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Determination of oxidized and reduced glutathione in pharmaceuticals by reversed-phase high-performance liquid chromatography with dual electrochemical detection

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Abstract

A sensitive and rapid reversed-phase HPLC method with electrochemical detection was developed for the analysis of glutathione in pharmaceutical preparations. The separation was achieved on an LC 18-DB (100×4.6 mm I.D.; 5 μm particle size) column. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid and acetonitrile (98:2, v/v). The effluent was monitored with dual electrochemical detection (applied potentials: E_1=+0.450 V; E_2=+0.750 V) in order to check simultaneously the declared amount of reduced glutathione and to quantify the related impurity oxidized glutathione. Limits of detection of 0.60 and 0.15 ng were achieved for the reduced and oxidized form, respectively. The method was validated and applied to the analysis of five commercial preparations containing reduced glutathione. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The tripeptide glutathione (L-γ-glutamyl-L-cysteinglycine, GSH) is the most abundant low-molecular-mass thiol in cells of many different organisms [1]. It plays an essential role in many important biological phenomena, including the synthesis of proteins and DNA, enzyme activity, metabolism and in protection of cells against toxic effects of oxidizing agents, free radicals, ionizing agents and certain exogenous compounds [2,3].

Glutathione sodium is used in the treatment of many types of intoxication of the human organism due to alcohol, drugs and heavy metals [4]. It has also been tried in idiophatic pulmonary fibrosis and in a number of other conditions including liver disorders, eczema, renal dysfunction and nephrotoxicity, corneal disorders and for mitigation of the side effects of antineoplastic therapy [5,6]. Furthermore, glutathione continues to be investigated in many different areas such as AIDS [7], Parkinson's disease [8] and anemia [9].

A number of analytical methods using HPLC have been developed for the analysis of glutathione (reduced and oxidized forms) based on different separation and detection techniques. Many of these methods include pre- or post-column derivatization with *o*-phthalaldehyde [10–12] or monobromobimane [13–17] or other fluorogenic reagents [18–20] and fluorimetric detection; otherwise GSH and the oxidized form GSSG can be converted to *N*-(2,4-dinitrophenyl) [21,22] or 4,4′-dithiodipyridine [23] derivatives and analysed by HPLC with UV detection.

Several methods have also been presented for the determination of both GSH and GSSG using HPLC

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with electrochemical detection (ED). Earlier methods were based on reduction of disulfides at an upstream electrode followed by detection of the thiols formed and pre-existent at the downstream gold/mercury electrode [24–29]. More recent papers report the use of two porous graphite detectors in series both set at positive potential: the first electrode is used for detection of GSH; oxidized glutathione is quantified with the second electrode held at a high positive potential. [30–35].

Very few of these reports concern the determination of glutathione in pharmaceuticals by HPLC: reduced glutathione is quantified using pre-chromatographic derivatization, removal of the excess reagent and reversed-phase HPLC separation followed by UV detection [36–38]. However, these methods seem to be rather complicated for application in the routine quality control of drugs; furthermore, none of the above-mentioned research reports the quantitation of the principal related impurity of GSH, GSSG.

In the paper of Gennaro et al. [39] a method using ion-interaction RP-HPLC with UV detection for the determination of both reduced and oxidized forms of glutathione in drugs is presented: in this method two different levels of dilution of the drug are used to quantitate the large amount of GSH and the low concentration of GSSG.

The purpose of this work was to develop a simple, rapid and sensitive method for simultaneous detection of reduced and oxidized forms of glutathione in order to check both the declared amount of reduced glutathione and the presence of oxidized glutathione in pharmaceuticals.

The method described was validated and applied to the analysis of glutathione-based commercial injectable preparations.

2. Experimental

2.1. Chemicals and reagents

Reduced glutathione (GSH) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany), and oxidized glutathione (GSSG) was from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Carlo Erba (Milan, Italy). Deionized, double-distilled water was used for the mobile phase preparation. Glutathione-containing drugs were commercial injectable formulations obtained from the national market.

2.2. Chromatographic system

The chromatographic apparatus consisted of a Series 410 BIO LC pump (Perkin-Elmer, Norwalk, CT, USA), a Rheodyne Model 7125 injection valve with a 20 μl sample loop (Rheodyne, Berkeley, CA, USA) and a Coulochem Model 5100A electrochemical detector (ESA, Belford, MA, USA) equipped with an analytical cell (Model 5014 B).

The working parameters for the electrochemical detector were +0.450 V (gain×5) for the first electrode and +0.750 V (gain×50) for the second; the signal of the upstream electrode was monitored on the first channel of the integration system to quantitate GSH; the signal generated by the downstream electrode, suitably amplified, was registered on the second channel to detect GSSG

For ED data collection and calculation, Waters Millennium Chromatography Manager software was used.

Isocratic separation was achieved at ambient temperature using a Supelcosil LC-18 DB column, particle size 5 μm, 100×4.6 mm I.D. (Supelco, Bellefonte, PA, USA). The mobile phase was 0.1% (v/v) TFA in water–acetonitrile (98:2, v/v). The run time was 8 min at a flow-rate of 1.2 ml/min.

The mobile phase, filtered through 0.45 μm filters (Gelman Sciences, MI, USA) before use, was continuously recirculated to the solvent reservoir and freshly prepared weekly.

2.3. Preparation of standard and sample solutions

Standard stock solutions of GSH and GSSG containing 0.3 and 0.1 mg/ml, respectively, were prepared in the mobile phase and stored in dark glass at +4°C for a week at most. The stability of the standard stock solution was checked over a period of 1 week, preparing and injecting daily a solution of each compound diluted in the mobile phase.

A working standard of both GSH and GSSG (6 and 0.15 μg/ml, respectively) was obtained daily by diluting the stock solutions with the mobile phase.

Sample solutions were prepared immediately be-

fore analysis by dissolving the powder for injection contained in one vial of the commercial preparation in the mobile phase and filling to 100 ml; this solution was then diluted to obtain an approximate concentration of thiol drug of 6 μg/ml. Solutions were then filtered through 0.45 μm nylon syringe filters.

3. Results and discussion

In order to obtain a good and rapid separation of reduced and oxidized glutathione, different columns and eluents described in former reports were used [23–25]. The main problems encountered using these elution methods were peak tailing or leading and, especially for GSSG which is eluted later, peak broadening. Therefore, an aqueous acetonitrile solution including 0.1% TFA was used as mobile phase; TFA is a weak hydrophobic ion-pairing reagent that also serves to maintain a low pH, thereby minimizing ionic interactions between the peptide and the stationary phase [40]. As a matter of fact, with the proposed method, adequate resolution, well-shaped peaks and short time of analysis were obtained.

The standard stock solution of GSH and GSSG prepared in this acidic mobile phase, stored at +4°C, showed no evidence of decomposition over a period of 1 week.

Fig. 1 shows typical chromatograms obtained by monitoring the signal of both channels after injection of 20 μl of a standard solution containing 6.1 μg/ml of GSH and 0.19 μg/ml of GSSG (retention times 2.3 and 5.7 min, respectively; run time 8 min);

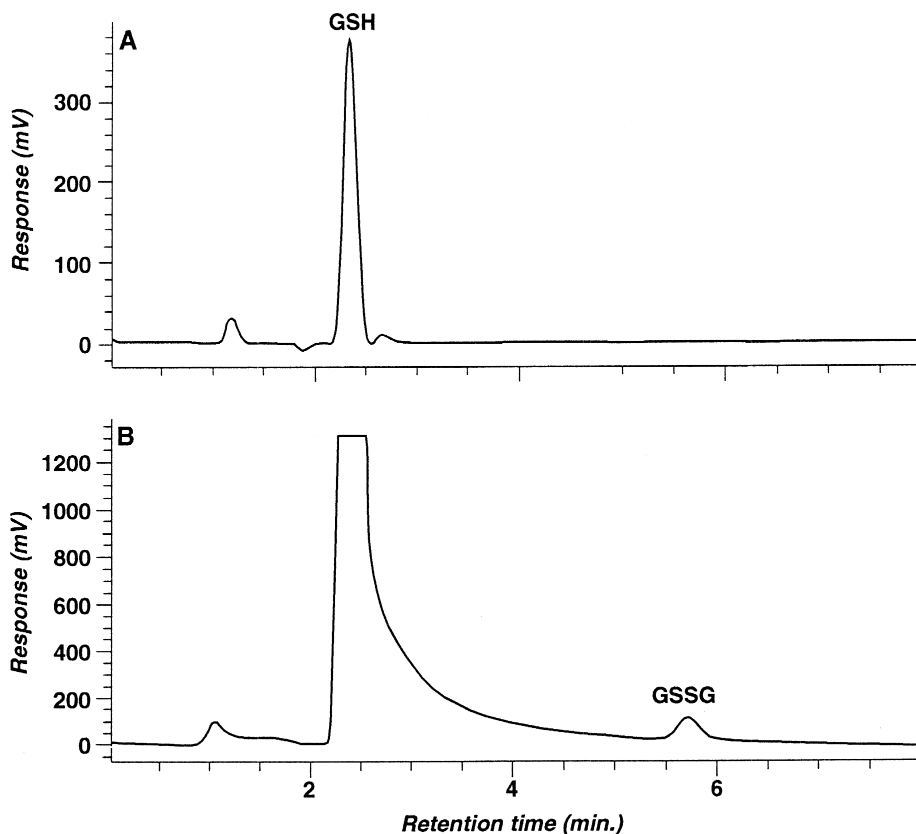

Fig. 1. Chromatograms obtained after injection of 20 μl of a standard solution containing 6.1 μg/ml of GSH and 0.19 μg/ml of GSSG in the mobile phase. (A) First electrode, $E=+0.450$ V, gain×5; (B) second electrode, $E=+0.750$ V, gain×50. Column, Supelcosil LC-18 DB, 100×4.6 mm I.D., 5 μm; mobile phase, 0.1% (v/v) TFA in water–acetonitrile (98:2, v/v); flow-rate, 1.2 ml/min.

almost identical chromatograms were obtained after injection of diluted drug solutions prepared as described in Fig. 2.

For the determination of the optimum working parameters for the electrochemical detection of GSH and GSSG the current–voltage curves were investigated.

The first curve was obtained by varying the upstream electrode potential from +0.200 to +0.650 V in 0.05 V steps while the potential of the downstream electrode was held at +0.750 V; to obtain the curve for GSSG the potential of the downstream electrode was changed from +0.500 to +0.800 V with the first electrode potential fixed at +0.300 V. The curves are shown in Fig. 2.

A response due to the oxidation of the thiol group of GSH was observed for a potential of greater than +0.200 V, while for GSSG the threshold potential was in the range +0.550 to +0.600 V. In accordance with previous work [30] the hydrodynamic voltammogram of GSH showed a further voltage-dependent increase before reaching the final plateau which was ascribed to the oxidation of the amine group.

On the basis of these results the potential of the first electrode was fixed at +0.450 V to detect GSH; the second electrode potential was set at +0.750 V in order to obtain the maximum sensitivity to quantitate the low amounts of GSSG.

Using the conditions described above, the detection and quantitation limits, linearity and repeatability were determined.

The detection limits at a signal-to-noise ratio of 3 were 0.60 ng for GSH and 0.15 ng for GSSG.

The detector response (E_1 for GSH and E_2 for GSSG) was linear in the range from 3 to 122 ng and from 0.65 to 13 ng for GSH and GSSG, respectively. Linear regression analysis of the data gave correlation coefficients (r) greater than 0.999 for both reduced and oxidised glutathione. Similar results were obtained when the detector response was expressed as peak heights instead of areas.

Inter- and intra-day repeatability was studied at three different concentrations; the results are shown in Tables 1 and 2: it can be seen that the relative standard deviations ranged from 0.6 to 3.4% for GSSG and from 0.5 to 2.7% for GSH.

Five preparations containing reduced glutathione which are commercially available in Italy were analysed using the proposed method.

The amount of active ingredient GSH and the

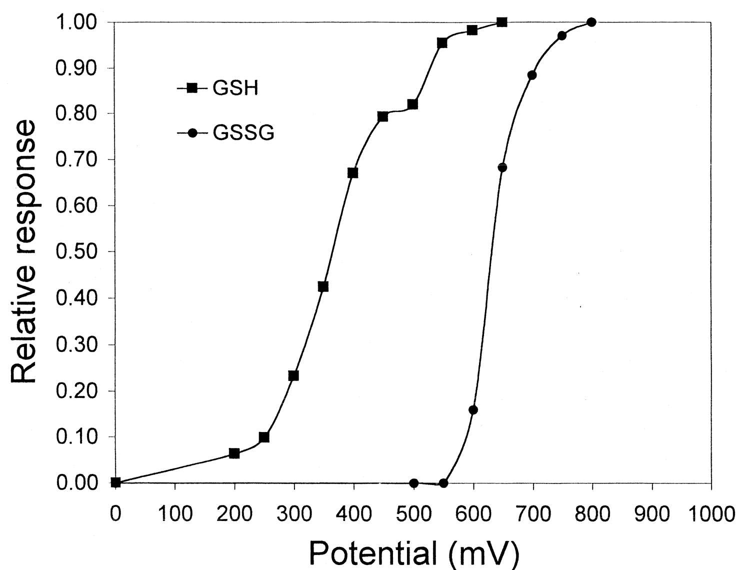

Fig. 2. Hydrodynamic voltammograms for GSH (upstream electrode) and GSSG (downstream electrode). The relative response is the ratio of the peak area measured at a given potential to that at plateau level. Values plotted are the mean of triplicate injections. The injected amounts were 30 ng for GSH and 10 ng for GSSG.

Table 1
Inter- and intra-day repeatability for GSH

Concentration (μg/ml)	RSD (%)			
	Day 1[a]	Day 2[a]	Day 3[a]	Overall[b]
0.771	0.70	0.96	0.99	2.0
1.52	0.49	0.83	0.58	2.2
3.04	0.49	1.20	0.73	2.7

[a] $n=5$.
[b] $n=3$.

Table 2
Inter- and intra-day repeatability for GSSG

Concentration (μg/ml)	RSD (%)			
	Day 1[a]	Day 2[a]	Day 3[a]	Overall[b]
0.0604	3.4	2.9	2.3	1.4
0.1208	2.0	2.7	2.7	0.4
0.604	1.3	0.9	0.6	2.4

[a] $n=5$.
[b] $n=3$.

weight/weight (w/w) percent of its related impurity GSSG were determined by employing the bracketing technique, i.e. injecting alternatively the working standard, the sample solution and the working standard solution again three times; the determination was repeated on five vials of the same batch for each preparation. Results are shown in Table 3. As can be seen the amount of active ingredient ranged from 96.7 to 103.8% of the declared amount. The w/w percentage of the related impurity GSSG varied from 1.3 to 2.0.

4. Conclusions

The HPLC separation system coupled with dual channel electrochemical detection described provides a sensitive, rapid and very simple method for routine analysis of glutathione-based commercial preparations allowing simultaneous dosage of the active ingredient and the quantitation of its principal related impurity, oxidized glutathione.

Table 3
Assay of GSH and related impurity GSSG in commercial dosage forms

Sample	Label claim (mg)	Found[a] mg (%)	RSD (%)	GSSG[a] (%, m/m)
A	600	580 (96.7)	2.6	1.7
B	600	594 (99.0)	2.5	1.3
C	600	597 (99.5)	1.0	1.8
D	600	612 (102.0)	0.8	1.8
E	600	623 (103.8)	1.6	2.0

[a] Mean of five determinations.

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