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Determination of homocysteine thiolactone, reduced homocysteine, homocystine, homocysteine–cysteine mixed disulfide, cysteine and cystine in a reaction mixture by overimposed pressure/voltage capillary electrophoresis

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ABSTRACT

An elevated level of thiol amino acid homocysteine is associated with several complex disorders. Homocysteine ability to bind proteins, thereby modulating their structure and function, is proposed to be one of the mechanisms of homocysteine induced pathogenecity. Homocysteine and homocysteine thiolactone bind to protein cysteine and lysine residues respectively. A major hurdle in studying protein homocysteinylation is the lack of suitable analytical techniques to determine simultaneously the concentrations of reduced and oxidized forms of homocysteine and cysteine (especially homocysteine–cysteine mixed disulfide) together with thiolactone formed during the reaction of homocysteine or thiolactone with proteins. Herein we report a capillary electrophoresis method to determine simultaneously the levels of these intermediates. For this 40 mmol/L Tris phosphate buffer at (pH 1.60) was used as running electrolyte, and the separation was performed by the simultaneous application of a CE voltage of 15 kV and an overimposed pressure of 0.1 psi. A rapid separation of these intermediates in less than 6 min with a good reproducibility of both peak areas (CV < 2%) and migration time (CV < 0.2%) was obtained. The applicability of our method was validated by incubating reduced homocysteine and albumin and measuring the reaction intermediates in the solution mixture.

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1. Introduction

Homocysteine (Hcy), a sulfur containing amino acid, is a key branch point intermediate in the methionine metabolism pathway. In healthy well nourished individuals the intracellular levels of Hcy are well regulated. However, deficiencies in micronutrients like vitamin B12, folate, vitamin B6, etc. and defects in genes that are directly or indirectly involved in the metabolism of Hcy results in elevated levels of this thiol amino acid [1,2]. Excess Hcy is exported out into circulation. Several clinical and epidemiologic trials have suggested that an elevated level of Hcy (hyperhomocysteinemia) is a risk factor for atherosclerosis and arterial and venous thromboembolism [3]. Recent studies indicate that hyperhomocysteinemia is also a risk factor for Alzheimer's disease and other disorders of cognitive dysfunction [4,5]. In circulation Hcy is present in different forms and plasma total Hcy is the sum of free Hcy and protein-bound Hcy [6]. Free Hcy is found as low molecular weight disulfide forms such as homocystine and homocysteine–cysteine mixed disulfide [7]. Less than 1% of total Hcy in circulation is present in free, reduced form [8,9]. The majority of the Hcy (>80%) in circulation is present in protein-bound form. Apart from this a small quantity of homocysteine (under 40 nmol/L) can be found in plasma as thiolactone form [10]. Protein-homocystamide (homocysteine-N-protein), the reaction product formed between a protein lysine residue and homocysteine thiolactone (HTL), is also found in circulation [11]. The ability of Hcy to bind to free cysteine residues of the protein or break critical accessible disulfide bonds can potentially lead to the modulation of structure and/or function of the protein. Several such examples have been reported in the literature. For instance, in vitro studies have shown that homocysteinylation of the Cys^9 residue of annexin II, the endothelial cell surface docking protein for tissue plasminogen activator, inhibits binding of tissue plasminogen

Abbreviations: CE, capillary electrophoresis; Hcy, homocysteine; Cys, cysteine; HSSH, homocystine; CSSC, homocysteine–cysteine mixed disulfide; CSSC, cystine.

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activator to annexin II [12], binding of Hcy to plasma fibronectin inhibits its ability to bind fibrin [13], binding of Hcy to factor Va, makes it resistant to inactivation by activated protein C [14]. Further, homocysteinylation appears to activate latent elastolytic metalloproteinase pro-MMP-2 by disulfide bond formation with the "Cys switch" on the propeptide [15]. It has also been shown that Hcy binds to LDL and homocysteinylation of LDL results in increased generation of reactive oxygen species in cultured endothelial cells [16–19], suggesting that LDL atherogenicity may be enhanced by apoproteinB-homocysteinylation [19]. Although there are several proteins that have been reported to bind Hcy, the mechanism of protein-S-homocysteinylation has not been studied in detail except for the mechanism of homocysteine's binding to albumin [6,20].

A major drawback in understanding the mechanisms of proteins homocysteinylation is the lack of methods suitable to measure simultaneously all the intermediates involved in the reaction, especially the free reduced and oxidized forms of Hcy and Cys-homocystine, homocysteine–cysteine mixed disulfide and cystine [21]. Currently, to determine the levels of all the metabolites present in the reaction mixture more than two analytical methods are to be used. Herein, we describe a rapid method for the simultaneous evaluation of HTL, Hcy, homocystine (HSSH), homocysteine–cysteine mixed disulfide (CSSH), cysteine (Cys) and cystine (CSSC) using capillary electrophoresis with overimposed pressure/voltage.

2. Materials and methods

2.1. Chemicals

Hcy, Cys, HSSH, CSSC, HTL, iodoacetamide (IAA), Tris, H_3PO_4, KH_2PO_4, $K_2HPO_4CuSO_4$, EDTA, albumin, trichloroacetic acid (TCA) were from Sigma (St Louis, USA). The 0.45 mL membrane filters (used to filter all buffer solution before CE analysis) were purchased from Millipore (Bedford, USA).

2.2. Calibration curves

Calibration curves for all the analytes that were available commercially were prepared from 0.01 to 2 mmol/L. However, for Hcy–Cys mixed disulfide which is not commercially available we prepared the calibration curve as follows: a mixture of 2 mmol/L of cysteine and homocysteine (dissolved in 100 mmol/L potassium phosphate, 0.2 mmol/L EDTA pH 7.4) was incubated in the presence of 50 μmol/L $CuSO_4$. It is known that in the presence of transition metal ions like copper, thiols oxidize. Therefore, incubation of Hcy and Cys in the presence of copper will lead to the formation of the three oxidized species – HSSH, CSSC and CSSH. The formation of oxidised species was monitored for 1 h. Aliquots (100 μL) were withdrawn at 0, 3, 6, 10, 15, 20, 30, 45, 60 min and mixed with 100 μL of 20 mmol/L of IAA to stop the reaction. The samples were injected on capillary electrophoresis to measure the concentration of Hcy, Cys, CSSC, HSSH. CSSH formation was then calculated on the basis of the stoichiometric reaction described below:

$$4\text{Cys} + 4\text{Hcy} \rightarrow \text{CSSC} + \text{HSSH} + \text{Cys} + \text{Hcy} + \text{CSSH}$$

Based on the concentration of CSSH obtained at various time points during the reaction a calibration curve was generated.

2.3. Capillary electrophoresis

An MDQ capillary electrophoresis system equipped with a diode array detector was used (Beckman Instruments, Fullerton, CA, USA). The system was fitted with a 30 kV power supply with a current limit of 300 μA. Analysis was performed in an uncoated fused-silica capillary (75 μm i.d. and 40 cm length, 30 cm to the detection window), injecting 35 nL of sample (7 s at 0.5 psi). Separation was carried out in a 40 mmol/L Tris buffer titrated with 1 mol/L phosphoric acid to pH 1.6, 15 °C, and 15 kV (240 μA) at normal polarity with an overimposed pressure of 0.1 psi from inlet to outlet. After each run, the capillary was rinsed for 0.5 min with 0.1 mmol/L HCl and equilibrated with run buffer for 1 min. Detection of analytes was performed at 190 nm except for HTL where the analysis was performed at 232 nm.

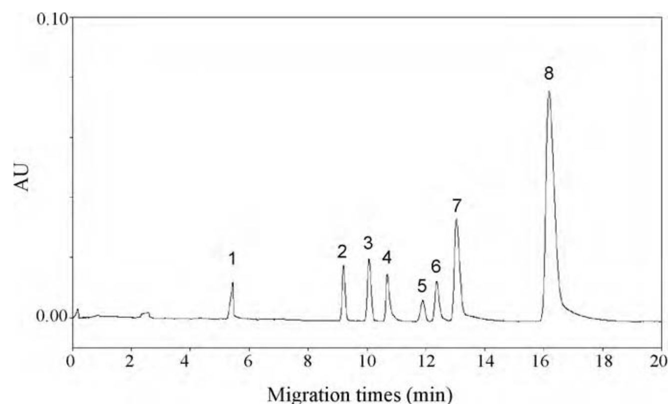

Fig. 1. Electropherogram of a standard mixture containing HTL (1), HSSH (2), HSCS (3), Hcy (4), CSSC (5), Cys (6), Hcy-IAA (7) and Cys-IAA (8). Electrophoretic conditions: 50 mmol/L Tris phosphate pH 1.7, 15 kV at 15 °C. Wavelength detection: 190 nm. Analyte concentrations: HTL, HSSH, HSCS and CSSC, 250 μmol/L; Hcy, 400 μmol/L; Hcy–IAA, 500 μmol/L; Cys, 600 μmol/L; Cys–IAA, 750 μmol/L.

3. Results and discussion

The objective of this study was to develop a method for separation of HTL, Hcy, HSSH, CSSH, Cys and CSSC using capillary electrophoresis. Further, since in some cases iodoacetic acid (IAA) is used to stop the reactions we also wanted to separate the adduct Hcy–IAA and Cys–IAA together with the other analytes. We had earlier developed methods using capillary electrophoresis for the measurement of small analytes as creatinine, creatine, guanidinio acetic acid, methionine, arginine, asymmetric dimethylarginine and symmetric dimethylarginine and had obtained good separations using Tris phosphate as run buffer [22–26]. Therefore, here also we used different concentrations of Tris phosphate as run buffer (25–75 mmol/L) and different pH values (1.6–2.0) to optimize the electrophoretic separation of analytes. Preliminary experiments were carried out by using a capillary with an effective length of 40 cm. Migration times of analytes increased with the increase in the pH of run buffer while the resolution between peaks improved by decreasing the pH and concentration of run buffer. Further, the sensitivity and efficiency of peaks also increased by decreasing the pH (data not shown). The best compromise between a good resolution, efficiency, sensitivity and fast migration times was reached using 50 mmol/L electrolyte run buffer at pH 1.7 (Fig. 1). However, in this case the total run time was about 20 min. To reduce the migration times we used the technique of overimposed pressure/voltage, in which a pressure from inlet to outlet was employed during the electric field application. The use of a hydrodynamic overimposed pressure during the whole electrophoretic run is commonly used in CE–MS systems to enhance the robustness of the method (in particular to provide stable electrospray conditions for successful coupling) and also to decrease run times [27,28]. This is due to the fact that during the electrophoresis, the buffer in the capillary moves toward the detection window increasing the mobility of the analytes. By applying a pressure of 0.1 psi we were able to decrease migration times considerably and

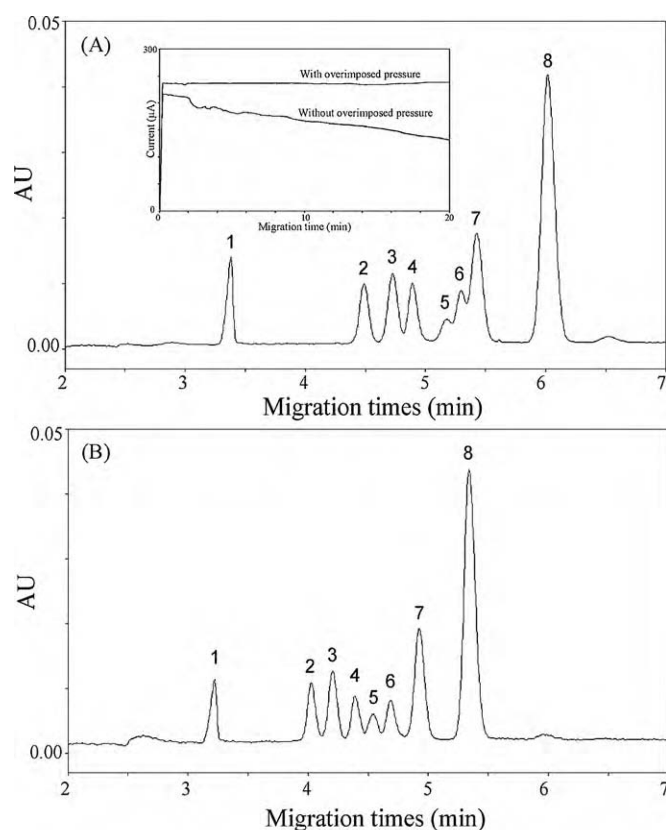

Fig. 2. Electropherograms of a standard mixture containing HTL (1), HSSH (2), HSCS (3), Hcy (4), CSSC (5), Cys (6), Hcy–IAA and Cys–IAA obtained in (A) 50 mmol/L Tris phosphate pH 1.7, 15 kV at 15 °C with an overimposed pressure of 0.1 psi from inlet to outlet and (B) 40 mmol/L Tris phosphate pH 1.6, 15 kV at 15 °C with an overimposed pressure of 0.1 psi from inlet to outlet. Wavelength detection: 190 nm. The inlet in panel A shows the effect of overimposed pressure/voltage on the current during electrophoresis.

the total run time was about 7 min (Fig. 2A) which is a significant improvement to the run time required when the overimposed pressure was not employed (20 min). However, under these conditions the peaks of CSSC, Cys and Hcy–IAA (peaks 5, 6 and 7 in Fig. 2A) were not resolved. Hence, the pH and concentration of run buffer were further modified and the optimal conditions were obtained using a 40 mmol/L Tris phosphate at pH 1.6 that allows a good separation of all peaks in less than 6 min (Fig. 2A). Moreover, using overimposed pressure, current generated during the electrophoresis was found to be stable reducing the frequency of voltage leak, due to the low pH used (inlet of Fig. 2A). Stabilization of current also improved inter-run reproducibility of migration times and peak area (Table 1).

Calibration curves for a standard water solution of thiols, obtained using five replicates, showed good regression coefficients (r^2 = 0.99 for all thiols) ensuring a linear response over the concentrations tested (Table 1). Precision tests, performed after repeated injection of the same sample, indicate a good reproducibility of method both for migration time (CV < 0.2%) and areas (CV < 2.0%) (Table 1). The LOD, calculated by 41-nL injections of a known solution of standard was between 1 and 4 μmol/L corresponding to an injected quantity between 40 and 160 fmol, with a signal-to-noise ratio of 3 (Table 1).

Since the reaction of Hcy with albumin has been mechanistically studied in detail, we evaluated the applicability of our method by incubating 1.5 mmol/L albumin with 4 mmol/L of reduced homocysteine in 100 mmol/L potassium phosphate, 0.2 mmol/L EDTA pH 7.4. As shown in Fig. 3 we were able to monitor the presence of dif-

Table 1
Calibration curves, reproducibility and limit of detection of overimposed pressure/voltage CE method.

	Calibration curves				Injection reproducibility (without overimposed pressure)		Injection reproducibility (with overimposed pressure)		Limit of detection (μmol/L)
	Range (mmol/L)	Slope	Intercept	r^2	Peak area (AU) Mean (CV)	Migration time (min) Mean (CV)	Peak area (AU) Mean (CV)	Migration time (min) Mean (CV)	
HTL[a]	0.02–2	21,943	−95	0.999	98,069 (4.77)	7.442 (1.73)	29,776 (1.89)	3.218 (0.15)	1
HSSH	0.02–2	41,119	−95	0.999	131,107 (4.46)	11.465 (1.51)	39,814 (1.60)	4.026 (0.11)	5
HSCS[b]	0.04–0.4	33,149	802	0.986	171,244 (4.97)	12.500 (1.61)	51,967 (1.62)	4.205 (0.10)	5
Hcy	0.04–2	12,525	266	0.999	115,049 (4.73)	13.278 (1.51)	24,141 (1.63)	4.426 (0.11)	12
CSSC	0.02–2	44,156	253	0.999	67,695 (4.82)	14.611 (1.65)	20,749 (1.62)	4.542 (0.12)	5
Cys	0.04–2	14,269	−42	0.999	108,850 (4.87)	15.224 (1.61)	23,565 (1.52)	4.692 (0.13)	12
Hcy–IAA	0.02–2	30,072	107	0.999	384,908 (4.73)	16.142 (1.56)	93,925 (1.38)	4.931 (0.13)	3
Cys–IAA	0.02–2	45,135	108	0.999	1,440,263 (4.74)	19.822 (1.60)	252,938 (1.34)	5.346 (0.14)	3

[a] Detection performed at 232 nm wavelength.
[b] Data obtained from preparation of HSCS as described in Section 2.

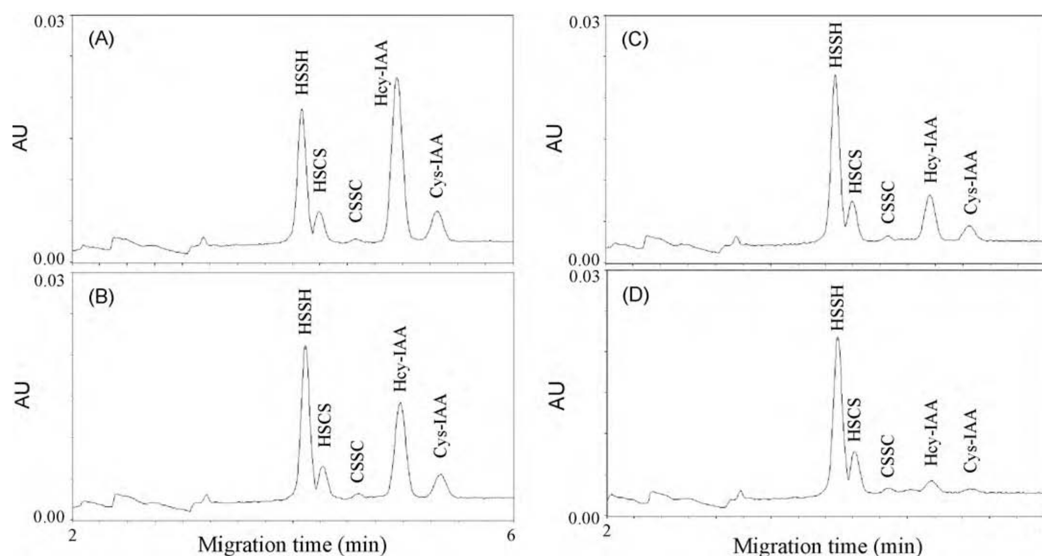

Fig. 3. Electropherograms obtained after incubation of 1.5 mmol/L albumin and 4 mmol/L of reduced homocysteine in 100 mmol/L potassium phosphate, 0.2 mmol/L EDTA pH 7.4 after 5 min (A), 10 min (B), 20 min (C) and 60 min (D). At the indicated time points aliquots were withdrawn are mixed with IAA (20 mmol/L final solution) and treated with TCA 10% to precipitate albumin. Supernatant was directly injected on capillary electrophoresis. Electrophoretical conditions: 40 mmol/L Tris phosphate pH 1.6, 15 kV at 15 °C with an overimposed pressure of 0.1 psi from inlet to outlet. Wavelength detection: 190 nm.

ferent analytes during the incubation of Hcy with albumin. It has been reported that Hcy binds to albumin in 2 steps. In the first step Hcy binds to the cysteine that is attached to Cys^{34} of albumin, producing HSSC and albumin thiolate anion. In the second step albumin thiolate anion reacts with Hcy in HSSC to form albumin bound Hcy [4]. Using our method we could clearly detect the intermediates in the reaction mixture (HSSH, HSCS, CSSC, Cys and unreacted Hcy) within 5 min of the reaction (Fig. 3 panel A). As expected after 10 and 20 min of the reaction (panel B and C) the concentration of the reduced species (Hcy and Cys) was found to constantly decrease until they almost disappeared at 60 min (panel D). This is in agreement with those described by Sengupta et al. [6].

4. Concluding remarks

We have developed a method to determine simultaneously thiols and their oxidized products using capillary electrophoresis. We believe that this method will be useful to mechanistically understand the binding of Hcy or HTL to proteins. Protein-bound homocysteine accounts for 70–80% of plasma total homocysteine in healthy individuals [29,30]. Protein homocysteinylation occurs through the post-translational acylation of free amino groups (protein-N-homocysteinylation, mediated by Hcy–thiolactone) or through the formation of a covalent disulfide bond, found primarily with cysteine residues (protein-S-homocysteinylation). The homocysteinylation of proteins is important as it could alter their structure and/or the function of the proteins [31]. For these reasons in the last few years considerable efforts have been made to understand the mechanisms involved in protein homocysteinylation [6,20]. The main hurdle in understanding the mechanism of the reaction was the lack of suitable methods to determine the intermediates formed in the reaction. The development of new specific and easier methods for the identification and quantification of the reaction intermediates would provide an impetus for further studies in the field. Using the capillary electrophoresis based method we were able to measure HTL, Hcy, HSSH, CSSH, Cys and CSSC simultaneously. The use of the technique of overimposed pressure/voltage allows to reduce migration times significantly and to improve the reproducibility of the analysis.

The novelty of the method here described is that it allows the simultaneous determination of the different thiol forms in a sample mixture, addressed to understand the reaction mechanisms involved both in protein-S-homocysteinylation and protein-N-homocysteinylation, by one single procedure, on the contrary to the other current strategies which should use more than two analytical methods to evaluate them.

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