Pre-Analytical Errors in Blood Homocysteine Assays

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Abstract: Blood homocysteine is a major biomarker for cardiovascular as well as other disorders. Several published studies reported inconsistent homocysteine levels with variable ranges. The reference intervals are variable hence the comparison of studies is difficult. Pre-analytical factors that influence homocysteine testing such as the storage, processing and transportation of blood samples, often pose severe problems in clinical settings. Total homocysteine levels were determined in 36 blood samples collected from normal healthy subjects by HPLC with fluorescence detection. The results indicate that delay separated samples stored at ambient temperature have significantly higher values than the delay separated samples stored at 2-8ºC. The rise was 41.9 % in 3 hours that amounts to a rate of ≈14 % per hour. It is concluded that there is a strong need for standardization of blood sample collection and processing in homocysteine assays. It is strongly advocated that homocysteine should never be measured in serum and that it is ideal to use plasma specimens preserved at 2-8ºC.

Introduction

Homocysteine is an endogenous amino acid derived from dietary methionine. Homocysteine is formed as a central metabolite during trans-methylation reactions. When methionine is in excess, homocysteine is directed to trans-sulphuration pathway whereas under the negative methionine balance homocysteine undergoes re-methylation [1-2]. Elevated blood concentration (>15µmol/L plasma) is associated with a wide range of disorders such as cardiovascular diseases, stroke, arterial thrombosis, neurological/psychiatric diseases and osteoporosis as well as complications during pregnancy[3-4]. Blood homocysteine level is now well accepted as an independent, potent biochemical marker with a strong indications / or increased risk of premature coronary artery disease, stroke and atherothrombosis among persons with normal cholesterol levels [4].The measurement of blood homocysteine has an important role in the risk stratification and monitoring of cardiovascular diseases as well as other disorders. Several published studies reported homocysteine levels in either serum or plasma and showed variable results [5-6]. The reference intervals are variable hence the comparison of studies is difficult. Pre-analytical factors that influence homocysteine testing such as the storage and transportation of blood samples, often pose severe problems in clinical settings [7-8]. In view of this, the present report highlights most common pre-analytical errors in homocysteine determination.

Materials and Methods:

The present work was done in the Department of Biochemistry, Maharashtra Institute of Medical Sciences and Research Medical College, Latur (Maharashtra State). 36 normal healthy subjects having age group 20 to 50 years were selected. All the subjects were free of any symptoms or any disease on clinical examinations and all routine laboratory tests done were within normal limits. The study was carried out under ethical consideration. Fasting blood samples were collected by vein puncture

in EDTA vacutainer using disposable syringe. The samples were divided into three aliquots. One aliquot was separated immediately (early separated plasma) while the other two aliquots were separated after a delay of three hours (delay separated plasma). Of these two delay separated samples, one was kept at room temperature (AT = ambient temperature) while other was stored in ice bath (2-8ºC). The early separated plasma samples were also stored at 2-8ºC until analysis. Total homocysteine level in the plasma samples was measured by HPLC with fluorescence detection method[7]. The reagent kits from M/s RECIPE, Sandstrasse, Germany[9] were used for quantitative determination. The statistical analysis was done by two tailed paired student 't' test.

Results:

Table-1:

#	Specimens	Number (n)	Total Homocysteine (Micromoles/L)	|t| paired test
I	Early separated plasma	36	10.81 ± 2.15	I vs II: 10.98❋
II	Delay separated, AT plasma	36	15.34 ± 3.76*	I vs III 1.986☼
III	Delay separated, cold preserved, plasma	36	11.95 + 2.12	II vs III 11.96❋

*❋P < 0.001; ☼ Not Significant

The results shown in the table indicate that there is a significantly higher mean value of homocysteine in delay separated samples stored at ambient temperature. The rise was 41.9 % in 3 hours that amounts to the rate of ≈14 % per hour. Whereas there is non significant difference in the values of homocysteine in delay separated samples stored at 2-8ºC.

Discussion

The measurement of homocysteine in blood has now become increasingly important for many medical specialties. Several aspects have to be taken into consideration regarding homocysteine testing. The pre-analytic period, especially the period after drawing the blood samples till separation of plasma / serum from blood cells by centrifugation or other processes, the time is of critical importance(10,11).The total homocysteine levels were significantly higher in plasma samples separated after 3 hours as compared with those early separated plasma samples. It is reported by several studies[10-12]that delay in separation of plasma from cells results in elevation of total homocysteine level in plasma. The present results also support the earlier reports[10-12].The rate of elevation was almost 14 % per hour which is much higher than the those reported earlier[7-8]. It is most likely due to the higher ambient temperature during the assays. The average daily temperature ranged from 30 to 42ºC during summer season. It is believed that the during the post phlebotomy period, blood cells produce and release homocysteine into plasma[10]. This is attributed to *in vitro* erythrocyte transmethylation reaction that is responsible for continuous

production and release of homocysteine into the plasma[11]. As erythrocytes continuously produce and export homocysteine into the plasma even after phlebotomy, it results in a falsely high homocysteine in plasma which may go even up to 50 % of the actual value *in vivo* at ambient temperature(12). During this period, these processes increase homocysteine concentrations at the rate of around 10% an hour. After 5 hours, the homocysteine concentration in plasma or serum may reach up to 50% higher than the actual value. The results shown in the table indicate that the difference in the values of delay separated (cold preserved) samples and early separated samples were statistically non-significant. If immediate centrifugation is not possible, then keeping the blood samples in refrigerator at 2-8ºC or even surrounded by ice until analysis, substantially reduces this pre-analytical error. However, after separation of plasma from the cells, homocysteine level remains stable at ambient temperature for at least 4 days[11-12]. Therefore, immediate separation of plasma after phlebotomy and keeping plasma in ice-bath can minimize the false increase in total homocysteine level. Hence the reliability of homocysteine results can be improved for proper clinical monitoring the diseases like coronary artery disease, stroke and athero-thrombosis[13]. Therefore, to measure homocysteine values reliably it is pivotal to either centrifuge the blood sample 'as soon as possible' (within 30 minutes after blood collection) or to store the whole blood sample immediately surrounded by crushed ice. This blood handling procedure, however, is often impractical in most clinical settings. It is essential to use plasma samples only for all homocysteine assays. It is concluded that there is a strong need for standardization of blood sample collection and processing methods so as to minimize the variations in the results due to common pre-analytical errors in homocysteine determination. It is strongly advocated that homocysteine should never be measured in serum and that plasma specimen is most ideal for all homocysteine assays.

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