Journal of Chromatography B, 879 (2011) 2567–2575

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of ascorbic acid, aminothiols, and methionine in biological matrices using ion-pairing RP-HPLC coupled with electrochemical detector

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ARTICLE INFO

Article history:
Received 20 April 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Column liquid-chromatography
Vitamin C
Aminothiols
Methionine
Electrochemical detection
Optimization
Validation

ABSTRACT

A novel highly sensitive ion-pairing reversed-phase high performance liquid-chromatography/electrochemical detection method for simultaneous determination of L-ascorbic acid, aminothiols, and methionine in biological matrices was developed, optimized, and validated. Reduced forms of the analytes were extracted from the sample matrices with 10% *meta*-phosphoric acid solution$_{(aqueous)}$. To determine the total vitamin C, the total aminothiols, and the total methionine, samples were treated with tris(2-carboxyethyl)phosphine solution in 0.05% trifluoroacetic acid solution$_{(aqueous)}$ subsequent to deproteination to reduce the oxidized forms of these compounds. Various analytes were separated on a C_{18} (250 × 4.6 mm, 5 μm) analytical column using methanol–0.05% trifluoroacetic acid solution$_{(aqueous)}$ (05/95, v/v), containing 0.1 mM 1-octane sulphonic acid as the ion-pairing agent) as the isocratic mobile phase pumped at a flow rate of 1.5 mL min^{-1} at room temperature. The column eluents were monitored at a voltage of 0.85 V. These analytes were efficiently resolved in less than 20 min using *n*-acetyl cysteine as the internal standard. The present method was specific for the analysis of these analytes and demonstrated acceptable values for linearity ($r^2 > 0.999$ in the range of 0.2–10,000 ng mL^{-1} for all the analytes), recovery (>96%), precision (%RSD ≤ 2.0), and sensitivity (on column limit of detection: 250–400 fg and limit of quantification: 0.8–1.25 pg), indicating that the proposed method could be efficiently used for determination of these analytes in the context of clinical research.

1. Introduction

Vitamin C, aminothiols (cysteine, homocysteine, and glutathione), and thioethers (e.g., methionine) have a number of physiological functions and the level of these compounds in various biological matrices such as plasma and erythrocytes are valuable markers in a number of clinical situations [1,2]. Vitamin C and glutathione are the two most active water-soluble anti-oxidants in many biological systems. They work in a cyclic manner to protect lipids, proteins, and other biomacromolecules from oxidative damage by neutralizing toxic peroxides and by stabilizing free radicals and other reactive oxidants. The ratios of their reduced (ascorbic acid and glutathione, respectively) to oxidized (dehydroascorbic acid and glutathione disulphide, respectively) forms are in fact good measures of the extent of oxidative stress to which the organism is exposed [3–8]. Cysteine and methionine, on the other hand, exert their anti-oxidant properties by acting as direct or indirect precursors for glutathione biosynthesis, respectively. Likewise, homocysteine occurs at a pivotal metabolic juncture between pathways of methionine remethylation and transulfuration, and hyperhomocysteinemia (elevated plasma homocysteine) generally reflects the relative activity of these two pathways. Mild to moderate hyperhomocysteinemia has become a useful clinical biomarker for cardiovascular diseases [9,10] and for folate, vitamin B_{12} (cobalamin), and/or vitamin B_6 deficiencies and several inborn folate, cobalamin, and/or methionine metabolic abnormalities [11]. Information regarding a connection between homocysteine metabolism and cognitive function, from mild cognitive decline (age-related memory loss) to vascular dementia and Alzheimer's disease has also been emerging [12]. Appropriate management of these clinical conditions thus depends on an understanding of the biochemical determinants of homocysteinemia and would be facilitated by a method that simultaneously quantify, within the same plasma sample, the vitamin C, the methionine, and the major aminothiols in both the transmethylation and transulfuration pathways.

Several papers have been published on the liquid chromatographic determination of ascorbic acid in combination with dehydroascorbic acid [13–25] and/or other compounds [25–31]. A

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doi:10.1016/j.jchromb.2011.07.013

review article has also been published on analysis of the ascorbic acid and its oxidized form [32]. Similarly, a large number of papers have been reported on the chromatographic analysis of glutathione in combination with glutathione disulphide [33–40] and/or other compounds including aminothiols and thioethers [1,41–44]. A review paper has also been reported on determination of the glutathione and its disulphide [45]. In fact, a method for simultaneous determination of aminothiols and ascorbic acid using capillary electrophoresis has also been reported [2]. To the best of our knowledge, except for the method reported by Khan et al. [46], no single paper has been reported so far that can simultaneously determine vitamin C, aminothiols, and thioethers. However, this paper also has a number of shortcomings including erroneous determination of cysteine and cystine concentrations due to elution with the solvent front, inability to determine the oxidized forms of homocysteine and methionine, and poor precision due to very high %residual standard deviation (%RSD) in intra- and inter-days reproducibility studies.

This study was thus designed to overcome the above mentioned shortcomings by developing a sensitive, precise, and accurate method for simultaneous determination of ascorbic acid, aminothiols, thioethers (e.g., methionine), and their oxidized forms in various biological matrices such as plasma and erythrocytes using ion-pairing reversed-phase high performance liquid-chromatography (RP-HPLC) coupled with electrochemical (EC) detector. The proposed method was also validated according to international guidelines.

2. Experimental

2.1. Chemicals and reagents

L-Ascorbic acid (purity ≥99%), L-cysteine (purity ≥98.5%), L-cystine (≥99.5%), DL-homocysteine (purity ≥95%), L-methionine (purity ≥99.5%), L-glutathione (purity ≥98%), (−)-glutathione disulphide (purity ≥99%), and *n*-acetyl-L-cysteine (purity ≥99%) were purchased from Sigma–Aldrich (via Analytical Measuring Systems Pvt. Ltd., Karachi, Pakistan). HPLC-grade acetonitrile (purity ≥99.9%), methanol (purity ≥99.9%), and 1-octane sulphonic acid, sodium salt monohydrate (OSA; purity ≥98%) and analytical-grade chemicals and reagents such as normal saline (0.9% aqueous sodium chloride), *ortho*-phosphoric acid (OPA; purity 85%), *meta*-phosphoric acid (MPA; purity 100%), monobasic potassium phosphate (KH_2PO_4; purity ≥99%), trifluoroacetic acid (TFA; purity ≥98%), formic acid (FA; purity 85%), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl; purity ≥99%) were purchased from either Sigma–Aldrich, Merck chemicals (via Science Centre, Rawalpindi, Pakistan), or Scharlau (via Musaji Adam & Sons, Karachi, Pakistan). Ultra-pure water was prepared by a Millipore ultra-pure water system (Milford, USA). All these chemicals and reagents were used without further purification accept mobile phases, which were vacuum filtered through 0.45 μm pore size filters.

2.2. Preparation of standard solutions

Primary stock solutions of the analytes were prepared in the aqueous component of the mobile phase and stored at −20 °C in amber glass vials. Secondary stock solution of the *n*-acetyl cysteine used as the internal standard (to give a final concentration of 200 ng mL^{-1}), to be added to all standard mixtures and sample matrices, was prepared by dilution of its primary stock solution with the mobile phase. Similarly, secondary stock solutions and working standard solutions (at eight concentration levels in the range of 0.2–10,000 ng mL^{-1} each containing 200 ng mL^{-1} *n*-acetyl cysteine as the internal standard) of all the analytes were also prepared by dilution of their primary standard solution with the mobile phase. Moreover, standard 1:1 calibration mixture containing 200 ng mL^{-1} each of ascorbic acid, cysteine, homocysteine, glutathione, methionine, and *n*-acetyl cysteine was also prepared.

2.3. Sample collection and preparation

Venous blood samples (about 3 mL) collected from healthy subjects in pre-cooled ethylenediaminetetraacetic acid (EDTA)-vacutainer tubes were immediately centrifuged at 2500 × *g* for 10 min at −10 °C to separate plasma and blood cells. To prepare erythrocytes samples, buffy coat was removed from the blood cells and these cells were then washed with normal saline. Both plasma and erythrocytes samples (50 μL) were then vortex-mixed with 3 mL (6 parts) 10% MPA solution$_{(aqueous)}$ and stored in amber colored eppendorf tubes at −80 °C until analyses. At the time of analysis, all these samples were thawed at room temperature and erythrocytes hemolysates were prepared by lysing the erythrocytes by freeze/thawing two to three times.

2.3.1. Direct determination of ascorbic acid, reduced aminothiols, and methionine

To determine the ascorbic acid, the reduced aminothiols (cysteine, homocysteine, and glutathione), and the methionine, pre-treated samples were vigorously vortex-mixed for 2 min at room temperature and subsequently centrifuged at 2500 × *g* for 10 min at −10 °C. The clear supernatants obtained were then directly injected into the HPLC system after dilution with the mobile phase.

2.3.2. Determination of vitamin C, total aminothiols, and total methionine/reduction of oxidized forms of the analytes

In case of determining the vitamin C (ascorbic acid plus dehydroascorbic acid), the total aminothiols (total cysteine, total homocysteine, and total glutathione), and the total methionine, the deproteination step is followed by reduction of the oxidized forms of these analytes, which is accomplished by the addition of TCEP·HCl solution to the samples. To optimize the reduction process, samples were treated with variable volumes of TCEP·HCl solution in either PBS, pH 7.4, or the acidified aqueous component of the mobile phase. The time required for complete reduction was also optimized.

2.3.3. Indirect determination of dehydroascorbic acid, oxidized aminothiols, and methionine sulfoxide

Concentrations of the dehydroascorbic acid, the oxidized aminothiols (cystine, homocystine, and glutathione disulphide), and methionine sulfoxide were calculated by subtracting ascorbic acid, cysteine, homocysteine, glutathione, and methionine concentrations from concentrations of vitamin C, total cysteine, total homocysteine, total glutathione, and total methionine, respectively.

2.4. Instrumentation

Chromatography was carried out on an HPLC system, consisting of an auto-sampler, pump, vacuum degasser, and peltier column oven (Perkin Elmer Series 200 HPLC system, Norwalk, USA) and the analytes were detected on an EC detector equipped with a single-channel EC flow cell, the Flexcell^{TM} having a three-electrode configuration of the HyREF^{TM} (maintenance free reference electrode), the auxiliary electrode, and the gold (Au) working electrode (DECADE II EC detector, Antec Leyden, Netherlands). The Flexcell had an effective volume of 0.5 μL. The chromatographic data

obtained was then analyzed on Perkin Elmer Totalchrom chromatography workstation (version 6.3.1.) interfaced with the HPLC system through Pe Nelson network chromatography interface (NCI) 900.

2.5. Chromatographic conditions

Analytes were separated on Supelco Discovery® HS C_{18} (250 mm × 4.6 mm, 5 μm; Bellefonte, USA) analytical column protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge. Different isocratic mobile phases, consisting of acetonitrile, methanol, or acetonitrile–methanol mixtures as the organic components, 50 mM KH_2PO_4 buffer, pH adjusted with 85% OPA (pH 2.0–3.5), or pH adjusted water with either 85% OPA, 85% FA, and TFA as the buffered/acidified aqueous components, and OSA (0.05–0.5 mM) as the ion-pairing agent pumped at various flow rates in the range of 0.5–2.0 mL min^{-1} were evaluated. Analyses were performed at various column oven temperatures in the range of 27–40 °C. The injection volume was tested in the range 5–20 μL.

To achieve best separation and detection, the preliminary selected parameters were evaluated on the basis of peak resolution ($R_{s;i,j}$) for the critical peak pairs "*i*" and individual tested values "*j*" of the studied parameters. The value "*j*" was selected as optimal when $R_{s;i,j}$ achieved at least the minimum acceptable value of 1.75.

2.6. Electrochemical detection parameters

To optimize the working potential for simultaneous determination of Ascorbic acid, reduced aminothiols, and methionine, the hydrodynamic voltammograms (current–voltage (*I*/*E*) curve) were constructed for all the analytes by plotting their respective detector response, both in millivolts (mV) and nanoamperes (nA), against the varying working potential in the range of 0.2–1.0 V. Similarly, other EC detection parameters such as polarity, temperature, filter, range and maximum compensation, and offset were also optimized.

2.7. Method validation

The proposed method was validated according to international guidelines with emphasis on specificity/selectivity, linearity within the expected concentration range, accuracy in terms of %recovery, precision (repeatability and intermediate precision), sensitivity, robustness, and stability of solutions [47,48].

2.7.1. Specificity/selectivity

To verify the specificity/selectivity of the method, separation of the peaks in the chromatograms of the blank solvent, the standard 1:1 calibration mixture, the blank sample matrices, and the sample matrices spiked with the standard 1:1 calibration mixture was observed.

2.7.2. Linearity

Linearity of the method was determined by spiking various standard mixtures into sample matrices (50 μL) that were then extracted and analyzed with triplicate injections. Response ratios were then plotted as a function of spiked concentrations of the analytes and the slope, the intercept, and the correlation co-efficient (r^2) were determined from the regression analysis.

2.7.3. Accuracy in terms of %recovery

Standard mixtures, at three nominal concentration levels, were spiked into samples matrices (50 μL; $n = 5$), extracted, and analyzed with triplicate injections. To calculate %recoveries, response ratios of the endogenous analytes in blank sample matrices were subtracted from total response ratios in spiked samples and results obtained were divided by response ratios of the analytes in the standard mixture and multiplied by 100.

2.7.4. Sensitivity

The sensitivity of the method was determined as the limit of detection (LOD) and the lower limit of quantification (LLOQ) using the signal-to-noise ratio (S/N) of 3 and 10, respectively. Both per mL and on-column sensitivities of the method were determined.

2.7.5. Precision

The injection repeatability was determined by injecting standard 1:1 calibration mixture at least 10 times. It was expressed by repeatability of peak area and retention time and determined as mean ± standard deviation (SD) and %RSD calculated from the data obtained. Similarly, to determine the analysis repeatability, 10 samples spiked with the standard 1:1 calibration mixture prepared individually from single plasma sample were analyzed. The result was expressed by repeatability of the recovered amount and determined as mean ± SD and %RSD calculated from the data obtained. In order to determine the intermediate precision (intra- and inter-days reproducibility), the spiked samples prepared for the accuracy/%recovery studies were analyzed three times a day at 09:00, 16:00, and 23:00 h and for three successive days. The result was expressed as reproducibility of the recovered amount and determined as mean ± SD and %RSD calculated from the data obtained.

2.7.6. Robustness

The robustness of the developed method was determined by studying the effect of small deliberate variations in system parameters, like organic component of the mobile phase (±1%), the mobile phase flow rate (±0.2 mL min^{-1}), the column oven temperature (±5 °C), and the working potential (±10 mV), on the performance of the method.

2.7.7. Stability

To evaluate short-term stability, peak areas of the analytes obtained at various time intervals were compared to their initial peak areas in standard solutions (stored in the freezer (at −20 °C) for 1 week) and spiked sample matrices (stored in the auto-sampler at room temperature for 4, 8, 12, and 24 h and after overnight storage in the refrigerator (at 4 °C) and the freezer).

3. Results and discussion

3.1. Optimization of sample preparation

Different measures were taken to ensure accurate and precise measurement of these potentially unstable compounds. These measures included handling and experimental procedures well protected from light and heat, collection of the blood samples in EDTA-vacutainer tubes that have chelating properties, acidification of the plasma and the erythrocytes samples with 10% MPA $solution_{(aqueous)}$ to prevent oxidation and hydrolysis of the analytes, immediate preservation of the plasma and the erythrocytes samples in the freezer until analyses, and reduction of the samples with water-soluble and odorless reducing agent, TCEP·HCl.

3.1.1. Direct determination of ascorbic acid, aminothiols, and methionine

Stabilization, deproteination, and extraction of the plasma and the erythrocytes samples with six parts of MPA (10% aqueous solution) obtained maximum recoveries of all the analytes. Samples were sufficiently diluted with the mobile phase before injection into the HPLC system.

3.1.2. Determination of vitamin C, total aminothiols, and total methionine/reduction of oxidized forms of the analytes

In order to determine vitamin C, total aminothiols, and total methionine, the deproteinized samples were treated with 10 μL (1/5 parts) of 20 mg mL^{-1} (70 mM L^{-1}) TCEP·HCl solution (in 0.05% TFA aqueous solution) by vigorous vortex-mixing for about 5 min at room temperature. These samples were then centrifuged at 2500 × *g* for 10 min at −10 °C before injecting the supernatant into the HPLC system. Representative chromatograms of various samples reduced with TCEP·HCl solution are given in Fig. 2C, F and I.

TCEP·HCl concentration in slightly molar excess is usually used for reduction of thiols, and variable volumes (10–100 μL) of its solution (100–120 mg mL^{-1}) have been used for reduction purposes [49,50]. However, using such a high concentration of TCEP·HCl solution resulted in a troublesome negative peak at about 4.8 min in the chromatogram (see in Fig. 2C, F and I). To overcome this problem, lower concentrations of the TCEP·HCl solution were tried and it was found that even 10 μL (1/5 parts) of the 10 mg mL^{-1} TCEP·HCl solution was sufficient to completely reduce 50 μL (1 part) of sample matrices spiked with standard 1:1 mixture containing 5 μg mL^{-1} each of ascorbic acid, cysteine, homocysteine, glutathione, methionine, and *n*-acetyl cysteine. But to ensure complete reduction, 10 μL (1/5 parts) of 20 mg mL^{-1} TCEP·HCl solution was used for reduction instead of using 10 mg mL^{-1}. On the other hand, authors have insisted the use of BPS: pH 7.4 as the neutral medium for catalyzing its reduction reaction [49,50]; however, according to our findings, the same reaction can occur quite efficiently in 0.05% TFA aqueous solution. Moreover, 5 min vigorous shaking of the sample matrices with TCEP·HCl solution at room temperature was sufficient to complete the reduction reaction.

3.2. Optimization of chromatographic conditions

3.2.1. Selection of the stationary phase

A routinely used RP column, Supelco Discovery® HS C_{18} (250 mm × 4.6 mm, 5 μm) was used for separation of the analytes. To avoid the reversible phase collapse (dewetting phenomenon) resulting from the prolonged use of highly aqueous mobile phases with the ordinary long-chain bonded alkyl stationary phases (octylsilane and octadecylsilane); column was occasionally conditioned with mobile phase having more than 50% organic modifier. Alternately, this problem can be avoided by using polar embedded or hydrophilic end-capped stationary phases [51–57].

3.2.2. Selection of the mobile phase and optimum flow rate

Mobile phase composition is very crucial for chromatographic analysis as it significantly influences retention, height, and character of the analytical signal. Different isocratic mobile phases, consisting of methanol, acetonitrile, or acetonitrile–methanol mixtures as the organic components, pH adjusted water with TFA, FA, or OPA or KH_2PO_4 buffer, pH adjusted with OPA as the buffered/acidified aqueous components, and OSA as the ion-pairing agent, were pumped at flow rates of 0.5–2.0 mL min^{-1}.

As far as organic component of the mobile phases is concerned, acetonitrile and methanol are the two most frequently used organic solvents in the RP-HPLC. Pure methanol in 5% concentration was chosen because ascorbic acid, the first desirable peak in the chromatogram was difficult to retain, and was unresolved from solvent front and other unretained components even with mobile phases containing less than 5% acetonitrile or acetonitrile–methanol (50/50, v/v) mixture as the organic components. Same was the case with cysteine that was unresolved from an unknown peak. Peak areas of all the target peaks also were comparatively more with methanol as compared to acetonitrile or acetonitrile–methanol (50/50, v/v) mixture. Influence of different concentrations of the methanol in the mobile phase (4–7%, v/v) on $R_{s;i,j}$ of the critical peak pairs (poorly retained hydrophilic components/ascorbic acid and unknown peak/cysteine) is shown in Fig. 1A.

Another important requirement for effective EC determination of the intended analytes was the presence of electrolytes (buffered aqueous component) in the mobile phase. The pH adjusted water with either 85% OPA or 85% FA (pH 2.0–3.5) was initially used as the acidified aqueous component of the mobile phase but it was unable to resolve the glutathione peak from the methionine peak even in more than 95% concentrations. The retention time of the analytes also was not reproducible with such mobile phases. This problem of retention time fluctuation, although, was controlled when water was replaced with 50 mM potassium phosphate buffer; however, the resolution problem still persisted. Finally, the problems related to the above mentioned buffered aqueous components were solved by using 0.05% TFA solution$_{(aqueous)}$ as the acidified aqueous component of the mobile phase. These superior properties of the TFA may be attributed to its ion-pairing properties. Influence of different concentrations (0.03, 0.05, 0.07, and 0.09%) of 85% TFA solution$_{(aqueous)}$ in the mobile phase on $R_{s;i,j}$ of poorly retained hydrophilic components/ascorbic acid and unknown peak/cysteine is shown in Fig. 1B.

In the absence of any ion-pairing agent, cysteine was eluting with the solvent front and other unretained compounds even in case of mobile phases containing only 1% methanol. Thus to increase the retention of the analytes on the stationary phase and to allow the use of higher % of the organic modifier in the mobile phase, OSA was used as the ion-pairing agent in the concentration range of 0.01–0.5 mM in the mobile phase (methanol–0.05% TFA aqueous solution 50/50, v/v). Resolution of cysteine from other extraneous components was still compromised until the OSA concentration was raised to 0.1 mM. Although, resolution of the cysteine was further improved above 0.1 mM OSA concentration; however, glutathione and methionine were strongly retained at higher OSA concentrations leading to longer run time. Thus OSA at a concentration of 0.1 mM in the mobile phase was optimal for the separation of ascorbic acid, aminothiols, and methionine. Selectivity of the mobile phase was also changed with the use of OSA, as the elution order was changed from cysteine, ascorbic acid, homocysteine, glutathione, methionine, and *n*-acetyl cysteine [46] to ascorbic acid, cysteine, *n*-acetyl cysteine, homocysteine, glutathione, and methionine. Influence of different concentrations of OSA in the mobile phase (0.05, 0.075, 0.1, 0.15, and 0.2 mM) on $R_{s;i,j}$ of poorly retained hydrophilic components/ascorbic acid and unknown peak/cysteine and retention times of glutathione and methionine is shown in Fig. 1C and D, respectively.

The selected flow rate of 1.5 mL min^{-1} was able to efficiently resolve all the analytical peaks in less than 20 min without building too much backpressure on the column (<2, 200 ± 50 psi).

3.2.3. Selection of the column oven temperature

Separation of the analytes was performed at various column oven temperatures in the range of 25–40 °C. Peak shape and height were improved and retention time decreased with increasing temperature. However, ascorbic acid peak was not efficiently resolved from the early eluting polar hydrophilic components at temperatures above 30 °C. So depending upon these parameters, room temperature was selected to be the optimum temperature for separation of all the analytes. Influence of different temperatures (27–40 °C) on $R_{s;i,j}$ of poorly retained hydrophilic components/ascorbic acid and unknown peak/cysteine is shown in Fig. 1E.

3.2.4. Injection volume

Injection of higher volume of the sample (10–20 μL) resulted in broader peaks and a troublesome solvent artifact that was interfer-

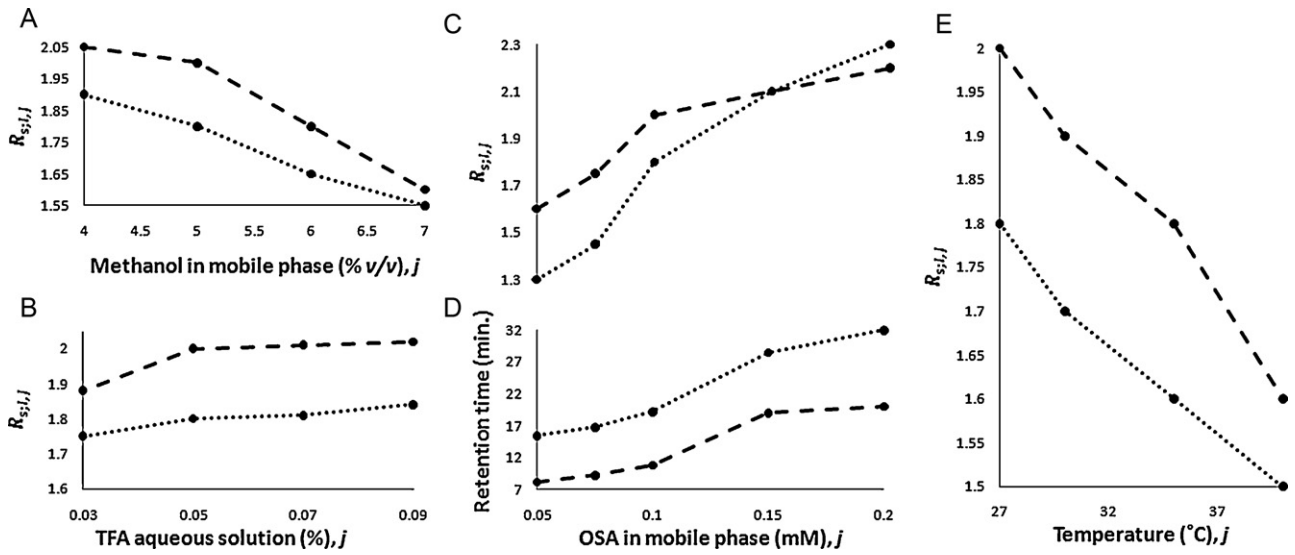

Fig. 1. Influence of (A) %methanol in mobile phase (v/v) on $R_{s;i,j}$; (B) concentration of the TFA aqueous solution (%) on $R_{s;i,j}$; (C) concentration of OSA in mobile phase (mM) on $R_{s;i,j}$; (D) concentration of OSA in mobile phase (mM) on retention time; and (E) column oven temperature on $R_{s;i,j}$. Dashed line represents ascorbic acid and dotted line represents cysteine, accept in (D), where dashed line represents glutathione and dotted line represents methionine. For other chromatographic conditions see Section 3.2.

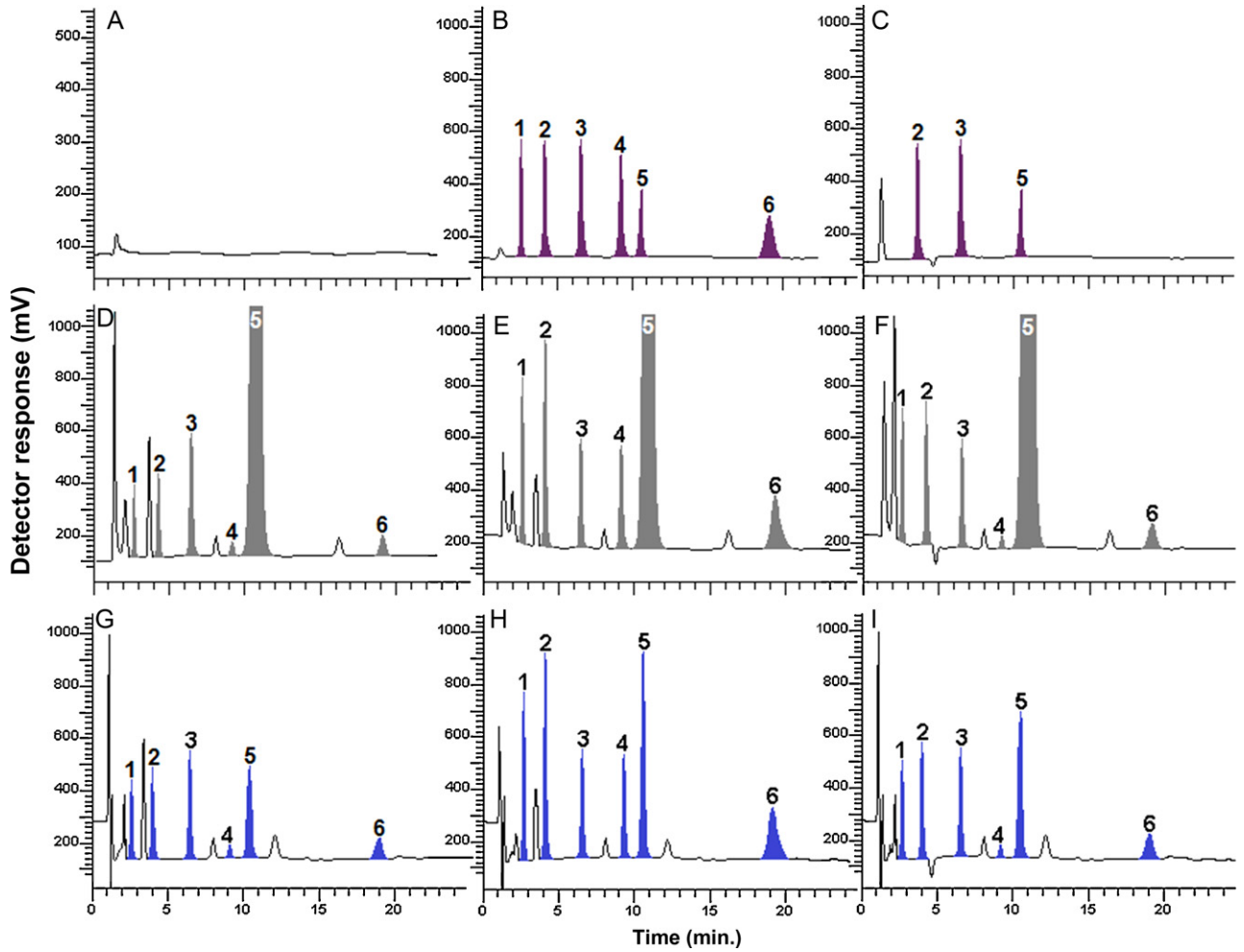

Fig. 2. Representative RP-HPLC chromatograms of different samples analyzed under optimum conditions. *Chromatograms*: (A) the blank mobile phase; (B) the standard 1:1 mixture; (C) the standard mixture containing 200 ng mL^{-1} each of the cystine, internal standard, and glutathione disulphide and reduced with TCEP·HCl solution; (D) the blank erythrocytes sample spiked with 200 ng mL^{-1} of the internal standard; (E) the erythrocytes sample spiked with the standard 1:1 mixture; (F) the blank erythrocytes sample containing 200 ng mL^{-1} of the internal standard and reduced with TCEP·HCl solution; (G) the blank plasma sample spiked with 200 ng mL^{-1} of the internal standard; (H) the plasma sample spiked with the standard 1:1 mixture; and (I) the blank plasma sample containing 200 ng mL^{-1} of the internal standard and reduced with TCEP·HCl solution. *Peaks*: (1) ascorbic acid (2.7 min); (2) cysteine (4.2 min); (3) internal standard (6.5 min); (4) homocysteine (9.3 min); (5) glutathione (10.75 min); and (6) methionine (19.2 min).

Table 1
Optimum electrochemical detection parameters for simultaneous determination of the ascorbic acid, the aminothiols, and the methionine.

Electrochemical detection parameters	Optimum values
Polarity	Positive
Working potential	0.85 V
Temperature	27 °C
Filter	0.01 Hz
Range and maximum compensation	20 nA and 2.5 μA
Offset	10%

ing with the peak of ascorbic acid, probably due to the elution of a very high concentration of some early eluting polar hydrophilic impurities with the mobile phase. So to overcome these problems, injection volume was kept at 5 μL.

3.2.5. Internal standard

Very few published methods for analysis of ascorbic acid and/or dehydroascorbic acid have reported the use of internal standard. Among the various compounds that have been used as internal standard for analysis of ascorbic acid are hydroquinone [58] and isoascorbic acid [19]. However, they may be oxidized during processing and storage leading to doubtful results [59]. Similarly, a number of compounds, including *n*-acetyl cysteine, 2-mercaptopropionylglycine, and cystamine have been used as internal standards in the analysis of glutathione and other thiols [60]. The use of *n*-acetyl cysteine, however, was preferred due to its comparatively greater stability and the resultant acceptable linearity of all the analytes.

3.2.6. Optimum chromatographic conditions

Of the various chromatographic conditions tested, best results were obtained with the Supelco Discovery® HS C_{18} (250 mm × 4.6 mm, 5 μm) analytical column flushed with methanol–0.05% TFA solution$_{(aqueous)}$ (05/95, v/v, containing 0.1 mM OSA) as the isocratic mobile phase pumped at a flow rate of 1.5 mL min^{-1} at room temperature after injecting 5 μL sample. Fig. 2 shows representative chromatograms, at optimum chromatographic conditions, of the blank mobile phase, the standard 1:1 calibration mixture, the blank plasma and erythrocytes samples spiked with the internal standard, the plasma and erythrocytes samples spiked with the standard 1:1 calibration mixture, and the samples reduced with TCEP·HCl solution$_{(aqueous)}$. Under the specified conditions, the mean retention times were 2.7, 4.2, 6.5, 9.3, 10.75, and 19.2 min for ascorbic acid, cysteine, *n*-acetyl cysteine, homocysteine, glutathione, and methionine, respectively.

3.3. Optimization of electrochemical detection parameters

As depicted in the hydrodynamic voltammograms for all the analytes given in Fig. 3, 0.85 V was selected as the optimum voltage for simultaneous determination of ascorbic acid, aminothiols, and methionine using a single-channel EC detector. The detector response to ascorbic acid was almost linear in the range of 0.5–1.0 V. Below 0.5 V, the detector response to it decreased considerably. On the other hand, detector response to aminothiols increased from 0.1 to 0.65 V where their maximum response was observed. Above 0.75 V, the detector response to these analytes again started to decline. As far as the methionine is concerned, its maximum response was observed at 0.85 V, which then became constant up to 1.0 V.

Various other optimum EC detection parameters for simultaneous determination of these analytes are summarized in Table 1.

Table 2
Calibration range, linearity, sensitivity, and accuracy in terms of %recovery of the method.

Parameters	Analytes				
	Ascorbic acid	Cysteine	Homocysteine	Glutathione	Methionine
Calibration range (ng mL^{-1})			0.2–10,000		
Linearity					
Spiked plasma samples					
Regression equation	$y = 0.0019x + 0.0272$	$y = 0.0021x - 0.0028$	$y = 0.0022x - 0.0143$	$y = 0.0019x + 0.0683$	$y = 0.0019x + 0.0670$
Correlation co-efficient, r^2	0.9999	0.9997	0.9996	0.9997	0.9996
Spiked erythrocytes samples					
Regression equation	$y = 0.0019x + 0.0593$	$y = 0.0020x + 0.0624$	$y = 0.0020x + 0.0243$	$y = 0.0021x + 0.0536$	$y = 0.0020x + 0.0623$
Correlation co-efficient, r^2	0.9998	0.9998	0.9997	0.9998	0.9994
Sensitivity					
Limit of detection, LOD					
Per mL (pg)	60	55	50	80	65
On-column (fg)	300	275	250	400	375
Lower limit of quantification, LLOQ					
Per mL (ng)	0.2	0.17	0.16	0.25	0.21
On-column (pg)	1.0	0.85	0.80	1.25	1.05
%Recovery					
Spiked concentration level 1 (0.2 ng mL^{-1} each)[a]					
Plasma	97.50 ± 1.50; 1.54	97.17 ± 1.44; 1.49	96.00 ± 1.32; 1.38	97.00 ± 1.5; 1.55	97.33 ± 1.76; 1.80
Erythrocytes	97.23 ± 1.50; 1.55	97.17 ± 1.89; 1.95	96.67 ± 1.76; 1.82	96.50 ± 1.00; 1.04	97.33 ± 1.61; 1.65
Spiked concentration level 2 (500 ng mL^{-1} each)[a]					
Plasma	96.67 ± 1.22; 1.26	96.93 ± 1.40; 1.45	97.27 ± 1.21; 1.24	97.00 ± 1.00; 1.03	96.33 ± 1.29; 1.33
Erythrocytes	96.60 ± 1.56; 1.62	96.67 ± 1.50; 1.55	96.73 ± 1.21; 1.25	97.13 ± 1.10; 1.13	96.47 ± 1.51; 1.57
Spiked concentration level 3 (5000 ng mL^{-1} each)[a]					
Plasma	98.67 ± 1.04; 1.05	98.17 ± 0.80; 0.82	98.13 ± 1.10; 1.12	98.13 ± 0.9; 0.92	98.40 ± 1.22; 1.24
Erythrocytes	98.90 ± 0.56; 0.56	98.87 ± 0.71; 0.72	98.17 ± 1.00; 1.02	98.17 ± 0.70; 0.72	98.67 ± 1.04; 1.05

%Recovery is expressed in terms of mean ± SD; %RSD.
[a] $n = 5$ (where n is the number of samples).

Table 3
Precision of the method.

Parameters	Analytes				
	Ascorbic acid	Cysteine	Homocysteine	Glutathione	Methionine
	Mean ± SD; %RSD				
Repeatability					
Injection repeatability					
Standard solution (200 ng mL^{-1} each)[a]					
Peak area	2,671,543 ± 41,700; 2	3,611,550 ± 61,138; 2	3,801,547 ± 74,302; 2	2,411,569 ± 51,272; 2	3,113,604 ± 66,909; 2
Retention time (min)	2.70 ± 0.04; 1.54	4.22 ± 0.05; 1.08	9.32 ± 0.08; 0.82	10.75 ± 0.04; 0.36	19.20 ± 0.19; 0.98
Analysis repeatability					
Spiked concentration level 4 (200 ng mL^{-1} each)[b]					
Plasma	195.0 ± 1.6; 0.8[c]	194.6 ± 3.8; 2.0[c]	195.8 ± 3.5; 1.8[c]	195.2 ± 2.6; 1.3[c]	195.4 ± 3.5; 1.8[c]
Intermediate precision					
Intra-day reproducibility					
Spiked concentration level 1 (0.2 ng mL^{-1} each)[b]					
Plasma	0.191 ± 0.004; 1.888[c]	0.187 ± 0.003; 1.343[c]	0.191 ± 0.004; 1.986[c]	0.192 ± 0.004; 1.878[c]	0.189 ± 0.004; 1.908[c]
Erythrocytes	0.190 ± 0.004; 1.852[c]	0.187 ± 0.004; 1.881[c]	0.184 ± 0.004; 1.905[c]	0.190 ± 0.004; 1.898[c]	0.193 ± 0.004; 1.823[c]
Spiked concentration level 2 (500 ng mL^{-1} each)[b]					
Plasma	486.3 ± 8.7; 1.8[c]	488.7 ± 5.7; 1.2[c]	485.3 ± 6.5; 1.3[c]	486.0 ± 6.6; 1.4[c]	488.0 ± 6.6; 1.3[c]
Erythrocytes	486.0 ± 8.5; 1.8[c]	484.7 ± 7.5; 1.6[c]	483.3 ± 6.7; 1.4[c]	485.3 ± 3.5; 0.7[c]	491.3 ± 6.0; 1.2[c]
Spiked concentration level 3 (5000 ng mL^{-1} each)[b]					
Plasma	4876.7 ± 64.3; 1.4[c]	4848.3 ± 63.3; 1.3[c]	4875.0 ± 50.0; 1.0[c]	4896.7 ± 60.5; 1.2[c]	4930.0 ± 50.8; 1.0[c]
Erythrocytes	4861.7 ± 32.5; 0.7[c]	4855.0 ± 57.7; 1.2[c]	4845.0 ± 26.5; 0.5[c]	4880.0 ± 42.7; 0.9[c]	4913.3 ± 55.1; 1.1[c]
Inter-days reproducibility					
Spiked concentration level 1 (0.2 ng mL^{-1} each)[b]					
Plasma	0.191 ± 0.004; 1.888[c]	0.187 ± 0.003; 1.637[c]	0.191 ± 0.004; 1.986[c]	0.189 ± 0.004; 1.908[c]	0.188 ± 0.004; 2.010[c]
Erythrocytes	0.189 ± 0.004; 1.855[c]	0.186 ± 0.004; 1.938[c]	0.184 ± 0.004; 1.905[c]	0.190 ± 0.009; 1.996[c]	0.193 ± 0.003; 1.554[c]
Spiked concentration level 2 (500 ng mL^{-1} each)[b]					
Plasma	486.3 ± 9.1; 1.9[c]	485.3 ± 8.5; 1.8[c]	483.3 ± 7.8; 1.6[c]	483.3 ± 8.7; 1.8[c]	483.3 ± 8.1; 1.7[c]
Erythrocytes	485.3 ± 8.5; 1.6[c]	483.3 ± 8.1; 1.7[c]	481.3 ± 7.5; 1.6[c]	481.3 ± 7.5; 1.6[c]	487.7 ± 6.7; 1.4[c]
Spiked concentration level 3 (5000 ng mL^{-1} each)[b]					
Plasma	4860.0 ± 81.8; 1.7[c]	4861.7 ± 55.8; 1.2[c]	4868.3 ± 46.5; 1.0[c]	4883.3 ± 40.7; 0.8[c]	4835.0 ± 42.7; 0.9[c]
Erythrocytes	8438.3 ± 53.0; 1.1[c]	4911.7 ± 80.0; 1.6[c]	4873.3 ± 54.8; 1.1[c]	4868.3 ± 46.5; 1.0[c]	4823.3 ± 30.6; 0.6[c]

[a] $n = 10$ (where n is the number of samples).
[b] $n = 3$ (where n is the number of samples).
[c] Quantity recovered in ng mL^{-1} (where n is the number of samples).

3.4. Method validation

3.4.1. Specificity/selectivity

The proposed method was selective and specific for ascorbic acid, aminothiols, and methionine as all target peaks were well resolved from each other, from other peaks of extraneous and endogenous substances in spiked plasma and erythrocytes samples, and from the peak of the solvent front, as depicted in the representative RP-HPLC chromatograms of various preparations given in Fig. 2.

3.4.2. Linearity

The proposed method was linear within the expected concentration range of 0.2–10,000 ng mL^{-1} for all the analytes in both spiked plasma and erythrocytes samples. Regression equations describing the calibration curves for spiked plasma and spiked erythrocytes samples and r^2 values of the analytes are summarized in Table 2.

3.4.3. Accuracy in term of %recovery

Recovery studies, summarized in Table 2, showed that the recovery of all the analytes was more than 96% at all the three nominal concentration levels for both plasma and erythrocytes samples with the selected extraction procedure.

3.4.4. Sensitivity

As depicted in Table 2, the proposed method was highly sensitive and could be efficiently used to determine the endogenous level of these analytes in the intended biological matrices.

3.4.5. Precision

Results of the repeatability (injection and analysis) and intermediate precision (intra- and inter-days reproducibility) are summarized in Table 3, showing complete agreement among the repeated injections (both retention times and peak areas), repeated analyses, and intra- and inter-days studies.

3.4.6. Robustness

Results of slight deliberate changes in various system parameters indicated that the proposed method was robust, as its performance was negligibly affected by minor changes in the mobile phase flow rate, the column oven temperature, and the

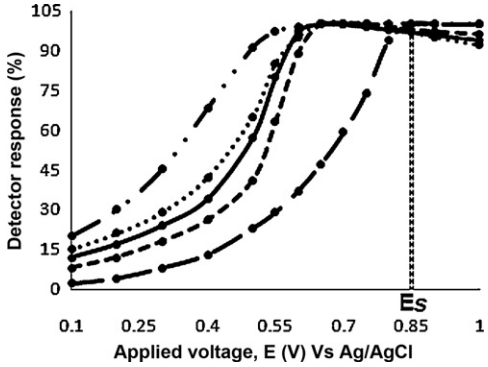

Fig. 3. Hydrodynamic voltammograms for the ascorbic acid, the aminothiols, and the methionine. Detector response (%) vs. voltage, E (V) relationship for the analytes: ascorbic acid (··–··); cysteine (. . . . . .); homocysteine (—); glutathione (- - - - -); and methionine (— —). While, E_s is the selected voltage, 0.85 V.

Table 4
Concentrations of vitamin C, aminothiols, and methionine in plasma and erythrocytes samples of normal human volunteers ($n = 15$).

Analytes	Concentration ($\mu g\,mL^{-1}$)	
	Plasma	Erythrocytes
Vitamin C		
Reduced	58 ± 9	40 ± 8
Total	64 ± 9	44 ± 9
Glutathione		
Reduced	10 ± 1	1055 ± 37
Total	18 ± 1	1073 ± 37
Cysteine		
Reduced	209 ± 10	17 ± 1
Total	279 ± 11	23 ± 1
Methionine		
Reduced	38 ± 7	38 ± 6
Total	42 ± 7	42 ± 7
Homocysteine		
Reduced	8 ± 1	0.9 ± 0.1
Total	11 ± 1	1.3 ± 0.1

All results are reported as mean ± SD.

working potential. However, the composition of the mobile phase was critical for efficient resolution of the analytes.

3.4.7. Stability

All analytes remained stable for at least 4 h when spiked plasma and erythrocytes samples were placed in the auto-sampler at room temperature. However, about 2.0 ± 0.5% ($n = 3$) ascorbic acid was lost after 8 h and about 12.6 ± 0.9% ($n = 3$) ascorbic acid, 3.5 ± 0.6% ($n = 3$) aminothiols, and 2.0 ± 0.3% ($n = 3$) methionine were lost after 24 h at room temperature. When the same samples were refrigerated at 4 °C, 1.3 ± 0.1% ($n = 3$) ascorbic acid was lost within 24 h. On the other hand, samples reduced with TCEP·HCl were stable for about 16 h at room temperature. Similarly, when frozen at −20 °C, spiked samples and standard solutions remained stable for at least 24 h and 1 week, respectively. These results indicated that in order to determine these analytes accurately, samples should be kept frozen until analysis.

3.5. Applicability of the method

The application of this method to the determination of these metabolically interrelated compounds would facilitate differential diagnosis and nutritional management of the many inborn errors of methionine metabolism and the various disease states associated with oxidative stress and abnormal one-carbon metabolism. The proposed method was actually a part of complex biochemical analyses of blood samples collected from human subjects (diseased as well as age- and gender-matched healthy volunteers) and would be used for assessment of the oxidative stress through monitoring of cysteine, methionine, glutathione, and vitamin C concentrations and ratio of their reduced to oxidized forms in plasma and erythrocytes. Results of the endogenous vitamin C, aminothiols, and methionine concentrations in plasma and erythrocyte samples of normal human volunteers measured by this method are shown in Table 4.

The same method could also be applied for the determination of these analytes in other sample matrices such as pharmaceuticals, dietary supplements, foodstuffs, and biological tissues. One should keep in mind, however, that the error in the quantification of the oxidized forms of the analytes should be slightly high because they are determined indirectly by subtracting their reduced forms from the total concentrations obtained after reduction of the samples.

4. Conclusion

Using isocratic RP-HPLC/EC detector, a novel highly sensitive, precise, and accurate method for simultaneous determination of ascorbic acid, aminothiols, methionine, and their oxidized forms in biological matrices was developed, optimized, and validated. Results of the various method validation parameters such as specificity/selectivity, linearity, sensitivity, accuracy in terms of %recovery, precision, and robustness indicated that the proposed method could be efficiently utilized for analysis of both the reduced and the oxidized forms of vitamin C, aminothiols, and methionine in various biological matrices in the perspective of clinical research.

Acknowledgement

The authors are thankful to the Higher Education Commission (HEC), Pakistan for funding this study.

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