Measurement of total homocysteine concentrations in acidic citrate using an enzymatic cycling method

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The sulphur-containing amino acid homocysteine (Hcy) has been long associated with diseases of the vascular system. Elevated plasma levels have been found in coronary heart disease, atherosclerosis, stroke, peripheral vascular disease, Alzheimer's disease and many other conditions. The amino acid links the methionine cycle between S-adenosylmethionine, vitamin B_{12} and folic acid. Reducing blood levels of Hcy by providing nutritional co-factors for its metabolism has been shown to reduce the risk of cardiac events.[1–4]

A number of methods have been described to assay Hcy levels in blood. These include high-performance liquid chromatography (HPLC), using fluorimetric[5] and electrochemical detectors,[6] as well as enzyme immunoassay,[7] the latter technique having been applied to automatic analysers.[8]

More recently, direct enzyme methods have been developed and adapted to clinical chemistry analysers. One such method, the Diazyme enzymatic assay,[9] has recently been introduced to the commercial market. In this method, oxidised Hcy is first reduced to free Hcy, which then reacts with a co-substrate, S-methyltransferase, to form methionine and S-adenosylhomocysteine (SAH).

The SAH is assessed by coupled enzyme reactions including SAH hydrolase, adenosine deaminase and glutamate dehydrogenase. The adenosine formed is hydrolysed immediately to inosine and ammonia. The ammonia reacts with glutamate dehydrogenase with a concomitant conversion of NADH to NAD^+. The decrease in NADH is measured by the change in absorbance at 340 nm. The concentration of Hcy in the sample is inversely proportional to the amount of NADH oxidised.[9] The reaction is illustrated in Figure 1[10]

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The value of assaying Hcy is offset by practical difficulties in sample collection. The Hcy concentration increases in whole blood by as much as 10% per hour unless samples are kept on ice and separated within 60 min.[10] The addition of 3-deazaadenosine to Vacutainer tubes has been reported to stabilise the whole blood Hcy for at least six hours at ambient temperature.[11] However, although the addition of 3-deazaadenosine is suitable for use when HPLC methods are utilised, it has been shown that any method using SAH hydrolase, including the Abbott FPIA enzyme immunoassay method, would be inhibited by the 3-deazaadenosine used as a preservative.[12]

To overcome this problem, tubes containing an acidic citrate solution at pH 4.2 have been used and have been found to stabilise the Hcy in blood for up to 30 hours without inhibiting the reaction. The acidic citrate was found suitable for preserving blood for assay by the Abbott FPIA enzyme immunoassay system,[13] but no claims have been made with regard to its suitability when direct enzyme methods are used.

It would seem logical to assume that other enzyme assay systems for Hcy that contain SAH hydrolase could also be used successfully to assay blood preserved in acidic citrate. However, the enzyme system used in the Diazyme assay kit contains a further enzymatic step not present in the Abbott FPIA enzyme immunoassay method. This is the catalytic conversion of α-ketoglutarate and ammonia to glutamate using glutamate dehydrogenase, with concomitant oxidation of NADH to NAD. The optimum pH for this reaction is 8.7.[14]

It is unclear whether or not the acidic nature of the citrate preservative will have an adverse effect on the reaction catalysed by glutamate dehydrogenase. Therefore, this study aims to evaluate the suitability of tubes containing acidic citrate for use in collecting blood when assaying plasma Hcy by the Diazyme enzyme cycling method.

Blood samples were collected at random from 37 volunteers who attended the venepuncture clinic at the haematology department at Bronglais Hospital, Aberystwyth, during the second week of October 2008. Local approval from the Dyfed Powys Research Ethics Committee was obtained and informed consent was given by all participants.

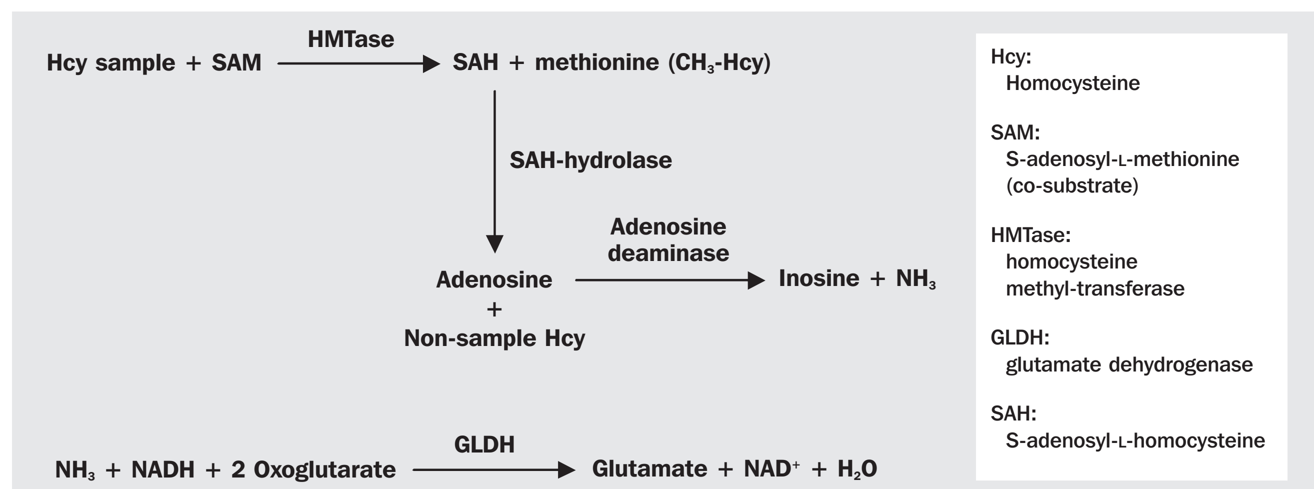

Fig. 1. The Diazyme enzymatic Hcy assay based on a co-substrate conversion product cycling principle.[10]

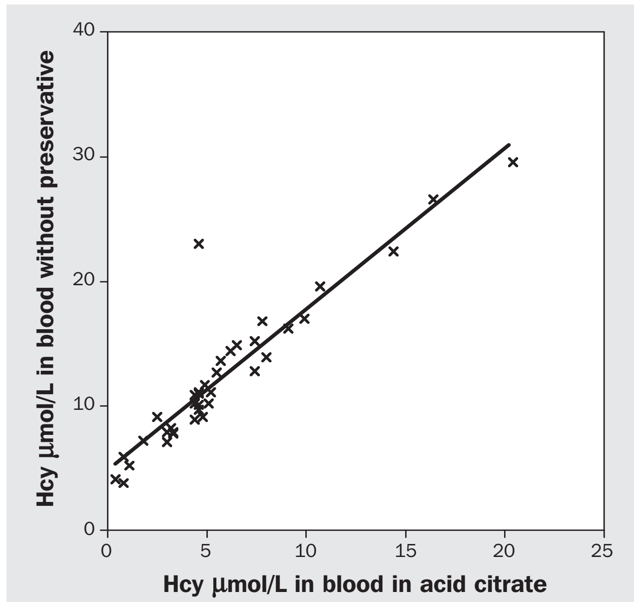

Fig. 2. Comparison of homocysteine (Hcy) results in blood collected in plain gel Vacuette with Hcy results in blood collected in tubes containing acid citrate as a preservative when assayed using the Diazyme enzymatic method (n=37, y =1.3x + 4.8).

A Diazyme homocysteine 2 reagent enzymatic assay kit (DZ568B) was obtained from Cambridge Life Science (Cambridge Life Science, Cambridgeshire Business Park). Blood was collected by venepuncture into a gel separator Vacuette tube containing no preservative, and into a Vacuette homocysteine detection tube containing acid citrate (Greiner Bio-One, Stroudwater Business Park, Stonehouse, Glos., UK).

Once collected, the contents of each tube were mixed and sent directly to the biochemistry laboratory where the pairs of blood samples were treated in an identical manner. Within 10 min of collection the blood samples were centrifuged for 10 min at 1500 x*g* and the serum and plasma frozen at –20˚C until required.[9] Homocysteine was measured on both samples utilising an Olympus AU400 analyser using the Diazyme kit for Hcy following a protocol supplied by the kit manufacturer.

The results obtained from the plasma samples were corrected (multiplying by a factor of 1.11) to allow for the dilution occurring as a result of the liquid acid citrate present in the tube.[13] The results were examined statistically to evaluate any differences between the pairs of Hcy results using Graphpad Prism 3 statistical computer package (version 5.1.2600) for Windows (Graphpad Software, San Diego, CA, USA).

Figure 2 shows that the homocysteine levels obtained from the acid citrate plasma samples were at least 4.8 µmol/L lower than those obtained using the unpreserved serum. At high levels, the results were markedly reduced and in some cases the plasma Hcy result appeared to be as much as 80% lower than the serum level.

Linear regression analysis showed y=1.3x + 4.8 and r^2= 0.846. The distribution of the results was found to be normal and hence suitable for analysis by the two-tailed paired t-test. This showed that the means of the two sets of Hcy results were significantly different (P<0.0001).

The results of this small study show that the Hcy results obtained from blood samples collected in the acid-citrate tubes were significantly lower than those for the bloods collected into plain gel Vacuette tubes.

As the unpreserved serum had been centrifuged and separated within 30 min, the difference in Hcy is far in excess of any expected change due to the preservative action of the acid citrate (±5%). The low pH of the citrate solution changed the pH of the reaction buffer used in the Diazyme enzyme kit to below that required for optimum glutamate dehydrogenase activity (D. Little, Greiner Bio-One, personal communication); thus, acid citrate tubes are not suitable for collecting blood for Hcy by the enzymatic cycling method described above.

It is suggested that a lower citrate concentration or a stronger buffer in the Diazyme system (or both) may resolve this problem. □

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