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Spectrophotometric determination of glutathione and of its oxidation product in pharmaceutical dosage forms*

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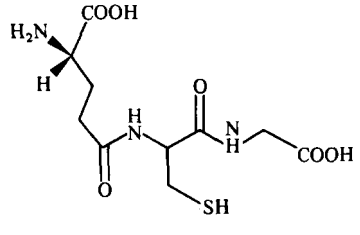
Abstract: A simple and sensitive spectrophotometric method suitable for the stability control of pharmaceutical dosage forms containing glutathione (γ-glutamyl-cysteinyl-glycine), GSH, is described. Besides GSH, the method quantitatively determines its oxidation product, GSSG. The colour reactions of GSH and GSSG with ammonium tetrachloropalladate have been investigated and the optimum reaction conditions, spectral characteristics and composition of the yellow water-soluble complexes have been established. The assay results of pharmaceutical formulations showed good accuracy and precision over the concentration range of 5×10^{-5}–6×10^{-4} M GSH.

Keywords: *Glutathione; oxidized glutathione; palladium(II) complexes; spectrophotometric determination; drugs assay.*

Introduction

Having been interested for some time in the development of new and improved methods for drugs analysis, we report on the assay of glutathione in pharmaceutical formulations.

Glutathione (GSH) is an ubiquitous tripeptide Glu-Cys-Gly (see Fig. 1) which directly or indirectly affects a number of cellular processes [1, 2]. Some of these biological activities, in particular the variety of detoxification reactions, have been exploited from some time in the treatment of poisoning [3] and more recently in radiotherapy and chemotherapy of neoplastic diseases [4]. With this in mind we have devised and implemented a simple and sensitive spectrophotometric method for the determination of GSH and of its oxidized form GSSG. A number of articles, reported in the literature, describe GSH determination in biological matrices (brain, plasma, liver, erythrocytes), mostly effected by means of HPLC [5–9]. Other investigations were carried out by means of electrochemical [10, 11] fluorimetric [12] and spectrophotometric [13, 14] methods.



N-(N-L-γ-glutamyl-L-cysteinyl) glycine

Figure 1
Molecular structure of the glutathione (GSH).

On the other hand, there are few reports of GSH analysis in pharmaceuticals. Among the official textbooks only Medicamenta [15] reports the monograph *Glutathione* and its determination by means of an iodometric titration. The method proposed is based on the use of reagent ammonium tetrachloropalladate which reacts both with GSH and with GSSG originating coloured complexes, to which spectrophotometric determination can be applied. This reagent has already been used by the authors [16] for the assay of other mercapto-compounds, such as penicillamine and mercaptopropionylglycine.

Experimental

Apparatus

A double-beam spectrophotometer (Jasco, UVIDEC 610) and a single-beam spectrophotometer (Jasco, UVIDEC 4) were used. pH values were measured with a Crison model 501 pH-meter.

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Materials

All chemicals were of analytical grade and were obtained from Fluka (Switzerland); deionized and double-distilled water was used. Stock solutions of GSH, GSSG and $(NH_4)_2PdCl_4$ (in HCl 1 N) were 2×10^{-2} M, and were used to prepare standard solutions by appropriate dilutions.

The examined pharmaceutical formulations containing GSH were commercially available in Italy and were the following: Tationil 300 and Toxepasi complex (from Boehringer Biochemia Robin) and Tad 300 (from Biomedica Foscana).

Continuous variation method (Job's method) [17, 18]

To serial volumes (from 1 to 5 ml) of a 2×10^{-3} M GSH solution, portions (from 5 to 1 ml) of a 2×10^{-3} M $(NH_4)_2PdCl_4$ solution were added and brought to 10 ml volume with H_2O. The absorbance values were measured after 5 min at 380 nm against the corresponding blanks, and reported in graph versus the [GSH]/[GSH]+[Pd] values (Fig. 2).

Calibration curves of GSH, GSSG

Serial volumes (from 0.5 to 6.0 ml) of 10^{-3} M standard solution of GSH were treated with 3 ml of a standard $(NH_4)_2PdCl_4$ solution in HCl 1 M (2×10^{-3} M for the low GSH concentration range and 6×10^{-3} M for the high GSH concentration range) and brought to volume with distilled H_2O in a 10 ml volumetric flask. The samples were analysed in a double-beam spectrophotometer, against a blank, at a $\lambda = 380$ nm. Absorbance readings were taken 5 min after sample preparation. The absorbance values obtained were reported against GSH concentrations, obtaining a good linear plot.

In preparing GSSG samples a similar procedure was followed, except that samples were heated at 75°C for 1 h before recording the spectra.

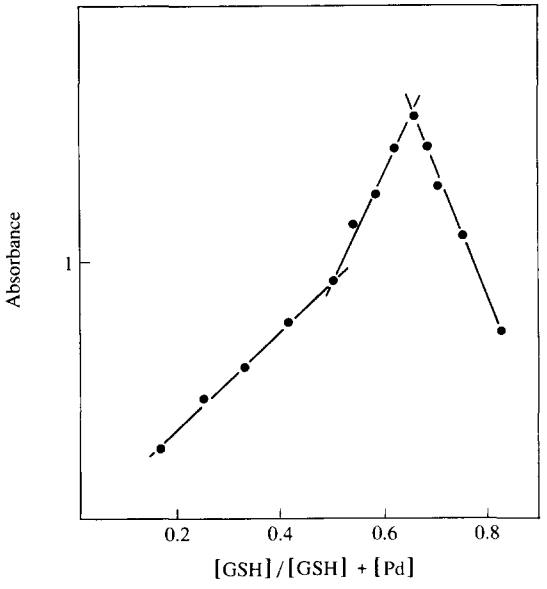

Figure 2
Absorbance versus molar fraction [GSH]/[GSH]+[Pd] (Job's plot).

Applications to pharmaceutical formulations

GSH determination in Tationil 300 (lyophilized Na–GSH powder for instant preparation of injectable solutions). The lyophilized content of a 300 mg sample was carefully ground in a mortar, weighed and dissolved in H_2O so as to obtain a GSH solution of 2×10^{-2} M concentration. Upon suitable dilutions and mixing with a 6×10^{-3} M $(NH_4)_2$ $PdCl_4$ solution the sample for spectrophotometric recording was obtained.

GSH determination in Tad 300 (lyophilized Na–GSH powder). The procedure described above was followed.

GSH determination in Toxepasi complex (lyophilized powders containing: reduced GSH, 100 mg, uridine-5'-diphosphoglucose, 20 mg, cyanocobalamine, 1000 mg). A procedure analogous to the one described above was followed for sample preparation.

Results and Discussion

The rationale of the devised spectrophotometric procedure is linked to the formation of a yellow hydrophilic complex resulting from the reaction of $(NH_4)_2PdCl_4$ (in HCl) with a mercaptan, in the present case GSH:

$$GSH + Pd(II) \rightleftarrows GSH—Pd(II). \quad (1)$$

Spectrophotometric measurements were carried out over the range of pH values 0.5–3, over 300–500 nm. The glutathione reacts with Pd(II) at all examined pH values, producing two stoichiometric different complexes, the relative yields of which do not depend on the pH value, but only on the molar ratio GSH:Pd. The composition of the complexes was deter-

mined by Job's continuous variation method. As one can see in Fig. 2, the plot shows clearly the presence of two complexes, corresponding to [Pd]/[GSH] molar ratios of 1:1 and 1:2, respectively, according to literature [11]. Since the first complex is formed very rapidly (the reaction is complete in a few minutes), unlike the second which requires some hours, we selected to carry out the reaction (1) with Pd(II) in excess. In Fig. 3, (a) shows the UV–vis spectrum of Pd:GSH (1:1) complex. Since excipients may interfere in the UV range, the band at 380 nm was chosen for the analytical procedure.

In order to obtain information about the equilibrium and the complexation kinetics of reaction (1), the effect of palladium concentration, on the absorbance values has been examined at constant [GSH]. The equilibrium position of reaction (1) was markedly shifted to the right: the absorbance values for a range of molar ratio Pd/GSH from 1:10 were the same. A molar ratio $\geqslant 3$ was chosen on the basis of kinetics considerations: at room temperature the complexation process reached equilibrium in 3–5 min. The stability of this complex was good; the absorbance values remained unchanged for at least 2 h.

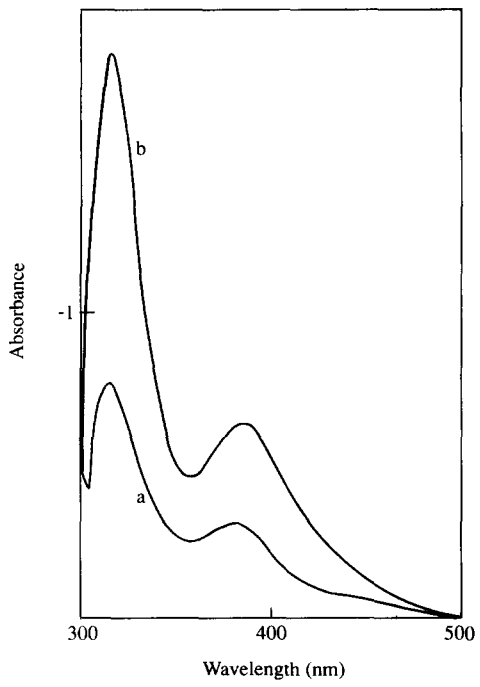

Figure 3
UV–vis spectra of (a) Pd–GSH complex, 2×10^{-4} M in 0.3 M HCl, and (b) Pd–GSSG complex, 2×10^{-4} M in 0.3 M HCl.

Under the optimal conditions, a calibration curve was made and a linear relationship was found over the range 5×10^{-5}–6×10^{-4} M of GSH; the regression equation ($y = 1505x + 0.0068$) had a correlation coefficient of 0.9999 ($n = 8$) and the relative standard deviation (RSD%) was 0.71% for a 3×10^{-4} M GSH solution ($n = 6$). The validity of the proposed method for the assay of the pharmaceutical dosage forms was tested by analysing a few commercial formulations containing GSH, alone or in association.

The results are shown in Table 1, where for comparison, the results obtained by iodometric titration [15] are also reported. The agreement is satisfactory but the spectrophotometric procedure appears better in terms of precision, accuracy and sensitivity, therefore quite suitable for quality control of pharmaceutical dosage forms.

The accuracy of the proposed method was assessed by recovery studies. Known volumes of standard solution of GSH were added to a known amount of the pharmaceutical form and the recovery ($n = 5$) was evaluated by the procedure described:

recovery = 100.1% (RSD% = 0.8%) for I;
recovery = 99.7% (RSD% = 0.9%) for II;
recovery = 99.1% (RSD% = 1.2%) for III.

One important characteristic of the method is

Table 1
Assay results of GSH and its oxidation product, GSSG, in pharmaceutical formulations ($n = 6$)

Pharmaceutical formulations	Spectrophotometric method GSH found (%)	RSD (%)	GSSG found (%)	Iodometric method GSH found (%)	RSD (%)
I-Tationil (lyophilized GSH as Na salt)	100.6	0.9	—	102.4	1.5
II-Tad 300 (lyophilized GSH as Na salt)	99.5	1.1	—	102.1	1.6
III-Toxepasi (lyophilized GSH in association)	98.9	1.3	—	101.8	1.8

the fact that it assays the stability of GSH formulations; besides GSH, the method quantitatively determines the oxidized form GSSG.

The oxidized glutathione also reacts with $(NH_4)_2PdCl_4$, in acidic medium, to give a yellow complex, whose spectrum [Fig. 3(b)] is similar to that of GSH–Pd complex but with higher values of the molar absorptivity. The stoichiometry of the Pd(II) complex with GSSG is 2:1 (2 Pd:1 GSSG) as shown in a recent investigation [19]. The formation of this complex is very slow, taking several hours to reach the equilibrium state, but it can be markedly accentuated by an increase in the reaction temperature. Under the optimum experimental conditions (heating for 1 h at 75°C; molar ratio $2 < [Pd]/[GSSG] < 10$), a calibration curve was constructed ($y = 3040x + 0.0147$; $r_c = 0.9997$; $n = 8$). The Lambert Beer law was observed over the concentration range $0.25–3 \times 10^{-4}$ M GSSG. The precision was satisfactory (RSD% = 0.92% for a 2×10^{-4} M GSSG solution; $n = 6$). The procedure was applied to the analysis of pharmaceutical forms (see Table 1), in order to control the presence of GSSG traces.

The samples, already treated with Pd(II) for GSH determination, were heated at 75°C for 1 h and examined spectrophotometrically. No absorbance change was observed indicating that the GSH drug was not altered. On the other hand, the addition to the samples of standard solutions of GSSG gave rise to a detectable increase in absorbance, having a determination limit value of 10 ppm GSSG. In conclusion, the spectrophotometric method, based on the use of Pd(II), seems suitable for the stability control of pharmaceutical dosage forms containing GSH.

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