“One pot” chloroformate derivatization of erythrocyte glutathione and its gas chromatography – mass spectrometry analysis

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"ONE-POT" ETHYL CHLOROFORMATE DERIVATIZATION AND LIQUID LIQUID EXTRACTION OF REDUCED GLUTATHIONE IN ERYTHROCYTE AND ITS QUANTITATIVE GCMS ANALYSIS

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Highlights:

- **Development of "one-pot" ECF-EtOH derivatization and LLE sample preparation method**
- **for GC/MS analysis of reduced glutathione (GSH) and hGSH in erythrocytes.**
- **Comprehensive validation of the novel analytical methodology.**
- **Feasibility for clinical investigation of the oxidative status: case studies.**

Abstract

A simple "one-pot" derivatization and liquid liquid extraction (LLE) procedure was developed for GC-MS analysis of reduced glutathione (GSH) analysis in erythrocytes. The metabolite was extracted by 5% (w/v) TCA, the supernatant treated with ECF and ethanol – pyridine media and the derivative separated and detected by gas chromatography – mass spectrometry using a short non – polar capillary GC column at a high column – head pressure. Total analysis time was 11 minutes. The process was optimized by a Design of Experiment. The method was validated showing a good linearity over the 25.4-813.4 μM concentration range, providing satisfactory results in terms of intra-day and inter-day precision as well as an optimal accuracy. The new method was evaluated in a pilot study involving patients with severe protein malnutrition. Comparing this group with a group of healthy subjects revealed significantly lower GSH concentrations in erythrocytes in the former, proving thus that the described GC-MS method could be employed for fast and simple GSH analysis in clinical studies.

Introduction

Oxidative stress has been defined as "tissue damage resulting from an imbalance between excessive generation of oxidant compounds and insufficient anti-oxidant mechanisms" [1,2]. The imbalance, due to an increased radicals production and/or a reduction of available scavengers, can cause oxidative reactions of many biological macromolecules such as DNA, lipids and proteins thus leading to irreversible structural and functional damages of cellular components [1,3]. Oxidative stress is known to play a key role in physiological cellular mechanism of ageing [4–6] and, on the other hand, is involved in the pathogenesis of several diseases through pro-inflammatory and

fibrogenetic effects that peroxidation products may exert [1,7–12]. Its measurement can be used in clinical practice to prevent damages [13]. Oxidative status can be evaluated by analytical methods

able to assess the total antioxidant capacity (TAC) like fluorescence methods such as Total Radical-tapping Antioxidant Parameter (TRAP), Oxygen Radical Absorbance Capacity (ORAC) and Ferric Ion Reducing Antioxidant Power (FRAP) [14,15]. However, the diagnostic benefit of this approach is controversial [16]. Direct measurement of active oxidant species is however a challenging task because of their high reactivity [17,18]. In clinical laboratories the evaluation of oxidative stress is often accomplished in an indirect way by measuring oxidative biomarkers such as (i) oxidative-damage products (e.g. 8-hydroxy-2-deoxyguanosine, malondialdehyde, hexanal, isoprostanes), (ii) antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase) or (iii) antioxidants (e.g. glutathione, vitamins C and E) [19]. Among these frequently used biomarkers, glutathione may be considered of particular interest because it is the most abundant endogenous antioxidant within the mammalian cells of all tissues. Therefore, red blood cells (RBC), for their high availability seem to be the best blood matrix for glutathione determination. Free glutathione is mainly observed within the cells in its reduced form (GSH) at a concentration of about 3mM [20] , while only a minimal percentage, about 2 µM [20] is detected in its oxidized form as disulfide (GSSG). In case of oxidative stress, the redox couple GSH-GSSG acts to maintain homeostasis: GSH is promptly oxidized to glutathione disulfide, hence the concentration values of its reduced form and the molar ratio GSH/GSSG decrease. Low GSH levels may be considered as an index of oxidative stress and disease risk [21], despite its concentration may be affected by nutritional and metabolic deficiencies [22]. There is evidence that changes in blood GSH concentration values are suitable representative of the redox status of other tissues. Values of GSSG and GSH/GSSG are reported to be less accurate indexes of the systemic oxidative balance since they are more affected

by ex-vivo artifacts [23–25]. Even though over the last few years a large number of methods for GSH detection have been published, consisting in electrophoretic or chromatographic techniques with numerous different analytical detectors, no agreement has been already reached about the analytical protocol to be performed [26,27]. Therefore, clinical laboratories lack reliable and time-saving analytical methodologies for routine GSH analysis.

In the present study, the instrumental conditions to detect and quantify GSH ethyl chloroformate derivative in erythrocytes with the highly-specific mass detector coupled with gas chromatography (GC-MS) were investigated. Furthermore a novel "*one-pot*" derivatization and LLE procedure was developed for GSH in erythrocytes, in accordance to P. Šimek and his coworkers' studies on chloroformates [28–31].

The sample preparation conditions were optimized by a full-factorial Design of Experiment (DoE) strategy and the new method was validated according with the recommendations of ISO/IEC 17025:2005**.** The proposed method allows to perform a fast, robust and specific determination of GSH in erythrocytes that might enlarge the number of oxidative biomarkers to be routinely monitored in clinical laboratories.

2. Experimental

2.1 Reagents and materials

Reduced glutathione (>98%), ethyl acetate (EA), ethylenediaminetetraacetic acid (EDTA), ethyl chloroformate (ECF), HPLC grade ethanol (EtOH), pyridine (Pyr) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich s.r.l. (Milan, Italy), while homoglutathione (hGSH) was purchased from Bachem AG (Bubendorf, Switzerland). TCA 5% w/v aqueous solution and EDTA

solution (1 mmol/L) were prepared in deionized water. EDTA solution was stored at 4°C and prepared every month. Moreover, a solution of EtOH and Pyr 4:1 v/v was prepared to adjust pH and catalyse derivatization reaction. Stock solution of GSH (3.25 mmol/L) and working solution of hGSH (6.72 mmol/L), the latter employed as internal standard, were prepared in deionized water saturated with nitrogen and stored immediately at -80°C.

2.2 Samples collection

In the present study the surplus fraction of venous blood from already analyzed samples relative to patients undergoing clinical tests in Clinical Biochemical Laboratory of Città della Salute e della Scienza University Hospital of Turin (Italy) were employed. EDTA tubes with whole blood samples were centrifuged (3500 rpm, 5 min, 4°C) and the fraction containing plasma was removed. Erythrocytes were frozen at -80°C for 30 minutes in order to ensure complete cell lysis. Then, red blood cell lysate was diluted (1:8 v/v) with cold EDTA solution and stored until analysis at -80°C.

2.3 Sample preparation

Lysate (600 µL) was added with 50 µL of internal standard working solution and 150 µL of deionized water. Proteins were precipitated by adding 600 µL of 5% (w/v) TCA solution. Samples were then vortex-mixed (15 s), let to stand for 10 min and then vortex-mixed (15 s) a second time. The precipitate was subsequently centrifuged (3500 rpm, 5 min, 4°C). Afterwards, "one-pot" ECF-EtOH derivatization catalyzed by pyridine and combined with simultaneous LLE was carried out in bain-marie at 22 ± 1°C . More in details, 600 µL of clear supernatant were added with 400 µL of

EtOH-Pyr solution, vortex-mixed (30 s), then added with 70 μL of ECF and 1 ml of EA and vortex-mixed (2 min) again. Samples were subsequently centrifuged (3500 rpm, 5 min, 4°C) and the organic extracts were evaporated to dryness under a nitrogen stream at 50°C. Finally, the residue was dissolved in 200 μL of EA for GC-MS analysis.

The chloroformate reagent converts the amino and thiol groups into carbamates and thiocarbonates, respectively, while the two carboxyls form mixed anhydrides being immediately converted by the EtOH-Pyr medium into ethyl esters [29]. The final product is (N, S)-ethoxycarbonyl-O-(bis)-ethylester GSH derivative.

2.4 Instrumentation

GC-MS analysis was carried out with a HP 6890/5973 Gas Chromatograph - Mass Spectrometer equipped with an Electron Ionization (EI) source (Agilent Technologies, Milan, Italy). A crosslinked 5MS® 0.25 mm x 0.25 μm capillary column was employed (CPS Analitica s.r.l., Milan, Italy) cut at a length of 5 m [32]. Injections (1 μL) were performed in splitless mode at 285°C. Column head pressure was set at 2.22 psi, with an oven temperature of 100°C and a helium flow of 0.9 mL/min. The GC oven temperature was initially set at 100°C, held at 100°C for 0.5 min, raised at a rate of 20°C/min up to 260°C, then at a rate of 60°C/min up to 290°C, where it was maintained for 1 min, for an overall runtime of 11 min. Temperatures were set at 250°C for the ionization source and at 280°C for the interface. GC-MS spectra of N,S-ethoxycarbonyl ethyl esters of GSH and hGSH were initially acquired in full-scan mode (mass range 50-600 m/z, source energy 70eV), and subsequently in Selective Ion Monitoring (SIM) mode. OpenChrom software [33] was employed for pre-processing the analytical raw data.

2.5 Validation

The analytical method was fully validated in accordance with the recommendations of ISO/IEC 17025:2005 international standard. The following parameters were investigated: selectivity, linearity range, limit of detection (LOD), limit of quantitation (LOQ), carry-over, accuracy, intra-day and inter-day precision and robustness. Venous blood sample was collected from 50 healthy volunteers and employed as working matrix for all the validation experiments.

2.5.1 Identification criteria and selectivity

The identification criteria for the analytes were defined according to well-known international guidelines [34,35]. Retention time deviations of 2–5% from the calibrators were considered acceptable for developed GC-MS method. Moreover, at least two qualifying ions for each analyte, in addition to the target ion, were evaluated to identify each specific target analyte. Furthermore, variations of ions intensities proved satisfactory when within ±20 %.

2.5.2 Linearity range, evaluation of LOD and LOQ and quantitation of unknown

The linear calibration model was checked through the analysis in replicates (n=2) of different venous blood samples spiked with working solutions at seven different concentrations (813.4, 610.1, 406.7, 203.3, 101.6, 50.8 and 25.4 μM). Area counts of GSH peak were normalized with the respective peak area of hGSH, which was employed as internal standard . . The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient, adjusted by taking into account the number of observations and independent variables (Adjusted R^2), was utilized to roughly estimate linearity. The appropriateness of the model was

assessed by evaluation of the residual plots and further significance tests, such as lack-of-fit test, F-test and Mandel's test. The assumption of homoscedasticity was also verified. LOD and LOQ were then estimated by means of Hubaux-Vos methodology [36]. Furthermore, LOD was also evaluated as the analyte concentration whose response provided a signal to noise ratio (S/N) value equal to 3, as determined from the least abundant among the qualifier ions and extrapolated from the S/N values of the three lowest concentrations of the calibration curve. The LOD values were subsequently confirmed with the analysis of spiked samples containing the target analyte at the concentration of its estimated LOD value. Similarly, the limit of quantification (LOQ) was estimated according to the S/N ratio, which had to be equal or greater than 10 [37]. Since no GSH-free matrix is easily available, a different calibration curve was performed for each unknown sample (standard additions method, two replicates per calibration point). Then, the obtained calibration curve was compared to the one from validation studies by means of significance tests. In case of positive comparison, the validation curve was employed to estimate the GSH amount in samples. . Otherwise,it was used the new calibration curve once tested in terms of linearity .

2.5.3 Precision and accuracy

Intra-day and inter-day precisions, expressed in terms of percentage variation coefficient (CV%), were evaluated for GSH. Intra-day precision was assessed by analysis of ten venous blood samples collected from presumed healthy subjects. On the other hand, inter-day precision was observed through evaluation of two samples (in triplicate) at different concentrations. Accuracy (expressed as bias %) was assessed only within-run on two replicates of different calibrators employed for the evaluation of linearity parameter. Standard acceptability criteria were assumed [38].

2.5.4 Carry-over

Carry-over effect was evaluated injecting an alternate sequence of five solvent blanks and five blood samples spiked with the target analyte at high concentration of the linear calibration range (600 µM). To ensure the absence of any carry-over effect for blanks, the S/N had to be lower than 3 for each monitored ion.

2.5.5 Robustness and stability

Robustness testing was conducted by introducing slight variations to previously selected analytical parameters and observing the resulting changes in term of quantitative response on venous blood samples spiked at the lowest validation level. A Youden approach was used [39,40], in order to minimize the number of experiments required. In particular, different mixing strategies and approaches were tested. With reference to the stability parameter, derivatized GSH was evaluated over the time and at different temperatures. Duplicate derivatized samples were injected after their preparation and stored for different times (i.e. t_0 = immediately, t_1 = 24 hours, t_2 = 7 days, t_3 = 30 days) and temperatures (T_1 = room temperature, T_2 = 4°C). t_3 was tested for the samples stored ad T_2, only.

2.6 Pilot study on patient- and control-groups

To complete the validation work, two small groups of individuals were compared: (i) a group of eight hospitalized patients with severe protein malnutrition, due to unstable intestinal failure (short-bowel syndrome or radiation enteritis), 3 females and 5 males, aged 43 – 84 and (ii) a control group of twelve non-smoker, healthy subjects, 7 females and 5 males, aged 28 – 64.

Informed consent was signed by all the hospitalized patients and control individuals. GSH molar content in red blood cells (RBC-GSH) was expressed per gram of Haemoglobin (HB) (µM GSH /g HB); in order to calculate RBC-GSH values the following formula was employed:

$$(\text{GSH [µM/L]} \times \text{Hematocrit [vol\%]}) / (\text{HB [g/dL]} \times 1000)$$

Results of the two groups were compared by means of Welch's t-test [41].

3. Results and discussion

3.1 Glutathione derivatization

The derivatization step of the highly polar GSH is mandatory to quantify it by means of GC-MS technique. Published methods [32,42] describe a laborious two-steps procedure: in the first step amino and thiol groups react with chloroformates, then products are extracted and in a second derivatization step, in acidic medium, the carboxylic groups are methylated. Glutathione N,S-ethoxycarbonyl methyl ester two-steps synthesis in biological samples is described by various authors. To shorten the pre-analytical time, we investigated in erythrocyte lysate, the feasibility of the simultaneous reaction of all disposable functional groups of the molecule with ECF as described by Šimek's group [28,30,31] in plasma for amino acids and dipeptides.

Ethylchloroformate causes a rapid derivatization of amino, thiol and carboxylic groups of the molecule with the synthesis of GSH and hGSH N,S-ethoxycarbonyl ethyl ester derivatives, as summarised in Figure 1.

Chloroformate derivatization was performed following Hušek's recommendations [43] in particular about volume ratio between EtOH and Pyr as well as between aqueous and organic portions. As stated by Hušek's, we experienced that, when medium was not neutralized, derivatization gave best results. Thus this procedure has been preferred. Stability studies performed on derivatives in EA let conclude that they can be stored up to 7 days at room temperature and up to one month at 4°C.Variations of GSH concentrations in any storage condition were lower than 10% and they are within the range of experimental error.

3.2 "One-pot" procedure optimization

In order to estimate glutathione in biological samples it is necessary to preserve the physiological balance between the redox couple GSH/GSSG during sample manipulation [10,12,13,22–25]. Stability of thiols at acidic pH is reduced in biological matrix because of its complexity [44]. Oxyhaemoglobin, free radicals and oxygen produced by metal catalysis are responsible of GSH oxidation at acidic pH in blood.

To minimize the formation of artefacts, optimal operative conditions were adopted, as suggested by previously cited authors: (i) low temperature, (ii) metal chelant (such as EDTA) and (iii) removal by gas bubbling (i.e. nitrogen) of the oxygen dissolved in distilled water used.

With the aim of avoiding contaminations that could shorten column lifetime, thus interfering with signal of the target analytes, samples were deproteinized prior to derivatization. As deproteinizing agent we choose TCA, which minimizes the supernatant protein and lipid content [28]. The TCA solution was prepared at a concentration fitted to the matrix in analysis.

We verified the feasibility of Šimek and co-workers "one-pot" procedure for GSH [28,30]. The simultaneous addition to the sample of ECF, EtOH with the pyridine catalyzer and an appropriate

organic solvent, provides for an extremely rapid derivatization in aqueous media and for immediate extraction of the synthesized derivative in the organic layer. To select the most suitable solvent for the extraction we investigate solvent mixtures with different polarity: Ethyl acetate (EA) versus Isooctane and Ethyl acetate (TMP-EA). To the organic phase we added the derivatization agent in a ratio 14:1 for Ethylacetate-ECF (EA-ECF) and 10:6:1 for isooctane-ethylacetate-ECF (TMP-EA-ECF). Better results were obtained with the former mixture. In order to evaluate the extraction yield, we performed a second extraction with an equal volume of EA-ECF, and we found that the organic phase of the second extraction did not present any detectable amount of GSH.

A full-factorial experimental design [45] approach was followed in order to develop and optimize the sample preparation leading to the "one-pot" derivatization and LLE of the GSH. Three experimental factors, which were supposed to influence GSH derivatization, were selected for this study and two different levels (each a "high" and a "low" one) were defined for these factors: reaction temperature ("high": 30°C, "low": 18°C), vortex time ("high": 2 min, "low": 1 min), and v/v ratio between EtOH and Pyr ("high": 4:1 EtOH/Pyr, "low": 3:1 EtOH/Pyr). The aim of this design was to maximize the extraction and the derivatization of GSH in order to raise the ratio between the areas of GSH and its internal standard hGSH. Eight experiments (i.e. 2^3 combinations) were performed in duplicate. Since no significant factors were observed among the investigated ones (see Supplementary Material), the analytical methodology was developed as described in section 2.3, in order to get the best compromise in terms of time and costs. The described procedure compared with traditional two-steps process is faster and gives better yields (data not shown).

3.3 GC-MS method

Kataoka and Capitan [32,46] underlined that elution of Glutathione N,S-ethoxycarbonyl methyl ester was possible only with short column (e.g. 10 m) and under specific instrumental conditions. We verified that GSH and hGSH N,S-ethoxycarbonyl ethyl ester derivatives can be eluted with reproducibility by a 5 m column setting a high column-head pressure. Our developed and optimized GC-MS method allowed to quantitatively detect GSH, together with its internal standard hGSH. The whole chromatographic run was completed in 11.0 min. Retention times of the target analytes ranged between 6.97 min (GSH) and 7.07 min (hGSH). As an example, Figure 2 shows the SIM chromatogram recorded from a venous blood sample spiked with GSH 810 μmol/L and hGSH 6.72 mmol/L.

All the monitored ions in SIM mode are depicted for each analyte in Figure 3.

3.4 Method validation

3.4.1 Identification criteria and selectivity

In order to achieve unambiguous identification, four ions (i.e. one target ion plus three qualifying ions) were monitored in SIM mode for each analyte, as summarized in Table 1. Hypothetical ionic

fragments are reported for GSH N,S-ethoxycarbonyl ethyl ester in Figure 4. The intra-assay precision for retention times, measured at low and high concentrations, showed random fluctuations within ±1.0%, confirming high repeatability not affected by analytes concentration. For each analyte, the relative abundance of the three selected ions, monitored in SIM mode, was found to vary by less than ±20%. These results meet the requirements for unambiguous identification of all analytes included in the assay. The SIM chromatograms obtained from different venous blood samples for hGSH and from solvent blank samples for GSH, showed no interfering signals (i.e., S/N ratio minor than 3) at the expected retention time. This demonstrates that the method is selective for the tested compounds and free from positive interferences from blood components and column bleeding.

3.4.2 Linearity and evaluation of LOD and LOQ

Seven levels of calibration standards of GSH in the range from 25 to 800 μM were obtained adding appropriate amounts of GSH stock solution to pooled erythrocyte lysate (600 μL). Adjusted R^2 values obtained from the calibration curve of GSH was equal to 0.9995, thus indicating good fit and linearity (see Supplementary Material). All the back calculations of standards were within 15% at each calibration level, and all the significance tests provided satisfactory results. LOD and LOQ values, according to Hubaux-Vos approach, turned to be equal to 4.29 and 8.58 μM, respectively. Comparable results were calculated from S/N values of the three lowest concentrations of the calibration curve. Positive detection (S/N>3) of analyte at its approximate LOD concentration was confirmed experimentally.

3.4.3 Precision and accuracy

Intraday and inter-day data on precision and accuracy provided satisfactory results. In particular, intra- and inter-day CVs%, were lower than 5% and 20%, respectively. Accuracy was satisfactory for all monitored calibrators (i.e. percent biases lower than ± 15% at all monitored concentrations). Results are included into the Supplementary Material.

3.4.4 Carry-over effect

No carry - over effects were observed under the conditions described in the experimental section.

3.4.5 Robustness and stability

Different mixing strategies (e.g. vortex and mechanic) were evaluated. It was found by means of t - test that no specific factor influenced the final results for GSH. With reference to the stability of the derivatized GSH, the tested samples provided a variation, in terms of concentration (μM), lower than 10% (for both robustness and stability results, see Supplementary Materials).

3.5 Pilot study on patient- and control-groups

RBC-GSH concentration values for the monitored subjects are reported in table 2. Further information about the investigated individuals are available in the Supplementary Material. The Welch's t-test performed on the μM/gHB values provided a p-value of $9 \cdot 10^{-5}$, thus strongly suggesting that the null hypothesis should be rejected. The significance probability indicates the probability of a result as large as this, by chance alone, is about 1 in $1 \cdot 10^{4}$. Even though the number of monitored individuals is low, this result suggests that GSH might be a useful marker for the identification of oxidative stress.

4. Conclusions

First time, a simple and fast "one pot" simultaneous ECF derivatization and LLE method was proposed for the determination of GSH in erythrocytes by GC-MS. The validated method was examined on the GSH analysis in erythrocytes and was proved suitable for routine purposes.

Further study is planned in a larger patient's cohort to assess the clinical usefulness of GSH concentration versus GSH/GSSG ratio in erythrocytes as a marker of oxidative stress.

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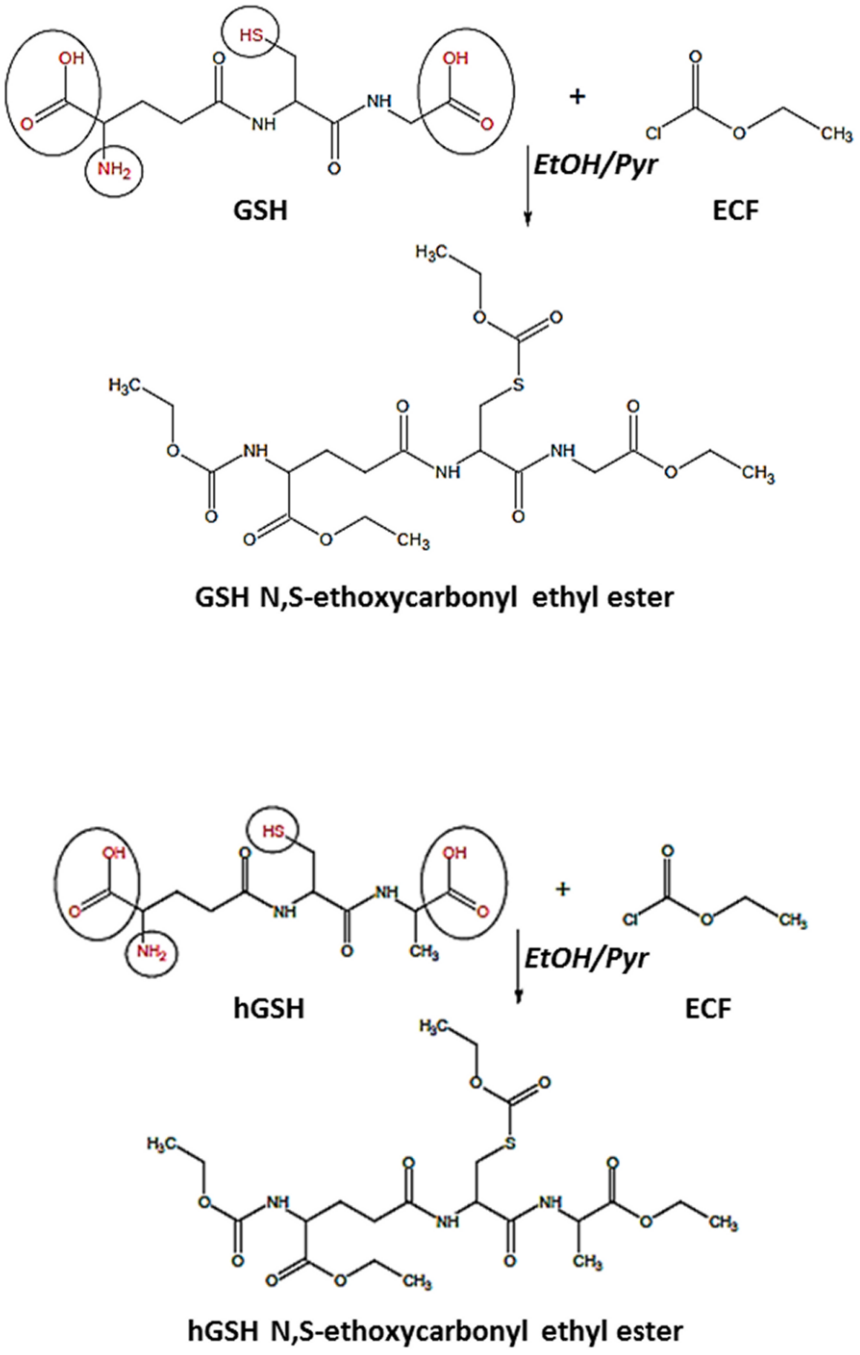

Figure 1. Schematic representation of the synthesis of both GSH (above) and hGSH (below) N,S-ethoxycarbonyl ethyl ester derivatives.

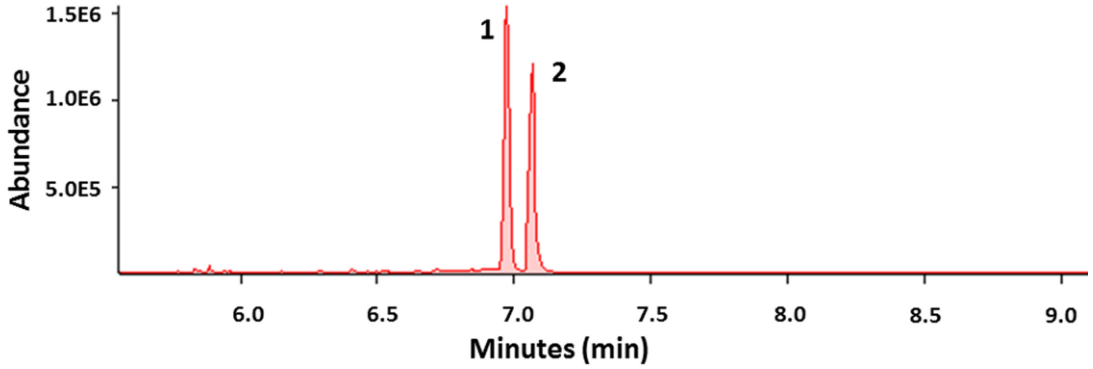

Figure 2. SIM chromatogram relative to GSH (406.7 µM) (1) and hGSH (2) N,S-ethoxycarbonyl ethyl ester derivative.

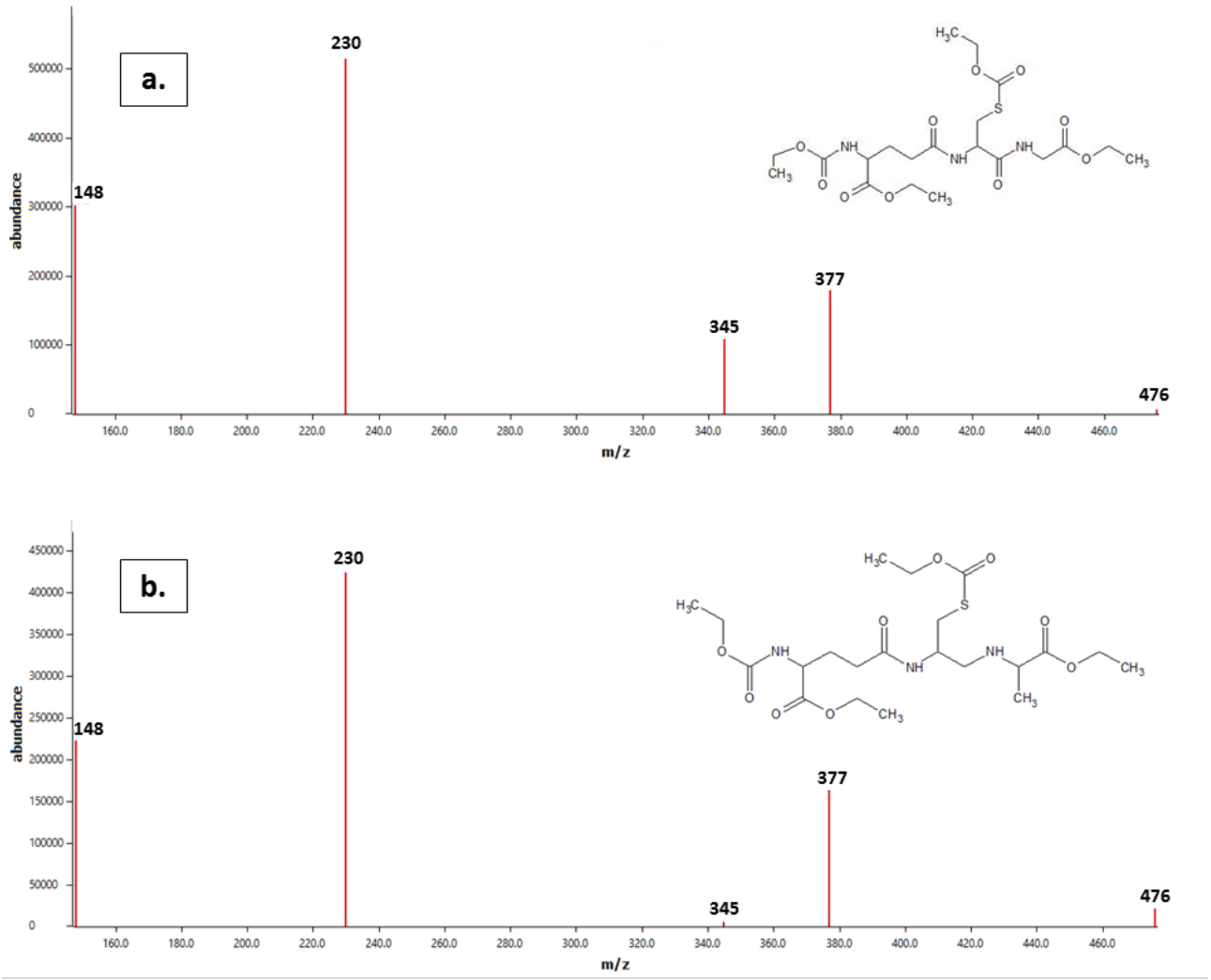

Figure 3.SIM spectra of both GSH (a.) and hGSH (b.) N,S-ethoxycarbonyl ethyl ester derivatives.

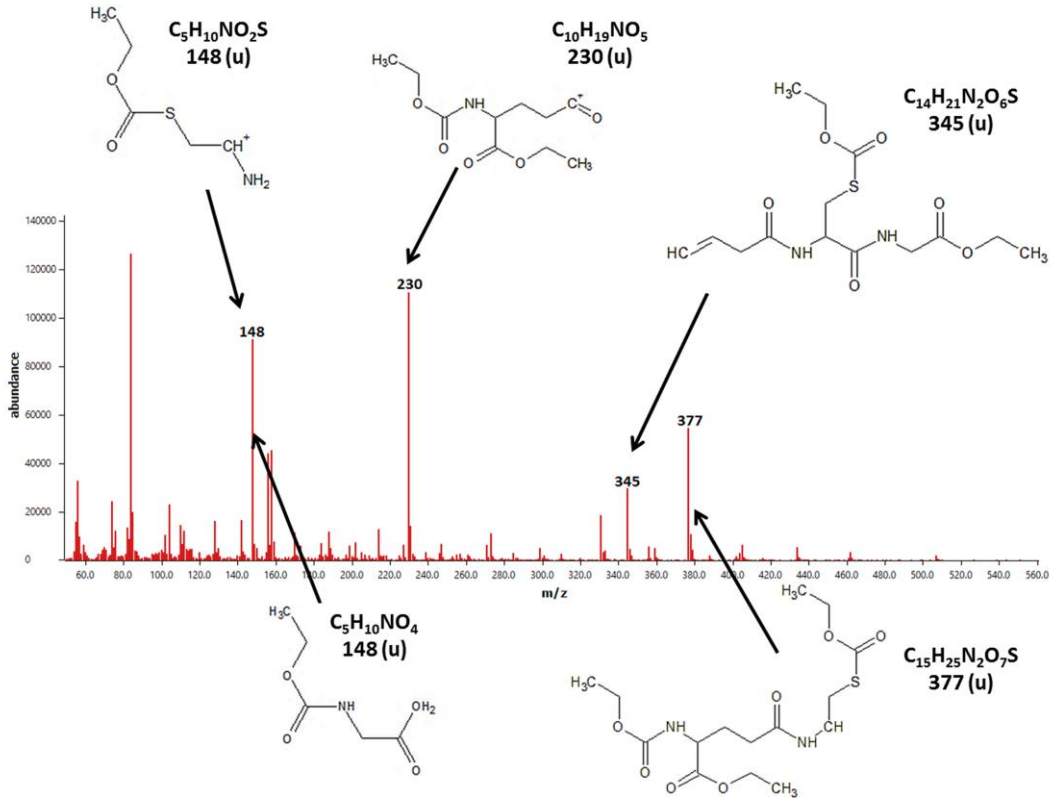

Figure 4. Hypothetical fragmentation pathways relative to the ions monitored in SIM mode for GSH N,S-ethoxycarbonyl ethyl ester.

Table 1. Selective ion monitoring (SIM) mode was employed for quantitative analysis. Retention times T_R, together with the monitored ions, are reported for both the GSH and the hGSH derivatives.

	GSH N,S-ethoxycarbonyl ethyl ester	**hGSH N,S-ethoxycarbonyl ethyl ester**
T_R (min)	6.97	7.07
MW (u)	507	521
target ion (m/z)	230	230
qualifying ions (m/z)	148-345-377	148-377-476

Table 2. RBC-GSH values relative to healthy control subjects (C) and hospitalized patients (P).

Individual	Class	GSH (µM/g HB)
1	C	1928.0
2	C	1718.7
3	C	1849.2
4	C	2039.4
5	C	1228.8
6	C	3040.9
7	C	2676.3
8	C	2280.3
9	C	2335.8
10	C	2343.4
11	C	1558.9
12	C	2200.4
13	P	991.0
14	P	437.1
15	P	1299.9
16	P	682.3
17	P	1115.1
18	P	1496.9
19	P	1638.5
20	P	862.4