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High performance liquid chromatography analysis of aliphatic thiols in alimentary supplements and pharmaceuticals using menadione as a new useful derivatization reagent

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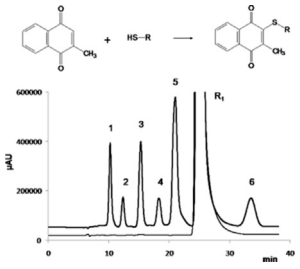
HIGHLIGHTS

- Menadione (MD) is proposed as a new derivatization reagent for thiol HPLC analysis.
- N-acetylcysteine/MD derivative was synthesized and characterized by ^{1}H NMR and IR.
- Derivatization reaction was carried out in mild and fast conditions.
- No interference of the complex matrix (alimentary supplements) was observed.
- N-acetylcysteine analysis was carried out without preliminary extraction procedures.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of menadione (MD) as a pre-column reagent for high performance liquid chromatography (HPLC) analysis of aliphatic thiols is proposed. The reaction was carried out for 5 min at room temperature and pH 8.5. The developed method was applied to the N-acetylcysteine (NAC) analysis of alimentary supplements and pharmaceutical formulations. The effect of the complex matrix was evaluated by the study of the thiol derivatization reaction both in standard and in placebo solutions. The yield of NAC-MD adduct was found to be quantitative at a reagent to thiol molar ratio of about 4 in comparison with an authentic specimen of synthesized NAC adduct, which was characterized by ^{1}H NMR, IR and UV. The routine chromatographic separations were performed on a Synergi MAX-RP column using a mobile phase consisting of methanol/triethylammonium (TEA) phosphate buffer (pH 3; 0.05 mol L^{-1}) 70:30 (v/v) at a flow-rate of 0.4 mL min^{-1}. UV-diode array detection was used setting the wavelength at λ = 260 nm. The validation parameters such as linearity, sensitivity, accuracy, precision, selectivity and ruggedness were found to be highly satisfactory. Similar linear responses were observed by standard and placebo solutions (determination coefficient: 0.9996). Limit of detection was about 0.019 μg g^{-1}. Intra-day precision (relative standard deviation, R.S.D.) was ≤0.81% for NAC to internal standard (IS) peak area ratio, ≤0.28% and ≤0.32%, respectively, for NAC and IS retention times (t_R), without significant differences between intra- and inter-day data. NAC recovery studies gave good results (100.12%) with R.S.D. = 1.05%.

1. Introduction

Sulphur, one of the major metabolic nutrients, is a typical non-metal element and is essential for the entire biological kingdom.

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Some of sulphur-containing antioxidant compounds are cysteine (Cys), N-acetylcysteine (NAC), methionine, taurine, glutathione (GSH), lipoic acid and mercaptopropionylglycine. Plasma thiols have pro-oxidant or antioxidant actions depending on the physiological circumstance, but are generally considered antioxidant. Since radiations cause damages to DNA through free-radical intermediates, thiols with a net-positive charge may protect against radiation poisoning due to concentrating in the microenvironment of DNA and scavenging free radicals [1]. NAC is a mucolytic agent and a precursor of L-Cys and reduced GSH. NAC is a source of sulfhydryl groups in cells and is a scavenger of free radicals, as it interacts with reactive oxygen species (ROS) such as hydroxyl radical and hydrogen peroxide [2]. Uses of NAC in different diseases including cancer, cardiovascular diseases, human immunodeficiency virus (HIV) infections, acetaminophen-induced liver toxicity and metal toxicity have been reviewed [3]. NAC can also prevent apoptosis and promote cell survival activating extracellular signal-regulated kinase pathway, a concept useful for treating certain degenerative diseases [4].

Liquid phase separation techniques, such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), are the most frequently used for the determination of organic substances in various matrices. Unfortunately, many substances of interest including thiols cannot be detected because they lack the structural properties necessary to produce signals compatible with common HPLC or CE detectors, such as UV absorbance and fluorescence. This problem can be overcome using derivatization reactions that add chromophoric or fluorophoric groups to the investigated molecules [5]. The variety of chemistries that can be adopted for either pre- or post-column derivatization in conjunction with HPLC is large because either aqueous or organic solvent-compatible reactions are possible [6]. The pre-column method seems to be recommended for the labelling because thiols might be decomposed during the separation in the analytical column. Recently, several reviews on analytical methods for thiols, including those with derivatization step, were published [5–15].

UV absorbance detector is known for its stability and low demand in terms of maintenance, and belongs to the standard instrumentation in all analytical laboratories [5,14,16]. UV absorbance detection is less specific and less sensitive in comparison with fluorescence or electrochemical detection, but extended path-length UV absorbance detectors may also provide satisfactory detection limits for underivatized thiols. However, UV derivatization reagent reacts with thiol functional group to produce a final UV higher absorbing derivative [5,12].

The choice of the derivatization reagent is important not only for the sensitive detection, but also for the stabilization of thiols, improvement of chromatographic properties and ionization responses (electrospray ionization-mass spectrometry), or introduction of a charge (CE) [5]. The best reagent reacts rapidly at room temperature, at weakly acidic pH to prevent oxidation of the analytes and specifically with thiols to set up stable products. Numerous reagents are available for the thiol derivatization. The majority of the reagents can be classified by type of the reactive moiety into four categories and are reviewed with some experimental details in excellent works [5]. The categories are: (i) compounds with activated halides (e.g. 2-haloquinolinium salts, benzofurazans; monobromobimane, mBrB) [17–23]; (ii) activated double bond compounds, such as enones (e.g. ethacrynic acid, naphtoylacrylic acids, 1,1′-[ethenylidenebis(sulfonyl)]bis-benzene, ESB; N-ethylmaleimide, NEM; 1,4-nahthoquinone, NPQ) [24–31], (iii) disulfides (e.g. Ellman reagent, 5,5′-dithio-bis-2-nitrobenzoic acid; DTNB; 4,4′-dithiodipyridine, 4-DPS) [32,33], and (iv) other compounds (e.g. 1,1′-thiocarbonyldiimidazole, TCDI, used for aminothiol derivatization) [34,35]. However, most reagents can involve different drawbacks such as e.g. (i) limited selectivity for thiol function (3-bromomethyl-propyphenazone, BMP) [36]; (ii) drastic reaction conditions (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, ABD-F and BMP) [20,36]; (iii) a time consuming derivatization procedure (4-ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate, SBD-F; TCDI and mBrB) [20,34,37]; (iv) low stability of the derivatives (mBrB and NEM) [22,37,38]; (v) need of extraction or hydrolysis step to remove reagent excess before analysis (ESB and TCDI) [29,34,35] and (vi) practical limitations (DTNB) [30,33].

Menadione (MD) is a synthetic chemical compound and is an analogue of NPQ with a methyl group in 2-position. MD is sometimes used as a nutritional supplement in the treatment and prevention of haemorrhage associated with vitamin K deficiency [39]. In the United States, MD supplements are banned by the US Food and Drug Administration (FDA) because of their potential toxicity in humans. Large doses of MD have been reported to cause adverse outcomes such as hemolytic anaemia [40], whereas low doses have yielded no reported cases of toxicity from MD in livestock or pets. However, as far as it is known, MD was not previously applied to analytical objects.

On the basis of the satisfactory results recently obtained using NPQ as a pre-column reagent for thiol function [31] and of the well-known disadvantages of the common reagents [20,22,29,30,33–38], in order to propose new and reliable derivatization reagents, the current work focuses on the study of MD, as a potential analytical reagent. In comparison with NPQ, the derivatization reaction with MD has the merit of producing fewer degradation products. This favourable aspect allowed to work at $\lambda = 260$ nm, instead of $\lambda = 420$ nm, necessary for NPQ; so the sensitivity of the developed method increased significantly. The effect of different parameters (temperature, pH and molar ratio reagent to thiol) affecting the derivatization reaction was accurately studied. The reaction yield was investigated, synthesizing NAC-MD adduct and characterizing it by ^{1}H NMR, IR and UV. The selectivity of MD towards the thiol function and the optimum chromatographic separations of thiol derivatives were examined. To demonstrate the practicality of the technique, the developed and validated HPLC method was applied to the quali-quantitative analysis of NAC in complex matrices, such as new and commercial alimentary supplements and pharmaceuticals.

2. Experimental

2.1. Materials

1-[3-Mercapto-2-(S)-methyl-1-oxopropyl]-(S)-proline (captopril), GSH, penicillamine, thioglycolic acid and MD (2-methyl-1,4-naphthoquinone), 2-naphthol as internal standard (IS), methanol Chromasolv®, chloroform and acetic acid were obtained from Sigma–Aldrich (Milan, Italy). NAC, D,L-homocysteine (Hcy), boric acid were obtained from Fluka (Milan, Italy). Triethylamine was purchased from Carlo Erba (Milan, Italy). TLC plates RP-18 F^{254s}(20 cm × 20 cm) were purchased from Macherey-Nagel (Düren, Germany). Purified water by a Milli-RX apparatus (Millipore, Milford, MA, USA) using 0.22 μm filters was employed for the preparation of all solutions, buffers and mobile phase. The formulation (effervescent tablets) was provided from E-Pharma Trento S.p.A. (Trento, Italy). The formulations (tablets and medicines in packets type I and type II) were purchased from a local pharmacy.

2.2. Solutions

All solutions were prepared freshly and stored at 2–8 °C during the day. NAC standard solutions (concentration as calibration ranges) in presence of IS (about 0.96 mg mL^{-1}) were prepared in a