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HPLC Determination Of Bioactive Sulfur Compounds, Amino Acids And Biogenic Amines In Biological Specimens

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Abstract

There is an increasing interest for analytical methods aimed to detect biological sulfur-containing amines, because of their involvement in human diseases and metabolic disorders. This work describes an improved HPLC method for the determination of sulfur containing amino acids and amines from different biological matrices. We optimized a pre-column derivatization procedure using dabsyl chloride, in which dabsylated products can be monitored spectrophotometrically at 460 nm. This method allows the simultaneous analysis of biogenic amines, amino acids and sulfo-amino compounds including carnosine, dopamine, epinephrine, glutathione, cysteine, taurine, lanthionine, and cystathionine in brain specimens, urines, plasma, and cell lysates. Moreover, the method is suitable for the study of physiological and non-physiological derivatives of taurine and glutathione such as hypotaurine, homotaurine, homocysteic acid and S-acetylglutathione. The present method displays good efficiency of derivatization, having the advantage to give rise to stable products compared to other derivatizing agents such as *o*-phthalaldehyde and dansyl chloride.

With this method, we provide a tool to study sulfur cycle from a metabolic point of view in relation to the pattern of biological amino-compounds, allowing researchers to get a complete scenario of organic sulfur and amino metabolism in tissues and cells.

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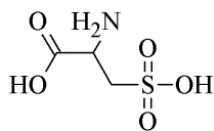
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Abbreviations: *HPLC*, High Performance Liquid Chromatography; *DABS*, 4-N,N-dimethylaminoazobenzene-4'-sulfonyl chloride; *DANS*, 5-dimethylaminonaphthalene-1-sulfonyl chloride; *OPA*, o-phthalaldehyde; *TDGA*, thiodiglycolic acid; *TCA*, trichloroacetic acid

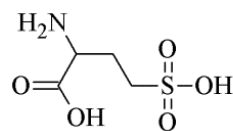
1 INTRODUCTION

Analysis of biogenic and sulfur-containing amines is becoming increasingly important in clinical and biochemical research (Curran et al. 2016). Taurine is a sulfur-containing β -amino acid present in different human body areas and is one of the most important and studied sulfurous organic bioactive molecules involved in human health (Jacobsen and Smith 1968). It is one of the end-products of cysteine metabolism and is excreted in urines. Taurine concentration in humans ranges from high millimolar in plasma, heart and brain, to micromolar amounts in tissue and body fluids such as urines (Schuller-Levis and Park 2003). Apart from its important physiological role as a neuroactive molecule (Wade et al. 1988), it displays a wide range of pharmacological effects including membrane stabilization, cytoprotective effects, antioxidant and anti-inflammatory action (Chaturvedi et al. 2015; Abdel-Moneim et al. 2015). In addition taurine is able to restore muscle function and performance in different pathological conditions (Chan-Palay et al. 1982a; Chan-Palay et al. 1982c; Chan-Palay et al. 1982b; De Luca et al. 2015). Recently, a number of studies have shown that taurine may have a beneficial effect against metabolic syndrome, preventing obesity regulating glucose metabolism and lowering cholesterol plasma concentration (Bai et al. 2016; Zhang et al. 2016b; Chen et al. 2016; Zhang et al. 2016a).

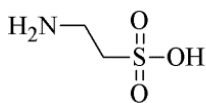
Moreover, many sulfurous amino compounds and taurine metabolic derivatives such as cysteic acid, homocysteic acid, hypotaurine and homotaurine (Fig. 1) have important biological roles (Jacobsen and Smith 1968) and researchers are in need of analytical methods for their accurate determination. Accumulation of sulfite, taurine, S-sulfocysteine and thiosulfate contributes to the severe neurological impairment in molybdenum cofactor deficiency, a severe autosomal recessive inborn error of metabolism (Atwal and Scaglia 2016).



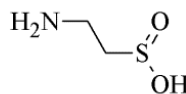
Cysteic acid



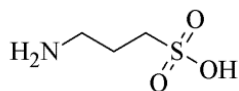
Homocysteic acid



Taurine



Hypotaurine



Homotaurine

Fig. 1. Taurine and other sulfur bioactive derivatives

For this purpose we developed a High Performance Liquid Chromatography (HPLC) analytical method for the determination of taurine, sulfur-containing molecules and other biogenic amines from different biological matrices. We improved a dabsyl chloride (DABS) pre-column derivatization method (Krause et al. 1995). DABS (4-N,N-dimethylaminoazobenzene-4' sulfonyl chloride), is an amine derivatizing agent (Fig. 2) able to give rise to stable products that can be easily monitored spectrophotometrically at 460 nm (Krause et al. 1995; Lin and Wang 1980).

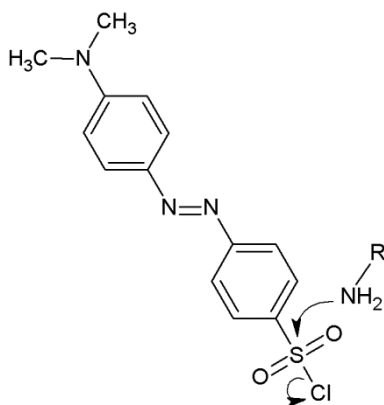


Fig. 2. Reaction of dabsyl chloride (DABS) with amines

2 MATERIALS AND METHODS

2.1 Chemicals

Amino acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gradient grade solvents used for chromatographic analyses were purchased from Carlo Erba Reagents (Milan, Italy). DABS was purchased from Supelco (595 North Harrison Road, Bellefonte, PA). All other reagents were analytical grade products from Sigma-Aldrich. S-Acetylglutathione (SAG) was purchased from GNOSIS S.p.A. (Desio, MB, Italy).

2.2 Derivatization procedure

The amino acid standards were dissolved in 0.1 M HCl containing 0.2% thiodiglycolic acid (TDGA) to prevent -SH oxidation. 1 μ L of amino acid standards at different concentrations were added to 14 μ L of reaction buffer (1M NaHCO₃, pH 8.6) and then to 25 μ L of 15 mM DABS in acetone. After vigorous vortexing, the standards were incubated at 40 °C for 30 minutes, vortexing at the first minute, twelfth minute and twenty-eighth minute. The resultant Dabsyl derivatives were put in an ice bath for five minutes and

then centrifuged at 14000g for 20 minutes. Supernatants were diluted 1:10 in mobile phase, filtered onto 0.2 µm filters, and then 50 µL were injected onto the column.

2.2.1 Treatment of plasma and urine samples

100 µL of fresh human plasma or urines were deproteinized by treatment with 100 µL of 10% TCA for 30 min at 4 °C and centrifuged at 14000g for 30 min at room temperature. The supernatant was then lyophilized. Dry material was resuspended in 15 µL of reaction buffer and 25 µL of 15 mM DABS and derivatized as described above.

2.2.2 Treatment of brain tissues and cultured cell samples

Selected mouse brain samples from either cortical or striatal regions (100 mg wet weight) and neuroblastoma cells (SH-SY5Y) pellet derived from 25 cm² flask were treated with 500 µL of 0.1 M HCl containing 0.2% TDGA, sonicated for 10 min (only for brain tissue), and then centrifuged at 14000g for 30 min. The supernatant was freeze-dried. 50 µL of reaction buffer and 100 µL of 15 mM DABS were added to the tube and derivatized as described above.

2.3 High-performance liquid chromatography (HPLC)

The apparatus consisted of a Waters HPLC 600 pump equipped with a controller, a Waters autosampler mod. 717 and a UV-Vis photodiode array detector mod. 2996. The chromatographic column was a reverse phase X-Bridge C18 column, 4.6 mm x 150 mm, 5 µm particle size, with a 10 mm guard column of the same material. Data analysis was performed using a dedicated application (Millennium³²). Elution was performed with a binary gradient system with sodium acetate (30 mM, pH 6.5) and acetonitrile in a ratio of 80:20 (v/v) (solvent A) and propan-2-ol with acetonitrile 50:50 (v/v)

(solvent B). The gradient was: 0-4 min, 5% B; 4-8 min, 20% B; 8-15 min, 25% B; 15-27 min, 60% B; 27-28 min, 100% B; 28-32 min, 100% B; 32-33 min, 5% B; 33-60 min, 5% B. The column was equilibrated for 20 min with 5% B at 1 mL/min and was maintained at 40 °C. Dabsylated products were monitored spectrophotometrically at 460 nm.

3 RESULTS AND DISCUSSION

A variety of pre-column amine derivatizing methods are reported in the literature, among them *o*-phthalaldehyde (OPA) and dansyl chloride (DANS) are the main derivatizing agents (Fig. 3) (Kang et al. 2006; Bertollini et al. 2012; Mou 1997).

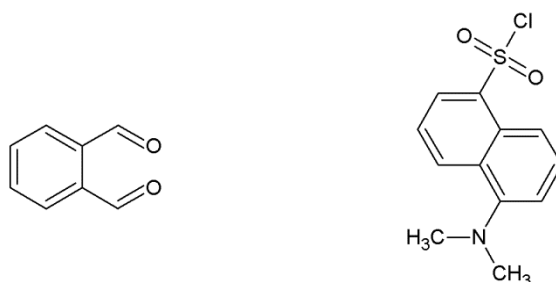


Fig. 3. Structures of OPA (left) and DANS (right)

OPA reacts in the presence of thiols specifically with primary amines above their isoelectric point. One of the limits of this procedure is that not all of the isoindolic fluorescent OPA derivatives are stable, moreover the analyses with this method could be hampered by interferences of unknown thiol moieties potentially present in biological samples. Thiols can displace the organic thiolic additive for the reaction generating different isoindolic adducts for the same investigated molecule (Kand'ar et al. 2007; Mopper and Delmas 1984).

DANS derivatization is a good versatile tool in terms of chromatographic separation and determination but the instability of the products and the need of a fluorimetric detector, as for OPA derivatization, makes this method inaccessible to a large number of research, commercial and clinical laboratories (Loukou and Zotou 2003). By contrast dabsyl chloride, 4-N,N-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS), is an amine derivatizing agent that provides a simple derivatization, good stability, good reproducibility and good analytical detection limit (Lin and Wang 1980; Krause et al. 1995). HPLC analyses with DABS show a good separation of a large number of amines and amino acids, which are detected in the visible region at 460 nm (Krause et al. 1995). DABS, like the other amine derivatization agents, can give rise to mono-dabsyl and bis-dabsyl derivatives in the presence of multiple amino groups groups. Furthermore with respect to OPA derivatization, DABS can react with primary and also with secondary amines.

In our experimental conditions the chromatographic method allows the separation and the analysis of a wide range of dabsylated amino-derivatives from different complex biological matrices such as plasma, brain tissue, urines and cell lysate samples. As shown in Fig. 4 the following molecules were analyzed in this elution order: Aspartate (Asp), Glutamate (Glut), reduced Glutathione (GSH), Serine (Ser), Threonine (Threo), Glycine (Gly), Hypotaurine (HTau), Taurine (Tau), γ -aminobutyric acid (GABA), Proline (Pro), Oxidized glutathione (GSSG), Methionine (Met), Phenylalanine (Phe), Lanthionine (LAN), Cystathionine (CYT), Cysteine (Cys), Carnosine (CAR), Dopamine (DPN) and Norepinefrine (NOR).

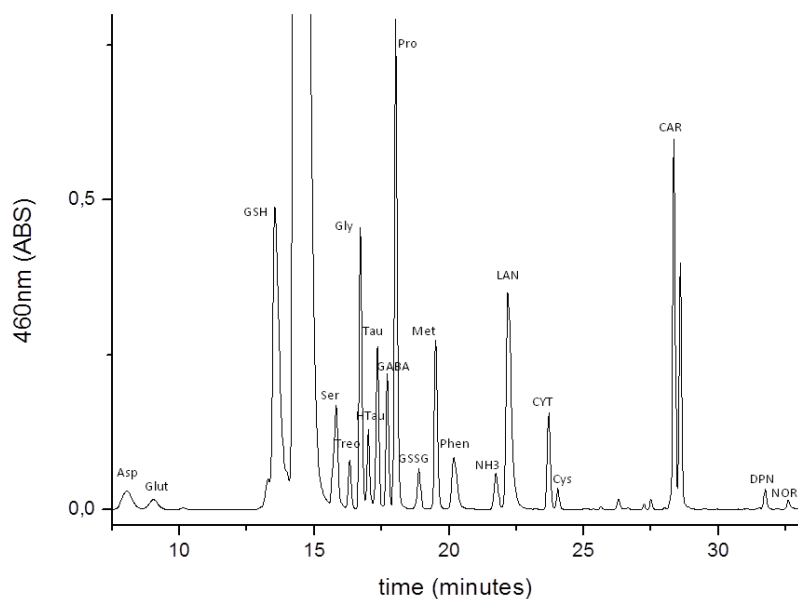


Fig. 4. HPLC chromatogram of selected dabsylated amines, amino acids and sulfurous amino compounds

Dabsylated amino acids and amphoteric molecules are eluted and separated according to their isoelectric point, from lower to higher value. DABS derivatization displays a good sensitivity (pmole level), linearity and efficiency of derivatization and has the important advantage of giving rise to stable derivatized products (24 h at 4 °C and at least 14 days at -20 °C) (Jansen et al. 1991; Kang et al. 2006; Krause et al. 1995).

With respect to other similar methods using DABS as a derivatizing agent, we introduced some important modifications of the procedure (Krause et al. 1995; Vendrell and Aviles 1986; Drnevich and Vary 1993).

Firstly, the derivatizing procedure was modified and the volumes of reaction were reduced to allow a better recovery of all the analytes from biological samples. The mobile phase was modified and no organic additives such as triethylamine or tetrahydrofuran were added to the hydrophilic phase (Solvent A, 30 mM sodium acetate pH 6.5 containing 20% CH₃CN). Many published methods in literature report the use of organic modifiers in the hydrophilic phase to avoid the interaction of the analytes with siloxan and silanol residues in non completely endcapped C18 stationary phases (Krause et al. 1995; Romero et al. 2000). In our setting the use of modifiers was not necessary because of the chemistry of the column. X-bridge columns have

replaced a certain number of siloxan groups with ethylene bridges reducing the hydrophilic interaction of the molecules with the stationary phase and improving the stability of the column. The ethylene bridge also reduces the number of free silanols, minimizing adverse interactions with the injected sample and improving both chromatographic resolution and peaks shape.

The second point is the separation of some strictly related compounds and acidic molecules that is strongly dependent on the pH of the mobile phase. In our setting it is important to maintain mobile phase at pH 6.5. Higher pH values lead to loss of chromatographic resolution for taurine and GABA, with concomitant improvement in aspartate and glutamate peak separation. Conversely, with lower pH values a more suitable separation of taurine and GABA is obtained, at the expenses of aspartate and glutamate peaks resolution. This could be explained by the increase of the total protonated forms of acidic compounds at lower pH values.

The third important factor that we want to underline is the condition of derivatization reaction with DABS. We set up our method on the basis of some considerations with respect to the basicity of the reaction environment and the temperature. We chose values of pH 8.6 and 40 °C for 30 minutes after a series of tests. Many papers report a reaction temperature of 70 °C with different reaction times. We tested the efficiency of different derivatizing reaction conditions by modifying reagents concentration, pH, temperature and reaction times, based on the chemical stability of the molecules of interests, and we found that some conditions are more appropriate than others for the detection of specific compounds. For example, our results indicated that for plasma samples it is necessary to increase the ionic strength of reaction buffer to guarantee the same pH value for all the samples. For the analysis of such biological samples (Fig. 5 and 6) after deproteinization with acid precipitants such as TCA, the use of a concentrated reaction buffer (0.5 to 1 M) is strongly recommended to ensure the buffering of the acidity.

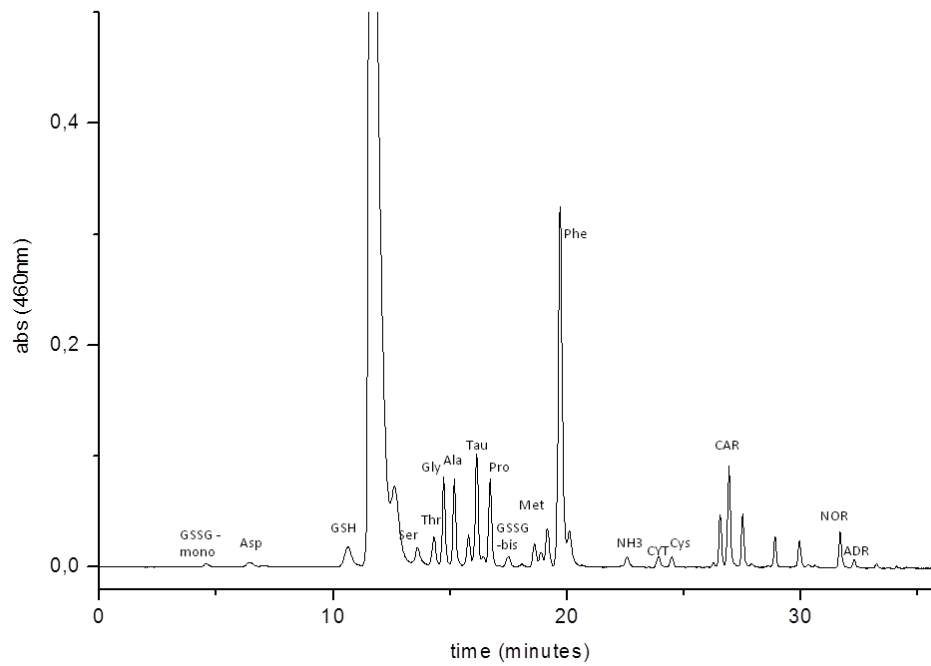


Fig. 5. HPLC chromatogram of dabsylated derivatives from human plasma

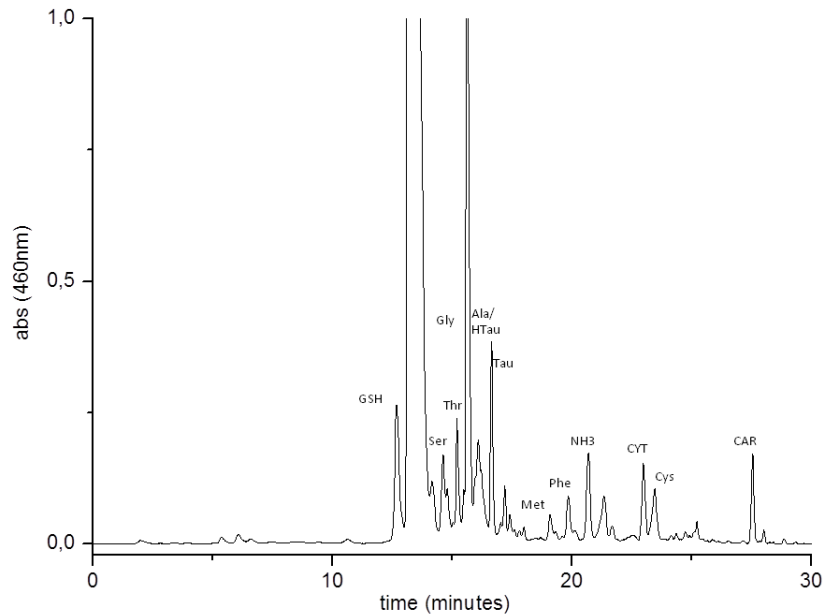


Fig. 6. HPLC chromatogram of dabsylated derivatives from human urine

As regards the pH of derivatization, we tested different reaction buffers (pH 8.0, 8.6 or 9.0). In our conditions the best derivatization efficiency of brain

tissue samples is obtained with a reaction buffer at pH 9.0, with a reaction time of 15 minutes at 70°C (Fig. 7).

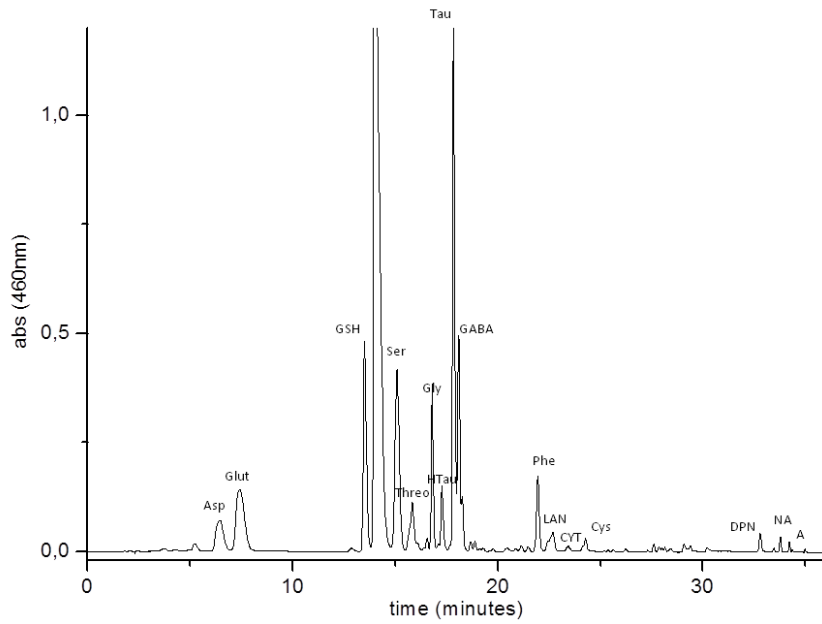


Fig. 7. HPLC chromatogram of dabsylated derivatives from brain tissue

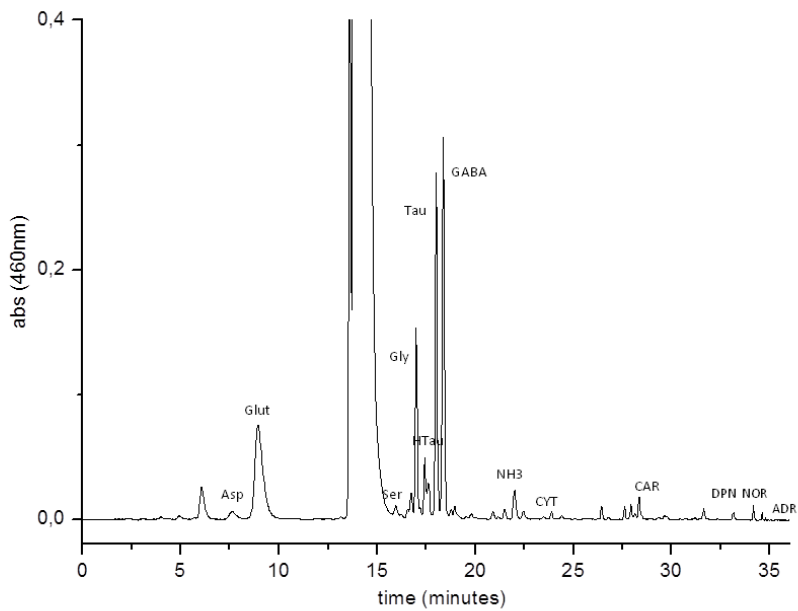


Fig. 8. HPLC chromatogram of dabsylated derivatives from neuroblastoma cell lysate

Same results can be obtained by lowering the pH of the buffer and increasing the time of derivatization at lower temperatures. It should be underlined that for some molecules such as catecholamines, pH 8.0 is more convenient because of the lability of these molecules and their tendency to be oxidized to form quinones with subsequent degradation or loss of derivatization. At variance, some authors reported the derivatization of these molecules on the –OH moiety at pH 11.5 by forming phenolate which acts as nucleophile (Cai et al. 2010). In our conditions, the derivatization efficiency of dopamine is strictly dependent on the pH value of the reaction buffer; indeed, at pH values higher than 9.0 the derivatization is not efficient probably because of the oxidation of dopamine to the *o*-quinonic form. For this reason in our final protocol we adopted a pH 8.6 for the reaction buffer.

The last parameters that we adjusted in the procedure of derivatization were temperature and time. We tested the efficiency and the effect of different reaction conditions on the deacetylation of an S-acetylated sulfo-amino derivative, the S-acetylglutathione (SAG) (Fig. 9). SAG is the thioester of GSH and its detection in biological specimens could be very important as regards the study of thioesters (Fig. 10).

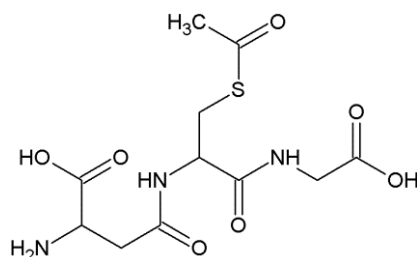


Fig. 9. S-acetylglutathione (SAG)

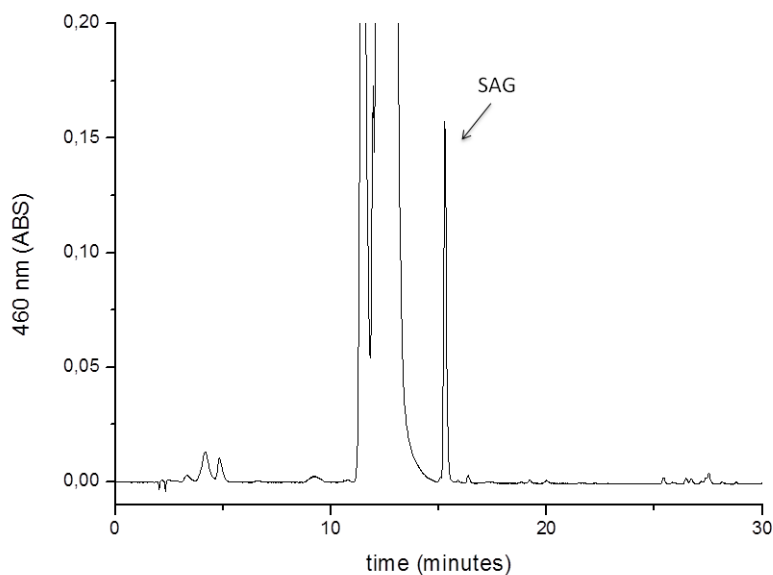


Fig. 10. HPLC chromatogram of dabsylated SAG

We tested different reaction conditions by analyzing the deacetylation of the molecule at different temperatures, pH and time of reaction. Dabsylation reaction carried out at pH 9 for 15 minutes at 70 °C gives a deacetylation of the thioester moiety of about 45%. Conversely, reaction conditions of 40 °C, 30 minutes and pH 8.6 reduces the extent of the deacetylation to about 8%. It is important to emphasize that reaction at pH values lower than 8.6 gives heterogeneous efficiencies of derivatization due to the fact that some amines have pKa values around 8.0. Hence, pH 8.6 is the best compromise that can ensure the homogeneous derivatization of all the studied amines and can minimize the degradation and the oxidation of labile molecules.

We also evaluated the derivatization of GSSG with DABS. As shown in Fig. 11, being GSSG a GSH dimer, its derivatization gives rise to the formation of two derivatives, the mono- and bis-dabsylated forms.

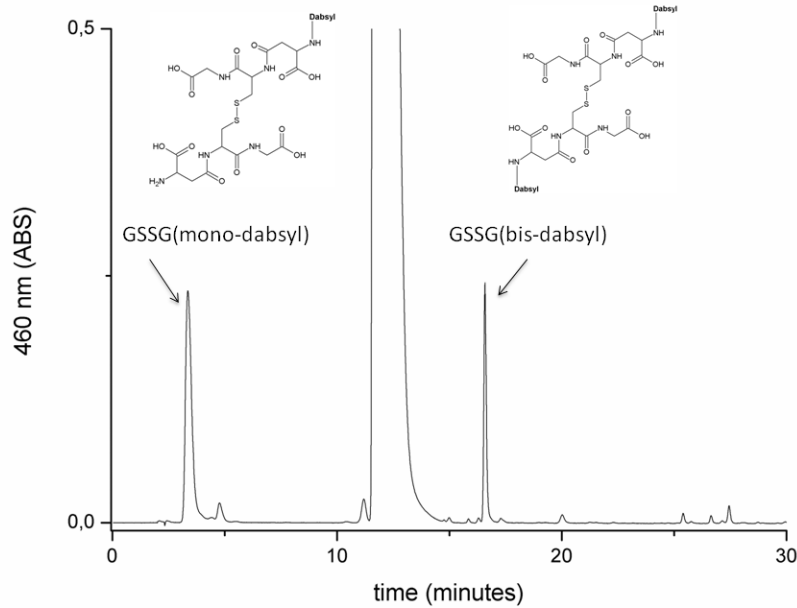


Fig. 11. HPLC chromatogram of Glutathione disulfide (GSSG)

Our data indicate that the dabsylation occurs in the same manner on the two amino groups and the rate of mono- and bis- dabsylated derivatives formation is constant in our reaction conditions.

As regards GSH and thiols in general, we used TDGA to protect thiol moieties from oxidation. The use of TDGA, together with the low temperature and the mild reaction conditions, allows the derivatization of GSH peptide to form a stable dabsyl derivative that can be monitored concomitantly with its bis-dabsylated oxidized form (GSSG) within the same chromatographic analysis. The simultaneous analysis of reduced/oxidized GSH forms, whose ratio is a useful index of oxidative stress (Zitka et al. 2012; Lakritz et al. 1997) enable the study of the redox state of cells or tissues.

GSH analysis shows the same linearity of derivatization and quantification as all the other studied amines, but it is important to underline that, when other thiols and sulfur-containing molecules are present in the derivatization process, some unknown byproducts are formed with a retention time that is strictly similar to GSH and in some cases not completely resolved from DABS peak.

We tested our chromatographic method by analyzing some important sulfur containing organic bioactive compounds. We analyzed simultaneously cysteic acid, homocysteic acid, hypotaurine, taurine and homotaurine (Fig. 12). As for GABA, taurine and homotaurine, resolution is improved at lower mobile phase pH, i.e. pH 6.45. This is due to the strictly related chemical structures and properties that homotaurine and GABA have.

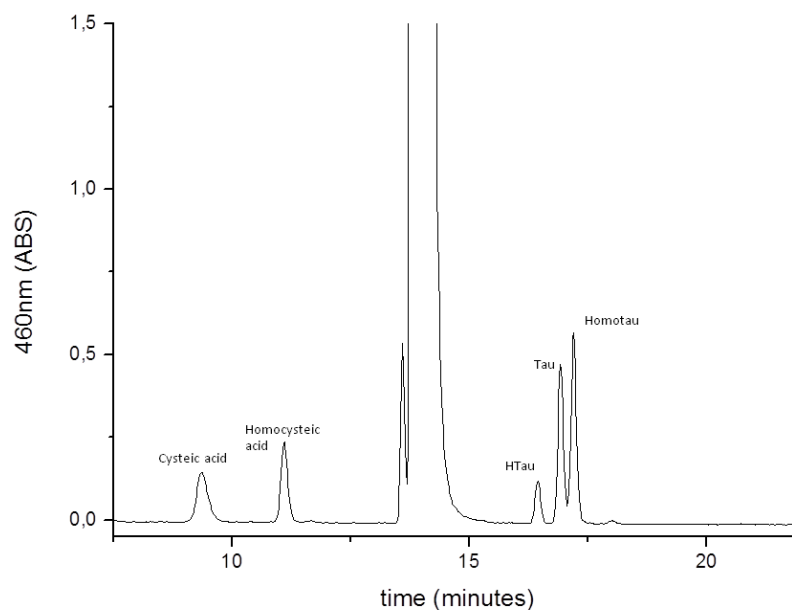


Fig. 12. HPLC chromatogram of Taurine and sulfur derivatives

As regards detectable sulfur-containing amines in the biological specimens analyzed, our data indicate that taurine relative content is higher in brain tissue samples with respect to all other biological samples analyzed. As described in the literature, the brain is one of the major body district in which taurine is present. Also in neuroblastoma cells the pattern of amino compounds (Fig. 8) reveals a relative content of taurine significantly higher with respect to other amines, whereas plasma and urine samples present lower relative amounts of taurine (Fig. 5 and 6).

It is well known that taurine is one of the end-products of cysteine metabolism. It is noteworthy, in brain tissues and neuroblastoma cells (Fig.

7 and 8) samples, we observed a lower amount of cysteine relative content along with higher amounts of taurine and hypotaurine, in agreement with cysteine metabolic cycle.

As regards the bioactive peptides carnosine and GSH, it can be noticed that urinary content of these two molecules is higher with respect to the other samples. GSH content in brain tissue samples is also relatively higher than in cells and in plasma, emphasizing the important role that this thiol tripeptide has at the cerebral level as a bioactive molecule.

4 CONCLUSION

The revised version of a relatively old analytical procedure, has enabled the improvement of a low-cost chromatographic method with high resolution and high diagnostic potential in order to investigate qualitatively and quantitatively many substances which can be implicated in the pathogenesis of several diseases.

This method represents a good tool to study sulfur biochemical cycle from a metabolic point of view in relation to the pattern of biologic amines and provides a complete scenario of organic sulfur and amino metabolism. It allows also the study of amine level variations in mice and human samples derived from different treatments or environmental conditions (pollutants, drugs, etc.).

The use of spectrophotometric detection makes this method accessible to a large number of research, commercial and clinical laboratories that do not have access to fluorimetric or mass spectrometric detectors. Apart from the obvious advantages of lower costs and larger number of samples that can be analyzed within a working day, more importantly, an undoubted advantage from a qualitative and methodological point of view is the possibility to simultaneously analyze in each single sample a large number of amines, aminoacids and sulfur-amino compounds including GSH, GSSG, cysteic acid, cysteine, taurine, hypotaurine, lanthionine and cystathionine.

In conclusion, the use of this chromatographic method on different complex biological matrices, could pave the way for a new diagnostic tool.

5 ACKNOWLEDGEMENTS

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