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CAPILLARY ISOTACHOPHORESIS DETERMINATION OF TRACE OXIDIZED GLUTATHIONE IN BLOOD

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A capillary isotachophoresis (CITP) method performed in a column-coupling apparatus has been developed for the simultaneous determination of glutathione (GSH) and glutathione disulfide (GSSG) concentrations in blood samples. The determination of GSSG and GSH concentrations in biological samples is important because of their roles in oxidative stress. Different concentrations of a leading ion in the coupled columns (concentration cascade) and a large volume (37 μl) of the injected sample facilitated a GSSG concentration of between 2 and 25 μmol/l. A reaction between iodoacetate and GSH under alkaline conditions was used to prepare the sample in order to avoid oxidation of GSH to GSSG. This step eliminated the main source of systematic errors with regard to the determination of the GSSG concentration. A linear relationship (R^2=0.9969) between the zone length of S-(carboxymethyl)glutathione (the product of the reaction between GSH and iodoacetate) and the concentration of GSH (40-120 μmol/l) was obtained. The method was applied to the analysis of bovine blood samples that had been diluted by a factor of ten with satisfactory results.

Keywords: glutathione, glutathione disulfide, isotachophoresis, bovine blood

1. Introduction

Glutathione (GSH), a thiol containing tripeptide, plays an important role in the antioxidant system of eukaryotic cells [1]. Upon oxidation, GSH is transformed into glutathione disulfide (GSSG) [1]. The concentrations of GSH and GSSG and their molar ratio are indicators of cell functionality as well as oxidative stress [2]. Different aspects of the determination of GSH and GSSG concentrations in biological samples, including sample pretreatments, were recently reviewed [3–5]. The main problems are related to the non-enzymatic oxidation of GSH when the pH exceeds 7, enzymatic conversion of GSH, a need for the removal of proteins prior to the analysis, blocking of free thiol groups, reduction of disulfides, and derivatization of thiol groups [3,6]. Among a wide variety of analytical methods, capillary electromigration (CE) methods, mainly CE coupled with different detectors (UV absorbance [7–10], fluorescence [11] and laser-induced fluorescence (LIF) [12, 13], electrochemical [14, 15] and mass spectrometry [16], have been used to determine the concentration of glutathione [5, 17].

The aim of this work was to develop a capillary isotachophoresis (CITP) method that involves minimal sample pretreatment for the simultaneous determination of GSSG and GSH concentrations in bovine blood samples. Determination of trace analytes in regular CITP, when the quantitation is based on the measurement of the zone length, is not very common. In this work, two key problems were solved: (1) lowering the quantitation limit of the CITP method, and (2) stabilization of GSH without protein precipitation to ensure the accurate determination of the GSSG concentration.

2. Experimental

An EA 202A electrophoretic analyzer (Villa Labeco, Spišská Nová Ves, Slovakia) was used for CITP separations. This fully automated CE system was equipped with two columns. In the first column, a fluoroplastic capillary tube with an inside diameter (ID) of 800 μm and contactless conductivity detection were used. In the second column, a fused silica capillary with an ID of 300 μm, contactless conductivity and UV photometric detectors were used.

The CITP separations were performed using the electrolyte systems shown in Table 1 at a constant driving current, and in the anionic mode (electrode E3 in Fig. 1 used as a cathode). At the beginning and end of the day, the separation and electrolyte units as well as the sample loop of the autosampler were rinsed with deionized water

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Table 1: Electrolyte systems.

Electrolyte	Parameter	ES1	ES2
LE1	Leading ion	Chloride	
	Concentration [mmol/l]	10	10
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	12	12
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.4	3.4
LE2	Leading ion	Chloride	
	Concentration [mmol/l]	5	2
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	6	2.4
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.8	3.9
TE	Terminating ion	Caproate	
	Concentration [mmol/l]	20	20
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	15	15
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	4.7	4.7

using built-in peristaltic pumps. Between analyses a relatively short rinsing procedure (ca. 1 min) with electrolyte solutions was used.

2.1 Samples

The bovine blood samples were collected using PVC taking set with an integrated needle HEMOS (Gama Group, České Budějovice, Czech Republic), and immediately transferred to the test tube containing K3-EDTA. The samples after dilution, required for hemolysis, were filtered prior to the analysis using a syringe filter with a glass fiber membrane and a pore size of 1 μm. During the dilution step a thiol-masking agent and NaOH were added to each sample.

3. Results and Discussion

3.1 Separation conditions

The combination of two columns with different IDs and the employment of a column-switching technique is beneficial for the CITP determination of analytes present in the multicomponent sample at low concentrations and/or at different concentration levels. The first (wider) capillary allowed the separation of sample constituents injected at a relatively high volume (37 μl). Typical macroconstituents, e.g. chloride and ethylenediaminetetraacetic acid (EDTA), migrated out of the separation path through

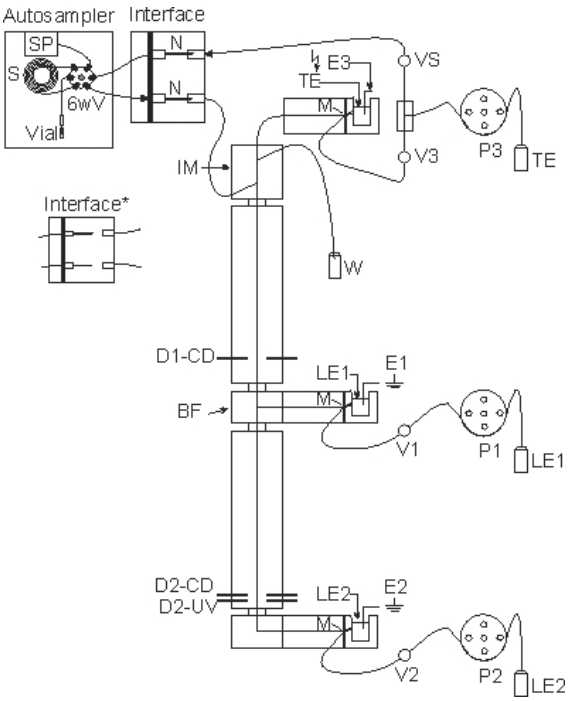

Figure 1: A scheme of the electrophoresis system. Autosampler: S – sample loop, SP – syringe pump, 6wV – 6-way valve. Interface: during the injection the septums are pierced by the needles (N). Interface*: during the separation the autosampler is disconnected from the analyzer. Separation and electrolyte unit: V1-V4 – pinch valves, E1-E3 – driving electrodes, P1-P3 – peristaltic pumps, IM – injection module, W – waste, D1-CD, D2-CD, D2-UV – contactless conductivity and absorbance detection cells, M – membrane, BF – bifurcation, TE, LE1, LE2 – terminating and leading electrolytes.

a bifurcation block, and as a result they were removed from the separation compartment. During this stage of the separation the driving current flowed between electrodes E1 and E3 (Fig. 1).

The very small isotachophoresis (ITP) zones of analytes created in the first column were insufficient for their concentrations to be determined. As a result of switching the direction of the driving current through both columns (by connecting electrodes E2 and E3, Fig. 1), the separated constituents were transferred to the second column. The signal from D1-CD was used to determine an appropriate time to switch the current (Fig. 1).

In the second (narrower) capillary the ITP zones of analytes were prolonged. In addition, due to the low GSSG concentration in the samples of blood (not in excess of tens of μmol/l), their ITP zone length was further extended by a reduction in the concentration of leading ions (ES2, Table 1) in the second column (Fig. 2). A higher degree of sensitivity in ES2 is also evident from the parameters of regression equations for the analytes (Table 2).

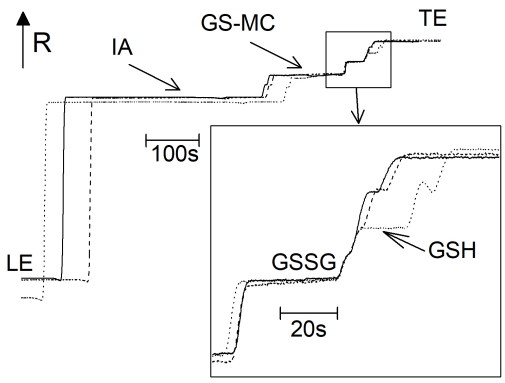

Figure 2: Isotachopherograms from the separations of GSSG and GSH performed in the electrolyte systems ES1 (a) and ES2 (b). The isotachopherograms were recorded by D2-CD (Fig. 1). The concentrations of GSSG and GSH in the injected sample were both 25 µmol/l.

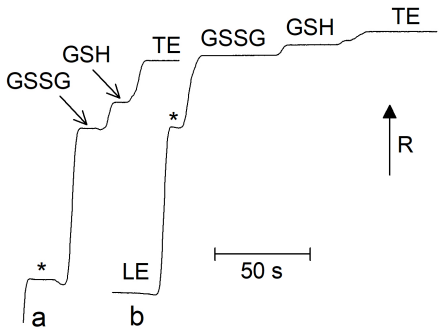

Figure 3: Isotachopherograms from the separation of the reaction mixture present in the electrolyte system ES2. The isotachopherograms were recorded by D2–CD. The mixture contained 15 µmol/l of GSSG, 80 µmol/l of GSH, 2 mmol/l of IA, 4 mmol/l of NaOH, and 10 % of TE. The sample was injected 5 mins (dot), 15 mins (dash) and 20 mins (solid) after the reagents were mixed.

3.2 Stabilization of glutathione

The stability of GSH and its oxidation to GSSG during the period between the collection of the sample and its analysis is the main source of systematic errors. In the sample of bovine blood a much higher concentration of GSSG and a lower concentration of GSH than expected was measured.

To avoid this problem, a thiol-masking agent was used, namely iodoacetic acid (IA) [18]. The substitution reaction between IA and GSH formed S-(carboxymethyl)glutathione (GS-MC).

Under the ITP separation conditions used, GS-MC migrated in front of GSSG (Fig. 3). The optimum conditions for the reaction between IA and GSH were determined by the ITP separations of reaction mixtures at different periods after the reagents were mixed. These experiments were conducted with both model and real samples. Under neutral and slightly alkaline conditions the reaction was very slow. An excess of IA (2 mmol/l) and the presence of NaOH (4 mmol/l) in the reaction mixture led to the fast (less than 20 mins.) and quantitative conversion of GSH to GS-MC (Figs. 3 and 4) without an increase in the concentration of GSSG.

For the purpose of a quantitative analysis, IA and NaOH was added to the sample of bovine blood immediately after its collection (during the sample dilution step required for hemolysis). Blood samples that had been diluted by a factor of ten were directly analyzed after their filtration through a syringe filter with a pore size of 1 µm. The concentrations of GSSG and GSH that were measured in the diluted blood samples were 4.4 µmol/l and 63.4 µmol/l, respectively. The average concentrations

Table 2: Parameters of regression equations.

Analyte	Range [µmol/l]	a [s l/µmol]	b [s]	R^2
GSSG[1]	5-50	0.67	1.45	0.999 7
GSSG[2]	2-25	1.66	2.01	0.9992
GSH[1]	10-50	0.48	0.25	0.9999
GSH[2]	10-50	1.24	0.60	0.9994
GSH (GS-MC)[2]	40-120	1.98	-6.60	0.9969

[1] Electrolyte system ES1 and [2] ES2 used for data evaluation. Regression equation: $Y = aX + b$.

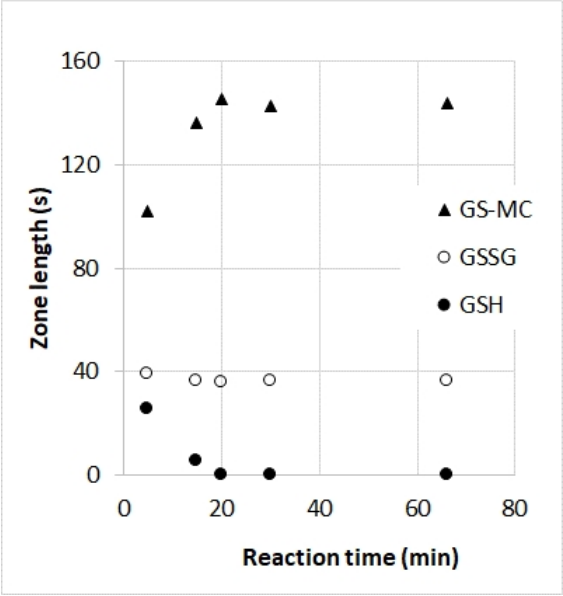

Figure 4: Dependence of the ITP zone length on the reaction time of the mixture containing 15 µmol/l GSSG, 80 µmol/l GSH, 2 mmol/l IA, 4 mmol/l NaOH and 10 % TE.

calculated from eight repetitive measurements of identical samples were in good agreement with those determined by enzymatic methods. The degrees of precision of the method, expressed by the relative standard deviation (RSD) values of the measured concentrations of GSSG and GSH, were 10.3% and 4.4%, respectively.

4. Conclusion

The sensitive and simultaneous determination of GSH and GSSG concentrations in entire samples of bovine blood is facilitated by the capillary isotachophoretic method developed. The simple and rapid preparation of blood samples, that only involves the masking of thiol group of GSH and the dilution and filtration of the sample, increases the accuracy of the GSSG concentration measured. No adverse effects caused by the proteins present in the real blood samples on the separation efficiency or detector response was observed. It can be assumed that this method is also suitable for the analysis of blood samples from other mammals.

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