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Journal of Chromatography A, 979 (2002) 255–260

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Assay of total homocysteine and other thiols by capillary electrophoresis and laser-induced fluorescence detection II. Pre-analytical and analytical conditions

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Abstract

In recent papers, we presented a new analytical method for thiol quantification in serum. This method was developed with capillary electrophoresis (CE) and laser-induced fluorescence (LIF) to analyze thiol-iodoacetamidofluoresceine (IAF) derivatives. Quantitative results for homocysteine, glutathione, cysteinylglycine, and cysteine were presented (Caussé E., et al., Clin. Chem. 45 (1999) 412). An exhaustive comparison of the quantitation of homocysteine in plasma, using high-performance liquid chromatography with either conventional fluorescence detection or fluorescence polarization immunoassay was also reported (Caussé E., et al., Electrophoresis 21 (2000) 2074). Sample preparation prior to derivatization with IAF had never been investigated. Recently we studied protein precipitation in serum with different organic agents (Caussé E., et al., J. Chromatogr. A 895 (2000) 173). In this work, we evaluated the conditions of protein precipitation in function of the amounts of acetonitrile and their influence on quantitation and quality of the electropherograms. Then, we looked at the variation of thiol concentrations in the haemolysis states and studied the thiol stability of blood samples cooled on ice.

Keywords: Homocysteine; Thiols; Iodoacetamidofluoresceine

1. Introduction

Hyperhomocysteinemia is a well-known risk factor for atherosclerotic vascular disease [1,2]. A 5-μmol/l increment in homocysteine (Hcy) elevates coronary artery disease risk by as much as cholesterol increases of 0.5 m*M* [2]. So, the analysis conditions (preanalytical and analytical) must be defined rigorously. Moreover, the measurement of thiol concentrations in human serum or plasma is often useful. Most of the plasma thiols are metabolically related. Homocysteine (Hcy) may either be catabolized to cysteine (Cys) or remethylated to methionine. Cysteine and γ-glutamylcysteine are precursors to glutathione (G-SH). Cysteinylglycine (CysGly) is derived from the breakdown of glutathione [3]. As previously reported [4–7], iodoacetamidofluoresceine (IAF) was used to derivatize plasma thiols which we studied by capillary

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01504-2

electrophoresis and laser-induced fluorescence detection (CE-LIF). Other studies related to plasma thiols using capillary electrophoresis and laser-induced fluorescence detection were published [8,9]. All of them used fluoresceine-based chromophores. These dyes are suitable for CE-LIF studies thanks to their negative charge above pH 9 and their high quantum yield of fluorescence at 488 nm using an argon ion laser.

Recently, we reported some differences with commonly used protein precipitation reagents [6]. Acetonitrile and sulfosalicylic acid offered the best yields to extract thiol. In this study, we evaluate different protein precipitation conditions in function of acetonitrile amounts, in terms of quality of electropherograms and quantitations. Then, we looked at the variations of the thiol concentrations in various haemolysed plasmas and the stability of blood samples cooled on ice.

2. Materials and methods

2.1. Chemicals

All chemicals where purchased from Sigma (St. Quentin Fallavier, France) except 6-IAF and tris(2-carboxyethyl)phosphine (TCEP) which came from Molecular Probes (Eugene, OR, USA).

2.2. Subjects

After an overnight fast, whole blood from 10 clinically healthy volunteers (five men and five women aged 22–52 years) was sampled into vacutainer blood-collecting tubes (six tubes for each subject), without additives (for preparation of serum) or containing EDTA as an anticoagulant (for preparation of plasma). All samples were cooled on ice (<4 °C) after collection. Afterwards, sera or plasmas were kept at -20 °C until they were analyzed. All volunteers were in good nutritional condition with normal protein intake. None took drugs.

2.3. Protein precipitation and thiol labeling

Plasma or serum samples (100 or 50 μl) were treated for 15 min at room temperature with 10 μl (or 5 μl) of TCEP (a thiol-reducing agent) 35 m*M*, as previously reported [4]. Using this procedure, all the different thiols were analyzed in their reduced form: homocysteine (tHcy; mixed and symmetric disulfides, including protein-bound Hcy), cysteine (Cys), cysteinylglycine (CysGly) and glutathione (GSH). All patient samples and plasma-based calibrators were incubated with TCEP and an internal standard (*N*-acetylcysteine (NA-Cys)) 5 μl at 1 m*M*) under the same conditions. The solution was deproteinized with 100, 200, 300 and 400 μl of acetonitrile, vortex mixed and centrifuged at 10 000 *g* for 15 min. Then to 100 μl of supernatant with 50 μl carbonate buffer, we added 50 μl of a 6-iodoacetamidofluorescein solution, 1 g/l in dimethylsulfoxide (DMSO). The mixture was incubated overnight in the dark at room temperature, to make sure the reaction had plateaued (>2 h) before the analysis. Each derivatized plasma was diluted 1000 times in water prior to analyzing it by capillary electrophoresis (CE).

2.4. Hemolysis

We studied the thiol leakage from the erythrocytes by inducing hemolysis by oxalate addition to the blood. Different quantities of the 1% oxalate in the serum (12–250 μl) were added to 250 μl whole blood in 12 tubes. After centrifugation, different degrees of hemolysis were observed and recorded on a cross scale from + for only slightly hemolysed plasma to ++++ for highly red-colored plasma. Then, the plasmas were treated as described above. Results are reported in Fig. 2.

2.5. Effect of delayed centrifugation of blood

We studied the thiol leakage effect from erythrocytes or active metabolism on the concentration of thiols in plasma. Whole blood (10 subjects) was collected in three pre-chilled (in ice-water) EDTA-vacutainer tubes and three dry vacutainers which were placed without delay in ice-water and chilled for a few minutes (T0), 1 h (T1) or 4 h (T4) before centrifugation at 3000 rpm at 4 °C for 10 min. Then, sera and plasmas were kept at -20 °C until analysis.

2.6. Capillary electrophoresis and laser-induced fluorescence detection

The samples were analyzed with a Phoresis 100

CE instrument (Zeta Technology, Toulouse–Ramonville, France) and a ZETALIF laser-induced fluorescence detector coupled to an argon ion 488 nm laser. The ZETALIF detector collected all the fluorescence above 515 nm. The different thiols were separated on a 50-μm I.D. fused-silica capillary (Polymicro Technology, Phoenix, AZ) with an effective length of 50 cm and a total length of 85 cm. The separation buffer consisted of 50 m*M* boric acid and 20 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) adjusted to pH 10 by addition of a sodium hydroxide solution (10 mol/l). The increase of the buffer pH gave better separations without any sodium dodecylsulfate. The separation voltage was +30 kV, resulting in an electrophoretic current of 35 μA. We injected hydrodynamically for 3 s at 50 mbar.

2.7. Statistical analysis

All data are given as mean±S.E.M. Statistical significance was assumed for $P<0.05$. Linear regression curves and correlation coefficients were calculated according to the least square method. Data obtained were analyzed by the Friedman test (non-parametric analysis of variance) [10]

3. Results and discussion

Fig. 1 presents a plasma separation of homocysteine, cysteine, cysteinyl–glycine and glutathione. Amino acids labeled with iodoacetamidofluoresceine (IAF) have two negative charges due to the fluoresceine nucleus and one or two due to the carboxylate functions of the amino acid or peptide. The pK_a values of α-amino functions were around 9–10 for free amino acids [11] and remain unknown for IAF derivatives. It is reasonable to assume that α-amino functions stay neutral at pH 10. The total charge of Cys containing species, i.e., CysGly, NACys, Cys, is −3. The −3 charged species migrate in function of their shape: the biggest (CysGly) has the lowest electrophoretic velocity and migrates first whereas Cys which is the smallest molecule has the highest electrophoretic velocity and migrates at the longest migration time of these three compounds. Normally, Hcy has a −3 charge. Its migration time is the shortest of the four species. However, it is a smaller species than CysGly. So it should migrate after this

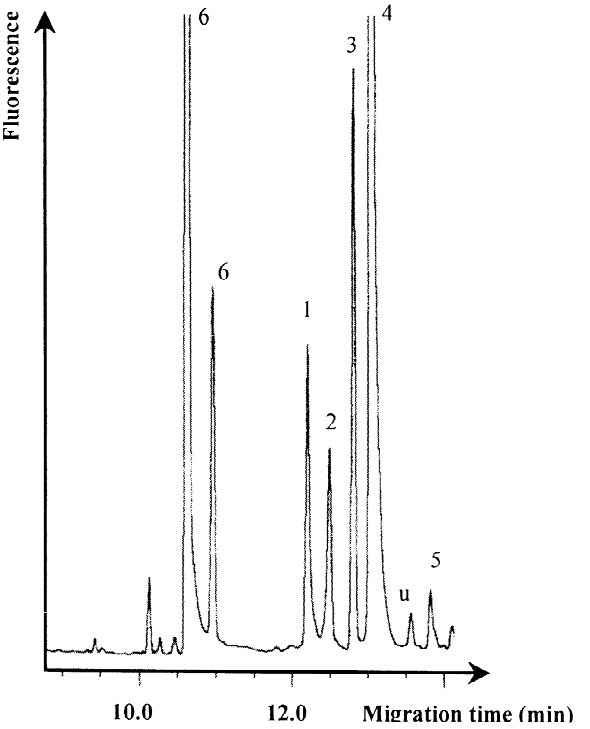

Fig. 1. Example of separation of 6-IAF-labeled thiols in a pathological sample by CE-LIF. Peaks: (1) homocysteine (Hcy), (2) cysteinyl–glycine (CysGly), (3) *N*-acetylcysteine, (4) cysteine (Cys), (5) glutathione (GSH), (6) 6-iodoacetamidofluorescein (IAF), (u) unknown. Pathological sample: Hcy, 32 μ*M*; Cys–Gly, 31 μ*M*; Cys, 280 μ*M*; G-SH, 4.1 μ*M*. Analytical conditions and sample preparation are indicated in Section 2.

molecule. This behavior may result from a higher pK_a value for the α-amino function of the IAF derivative which would add a small + charge to this compound, compared to the other −3 charged species. IAF-homocysteine has less than three negative charges. GSH is the γ-glutamylcysteinylglycine species that migrates the most slowly. When it is derivatized with IAF, it is charged −4 at the pH we use. Therefore it is the slowest species to migrate.

3.1. Acetonitrile precipitation

In a previous work, we noticed that acetonitrile precipitation and sulfosalicylic acid precipitation give similar results. However, sample preparation before CE analysis was shorter with acetonitrile because no neutralization step is required before adding the dye. Moreover, some years ago, Shihabi

[12] proposed to use acetonitrile for protein precipitation and CE analysis of serum theophilline. He indicated that the best results for protein precipitation were obtained with 60% acetonitrile. Previously, we used the same amount of acetonitrile to precipitate protein for thiol extraction [5]. In this study we analyzed 100 μl of a serum, adding 100, 200, 300 and 400 μl of acetonitrile after TCEP treatment, i.e., 48–79% acetonitrile. The effect on peak height (PH) and theoretical plate number (*N*) of the different thiols are presented in Table 1. This work was repeated four times. The mean values we observed for *N* and PH are indicated. The relative standard deviations are lower than 12% for *N* and 6% for PH. GSH was difficult to identify for an acetonitrile concentration above 66%. As we can notice, when the amount of acetonitrile increases, the peak height of all the thiols decreases. On the contrary, when the acetonitrile concentration increases, the theoretical plate number increases. It may result from ''cleaner'' samples, containing fewer salts. Therefore we chose to use 48% acetonitrile to achieve protein precipitation. Lower amounts of acetonitrile did not give better results.

3.2. Hemolysis

Fig. 2 shows the haemolytic effect on thiol assays. We observed stable Hcy values in all samples. However, it is well known that erythrocytes can contribute significantly to homocyteine accumulation in blood samples, because all the enzymes required to metabolize methionine in homocysteine are expressed in erythrocytes. Intracellular erythrocyte homocysteine content can therefore be expected to increase as a result of continued methylation [13]. Cell culture studies have also shown that homocysteine does not accumulate intracellularly and is released in the extracellular fluid [14]. On the contrary, the intra-erythrocytic Hcy level was not modified in hemolytic samples. Therefore our measures confirm previous HPLC studies [15].

3.3. Effect of delayed centrifugation of blood

Plasma rather than serum is recommended to facilitate rapid centrifugation. The most widely used anticoagulant is EDTA. However, in serum or plasma, it was shown that total homocysteine increases when separation from cells is delayed [15,16]. The absolute increase in plasma homocysteine is independent of the initial plasma concentration. This may cause larger proportionate errors in samples with normal plasma homocysteine content, whereas the relative increase is less likely to alter the clinical interpretation at higher basal concentration [17]. So we determined the mean concentrations of different thiols in whole blood cooled on ice with delayed centrifugation (0, 1 or 4 h after blood collection). The results are shown in Table 2. The 10 subjects we studied, showed a similar pattern. We observed a small non-significant increase after 4 h in ice for homocysteine, especially in serum. This slight increase was also observed by other authors who indicated that the rate of increase is approximately 1 μmol/l per h during the first 24 h at 22 °C [15,16]. In contrast, GSH is significantly increased after 4 h in EDTA. Consequently, as previously described [15], if immediate centrifugation is not possible, the increase in homocysteine is reduced by keeping the whole blood on ice until separation is done (maximum 2 h). We confirm these results with our method.

Table 1
Theoretical plate number (*N*) and peak height (PH) of plasma thiols for different concentrations of acetonitrile for protein precipitation ($n=4$)

Acetonitrile (%)	Hcy		CysGly		NACys		Cys		C-SH	
	N	PH	*N*	PH	*N*	PH	*N*	PH	*N*	PH
48	110 600	303.4	159 200	457.4	241 300	426.7	127 800	1768.1	137 750	134.9
66	113 700	153.9	190 400	107.1	245 200	155.7	173 500	694.2	UD	26.7
74	109 100	129.8	189 900	45.6	245 300	120.8	172 400	431.4	UD	11.1
79	81 800	89.7	145 600	35.6	240 900	125.2	150 550	373.8	UD	5

The relative standard deviations are lower than 12% for*N* and 6% for PH.
PH, peak height in milli relative fluorescence units (mrfu); UD, the measurement could not be made.

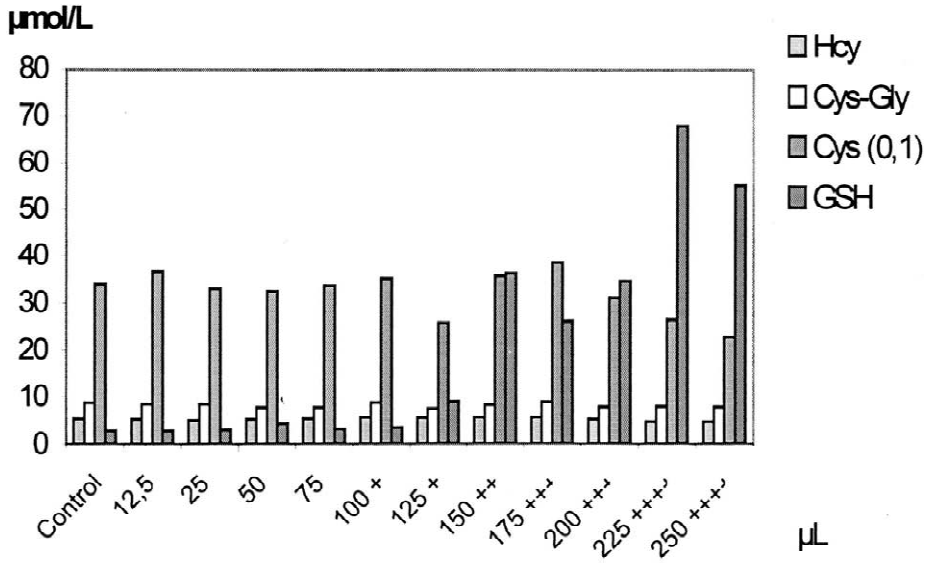

Fig. 2. Hemolytic effect on thiol assays: concentration of thiols (μM) in plasma versus addition into the same whole blood of different quantities (μl) of ammonium oxalate solution (1%). Concentration of cysteine is divided by 10. The cross scale is given from + for only slightly haemolysed plasma to ++++ for highly red-colored plasma. Analytical conditions and sample preparation are indicated in Section 2.

These observations are identical for CysGly and Cys but more care must be taken for GSH, whose determination should be done quickly after blood collection.

4. Conclusions

In conclusion, the study of several pre-analytical steps for the analysis of plasma thiols revealed that the best conditions to precipitate protein and obtain good plasma thiol separation is 48% acetonitrile. We verified that homocysteine and CysGly degrades slowly during the blood sampling and analysis phases. However, GSH and cysteine degrade faster, which may affect the analysis. As reported by several authors, we showed that the haemolysis does not significantly affect the concentration of homocysteine. But it has different effects on cysteine, cysteinyl–glycine and glutathione.

Acknowledgements

This work was supported by grants from the Conseil Régional Midi-Pyrénées and Picometrics.

Table 2
Effect of the delayed centrifugation on thiols concentrations in plasma or serum from 10 volunteer subjects (means±S.E.M., µmol/l)

	Serum				EDTA			
	T0	T1	T4		T0	T1	T4	
Hcy	8.56±0.67	8.57±0.59	9.26±0.75	NS	8.35±0.70	8.31±0.61	8.55±0.73	NS
CysGly	29.5±1.54	30.2±1.61	32.7±2.20	NS	30.8±1.56	30.8±1.72	31.5±1.72	NS
Cys	220±9.3	225±7.2	236±12.9	NS	220±8.4	220±7.0	218±7.3	NS
G-SH	6.51±0.50	6.40±0.50	6.97±0.57	NS	7.01±0.51	7.34±0.42	8.79±0.66	$P<0.05$

Cys (cysteine); CysGly (cysteinyl–glycine); G-SH (glutathione); Hcy (homocysteine).

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