Hyperhomocysteinemia and venous thrombosis : studies into risk and therapy

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Chapter 4

Measurement of total homocysteine concentrations in acidic citrate- and EDTA-containing tubes by different methods

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Abstract

In epidemiological studies, blood handeling for measurement of homocysteine is cumbersome because at room temperature homocysteine production in whole blood continues after blood collection. Acidic citrate stabilizes homocysteineproduction in whole blood at room temperature. In a previous study baseline differences in homocysteine concentration were found between EDTA and acidic citrate anticoagulated blood. This study was performed to further explore this difference in homocysteine concentration.

Blood from 208 volunteers was collected in tubes ontaining EDTA and acidic citrate as anticoagulant. The blood was processed within 30 minutes. Homocysteine determination in the plasma was done with 2 HPLC methods [HPLC(a) and (b)] and with an automated FPIA method.

The mean differences in homocysteine between acidic citrate blood and EDTA blood with HPLC(a), HPLC(b) and FPIA were 1.8 µmol/l (95% CI 1.6 to 2.1 µmol/l), -2.8 µmol/l (95% CI –3.1 to -2.5 µmol/l) and 0.1 µmol/l (95% CI 0.0 to 0.3 µmol/l) resp.

With all three measurement methods homocysteine concentrations in acidic citrate blood correlated well with homocysteine concentrations in EDTA blood. Regression analyses showed a slopes and intercept of 1.01 and 1.7 for HPLC(a), 0.75 and 0.7 for HPLC(b) and 0.95 and 0.7 for the FPIA.

We conclude that acidic citrate can be used for measurement of homocysteine with all three measurement methods. However, when using these methods, new reference values need to be established, because there is a difference in individual concentrations in acidic citrate and EDTA blood.

Introduction

In the past decade several studies have shown an association of hyperhomocysteinemia with arterial vascular disease[1-3], venous thrombosis[4], pregnancy complications[5] and Alzheimers disease[6,7]. Blood collection for homocysteine measurement is usually done in tubes containing EDTA as anticoagulant. The tubes have to be put on melting ice immediately and have to be centrifugated within 1 or 2 hours. At room temperature there is an increase in homocysteine in whole blood[8-12]. This procedure with tubes being placed on ice and centrifugated within 1 or 2 hours is cumbersome in a clinical setting, but in particular in large epidemiological studies. Therefore several methods are proposed to stabilize homocysteine in whole blood.
In a previous study we found that citrate with a low pH (pH=4.3, after blood collection ~5.9) stabilizes plasma homocysteine concentrations in whole blood for 6 hours when the blood is stored at room temperature[12]. However, we found a small difference in absolute homocysteine concentration measured in acidic citrate anticoagulated blood compared to EDTA.
The purpose of the current study is to further explore the differences in homocysteine concentrations between EDTA and acidic citrate anticoagulated blood and to investigate whether the differences are the same for different methods for measurement of homocysteine.

Methods

Blood was collected from volunteers who were selected from a general practice in Beverwaard, Rotterdam, the Netherlands. Thousand people were randomly selected from the practice and addressed by mail to participate as controls in a case-control study. A total of 258 participated. We draw additional blood tubes to perform our comparison study. All volunteers gave their informed consent according to the revised Helsinki declaration.
Blood was collected by venipuncture in 10 ml tubes containing EDTA (Vacutainer^{R}, Bencton&Dickinson, U.S.A.), and 5 ml tubes with acidic citrate (Stabilyte^{R}, Biopool, Sweden) as anticoagulant. Care was taken that the acidic citrate tubes were completely filled in order to obtain a blood:anticoagulant ratio of 9:1 in every sample. Blood collected in the EDTA tubes was stored at 0°C (melting ice) immediately after blood sampling until processing. Blood in the tubes with acidic citrate was kept at room temperature. All samples were processed within 30 minutes. They were centrifugated for 10 minutes at 2000 *g*

in a non-cooled centrifuge. The plasma was divided into aliquots and stored at –30°C until determination of the homocysteine.

The homocysteine was measured with three different methods:

1. HPLC(a): Automated high-performance liquid chromatography (HPLC) with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor (Gilson Medical Electronics Inc., Middleton,WI), Spectra-Physics 8800 solvent delivery system and Spectra-Physics LC 304 fluorometer (San Jose,CA)), according to the method described by Fiskerstrand *et al.*[13] with some modifications[14]. The reagents used for the reduction are NaBH4 and DTE. For the derivatization procedure we used ethylmorpholine buffer and monobromobimane.
2. HPLC(b): Automated high-performance liquid chromatography with reversedphase seperation and fluorescence detection according to Araki *et al.*[15] and modified by Ubbink *et al.*[16]. With this method homocysteine is reduced with tri-N-butylphosphine and the derivatization is done with SBD-F in borate buffer.
3. FPIA: A commercially available fluorescence polarization immunoassay (FPIA)(IMx Homocysteine, Abbott Diagnostics)[17]. This method, as the above mentioned HPLC methods, begins with the reduction of homocysteine using DTT. The homocysteine is enzymetically converted to SAH using adenosine and SAH hydrolase. Subsequent steps are adding mouse monoclonal antibodies and a fluoresceinated tracer before the homocysteine measurement. The assay is fully automated and can easily be applied in laboratories who do not have HPLC equipment but do have the means for an FPIA.

Homocysteine concentrations measured in acidic citrate were corrected for the amount of fluid present in the tube prior to the blood collection by multiplying the measured concentration with 10/9. We did not correct for the fluid present in the EDTA tubes prior to collection (i.e., 0.117 ml), since the difference is negliable (±1%). All homocysteine measurements with HPLC(a) were corrected by subtracting 2.4 µmol/l from the measured concentration, according to the results as descibed by de Bree *et al.*[11].

Statistics

The mean homocysteine concentrations in acidic citrate and EDTA were compared by paired-samples T-tests.
We studied the association of homocysteine concentrations in acidic citate plasma and EDTA plasma with liniair regression for HPLC(a), HPLC(b) and FPIA.

Furthermore, to study the differences in individual values we plotted the mean difference of two individual measurements against the difference, according to the method described by Bland and Altman[18]. This method gives information about the spread of the differences between the individual values. We calculated the mean proportional bias (i.e., the mean difference in percentages) and the corresponding 95% confidence intervals (expressed as 'limits of agreement') after log-transformation of the homocysteine concentrations.

Results

Blood was obtained from 258 volunteers. From 50 volunteers there was an insufficient amount of plasma to perform all six analyses. Of 208 persons (79 male, 129 female; age 23-88, median 65), all six measurement were available for the analyses.

Homocysteine concentrations in EDTA and acidic citrate samples with the 3 measurement methods are shown in boxplots in Figure 4.1. With HPLC(a) mean homocysteine measured in acidic citrate was 1.8 µmol/l (95% CI 1.6 to 2.1 µmol/l) higher than in EDTA. Mean homocysteine in the acidic citrate was lower than in the EDTA samples (mean difference -2.8 µmol/l (95% CI 2.5 to 3.1 µmol/l) when measured with HPLC(b). The mean difference in homocysteine concentration was the smallest with FPIA: 0.1 µmol/l (95% CI 0.0 to 0.3 µmol/l).

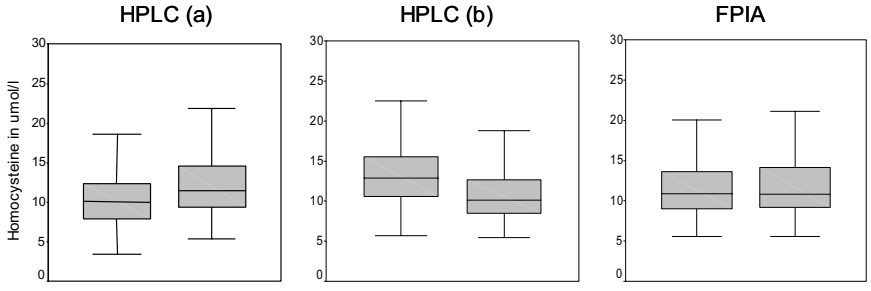

Figure 4.1 Boxplots of homocysteine measured in EDTA vs. acidic citrate anticoagulated blood. Measurement with 2 HPLC methods and FPIA.

The regression analysis of the homocysteine concentrations measured with HPLC(a) shows a slope of 1.01 (95% CI 0.96 to 1.07) and an intercept of 1.7 (95% CI 1.1 to 2.4). With HPLC(b) the slope was 0.75 (95% CI 0.72 to 0.78)

and the intercept at 0.7 (95% CI 0.2 to 1.2), meaning that homocysteine are approximately 25% lower measured in acidic citrate than in EDTA. With FPIA we calculated a slope of 0.95 (95% CI 0.92–0.98) with an intercept of 0.7 (95% CI 0.3 to 1.2). The plots are shown in Figure 4.2a-c.

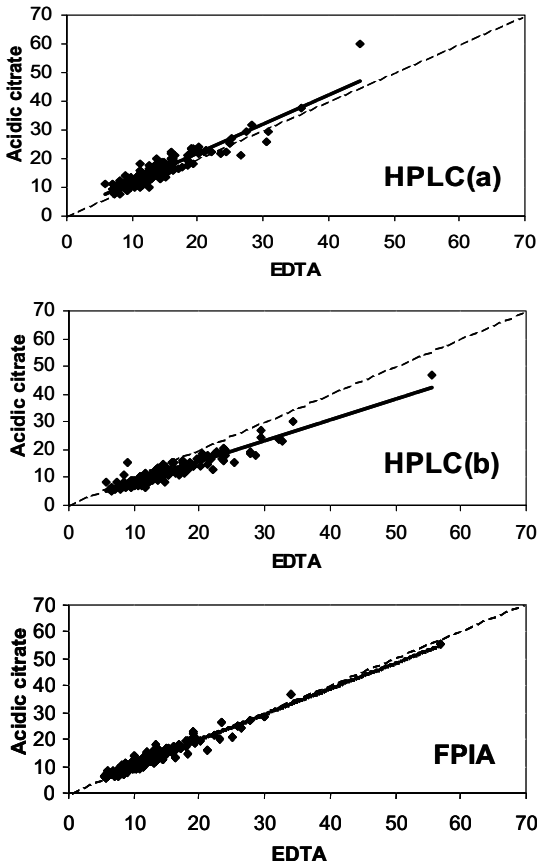

Figure 4.2 Total homocysteine (tHcy) in µmol/l in acidic citrate versus EDTA samples for HPLC(a), HPLC(b) and FPIA. (- - - = line of equality, —— = regression line)

The agreement of the individual concentrations is shown in the Bland-Altman plots. They show the spread of the differences between the individual measurements (Figure 4.3). It shows us that the acidic citrate values agree the most in the FPIA method. This is also shown in Table 4.1 in which the upper and lower levels of agreement are shown after log-transformation. The limits of agreement are the broadest with HPLC(b). This is a result of the fact that the

homocysteine concentrations in the acidic citrate tubes are approximately 75% of the concentrations in EDTA. With increasing concentrations the difference between individual concentrations gets higher but also the mean difference increases, so analysis of the limits of agreement results in a broad interval between the limits of agreement.

Table 4.1 Difference between homocysteine concentrations in acidic citrate and EDTA.

	Mean difference in µmol/l (EDTA – acidic citrate)	95% CI of the mean	Mean proportional bias	Lower limit of agreement	Upper limit of agreement
HPLC(a)	-1.8	-2.1 to -1.6	-16%	-40%	15%
HPLC(b)	2.8	2.5 to 3.1	24%	-4%	62%
FPIA	-0.1	-0.3 to 0.0	2%	-19%	16%

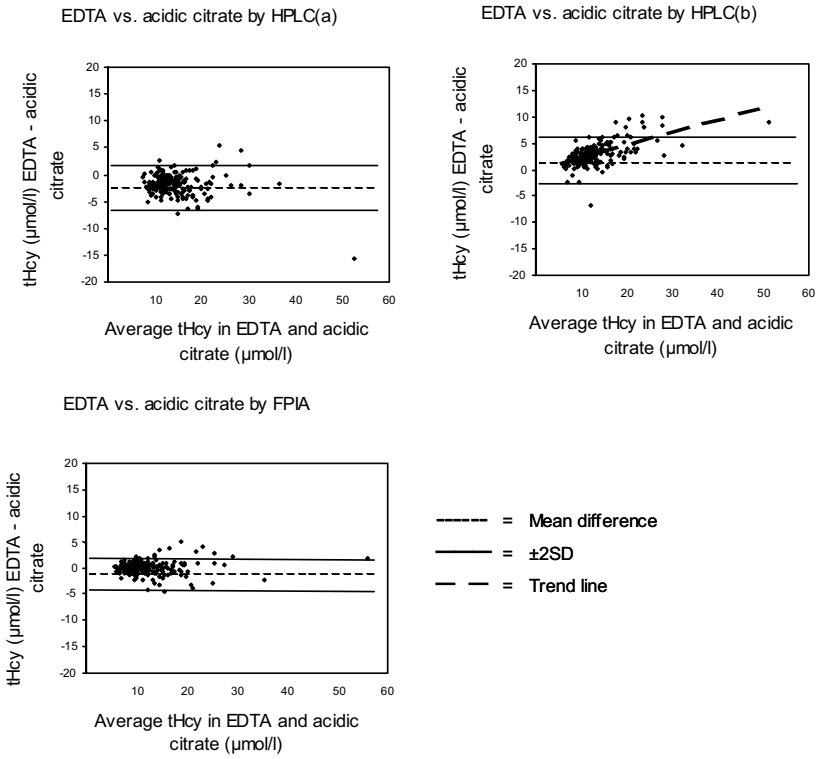

Figure 4.3 Bland-Altman plots of homocysteine measured in EDTA vs. acidic citrate anti-coagulated blood. Measurement with 2 HPLC methods and FPIA.

Discussion

Large epidemiological studies require blood collection techniques that are easily applicable in a field setting. Difficult blood collection techniques are sensitive to blood handling mistakes, which causes loss of information. For homocysteine measurement, storage of whole blood collected in EDTA prior to centrifugation should be done at 0°C to prevent elevation of homocysteine due to the homocysteineproduction by the blood cells, mainly the erythrocytes. Several alternative collection media have been proposed. Fluoride stabilized homocysteine for 2 hours at room temperature in several studies[8,12,19], but this could not be reproduced in other studies[20-23]. Hughes *et al*. also showed that sodium fluoride causes additional dilution defects, leading to lower plasma homocysteine concentrations at baseline and advises not to use NaF as anticoagulant for homocysteine measurement[20]. Probst *et al*. developed a collection device in which whole blood is lysed. Homocysteine remained stable in this lysate for 48 hours at room temperature[24]. Hill investigated 3-deazaadenosine (3DA) and found that EDTA blood with added 3DA stabilized homocysteine for 72 hours when stored at 2-8°C[25]. However, when the blood was stored at room temperature, homocysteine concentrations increased.
We previously reported that acidic citrate stabilizes homocysteine in whole blood at room temperature for 6 hours[12]. Our finding was confirmed by Salazar *et al*.[26]. They found that homocysteine stayed stable up to 6 hours when the measurement was done by HPLC. They used a HPLC method[27] that differed form ours. However, when they measured with FPIA, they found an increase in homocysteine in both acidic citrate and EDTA stored at 0°C[26].

The aim of the current study was to compare homocysteine measurement in acidic citrate plasma with homocysteine concentrations in EDTA plasma. We found that homocysteine measured in blood with acidic citrate as anticoagulant correlates well with homocysteine measured in blood with EDTA as anticoagulant, regardless of the method of measurement used. This is derived from the narrow 95% confidence intervals in the regression analyses. We also found that the absolute individual homocysteine concentrations in both media may differ. The level of agreement was the best with the FPIA method.
We found differences between the mean homocysteine concentrations in acidic citrate compared with EDTA. This difference is not the same for each measurement method, thus depends on the method used. With HPLC(a) the difference is constant, meaning that the homocysteine measured in acidic citrate is approximately 1.7 µmol/l higher than in the EDTA samples. With HPLC(b) the difference is dependant of the level of the homocysteine, the absolute difference rises with increasing concentrations of homocysteine. On

average the homocysteine in the acidic citrate samples is 25% lower than in the EDTA samples. The difference in mean homocysteine was the smallest with the FPIA (0.1 µmol/l), with a regression line close to the line of equality.
The difference we measured with HPLC(a) is comparable to the difference we found previously in a stability study in which homocysteine was 1.3 µmol/l higher in acidic citrate samples[12]. The same HPLC method was used in this study. With HPLC(b) we measured much lower homocysteine in the acidic citate samples. Salazar *et al*. found a smaller difference of 5% between homocysteine in EDTA and acidic citrate samples measured with HPLC, with homocysteine being lower in acidic citrate samples than in the EDTA samples[26]. We have hypothesized about the mechanisms causing homocysteine being higher in acidic citrate than in EDTA samples when measured with HPLC(a), and being lower with HPLC(b). We thought it could be a result of the acidic environement interacting with the measurement method. Therefore we added acid to EDTA plasma and did 10 additional measurements with HPLC(a), but the homocysteine concentrations in the regular EDTA plasma and the acidic EDTA plasma were comparable (data not shown). It may be that the reagents used in the measurement interact with homocysteine from acidic citrated plasma and influence the measurements. Since both HPLC methods use different reagents this could lead to the differences we found.
The difference found between homocysteine in EDTA versus acidic citrate samples with the FPIA was small. In the same study as mentioned above, Salazar also used the same FPIA method as in our study and also found that homocysteine in acidic citrate samples did not differ significantly from the homocysteine concentrations in EDTA samples[26]. Palmer-Toy *et al*. compared homocysteine in EDTA and citrate (i.e., non-acidic citrate) anticoagulated blood measured with FPIA[28]. They also found no difference between homocysteine in the collection media when they corrected the homocysteine values from the citrate samples with a correction factor. This correction factor corrected for hematocrit, based on gender and the added amounts of anticoagulant present in the collection tube before blood sampling. We used the same correction factor to transform our homocysteine values from the acidic citrate samples measured with FPIA and did the regression analysis for the FPIA method again, but this did not influence the results.

Based on the results of this study, we conclude that tubes with acidic citrate can be used in epidemiological studies since homocysteine concentrations correlate highly with those measured in EDTA plasma. Reference values need to be established when using acidic citrate tubes since individual values differ from those measured in EDTA samples and the difference is dependent on the measurement method being used. Individual values differ between EDTA and acidic citrate samples, therefore the two media cannot be compared directly.

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