Hyperhomocysteinemia and venous thrombosis : studies into risk and therapy

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Chapter 3

Acidic citrate stabilizes blood samples for assay of total homocysteine

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Introduction

Homocysteine is a sulfhydryl-containing amino acid, formed by demethylation of the essential amino acid methionine. Homocysteine is either transsulfurated to cysteine or is remethylated to methionine by methionine synthase. Excess intracellular homocysteine is likely to be transported to the extracellular compartment[1]. Increasing evidence indicates that homocysteine is implicated in the pathogenesis of thromboembolic diseases. Several case control studies have shown a relationship between increased total plasma homocysteine (tHcy) concentrations and an increased risk of arterial[2-4] and venous thrombosis[5–8]. An increase of the tHcy concentration of 5 µmol/l is associated with 1.5–1.9 times increased risk for coronary artery or cerebrovascular disease[9].These values indicate that small differences may be of clinical importance. Therefore, practical standardized conditions for handling blood specimens for tHcy determination are required. In most studies, blood is drawn in tubes containing K3EDTA. The whole-blood sample is immediately put on crushed ice and then centrifuged as soon as possible to prevent an increase of tHcy concentrations. This tHcy increase is caused by ongoing homocysteine metabolism in blood cells, the majority of which are red blood cells[10,11]. This blood handling procedure is not practical, particularly when larger studies are conducted outside a hospital setting; even in a routine clinical setting, this protocol might be hard to put into practice. To find an alternative, more suitable blood-collection medium, we investigated the effect of different blood-collection media on tHcy production when whole blood is kept at room temperature for 6 hours.

Methods

Blood was drawn by venipuncture of the antecubital vein from laboratory coworkers or from consecutive patients who visited the outpatient clinics of the Leyenburg Hospital in The Hague for various reasons, unknown to the authors. Informed consent was obtained in accordance with the current revision of the Helsinki declaration of 1975. Two studies were performed. A pilot study was done with blood from 11 patients and 11 laboratory coworkers (12 men and 10 women; ages 18–63 years). Blood was drawn in tubes with 1.8 g/l K3EDTA (Vacutainer Tube; Becton Dickinson), in tubes with 2.5 g/l sodium fluoride and 2 g/l potassium oxalate as anticoagulant (Vacutainer Tube), in tubes with 0.5 mol/l acidic citrate (Biopool StabilyteTM), and in tubes with a mixture of the sodium fluoride, potassium oxalate, and acidic citrate. Care was taken that all tubes were completely filled by blood. The EDTA-treated blood was put on crushed ice immediately after venipuncture. The other blood samples were

kept at room temperature. The results of this pilot study indicated that tHcy concentrations remained stable in acidic citrate. We conducted a second study to explore this phenomenon more extensively. This main study was done in 30 laboratory coworkers (17 men and 13 women; ages 18–52). Blood was taken in tubes with EDTA and acidic citrate (as described above). From every volunteer, one-half volumes of the tubes containing EDTA were kept at room temperature; the other half were put on crushed ice immediately after sampling. From the tubes with acidic citrate, one-half volumes were kept at room temperature, and the other half were stored in water of 37°C. In both study groups, the blood was centrifuged for 10 min at 2000*g* as soon as possible (within 15 min) after sampling ("0 h") and 2, 4, and 6 h after the venipuncture.

After separation, the plasma was stored at -20°C until determination of the tHCy concentration at the Laboratory of Pediatrics and Neurology of the University Hospital Nijmegen (by H.J.B. and S.V.) by automated HPLC with reversed-phase separation and fluorescent detection [Gilson 232–401 sample processor (Gilson Medical Electronics, Inc.), Spectra-Physics 8800 solvent delivery system, and Spectra-Physics LC 304 fluorometer], according to the method described by Fiskerstrand *et al.*[12] with some modifications[13]. The tHcy concentrations from the tubes containing the acidic citrate were corrected for the dilution caused by the fluid already present in the tube before blood collection.

Paired-sample *t*-tests were used to calculate the significance of the increase of tHcy concentrations in the collection media. Paired-sample *t*-tests were also used to calculate the significance of the differences between the tHcy concentrations in the different collection media at baseline. Results of the *t*-tests are given as the intervals that show $P<0.05$ significance [95% confidence intervals (CI)].

Results

Results of the pilot study are shown in Table 3.1. tHcy concentrations in blood taken in tubes containing sodium fluoride rose markedly after 2 h (0.9 µmol/l; 95% CI 0.5 to 1.3 µmol/l). In the tubes containing sodium fluoride with acidic citrate, tHcy concentrations remained stable for 4 h. After 6 h, there was a slight increase of 0.6 µmol/l (95% CI 0.0 to 1.1 µmol/l). tHcy concentrations in blood taken in tubes containing EDTA that were stored at 0°C and tHcy concentrations taken in tubes with acidic citrate that were kept at 21°C did not rise markedly for 6 h [0.0 µmol/l (95% CI -0.5 to 0.6 µmol/l) and 0.4 µmol/l (95% CI -0.5 to 0.9 µmol/l), respectively]. Results of the main study are shown in Figure 3.1 and in Table 3.1. tHcy concentrations in the EDTA-containing tubes that were put on ice did not rise markedly for 6 h (0.3 µmol/l; 95% CI -0.1 to

0.7 µmol/l). tHcy concentrations in the EDTA-containing tubes that were stored at room temperature rose 2.0 µmol/l after 2 h (95% CI 1.6 to 2.4 µmol/l) and up to 4.7 µmol/l (95% CI 4.1 to 5.3 µmol/l) after 6 h. At room temperature, the tHcy concentrations in acidic citrate did not rise markedly for 6 h after collection (0.3 µmol/l; 95% CI -0.2 to 0.7 µmol/l), whereas tHcy concentrations in blood collected in acidic citrate stored at 37°C increased markedly after 4 h (0.9 µmol/l; 95% CI 0.5 to 1.3 µmol/l).

We found a difference of 1.3 µmol/l (95% CI 0.9 to 1.6 µmol/l) between tHcy concentrations measured in blood sampled in tubes with EDTA and stored at 0°C and tHcy concentrations measured in blood sampled in tubes with acidic citrate kept at room temperature. Such significant differences were present at all measurement times. When the results of the pilot study and the main study were combined, this difference decreased but was still significant (0.8 µmol/l; 95% CI 0.4–1.1 µmol/l; range, -3.1 to 2.2 µmol/l).

Table 3.1 Mean (±SD) increase in homocysteine (in µmol/l) in whole blood in different collection media.

		Pilot study (n=22)				Main study (n=30)			
			Increase after				Increase after		
Storage medium	Temp. °C	Baseline	2 h	4 h	6 h	Baseline	2 h	4 h	6 h
EDTA	0	12.5 (2.4)	0.1 (1.1)	0.4 (0.9)	0.1 (1.2)	12.7 (3.3)	0.1 (1.3)	0.1 (1.3)	0.3 (1.1)
Acidic citrate	21	12.6 (2.6)	0.1 (1.1)	0.4 (0.8)	0.4 (1.1)	14.0 (3.6)	0.0 (1.1)	0.0 (1.1)	0.3 (1.2)
NaF	21	11.3 (1.8)	0.9 (0.9)	1.6 (0.8)	1.7 (1.3)				
Acidic citrate and NaF	21	11.6 (1.9)	0.1 (0.9)	-0.1 (1.0)	0.6 (1.2)				
EDTA	21					13.3 (3.4)	2.0 (1.0)	3.5 (1.4)	4.7 (1.7)
Acidic citrate	37					13.9 (3.5)	0.0 (1.0)	0.9 (1.1)	1.2 (1.5)

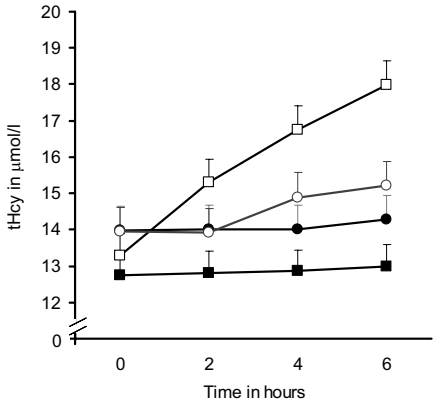

Figure 3.1 Increase of mean tHcy concentrations in whole blood of 30 laboratory coworkers processed 0, 2, 4, and 6 h after collection. Blood was collected in tubes containing EDTA and stored at 0°C (■) or at room temperature (□), and blood was collected in tubes with acidic citrate and stored at room temperature (●) or stored at 37°C (○); bars, SEM of tHcy concentrations.

Discussion

Blood cells produce homocysteine, which can lead to falsely increased plasma homocysteine concentrations. Thus, in the ideal setting, blood cells should be separated from plasma immediately after collection. In EDTA- containing blood, we found that tHcy concentrations increased at room temperature, which has been demonstrated before[10,14]. Even storage at 4°C has been associated with a smaller but steady increase of the tHcy concentration[10]. However, we found that tHcy concentrations remained stable for 6 h when EDTA-containing blood was stored at 0°C, which confirms the findings of Kittner *et al.*[15].

In a study by Møller and Rasmussen[16], heparin containing tubes with sodium fluoride added to a concentration of 2 or 4 g/l of blood prevented the increase of tHcy for only 2 h. In our pilot study and also in the study by Ubbink *et al.*[10], comparable results of increasing tHcy concentrations were found by using 2.5 g/l sodium fluoride.

This investigation is the first concerning the stability of tHcy concentrations in whole blood containing acidic citrate. This tube contains citrate at a low pH of 4.3 (pH±5.9 after blood collection) and was originally developed for the determination of fibrinolytic markers. During studies on fibrinolytic markers in relation to homocysteine, we found that tHcy might not increase in these samples at room temperature (data not shown). In our pilot study, we tested the combination of acidic citrate with sodium fluoride but found no difference between the tubes containing just acidic citrate and the tubes combining acidic citrate with sodium fluoride. Therefore, the stability of tHcy could be solely attributed to the acidic citrate fluid, making the addition of sodium fluoride unnecessary.

We performed this study to find a collection medium that is suitable in epidemiological field conditions. We therefore also tested how tHcy concentrations behave when whole blood, taken in acidic citrate tubes, is stored at a temperature higher than room temperature. When blood was stored at 37°C, tHcy concentrations were increased after 4 h. We do not know the mechanism by which the acidic citrate keeps tHcy concentrations stable. It is possible that the enzymes involved in homocysteine metabolism are blocked at this low pH. This blockage, however, is temperature dependent because tHcy concentrations rise when blood is stored at 37°C. Baseline tHcy concentrations in the EDTA-containing tubes kept at room temperature and centrifuged at t=0 h were higher than the concentrations in EDTA tubes put on ice and centrifuged at 0 h. As mentioned earlier, there was a time interval between the venipuncture and the centrifugation of the blood at t=0 of, at most, 15 min. When cooling of the blood instantly stops the export of homocysteine, tHcy concentrations increase in blood that is kept at room temperature from the time between the blood samples being taken and the separation of plasma from

blood cells because of ongoing export, explaining the difference at $t=0$. We also found a difference between the mean baseline concentrations of tHcy measured in EDTA- treated blood that was stored at 0°C and tHcy concentrations in blood taken in acidic citrate. This difference can only partly be explained in the same way as above (i.e., a rise of tHcy concentrations in the time interval between blood sampling and separation). Other factors, such as a higher osmolarity of EDTA than acidic citrate, leading to higher plasma volumes, could be responsible for the difference. This, however, needs needs further investigation. This study was conducted to find an alternative collection medium for the determination of tHcy concentrations in plasma. When blood is collected outside a hospital or laboratory setting (e.g., in a large epidemiological study), it is not always possible to put blood tubes on crushed ice. Even within hospitals, it can easily be forgotten or found impractical to put the tubes on crushed ice before processing. Therefore, this method can be prone to inappropriate handling of blood for determination of tHcy concentrations. This study shows that acidic citrate is a good alternative when screening patients in an epidemiological field study because tHcy concentrations stay stable for 6 h. However, because of the differences found between the mean baseline tHcy concentrations in EDTA on ice and in acidic citrate at room temperature, new reference values need to be established before tHcy concentrations obtained in EDTA tubes and stored on crushed ice can be replaced by acidic citrate tubes for determination of tHcy concentrations in individual patients.

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