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Determination of reduced and oxidized homocysteine and related thiols in plasma by thiol-specific pre-column derivatization and capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A new sensitive and rapid capillary electrophoresis (CE) assay for measuring reduced and oxidized thiols in human plasma has been developed. To prevent oxidation of the thiols, whole blood was immediately centrifuged after collection and the plasma proteins were precipitated with perchloric acid. The reduced thiols in the supernatant were derivatized quantitatively at 25°C, pH 7.5 with a fluorescent reagent, fluorescein-5-maleimide (FM). The total plasma concentration of thiols, including the fraction coupled to proteins, was assayed after an initial reduction of the disulfide linkage in plasma with dithiothreitol. The separation of FM-thiols was performed in an acetonitrile/10 m*M* sodium phosphate–50 m*M* SDS buffer [25:75 (v/v); pH 7.0] using a fused-silica capillary (57 cm×75 μm I.D.) at 45°C. A 3-mW argon-ion laser (λ_{ex} 488 nm/λ_{em} 520 nm) was employed for FM-thiol detection. With the electric field of 530 V/cm, the time needed for the separation of FM-homocysteine, FM-glutathione and FM-*N*-acetylcysteine was less than 8 min. The lower limit of detection was 3 μ*M* for the total thiols and 10 n*M* for the reduced thiols. The method was applied to the determination of homocysteine levels in plasma from patients with end-stage renal disease. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Homocysteine; Thiols

1. Introduction

Homocysteine (Hcys), a thiol-containing amino acid, is formed in numerous transmethylation reactions by demethylation of methionine (Fig. 1). Hcys is then either remethylated, again forming methionine or is irreversibly catabolized in a series of reactions to form cystathionine and cysteine [1]. In most tissues, the remethylation of Hcys to

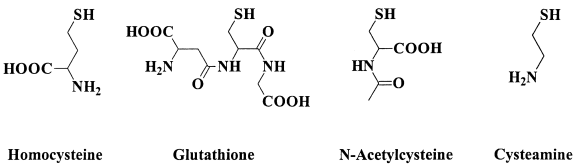

Fig. 1. Structure of the solutes.

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methionine is dependent on the cofactor activity of folate and vitamin B_{12} [2].

Clinical studies have identified hyperhomocysteinemia as a risk factor for cerebrovascular, peripheral vascular and coronary heart diseases [3–5]. Furthermore, plasma Hcys concentration is a sensitive marker for the diagnosis and follow-up of folate and vitamin B_{12} deficiencies. Thus, the routine determination of Hcys plasma levels is often desirable.

In biological systems, thiols easily undergo oxidation to various disulfides. In plasma, almost all Hcys is in the oxidized dimeric form (homocystine), either bound to protein (70–80%) or to low-molecular-mass sulfhydryls (20–30%) – and only traces are found in reduced sulfhydryl form (homocysteine) [6,7]. The terms total and reduced Hcys (tHcys and rHcys, respectively) are commonly used to define the different homocysteinyl derivatives. tHcys refers to all Hcys moieties, homocystine and homocysteine. rHcys corresponds to the fraction of Hcys that is exclusively in its sulfhydryl form (homocysteine).

The increasing interest for monitoring Hcys levels in biological fluids has led to the development of several chromatographic methods, including gas chromatography–mass spectrometry [8], ion-exchange chromatography [9], high-performance liquid chromatography (HPLC) coupled to UV, fluorescence or electrochemical detection [10–13]. Sensitive and reliable assays were obtained by labeling Hcys with a thiol-specific fluorescent reagent, followed by HPLC and fluorescence detection [6,14–18]. Among the most widely used fluorescent probes, monobromobimane [19,20], 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD) [21], and 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [22,23] have demonstrated a good selectivity and a high fluorescence yield, in spite of the instability of their thiol adducts and the complex HPLC conditions often required for their separation. Recently, 4-aminosulfonyl-7-fluoro-2, 1, 3-benzodiazole-4-sulfonate (ABD-F) was used for the derivatization of plasma Hcys before analysis with capillary electrophoresis (CE) and UV detection [24]. This rapid and simple method pointed out the promising applications of CE in the determination of plasma thiols. Although there are numerous advantages of this procedure, the method lacked the sensitivity required to quantify the different homocysteinyl derivatives such as rHcys, which is only found in trace amounts in human plasma.

Most of the available assays for the measurement of Hcys have practical limitations in terms of sensitivity or time-consuming procedures. Moreover, few of them are selective enough to provide a suitable determination of the different homocysteinyl compounds. In this paper, we report the development and the validation of a simple, sensitive and selective CE method using laser-induced fluorescence (LIF) detection for the determination of tHcys and rHcys in plasma. Fluorescein-5-maleimide (FM) was used as a fluorescent reagent. Fluoresceine has the strongest absorption and highest fluorescence quantum yields for instrumentation using the 488 nm spectral line of an argon-ion laser and a filter collecting the emission at 520 nm. Maleimides are excellent reagents for thiol-selective modification, quantification and analysis [25–27]. As a result, FM, which is commercially available, was a promising candidate for the derivatization of Hcys and related compounds. The resulting assay has been fully validated for tHcys determination and applied to a study in hemodialysis patients of high-dose folic acid therapy as a treatment for hyperhomocysteinemia.

Two other thiols of clinical interest are *N*-acetylcysteine (NAC) and the tripeptide glutathione (GSH), L-γ-glutamyl-L-cysteinylglycine (Fig. 1). NAC is widely used as a mucolytic agent [28], in cancer chemotherapy [29] and as an antidote in paracetamol overdosage [30]. The properties of NAC as a protective agent in paracetamol overdosage and in chemotherapy depend on its function as a precursor amino acid for GSH synthesis [31]. GSH acts as an enzyme cofactor [32], a detoxification agent in case of high concentrations of poisonous heavy metals [33], a protective agent against irradiation damage [34], and as an antioxidant [35]. These two thiols can also be assayed by this method.

2. Experimental

2.1. Chemicals

Homocysteine, homocystine, reduced and oxidized glutathione, *N*-acetylcysteine, cysteamine, cystamine,

ethylenediaminetetraacetic acid and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). The derivatizing agent, fluorescein-5-maleimide, was purchased from Molecular Probes (Eugene, OR, USA). The source for HPLC grade acetonitrile was Mallinckrodt (Paris, Kentucky, USA). Sodium hydroxide, sodium phosphate, *o*-phosphoric acid and dodecyl sodium sulfate (SDS) were purchased from Fisher (Jessup, MD, USA). Perchloric acid was purchased from J.T. Backer (Phillipsburg, NJ, USA). Pooled control human plasma was supplied by the Blood Bank of Georgetown University Medical Center.

2.2. Instrumentation

The CE–LIF instrumentation used for the development and the validation of the method was a Beckman P/ACE 5500 system (Beckman, Fullerton, CA, USA) comprising a Beckman P/ACE auto-sampler and a Beckman P/ACE LIF detector (λ_{ex} 488 nm, λ_{em} 520 nm). The data acquisition was carried out with an IBM/PC Pentium 133 MHz equipped with the Beckman P/ACE Station software (version 1.0). The separation of FM-adducts was accomplished on a Beckman bare fused capillary 57 cm× 75 μm I.D.

2.3. Chromatographic procedures

Baseline separation of the three compounds was achieved with an electrophoretic buffer consisting of acetonitrile: [10 m*M* potassium phosphate monobasic buffer/50 m*M* SDS/pH 7.0] (25:75, v/v). The experiment was carried out at 45°C with a 530 V/cm electric field, a 5 s water plug and a 1 s injection (pressure). A 3-mW argon-ion laser (λ_{ex} 488 nm/λ_{em} 520 nm) was employed for the detection of thiol derivatives.

2.4. Clinical samples

Blood samples were obtained from healthy volunteers and patients with end-stage renal disease (ESRD) enrolled in a study of high-dose folic acid therapy for hyperhomocysteinemia. The study was approved by the Georgetown University Medical School's Ethics Committee and informed consent was obtained from the volunteers and ESRD patients.

2.5. Blood collection

Venous blood samples were collected in chilled EDTA Vacutainer tubes (Becton Dickinson, Grenoble, France) and were immediately centrifuged at 4°C. After centrifugation, the plasma proteins in 0.5 ml of plasma were directly precipitated with 0.1 ml of 2.34 *M* perchloric acid containing 2 m*M* EDTA. The supernatant, as well as an aliquot of untreated plasma, were stored at −80°C.

2.6. Extraction procedure for total homocysteine (tHcys), total glutathione (tGSH) and total N-acetylcysteine (tNAC)

Aliquots of untreated plasma (0.5 ml) were spiked with 50 μl of the internal standard (I.S.) solution (cystamine, 500 μ*M* in 0.1 N HCl), vortexed for 30 s, mixed with 250 μl of 8 m*M* DTT (dithiothreitol) in 0.9% NaCl and the mixture was incubated at 37°C in the dark for 15 min. A 100 μl volume of 2.34 *M* perchloric acid containing 2 m*M* EDTA were added and the precipitate was shaken for 30 s, the mixture was then centrifuged at 4°C and 2400 *g* for 10 min and 100 μl of the acidic supernatant was collected in a 10×75 mm disposable boro-silicate culture tube. FM [1 ml, 0.42 mM] in phosphate buffer [50 m*M*, pH 7.5]) was added, the solution was vortex-mixed for 30 s and left in the dark at room temperature for 15 min. The samples were diluted 100-fold with Nanopure water and stored in the fridge at 4°C.

2.7. Extraction procedure for reduced homocysteine (rHcys), reduced glutathione (rGSH) and reduced N-acetylcysteine (rNAC)

To 0.2 ml of acidic supernatant were added 30 μl of the internal standard solution (cysteamine, 5 μ*M* in 20 m*M* EDTA), 50 μl of 1 *M* NaOH and 1 ml of 10^{-5} *M* FM in phosphate buffer [50 m*M*, pH 7.5]. The resulting solution was vortex-mixed for 30 s and left in the dark at room temperature for 15 min. The samples were diluted ten-fold with Nanopure water and stored in the fridge at 4°C.

2.8. Validation study

2.8.1. Preparation of spiked plasma standards

A 500 μ*M* solution of homocystine, oxidized GSH and oxidized NAC was prepared in 0.1 N HCl. Standards in water were obtained by serial dilution of this stock solution. Aliquots of 0.5 ml blank human plasma were spiked with 50 μl of each standard. The plasma calibration curve consisted of standards at 5, 10, 30, 50 and 80 μ*M* for tHcys and 10, 30, 50, 80 and 100 μ*M* for tGSH and tNAC.

2.8.2. Standard curves

The calibration curves were constructed by plotting the peak area ratios (tHcys/I.S.), (tGSH/I.S.) and (tNAC/I.S.) versus the concentration in μM.

2.8.3. Accuracy, precision and specificity

Intra- and inter-day validation studies for precision and accuracy were performed on five replicates of spiked plasma standards containing I.S. at 100 μ*M* as well as tHcys, tGSH and tNAC at three levels: 8, 22.5 and 45 μ*M* for tHcys, 22.5, 45 and 90 μ*M* for tGSH and tNAC. The analysis was repeated on three separate days. Five samples of blank plasma were run each day. The mean residual value obtained with blank plasma for tHcys/I.S., tGSH/I.S. and tNAC/I.S. was subtracted to the area/I.S. obtained for spiked plasma. Specificity was checked by extracting five replicates of blank plasma with and without Hcys, GSH, NAC and I.S.

3. Results and discussion

3.1. Method development

3.1.1. Derivatization

The reduced thiols in plasma, including Hcys, GSH, NAC and I.S., were derivatized with FM after protein precipitation with perchloric acid. The reaction involves addition of the thiol across the double bond of the maleimide to yield a thioether (Fig. 2). The pH is crucial for a specific conjugation with sulfhydryl groups when performing the derivatization with FM. At alkaline pH values, selectivity for sulfhydryl groups decreases and amino groups begin to react with maleimide. Maximum

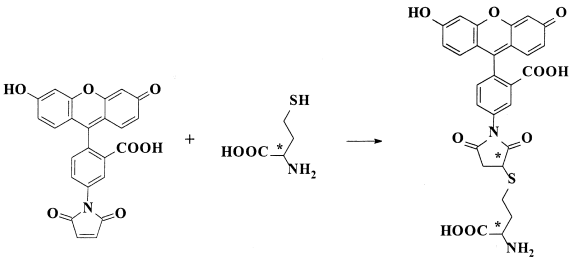

Fig. 2. Formation of the fluorescent FM-Hcys adduct (*chiral center).

reaction yields were obtained when the pH value was between 6.9 and 7.6. At pH 7.5, FM-derivatives were stable for 1 week at 0°C in the dark and at least 12 h in the CE tray. The FM excess required for the assay of rHcys did not exceed 10%.

The total thiols in plasma, including the fraction linked to proteins, were derivatized with FM after an initial reduction of the disulfide bonds with DTT and protein precipitation with perchloric acid. A sufficient amount of DTT was necessary to reduce Hcys, GSH, NAC, I.S. and the disulfides naturally occurring in human plasma. The efficiency of the reduction was elucidated by varying the amount of DTT (250 μl of 2–12 m*M* DTT per 0.5 ml of plasma) with a constant reaction time (15 min) at 37°C (Fig. 3). A concentration below 4 m*M* was not sufficient to reduce the total amount of disulfide bonds in plasma. The minimum value varied from 2.8 to 3.6 m*M* according to the plasma studied. This range may be related to the concentration of albumin which possesses 17 intramolecular disulfide bonds. A concentration above 10 m*M* led to the appearance of new interfering peaks preventing the correct quantifi-

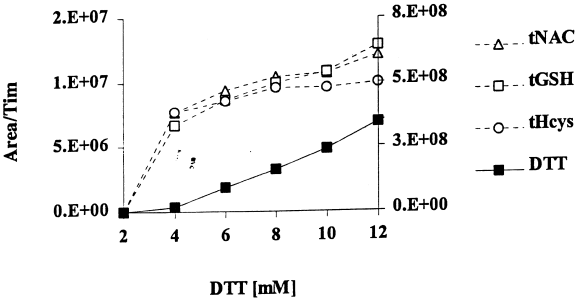

Fig. 3. Effect of DTT concentration (DTT values on the second *y*-axis).

cation of Hcys and NAC. As a result, a concentration of 8 m*M* DTT was used throughout the study to achieve an optimum reduction rate. Because of the two thiol groups present in DTT, a large amount of FM was required in order to derivatize both the excess of reducing agent and the thiols of interest (Fig. 4). A concentration of 0.42 m*M* FM was used to carry out the derivatization.

3.1.2. Optimization of capillary electrophoresis

The CE separation of the FM-derivatives was obtained by micellar electrokinetic chromatography using sodium dodecyl sulfate. Without any surfactant in the electrolyte, peaks from the FM-adducts and the FM in excess overlapped. The CE separation conditions were first examined at the basic electrolyte conditions (borate buffer, 20 m*M*, pH 8.0–10.5). Fig. 5 shows the electropherogram of FM-thiols obtained at basic conditions. The peak of each thiol was split into two, so quantitative analysis of FM-thiols was not convenient. The presence of two peaks per FM-thiol may be explained by the formation of a diastereoisomeric mixture during the reaction of derivatization (Fig. 2).

Acidic conditions (phosphate buffer, 20 m*M*, pH 2.0–6.0) were then investigated and led to the precipitation of the FM adducts on the capillary wall. FM is poorly soluble below pH 6.0. Adding an organic solvent to the electrolyte did not improve the solubility of the FM-derivatives. Finally, a pH value between 6.5 and 7.5 proved to be a convenient choice for the separation.

At pH 7.0, FM-Hcys, FM-GSH and FM-NAC were eluted after the electroosmotic flow (EOF), indicating that the predominant forms of the studied

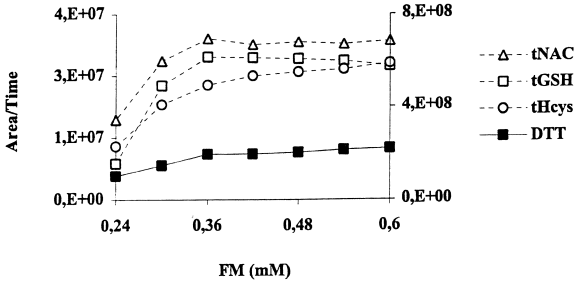

Fig. 4. Effect of FM concentration (DTT values on the second *y*-axis).

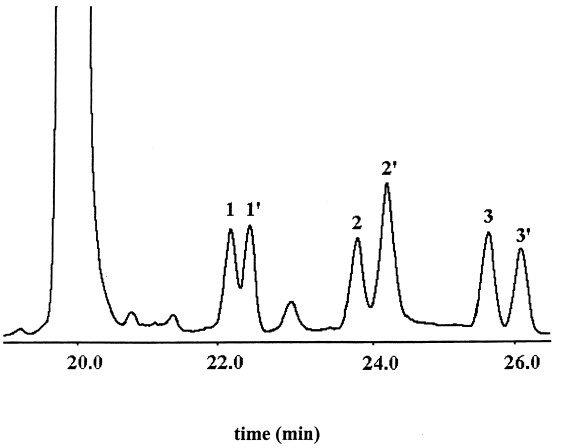

Fig. 5. Electropherogram of the FM-thiols obtained at basic conditions: (1,1′) glutathione (2 μ*M*), (2,2′) *N*-acetylcysteine (2 μ*M*) and (3,3′) homocysteine (2 μ*M*) (conditions: fused capillary (57 cm×75 μm I.D.), 20 m*M* borate buffer–50 m*M* SDS, pH 9.5, 30°C, 16 kV, 1 s hydrodynamic injection).

FM-thiols have negative charges in neutral buffer. As shown in Fig. 6A, the use of an organic solvent was required to separate Hcys from I.S., as well as to get rid of two small interferences. Among the different organic modifiers tested, acetonitrile provided the most suitable separation in the shortest analysis time (Fig. 6B–D).

In this study, cysteamine (2-mercaptoethylamine) was used as an internal standard (Fig. 1). Cysteamine (I,S,) has sufficient chemical similarities to Hcys to make it suitable as an internal standard. In addition, the plasma concentration of naturally occurring total cysteamine is extremely low (<0.1 μ*M* for total cysteamine) [36] and does not interfere with the use of cysteamine as an internal standard at a concentration of 100 μM.

3.2. Method validation

Fig. 7 shows a typical electropherogram of the FM-thiols in human plasma spiked with a mixture of Hcys, oxidized GSH, oxidized NAC and cystamine. The intense peak at 6.8 min corresponds to the DTT excess. The peak related to the FM excess was eluted after 11 min. In order to increase the throughput of the assay, each run was interrupted after 8 min and the FM excess was eluted by washing the capillary with 0.1 N NaOH. The migration times of FM-Hcys,

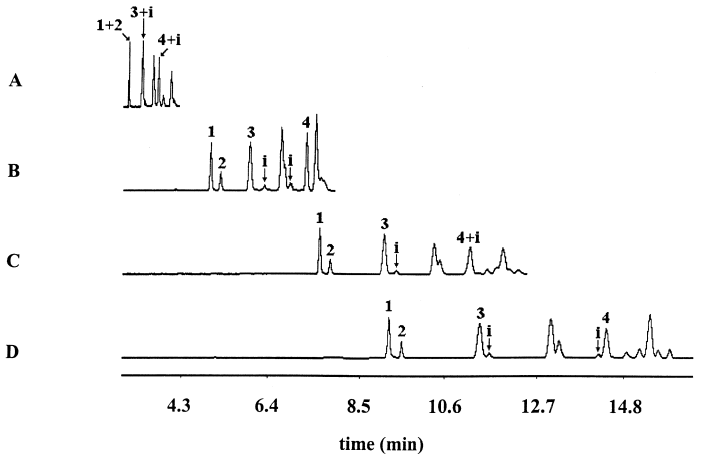

Fig. 6. Electropherogram of the FM-thiols obtained with different organic modifiers: (A) no modifier, (B) acetonitrile, (C) ethanol, (D) dimethylformamide, (1) homocysteine (2 μ*M*), (2) internal standard (2 μ*M*), (3) glutathione (2 μ*M*), (4) *N*-acetylcysteine (2 μ*M*), (i) interference (conditions: fused capillary (57 cm×75 μm I.D.), organic modifier/10 m*M* phosphate buffer–50 m*M* SDS [25:75 (v/v); pH 7.0], 45°C, 25 kV, 5 s water plug, 1 s hydrodynamic injection).

FM-IS, FM-GSH and FM-NAC were 5.25, 5.5, 6.3 and 7.8 min, respectively. The limit of detection for the mixture FM-Hcys, FM-GSH and FM-NAC was 3 μM.

The standard curves, calculated from the unweighted regression of peak area ratios of tHcys/I.S., tGSH/I.S. and tNAC/I.S. to concentration (μ*M*) were linear over the range of 5 to 80 μ*M* for tHcys and 10 to 100 μ*M* for tGSH and tNAC. The linear regression equations for FM-Hcys, FM-GSH and FM-NAC were $y = 0.0063x + 0.0046$ ($R^2 = 0.9992$), $y = 0.0067x - 0.0268$ ($R^2 = 0.9985$), and $y = 0.0097x - 0.0409$ ($R^2 = 0.9984$), respectively.

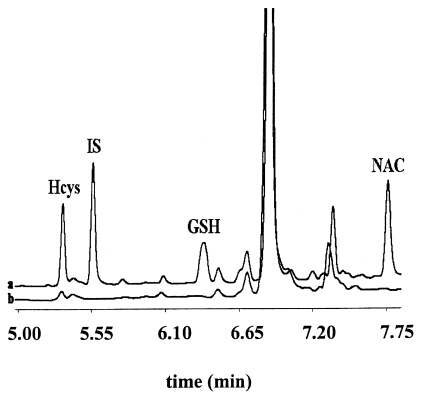

Fig. 7. Electropherograms of the FM-thiols: (a) human plasma spiked with homocystine (40 μ*M*) (Hcys), oxidized glutathione (40 μ*M*) (GSH), oxidized *N*-acetylcysteine (40 μ*M*) (NAC) and cystamine (50 μ*M*) (I.S.); (b) blank plasma (conditions: fused capillary (57 cm×75 μm I.D.), acetonitrile/10 m*M* phosphate buffer–50 m*M* SDS [27:73 (v/v); pH 7.0], 45°C, 25 kV, 5 s water plug, 1 s hydrodynamic injection).

The results of the validation for tHcys, tGSH and tNAC are given in Tables 1–3, respectively. Precision (C.V.%) ranged from 1.0 to 6.6 for Hcys, from 0.9 to 6.3 for GSH and from 2.3 to 11.8 for NAC. Accuracy was within ±9% of theoretical values at the high and medium levels and ±12% at the low level for Hcys. Accuracy was within ±6% of theoretical values at the low and medium levels and ±8% at the high level for GSH. Accuracy was within ±3.1% of theoretical values at the high level and ±8% at medium and high level for NAC.

Fig. 8 shows a typical electropherogram of the FM-thiols in human plasma spiked with a mixture of rHcys, rGSH, rNAC and reduced I.S. All the peaks of interest were well separated within 8 min. Migration times were similar to the ones obtained for total thiols. The limit of detection for the mixture FM-Hcys, FM-GSH and FM-NAC was 10 n*M*. Within the average injection volume of 6 nl (0.5 p.s.i., 1 s), 60 attomoles of FM-Hcys were detected. The method was linear over a 70 to 1000 n*M* range for rHcys and rNAC. The linear regression equations for FM-Hcys

Table 1
Intra- and inter-day variabilities for the assay of total homocysteine in plasma

tHcys level (μ*M*)	Intra-day					Inter-day				
	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)
8	8.9	5	0.3	3.8	111.7	8.3	12	0.5	6.6	103.8
22.5	24.2	5	1.3	5.4	107.7	22.3	14	1.3	5.9	99.3
45	43.5	5	0.5	1.0	96.7	41.1	11	2.6	6.4	91.2

Table 2
Intra- and inter-day variabilities for the assay of total glutathione in plasma

tGSH level (μ*M*)	Intra-day					Inter-day				
	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)
22.5	23.3	5	1.0	4.2	103.5	22.4	14	0.8	3.6	99.6
45	43.7	5	1.2	2.8	97.2	42.9	12	2.7	6.3	95.4
90	97.0	5	0.9	0.9	107.8	87.8	13	4.1	4.7	97.5

Table 3
Intra- and inter-day variabilities for the assay of total *N*-acetylcysteine in plasma

tNAC level (μ*M*)	Intra-day					Inter-day				
	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)	Mean observed concentration (μ*M*)	*n*	SD	C.V. (%)	Accuracy (%)
22.5	24.2	5	1.3	5.2	107.7	23.5	10	2.8	11.8	104.6
45	48.1	5	1.9	3.9	106.9	46.0	11	2.9	6.2	102.1
90	92.1	5	2.1	2.3	102.4	92.8	12	5.0	5.4	103.1

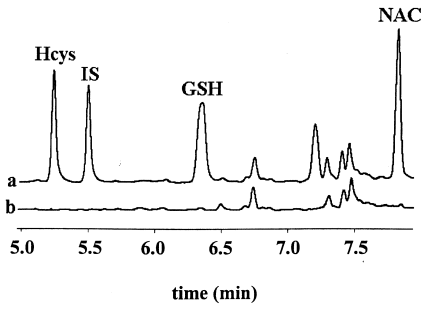

Fig. 8. Electropherograms of the FM-thiols: (a) human plasma spiked with homocysteine (2 μ*M*) (Hcys), reduced glutathione (2 μ*M*) (GSH), reduced *N*-acetylcysteine (2 μ*M*) (NAC) and cysteamine (2 μ*M*) (I.S.), (b) blank plasma (conditions: fused capillary (57 cm×75 μm I.D.), acetonitrile/10 m*M* phosphate buffer–50 m*M* SDS [27:73 (v/v); pH 7.0], 45°C, 25 kV, 5 s water plug, 1 s hydrodynamic injection).

and FM-NAC were $y = 0.0004x - 0.0014$ ($R^2 = 0.9977$), and $y = 0.0008x - 0.0063$ ($R^2 = 0.9985$), respectively.

rHcys and rNAC levels in plasma are in the nanomolar range. Up to 30% of total plasma GSH is in the reduced form, so the expected levels of rGSH are in the micromolar range. The use of a large amount of FM to extend the domain of linearity would compromise the sensitivity of the method for rHcys and rNAC. Therefore, a polynomial equation was used to construct the standard curve for rGSH in plasma over a 0.5 to 5 μ*M* range: $y = -0.0371x^2 + 0.7163x - 0.0226$ ($R^2 = 0.9999$).

In order to determine the reproducibility of the assay for reduced thiols, a standard mixture of FM-thiols extracted from plasma was injected repeatedly

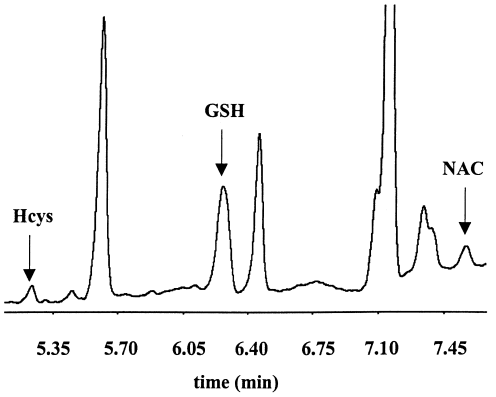

Fig. 9. Electropherogram of reduced thiols in plasma for a healthy volunteer: [rHcys]=0.09 μ*M*, [rGSH]=0.64 μ*M*, [rNAC]=0.13 μM.

ten times at the same conditions. Coefficients of variation (C.V.) were less than 5%. Accuracy for the LOQ was within ±12% for rHcys and rNAC and ±11% for rGSH. A typical electropherogram of reduced thiols in plasma of a healthy volunteer is presented in Fig. 9.

4. Application

The assay for tHcys in plasma was applied to samples from a clinical study of high-dose folic acid therapy for hyperhomocysteinemia in hemodialysis patients. Patients with end-stage renal disease (ESRD) receiving regular hemodialysis therapy have an elevated annual mortality rate, >20%, from cardiovascular system diseases. This dramatic can not be readily ascribed to traditional risk factors such as hypercholesterolemia or hypertension.

Recently, studies have focused on unconventional risk factors such as elevated tHcys levels. Preliminary data support the evidence of an association between plasma tHcys and cardiovascular diseases. Fig. 10A shows a typical electropherogram of total thiols in plasma of a healthy volunteer, derivatized with FM. Among the group of healthy volunteers analyzed ($n=20$), a mean tHcys concentration of 7.41±1.82 μ*M* was observed. Fig. 10B shows a typical electropherogram of total thiols in plasma of an ESRD patient, derivatized with FM. In the first group of patients analyzed ($n=33$), a three-fold

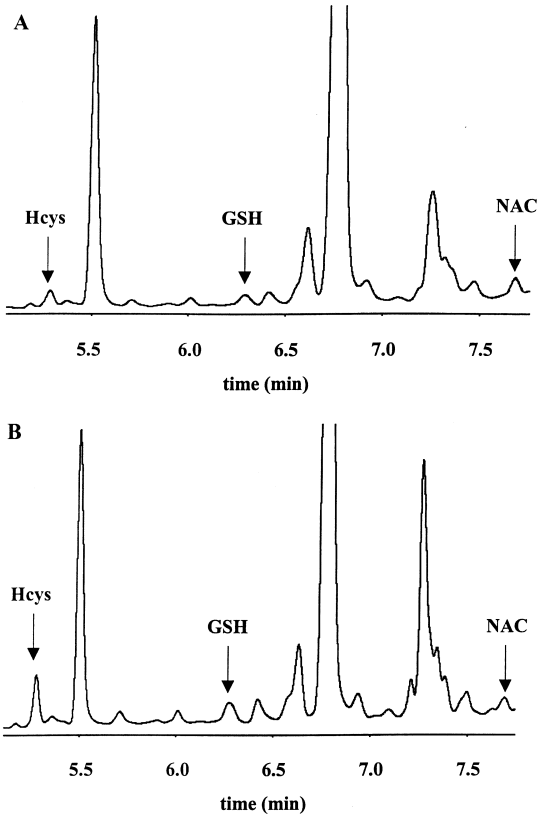

Fig. 10. Electropherograms of total thiols in plasma; (A) Healthy volunteer: [tHcys]=8.50 μ*M*, [tGSH]=11.33 μ*M*, [tNAC]=11.54 μ*M*, (B) ESRD patient: [tHcys]=25.95 μ*M*, [tGSH]=21.65 μ*M*, [tNAC]=10.60 μM.

higher mean concentration of tHcys was obtained (21.58±5.83 μ*M*) compared to the healthy subjects. A 4-month trial period involving 80 ESRD patients and 90 healthy volunteers will determine the ability of folic acid or vitamin B_6+B_{12} supplements to normalize plasma tHcys concentration.

5. Conclusion

A CE–LIF method has been developed for the determination of rHcys and tHcys in plasma. Fluorescein-5-maleimide was used as a fluorescent probe. Although fluoresceine isothiocyanate is a well-known derivatizing agent for amino acids, as far as we know, fluorescein-5-maleimide has never been used for the specific derivatization of sulfhydryls in a

chromatographic assay. The described procedure is simple, rapid and reproducible for the analysis of homocysteine, glutathione and *N*-acetylcysteine in plasma. The sensitivity of the assay allowed the monitoring of homocysteine level in healthy subjects as well as in patients with end-stage renal disease.

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References

[1] S.H. Mud, H.L. Levy, F. Skovby, Disorders of Transsulfuration, in: C.G. Scriver, A.L. Beudet, W.S. Sly, D. Vall (Eds.), The Metabolic Basis of Inherited Disease, 6th ed, McGraw Hill, New York, 1989, pp. 693–734.
[2] S.S. Kang, P.-W.-K. Wong, M. Norusis, Metabolism 36 (1987) 458.
[3] P.M. Ueland, H. Refsum, L.E. Brattström, Plasma Homocysteine and Cardiovascular Disease, in: R.B. Francis Jr. (Ed.), Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function, Marcel Dekker, Inc, New York, 1992, p. 183.
[4] S.S. Kang, E.L. Passen, N. Ruggie, P.W.K. Wong, H. Sora, Circulation 88 (1993) 1464.
[5] J. Danesh, S. Lewington, J. Cardiovasc. Risk 5 (1998) 229.
[6] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[7] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[8] S.P. Stabler, P.D. Marcell, E.R. Podell, R.H. Allen, Anal. Biochem. 162 (1987) 185.
[9] A. Andersson, L. Brattstrom, A. Isaksson, B. Israelsson, B. Hultberg, Scand. J. Clin. Lab. Invest. 49 (1989) 445.
[10] B. Chadefaux, M. Coude, M. Hamet, J. Aupetit, P. Kamoun, Clin. Chem. 35 (1989) 2002.
[11] B. Vester, K. Rasmussen, Eur. J. Clin. Chem. Clin. Biochem. 29 (1991) 549.
[12] A. Andersson, A. Isaksson, L. Brattstrom, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[13] D.L. Rabenstein, G.T. Yamashita, Anal. Biochem. 180 (1989) 259.
[14] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (1989) 1180.
[15] D.W. Jacobsen, V.J. Gatautis, R. Green, Anal. Biochem. 178 (1989) 208.
[16] T. Toy'oka, F. Furukawa, T. Suzuki, Y. Saito, M. Takahashi, Y. Hayashi, Biomed. Chromatogr. 3 (1989) 166.
[17] K. Nakashima, C. Umekawa, H. Yoshida, S. Nakatsuji, S. Akiyama, J. Chromatogr. 414 (1987) 11.
[18] T. Toyo'oka, K. Imai, J. Chromatogr. 282 (1983) 495.
[19] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[20] G.L. Newton, R. Dorian, R.C. Fahey, Anal. Biochem. 114 (1981) 383.
[21] N.P.B. Dudman, X.W. Guo, R. Crooks, L. Xie, J.S. Silberberg, Clin. Chem. 42 (1996) 2028.
[22] J.B. Ubbink, W.J. Hayward Vermaak, S. Bissbort, J. Chromatogr. 565 (1991) 441.
[23] K. Kuo, R. Still, S. Cale, I. McDowell, Clin. Chem. 43 (1997) 1653.
[24] S.H. Ho Hang, J.-W. Kim, D.S. Chung, J. Pharm. Biomed. Anal. 15 (1997) 1435.
[25] C.W. Wu, L.R. Yarbrough, F.Y.H. Wu, Biochemistry 15 (1976) 2863.
[26] M. Johansson, D. Westerlund, J. Chromatogr. 385 (1987) 343.
[27] N. Ercal, S. Oztezcan, T.C. Hammond, R.H. Matthews, D.R. Spitz, J. Chromatogr. B 685 (1996) 329.
[28] E.A. Mitchell, R.B. Elliott, Aust. Paediatr. J. 18 (1982) 40.
[29] L.R. Morgan, M.R. Holdiness, L.E. Gillen, Semin. Oncol. 10 (1983) 56.
[30] L.F. Prescott, A. Ballantyne, J. Park, P. Adriaenssens, A.T. Proudfoot, Lancet 2 (1977) 432.
[31] G.B. Corcoran, B.K. Wong, J. Pharmacol. Exp. Ther. 238 (1986) 54.
[32] E. Marmstal, B. Mannervik, Biochem. Biophys. Acta 566 (1979) 362.
[33] E. Boyland, L.F. Chasseaud, Adv. Enzymol. 32 (1969) 173.
[34] G.L. Jensen, A. Meister, Proc. Natl. Acad. Sci. USA 80 (1983) 4714.
[35] B. Halliwell, J.M.C. Gutteridge, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Clarendon Press, Oxford, 1989, p. 126.
[36] L.A. Smolin, J.A. Schneider, Anal. Biochem. 168 (1988) 374.