Simultaneous aminothiol determination in Down syndrome individuals using a modified HPLC method

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Submitted: 11 May. 2010; Revised: 25 Jul. 2010; Accepted: 31 Jul. 2010

Abstract
A modified high performance liquid chromatography method has been established for simultaneous measurement aminothiol levels in the plasma. This method is simple, precise and sensitive for the determination of total cysteine, cysteinyl-glycine, homocysteine and glutathione. Using this modified method, the simultaneous separation of aqueous standard calibration curves has shown high linearity over the analytical range. The different eluted aminothiol peaks have been distinctly separated without any interference related to the retention time of the analytes. Thiol status was examined in plasma prepared from 19 Down syndrome children and similar number of age and sex matched healthy control group. The average levels of the four aminothiols in the Down syndrome and the control groups have been significantly not different. This suggested that the total amounts of these aminothiols as such are not directly correlated to the Down syndrome phenotype.

Keywords: total aminothiols; HPLC; Down syndrome.

INTRODUCTION

Due to the association between the changes of different aminothiol levels in the body fluids and the maintenance and integrity of cellular systems (Benkova et al., 2008), the determination of aminothiols in biological samples has attracted great interest in the medical as well as the scientific fields. Numerous methods for measuring aminothiol levels in biological samples have been reported (Jones et al., 1998; Raggi et al., 1998; Reeve et al., 1980), but the overwhelming majority of the published methods have been validated for the determination of only one or two types of thiols at a time. In addition, each of these methods has its own basic limitations in terms of HPLC equipment type, cost, complexity, sample processing and run times.

Low molecular weight thiol-containing amino acids, such as homocysteine (Hcys), cysteine (Cys), cysteinyl-glycine (Cys-gly) and glutathione (GSH) have a facile electron-donating capacity linked to their sulfhydryl (−SH) group and substantially influence the cellular redox state and cellular capacity to detoxify toxic compounds, free radicals and reactive oxygen species (Kemp et al., 2008; Zhang et al., 2008). Furthermore, thiol oxidation, and thiol-disulfide equilibrium have important roles in cellular processes (Kemp et al., 2008; Kidd, 1997). Disorders in the metabolism of Cys can result in the precipitation of Cys causing cystinuria (Brauers et al., 2006) while the level of glutathione, reflects the oxidative stress status and its association with increased lipid peroxidation and aging (Chitre et al., 2008; Rebrin et al., 2008). Furthermore, moderate elevation in plasma Hcys levels became a useful clinical biomarker for deficiencies in folic acid, cardiovascular diseases and several inborn errors of folate or methionine metabolism (Cui et al., 2008; Freitas et al., 2008; Nekrassova et al., 2003; Stanislawksa-Sachadyn et al., 2008).

Individuals suffering from Down Syndrome (DS) are expected to be under oxidative stress; due to the imbalance in the production of large amounts of reactive oxygen free radicals that are not accompanied by equivalent increase in the production of the various cellular antioxidants (Sinet, 1982; Zitnanova et al., 2004). The role of biothiols in the protection of cells against the damage induced by free radicals has been reported (Włodek, 2002), thus, measurement of these compounds in DS may elucidate a clear interpretation of their clinical along with their abnormal features.
MATERIALS AND METHODS

Subjects

Nineteen DS children (ages between 5 and 16 years old), registered at "Almanar School for Thinking Development" have participated in this study along with an age and sex matched group of nineteen normal healthy control children. The work was conducted in accordance with the declaration of Helsinki. An informed consent was obtained from the parents of the children in all cases prior to blood collection.

Apparatus

The chromatographic system (Shimadzu, Japan) for HPLC analysis was consisted of a pump LC-10A DVP, UV-vis detector SPD-10 AVP, auto injector SIL-10A DVP, degasser DGV-12 A and system controller SCL-10 AVP. Data were analyzed by Shimadzu class-VP software Ver 6.12 SP4 and HPLC system connected to an IBM computer. Separations were achieved using LC-18 column (150×4.6 mm, 5 μm) (Thermo Hypersil Gold, USA), loop 20 μl.

Chemicals

Reduced L-glutathione, L-cysteine, cysteinyl – glycine, 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), homocysteine and all other reagents (analytical grade) were purchased from Sigma; dithiothreitol (DTT) and 5-sulfosalicylic acid dehydrate were purchased from Fluka (Switzerland); HPLC grade acetonitrile was purchased from GCC (UK).

Preparation of the standards

Daily fresh 100 ml standard stock solution was prepared with 750 μM of cysteine, 80 μM of cysteinyl – glycine, 45 μM of homocysteine and 30 μM of glutathione in deionized water. Five different serial working dilutions were then prepared from the previous standard stock solution to give five final concentrations (0.1×, 0.05×, 0.01×, 0.005× and 0.0005×) of the analytes standards. Stock of 10 mM DNTP was prepared by dissolving 39.6 mg of DNTP in 10 ml of 0.5 M K₂HPO₄ (pH 7.2). Phosphoric acid (7 M) solution was prepared by dissolving 39.6 mg of DNTP in 10 ml of 0.5 M K₂HPO₄ (pH 7.2). Phosphoric acid (7 M) solution was prepared by dissolving 39.6 mg of DNTP in 10 ml of 0.5 M K₂HPO₄ (pH 7.2). The separation of the standard analytes in the thiol chromatogram at pH 3.8 (Fig. 2). The eluted peaks have been distinctly separated without any interference related to the retention time of the different analytes. The retention times (minute) are 2.6, 3.4, 5.7 and 6.7 for Cys, Cys–gly, Hcys and GSH respectively. The upper and lower analytical ranges obtained by this method are similar to those reported earlier (Katrusiak et al., 2001).

The separation of the standard analytes in the thiol chromatogram at pH 3.8 (Fig. 2). The eluted peaks have been distinctly separated without any interference related to the retention time of the different analytes. The retention times (minute) are 2.6, 3.4, 5.7 and 6.7 for Cys, Cys–gly, Hcys and GSH respectively. The upper and lower analytical ranges obtained by this method are similar to those reported earlier (Katrusiak et al., 2001).

Preparation of plasma from blood samples

Plasma samples were prepared using equal volume of 9 % 5- sulfosalicylic acid dehydrate containing 0.2 mM EDTA followed by centrifugation at 10000 Xg for 5 minutes using Sigma centrifuge. The resulted supernatant was stored at – 60 °C until further analysis.

Analytical procedure

A total of 130 μl of the previously prepared clear supernatant or standards solution were used, and the followings were sequentially added: 0.5 ml of 0.5 M Tris–HCl buffer, pH 8.9 and 20 μl of 10 mM of DTT. After ten minutes of incubation at room temperature, 350 μl of 10 mM DTNB in 0.5 M K₂HPO₄ (pH 7.2) were added and the reaction tubes were incubated at room temperature for additional five minutes. The mixtures were then re-acidified by the addition of 50 μl of 7 M H₃PO₄ (Katrusiak et al., 2001). The separation was performed at room temperature after filtration through 0.25 μm sterile Millipore filters. The solution was injected into the HPLC through a 20 μl loop with a flow-rate of 1 ml/min. The mobile phase A and B were composed of a v/v mixture of 100 mM K₂HPO₄ pH 3.8 and mixed with either 6 % (mobile phase A) or 40 % (mobile phase B) of acetonitrile and the detection wavelength was 330 nm.

RESULTS

The simultaneous separation of aqueous standard curves of the four examined aminothiols showed high linearity over the analytical range, with goodness of fit higher than 0.99. Fig. 1 [Supplementary data] shows HPLC calibration curves of the aqueous standard mixtures of the studied aminothiols, which had a linear relationship between the different analyte concentrations (Y) and the peak areas (X). These curves show that the goodness of fit of individual aminothiols and their equation regression lines are respectively equal to: 0.999757, Y = 0.000264871X + 0 for Cys, 0.999519, Y = 0.000294764X+ 0 for Cys–gly, 0.997688, Y = 0.000274269X + 0 for Hcys 0.999815, Y = 0.000260598X + 0 for GSH.

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The average levels of the total aminothiols Cys, Cys-gly, Hcys and GSH in the plasma of DS group are 91.69, 11.39, 9.21, 11.86 μmole/L compared to 84.06, 11.61, 8.21, 3.51 μmole/L in the plasma of the control group respectively (Table 1 [Supplementary data]). The means of the levels of total aminothiols were significantly (P > 0.05) not different in the DS group from those of the controls.
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**DISCUSSION**

Several methods for the analysis of total aminothiols from different biological sources have been reported in the literature. Among the currently available methods is the derivatization of aminothiols with UV-absorbing reagents, which offers a readily accessible and highly reproducible HPLC method (Katrusiak et al., 2001; Raggi et al., 1998; Zhloba and Blashko, 2004). In this study, a modified method has been established to adapt the automated HPLC apparatus for usage in simultaneous measurements of the total levels of cysteine, cysteinyl–glycine, homocysteine and glutathione in the plasma. The method has been modified to adapt the available automated Shimadzu HPLC apparatus. This modification has included the use of LC-18 column (150×4.6 mm, 5 μm) instead of LC-18T column (15×4.6 mm, 3 μm) used earlier (Katrusiak et al., 2001). The mobile phases A and B have been achieved by 6 % acetonitrile (v/v, 100 mM KH₂PO₄, pH 3.8) and 40 % acetonitrile (v/v, 100 mM KH₂PO₄, pH 3.8) respectively and the incubation time with the reducing agent DTT has been extended to 10 minutes to assure complete reduction of the studied oxidized aminothiols. These modifications have been proved to result in a simple, sensitive, precise, fully validated and reproducible separation procedure for the total aminothiols measurement.

The adapted method was used to examine if there are any differences in the total levels of the four aminothiols in a group of DS individuals and an age and sex matched controls. The average levels of the four aminothiols in the DS and the control groups were significantly not different in the groups of DS and the controls. This indicated that the total amounts of these aminothiols as such are not directly related to the thiol redox equilibrium, therefore, the relative quantities of the reduced and oxidized forms of these aminothiols are the factors that are related to the redox equilibrium rather than the total amounts of these aminothiols. Thus, the ratio between the oxidized and the reduced status in particular could be the critical factor as an oxidative stress biomarker and worth to be studied in the future.

**Acknowledgement**

The authors would like to thank Mr. Faruq Zghol (Pharmaceutical Research Center, Jordan University of Science and Technology) for the technical assistance. This work was supported by a grant (Grant number: 20/2003) from Yarmouk University, Irbid, Jordan.

**References**


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