Quantitation of reduced glutathione and cysteine in human immunodeficiency virus-infected patients

Plasma viral load (VL) values and CD4⁺ cell count are employed clinically for initiation of therapy in the treatment of patients infected with human immunodeficiency virus (HIV), as previous clinical studies have shown a marked prevalence of acquired immunodeficiency syndrome (AIDS) development in seropositive individuals with VL values over 30,000 copies/mL. Many studies have shown that reduced glutathione (GSH) and cysteine (Cys) deficiency play an important role in the infection. We have developed capillary zone electrophoresis (CZE)-based assays and have used them to investigate the relationship between plasma and intracellular thiol levels and HIV-1 viremia in plasma. Blood samples from healthy volunteers and seropositive patients undergoing different antiretroviral regimes were analyzed in the study. The VL assay was based on CZE-UV detection of viral RNA at 260 nm. Determination of endogenous reduced Cys and GSH was achieved by CZE-UV detection of their mercurial complexes at 200 nm. We found that a decrease in GSH and Cys levels may be associated with disease progress. In fact, reduced GSH and Cys levels appear progressively reduced with increasing VL.

Keywords: Capillary electrophoresis / Cysteine / Glutathione / Human immunodeficiency virus / Viral load
DOI 10.1002/elps.200305848

1 Introduction

Plasma viral load (VL) values and CD4⁺ cell count are employed clinically for initiation of therapy in the treatment of patients infected with human immunodeficiency virus-positive (HIV+) patients. Previously, clinical studies have shown that HIV+ individuals with VL values over 30,000 copies/mL are at increased risk to develop acquired immunodeficiency syndrome (AIDS). However, the correlation between CD4⁺ cell count and VL values is not as easy to define. In fact, for a given CD4⁺ cell count range, associated VL values can change by two orders of magnitude.

G-glutamylcysteinylglycine (reduced glutathione, GSH) is involved in many biological processes [1, 2] and together with cysteine (Cys) may play an important role in the progress of HIV-related disease. Many studies have shown evidence of a GSH deficiency in HIV-infected patients: measured concentrations of GSH are in fact lower in plasma and peripheral blood mononuclear cells (PBMC) of HIV+ individuals [3–5]. In vitro studies have shown that low intracellular GSH levels impair T cell function [1], promote HIV expression [6] and nuclear factor kB (NF-kB) activation [7, 8], and increase sensitivity to tumor necrosis factor-induced cell death [9]. Clinical reports have shown that HIV-infected individuals with lower GSH levels have a poor survival rate. In fact, the probability that HIV-infected subjects will die within 2–3 years is dramatically higher when CD4 T cells have low GSH levels [10]. Survival is improved when GSH was replenished and maintained, which has led many researchers to evaluate sulfur supplementation as an experimental therapy to increase GSH levels in HIV+ patients. Additionally, increased Cys and related thiols in Molt-4 and U937 cells inhibit HIV-1 replication in persistently infected cells. Cys and N-acetyl cysteine (NAC) also inhibit NF-kB activity and chloramphenicol acetyl-transferase (CAT) gene expression under control of NF-kB binding sites in uninfected cells. This could suggest that GSH and Cys deficiency in HIV+ individuals may cause an overexpression of NF-kB-dependent genes enhancing HIV-1 replication [6]. Although the mechanism responsible for HIV+ related GSH deficiency has not yet been determined, a possible hypothesis is that a persistent increased oxidative stress leads to an accelerated rate of consumption of GSH not matched by an increased rate of synthesis.
A variety of methods for determining total and reduced thiols by capillary zone electrophoresis (CZE) have been reported [11–30]. Most of these studies use CZE interfaced with mass spectrometry (MS), electrochemical, or laser-induced fluorescence (LIF) detectors to enhance detection limits. Other studies have used Eilmann’s or other derivatizing agents to detect micromolar levels using UV to avoid more sophisticated or expensive instrumentation. In order to evaluate the relationship between sulfur loss and disease progress, and the effectiveness of sulfur supplementation therapies, a reliable and rapid (CE) assay to determine low plasma and intracellular GSH concentrations in clinical samples in HIV+ patients would be of interest. In this paper, rapid CZE methods for determining plasma VL and for the quantitation of GSH and Cys in erythrocytes and plasma have been developed, and/or optimized. The assays were applied to a preliminary investigation of the relationship between GSH and Cys levels and HIV-1 VL in HIV+ patients undergoing different treatment regimens.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade. L-Cys and L-GSH were purchased from Sigma (St. Louis, MO, USA). Stock solutions (5.0 mmol/L) were prepared by dissolving an appropriate amount of each thiol in dilute (1:10) running buffer. Solutions were stable for at least one week when kept frozen (−18°C) and protected from light. Working standard solutions were freshly prepared by diluting the corresponding stock solution in dilute (1:10) running buffer. A stock solution of 1000 ± 5 μg/L of inorganic Hg(II) prepared from HgCl₂ was purchased from BDH Laboratory Supplies (Poole, England). Working mercury solutions were prepared by serial dilutions of the stock solution, just before use. Chloroform-isoamyl alcohol mixture “Ready Red”, obtained from AppliGene, and 2 M sodium acetate, pH 4.0 were stored at 4°C. Extracting solution containing 5 M guanidinium thiocyanate (GdSCN), 0.1 M mercaptoethanol, 25 mM citrate, pH 7.0 was stored at +4°C. All the working solutions of Hg(II)/thiol complexes to be processed by CZE were prepared freshly in running buffer diluted 1:10. Thiol-containing samples were derivatized prior to the electrophoretic run by adding a threefold molar excess of Hg(II) over the total thiol concentrations found in plasma. Since the reaction of inorganic Hg(II) with sulfhydryl groups is extremely rapid at room temperature, no incubation time is required before measurements. All solutions and buffer systems were filtered through 0.22 μm filters (Millex; Millipore, Billerica, MA, USA) before electrophoretic injections. The running buffer for VL analysis was basic protein analysis buffer (30 mM borate, pH 8.30), obtained from Bio-Rad (Hercules, Ca, USA) and was used as supplied. Running buffer for thiol analysis was prepared by dissolving 40 mM NaH₂PO₄ (Aldrich Chemicals, Milwau-kee, WI, USA) in Milli-Q (Millipore, Bedford, MA, USA) water and adjusting to pH 2.3 with 1.0 M hydrochloric acid. Plasma samples with known HIV-1 and hepatitis C virus (HCV) VL were obtained from ProMedDX (Norton, MA, USA) and employed for CZE method optimization and calibration. Blood samples were obtained from seven healthy volunteers and 19 HIV+ infected patients undergoing different antiretroviral regimens. All were treated as described in Section 2.2. Milli-Q water was employed in all CZE operations while double-distilled sterile water was employed in all RNA procedures.

2.2 Sample preparation

Blood samples from volunteers were processed as described in previous studies [31]. In brief, venous whole blood samples were collected using chilled EDTA vacutainers and centrifuged at 1500 × g for 5 min at 4°C. The plasma layer was removed, treated with perchloric acid (PCA) to precipitate plasma proteins. The resulting solution was then stored at −70°C until used. The entire procedure was performed at 2–8°C to preserve GSH and Cys from oxidation.

2.2.1 Sample preparation for the determination of HIV-1 VL

All the plasma samples (10 mL) were treated according to the acid guanidinium phenol chloroform (AGPC) extraction protocol for viral RNA extraction (described below). After extraction the resulting solution was injected hydro-dynamically onto a polyacrylamide-coated capillary. Viral RNA concentration was determined by a calibration curve obtained using plasma samples with known viremia. AGPC extraction: extraction solution (5 mL) was added to each 1 mL of plasma and the mixture vortexed. 2 M sodium acetate pH 4.00 (500 μL), phenol (5 mL), chloroform-isoamyl alcohol (1 mL) were then added. After addition of each component the resulting solution was mixed without vortexing. The mixture was then incubated on ice for 30 min, vortex-mixed for 1 min, and incubated on ice for an additional 15 min. After incubation, the mixture was centrifuged at > 3000 × g for 20 min at 4°C. The aqueous phase was transferred to a fresh corex glass tube and isopropanol (5 mL) added. After vortex-mixing for 1 min, the mixture was stored at −30°C for 1 h and then centrifuged at > 3000 × g for 20 min at 4°C. The RNA pellet was washed with 70% ethanol, centrifuged at > 3000 × g for 20 min at 4°C, dried, and finally dissolved in the appropriate amount of water (100 μL).
2.2.2 Sample preparation for the determination of intracellular GSH level

After separation by centrifugation over Ficoll-Paque at 400 × g for 30 min at room temperature, erythrocytes were recovered from the bottom pellet, washed with isotonic solution and checked for viability and cell yield. Packed erythrocytes were then completely lysed by repeated alternate freezing at −70°C and thawing in a water bath at 60°C. Proteins were precipitated by adding an equal volume of 0.1 M trichloroacetic acid solution to the lysed cells, vortexing, and discarding the precipitate. The supernatant containing GSH was filtered through a 0.22 μm Millex filter and analyzed after suitable dilution with 1:10 running buffer. Calibration for GSH analysis by CZE was performed by the standard addition method, by using an uncoated capillary.

2.2.3 Sample preparation for the determination of plasmatic Cys and GSH levels

Plasma proteins were precipitated from plasma with a PCA solution containing EDTA. The supernatant was filtered through 0.22 μm Millex filter and stored at −80°C until analyzed by dilution with 1:10 running buffer. All samples were derivatized adding excess Hg(II) solution prior to the electrophoretic run. Since the detection of the complex is not affected by the presence of a large excess of Hg (we tested Hg:Thiol ratios from 1:2 to 5:1), unknown amounts of reduced thiols in a sample can be detected regardless of the stoichiometric ratio. The reaction of inorganic Hg(II) with sulfhydryl groups is also rapid at room temperature, thus no incubation time is required before measurements. Quantitative determinations by CZE of both the thiols (as Hg(Cys)_2 and Hg(GSH)_2 complexes at 6.5 and 4.8 min, respectively) were performed by the standard addition method, by using an uncoated capillary.

2.3 CE apparatus and operating conditions

A Bio-Rad Biofocus capillary electrophoresis system, equipped with UV-variable wavelength detector was used. Electropherograms were collected using Biofocus Integrator Software from Bio-Rad, and data were analyzed using Origin 6.0 Professional and MS Excel 2000 and XP software. CE of thiol complexes was performed using a 24 cm × 50 μm uncoated fused-silica capillary. Samples were introduced into the capillary by hydrodynamic injection at 5 psi for 1 s. Direct UV detection was employed at a wavelength of 260 nm. Direct UV detection was employed at a wavelength of 260 nm. All operations were conducted at 15°C, using a constant voltage of 18 kV. Before each run, the following preinjection procedure was carried out: 1 min purge with capillary wash solution, 0.5 min purge with Milli-Q water, 1.5 min purge with running buffer. Quantification was based on the average of at least five runs. VL calibration was accomplished by mean of the Food and Drug Administration (FDA) approved RT-PCR assay (Roche, Basel, Switzerland).

3 Results and discussion

3.1 HIV-1 viral RNA determination

Figure 1 shows the electropherogram of an infected plasma sample in which a single peak due to extracted viral RNA is present. The analyses of certified samples with known viremia due to HIV-1 and HCV were tested in order to verify the possible interference due to the HCV

![HIV-1 RNA](image)

Figure 1. Electropherogram of a plasma sample showing the peak due to HIV-1 RNA. \( t_{\text{mHIV-1RNA}} = 6.18 \) min. VL value obtained by extrapolation of the calibration curve: 19,023 copies/mL. Electrophoretic conditions: 50 μm × 36 cm Bio-Rad polyacrylamide-coated capillary cartridge; multi-wavelength UV detector, 260 nm; hydrodynamic injection, 5 psi · s on a 30 mm Bio-Rad basic protein analysis borate buffer, pH 8.5; constant voltage, 18 kV; temperature of the capillary and carousel compartment, 15°C.
RNA. Two well-separated peaks in the electropherograms (Fig. 2) show that the separation and quantification of HIV RNA is not affected by the presence of HCV RNA. Also, because of different molecular sizes [31] it is unlikely that other viral RNAs will affect HIV RNA determination.

In order to assess the reliability of CE for VL, plasma samples were evaluated for VL by the CZE method in tandem with the Roche Amplicor method. The CZE method for VL was calibrated by injecting processing standard infected plasmas with VL ranging from 3264 and 1721993 copies/mL. The calibration curve was prepared by plotting the integrated peak area vs. VL. The calibration parameters are reported in Table 1. Under these conditions, the CZE-based method has a limit of detection (LOD) of 50 copies/mL. Precision was evaluated by injecting a sample with known VL five times per day over a period of three days. The relative standard deviation (RSD) values of migration time and integrated peak area were 1.2–1.8% and 2.2–4.0%, respectively.

### 3.2 Quantification of thiols in erythrocytes and plasma

The CZE method has been developed for the determination of thiols (Cys and GSH) at low micromolar concentrations. While erythrocyte GSH is present at mM levels,
plasma GSH and Cys concentrations are in the micromolar range. For this reason, a novel procedure based on the derivatization of thiols with inorganic Hg prior to CZE analysis has been developed and optimized. The affinity of inorganic and organic Hg (Hg\(^{2+}\) and RHg\(^{+}\)) for thiols, such as GSH and Cys, is known to be very high in the pH range of 1–13 [32]. Inorganic Hg is highly water-soluble and reacts readily with thiols without requiring excess reagent or long incubations. Due to the high specificity for \(-\text{SH}\) groups, complexes, such as Hg(\text{RS})\(_2\), with a 1:2 stoichiometric ratio are formed [32–34]. During our preliminary studies, we have found that under our electrophoretic conditions the only complex formed is Hg(Thiol)\(_2\). In fact, when the Hg:Thiol ratio is larger than 1:2, the area and height of the electrophoretic peak of the complex do remain unchanged, regardless of the excess Hg added. Molar ratios of Hg:thiol ranging from 1:2 to 5:1 were tested in these preliminary experiments and no difference in the yield of the derivatization reaction was observed. Since excess Hg had no effect on the assay, a concentration of Hg that was 3-fold higher than the total thiol concentration normally found in the plasma was employed in all assays.

The detection and the determination of reduced thiols via their Hg complexes has the advantage of enhancing the sensitivity because the complex has a higher absorbance at 200 nm compared to that of the free thiol. Preliminary CZE experiments have been performed on standard solutions of Hg(Cys)\(_2\) and Hg(GSH)\(_2\) complexes in order to optimize the electrophoretic conditions, to improve peak shape and efficiency, and to avoid spikes that could affect detector response. The best results, in terms of reasonable migration time and efficiency, were obtained using 40 mM NaH\(_2\)PO\(_4\) buffer, adjusted to pH 2.3 with HCl.

In Table 1, calibration data for Cys, GSH, and their respective Hg(II) complexes are reported. Peak area and migration time showed a good reproducibility for both derivatized and undervatized thiols. In all the cases excellent linearity was achieved \((R^2 > 0.99)\). A lower LOD value of about one order of magnitude and a larger dynamic range for complexed than for the uncomplexed thiols were also found. The recoveries of organomercurial species in the spiked samples ranged between 97.1 and 99.5%. In addition, due to a lower charge, both Cys and GSH-Hg complexes migrate at a faster rate than the corresponding uncomplexed thiol.

To verify that prolonged storage at \(-80^\circ\text{C}\) was not affecting the availability of reduced thiols, recovery studies were performed. The results of these studies showed that when the centrifugation and separations were done at 4°C and the plasma sample was then immediately analyzed, the recovery was 99.1% for Cys and 98.3% for GSH; when the same plasma sample was frozen at \(-80^\circ\text{C}\) for 10 days, the recoveries were 98.5% for Cys and 98.0% for GSH.

### 3.3 Application to blood samples

After optimization of the method, calibration curves were generated for blood samples from healthy volunteers for intracellular GSH concentrations and plasma Cys and GSH concentrations (Table 1). Figure 3 shows the electropherogram that was obtained using a sample of lysates from human erythrocytes without derivatization. Based on calibration data (Table 1), the concentration of GSH in the sample injected was 0.126 mM, corresponding to an intracellular GSH concentration of 2.52 mM. The average intracellular GSH concentration for erythrocytes in all the samples examined was 2.65 ± 0.17 mM, in agreement with published data [15]. Because the GSH level in erythrocytes from healthy subjects is high enough, complex formation with Hg is not needed for detection. However, plasma GSH is found at very low concentration levels making complexation with Hg(II) necessary for detection.

![Figure 3. Electropherogram of an erythrocyte sample showing the peak due to intracellular GSH complex.](image)
The mean concentration of Cys and GSH in the plasma samples was $20.3 \pm 6.5$ and $5.9 \pm 1.8 \mu M$, respectively, well within the linear range for this assay.

### 3.4 GSH and Cys levels in HIV-1 infected patients

In both red blood cells and plasma Hg complexes of GSH and Cys are easy to detect even in a complex biological matrix. Since the LOD is lower with this assay than those obtained with other CZE UV-based systems, it is possible to monitor patients undergoing thiol depletion caused by a disease – like HIV. Although concentrations in the nanomolar range can only be detected by using LIF, the method herein described, unlike other CZE-UV and MEKC-UV methods, has adequate sensitivity to detect physiologic levels of GSH and Cys [35, 36].

Using the CZE-UV method we quantified the VL, GSH, and Cys in patients undergoing different therapeutic regimes and group of control subjects (Table 2). As indicated for patients being treated with various therapies, such as highly active antiretroviral therapy (HAART) and antiretroviral therapy (ART), there was an increase in plasma GSH and Cys compared to untreated patients. However, daily supplementation with 600 mg NAC increased GSH and Cys concentrations to the low-normal range, in agreement with published data [37]. This indicates that NAC supplementation as a potential sulfur replenishment medication should be further investigated. As expected, depletion of naturally occurring sulfur-containing compounds appears to be related to the disease progression. In fact, higher viral load values correspond to lower GSH and Cys availability, both intra- and extracellular (Fig. 5). The relationship linking GSH and Cys levels to VL is not understood and is beyond the scope of the present study.

In conclusion, CZE-UV methods for the determination of HIV-1 VL and intracellular and plasma thiol concentration have been developed. The methods were applied to the investigation of the relation between thiol levels and VL in HIV-infected patients, and the results obtained suggested an inverse relationship between thiols and viremia. The results in this paper show that CE can be considered as a useful and innovative tool for the modern infectious diseases facility.

The authors wish to thank Dr. Ann F. Fisher and Dr. John R. Petersen for their valuable comments and contribution.
Table 2. Results obtained testing a pool of patients undergoing different therapeutic regimes and control subjects

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<th>Therapya)</th>
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<th>Plasma Cys (µM)</th>
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VL, GSH and Cys values found, and the patient therapy are shown.
a) The legend HAART is employed for patients treated with zidovudine (AZT), didanosine and saquinavir, and the acronym ART for those taking AZT only (at the time when the samples were collected, some clinics were still employing AZT monotherapy in HIV patients).
b) Values obtained in tandem by Roche Amplicor and CZE methods.

Figure 5. Progressive thiol depletion with VL increase. (○) [GSH] in erythrocytes (mM); (X) [GSH] in plasma (mM); (△) [Cys] in plasma (mM). Coefficients of determination: $R^2_{pl.GSH} = 0.1936$; $R^2_{pl.Cys} = 0.0637$; $R^2_{ery.GSH} = 0.4319$. 

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4 References


