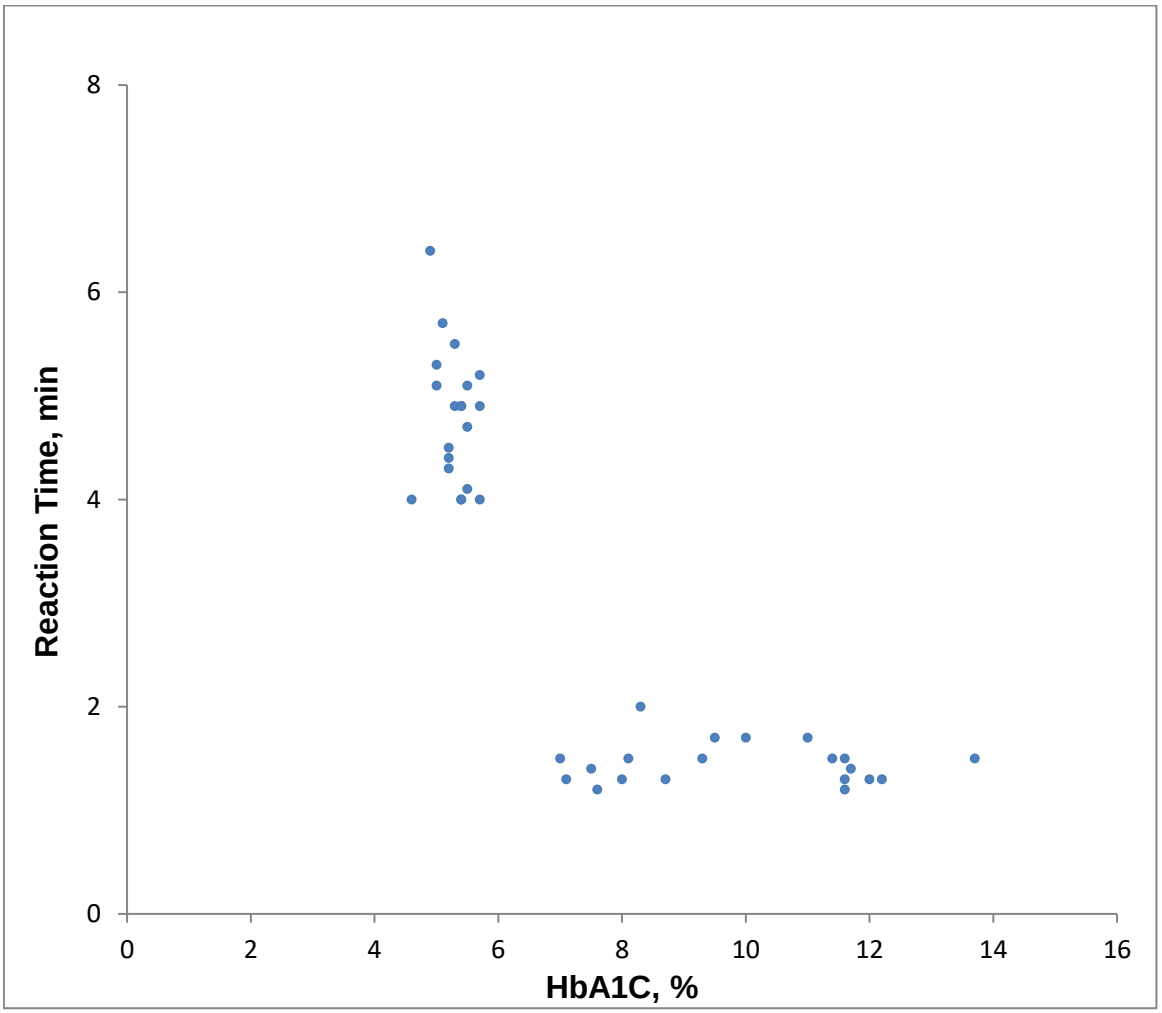
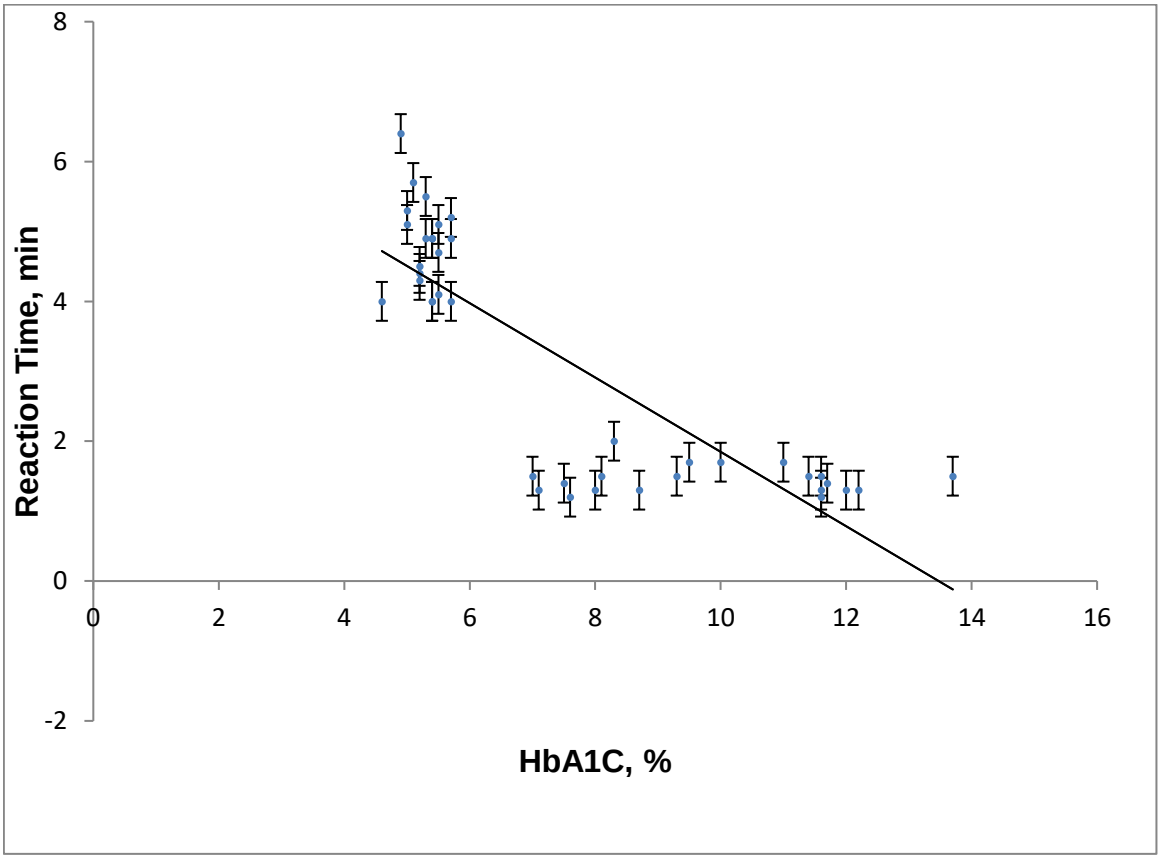


Figure 4: Linear regression line y = 7.17-0.53x of hemoglobin oxidation times (in min) with 95% confidence interval error bars for butyl nitrite



The product moment correlation coefficient (r) was found to be - 0.87 and -0.83 for the amyl nitrite and butyl nitrite studies, respectively. According to Colton [11] correlations from 0 to 0.25 indicate little or no relationship, those from 0.25 to 0.50 indicate a fair degree of relationship, those from 0.50 to 0.75 a moderate to good relationship, and those above 0.75 a very good to excellent relationship. Sometimes one encounters correlations of 0.95 or higher. When such correlations arise, particularly in the biological field and where human data are involved, one should immediately be suspect. Correlations this high are almost too good to be true. Therefore, the correlation coefficients obtained of – 0.87 and -0.83 indicates a very good to excellent relationship between percentage of HbA1C and the hemoglobin oxidation times and appears believable. Of course the negative sign indicates that the two variables have an inverse relationship; namely, this means that as the amount of HbA1C (%) increases the hemoglobin oxidation time then diminishes.

Thus, these studies demonstrate that not only is susceptibility of blood oxidation by amyl nitrite enhanced in Type 2 diabetes but that the hemoglobin oxidation times can be predicted by the equation Y = 4.43 - 0.25X based solely on the percentage of HbA1C. In a like fashion butyl nitrite oxidation times can be predicted by the equation Y = 7.17 - 0.53X. In these least-squares regression lines the Y intercept represents the predicted mean of the dependent variable, Y, when the independent variable, X, is zero. The more important quantity is the slope, which is interpreted as the estimated mean change in the dependent variable for a unit change in the independent variable. Thus, in the first study hemoglobin oxidation time decreases an average of 0.25 minute for each percentage increase of HbA1C over the range of HbA1C studied for amyl nitrite whilst it decreases an average of 0.53 minute for each percentage increase of HbA1C over the range of HbA1C studied for butyl nitrite. Obviously the butyl nitrite curve is twice as steep. For amyl nitrite, the 95 percent confidence limits on the slope (b) are obtained as b ± 1.96(Est SE (b)) = -0.25 ± 1.96 x 0.0230 [11]. These limits are interpreted as follows: based on the sample, the chances are 19 in 20 that, in the underlying population, the slope of the regression line of hemoglobin oxidation time on HbA1C percentage is somewhere in the range of -0.20 to -0.30 min per percent of HbA1C. For butyl nitrite, the 95 percent confidence limits on the slope of the regression line is in the range of -0.42 to -0.65 min per percent of HbA1C indicative of a steeper slope.

For amyl nitrite the 95 percent confidence limits on the intercept (a) are obtained as a ± 1.96(Est SE(a)) = 4.43 ± 1.96 x 0.207. This means one would expect a Y-intercept value between 4.02 and 4.84 which does not appear unreasonable for 100% HbA0. For butyl nitrite the 95 percent confidence limits on the intercept (a) are calculated as 7.17 ± 1.96 x 0.472. This means one would expect a Y-intercept value between 6.24 and 8.10. Again this does not appear to be an unreasonable hemoglobin oxidation time for someone with no HbA1C. Nevertheless, in drawing inferences regarding the intercepts for amyl nitrite and butyl nitrite, one should note first whether the intercept falls within the range of the observed data points. Since the data points cluster far beyond X = 0, the determination of the intercept involves considerable extrapolation of the straight line. Hence, inference regarding the intercept is not illustrated with the data at hand for amyl nitrite or butyl nitrite. Actual data would need to be present closer to the Y intercept to better validate these calculated Y intercept values.

The aforementioned linear regression equations could be of good predictive value as to the susceptibility of hemoglobin induced oxidation by alkyl nitrites. For example in an individual with 10% HbA1C (a diabetic) the calculated hemoglobin oxidation times of 1.9 min were obtained for both amyl nitrite and butyl nitrite. In fact sample 2D from Table 1 (i.e., the amyl nitrite study) had an HbA1C = 10.2% and this patient had an experimental hemoglobin oxidation time of 1.6 min, while sample 3D' from Table 2 (i.e., the butyl nitrite study) had an HbA1C = 10% and had an experimental hemoglobin oxidation time of 1.7 min. Both of the calculated values were quite close to the experimental values obtained. Another example would be a patient with 5% HbA1C (a non diabetic or normal patient) who had calculated hemoglobin oxidation times of 3.2 min and 4.5 min for amyl nitrite and butyl nitrite, respectively. In fact sample 4N from Table 1 (amyl nitrite study) had an HbA1C = 5.1% and had an experimental oxidation time of 2.6 min while sample 9N' from Table 2 (butyl nitrite study) had an HbA1C = 5% and had an experimental oxidation time of 5.1 min. Again both of the calculated values are close to the experimental values obtained.

Interestingly, the enhanced susceptibility to either amyl nitrite or butyl nitrite induced oxidation reactions occurred in Type 2 diabetics blood which implies that HbA1C oxidation into

methemoglobin is a direct function of the amount of HbA1C present as opposed to metabolic differences in the type 1 and type 2 diabetes [8]. The product moment correlation coefficients (r) were independently calculated correlating HbA1C with oxidation times for all samples in the amyl nitrite and butyl nitrite studies. Because these results gave absolute r values greater than the absolute value of 0.75 this means that not only is there a strong relationship between HbA1C (%) and the hemoglobin oxidation times (min) but this finding also gives more statistical evidence to allow us to predict the oxidation time of the blood with confidence. In fact, the straight line equations obtained allows for a more quantitative prediction of how long the oxidation reaction takes. Thus, any Type diabetic 2 used in these studies simply has a significantly greater percentage of HbA1C ($P<0.05$) that determines through these empirical equations that the oxidation times are significantly shorter than in normal blood ($P<0.05$) [9]. This finding appears to be well supported by the fact that glycation of hemoglobin is an irreversible chemical reaction that is non-enzymatic in nature irrespective of the type of diabetes. This means that the hemoglobin (HbA0) is permanently altered to form a less stable HbA1C molecule. Thus, these preliminary findings indicate that diabetics have hemoglobin that exhibits greater overall oxidative stress to alkyl nitrites such as amyl nitrite or butyl nitrite owing to a higher percentage of HbA1C. In fact HbA1C has been reported to be more thermolabile than non glycated Hemoglobin (HbA0) meaning that compared to HbA0 the HbA1C is more rapidly auto oxidized [8]. This supports the view that structural modification of hemoglobin due to glycation causes the HbA1C to become less stable and more prone to oxidation by glycation-induced structural modification of hemoglobin which leads to a functional modification resulting in oxidative stress in diabetic patients [12]. Of future interest would be to ascertain whether additional alkyl nitrites would give similar linear regression equations and correlation coefficients and thus to see if this is a general trend. Were this the case this would indicate a possible contraindication for the use of this class of drugs for individuals with type 2 diabetes mellitus.

References

1. Longo DL, Kasper DL, Jameson JL, Fauci AS, Hauser SL, et al. (2012) Harrison's Principal's of Internal Medicine. McGraw Hill (18th ed) 1: 857-858.
2. Tarburton J (2013) Amyl nitrite induced hemoglobin oxidation studies in diabetics and nondiabetics blood. J Diabetes and Metabolism 4: 258-260.
3. Tarburton JP (2014) Hemoglobin oxidation studies in diabetics blood comparing the effect of sodium nitrite vs. amyl nitrite. Diabetes Research and Treatment 1:112-117.
4. Dixon DS, Reisch RF, Santinga PH (1981) Fatal methemoglobinemia resulting from ingestion of isobutyl nitrite a "room odorizer" widely used for recreational purposes. J Forensic Science 26: 587-593.
5. Pruijm MT, de Meijer PH (2002) Methemoglobinemia due to ingestion of isobutyl nitrite ('poppers'). Ned Tijschr Geneeskd 146: 2370-2373.
6. Moussa SA (2007) Biophysical changes in red blood cells and hemoglobin components of diabetic patients. Journal of Genetic Engineering and Biotechnology 5: 27-32.
7. National Diabetes Fact Sheet (2011) U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.
8. Moussa SA (2008) Oxidative stress in diabetes mellitus. Romanian J Biophys 18: 225-236.
9. Tarburton, JP (2015) Hemoglobin Oxidation Studies in Diabetics Blood and Normal Blood: The Effects of Butyl Nitrite and Pentyl Nitrite. Diabetes Research and Treatment 2: 127-137.
10. Weissbluth M (1974) Hemoglobin: Cooperativity and electronic properties. In: Kleinzellar A, Springer GF, Whitman HG (eds), Molecular Biology, Biochemistry and Biophysics, Vol 15 Sringer-Verlag, New York 136-141.
11. Colton T. (1974) Statistics in Medicine. Little, Brown and Co. Boston, MA. pp. 190-202 and 207-211.
12. Sen S, Kar M, Roy A, Chakraborti AS (2005) Effect of nonenzymatic glycation on structural and functional properties of hemoglobin. Biophys Chem 113:289-298.