

Quantitation of Glutathione as a Urinary Autism Biomarker

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Overview

Autism cases have been rising in the last few decades. A Center for Disease Control report released in March 2012 reports 1 in 88 cases in 2008 up from 1 in 110 in 2006, with boys five times more likely than girls to have it. Are these increases due to a higher incidence, a better tracking system, increased number of parents getting their children tested, or a combination of all these factors? Theories have linked autism to genetics, infections, social conditions, exposures to toxicants, parents having children later in life, as well as vaccinations but there have been no substantial evidence proving any of these claims.

Currently, diagnosis is made via observation by medical practitioners. Early diagnosis is essential because children can receive physical therapy, speech therapy, and occupational therapy which all help in reducing their learning and social impairment and they can go on to lead normal lives. Currently children are diagnosed at age three with some as early as fifteen months. However, in most cases children are diagnosed late with the majority diagnosed between ages 4 and 5. At this stage they are not susceptible to treatment. It would then be beneficial to have a system of testing whereby diagnosis would be biological with early intervention occurring right after birth.

Introduction

Oxidative stress has been linked to autism as well as a number of other conditions including Parkinson's, Alzheimer's, Sickle cell, and cancer. Oxidative stress is an imbalance between the reactive oxygen species production and the body's ability to eliminate or repair the damage done. This causes an increased production of oxidizing species. To counteract this effect, the human body has a large production of antioxidants to protect itself against free radical damage by neutralizing them.

Glutathione is one such species which helps to maintain the reduced state of the body. It protects cells from toxicity by scavenging free radicals thus protecting cells from the toxic effects of reactive oxygen compounds. When cells are exposed to oxidative stress, there is an increase in oxidized glutathione, and as a result, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is decreased. This makes glutathione measurement very important as an indicator for oxidative stress, and why it is therefore being studied as a potential biomarker for autism.

Standards, controls, and samples were analyzed via ESI-LC-MS using a Thermo LCQ Advantage Ion Trap (San Jose, CA) via an auto sampler. Artificial urine was used for method development, followed by analysis of actual urine samples to determine the range at which GSH is present in autistic and control samples. X-calibur software and Excel were used to interpret data and create calibration curve plots for GSH quantitation.

Experimental

Materials:

- Reduced Glutathione (GSH) - Glutathione Reduced Ethyl Ester (GSH-Et)
- HPLC grade water - HPLC grade acetonitrile
- Artificial urine - Healthy adult urine

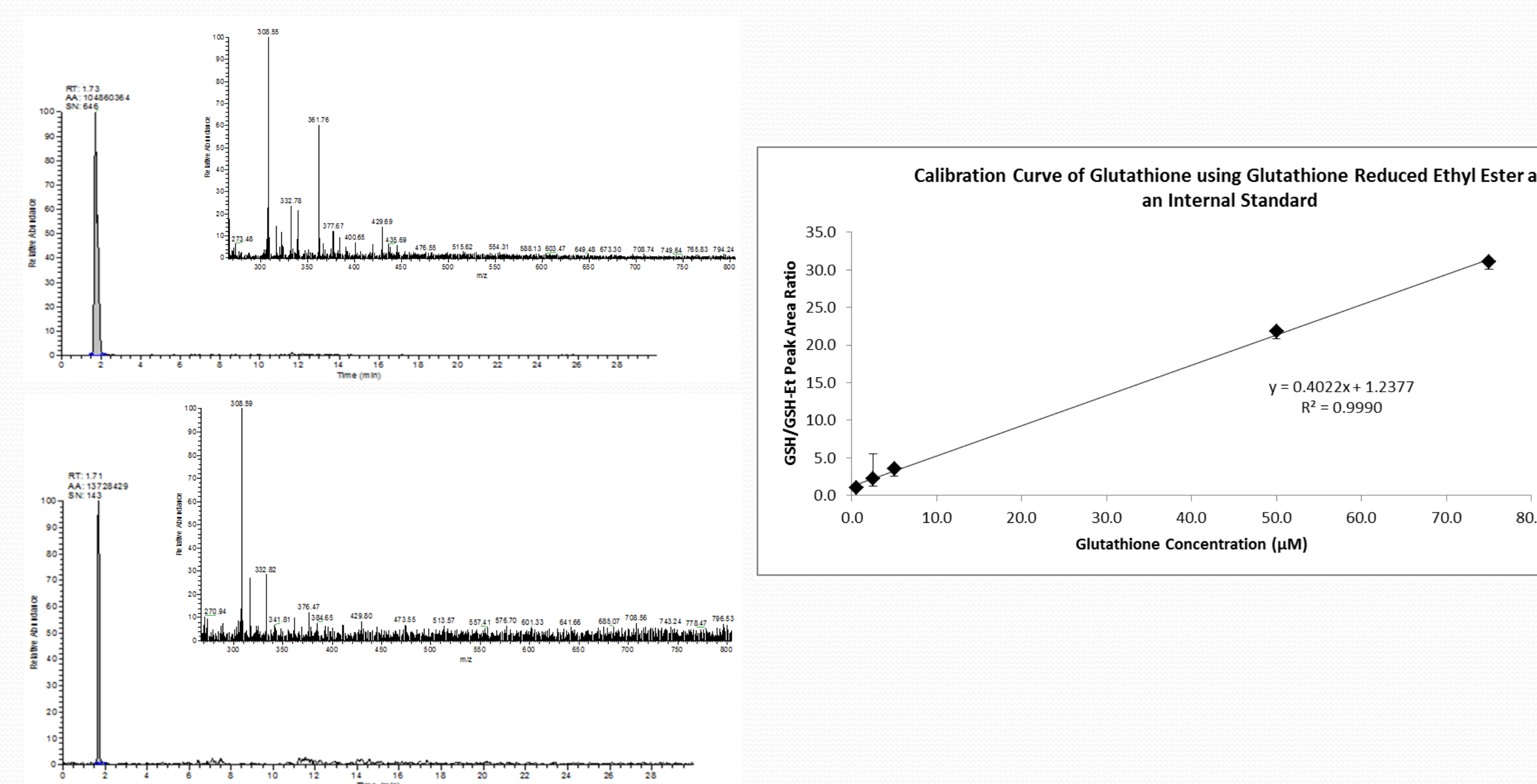
LC-MS

1. Urine samples are thawed to room temperature
2. 1.2ml is pipetted into a centrifuge tube
3. Sample is spiked with glutathione reduced ethyl ester
4. Sample is filtered through 0.45 μm membranes
5. 1ml of sample is decanted into a sample vial
6. Sample is then dried down and reconstituted in acetonitrile/water
7. 20 μL of sample is injected onto an LCQ Advantage Ion Trap (Thermo Scientific) via an auto sampler in positive ionization mode
8. A C18, 2.1 x 10mm online desalting column is used in line to the HPLC column and a C18, 3.9 x 150mm, 4 μM HPLC column is used for compound separation
9. Flow rate is 600 $\mu\text{L}/\text{min}$
10. Column and tray temperatures are 50 $^{\circ}\text{C}$
11. Gradient elution is used with mobile phase A (water with 0.3% formic acid) and mobile phase B (acetonitrile with 0.3% formic acid) as follows: 0 - 1 minute 1% B, 1 - 3 minutes 2% B, 3 - 9 minutes 2 - 45% B, 9 - 15 minutes 45 - 1% B with 10 minute column re-equilibration

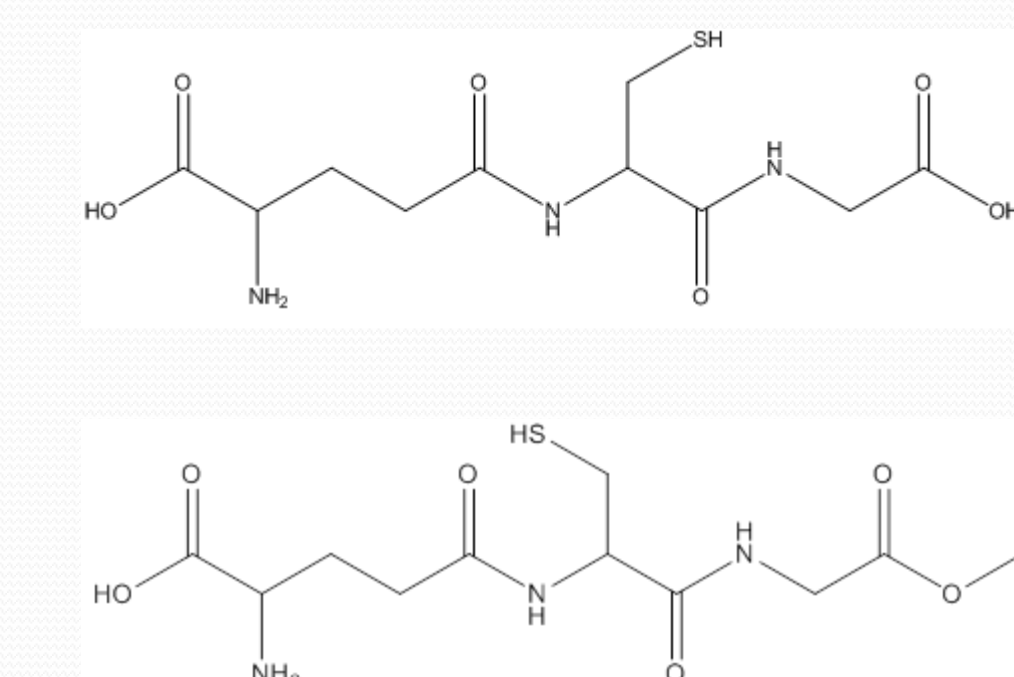
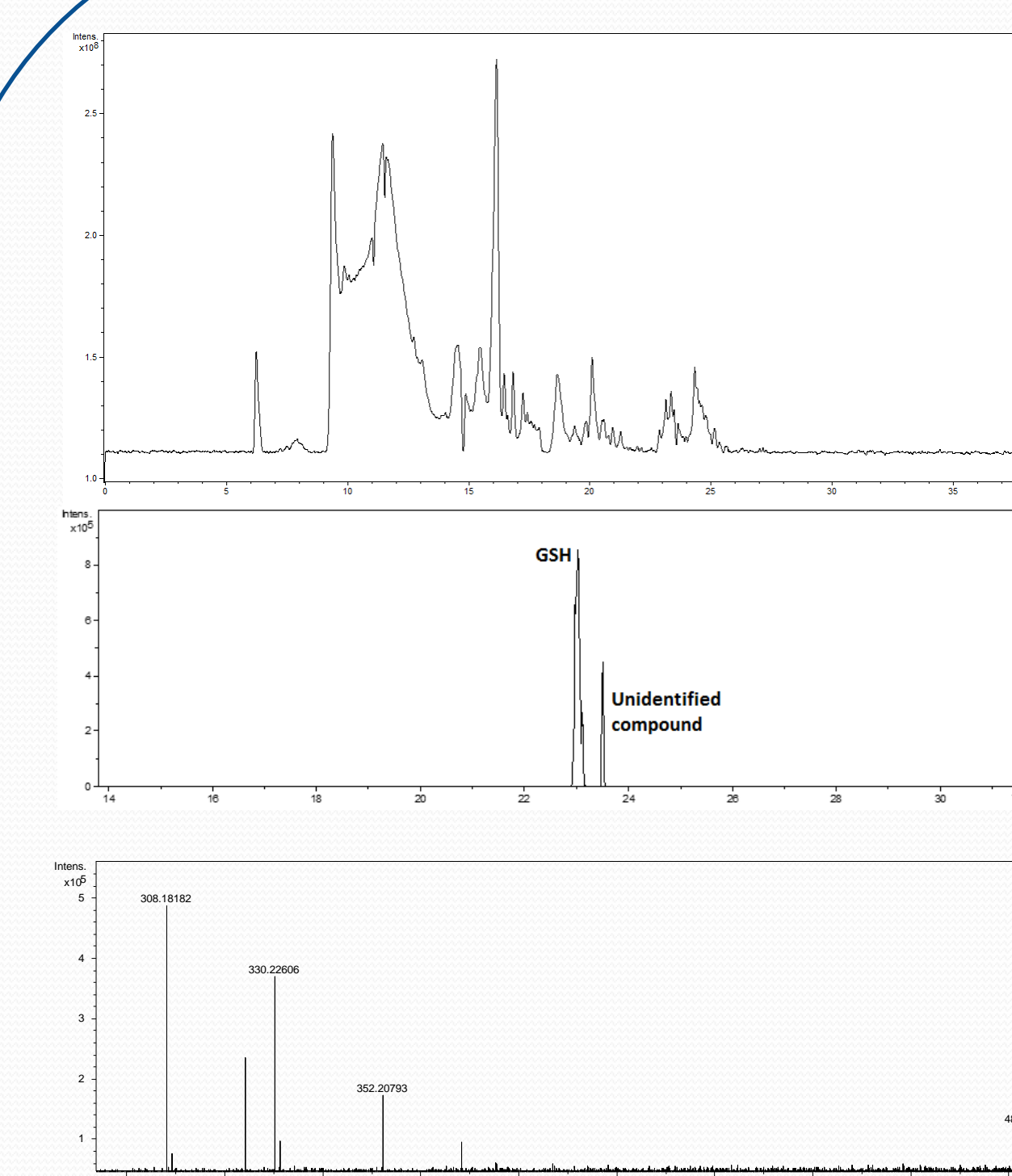
Nano-LC-MS

1. Similar sample preparation procedure as LC-MS steps 1 through 6 above
2. 1 μL of sample is injected onto a Dionex Acclaim[®] PepMap RSLC 75 μM x 15cm, C18, 2 μm , 100 \AA nano LC column for chromatographic separation with a Dionex Acclaim[®] PepMap 100 75 μM x 2cm, C18, 3 μm , 100 \AA trap column in line to trap particulates
3. Flow rate was 0.5 $\mu\text{L}/\text{min}$
4. Column temperature operated at room temperature
5. Tray temperature was operated at 4 $^{\circ}\text{C}$

Results



Extracted Ion Chromatogram of GSH analyzed using LC-MS: (Top Left) Artificial urine, (Bottom Left) Healthy adult urine. (Right) Calibration curve in artificial urine



Structures of (Top) GSH, (Bottom) GSH-Et

Healthy adult urine analyzed via high resolution nano-LC-MS. (Top) Total Ion Chromatogram, (Middle) Extracted Ion Chromatogram of GSH, (Bottom) Mass spectrum of GSH

Conclusion

It was shown that glutathione could be successfully detected and quantified in urine. There was no loss of sample following the filtration and desalting steps prior to eluent flow to the source.

3 autistic and 5 control samples were tested using LC-MS. The concentration averages of GSH for the autistic and control samples were 5.9nM and 7.0 μM respectively, showing that the autistic samples had depleted amounts of GSH in their urine compared to the control samples.

Accumulation of additional data from a larger sample pool is needed in order to more accurately determine biologically significant levels of GSH in both controls and autistics. High resolution nano-LC-MS is being utilized to probe for accurate mass as well as for further urinary autism biomarkers for additional quantitative studies.

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