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Assays for total homocysteine and other thiols by capillary electrophoresis–laser-induced fluorescence detection I. Preanalytical condition studies

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Abstract

In recent papers, we presented a new analytical method for thiol quantification in serum. It is based on the use of capillary electrophoresis and laser-induced fluorescence to analyze thiol 6-iodoacetamidofluoresceine (IAF) derivatives. Quantitative results of homocysteine, glutathione, cysteine–glycin, and cysteine were shown (Clin. Chem. 45 (1999) 412). A comprehensive comparison of the quantitation of homocysteine in serum, using high-performance liquid chromatography/conventional fluorescence detection and fluorescence polarization immunoassay was also used (E. Caussé et al., Electrophoresis 21 (2000) 2074). Sample preparation prior to derivatization with IAF had never been investigated. In this work we present the results of quantitation of thiols in serum and plasma with three different anticoagulants widely used: ethylenediaminetetraacetic acid (EDTA), heparin, and sodium citrate. We show that serum and EDTA plasma gave the same results. Then serum protein precipitations by acetonitrile, acetone, sulfosalicylic acid, perchloric acid and trichloracetic acid, prior to derivatization by IAF, were also investigated. Their influence on the concentrations of the thiols were determined. Sulfosalicylic acid and acetonitrile precipitations are well adapted, whereas acetone cannot be used.

Keywords: Homocysteine; Thiols; Iodoacetamidofluorescein; Cysteine; Amino acids; Glutathione

1. Introduction

Hyperhomocysteinemia is a well-known risk factor for atherosclerotic vascular disease [1,2], but the strength of the relationship and the interaction of serum/plasma homocysteine (Hcy) with other risk factors are unclear [3]. A 5-μmol/L Hcy increment elevates the risk of coronary artery disease by to the same extent as cholesterol increases of 0.5 mmol/L [2]. The measurement of thiol concentrations in human serum or plasma is therefore often useful. Most plasma thiols are metabolically related. Hcy may either be catabolized to cysteine (Cys) or remethylated to methionine. Cysteine and γ-glutamine–cysteine are precursors of glutathione (GSH). Cysteine–glycine (Cys–Gly) is derived from the breakdown of GSH [4].

Recently, different studies related to plasma thiols using capillary electrophoresis (CE) and laser-induced fluorescence (LIF) detection were published in

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the literature [5–8]. All those studies used fluorescein based fluorophores. These reagents were adapted to CE–LIF studies because of their negative charge at pH above 9 and their high quantum yield of fluorescence at an excitation wavelength of 488 nm with an argon ion laser. These methods are detailed with their advantages or disadvantages in Table 1.

The analysis (preanalytical and analytical) conditions must be followed up rigorously. Several studies on the biological variation of plasma Hcy have been published [9] but sample treatment is unclear [10]. Fasting blood samples have been traditionally recommended for plasma Hcy measurement [11]. The relationships between Hcy and other thiols with these changes were poorly reported [12].

On the other hand, in most of the methods previously developed for plasma analysis of thiols, different conditions were used for protein precipitations. Only a few reports indicated the differences observed with the different commonly used reagents, e.g. acetonitrile, acetone, sulfosalicylic acid, perchloric acid and trichloracetic acid [8].

In this work, 6-iodoacetamidofluorescein (6-IAF) was used to derivatize Hcy, Cys, Cys–Gly and GSH (thiol-containing molecules) and the derivatives were studied using CE–LIF.

We examined the variation of thiol concentrations under different anticoagulant conditions. Then, we evaluated the effects of the different protein precipitation reagents on the electropherograms and quantitations which were obtained.

2. Materials and methods

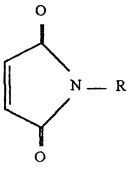
2.1. Chemicals

All chemicals were purchased from Sigma (St. Quentin Fallavier, France), except 6-IAF and the agent tris(2-carboxyethyl)phosphine (TCEP) that came from Molecular Probes (Eugene OR, USA) [8,13].

2.2. Clinical samples

After an overnight fast, whole blood from 10 apparently healthy volunteers (five men and five women, aged 22–52 years) was collected into evacuated blood-collecting tubes (four tubes for each subject), without additives (for preparation of serum) or containing ethylenediaminetetraaceticacid (EDTA), or heparin, or citrate as anticoagulant (for preparation of plasma). All the samples were kept cold in ice (<4°C) after collection and were immediately centrifuged at 4°C, 3000 *g*, 10 min. Serum and plasma supernatants were transferred to new vials and stored at −20°C until the analysis. All volunteers were in a good nutritional state with a normal protein intake. None took drugs.

Table 1
The different fluorescein-based reagents used in CE–LIF studies of homocysteine and plasma thiols[a]

Reactive	Chemical function	Qualities of the derivatives	Ref.
Fluorescein isothiocyanate	R–S=C=N	High sensitivity, poor selectivity	[5]
Bromomethylfluorescein	R–CH_2-Br	High sensitivity, high stability, low selectivity (SH and COOH functions can be labeled)	[6]
Fluoresceinmaleimide		High sensitivity, high stability, high selectivity, but diastereoisomers	[7]
Iodoacetamidofluorescein	R–NHCOCH$_2$I	High sensitivity, high selectivity	[8]

[a] R=Fluorescein nucleus.

2.3. Sample preparation

Plasma or serum samples (100 μL or 50 μL) were treated for 15 min at room temperature with 10 μL (or 5 μL) of 35 m*M* TCEP (a thiol reducing agent), as previously reported [9]. Using this procedure, all the different thiols were analyzed in their reduced form: homocysteine (tHcy; mixed and symmetric disulfides, including protein-bound Hcy), Cys, Cys–Gly and GSH. All patient samples and plasma-based calibrators were incubated with TCEP and an internal standard [*N*-acetylcysteine (NA-Cys)], 5 μL at 10 mmol/L) under the same conditions. The solution was deproteinized with 100 μL of precipitating reagent (i) sulfosalicylic acid (0.8 mol/L, SSA), (ii) perchloric acid (10%, PCA) (iii) trichloracetic acid (4%, TCA), (iv) acetonitrile (pure) or acetone (pure), with vortex mixing, followed by centrifugation at 10 000 *g*, 15 min. We mixed 100 μL of the supernatant with carbonate buffer (50 μL, 0.3 mol/L, pH 9.5) and sodium hydroxide, 5 mol/L (10 μL for SSA and PCA, but 5 μL only for TCA; none for acetonitrile or acetone). Then, we added 50 μL of a 6-IAF solution (1 g/L) in dimethyl sulfoxide (DMSO). The mixture was incubated overnight in the dark, at room temperature, to allow analysis after the reaction has plateaued (>2 h) and is stable for several days [8]. Each derivatized plasma was analyzed after a 5000-fold dilution.

2.4. CE–LIF detection

The analyses were performed on a ZETA CE instrument (Picometrics, Toulouse–Ramonville, France). It was equipped with a ZETA LIF detector and an argon ion 488-nm wavelength laser. The ZETA LIF detector collects the fluorescence above 515 nm. The thiols were separated on a 50-μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 50 cm and a total length of 85 cm. The separation buffer consisted of 50 mmol/L boric acid and 20 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) adjusted to pH 10 by addition of a sodium hydroxide solution (10 mol/L). The pH increase of the buffer allowed us to get better separations without sodium dodecylsulfate. The separation voltage was +30 kV, resulting in an electrophoretic current of 35 μA. These conditions are an improvement when compared to the previous study [8], in which sodium dodecylsulfate was used at a lower pH buffer. Nice electropherograms were obtained [8].

2.5. Statistical analysis

All data were given as means±standard error of the mean (SEM). Statistical significance was assumed for $P<0.05$ and calculated using an unpaired Friedman test. Linear regression curves and correlation coefficients were calculated according to the least squares method.

3. Results and discussion

Measurements of thiols are difficult because they are highly reactive and easily oxidized during sample preparation. Moreover, an increase in Hcy would be expected postprandially because of the intake of methionine from proteins and reported results showed small and rather variable effects. Furthermore, it was recommended that the patient should fast, as was the case here. Consequently, all the factors which can influence the concentration of plasma thiols must be examined and preanalytical and analytical conditions must be followed rigorously.

3.1. Effect of anticoagulant

Our aim was to evaluate the influence of different anticoagulants on the plasma thiol quantitation. Table 2 shows concentrations (means±SEM in μmol/L) of the different thiols obtained using serum or plasma in which anticoagulant was EDTA, heparin or sodium citrate. All our samples were centrifuged immediately after blood sampling. The sera and plasmas were stored at −20°C until they were analyzed. For each subject, the four samples were analyzed on the same day. With our method, no significant difference between serum, EDTA and heparin for Hcy was detected. However, we observed significantly lower levels for Cys–Gly, and Cys with sodium citrate and a higher level for GSH. We can infer that we can collect blood with EDTA as anticoagulant or without anticoagulant (serum). Frantzen et al. [14] found nearly identical Hcy con-

Table 2
Effect of the different anticoagulants on the quantitation of the different thiols. (mean±standard error of mean, expressed in μmol/L)

	Serum	EDTA	Citrate	Heparin	Significance[a]
Hcy	8.56±0.67	8.35±0.70	8.01±0.61	8.50±0.68	NS
Cys–Gly	29.5±1.54	30.2±1.61	27.6±2.13	28.6±1.24	$P<0.05$
Cys	220±9.3	220±8.4	197±8.3	215±8.7	$P<0.05$
GSH	6.51±0.50	7.01±0.51	7.38±0.5	6.77±0.58	$P<0.05$

[a] NS: not significant; significant results between sodium citrate and other anticoagulants.

centrations (with immunoassay method) in serum and plasma, independent of the type of anticoagulant. However, the results vary with the analytical method used. Houzé et al. [15] had similar Hcy levels with EDTA and citrate, but higher levels (+25%) with heparin and (+10%) with serum. On the contrary, Salazar et al. [16] indicated that Cys, Hcy and GSH concentrations measured with HPLC remained stable whatever the anticoagulant used: EDTA at 0°C or acidic citrate at room temperature. Collection on EDTA medium is not possible, in particular for epidemiological surveys. Plasma rather than serum is recommended to facilitate rapid centrifugation. The most widely used anticoagulant is EDTA. In our previous studies the analyses were performed on serum. However, serum is not very convenient compared to plasma. There must be a very short time between sampling and analysis. This study shows that when this is not possible, EDTA plasma can be used as well, without interference on thiol concentrations. Other anticoagulants give different results ($P<0.05$).

3.2. Protein precipitation

One of the least described steps of plasma or serum studies is the precipitation step of the plasmatic proteins. We tested different conditions described in the literature to clearly identify, in our case, the simplest way to precipitate proteins. Generally, two methods can be used: (i) acidic method, i.e. SSA, TCA, PCA, (ii) organic solvent methods i.e. acetonitrile or acetone. The use of acetonitrile is well documented for plasma CE applications [10]. Table 3 shows the calibration equations obtained from serum added with known amounts of Hcy for the acidic and organic solvent conditions for protein precipitation. In Table 4 we present the same work on Cys, Cys–Gly and GSH. All those results present the ratio of the areas of the peaks of the thiols to the internal standard (*N*-acetylcysteine), as a function of the concentration of thiols in the serum. Fig. 1 presents the electropherograms obtained for each protein precipitation method from the same serum at a concentration of Hcy of 20 μ*M* (diluted 5000 times prior to injection). The separation of Hcy, Cys–Gly are quite good for PCA, TCA, SSA and acetonitrile precipitations, even if PCA precipitation give better resolution. For GSH identification, the best separations were achieved with acetonitrile and SSA precipitations. Moreover, better extraction yields are obtained using SSA and acetonitrile, making quantifications easier than with PCA and TCA. We had some difficulties of GSH identification with PCA and TCA precipitations which made it necessary to spike the sample with standard GSH.

On the other hand, we can see that acetone is particularly useless for homocysteine studies, because no peak was detected in the range of concentration we were expecting, and no calibration curve could be obtained. Moreover, we can see that all the other protein precipitation methods lead to obtaining quite the same calibration curves. TCA seems to give a higher intercept, indicating a better Hcy recovery from the sample.

A disadvantage, in acidic precipitation, is that we have to increase the pH above 9, prior to IAF

Table 3
Intercepts, slopes and coefficients of correlation (r^2) of the calibration curves of homocysteine in serum, using different protein precipitation methods[a]

	SSA	TCA	PCA	Acetone	Acetonitrile
Intercept	0.1917	0.3452	0.1719	none	0.1900
Slope	0.0264	0.0266	0.0315	none	0.0225
r^2	0.9854	0.9851	0.9912	none	0.9655

[a] RSDs of intercepts are lower than 3.2% ($n=3$), RSDs of slopes are lower than 1.7% ($n=3$).

Table 4
Intercepts, slopes and coefficients of correlation (r^2), on the calibration curves of homocysteine, cysteine, cysteinyl–glycine and glutathione in serum using different protein precipitation methods[a]

	Hcy	Cys	Cys–Gly	GSH
TCA Intercept	0.3452	1.7780	0.2219	None
TCA slope (μM^{-1})	0.0266	0.0189	0.0131	None
TCA r^2	0.9851	0.8469	0.9739	None
Acetonitrile Intercept	0.1900	3.7563	0.2969	0.0897
Acetonitrile slope (μM^{-1})	0.0225	0.0252	0.0128	0.0097
Acetonitrile r^2	0.9655	0.9883	0.9887	0.9534
Ratio acetonitrile/TCA[b]	0.543	1.967	1.11	None
Ratio acetonitrile/SSA[b]	0.764	1.756	0.655	0.699

[a] RSDs are 5.7% (n=3), RSDs of Intercepts are lower than 3.2% (n=3), RSDs of slopes are lower than 1.7% (n=3).
[b] Ratio of peak areas of each compound at 20 μM.

labeling and CE separation. In the previous studies [8,13], we used SSA to precipitate protein. Here, acetonitrile seems to be more convenient, because no pH adjustment is required after protein precipitation.

The comparison between TCA precipitation and acetonitrile (Table 4) leads to the conclusion that TCA is well adapted to Hcy quantitation only. Actually, Cys and Cys–Gly calibration curves are lower than the ones obtained with acetonitrile, whereas GSH does not give useful results and cannot be used for quantitative studies. Our previous work [8,13] showed the good behavior of SSA for protein precipitation. In Table 4, we compare SSA results with acetonitrile, particularly the acetonitrile/SSA ratio. Table 4 presents the ratio of the peak areas of Hcy, Cys, Cys–Gly and GSH, in the same serum at a concentration of 20 μM using acetonitrile precipitation versus SSA precipitation. We can see that these ratios are more favorable for SSA precipitation, in all cases except for Cys. However, SSA precipitation requires an additional step to increase the pH prior to derivatization, a step not required with

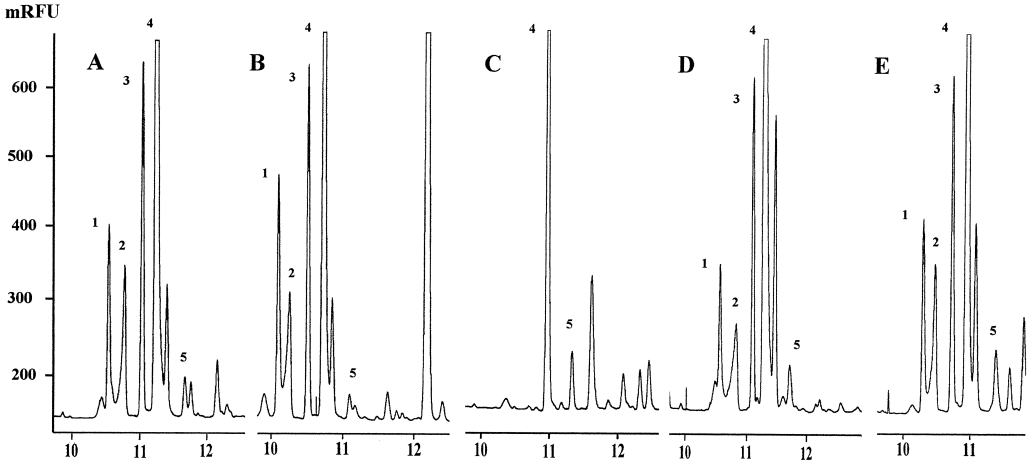


Fig. 1. Electropherograms of thiols in the same spiked serum obtained after different methods of precipitation: (A) perchloric acid (PCA); (B) trichloroacetic acid (TCA); (C) acetone; (D): acetonitrile; (E) sulfosalycylic acid (SSA). 1=Homocysteine (30 μmol/L), 2=cysteine–glycine (60 μmol/L), 3=*N*-acetylcysteine, 4=cysteine (150 μmol/L), 5=glutathione (30 μmol/L). mRFU: milli-relative fluorescence units. Electrophoretic conditions: boric acid 50 m*M*, CAPS 20 m*M* at pH 10, +30 kV (25 μA). Each derivatized sample is diluted 5000-fold before analysis. *x*-Axis=migration time in min.

acetonitrile precipitation, keeping the sample process easier and more accurate (less errors in pipeting).

4. Conclusion

In this work we show that quantitation of thiols in serum or plasma using EDTA gives similar results, whereas plasma obtained with heparin or sodium citrate can give different results. This can affect the clinical interpretation of results because the concentration of Hcy between healthy subjects and pathologic patients is very close. The best way to complete protein precipitation is to use acetonitrile and sulfosalicylic acid. Acetonitrile results in lower peaks for the same quantities of thiols. However, it is easier to use because it does not require an increase in pH prior to derivatization.

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