Capillary electrophoresis method optimized with a factorial design for the determination of glutathione and amino acid status using human capillary blood

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ABSTRACT

Plasma aromatic and sulfur containing amino acids are good indicators of protein anabolism/catabolism, while blood reduced and oxidized glutathione reflect oxidative status in an organism. Using a full factorial design for screening important variables (pH, concentration, temperature) we developed a capillary zone electrophoresis method permitting their measurements in the single run, without any derivatization procedures. The best separations were obtained within less than 30 min employing a 10 mmol/l phosphate buffer, pH 2.8, 18 °C, 15 kV voltage. Fairly good precision with a linear relationship between peak area and concentrations ($r = 0.995$–0.999) were obtained. The method was used to analyze human capillary blood.

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1. Introduction

Thiols and in particular glutathione (GSH) have an important role in overall metabolism including cell homeostasis, radioprotection, and antioxidant defense [1]. In the presence of free oxygen radicals it is oxidized to disulfide form (GSSG). Thus measuring both forms of this peptide seems to be interesting in investigating oxidative/antioxidative status in various physiological and pathophysiological conditions [1,2]. Since blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both GSH and GSSG in blood has been considered essential as an index of the whole subject oxidative status and as a useful indicator of disease risk in humans [3].

Sulfur containing free amino acid, such as cysteine (CyS) or homocysteine (hCyS), have important roles in various metabolic conditions, too [4–6]. Their oxidation and/or interactions produce amino acids with disulfide bounds such as homocystine (hCySS), cystine (CySS) or mixed disulfides (hCyS–CyS) [7]. Thus the levels of thiol containing amino acids present in the physiological fluids could be important in defining metabolic status of an organism. Also the presence of other free amino acids in biofluid could reflect metabolic disturbances in various physiological and pathophysiological conditions [8–11]. Namely, free aromatic amino acids such as: histidine (His), tryptophane (Trp), phenylalanine (Phe) and tyrosine (Tyr) particularly reflect disturbance in protein anabolism/catabolism ratio in an organism [12,13]. Considering these findings it would be very useful to have method permitting simultaneous measurement of these amino acids and both forms of glutathione in the single run.

It is well known that various methods could be used for amino acid and peptide determinations in physiological fluids, including gas chromatography, high pressure liquid or ion-exchange chromatography [8,14,15]. However, during the last decade increased use of capillary electrophoresis (CE) occurred while a lot of attempts have been focused on amino acids and peptides separations [16–18]. Main advantages of CE are low consumptions of buffers and solvents; requirement of a small sample volume and fast separation of anionic, cationic and neutral compounds under proper conditions [17,19,20]. As the variables related to the carrier electrolyte and the instrumental parameters frequently have a complex effect on the separation by CE there are increasing use of multivariate experimental design to find optimal CE conditions for the separation of various compounds [21]. Also during the last decade CE has become an important tool for the analysis of a wide variety of biological molecules including amino acids and peptides [17,19,20]. Since free amino acids generally possess very limited chromophores derivatization procedures prior CE are usually used [22,23], but there are also attempts to analyze amino acids directly, without their derivatization employing measurement of indirect

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absorbance or use other detection capabilities [17,20]. Considering possibility of an in vitro thiols/disulfides interconversion during derivatization procedure it seems more convenient to analyze their native forms present in physiological fluids. Namely, direct detection and omitting derivatization procedure prior CE might better reflect in vivo oxidation. It has been shown that CE is suitable for determination of amino acids containing aromatic moieties as well as for separation of oxidized and reduced glutathione from thiol containing amino acids with direct UV detection and without prior derivatization [20].

In this paper, we present development of a CE procedure permitting simultaneous measurement of glutathione and glutathione disulfide as well as aromatic and thiol/disulfide containing amino acids employing direct detection without any derivatization. The full factorial design has been applied for defining optimal conditions for their separation during its development. The method was evaluated with respect to its repeatability and linearity and was finally tested to analyze human capillary blood.

2. Experimental

2.1. Instrumentation and equipment or capillary electrophoretic system

The CE analysis was performed on a P/ACE 5010 System (Beckman, Palo Alto, CA, USA) with P/ACE system software controlled by an IBM computer. This system contains built-in 200-, 214-, 254- and 280-nm narrow-band filters for on-line detection and quantification. In the present method 200 nm on-line direct detection was used. The instrument was operating at a thermostated temperature which allows a minimum capillary temperature of 15 °C as according to the specification of the manufactures. The lowest temperature used in the present study was 18 °C. Also during the development of the method higher temperatures were used, too.

Electrophoresis was performed in uncoated fused-silica capillary tubing (Beckman, PN 338454) total length 47 cm (40 cm from the window to inlet) and 75 μm I.D. The capillary was assembled in the cartridge with 100 μm × 800 μm aperture and detection window located at 6.5 cm from the capillary outlet.

The carrier electrolyte consisted of disodium monophosphate prepared by dissolving an appropriate amount in water and titrated to the desired pH with phosphoric acid. Working electrolytes were prepared by dilution of stock solution daily. Accordingly, electrolyte concentrations given refer to the concentration of sodium phosphate. All carrier electrolytes were filtrated through 0.45 μm cellulose acetate filters (Millipore). Used water was Milli-Q.

At the start of work each day, the capillary was conditioned by flushing with 0.1 mol/l sodium hydroxide (2 min), followed by deionised water (2 min), and finally with carrier electrolyte (2 min). Also at the beginning of each run the capillary was flashed with carrier electrolyte, while at the end of each run it was flashed with 0.1 mol/l sodium hydroxide (2 min), followed by deionised water (2 min).

2.2. Chemicals and solutions

All chemicals were of analytical grade. Disodium hydrogen phosphate dihydrate and phosphoric acid (85%) were purchased from Serva (New York, USA) and Merck (Darmstadt, Germany), respectively. Carrier electrolyte was disodium hydrogen phosphate dihydrate dissolved in water and titrated with phosphoric acid to the desired pH. Stock solution was prepared at the 100 mmol/l concentration. Running electrolyte at concentration of 4, 7, and 10 mmol/l were prepared by dilution carrier electrolyte with low conductivity deionised Milli-Q water.

Individual amino acids were from several suppliers. L-Histidine·HCl, DL-tryptophane, L-phenylalanine, L-tyrosine, DL-homocystine, L-cystine, L-carnosine were obtained from Serva (Heidelberg, Germany). DL-Homocysteine and L-cysteine·HCl were obtained from Fluka (Switzerland) and Merck (Darmstadt, Germany), respectively. Reduced glutathione as well as oxidized glutathione hydrated were products of ICN Biomedicals (USA).

Individual stock solutions of the examined amino acids and peptides were prepared in Milli-Q water at the concentration of approximately 10 mmol/l. Working solution A (mixture of carnosine, His, Trp, Tyr, Phe, hCySS, CySS and GSSG) at different concentrations were prepared weekly, while GSH was prepared daily and included in the previous mixture (working solution B).

2.3. Method development

Development of the method was aimed at optimizing the separation of the examined compounds. Screening was done by applying a factorial design to identify variables that have an impact on the separation. The resolution values of the compounds in the electropherograms were used as responses in the optimization.

The resolution (R) was calculated using the following equation:

$$R = \frac{2(t_2 - t_1)}{w_{b1} + w_{b2}}$$

where t_1 and t_2 represent migration times of peaks 1 and 2, while w_1 and w_2 are baseline widths of the corresponding peaks.

Migration times, peak widths and peak area were recorded, while peak identification for each analyte was carried out by the injection of individual amino acids and/or by spiking samples with known standards.

2.4. Biological samples

The developed method was tested by human capillary blood. Capillary blood sample (10 μl) was rapidly mixed with cold water (40 μl) and hemolyzate was immediately ultrafiltrated to remove proteins using a micropartition device with molecular 10 kDa (minicentrifuge filter probes, Nalge Nunc International, USA) by centrifugation at 21,000 × g, 20 min at 4 °C in a microlaboratory refrigerated centrifuge MPW-350R (MPW Med. Instruments, Warsaw, Poland). The ultrafiltrate was diluted three times with water and was analyzed by CE.

2.5. Statistical evaluation of the method

Within run precision of migration times and peak area measurements were determined. Values were expressed as means ± SD with coefficients of variation (CV). Linearity was assessed from the analysis of standard amino acids/peptides mixture at concentrations ranging from 0 to 200 μmol/l.

3. Results and discussion

Initially several preliminary trials and error experiments were carried out to determine analytical conditions that could be used as a starting point in the screening analysis. During that the electrophoretic behaviors of investigated substances as well as their photometric characteristics were investigated employing phosphate buffers differing in pH and concentrations. At the very beginning we used the capillary 57 cm long (I.D. 75 μm), employing 15 kV voltage (field strength 263 V/cm), and 10 mmol/l running electrolyte at pH 2.04. It permitted separations of carnosine, His, hCySS, CySS and Trp, while Phe and Tyr as well as GSH and GSSG comigrated (Fig. 1A). Considering that it was achieved within almost
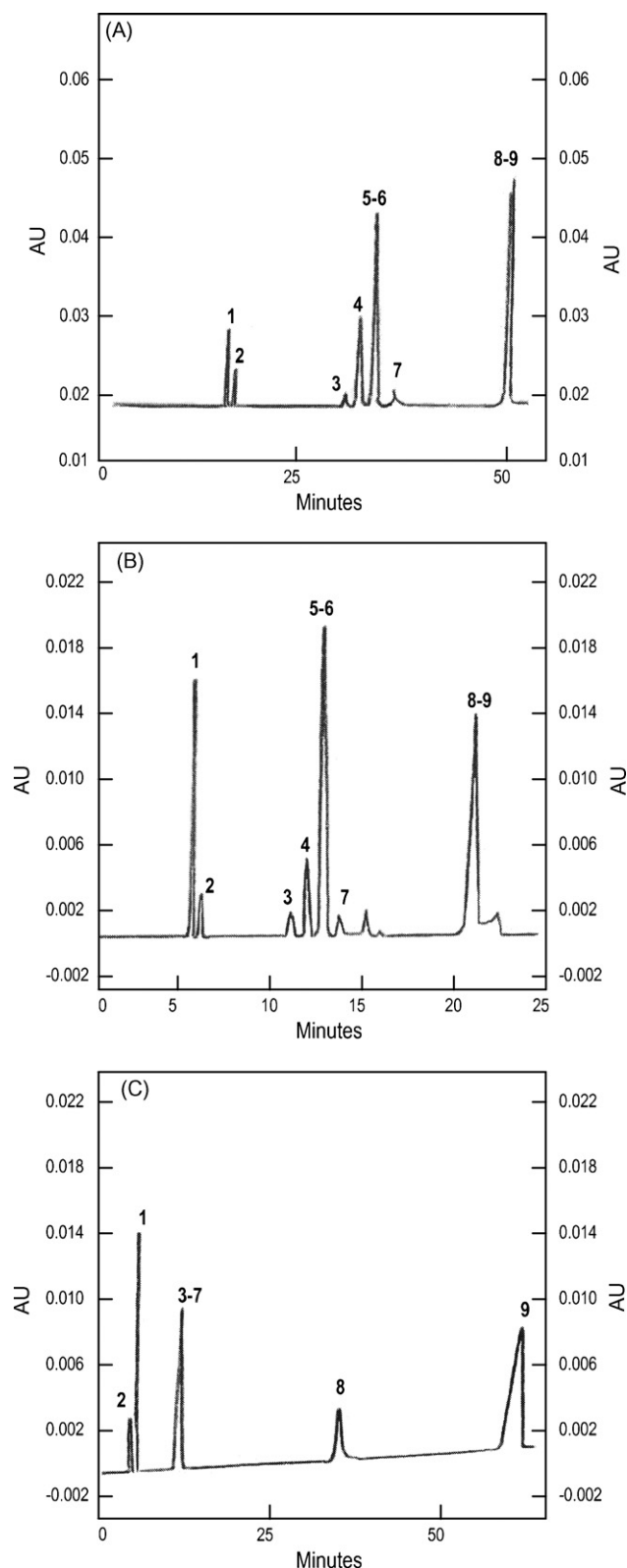
Fig. 1. Electropherogram of the test mixture of the studied compounds (all 50 μmol/l) recorded in the preliminary experiments. Peaks: (1) carnosine, (2) His, (3) hCySS, (4) Trp, (5) Phe, (6) Tyr, (7) CySS, (8) GSH, and (9) GSSG. (A) Capillary: total length 57 cm, effective length 51.5 cm, I.D. 75 μm. Injection: overpressure 10 s. Voltage: 15 kV. Temperature: 18 °C. Detection: UV at 200 nm. Carrier electrolyte: 10 mmol/l phosphate pH 2.04. (B) Capillary: total length 47 cm, effective length 41.5 cm, I.D. 75 μm. Injection: overpressure 10 s. Voltage: 15 kV. Temperature: 18 °C. Detection: UV at 200 nm. Carrier electrolyte: 10 mmol/l phosphate pH 2.04. (C)

Table 1
Levels of the design variables in the two-level factorial design with centre point

Variable	High level (+)	Low level (−)	Centre point (0)
Temperature (°C)	24	18	21
PH	3	2	2.5
Concentration (mmol/l)	10	4	7

50 min we supposed that employing shorter capillary (47 cm total length, I.D. 75 μm) and greater field strength (319 V/cm) would make it faster. Obtained results have documented that shorter capillary permitted much faster analysis (Fig. 1B and C). Thus in the further experiments we proceeded with shorter capillary. During the preliminary studies we also tested injection time (5, 10 and 12 s). Considering observed increase in peak area with injection time as well as the fact that separation efficiencies are reduced with increased injection time we proceeded with 10 s injection time. As we observed that both hCyS and CyS are easily oxidizable compounds we did not include them in the standard mixture while performing experiments for the factorial design.

The best analytical conditions of these tested in the preliminary experiments are illustrated by the electropherograms shown in Fig. 1. Compounds were identified by their separate injections and confirmed by addition to the standard mixture of examined compounds by increase of peak area of corresponding compound. The results obtained during these preliminary investigations have shown that lower pH improved separations of almost all examined compounds, except glutathione and glutathione disulfide (Fig. 1B). Namely, regardless of the temperature or ionic strength of the running solution, these peptides co-migrated at the pH lower than 2.5. Although higher pH improved separations of these peptides, it had opposite effects on the resolution of the examined amino acids (Fig. 1C).

3.1. Experimental design

To evaluate the influence of capillary temperature, buffer pH and its concentrations on the separation of the examined compounds we used a two-level full factorial design (2^3). Factors and their "low"(−) and "high" (+) levels are summarized in Table 1. The eight runs of the design were carried out in a randomized order to transform systemic error into randomized one. To estimate the experimental error, two or three replications of factor combinations were performed leading to a total of 20 experiments. Considering eight analyses of centre points, the total number of 28 analyses were performed.

The model equations for describing full factorial design and the final factorial influences are a linear model of the form: $y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1\ x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{123} x_1 x_2 x_3$, where y represents the estimate response, b_0 is the average experimental response, the coefficients b_1–b_3 are the estimated effects of the factors considered: b_1 the effect of factor A, b_2 the effect of factor B and b_3 is the effect of factor C. The extent to which these terms affect the performance of the method is called the main effect. The coefficients b_{12}–b_{123} are called the interaction terms [24].

Visual inspections of the obtained electropherograms have shown that there were two pairs of the peaks and a group of five amino acids (hCySS, Trp, Phe, Tyr, CySS) difficult to separate. Under examined condition Phe and Tyr could not be separated. Thus, the response variables in focus were the resolution values of the fol-

(C) Capillary: total length 47 cm, effective length 41.5 cm, I.D. 75 μm. Injection: overpressure 10 s. Voltage: 15 kV. Temperature: 18 °C. Detection: UV at 200 nm. Carrier electrolyte: 10 mmol/l phosphate pH 3.66.

Table 2
The resolution values of the different pairs of peaks

Run	Resolution values				
	His and carnosine	hCySS and Trp	Phe/Tyr and Trp	Phe/Tyr and CySS	GSH and GSSG
1	1.794	1.502	1.282	1.413	0.000
2	1.676	1.844	1.405	1.544	0.000
3	−1.186	0.000	0.000	0.000	5.307
4	−0.989	0.000	0.000	0.000	4.940
5	3.142	2.391	0.544	3.623	0.000
6	3.171	2.806	2.476	2.444	0.307
7	−1.079	0.000	0.000	0.000	3.585
8	−1.440	0.000	0.000	0.000	5.543
Centre	−0.692	0.640	1.085	1.219	2.635
Centre	0.000	0.542	1.040	1.122	1.684
Centre	0.000	0.829	1.404	1.566	2.428

lowing pairs of peaks: His and carnosine (a); hCySS and Trp (b); Trp and Phe/Tyr peak (c); Phe/Tyr peak and CySS (d); GSH and GSSG (e).

Obtained resolution values of the different pairs of peaks are presented in Table 2 indicating that the lowest pH associated with the highest concentration of the running buffer and the lowest temperature (runs 5 and 6) produce the best resolutions of almost all the examined compounds. Thus these conditions produce the highest total resolution values, but do not permit resolution between GSH and GSSG. On the other hand, under centre point conditions all compounds were separated but the total resolution value was not the greatest. It was mainly due to the fact that individual resolution values were ranging between 1 and 2. Also it has to be pointed out that under centre point conditions resolutions between His and carnosine as well as between GSH and GSSG were rather pure if higher concentrations of these compounds (above 100 μmol/l) were considered.

3.2. Separation of His and carnosine

The factorial design has shown that the pH of the carrier electrolyte was the variable that had the largest impact on the resolution of carnosine and histidine within the examined experimental design. The negative effect of pH on the resolution of these compounds suggests that decreased pH improved their separation. Also the pH increase of running electrolyte was associated with decreased migration times of both compounds. The effects of electrolyte pH on the resolution of these two peaks can be seen in the electropherograms in Fig. 1. It has to be pointed out that pH mainly effect upon His migration. Namely, faster migration of His compared to carnosine was obtained at higher pH. Thus, His migrated before carnosine in running electrolyte with pH > 3.3, while at pH < 2.3 it migrated after carnosine. However, in the condition of centre point (pH 2.5, 7 mmol/l) they co-migrated. Obtained results indicated that temperature had very little effects on resolutions of these two compounds. However, the concentration of running electrolyte had positive effects on their separation, but its effects are much less compared to the pH effects. The following equation was calculated:

$$y = 0.636 - 0.063x_1 - 3.619x_2 + 0.625x_3 - 0.019x_1x_2 - 0.103x_1x_3 - 0.797x_2x_3 - 0.176x_1x_2x_3.$$

3.3. Separation of Trp, Phe, Tyr, hCySS and CySS

The results obtained with factorial design suggest that these five amino acids are better separated in the running electrolyte with the lowest pH. Phe and Tyr have not been separated from each other, but co-migrated under all examined conditions. Thus, in order to examine the influence of the three investigated variables we ana-

lyzed three pairs of amino acids, while Phe/Tyr peak was considered as one. Obtained results indicate the greatest effect of pH on the separation of all pairs of amino acid peak.

However, the separation of hCySS and Trp was significantly influenced by the concentration of the electrolyte and to a lesser extent by the temperature. They positively effected upon the separation of these amino acids, while negative relationship with pH was obtained. The following equations were obtained:

(a) $y = 1.068 + 0.189x_1 - 2.136x_2 + 0.463x_3 - 0.189x_1x_2 + 0.018x_1x_3 - 0.463x_2x_3 - 0.018x_1x_2x_3$

(b) $y = 1.128 - 0.262x_1 - 2.256x_2 + 0.777x_3 + 0.262x_1x_2 - 0.328x_1x_3 - 0.777x_2x_3 + 0.328x_1x_2x_3$

(c) $y = 0.713 + 0.262x_1 - 1.427x_2 + 0.083x_3 - 0.514x_1x_2 + 0.452x_1x_3 - 0.083x_2x_3 - 0.452x_1x_2x_3$ representing resolution values for hCySS and Trp (a), Phe/Tyr peak and CySS (b) as well as Phe/Tyr peak and Trp (c), respectively.

3.4. Separation of GSSG and GSH

The greatest impact on the separation between GSH and GSSG had pH of the running electrolyte. Namely, their separations were improved with increasing pH of the buffer. The other variables (i.e. temperature and concentration) had little effect on the separation between these peptides. The effect of pH on the resolution of GSH and GSSG is also demonstrated by electropherograms shown in Fig. 1. These compounds co-eluted at low pH, while at the pH above 3 their separations progressively increased with pH. When all variables were at their low level (pH 2.2, electrolyte concentration 4 mmol/l, temperature 18 °C) GSH and GSSG co-eluted. Additional experiments have shown that these compounds co-migrated when pH is bellow 2.6. The following equation was calculated for factorial design:

$$y = 2.460 + 0.474x_1 + 4.767x_2 - 0.203x_3 + 0.321x_1x_2 + 0.658x_1x_3$$
$$- 0.357x_2x_3 + 0.505x_1x_2x_3.$$

3.5. Final optimizations

The analytical conditions that accomplished the best separation in the factorial design were used as a starting point in the final optimization of the conditions. The results obtained in the factorial design indicated the greatest effect of pH on the separations of all examined compounds. As these experiments have shown that temperature (factor A) had almost no influence on the separations of the investigated compounds, in the following experiments we kept it constant at 18 °C level. Considering that factorial design have shown that concentration (factor C) of the running buffer little influenced upon examined separations in the following experiments we kept it
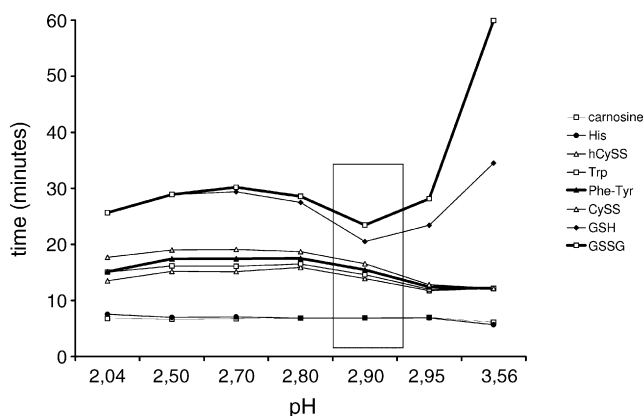
Fig. 2. Effects of pH on the separation of the examined compounds.

at 10 mmol/l level. Thus in the final optimization we varied pH and additionally examined following pH: 2.50, 2.70, 2.80, 2.85, 2.90, and 2.95 of the running electrolyte, considering also pH tested in factorial design procedures. The effects of the running buffer acidity on the migration times of the examined analytes are presented in Fig. 2.

At 10 mmol/l concentration and 18 °C temperature, the running electrolyte with pH around 2.90 has produced the best separations of the most examined compounds (Fig. 3). It was achieved within less than 30 min at 15 kV voltage. At higher voltage separations were faster, but with less sensitivity (smaller peak areas). These conditions were considered as optimal and were used in further examinations of this method. It has to be pointed out that under this condition carnosine and His has not been well separated and in further experiments we omitted carnosine from the standard mixture.

3.6. Sensitivity and linearity

Sensitivity of the method was evaluated by determining limits of detection (LOD) and limits of quantification (LOQ). The LOD and LOQ in standard mixtures of each compound are based on signal-to-noise ratio of 3 and 10, respectively. Obtained results are summarized in Table 3. The smallest LOD and LOQ are obtained for GSSG ranging from 1 to 4 μmol/l, respectively. The highest values were obtained for hCySS and CySS ranging around or even above 100 μmol/l. On the other hand both GSH and examined aromatic
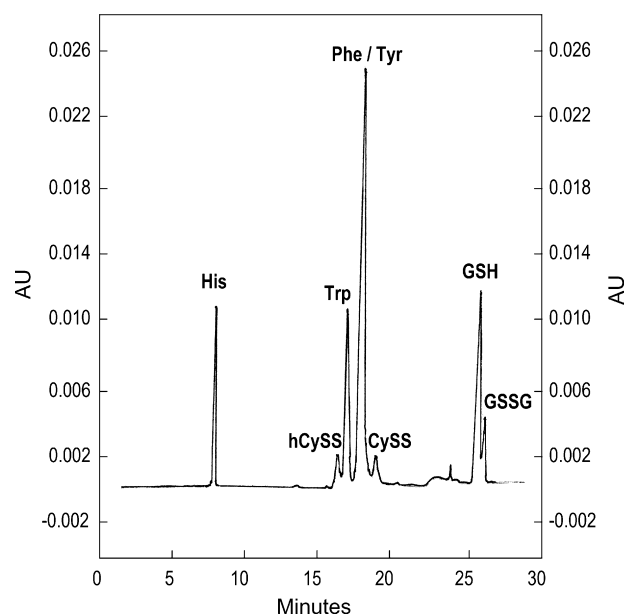

Fig. 3. Electropherogram recorded under the optimized conditions. Carrier electrolyte: 10 mmol/l phosphate, pH 2.90. Capillary: total length 47 cm, effective length 41.5 cm, I.D. 75 μm. Injection: overpressure 10 s. Voltage: 15 kV. Temperature: 18 °C. Detection: UV at 200 nm. Concentrations of His, hCySS, Trp, Phe, Tyr, CySS, and GSH are 70 μmol/l each, while GSSG is 7 μmol/l.

amino acids had both LOD and LOQ in the range between 10 and 30 μmol/l, respectively.

The linearity of the analytical response to the target compounds was tested in the concentration range 0–200 μmol/l for all compounds except GSSG ranging 0–20 μmol/l. Concentration and peak area correlated linearly in the examined range. The linearity, obtained with the mean values from five determinations, was expressed by equations presented in Table 3.

3.7. Repeatability studies

Table 4 summarizes the repeatability of migration time, peak area and corrected peak area (i.e. peak area divided by migration time) for each of the well-separated peaks under optimized conditions. Within run precision of migration times for His, hCySS, Trp, Phe/Tyr and CySS were below 2%, while higher variations were
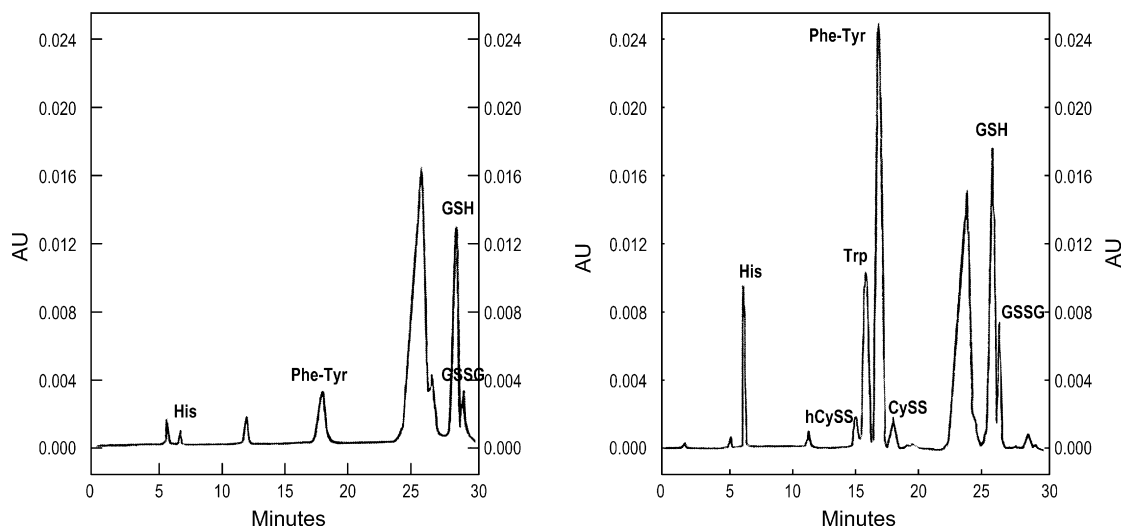

Fig. 4. Electropherograms of human capillary blood (diluted 12 times) without (left) and spiked (right) with standard mixture of examined compounds.

Table 3
LOD, LOQ, equations and correlation coefficients for the examined compounds in the examined concentration ranges

Compound	LOD (μmol/l)	LOQ (μmol/l)	Equation	r	P_a
GSSG	0.9	3.0	$y = 23744.0 + 5600.3x$	0.991	0.061
Phe/Tyr	3.6	11.9	$y = 56802.4 + 4231.9x$	0.998	0.538
Trp	4.2	13.8	$y = 1870.6 + 3793.4x$	0.998	0.998
GSH	5.0	16.6	$y = 9302.6 + 80.7x$	0.999	0.101
His	8.2	27.5	$y = 517.0 + 586.4x$	0.998	0.326
hCySS	24.7	82.3	$y = 2027.2 + 17.6x$	0.999	0.823
CySS	36.6	121.9	$y = 7758.4 + 666.1x$	0.982	0.535

y, peak area; x, concentration; P_a, P for intercept.

Table 4
Summary of the repeatability studies

Compound	μmol/l	Time (min) Within run	Area Precision	Corr.area ($n = 3$)
His	50	7.11 ± 0.03 (0.4)	20351 ± 1113 (5.5)	2861 ± 163 (5.7)
hCySS	50	15.23 ± 0.18 (1.2)	29754 ± 354 (1.2)	1954 ± 11 (0.6)
Trp	50	16.04 ± 0.2 (1.2)	185787 ± 7457 (4.0)	11586 ± 419 (3.6)
Phe/Tyr	50	17.08 ± 0.23 (1.3)	487162 ± 25327 (5.2)	28520 ± 1538 (5.4)
CySS	50	18.45 ± 0.27 (1.5)	29443 ± 4636 (15.7)	1596 ± 250 (15.6)
GSH	50	23.49 ± 1.76 (7.5)	131247 ± 5554 (4.2)	5603 ± 360 (6.4)
GSSG	5	25.44 ± 2.04 (8.1)	59161 ± 7903 (13.4)	2320 ± 169 (7.3)

Compound	μmol/l	Time (min) Between run	Area Precision	Corr.area ($n = 4$)
His	50	7.05 ± 0.09 (1.3)	19698 ± 1193 (6.1)	2794 ± 147 (5.3)
hCySS	50	15.12 ± 0.2 (1.3)	29139 ± 2093 (7.2)	1926 ± 136 (7.0)
Trp	50	15.91 ± 0.22 (1.4)	184036 ± 5911 (3.2)	11563 ± 300 (2.6)
Phe/Tyr	50	16.95 ± 026 (1.5)	470054 ± 29644 (6.3)	27730 ± 1536 (5.5)
CySS	50	18.16 ± 0.4 (2.4)	32757 ± 3109 (9.5)	1806 ± 195 (10.8)
GSH	50	23.16 ± 2.41 (10.4)	135024 ± 14018 (10.4)	5846 ± 474 (8.1)
GSSG	5	25.60 ± 2.36 (9.2)	59574 ± 5079 (8.5)	2341 ± 268 (11.4)

Values are given as means ± SD (CV in parenthesis).

obtained for both GSH and GSSG peaks. Variations of corrected peak areas for the most of the examined compounds were below 10%.

3.8. Testing the method on blood sample analysis

The method was tested on human capillary blood samples (Fig. 4A and Table 5). Separation of the examined compounds were also tested by spiking physiological fluid with the standard mixture (Fig. 4B). Obtained results indicate that examined compounds were completely separated under optimized conditions. This method permitted GSH, GSSG as well as aromatic amino acids (i.e. His, Trp and Phe + Tyr) measurement in blood hydrolysates. However, due to the detection limits of the method it was not possible to measure disulfide containing (hCySS and CySS) amino acids in these samples under used experimental procedure (Table 5). Namely, LOQ values were 82.3 and 121.9 μmol/l for hCySS and CySS, respectively (Table 3). However, LOD of the other compounds were below 10 μmol/l permitting measurements of the other compounds well within run precision. Finally we calculated the concentration of each compound considering dilution factor (Table 5). The level of

Table 5
Concentrations of examined compounds in capillary blood samples

Compound	Determined concentration (μmol/l)
His	148.8 ± 6.0 (4.0)
hCySS	0.0 ± 0.0 (0.0)
Trp	123.6 ± 2.8 (2.2)
Phe/Tyr	282.6 ± 2.9 (1.0)
CySS	0.0 ± 0.0 (0.0)
GSH	1078.1 ± 17.0 (1.6)
GSG	110.6 ± 7.3 (6.6)

Values are given as mean ± SD of three analysis. CV (%) are given in parenthesis.

GSH in capillary blood samples reached 1078.1 μmol/l, while GSSG level was 110.6 μmol/l. The others have reported similar levels of GSH and GSSG in blood samples for healthy subjects reaching from 916 to 1383 μmol/l and from 1 to 332 μmol/l, respectively [25,26]. Thus the values for GSH and GSSG obtained in the present study are in the range reported by others for healthy subjects. Some differences probably are consequence of the fact that blood GSH/GSSG determination is frequently affected by methodological flews with consequent high variability in results and even over estimation of GSSG [25]. They considered that during acid deproteinisation hemoglobin can deliver electrons to oxygen or other molecules, yielding free radicals, particularly during sample acidifications. In the present study we used ultrafiltration for protein removal, preventing sample acidification and concomitantly reducing artificial increase of GSSG. In addition the levels of examined aromatic amino acids in whole blood hydrolysates obtained in the present study are significantly higher from reported plasma values [12–14] confirming the fact that their intracellular levels are above extracellular ones. Recently, the others [23] have also reported CE as a suitable tool for the quality control of amino acids and glutathione, although they analyzed samples after derivatization and testing the method for analysis in the pharmaceutical field. The method obtained in the present study permits simultaneous measuring of GSH and GSSG and some amino acids by CE in biological fluids. It has to be pointed out that this method needs no derivatization procedures, permitting analyses of native compounds in the blood samples.

4. Conclusion

The method employing CE for the analysis of both reduced and oxidized glutathione as well as aromatic (His, Trp, Phe/Tyr) and sulfur containing (hCySS, CySS) amino acids was developed. The

compounds were separated in the single run without any derivatization procedure representing one of the main advantages of this method. Under optimized conditions their separations were achieved within less than 30 min. Finally, this study confirms factorial design as suitable procedure in screening important variables leading to the best separations of the compounds present in a mixture. Also it has been shown that optimized conditions could be suitable for blood analysis, permitting screening of oxidative and protein status of an organism.

Acknowledgement

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