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Separation of reduced and oxidized glutathione in a pharmaceutical preparation by ion-interaction reversed-phase HPLC

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Abstract: A new method is presented for the simultaneous determination of reduced and oxidized glutathione by ion-interaction reversed-phase HPLC with octylamine orthophosphate as the interaction reagent, 5-μm Spherisorb ODS-2 as the stationary phase and UV detection at 230 nm. Lower limits of detection of 2.50 μg ml^{-1} have been achieved for both the oxidized and the reduced forms. The method has been applied in the analysis of a commercial pharmaceutical preparation.

Keywords: *Ion-interaction reversed-phase HPLC; glutathione determination; separation of reduced and oxidized glutathione; reduced glutathione; antioxidants; radical scavengers.*

Introduction

L-γ-glutamyl-L-cysteinyl-glycine (Fig. 1), generally known as reduced glutathione, is one of the principal non-proteic thiolic compounds naturally present in living plant and animal cells [1]. Glutathione plays an essential role in cellular metabolism and has important functions in many biological processes such as enzyme activity control [2] and amino-acid transport in tissue cells [1, 3].

Moreover it is very efficient in fighting many types of intoxication of the human organism, such as alcoholism and the side-effects from medical treatment with chemotherapeutic, antituberculosis, antimalarial and anticancer drugs [4–7].

Glutathione is also known as a 'radical scavenger' because it acts as the natural defence of the body against cell damage from ionizing radiation, free radicals, peroxides and other oxidizing agents [2, 8–10].

When acting as a reductant, glutathione forms the so-called oxidized glutathione [11] (Fig. 1). The concentration ratio between reduced and oxidized glutathione is a very useful parameter in the diagnosis of glutathione metabolism disorders [2]. In addition, recent studies [5] have shown that precursor amino-acids such as glutamic acid and glutamine can affect regulation of the glutathione level in cells.

Methods of the analysis of reduced glutathione generally use electroanalytical techniques [10] or HPLC with electrochemical detection [3, 8, 9, 12, 13]. Only some HPLC methods utilize UV or fluorescence detection [4, 14]. Because of growing interest in the biological role of sulphydryls and disulphides, some investigations deal with the separation of reduced and oxidized glutathione, employing HPLC with electrochemical detection [11, 15] or HPTLC [2].

In the present work, a HPLC method for the simultaneous determination of reduced and oxidized forms of glutathione is presented; the technique is ion-interaction reversed-phase chromatography, with octylammonium orthophosphate as the interaction reagent, a C-18 reversed-phase column as the stationary phase and UV detection at 230 nm. The method has been applied in the analysis of a commercial pharmaceutical preparation [16] containing reduced glutathione.

Experimental

Apparatus

Analyses were carried out with a Merck-Hitachi (Merck, Germany) Lichrograph

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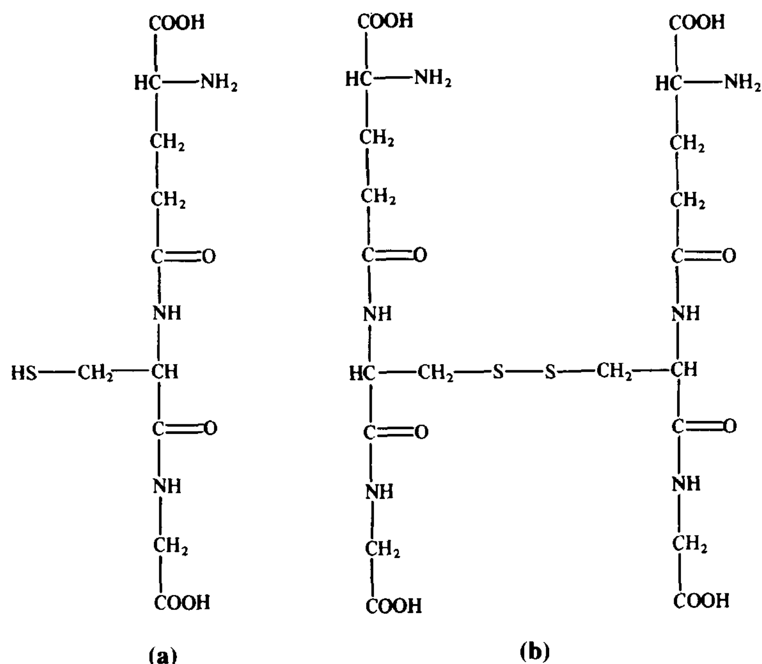

Figure 1
Structural formulae of (a) reduced glutathione and (b) oxidized glutathione.

chromatograph model L-6200, equipped with a two-channel Merck-Hitachi Model D-2500 Chromato-Integrator (Merck) interfaced with an UV/UV–vis detector L-4200 and a L-3720 conductivity detector (Merck).

For pH measurements, a Metrohm 654 pH-meter (Metrohm, Switzerland) equipped with a combined glass–calomel electrode was employed, and for the evaluation of absorptivity values a Hitachi 150-20 (Hitachi, Japan) spectrophotometer operated at 230 nm was used.

Chemical and reagents

Ultra-pure water from Millipore Milli-Q (Millipore, France) was used for the preparation of solutions.

Octylamine and oxidized glutathione were Fluka analytical grade reagents; reduced glutathione was a Merck reagent. All other reagents were C. Erba analytical grade chemicals.

Chromatographic conditions

5-μm Spherisorb ODS-2 in a 250 × 4.6 mm i.d. column was used as the stationary phase, characterized by a 12% carbon load (0.5 mM g^{-1}, w/w) and was fully endcapped.

0.0050 M octylammonium orthophosphate for use as the interaction reagent was prepared [17–20] by dissolving a weighed amount of octylamine in ultra-pure water and adjusting the pH of the solution to 6.4 ± 0.4 by addition of orthophosphoric acid. Under these pH conditions, taking into account the value of the acidic formation constant, octylamine is present as octylammonium ion but the composition of the salt is not exactly stoichiometric; however, for simplicity, the term octylammonium orthophosphate will be applied to this salt. The solution was freshly prepared every third day.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained. The repeatability of measurements of retention time was within 2% for sequential measurements and the reproducibility was within 4%. The column was regenerated with ultra-pure water for 10 min at a flow-rate of 0.3 ml min^{-1} and then with water–methanol (1:1, v/v) overnight at a flow-rate of 0.1 ml min^{-1}.

Preparation of standard solutions and samples

The prepared standard solutions (1000 μg ml^{-1}) of reduced and oxidized glutathione were kept in dark-glass flasks and stored at 4°C.

The sample was prepared by dilution with

ultra-pure water and filtration through a 0.2-μm Anotop 25 Plus syringe-filter.

Results and Discussion

Reversed-phase ion-interaction HPLC was employed. The interaction reagent 0.005 M octylammonium phosphate (at pH 6.4) was freshly prepared and was the only component of the mobile phase; it was eluted under isocratic conditions through a reversed-phase ODS-2 stationary phase. The interaction reagent determines the so-called 'dynamic functionalization' of the column itself; the stationary phase is modified by the flowing interaction reagent. Sorption effects and electrostatic forces result in the formation of an electrical double layer on the surface. Previous results on the separation of anionic species and amines [17–20] showed that injected anions and amines give rise to ion-pairs which in turn can be adsorbed on the stationary phase and retained; anions form an adsorbable species with the ammonium ion of the interaction reagent whereas amines give rise to ion-pairs with the anion.

In this work, the use of octylammonium orthophosphate as the interaction reagent (flow-rate = 1.0 ml min^{-1}), a reversed-phase ODS-2 column as the stationary phase and UV-detection at 230 nm permitted the separation of the reduced and oxidized forms of glutathione, as well as of related compounds such as cysteine and glutamic acid which are of relevant interest [5] in glutathione metabolism.

Capacity factors K' and estimates of their standard deviation are: cysteine 0.5 ± 0.1; glutamic acid 2.0 ± 0.1; oxidized glutathione 4.9 ± 0.2; and reduced glutathione 7.6 ± 0.2. Dead time t_o (2.20 min) was evaluated from elution of $NaNO_3$ and unretained Na^+ was detected by conductance. The molar absorptivity values at 230 nm for reduced and oxidized glutathione are $2.8 \pm 0.2 \times 10^2$ and $1.25 \pm 0.04 \times 10^3$ mol 1 cm^{-1}, respectively.

Figure 2 shows the separation of a mixture of oxidized (peak a) and reduced (peak b) glutathione, both at a concentration of 50.0 μg ml^{-1}, obtained at a flow-rate of 1.5 ml min^{-1} (t_o = 1.43 min). The peaks show comparable

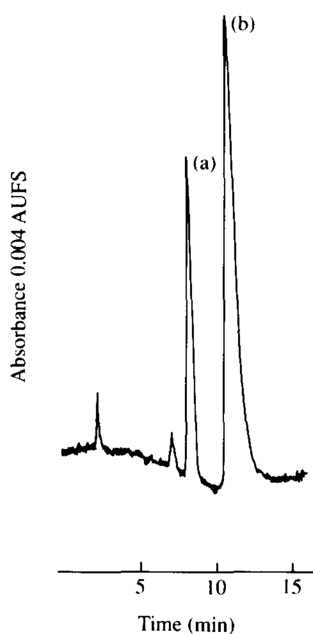

Figure 2
Separation of a mixture of (a) oxidized glutathione (50.00 μg ml^{-1}) and (b) reduced glutathione (50.00 μg ml^{-1}). Stationary phase: Spherisorb ODS-2 (250 × 4.6 mm i.d.). Ion-interaction reagent: 0.005 M octylamine orthophosphate. Flow-rate 1.5 ml min^{-1}. Injection 100 μl. UV-detection 230 nm.

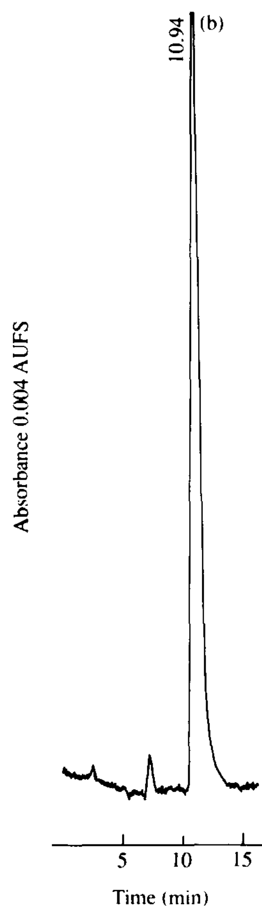

Figure 3
Chromatogram recorded for a solution of the pharmaceutical formulation 'Tationil' diluted 1/1000, v/v. Chromatographic conditions as in Fig. 2.

areas, even if the absorptivity of oxidized glutathione at this wavelength is more than four times that of the reduced form; this behaviour was confirmed by other experiments performed with mixtures of the analytes in concentrations of 3.00–500.0 μg ml^{-1}. Furthermore, the oxidized form eluted before the reduced form; the results conflict with previous results of the authors obtained under similar experimental conditions where similar structures were retained in proportion to their molecular mass [20].

In order to explain this behaviour, some correlation can be proposed between the retention time and the molecular structure of the analytes, taking into account the values for the acidic formation constants of the functional groups involved in retention. As mentioned above, under the chromatographic conditions employed here, the retention of analytes occurs through the formation of an ion-pair, which the analyte can form either with the cation (the protonated amine) or with the anion of the interaction reagent. The acidic dissociation constants of reduced glutathione are: $pK^{H}_{1} = 2.12$; $pK^{H}_{2} = 3.53$; $pK^{H}_{3} = 8.66$; and $pK^{H}_{4} = 9.12$ [21]. Under the experimental pH conditions (pH 6.4), the reduced glutathione molecule could be retained either through the carboxylate groups (so giving rise to an ion-pair with octylammonium) or through the —NH_2 (which being protonated at pH 6.4, can form an ion-pair with orthophosphate). In contrast, the —SH group ($pK^{H} = 8.66$) does not seem to be present under suitable conditions for participating in retention. The same considerations hold also when the retention of oxidized glutathione is con-

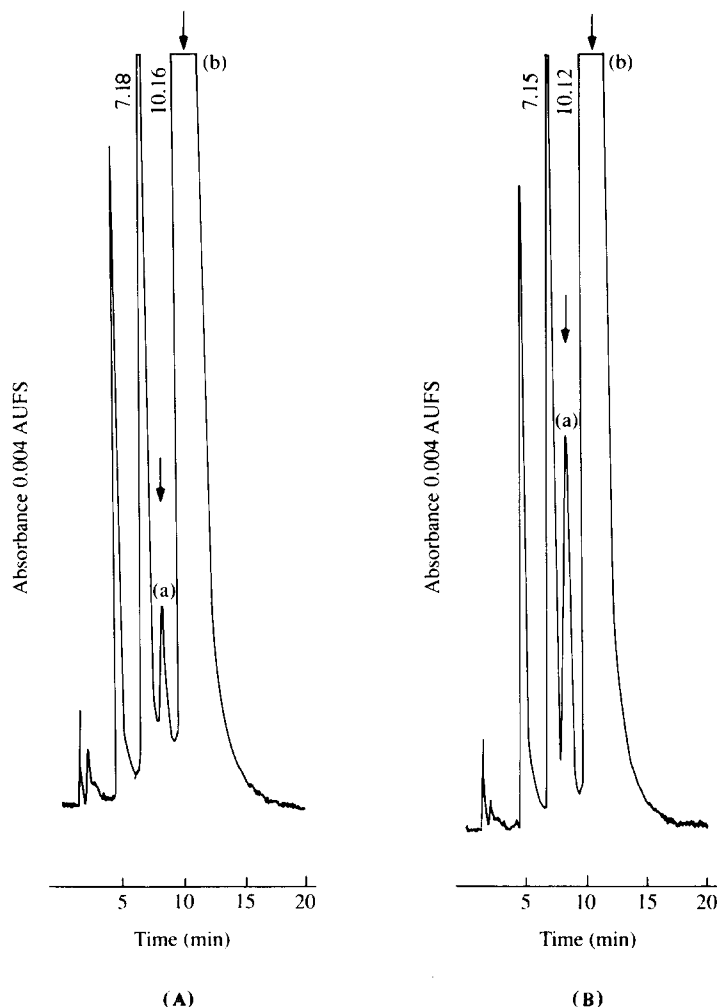

Figure 4
Chromatograms recorded for a solution of the pharmaceutical preparation 'Tationil' diluted 1/20, v/v (A), and with added 50.00 μg ml^{-1} of standard oxidized glutathione (B). Conditions and peaks of identification as in Fig. 2.

sidered (Fig. 1) so that the different sensitivity observed for the two forms of glutathione seems therefore not to be ascribable to a different retention mechanism.

It is concluded that the method presented here for the separation of reduced and oxidized glutathione possesses advantages of high resolution, speed and simplicity.

Application to a pharmaceutical preparation

The method was applied to the analysis of a glutathione-based commercial preparation [16] in order to check both the declared amount of reduced glutathione and the presence of oxidized glutathione.

Figure 3 shows a chromatogram recorded for a solution of the drug diluted 1/1000, v/v, in which only the peak due to reduced glutathione can be seen. Quantitative analysis (based on a standard calibration curve) resulted in a content of 104.1 ± 5.1 g l^{-1} for the drug, within the acceptable 5% deviation from that declared (300 mg 3 ml^{-1}). In order to check for the presence of oxidized glutathione, chromatograms were recorded for injections of the drug diluted 1/20, v/v. As shown in Fig. 4(A), some oxidized glutathione, confirmed by standard addition [Fig. 4(B)], is also present in the preparation at a concentration of 0.47 ± 0.06 g l^{-1} (i.e. about 0.5% of the total).

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