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A new HPLC method for the simultaneous determination of ascorbic acid and aminothiols in human plasma and erythrocytes using electrochemical detection

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ABSTRACT

A new, simple, economical and validated high-performance liquid chromatography linked with electrochemical detector (HPLC–ECD) method has been developed and optimized for different experimental parameters to analyze the most common monothiols and disulfide (cystine, cysteine, homocysteine, methionine, reduced (GSH) and oxidized glutathione (GSSG)) and ascorbic acid present in human plasma and erythrocytes using dopamine as internal standard (IS). Complete separation of all the targets analytes and IS at 35 °C on Discovery HS C18 RP column (250 mm × 4.6 mm, 5 μm) was achieved using 0.05% TFA:methanol (97:3, v/v) as a mobile phase pumped at the rate of 0.6 ml min^{-1} using electrochemical detector in DC mode at the detector potential of 900 mV. The limits of detection (3 S/N) and limits of quantification (10 S/N) of the studied compounds were evaluated using dilution method. The proposed method was validated according to standard guidelines and optimization of various experimental parameters and chromatographic conditions was carried out. The optimized and validated HPLC–ECD method was successfully applied for the determination of the abovementioned compounds in human plasma and erythrocytes. The method will be quite suitable for the determination of plasma and erythrocyte profile of ascorbic acid and aminothiols in oxidative stress and other basic research studies.

1. Introduction

Human bodies are continuously exposed to oxidative stress stimuli both from external as well as internal sources. Mitochondrion is the major internal source of reactive oxygen substances (ROS) generation, mainly due to the uncontrolled leakage of electrons. The deleterious effects of these reactive oxygen substances are combat by several antioxidant defenses of the body. Thiols are endogenous antioxidants molecules which contain the sulfhydryl group (–SH) attached to a carbon atom and are the most reactive reducing substances found in the cell that operate at physiological pH to counteract free radicals and ROS that help the body cells maintaining a reducing state, regardless of the oxidizing environment of the body. Similarly, the water soluble vitamins also provide antioxidant defense to the body against free radicals and ROS [1–3]. Vitamin C scavenges various reactive oxygen species including singlet oxygen, superoxide and hydroxyl radicals, inhibits oxidation of low density lipoprotein (LDL) [4,5], and also responsible for the mitochondrial reduction of α-tocopherol [6]. Thiol compounds such as cystine, cysteine (Cys), methionine, N-acetylcysteine (NAC), homocysteine (Hcy), reduced (GSH) and oxidized glutathione (GSSG) have a pivotal role in the human body, e.g. protection against reactive oxygen and nitrogen species, control of gene expression, heavy metal detoxification, markers of various health disorders and signal transduction [7–9]. Glutathione (L-γ-glutamyl-L-cysteinglycine, GSH) is tripeptide non-protein thiol and is the key member of body antioxidant network system playing a vital role in cellular metabolism. It is a cofactor of enzymes such as glutathione peroxidase (GPx) and dehydroascorbate reductase. It recycles both vitamins C and E to their reduced form making antioxidant network functional [10–13]. The capability of glutathione to regenerate other antioxidants depends on the GSH/GSSG ratio of the body. Body GSH and GSSG level may be focused as a useful biomarker in oxidative stress and free radical induced pathologies [14–17]. Homocysteine (Hcy) is an intermediate sulfur-containing amino acid that plays a vital role in the methionine–Hcy–cysteine pathway via donation of methyl group in methionine metabolism. It is produced from methionine during processing of dietary protein and can be reconverted to methionine through the remethylation pathway. Its high serum concentration has been identified as a risk factor for many diseases including cardiovascular, cerebrovascu-

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lar, peripheral vascular, thrombotic disease and Parkinson's disease [9,18–20]. In normal humans, the homocysteine level has been found in the range of 5–15 μmol/l [21]. Low level of cysteine may be found in subjects whose transsulfuration pathway is disturbed. Since cysteine is the rate-limiting factor of glutathione biosynthesis, as a result the glutathione production may be impaired [22]. It has been concluded from *in vitro* studies that homocysteine has both antioxidant as well as pro-oxidant effects on LDL oxidation [23]. N-acetylcysteine protects against oxidative damage through its antioxidant effect via its reducing thiol group both *in vitro* and *in vivo* [24].

A number of analytical methods including both stationary (voltammetry, polarography, chronpotentiometry) and flow technologies such as LC, GC, and CE have been developed for the evaluation of these thiol compounds. As for as LC is considered there are numerous detection modes, including high-performance liquid chromatography (HPLC) linked with UV, MS, and electrochemical detection (ED) had been used for the determination of active thiol compounds. There are certain advantages and limitations associated with each method. The most extensively used method in biological samples is HPLC–UV, associated with low sensitivity, less specificity, and derivatization is necessary for reaching of lower detection limits [9,25]. It may be difficult to quantify all thiol compounds in a single chromatogram using MS detection. In comparison electrochemical detection is an attractive alternate that has advantages of high sensitivity, simplicity, and relatively low cost [7,8]. New methodologies with higher sensitivity and selectivity, such as high-performance liquid chromatography coupled to a variety of detection techniques, including UV–Vis, DAD (diode array detection), fluorometry, electrochemical detection, and mass spectrometry (MS), have been reported for quantification of low concentration of GSSG accurately compared with non-separative techniques, such as UV–Vis spectroscopy, spectrofluorometry, and amperometry [26]. Many HPLC methods using absorbance, fluorescence, and electrochemical detection have been reported for the analysis of ascorbic acid and thiols individually. However an HPLC linked to electrochemical detector method for simultaneous determination of ascorbic acid and thiols has not been reported so for [27]. An HPLC–UV method has been reported for the determination of GSH/GSSG in erythrocytes simultaneously applying standard addition method. Although the UV absorbance is simple but inferior in terms of sensitivity and selectivity compared with electrochemical and fluorometric detection. The glutathione levels are much higher in erythrocytes than plasma however, it is difficult to determine these in erythrocytes due to the presence of ferric ions (Fe^{3+}), that oxidize GSH to GSSG [28]. Simultaneous determination of reduced and oxidized glutathione (GSH/GSSG), and cysteine in maize kernels using high-performance liquid chromatography with electrochemical detection has been reported [8]. Liquid chromatography–electrospray ionization/mass spectrometry methods for quantification of GSH/GSSG in plant tissues extracts [26], and simultaneous determination of glutathione, cysteine, methionine, homocysteine, and their disulfides in biological samples have been reported [29,30]. High-performance liquid chromatography coupled with UV and fluorescence detector have been used for quantification of cysteine, homocysteine and related thiols [31], and total homocysteine in plasma [32–35] and serum [36] along with GC–MS method in human plasma [34].

To our knowledge, the proposed method is the first one that represents the simultaneous determination of ascorbic acid, GSH/GSSG, homocysteine, methionine, cysteine, and N-acetyle cysteine in human plasma and erythrocytes. The proposed HPLC–ECD method was developed for the evaluation of vitamin C and thiol compounds in biological fluids and validated according to standard guidelines [37]. Moreover, various experimental parameters were optimized in order to achieve a highly sensitive, more accurate, rapid, and inexpensive method for the evaluation of these compounds in biological fluids. The described method was found quite suitable for the analysis of above mentioned compounds in human volunteers as well as in clinical practice.

2. Experimental

2.1. Chemicals

Cystine, cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione, homocysteine (Hcy), methionine, N-acetylcysteine (NAC), ascorbic acid (vitamin C), and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (Oslo, Norway). Dopamine (purity 98.5%) was provided by Fluka-Chemika (Switzerland). HPLC grade acetonitrile (>99.9%; v/v) and methanol (>99.9%; v/v) and analytical grade other chemicals including potassium dihydrogen phosphate, phosphoric acid, dichloromethane, ethyl acetate, diethylether and hydrochloric acid were also purchased from Sigma–Aldrich (Oslo, Norway). Purified water was prepared with Millipore (Milford, USA) distillation apparatus and used for buffer preparation. The plasma was obtained from volunteers in the Pharmaceutical Laboratory at Department of Pharmacy, University of Peshawar (Pakistan).

2.2. Equipments

The study was carried out using a Perkin Elmer HPLC system (Norwalk, USA) consisted of a pump (series 200), on-line vacuum degasser (series 200), autosampler (series 200), column oven (series 200), linked by a Pe Nelson network chromatography interface (NCI) 900 with a DECADE II Electrochemical Detector (Antec Leyden, Netherlands), with Flexcell flow cell has an effective volume of 0.5 μl and consists of three electrode configuration with a working glassy carbon electrode, a HyREF reference electrode (REF) and the auxiliary electrode (AUX), KCl Ag/AgCl. The whole HPLC system was controlled by Perkin Elmer Total Chrom Workstation Software (version 6.3.1). The data was acquired and quantified using this software. The separation was performed using Discovery HS C_{18} RP chromatographic column (250 mm × 4.6 mm, 5 μm; Bellefonte, USA), protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge.

2.3. Chromatographic conditions

Chromatographic analysis of all the analytes was performed using electrochemical detector in DC mode applying the detector potential of 900 mV. The mobile phase consisting from 97% to 0.05% (aq) (v/v) TFA (solvent A) and 3% methanol (solvent B) was pumped at the rate of 0.6 ml min^{-1} keeping the column oven and detector temperature at 35 °C. Dopamine (1 μg/ml) was used as an internal standard for the determination of ascorbic acid and thiol compounds. The sample (5 μl) was injected into HPLC system by autosampler.

2.4. Preparation of standard solutions

The stock solutions of quality control standards for thiol compounds and ascorbic acid each 1 mg/ml were prepared in 0.05% TFA and stored at −20 °C in the dark. The stock solutions of all the analytes were further diluted with the same solvent to obtain their respective working standard solutions on daily basis keeping the IS concentration constant (1.0 μg/ml).

2.5. Blood collection and Plasma sample preparation

Blood was collected in the ethylene diamintetraacetic acid (EDTA) tube (≈5 ml), from the healthy volunteers (Pharmacy Grad-

uates in Department of Pharmacy, University of Peshawar, aged 20–25 years) who had given their informed consent. The study protocol was approved by the ethical committee of Department of Pharmacy, University of Peshawar. The collected blood was centrifuged at 2000 × *g* for 15 min at 4 °C to separate the plasma and erythrocytes. The plasma and erythrocytes were stored at −80 °C until analysis. At the time of analysis the plasma was thawed at room temperature and spiked with appropriate concentrations of vitamin C and aminothiols to prepare their corresponding dilutions. An equal volume (10 μl) of internal standard (1 mg/ml) was added to each sample (10 ml tube) to make IS concentration 1 μg/ml in each sample. Following extraction the samples were analyzed and calibration curves were constructed for each analyte in their respective concentration ranges.

2.6. Liquid–liquid extraction from plasma and erythrocytes

Plasma (150 μl) and an equal volume of freshly prepared 10% meta-phosphoric acid solution (protein precipitant) were transferred to plastic Eppendorf tube (≈2 ml), and 50 μl internal standard solution (10 μg/ml of dopamine) was added. The mixture was vortexed for 2 min. Then mobile phase (150 μl) was added and centrifuged for 10 min at 14,000 rpm at 4 °C. After centrifugation the clear supernatant was separated, transferred to autosampler vial, followed by injection (5 μl) into HPLC system.

Similarly, the erythrocytes were lysed by freezing and thawing several times to ensure complete cell lysis. Then erythrocytes (150 μl) and an equal volume of freshly prepared 10% meta-phosphoric acid solution were transferred to plastic Eppendorf tube (≈2 ml), and 50 μl internal standard solution (10 μg/ml of dopamine) was added. The mixture was vortexed for 2 min. Then mobile phase (150 μl) was added and centrifuged for 10 min at 14,000 rpm at 4 °C. After centrifugation the clear supernatant was separated, transferred to autosampler vial and 5 μl sample was injected into HPLC system.

2.7. Chromatographic conditions optimizations

Various chromatographic conditions such as mobile phase composition, detector potential, flow rate, extraction solvent, internal standard, and column oven temperature were optimized for the analysis of ascorbic acid and aminothiols using RP-HPLC–ECD system in isocratic mode.

2.7.1. Detector potential optimization

First of all, working electrode potential of the detector was varied in the range of 500–1000 mV, for the selection of optimum potential to achieve better sensitivity of all the analytes. The voltammogram was constructed between applied potential and detector response. The optimum detector potential at which all analytes showed good peak response was selected for the evaluation of these analytes. The analysis was performed using both isocratic and gradient mobile phase comprised of 0.05% TFA and methanol (97: 3; v/v).

2.7.2. Mobile phase optimization

The composition of the mobile phase is very crucial because it significantly influences the peak response and retention of the analytes. The presence of an electrolyte such as TFA in the mobile phase is essential for effective electrochemical determination of thiol compounds. The effect of concentration of TFA as well as its strength on the retention and peak response was studied. Similarly organic solvents most commonly used for the thiol compounds determination include acetonitrile and methanol.

Various organic solvents like methanol, acetonitrile, n-hexane, tetrahydofurane (TFA) (0.05% and 0.1%) and various buffers including phosphate buffer (KH_2PO_4) in the range of 0.010–0.100 M and 50 mM NaH_2PO_4 in different compositions were applied both in isocratic and gradient mode for the analysis of thiols and ascorbic acid. The peak response of the studied compounds was recorded against all combinations of the mobile phase. The mobile phase composition that analyzed the target peaks in a shorter run time with greater sensitivity and peak resolution was selected.

2.7.3. Flow rate optimization

Flow rate of the mobile phase is another experimental parameter that markedly influences the elution of analytes. Thiol compounds were analyzed with different flow rates in the range of 0.500–0.900 ml min^{-1} and its effect on separation and peak shape was studied. The flow rate that showed greater sensitivity and good resolution of all target peaks was selected. The selected flow rate was then optimized under various chromatographic conditions.

2.7.4. Temperature optimization

The temperature of column oven and detector was varied in the range of 25–45 °C to study its effects on the chromatograms of analytes. The effects of temperature on the sensitivity, retention time, and peak resolution of all the compounds were studied from the chromatograms of these analytes. The changes observed in the chromatograms with respect to temperature were recorded.

2.7.5. Extraction solvent/procedure optimization

Aminothiols, ascorbic acid and dopamine (IS) were extracted from both plasma and erythrocytes using liquid–liquid extraction procedure. In liquid–liquid extraction various organic solvents including ethyl acetate (EA), dichloromethane (DCM), diethyl ether (DEE), acetonitrile, and a 50:50 mixture of DCM:DEE were used to obtain the maximum recovery of both analytes and IS. To plasma (100 μl) was added an equal volume of 10% metaphosphoric and spiked with appropriate volume of standard solutions equal to 0.5, 1.0, and 2.0 μg/ml of each analyte and 1 μg/ml IS. The samples were then vortexed, centrifuged, extracted and analyzed. The recoveries were determined at three concentration levels of each analyte. Similarly, liquid-phase extraction was applied to erythrocytes keeping the internal standard concentration 1 μg/ml and recoveries of analytes were determined.

2.7.6. Selection of internal standard

Different compounds were tried to be used as internal standard. Among all these compounds vitamin A palmitate, retinyl acetate, tocopherol acetate, naproxen sodium and dopamine were assessed to be used as an internal standard. Among these compounds the one that showed good resolution and instrumental response with the above mentioned analytes was selected as internal standard. Then dopamine was used as an internal standard in the present studies.

2.8. Method validation

The specificity, accuracy, precision, sensitivity, linearity, recovery, limits of detection (LOD) and limit of quantitation (LOQ), robustness, and stability of samples were evaluated in order to validate the developed method.

The specificity of the chromatographic method was evaluated by analysis of the analytes in mobile phase, blank plasma, and 1:1 mixture (containing 1 μg/ml of each analyte and internal standard) and plasma samples spiked with 1 μg/ml each of analyte and internal standard.

The accuracy of the method was determined by % recovery method. The % recoveries of the analytes were determined at three concentrations levels by spiking the plasma (250 μl) with appropriate concentration of each analyte and IS. Each sample was injected

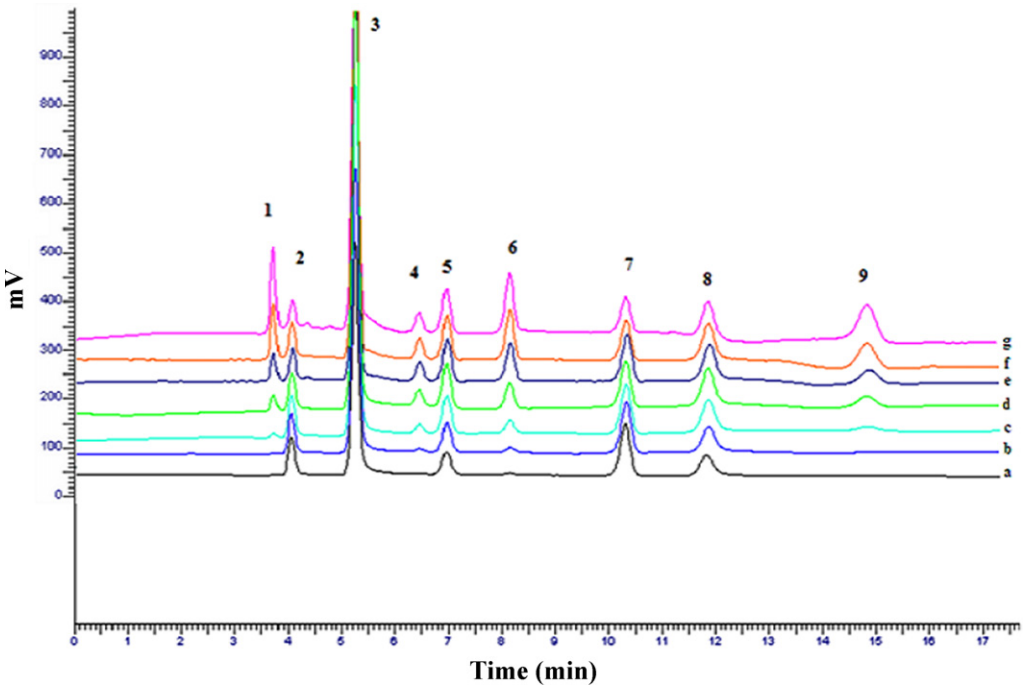

Fig. 1. Hydrodynamic voltammogram showing the effect of various applied detector electrode potentials in order of: a, 700; b, 750; c, 800; d, 850; e, 900; f, 950; and g, 1000 mV on the peak response of vitamin C and aminothiols. *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH) 6, methionine; 7, dopamine; 8, N-acetylcysteine; and 9, Glutathione oxidized (GSSG). The chromatograms were obtained using mobile phase 0.05% TFA:methanol in the ratio of 97:3 at a flow rate of 0.6 ml min^{-1} at column oven and detector temperature of 35 °C. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected.

in triplicate and recovery was determined according to the following equation:

$$\text{Recovery} = \frac{[\text{C}] \times 100}{[\text{A}] + [\text{B}]} \quad (1)$$

where, A = response ratio of the analyte with reference to IS in the mobile phase; B = response ratio of analyte with reference to IS in the control plasma; C = response ratio of the analyte with reference to IS in spiked plasma.

The linearity of the method was assessed from the calibration curves constructed at six concentration points of each analyte in the mobile phase and spiked plasma. Calibration curves were constructed by plotting the response ratios (ratios of peak areas of analytes to internal standard) versus concentration of each analyte using a linear least squares regression. The resulting plot slope (m), intercept (b), and correlation coefficient (r) were calculated from the regression equation using Microsoft Excel 2007.

Precision study was carried out on the basis of injection repeatability and analysis repeatability of spiked plasma samples. Injection repeatability was determined by repeated injection ($n = 10$) of plasma sample spiked with standard mixtures equivalent to 1 μg/ml of each analyte and internal standard into HPLC system. The retention time and peak area repeatability data obtained as mean, standard deviation (±SD) and covariance (% RSD), were expressed as a measure of precision of the method. Analysis repeatability was determined by analyzing plasma samples ($n = 5$) spiked with appropriate volume of standard mixtures equivalent to 1 μg/ml of each analyte and internal standard, prepared individually from same human plasma and the results were obtained as repeatability of recovered amount, expressed by mean, standard deviation (±SD), and covariance (% RSD).

The intra-day and inter-day variations were studied by analyzing plasma samples spiked with standard solutions of each analyte and IS (1 μg/ml), at 8:00, 16:00, and 24:00 h, for one week at alternate days. The results were expressed as mean, standard deviation (±SD), and covariance (% RSD). The recovered amounts were calculated in the form of concentration by the following equation:

$$C = \left(\frac{X}{Y}\right) \times \left(\frac{A}{B}\right) \times C_S \times F_D \quad (2)$$

where X and Y are peak areas of the analyte in plasma samples and 1:1 mixture, respectively; A and B are peak areas of the internal standard in 1:1 mixture and plasma samples, respectively; C_S is the concentration of analyte in the 1:1 mixture; and F_D is the dilution factor.

The sensitivity of the method was evaluated by quantifying the limit of detection (LOD) and limit of quantification (LOQ) for each analyte. The limit of detection (LOD) of the analyte is the concentration at which signal-to-noise ratio (S/N) is three and limit of quantification (LOQ) is the minimum concentration of analyte that can be determined at an acceptable precision and accuracy under rated conditions of analysis by a given method. For LOD and LOQ quantification dilutions of each analyte were prepared in the range of 0.1–30 ng/ml. The LOD was then determined from the peaks by the software at signal-to-noise ratio (S/N) of three, while LOQ was determined by measuring the analyte response with precision and accuracy of <20%, respectively.

The robustness/ruggedness of the reported method was assessed by bringing small deliberate changes in the various chromatographic conditions, like mobile phase composition (±2%), column oven temperature (±5 °C), and flow rate of mobile phase (0.2 ml min^{-1}).

Stability studies of standard stock solutions stored at 25 °C and 4 °C for one week were carried out. Similarly, the stability studies of spiked plasma samples stored at −20 °C were carried out for one week. Each sample was injected in triplicate and the % stability was calculated by the following equation:

$$\%\,\text{stability} = \frac{S_t}{S_0} \times 100 \quad (3)$$

where S_t is stability of analyte at time t, and S_0 is stability at initial time.

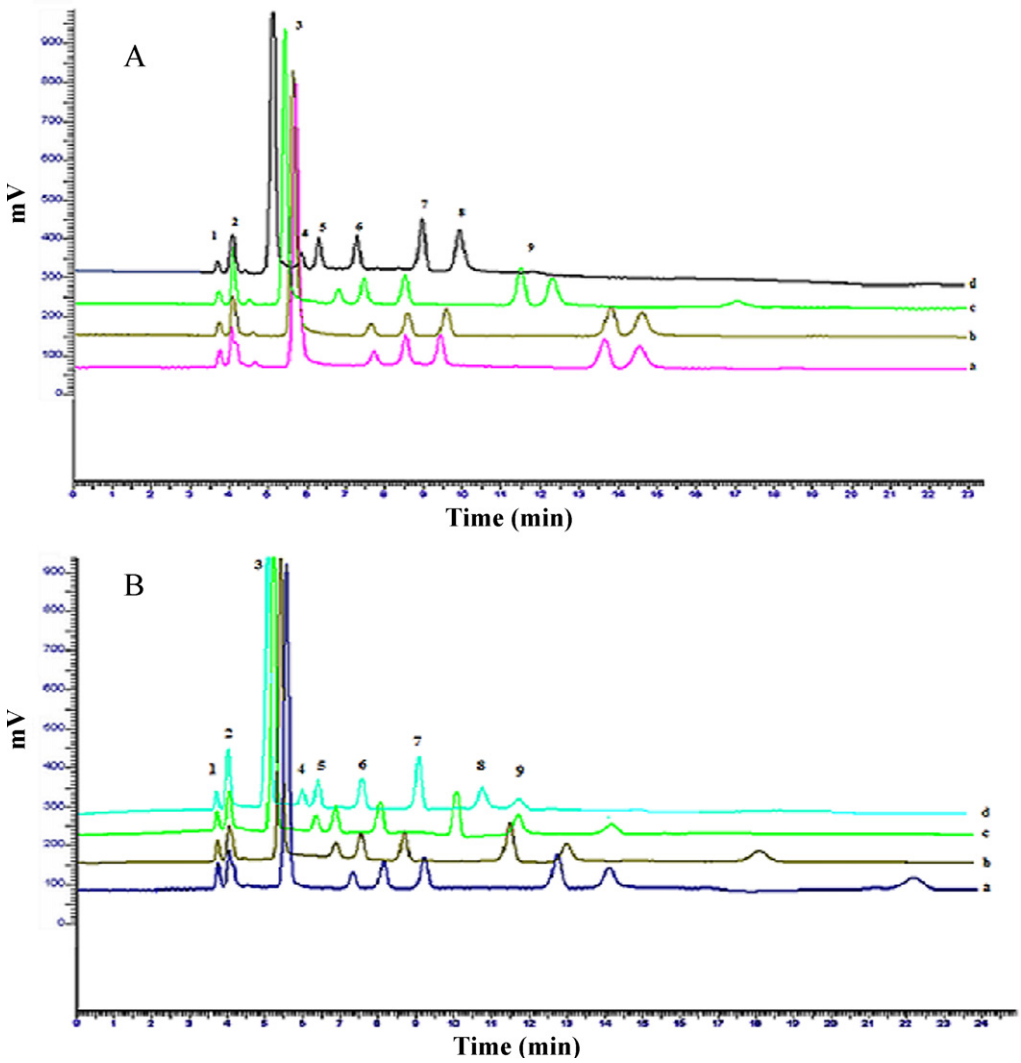

Fig. 2. (A) Influence of different 0.05% TFA:acetonitrile ratios (a, 99:1; b, 99:2; c, 99:3; d, 99:4) on the elution of studied compounds. The chromatograms were obtained using mobile phase 0.05% TFA:acetonitrile at a flow rate of 0.6 ml min^{-1} and at detector potential of 900 mV. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected. (B) Influence of different 0.05% TFA: methanol ratios (a, 99:1; b, 99:2; c, 99:3; d, 99:4) on the elution of studied compounds; *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; 9, glutathione oxidized (GSSG). The chromatograms were obtained using mobile phase 0.05% TFA:methanol at a flow rate of 0.6 ml min^{-1} at detector potential of 900 mV. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected.

3. Results and discussion

The present method developed for determination of aminothiols and ascorbic acid both in human plasma and erythrocytes using dopamine as an internal standard is rapid, robust and easy to automate. Complete separation of all target compounds and internal standard was achieved with good instrumental response. Some endogenous compounds have been co-extracted with the applied extraction procedure but none of these interfered with the target peaks. The various chromatographic conditions and experimental parameters were optimized and the method was validated in accordance with standard guidelines [37]. The suggested method is novel in the sense that simultaneous determination of ascorbic acid and seven aminothiols both in plasma and erythrocytes has been performed for the first time in a shorter analysis time using a simple method in comparison with other reported methods where fewer compounds have been determined in a single run with a longer analysis time in plasma alone using laborious, complex and expensive methods [7,8,30,31,38].

3.1. Optimization of chromatographic conditions and experimental parameters

The various experimental parameters and chromatographic conditions were optimized and the method was found suitable for the analysis of the above mentioned compounds in human plasma and erythrocytes.

3.1.1. Optimization of working electrode applied potential

The influence of working electrode potential on the current responses of all the analytes was recorded as shown in the full scan voltammogram of studied compounds (Fig. 1), keeping all other parameters constant. The optimum peak response was observed at 900 mV for all the compounds. The response of cysteine and dopamine decreased above this potential. The most suitable working electrode potential for simultaneous quantification of all the analytes was selected as 900 mV.

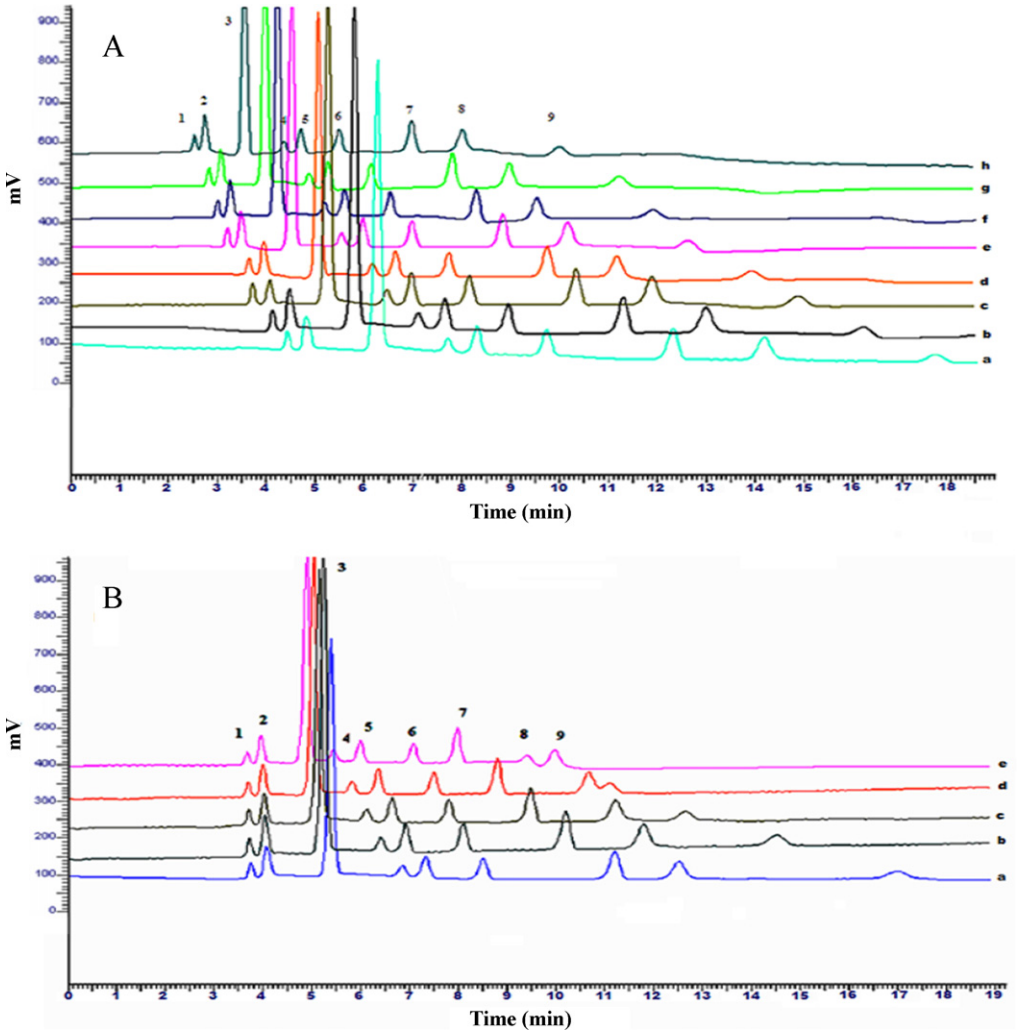

Fig. 3. (A) Effect of different flow rates (a, 500; b, 550; c, 600; d, 650; e, 700; f, 750; g, 800; and h, 900 ml min^{-1}) of mobile phase consisted from 0.05% TFA:methanol (97:3, v/v), on the elution of different analytes in order of: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; 9, glutathione oxidized (GSSG). (B) Effect of different column oven temperatures on the elution of studied compounds in order of: a, 25; b, 30; c, 35; d, 40; and e, 45 °C. *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; and 9, glutathione oxidized (GSSG). The chromatograms were obtained using mobile phase 0.05% TFA:methanol in the ratio of 97:3 at a flow rate of 0.6 ml min^{-1} at detector potential of 900 mV. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected.

3.1.2. Selection of mobile phase composition

Although symmetric and well resolved peaks of individual's compounds were obtained with mobile phase comprised of 0.05% (aq) (v/v) TFA and ACN in the ratio of 97:3, however peaks overlapping of dopamine and N-acetylcysteine were observed in the simultaneous run (Fig. 2A). Therefore acetonitrile was replaced with methanol in same ratio (97:3) in the mobile phase that resulted in well separated peaks of the studied compounds and sensitivities of Cys, GSH and GSSG were also increased (Fig. 2B). Therefore mobile phase consisted of 0.05% TFA and methanol (97:3) was selected the most optimum for chromatographic analysis of the studied compounds.

3.1.3. Selection of TFA concentration

Concentration of TFA in the mobile phase greatly affects the separation of thiol compounds. Changes in the retention times, peaks resolution and sensitivities of the mentioned compounds were observed although good separation was achieved at all concentrations. Peaks were broad and not well resolved at higher TFA (0.1%) concentration as compared with TFA concentration of 0.05%.

3.1.4. Effect of TFA and acetonitrile/methanol concentration

The 0.05% TFA and ACN/methanol ratios 99:1, 99:2, 99:3, 99:4, 99:5 were tested to show its effect on analysis of studied compounds. It was observed that peak widths decreased (up to 3% ACN/methanol content), then increased as shown in Fig. 2A and B, while retention times of methionine, dopamine, N-acetylcysteine, and GSSG changed significantly. Peak heights of the studied compounds increased up to 3% methanol content (Fig. 2B).

3.1.5. Effect of flow rate

The effect of mobile phase flow rate on the peak separation and resolution was studied as shown in Fig. 3A. Heights of the analyte peaks increased and their respective width and retention times decreased with increasing flow rate of the mobile phase, however, peaks of cystine and cysteine co-eluted and their resolution decreased at higher flow rates as indicated in Fig. 3A. Optimum separation and peak resolution of all the compounds were achieved at 0.6 ml min^{-1}.

3.1.6. Column oven and detector temperature optimization

Significant changes were observed in the chromatographic detection and separation of studied compounds with variation in

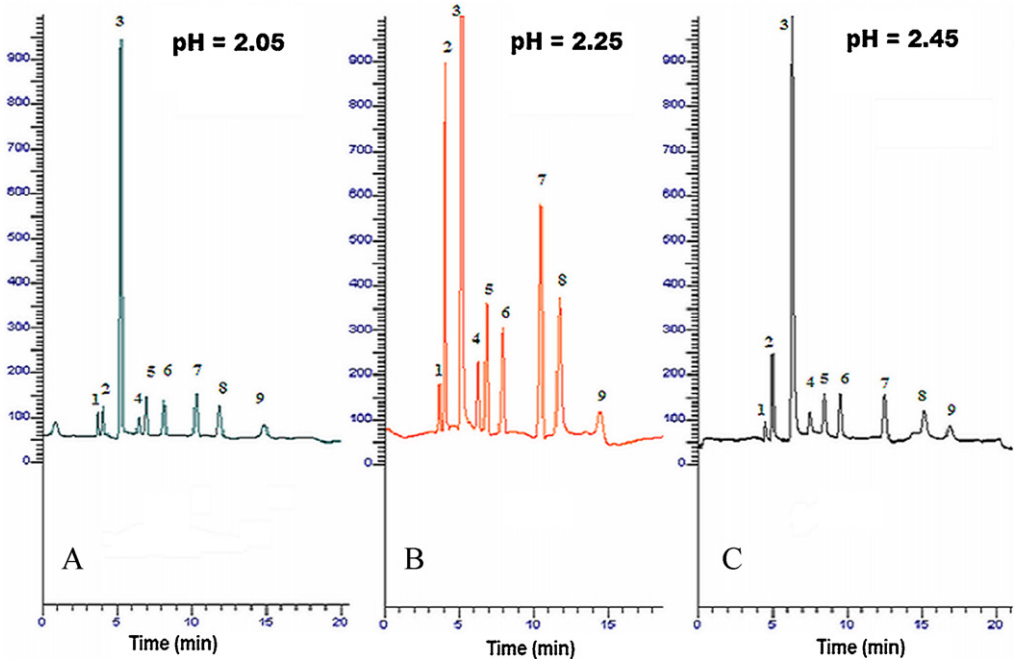

Fig. 4. Influence of various pH of mobile phase on the elution of different analytes. (A) pH 2.05; (B) pH 2.25; (C) pH 2.45. *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; 9, glutathione oxidized (GSSG). The chromatograms were obtained at detector potential of 900 mV using mobile phase 0.05% TFA:methanol in the ratio of 97:3 at a flow rate of 0.6 ml min^{-1} and at column oven and detector temperature of 35 °C.

the detector and column oven temperature. Maximal peak heights of the compounds were recorded at temperature of 40 °C, however peaks overlapping of N-acetylcysteine and GSSG occurred due to their co-elution as shown in Fig. 3B. For that reason column and detector temperature 35 °C was selected optimal for simultaneous determination of the studied compounds.

3.1.7. *Effect of pH on separation of ascorbic acid and thiols*

The pH affects the separation of thiol compounds and ascorbic acid significantly as shown in Fig. 4A–C. The resolution and sensitivity of all the analytes increased and their retention times decreased at pH 2.25 (Fig. 4B) as compared with pH 2.45 (Fig. 4C).

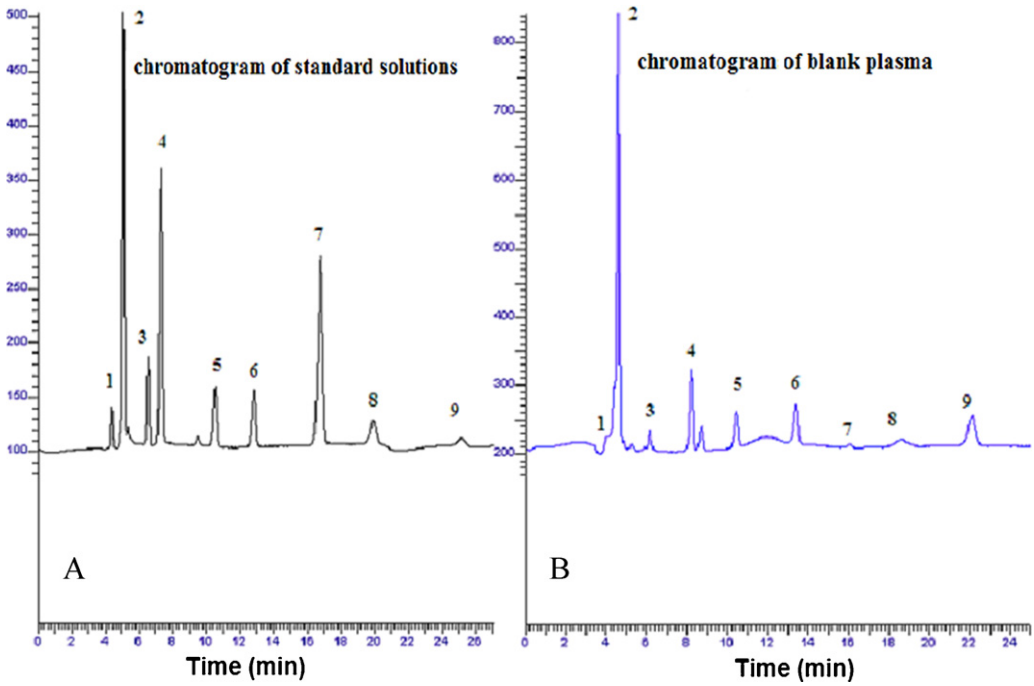

Fig. 5. Representative chromatograms showing: (A) standard solutions (1 μg/ml of each analyte and IS) and (B) blank plasma profile of the studied compounds. *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; 9, glutathione oxidized (GSSG). The chromatograms were obtained using mobile phase 0.05% TFA:methanol in the ratio of 97:3 at a flow rate of 0.6 ml min^{-1} at detector potential of 900 mV. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected.

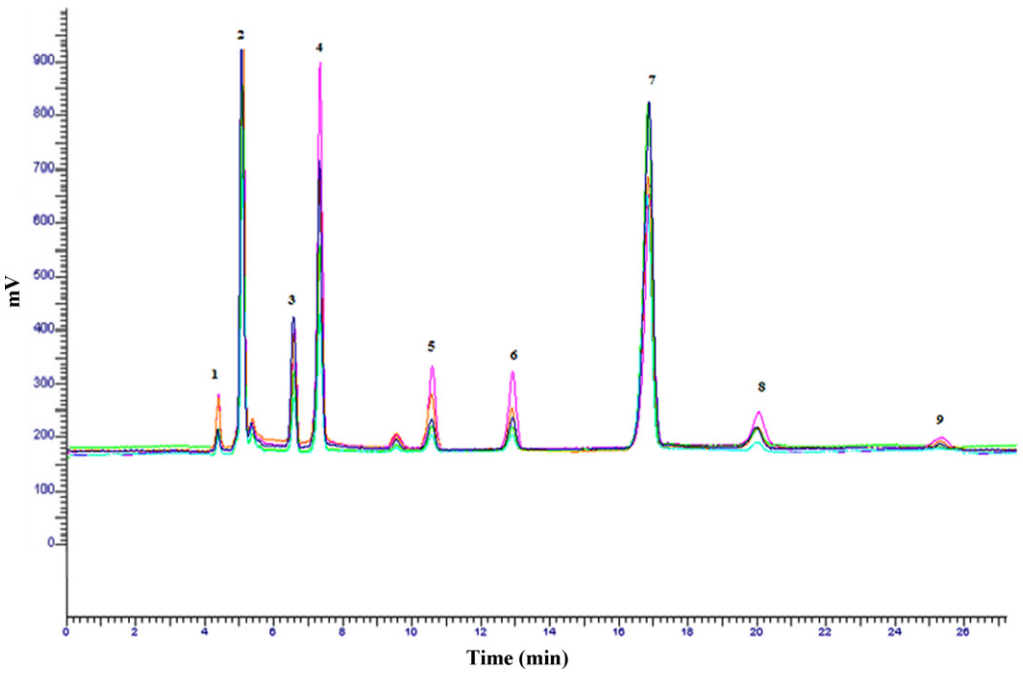

Fig. 6. Overlay chromatogram showing spiked plasma samples with various concentration of vitamin C and aminothiols. *Peaks*: 1, cystine; 2, cysteine; 3, vitamin C (ascorbic acid); 4, homocysteine; 5, glutathione reduced (GSH); 6, methionine; 7, dopamine; 8, N-acetylcysteine; 9, glutathione oxidized (GSSG). The chromatograms were obtained using mobile phase 0.05% TFA:acetonitrile at a flow rate of 0.6 ml min^{-1} and at detector potential of 900 mV. Concentration of all the analytes was 1 μg/ml and 5 μl sample was injected.

3.1.8. Internal standard selection/optimization

Internal standard was selected on the basis of its sensitivity, specificity, stability and compatibility with other analytes and extraction procedure. Retinyl acetate, retinyl palmitate, tocopherol acetate, naproxen sodium, and dopamine were evaluated as internal standard with aminothiols and ascorbic acid. Among all these compounds dopamine showed the better results in term of sensitivity, good recovery and reten-

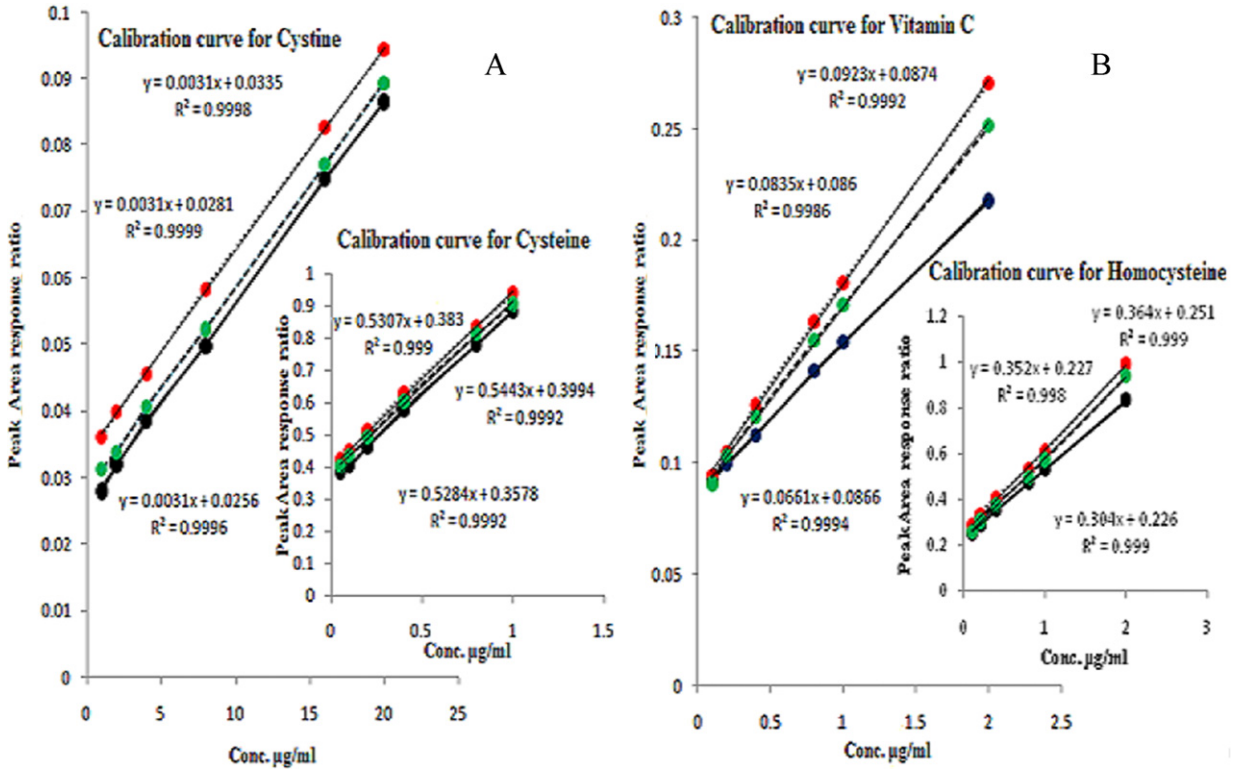

Fig. 7. (A) Calibration curves of cystine and cysteine. (B) Calibration curves of ascorbic acid (vitamin C), and homocysteine. Solid line (—) represents standard solutions, dotted line (. . .) represents spiked plasma samples, dashed line (- - -) represents spiked plasma samples corrected for the blank plasma. *Note:* Each point is a mean of triplicate injections.

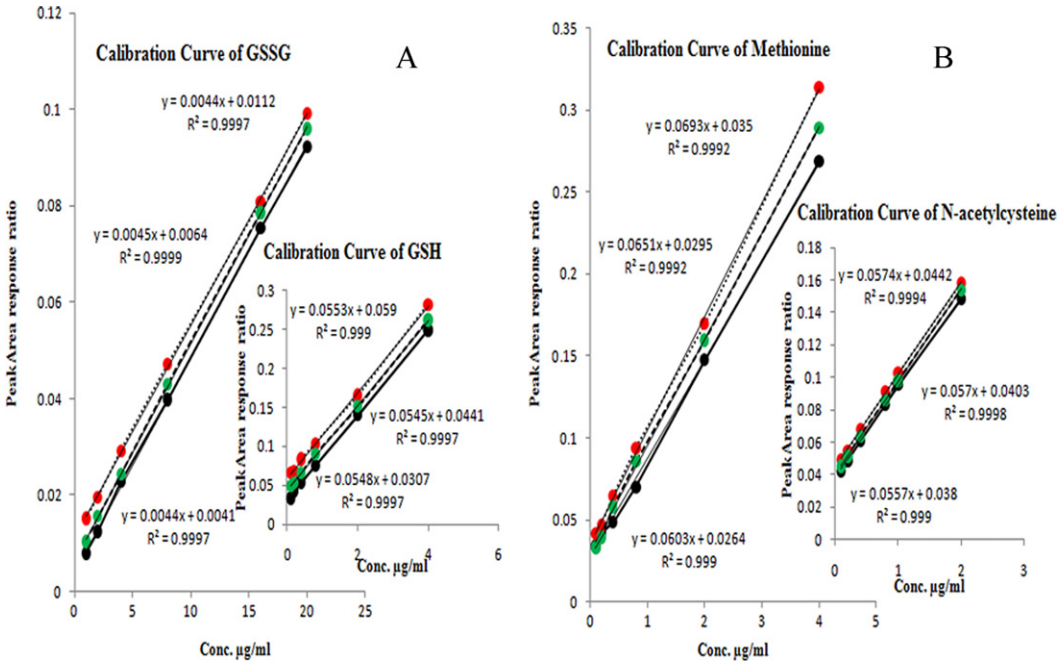

Fig. 8. (A) Calibration curves of glutathione oxidized (GSSG) and glutathione reduced (GSH). (B) Calibration curves of methionine and N-acetylcysteine (NAC). Solid line (—) represents standard solutions, dotted line (. . .) represents spiked plasma samples, dashed line (- - -) represents spiked plasma samples corrected for the blank plasma. *Note:* Each point is a mean of triplicate injections.

tion time compared with the other internal standards under study.

3.1.9. Extraction solvent/procedure optimization

Various solvents were tried for the extraction of aminothiols and ascorbic acid from plasma and erythrocytes as mentioned in above section, however the best recoveries of all the analytes were achieved with direct extraction method using 10% meta-phosphoric acid for deproteination and mobile phase as extraction solvent. To plasma and erythrocytes (150 μl) an equal volume of 10% meta-phosphoric acid was added for deproteination. The mixture was vortex-mixed and extracted with the mobile phase. After centrifugation at 14,000 rpm, for 10 min at 4 °C, the supernatant (5 μl) was injected into HPLC system.

3.2. Method validation

The method was validated by evaluating the linearity, precision, specificity, sensitivity, recovery, limits of detection and limits of quantitation, robustness, stability of solutions and system suitability parameters. Our laboratory results showed that the method is fully validated and accurate for the determination of aminothiols and ascorbic acid in plasma using electrochemical detection. Complete separation of the target peaks was achieved, by analyzing the standard solutions, blank plasma and spiked plasma samples. Representative chromatograms of 1:1 mixture (1 μg/ml of each analyte and internal standard), and blank plasma along with chromatograms overlay are shown in Figs. 5A, B and 6, respectively.

The calibration curves of thiols and ascorbic acid standard solutions, spiked plasma samples, and spiked plasma samples corrected for blank plasma constructed at six concentration levels show good linearity in their respective reported ranges as given in Table 1 (Figs. 7 and 8). Regression equation and correlation co-efficient (r) calculated from the calibration curves of standard solutions, spiked plasma samples, and spiked plasma samples corrected for blank plasma for the mentioned compounds are shown in Table 1.

Accuracy of the method determined on the basis of percent recovery at appropriate concentration, for all the analytes are shown in Table 1.

The repeatability (injection repeatability, analysis repeatability), and intermediate precision (intra-day, inter-day studies) are presented in Table 2.

Recoveries of all the analytes both from plasma and erythrocytes were measured at three concentration levels using liquid–liquid extraction. The % recoveries of all the analytes at their respective concentrations and the absolute recovery of the internal standard at a single concentration of 1 μg/ml are shown in Table 1.

Sensitivity of the method was determined on the basis of quantification of LOD and LOQ values of studied compounds. The LOD and LOQ values as presented in Table 1.

The stability studies showed that the standard solutions and spiked samples of studied compounds were stable for at least one week when stored at −20 °C, in comparison with room temperature 25 °C. The degradation rate of standard solutions stored at 25 °C was higher than the samples stored at 4 °C, and −20 °C, respectively. Standard solutions of ascorbic acid were more unstable as compared with other analytes. The % loss of standard solutions stored at 25 °C and 4 °C are shown in Table 3.

Method robustness evaluated by bringing minor changes in different chromatographic conditions such as mobile phase composition, column oven temperature, flow rate and detector voltage, resulted negligible changes in the peak area and retention time of the analytes.

4. Application of the method

Aminothiols and ascorbic acid in reduced, oxidized, and protein-bound form are the crucial components of body extracellular antioxidant defense system. The accurate evaluation of reduced:total ratios of plasma aminothiols and ascorbic acid will provide an indication that antioxidant intervention is necessary. The proposed optimized HPLC–ECD method was successfully applied for the quantification of ascorbic acid and aminothiols in

Table 1
Concentration range, linearity, accuracy, repeatability, recovery, and sensitivity of the developed method.

S.No.	Parameters	Cystine	Cysteine	Vitamin C	Homocysteine	GSH	Methionine	NAC	GSSG
1	Concentration range (μg/ml)	1–20	0.05–1	0.1–2	0.1–2	0.1–4	0.1–4	0.2–2	1–20
2 Linearity									
(a) Sandard solutions									
	Regression equation	$y = 0.003x + 0.034$	$y = 0.544x + 0.399$	$y = 0.066x + 0.087$	$y = 0.305x + 0.226$	$y = 0.055x + 0.031$	$y = 0.060x + 0.026$	$y = 0.056x + 0.038$	$y = 0.004x + 0.004$
	Correlation coefficient, r	0.9998	0.9992	0.9994	0.9996	0.9997	0.9990	0.9990	0.9997
(b) Spiked plasma samples									
	Regression equation	$y = 0.003x + 0.028$	$y = 0.531x + 0.383$	$y = 0.092x + 0.087$	$y = 0.352x + 0.227$	$y = 0.054x + 0.059$	$y = 0.069x + 0.035$	$y = 0.057x + 0.044$	$y = 0.004x + 0.011$
	Correlation coefficient, r	0.9999	0.999	0.9992	0.9987	0.9990	0.9992	0.9994	0.9997
(c) Corrected plasma samples									
	Regression equation	$y = 0.003x + 0.026$	$y = 0.528x + 0.358$	$y = 0.084x + 0.086$	$y = 0.365x + 0.252$	$y = 0.055x + 0.031$	$y = 0.065x + 0.030$	$y = 0.057x + 0.040$	$y = 0.005x + 0.006$
	Correlation coefficient, r	0.9996	0.9992	0.9986	0.9999	0.9997	0.9992	0.9998	0.9999
3 Accuracy									
	(% Recovery ± SD; % RSD)								
	0.25 μg/ml	92.83 ± 2.01; 2.16	95.92 ± 1.32; 1.38	100.21 ± 0.88; 0.88	95.88 ± 1.34; 1.39	98.61 ± 1.41; 1.43	99.22 ± 1.43; 1.44	95.80 ± 0.59; 0.61	92.28 ± 0.88; 0.95
	2.00 μg/ml	93.08 ± 1.00; 1.07	97.91 ± 1.42; 1.45	97.05 ± 0.32; 0.33	100.42 ± 1.48; 1.47	99.23 ± 0.87; 0.88	98.09 ± 1.70; 1.73	99.00 ± 0.41; 0.42	96.31 ± 1.64; 1.70
4 Recovery									
	(Recovery from plasma)								
	(Amount recovered ± SD;% RSD)								
	0.25 μg/ml	0.239 ± 0.006: 2.39	0.236 ± 0.007: 2.99	0.248 ± 0.002; 0.88	0.239 ± 0.004; 1.53	0.236 ± 0.001; 0.23	0.246 ± 0.003; 1.20	0.25 ± 0.002; 0.87	0.23 ± 0.003; 1.44
	0.50 μg/ml	0.465 ± 0.005: 1.07	0.499 ± 0.007: 1.44	0.488 ± 0.003; 0.66	0.491 ± 0.007; 1.47	0.496 ± 0.044; 0.88	0.461 ± 0.006; 1.39	0.49 ± 0.006; 1.17	0.48 ± 0.021; 4.39
	2.00 μg/ml	2.000 ± 0.019: 0.97	1.771 ± 0.030: 1.68	1.980 ± 0.016; 0.78	1.925 ± 0.033; 1.77	1.777 ± 0.078; 4.39	1.828 ± 0.053; 2.88	1.96 ± 0.031; 1.57	1.92 ± 0.044; 2.29
	(Recovery from erythrocytes)								
	2.00 μg/ml	1.982 ± 0.047; 2.37	1.846 ± 0.038; 2.05	1.863 ± 0.024; 1.28	1.795 ± 0.054; 3.00	1.82 ± 0.072; 3.94	1.96 ± 0.076; 3.86	1.86 ± 0.076; 4.10	1.88 ± 0.065; 3.45
5 Sensitivity									
	Limit of detection (ng/ml)	12	0.6	0.8	0.4	9	1	0.7	25
	Limit of quantification (ng/ml)	43	1	2	0.8	30	3.5	2	96

Table 2
Repeatability (injection repeatability and analysis repeatability) and Intermediate precision (intra-day and inter-day studies) performed for vitamin C (ascorbic acid) and aminothiols.

Precision studies									
Parameters	Cystine Mean ± SD; % CV	Cysteine Mean ± SD; % CV	Vitamin C Mean ± SD; % CV	Homocysteine Mean ± SD;% CV	GSH Mean ± SD; % CV	Methionine Mean ± SD; % CV	Dopamine Mean ± SD;% CV	NAC Mean ± SD; % CV	GSSG Mean ± SD;% CV
A. *Repeatability*									
a. Injection repeatability									
Retention time	4.08 ± 0.05; 1.33	4.61 ± 0.01; 0.18	5.76 ± 0.04; 0.69	6.36 ± 0.05; 0.76	8.72 ± 0.03; 0.32	10.50 ± 0.04; 0.41	13.30 ± 0.08; 0.57	15.36 ± 0.03; 0.19	18.45 ± 0.10; 0.53
Peak area (1 ug/ml)	73,972 ± 768; 1.0	1,518,442 ± 69,115; 4.6	2,745,341 ± 17,826; 2.4	944,206 ± 53,511; 5.7	369,066 ± 10,958; 3.0	289,026 ± 1198; 0.4	1,242,375 ± 7309; 0.6	228,085 ± 1002; 0.4	105,820 ± 4911; 4.6
b. Analysis repeatability									
Amount spiked	2 ug/ml	0.5 ug/ml	0.5 ug/ml	0.5 ug/ml	0.5 ug/ml	0.5 ug/ml	1 ug/ml	0.5 ug/ml	2 ug/ml
Amount recovered	1.91 ± 0.048; 2.51	0.47 ± 0.017; 3.69	0.49 ± 0.003; 0.51	0.49 ± 0.007; 1.49	0.48 ± 0.016; 3.38	0.45 ± 0.006; 1.38	0.88 ± 0.027; 3.08	0.48 ± 0.013; 2.72	1.88 ± 0.032; 1.68
B. *Intermediate precision*									
a. Intra-day studies									
0.5 μg/ml	0.45 ± 0.026; 5.78	0.45 ± 0.017; 3.72	0.48 ± 0.016; 3.43	0.45 ± 0.009; 2.01	0.46 ± 0.014; 3.00	0.46 ± 0.021; 4.55		0.43 ± 0.025; 5.89	0.45 ± 0.022; 4.87
1 μg/ml	0.94 ± 0.034; 3.64	0.90 ± 0.044; 4.89	0.83 ± 0.031; 3.70	0.87 ± 0.099; 11.40	0.76 ± 0.060; 7.90	0.94 ± 0.054; 5.75	0.96 ± 0.034; 3.55	0.88 ± 0.068; 7.72	0.86 ± 0.025; 2.90
2 μg/ml	1.89 ± 0.064; 3.37	1.50 ± 0.034; 2.06	1.57 ± 0.020; 1.29	1.92 ± 0.048; 2.49	1.69 ± 0.118; 7.01	1.85 ± 0.053; 2.87		1.64 ± 0.151; 9.20	± 0.035; 1.99
b. Inter-day studies									
0.5 μg/ml	0.38 ± 0.122; 31.83	0.36 ± 0.164; 45.59	0.41 ± 0.094; 22.90	0.39 ± 0.079; 20.38	0.41 ± 0.092; 22.54	0.40 ± 0.064; 16.30		0.33 ± 0.117; 35.77	0.31 ± 0.138; 44.31
1 μg/ml	0.84 ± 0.261; 30.96	0.73 ± 0.315; 42.95	0.83 ± 0.111; 13.42	0.65 ± 0.205; 31.74	0.70 ± 0.194; 27.50	0.77 ± 0.069; 9.05	0.85 ± 0.045; 5.32	0.83 ± 0.113; 13.52	0.68 ± 0.21; 31.05
2 μg/ml	1.69 ± 0.448; 26.46	1.25 ± 0.661; 53.03	1.36 ± 0.331; 24.29	1.42 ± 0.475; 33.38	1.61 ± 0.176; 10.94	1.83 ± 0.178; 9.76		1.55 ± 0.227; 14.67	1.57 ± 0.211; 13.46

Table 3
Stability studies of standard solutions of vitamin C and aminothiols performed at room temperature (25 °C) and refrigerator temperature (4 °C).

Standard solutions (2 μg/ml)	Retention time (min)	Room temperature (25 °C)			Refrigerator temperature (4 °C)		
		Peak area	Peak area	Percent loss	Peak area	Peak area	Percent loss
Cystine	4.1	1,022,812	142,752	86.04	1,022,812	442,752	56.71
Cyseine	4.6	2,818,120	1,529,989	45.70	2,818,120	1,929,989	31.51
Vitamin C	5.7	694,530	111,106	84.00	694,530	311,106	55.20
Homocysteine	6.3	2,660,479	1,463,243	45.00	2,660,479	186,3243	29.96
Glutathione reduced (GSH)	8.7	796,207	685,545	13.89	796,207	695,545	12.64
Methionine	10.4	836,456	451,565	46.01	836,456	651,565	22.10
Dopamine	13.4	319,0745	2718,223	14.80	3,190,745	2,918,223	8.54
N-acetyle cysteine (NAC)	15.7	472,635	334,219	29.28	472,635	384,219	18.70
Glutathione oxidized (GSSG)	18.4	194,275	154,835	20.30	194,275	164,835	15.15

Table 4
Plasma and Erythrocytes ascorbic acid and aminothiols profile of healthy volunteers ($n = 15$) using the developed method.

Analytes (μg/ml)	Human blood plasma ($n = 15$)			Human erythrocytes (RBCs) ($n = 15$)		
	Mean ± SD; %RSD	Minimum value	Maximum value	Mean ± SD; %rsd	Minimum value	Maximum value
Cystine	9.39 ± 2.25; 23.96	7.26	11.47	6.64 ± 0.80; 12.05	4.94	7.42
Cysteine	28.10 ± 3.62; 12.88	22.29	29.87	4.84 ± 0.57; 11.78	4.12	5.35
Ascorbic acid (Vitamin C)	4.29 ± 0.36; 8.39	3.28	5.07	6.24 ± 1.18; 18.91	4.67	7.04
Homocysteine	1.31 ± 0.62; 47.32	0.89	1.53	1.74 ± 0.36; 20.68	1.25	3.09
Glutathion reduced (GSH)	2.07 ± 0.87; 41.99	1.67	3.92	9.23 ± 1.93; 20.91	8.12	10.55
Methionine	5.74 ± 0.93; 16.20	3.76	6.58	4.91 ± 1.40; 28.51	3.13	5.19
N-acetylcysteine	0.73 ± 0.06; 08.22	0.35	1.67	1.50 ± 0.10; 06.61	0.82	1.95
Glutathion oxidized (GSSG)	0.88 ± 0.05; 05.68	0.54	1.34	9.06 ± 1.72; 18.98	7.98	10.43

human plasma and erythrocytes (Table 4). We analyzed the diluted samples of human plasma as well as erythrocytes and got the peak response of all the target compounds. This method is a part of biochemical analysis of blood samples collected from healthy human volunteers and patients with diabetes and cardiovascular diseases. This method will be applied for the assessment of oxidative stress through measuring plasma concentration of ascorbic acid and aminothiols in healthy volunteers and patients with diabetes and cardiovascular diseases. Since the ascorbic acid and aminothiols are quantified in the same sample simultaneously, the suggested method could be easily applied in clinical set up and basic research studies to determine the etiologies of various diseases and metabolism abnormalities. Similarly, the method can also be used as a tool to evaluate the mechanism and effectiveness of nutritional/dietary intervention strategies. For basic research applications, tissue aminothiol levels can be assessed to understand the abnormal one-carbon metabolism and homocysteine toxicities in oxidative stress related pathophysiological conditions such as cardiovascular, neurologic, renal disorders, hepatic diseases, and various vitamin deficiencies. The method can also be easily applied for the quantification of intracellular aminothiols in blood cells and tissue extracts.

5. Conclusion

The reported optimized and validated HPLC–ECD method for the determination of aminothiols and ascorbic acid in human plasma was rapid, simple, economical, accurate, sensitive, precise, selective and reproducible. The method was optimized using various chromatographic parameters and validated according to standard guidelines [37]. Various parameters such as mobile phase composition, stationary phase, flow rate, detector voltage, column oven and detector temperature, and internal standard were evaluated and selected on the basis of repeated trials. The selected method was also validated on the basis of specificity, sensitivity, linearity, stability, precision, recovery, robustness and system suitability. This is a suitable method for biochemical and molecularly biological study of aminothiols. This method has a potential value in the field of biochemistry and medicine. The aminothiols and ascorbic acid were well separated and detected in human plasma and erythrocytes using this method.

The suggested HPLC–ECD method is not only applicable for routine analyses, but will also be suitable for understanding the aminothiols metabolism abnormalities, improvement of diagnoses, interpretation of nutritional intervention, and mechanistically-based research studies. The method will provide a tool for understanding of abnormal one-carbon metabolism and will aid in the designing of new remedial strategies.

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