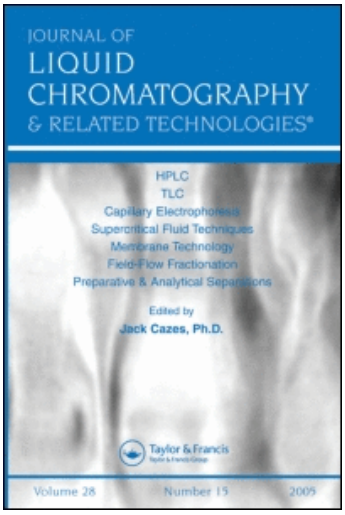

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Study of the Reaction of Glutathione with 2,2,6,6-Tetramethyl-4-Hydroxy Piperidine Nitroxide by RP-HPLC

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STUDY OF THE REACTION OF GLUTATHIONE WITH 2,2,6,6-TETRAMETHYL-4-HYDROXY PIPERIDINE NITROXIDE BY RP-HPLC

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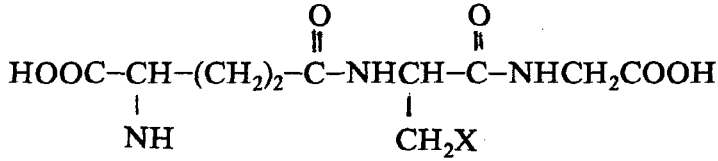
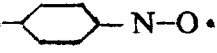
ABSTRACT

In this paper, the quantitative determination of the oxidation products from the reaction mixture of glutathione with 2,2,6,6–tetramethyl–4–hydroxy piperidine nitroxide was carried out satisfactorily by RP–HPLC with an external standard method. The relative error of the quantitative analysis was less than ± 2.0%, the standard deviation was less than 0.68 and the recovery rate was almost above 99%.

INTRODUCTION

Glutathione (GSH) possesses biological activity [1], playing an important role especially in the maintenance of mercapto groups in some proteins and cell protection membranes. Consequently, studies of the structure and properties of GSH have always been one of the most active subjects in biochemistry. However, very few analytical methods for GSH and its oxidation products, such as glutathione sulfonic acid (GSO_3H) and glutathione disulfide (GSSG), have been studied in the past. An indirect determination method for derivative preparation was reported by J. Reeve et al [2]. The direct determination for the reaction mixture of GSH with 2,2,6,6–tetramethyl–4–hydroxy piperidine nitroxide (1) by RP–HPLC has not been reported so far. In this paper, the quantitative determination of the oxidation products from the reaction mixture of GSH and 1 was carried out satisfactorily by RP–HPLC with an external standard method. Upon reaction of GSH with excessive 1 in different PH buffers, the main components in the product were glutathione sulfonic acid (GSO_3H), glutathione disulfide (GSSG) and excess reactant 1. The structures of all reactants and products were the following:



1: 

GSH: X = –SH

GSO_3H: X = $-SO_3H$

GSSG: X = –SSG

EXPERIMENTAL

APPARATUS

Chromatographic experiments were performed with a Model Hitachi 635–AR high–performance liquid chromatograph equipped with a Hitachi 200–10 double–beam UV detector.

REAGENTS AND STANDARD SAMPLES

The standard samples of 1, GSO_3H, GSSG were prepared following published procedures[3–5]. Chemicals were obtained from a variety of suppliers and their aqueous solutions were prepared in redistilled water.

RESULTS AND DISCUSSION

CONDITIONS FOR QUANTITATIVE ANALYSIS

The choice of solvent was carried out by a trial and error method on the basis of TLC tests with YWG–$C_{18}H_{37}$, 10μ, 200 × 4mm, chromatographic column, using methanol / 0.22% acetic acid (11:100 v / v) as mobile phase; good resolution was obtained, but the retention time of 1 was too long (42 minutes). In order to shorten analysis time, mobile phase flow rate was changed from 0.6ml / min to 2.5ml / min at 8 minutes after in-

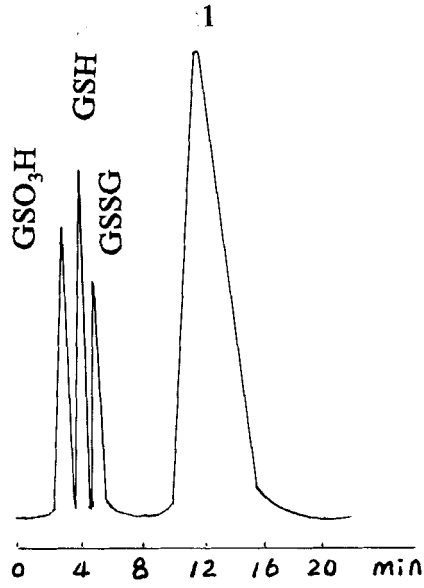


Figure 1. Chromatogram of a standard mixture.
colum: 200 × 4mm, YWG–$C_{18}H_{37}$, 10μ; detector: UV, 254nm; mobile phase: methanol / 0.22% acetic acid (11:100 v / v); flow rate: 0.6ml / min (0–8min), 2.5ml / min (8–20min).

jection of sample. All components passed through the column within 20 minutes under this condition, and it was possible to determine GSH GSO_3H and GSSG within 8 minutes (Figure 1).

QUALITATIVE ANALYSIS

With reaction mixture and standard mixture separated under the same chromatographic condition, respectively, two

corresponding peaks with the same retention were scanned on–line with a fast scanning UV detector. The resultant UV spectra were identical.

QUANTITATION ANALYSIS

Preparation of the Mixture (A) of Standard GSO_3H, GSSG

Various amounts of GSO_3H, GSSG were weighted exactly and put in eight 5mL volumetric flasks. Then 0.5mL buffer solutions (HAC–NaAC–$NaBO_3$) with different pH values were added, respectively, which were consistent with those of reaction mixtures. The solutions were finally diluted to the 5.0mL graduation with water.

Preparation of Sample Solution (B)

0.5mL reaction mixtures of 1 and GSH in various ratios were put in eight 5mL volumetric flasks, respectively, then diluted to the graduation with water.

Sample Determination

Under the chromatographic conditions of Figure 1. A and B solutions were alternately injected. The contents of GSO_3H and GSSG in B solution were determined by simple point correction method. The results are given in Table 1. It was indicated from Table 1 that the relative contents of two oxidation products had the opposite trends with increasing pH of the system. In strongly acidic solutions, GSO_3H was the main product, but at pH > 9, GSSG was the main product (Figure

Table 1. Quantitative Results of Products GSO_3H and GSSG by HPLC

pH / concentration	2.0	2.5	3.8	5.0	6.0	7.5	8.5	9.5
$GSO_3H \times 10^2$(M)	6.44	5.45	4.66	2.93	2.17	1.38	0.71	0
$GSSG \times 10^2$(M)	0	0.36	1.13	1.59	1.92	2.81	3.28	3.50
molar percentage of GSSG	0	6.2	19.4	35.1	47.0	67.1	82.3	100
molar percentage of GSO_3H	100	93.8	80.6	64.9	53.0	32.9	17.7	0

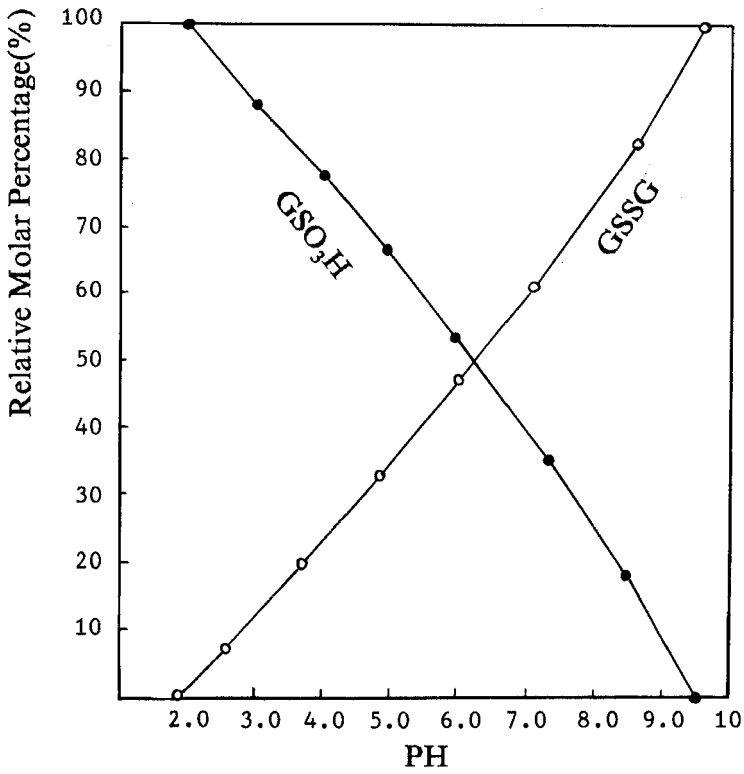


Figure 2. Correlation of the distribution of GSO_3H and GSSG with PH

Table 2. Results of Recovery Test

components	prepared values (mg / ml)	measured values (mg / ml)	recovery (%)	relative error (%)
GSO_3H	2.24	2.24	100.0	± 1.98
	2.02	1.92	95.1	
	1.12	1.11	99.1	
	0.44	0.45	102.3	
	0.22	0.22	100.0	
GSSG	0.44	0.45	102.3	± 1.09
	0.66	0.64	97.0	
	1.10	1.11	100.9	
	1.93	1.96	99.0	
	2.20	2.21	100.5	

Table 3. Results of Reproducibility Tests

Components	X[a]	SD[b]	CV%[c]
GSO_3H	43.9	0.32	0.73
GSSG	133.1	0.63	0.51

a: X was average values of content

b: SD was standard deviation

c: CV was coefficient of variation

2.). The result was not consistent with that observed previously. This method was available to study the oxidation of strong polar peptides and their products.

Recovery and Reproducibility

Tests for recovery and reproducibility were conducted in order to examine the reliability of the method. Table 2 showed that the recovery rate was almost above 99% with a relative error of below ± 2%. The results observed in five repeated tests for each standard sample are given in Table 3, SD < 0.68.

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