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HPLC-FLD-DAD CHARACTERISTICS OF THE S-NITROSOGLUTATHIONE DERIVATIVES OBTAINED BY O-PHTHALALDEHYDE

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S-nitrosothiols (RSNO) are considered to be natural depo and a transport form of nitric oxide (NO); their physiological activity is realized by releasing NO, which stimulates vasodilatation, prevents platelet aggregation and damages originating from ischemia/reperfusion injury. The main reservoir of non-protein RSNO is S-nitrosoglutathione (GSNO) created by S-nitrosylation of glutathione (GSH), most widespread endogenous non-protein thiol. The aim of this study was to set up an efficient GSNO synthesis and to examine the possibility of creating fluorescent derivatives of GSNO with o-phthalaldehyde (OPA), perform their spectroscopic characterization and behavior in the system of high performance liquid chromatography (HPLC). Synthesized GSNO and its OPA derivative were analyzed by HPLC system by using fluorescent (FLD) and "diode array" (DAD) detection. We developed the synthetic procedure by which GSNO was obtained in a solid, anhydrous form with a good yield. After UV characterization of the obtained GSNO, derivation of GSH and GSNO with OPA reagent, with or without mercaptoethanol (ME), was performed, and the derived products were analyzed by HPLC-FLD-DAD system. We optimized the reaction conditions that had lead to the formation of a stable and fluorescent tricyclic isoindole derivative of GSNO with OPA-ME reagent, which is essential for the development of HPLC method that would be used for the quantification of GSNO in real biological systems. *Acta Medica Medianae 2010;49(1):27-32.*

Key words: S-nitosoglutathione, HPLC, DAD, FLD, o-phthalaldehyde

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Introduction

Nitric oxide (NO) is produced *in vivo* from arginine by catalytic effect of NO-synthase. It represents an important intracellular signaling molecule. It participates in the regulation of numerous physiological processes in the body, and also acts as a mediator in many molecular mechanisms which cause pathological conditions (1, 2). It is a very reactive free radical. Since it is lyophilic, it easily diffuses through cell membranes and forms complexes with many 'target' molecules in almost all organ systems. There is a disagreement concerning the participation of NO in physiological and pathophysiological conditions. The type of chemical reaction it is subjected to, which depends on its flux and surrounding microenvironment, determines the role of NO as a signaling molecule or strong cytotoxin (3, 4).

NO participates in at least three basic metabolic routes. It reacts in the circulation with the heme from hemoglobin whereby nitrates and methemoglobin are produced. In redox reactions it is oxidated to nitrosium cation (NO^+) or is reduced to nitrosyl anion (NO^-), which results in the formation of several reactive types of nitrogen, including NO_2, N_2O_3 and/or peroxynitrite anion ($ONOO^-$). Its primary role is in the activation of the guanyl cyclase enzyme (sGC) by binding to the heme component of its active center thereby directly stimulating the emergence of the cyclic guanosine-monophosphate (cGMP), one of the most important mediators in the local and global cell signaling. The interaction between NO and sGC determines many biological functions of NO, first of all the relaxation of smooth muscles and vasodilatation, regulation of platelet aggregation, neurotransmission, etc. (5).

It is commonly believed today that compounds with thiol function (RSH) are the major targets of nitric oxide (6). Namely, nitrosonium cation (NO^+), obtained by oxidation of nitric oxide, may react with numerous nucleophiles such as amidic and carboxyl function, hydroxyl group, aromatic rings. However, in biological systems, the most significant reaction is with the thiols whereby S-nitrosothiols are produced (RSNO) (7). These compounds are considered to be natural depots and transport forms of nitric oxide. It has been proved that they participate in the control of NO flux through a cell, thus regulating the NO availability for numerous metabolic processes (8). According to their chemical structure, they are primarily the derivatives of amino acids, peptides and proteins

and the majority of them are produced endogenously, such as S-nitroso-albumin, S-nitrosocysteine and S-nitrosoglutathione (GSNO). Apart from that, the synthetic RSNO derivatives, such as S-Nitroso-N-acetylpenicillamine (SNAP) and S-nitroso-captopril, are produced for therapeutic purposes (9). These compounds achieve their pharmacological effect by releasing nitric oxide, thus stimulating vasodilatation, preventing platelet aggregation and damage originating from ischemia/reperfusion (10).

Due to its complex chemical structure, the biological effect of S-nitrosothiol may be connected with both heterolytic and homolytic cleavage mechanisms. Their biological activity causes the release of NO and/or the transfer of nitric oxide among different thiols by the process called transnitrosylation. It is believed that transnitrosylation is responsible for the activity of many RSNO *in vivo* (3).

S-nitrosoglutathione (GSNO) is the main reservoir of non-protein S-nitrosothiols and it is obtained by S-nitrosylation of glutathione (GSH) (10). Contrary to other nitric oxide donors, GSNO is an arterioselective vasodilatator and it can protect the brain by improving the cerebral circulation and reducing the inflammation during a stroke (11). Therefore, analogous compounds with similar or better therapeutic effectiveness without damaging side effects are needed (12).

Due to its important role and ambiguous mechanisms which are at the basis of its origin and function, it is necessary to provide a sensitive and specific method for determining GSNO in physiological samples. The GSNO standard is a prerequisite for developing a solid analytical method. However, since it is chemically unstable, it should be prepared fresh and in sufficient amount using a quick and reliable synthetic procedure. The aim of this procedure was to optimize the reaction conditions of S-nitrosoglutathione synthesis, perform its spectroscopic characterization and examine its behavior in the system of high performance liquid chromatography (HPLC). After that, the work aims at exploring the possibilities of producing GSNO fluorescent derivatives with *o*-phthalaldehyde (OPA) having in mind that fluorometric methods are very sensitive and can be used in determining low concentration compounds (several μM) which is most often the case with S-nitrosoglutathione.

Material and Methods

Reagents

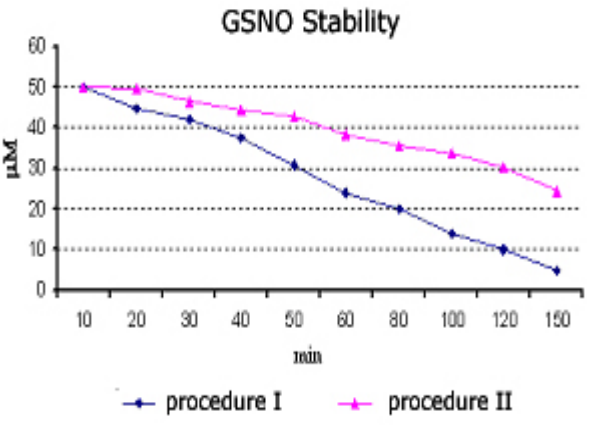
Glutathione (GSH), sodium nitrite, 2-mercaptoethanol (ME), *o*-phthalaldehyde (OPA), diethylene triamine pentaacetic acid (DTPA) were ordered from Sigma-Aldrich Chemical Co, whereas methanol and acetonitrile (HPLC grade) were obtained from Merck. Two different OPA reagents were prepared, each containing 50mM OPA in a borate buffer (100 mM, pH-9.4) in 10% methanol. The OPA reagent A did not contain other chemicals, whereas OPA reagent B contained 15 mM ME. GSH solution (1 mM) was prepared daily in distilled water which contained 0.5 mM DTPA, whereas GSNO solution (1 mM), that was synthesized using procedure II, was also prepared on a daily basis in diluted HCl (10 mM). Immediately before derivatization with OPA reagent, GSH and GSNO mother solutions were diluted to a concentration of 50 μM in a phosphate buffer pH-7.4.

OPA Derivatization

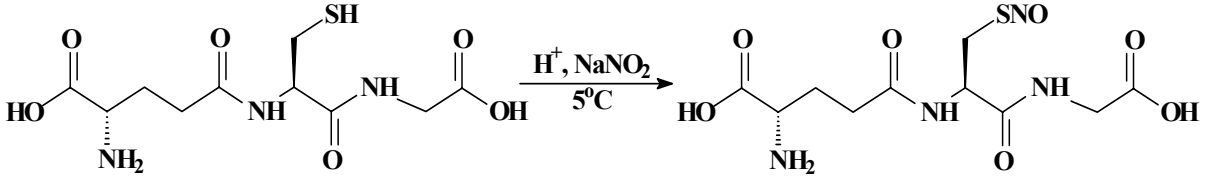
The derivatization of GSH and GSNO with OPA reagent was performed at room temperature using vortex – mixer to join 500 μL solution of 50 μM GSH or GSNO with 100 μL of appropriate OPA reagent. 20 μL of aliquot were used in HPLC analysis. GSH and GSNO derivatisation products were analyzed by HPLC technique with UV and FLD detection and they are presented in Results and Discussion section.

GSNO Synthesis

S-nitrosoglutathione was synthesized in two ways using the original method by Hart (13) with certain modifications. The first procedure includes the reaction of equimolar amounts of glutathione and sodium nitrite (100 mM) in 0.25 M HCl in the presence of 0.1 mM DTPA which served as a chelate reagent, for 30 minutes at a constant temperature of 5^0C (equation 1). The red solution of the final product is neutralized by 5 M NaOH solution and diluted in 20 mM phospfate buffer (pH-7.4).



Graph 1. The stability of GSNO synthesized according to procedure I (in aqueous solution) and procedure II (sedimentation). The reduction of GSNO concentration was monitored in a phosphate buffer (pH-7.4) by spectrophotometric absorbance determination at λ_{max}-334nm.



(1)

The effectiveness of S-nitrosylation was tested by HPLC system A. Molar concentration of GSNO was calculated according to Beer-Lambert law ($A=\varepsilon \bullet c \bullet l$) using the molar extinction coefficient (ε_{334nm}) of 0.85 $mM^{-1}cm^{-1}$. The concentration of synthesized GSNO, as well as the monitoring of its stability, was performed by spectrophotometric determination of absorbance (λ_{max}=334 nm) on a Beckman DU 330 spectrophotometer. The other synthesis method (procedure II) is also based on the reaction of glutathione with nitrite in an acidic environment but the conditions of the reaction were changed so that GSNO red residue was obtained in an anhydrous form. In short, aliquots of $NaNO_2$ (0.276 g; 4 mmol) were added to an iced solution of L-glutathione (1.228 g; 4 mmol) mixed in water (5 mL) containing 2.5 M HCl (1.5 mL), constantly monitoring the temperature of the reaction mixture (5^0C). After 30 minutes, the red residue of the obtained GSNO was treated with acetone (2.5 mL) and then with acetonitrile (2 mL) and mixed for 20 minutes. A fine pale red powder was segregated which was then separated using a vacuum filtration and rinsed with icy water (5 x 1 mL), acetone (3 x 10 mL) and ether (3 x 10 mL). The synthesized GSNO (0.98g, 2.9 mmol,72%) was kept at -20^0C. The identification of the obtained product was performed by HPLC method A, based on the characteristic UV spectrum, with absorption maximum at 334nm.

DAD and FLD HPLC analysis

HPLC analysis was done using Agilent 12000 Series equipped with fluorescent (FLD) and diode array (DAD) detectors. Chromatographic separation and analysis of the synthesized GSNO was done using the HPLC system A: Zorbax Eclipse XDB-C18 RR column 4.6mm x 50mm, 1.8µm; isocratic mobile phase (1 mL/min) 90% citrate buffer (50 mM, pH-6), 5% methanol and 5% acetonitrile. The OPA derivatives of GSH and GSNO were analyzed by HPLC system B: Zorbax SB-C18 column 4.6mm x 50mm, 3.6µm; isocratic mobile phase (1 mL/min) 92% phosphate buffer (50 mM, pH-6) and 8% methanol.

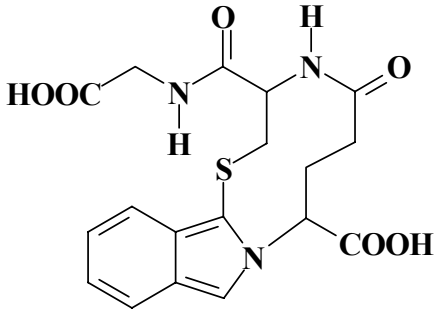
Results and Discussion

The synthesis of GSNO includes nitrosylation of reduced glutathione where NO^+ group donor is nitrous acid obtained *in situ* from sodium nitrite in an acidic environment (equation 2 and 3).

$$HNO_2 + H^+ \longrightarrow NO^+ + H_2O \quad (2)$$

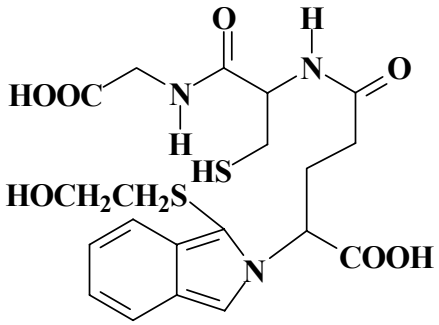
$$RSH + NO^+ \longrightarrow RSNO + H^+ \quad (3)$$

Depending on the concentration of thiol and other components of the reaction mixture, the conditions may be adjusted so as to obtain either an aqueous GSNO solution (procedure I) or GSNO residue (procedure II). HPLC chromatogram of GSNO obtained by procedure I shows that the effectiveness of S-nitrosylation is 82% without the detectable amounts of the starting reagents, glutathione and/or nitrites. The GSNO solution obtained in this way can be used in numerous chemical systems but its use is limited in biological models. The reason is that in an acidic solution, GSNO releases a certain amount of nitric oxide which would interfere with the interpretation of the results referring to the metabolic role of the GSNO introduced exo-genously. Namely, NO itself does not react with reduced thiols in physiological conditions but if there is even a very small amount of oxygen it will oxidize to anhydride of nitrous acid N_2O_3 which is a strong nitrosylating reagent (3). However, although it is neutralized and kept in a phosphate buffer at pH-7.4 in the presence of DTPA as a chelate reagent, at room temperature GSNO disintegrates within one hour to half of its starting concentration (Graph 1). An alternative synthesis (procedure II) with the use of acetone and acetonitrile, has provided a good yield of solid GSNO in anhydrous form. HPLC analysis determined that it could be used without subsequent purification (Figure 1). Besides, GSNO solution prepared by dissolving an appropriate amount of anhydrous form in a phosphate buffer (pH-7.4) showed a greater stability at room temperature – the starting concentration halves only after 2.5 h (Graph 1).



a) OPA – GSH derivat

a) <u>In the absence of ME</u>, GSH reacts with OPA and forms a tricyclic isoindole derivative (OPA-GSH).

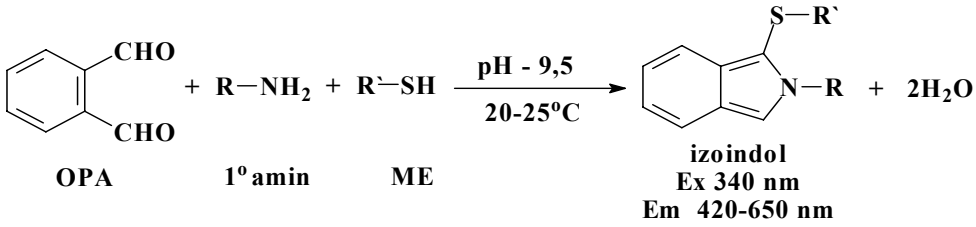


b) OPA – GSH – ME derivat

b) <u>In the presence of ME</u>, OPA-GSH derivative is formed, but an isoinodole derivative may also appear, which carries one GSH molecule and ME (OPA-GSH-ME). The slow intramolecular nucleophilic attack by glutathione mercapto-group on carbon atom from 2-mercaptoethanol leads to the formation of OPA-GSH derivative.

*Figure **2.** Chemical structures of UV-absorptive and fluorescent derivatives which are formed by glutathione (GSH) reaction with OPA in a borate buffer at pH 9.5 without **(a)** and with **(b)** ME.*

A potential biological role of GSNO as a potent NO donor initiated a great number of researches dealing with the development of the analytical methods for determining its physiological concentration (14, 15). Although there is no consensus about the referent GSNO concentrations in physiological fluids, it is considered that they range from 25 nM to 80 nM in human plasma (15). Since molar absorptivity of S-nitroso GSNO group is small (around 0.8 $mM^{-1}cm^{-1}$ at 334 nm), the UV detection of authentic GSNO after chromatographic separation enables the determination of molecular concentrations. Fluorescent detection is an alternative procedure which is more sensitive and may detect nanomolar concentrations of a tested compound. The glutathione itself and/or its metabolite GSNO are not fluorescent, so it is necessary to convert them into appropriate derivatives. One of the most commonly used procedures for determining amino acids and peptides by fluorescence spectroscopy is derivatization of the primary amino group with *o*-phthalaldehyde (OPA) in the presence of mercaptoethanol (ME) (16), whereby an isoindole structure is obtained which, in the fluorescent spectrum, has a characteristic extinction peak at λ_{ex}=340nm and an emission peak at λ_{em}=420 (equation 4).



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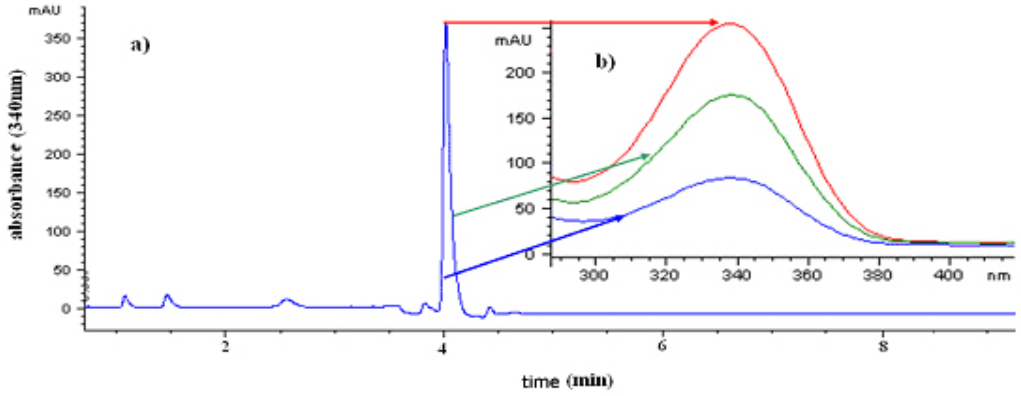
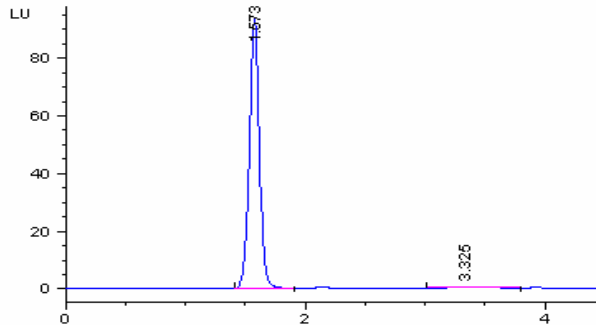
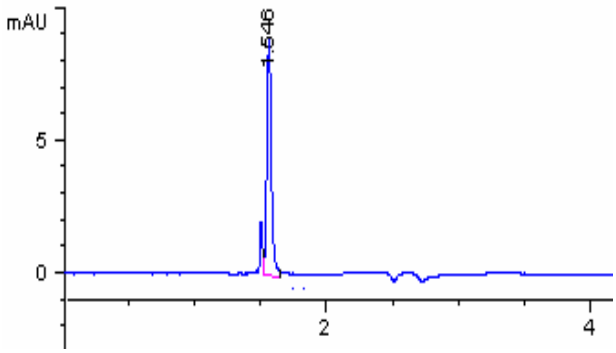


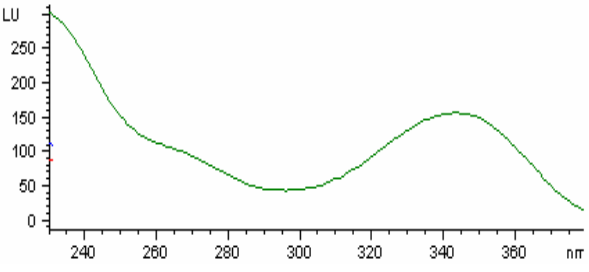
Figure 1. a) a) HPLC chromatogram of GSNO solution synthesized according to procedure II b) absorption spectrum of S-nitrosoglutathione peak



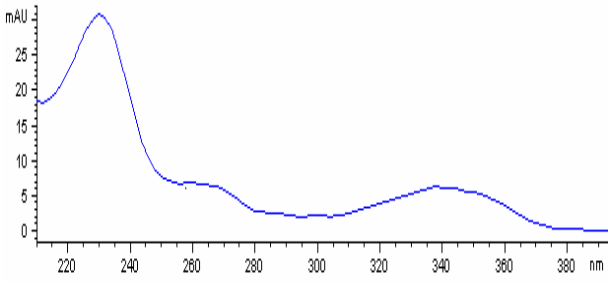
a) chromatogram with fluorescent detection (345 nm/445nm, extinction/emission)



c) chromatogram with UV detection (345 nm)



b) Corresponding emission spectrum



d) corresponding UV absorption spectrum

Figure 3. HPLC chromatograms of GSNO-OPA-ME. 500μL GSNO (50μM) + 100μL OPA reagent B (OPA + ME), injected 20μL

Table 1. The surface and characteristics of the peaks of two GSNO derivatives with OPA-ME reagent

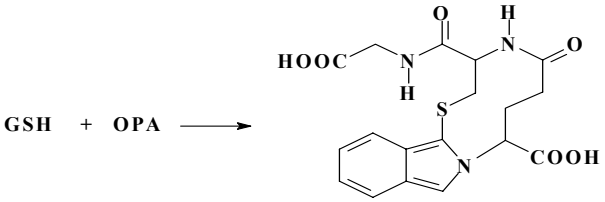
Derivatization time	Type of derivative	RT (min)	Peak area	% of total area	Derivatization time
5min	GSH-OPA GSH-OPA-ME	1.573 3.325	510.2 70	88.7 11.3	1.1 0.48
15min	GSH-OPA GSH-OPA-ME	1.572 3.330	542.2 69.4	94.3 11.9	1.047 0.488
30min	GSH-OPA GSH-OPA-ME	1.57 3.32	556 19	96.7 3.5	1.037 0.482
45min	GSH-OPA GSH-OPA-ME	1.573 3.325	553 14.5	97.5 2.552	1.033 0.484
60min	GSH-OPA GSH-OPA-ME	1.573 3.325	563 3.4	99.5 0.3	1.029 0.48

Neuschwander-Tetri and Roll (17) showed that in the case of GSH, the presence of ME is not necessary because this peptide already possesses a free SH group. Therefore, depending on the content of derivatization reagent, GSH may provide two derivatives. Tsikas et al. (14) used this specific glutathione behavior in the reaction of its OPA derivatization in order to develop a method for determining GSNO in the human plasma.

Having in mind the publications which deal with fluorometric determination of GSH and GSNO OPA derivatives, in our work, we repeated these procedures with certain modifications and analyzed the obtained products with our HPLC system B. The GSH reaction solely with OPA provided 1-alkylthio-2-alkylisoindole, a tricyclic isoindole derivative (OPA-GSH) (Figure 2a). Retention time (RT) of this compound with the conditions of our HPLC system B (Zorbax – SB C18 column 4.6mm x 50mm, 3.6μm; isocratic mobile phase (1 mL/min) 92% phosphate buffer (50 mM, pH-6) and 8% methanol) is 1.57 min. The reaction between GSH and OPA reagent A almost immediately (1min) produced a fluorescent GSH-OPA derivative with a characteristic extinction at λ_{ex}=345 nm and emission at λ_{em}=445 and UV spectrum maximum at 345 nm. It is interesting that a GSH reaction with OPA reagent B (in the presence of ME) is slower than with OPA reagent A, which shows that 2- mercaptoethanol interferes with the reaction between GSH and OPA and primarily leads to the formation of GSH-OPA-ME derivatives with retention time of 3.325.

Contrary to that, GSNO has a very slow reaction solely with OPA (without ME, reagent A): after 120 minutes of incubation less than 10% of GSNO was converted to a derivative with retention time of 1.57. The reason for this is thiol function obstruction by NO group and inability of a fast formation of GSH-OPA derivatives. However, using OPA reagent B, i.e. in the presence of 2-mercaptoethanol, the formation of a UV-absorptive and active fluorescent derivative with RT-1.57 is much faster (Table 1, Figure 3). In case of GSNO, 2- mercaptoethanol has a specific function: not to participate in the formation of fluorescent derivative as in the case of a general reaction between primary amines with OPA and ME, but to take over the NO group from S-nitrosoglutathione through S-transnitrosylation whereby GSH and 2-(nitrosomercapto)- ethanol are released (equation 5).

$$GSNO + HOCH_2CH_2SH \longrightarrow GSH + HOCH_2CH_2SNO \quad (5)$$



(6)

The optimal time for a complete, quantitative GSNO derivatization is 60 min (Table 1). It has been noticed that at the beginning of the reaction, besides the desired derivative at RT-3.325 min, a peak of GSH-OPA-ME compound appears, but after 60 min it completely disappears. The reason for this is probably in the fact that the intramolecular nucleophilic attack by cysteine SH group of the OPA-derivatized glutathione at the second aldehyde function of *o*-phthalaldehyde is preferred in relation to 2- mercaptoethanol (Figure 2b). Besides, it is possible that the GSH-OPA-ME derivative is unstable due to steric hindrances. Based on this unique reaction between GSH and OPA, whereby a highly stable and fluorescent tricyclic isoindole derivative is formed, we have developed HPLC conditions which may serve as a basis for further development of the HPLC method for the determination of GSNO in physiological samples.

Conclusion

Having in mind the great significance of S-nitrosoglutathione as a potential physiological vasodilator and inhibitor of platelet aggregation, we have developed a synthetic procedure for obtaining this compound in a solid, anhydrous form with satisfactory yield. Since S-nitro-soglutathione appears in nanomolar concentrations in physiological samples, it was necessary to introduce a highly sensitive HPLC method with fluorometric detection. Therefore, it was necessary to derivatize the GSNO with *o*-phthalaldehyde in the presence of mercaptoethanol and optimize the conditions of this reaction which led to the formation of a stable and fluorescent tricyclic isoindole derivative, thus creating a prerequisite for the development of a HPLC method which would be used for quantification of GSNO in real biological systems.

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HPLC-FLD-DAD KARAKTERISTIKE DERIVATA S-NITROZOGLUTATIONA DOBIJENIH SA O-FTALALDEHIDOM

Nataša Trutić, Radmila Pavlović, Goran Nikolić, Maja Srećković i Tatjana Jovanović

S-nitozoglutation (GSNO) nastaje S-nitrozilacijom najrasprostranjenijeg endogenog neproteinskog tiola, glutationa (GSH). Predstavlja glavni rezervoar neproteinskih S-nitrozotiola (RSNO) koji se smatraju prirodnim depoom i transportnim oblikom azot monoksida (NO). Njihova fiziološka uloga je da oslobađaju NO koji stimuliše vazodilataciju, sprečava agregaciju trombocita i nastajanje oštećenja u ishemiji/reperfuziji. Cilj rada bio je pronaći specifičnu i dovoljno osetljivu metodu za određivanje GSNO u fiziološkim uzorcima. Zato je bilo neophodno obezbediti čist GSNO standard, što je predstavljalo problem, s obzirom na to što GSNO nije hemijski stabilan. Trebalo je pronaći brz i pouzdan sintetički postupak kojim bi se dobila potrebna količina GSNO i njegovih fluoroscentnih derivata sa o-ftalaldehidom (OPA). Takođe, trebalo je izvršiti spektroskopsku karakterizaciju dobijenih derivata i ispitati njihovo ponašanje u sistemu tečne hromatografije visokih performansi (HPLC). Postavili smo sintezu kojom smo dobili GSNO u čvrstom, anhidrovanom obliku sa zadovoljavajućim prinosom. Nakon UV karakterizacije dobijenog GSNO izvršena je derivatizacija GSH i GSNO sa OPA reagensom sa i bez merkaptoetanola (ME) i nastali proizvodi su analizirani HPLC uz "diode array" (DAD) i fluorescentnu (FLD) detekciju. Stvorili smo optimalne uslove reakcije koja je dovela do formiranja stabilnog i fluorescentnog tricikličnog izoindolnog derivata GSNO sa OPA-ME reagensom, čime je stvoren preduslov za razvoj HPLC metode, kojom bi se izvršila kvantifikacija GSNO-a u realnim biološkim sistemima. Acta *Medica Medianae 2010;49(1):27-32.*

Ključne reči: S-nitrozoglutation, HPLC, DAD, FLD, o-ftalaldehid