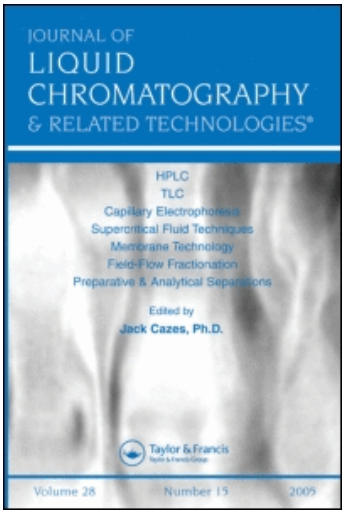

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ANALYTICAL SEPARATION OF REDUCED AND OXIDIZED FORMS OF GLUTATHIONE FROM AMINO ACID MIXTURES BY OVERPRESSURED THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A new method has been developed for the separation of reduced and oxidized forms of glutathione from amino acid mixtures. The samples were spotted on Kieselgel plates and developed in phenol-water = 7:3 containing SDS. The separation was performed in pressurized ultramicro chamber. The running time was shorter than that in a normal chamber, decreasing the possibility of diffusion. In addition to the two forms of glutathione, nine different kinds of amino acids could be separated. The method affords a possibility for rapid analysis of two forms of glutathione both in the clinical and industrial practice.

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INTRODUCTION

The tripeptide glutathione, γ-glutamylcysteinylglycine, has an important role in biochemical processes, such as the maintenance of the sulfhydryl state of cysteinyl side-chains of proteins, regulation of enzyme activity by disulphide interchange, detoxifying processes, removal of peroxides generated in the metabolism (1). Glutathione is present mainly in its reduced form in the tissues and the oxidized one is a lower concentration.

There are several chromatographic methods for determination of glutathione. It has been separated with two-dimensional paper chromatography using phenol and collidine as solvents, or one-dimensional chromatography using propanol-water mixture after blocking of the sulfhydryl groups with N-ethylmaleimid (2). Glutathione and its derivatives could be separated from other thiols and ninhydrin-positive compounds by HPLC analysis (3-4) or in amino acid analyser (5).

Murata and his cowerkers (6) have published a procedure for the continuous production of glutathione from its constituent amino acids using immobilized whole cells of Saccharomyces cerevisiae entrapped in poliacrylamide gel. The cell membrane has become permeable for glutathione releasing it continually from the cells. An intensive amino acid synthesis accompanying the tripeptide production has been found.

The aim of the present study was the separation and determination of reduced (GSH) and oxidized glutathione (GSSG) in a biological mixture. The overpressured thin-layer chromatographic technique (OPTLC) reported by Thihák et al. (7-8) has been adopted. The method proved to be suitable for the separation of two forms of glutathione in phenol--water solvent system which has high viscosity.

MATERIALS AND METHODS

Chemicals for general use were analytical grade and purchased from Reanal Factory of Laboratorial Chemicals (Budapest, Hungary). Amino acid calibration mixture was purchased from BIO-RAD Laboratories (Richmond, California, USA), phenol was obtained from Loba Chemie (Wien, Austria) and it was vacuum destilled from magnesium. The Kieselgel 60 F_{254} and HPTLC chromatoplates were purchased from Merck (Darmstadt, FRG).

The development of sheets was performed in Crompres[10] pressurized ultramicro chamber obtained from Labor MIM (Budapest, Hungary). The concentration of glutathione was measured with Telechrom OE-974 videodensitometer, Chinoin Pharmaceutical Work, Ltd. (Budapest, Hungary).

EXPERIMENTS

Saccharomyces cerevisiae IFO 2044 cells were entrapped in polyacrylamide gel according to the method of Murata et al. (6).

For the production of glutathione the immobilized cells were incubated in a mixture containing 0.5 M glucose, 0.01 M $MgCl_2$, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (pH 7.0), at 303 K.

The amino acids and glutathione were dissolved in 0.1 M potassium phosphate buffer (pH 5.5), their concentration varied from 1.7 to 2.3 mg/ml, each spot represents about 3-4 µg amino acid.

The chromatoplates were developed in phenol-water 7:3 (w/w) solvent system containing sodium dodecyl sulfate (SDS).

The concentration of SDS was varied between zero and one percentage. The chromatography was performed in Chrompres[10], in linear arrangement, at 303 ^{o}k. The development required about 50 minutes with a longer migration distance, setting a filter paper stripe (20x13 cm) on the top of chromatoplates. The development was conducted until the solvent saturated the filter paper. The membrane pressur was 1.2 MPa. Flow rate was varied from 3.08×10^{-3} cm^3/sec to 2.75×10^{-3} cm^3/sec, depending on the concentration of SDS in the solvent.

To remove the phenol from the layers, the plates were dried at 413 K, in -0.9 kp/cm^2 vacuum for 30 minutes.

The identification of amino acids happened with ninhydrin reagent sensitivised with polychromatic γ-collidine. The composition of staining reagent was the following:

50 ml solution A + 50 ml solution B + 2 ml γ-collidine. Solution A contained 2 g of ninhydrin in 100 ml acetone and solution B 1g of cupper acetate dissolved in 245 ml of deionized water, to which were added 5 ml of glacial acetic acid and 250 ml of acetone.

RESULTS AND DISCUSSION

The migrations of glutathione and different amino acids were investigated in phenol-water = 7:3 solvent system containing SDS in different concentrations, on Kieselgel 60 F_{254} plates. Without SDS the GSSG has been separated from the neighbour amino acids, however the GSH has migrated close to the threonine and asparagine. The relative migration distances of glutathione and amino acids are shown in the Table I. The relative migration distances are refered to valine because

Table I.

Effect of SDS on the relative migration distances of glutathione and amino acids referred to valine in phenol-water = = 7:3 solvent system containing SDS in different concentration.

	Relative migration distance		
	Phenol-water = = 7:3	Phenol-0.5% SDS = 7:3	Phenol-1% SDS = 7:3
Lys	0.036	0.119	0.245
Arg	0.094	0.238	0.570
GSSG	0.144	0.081	0.070
His	0.194	0.238	0.290
Asp	0.244	0.163	0.115
Cys_2	0.244	0.325	-
Glu	0.300	0.238	0.200
Cys	0.344	0.263	0.245
Ser	0.344	0.344	0.355
Gly	0.444	0.431	0.475
Thr	0.544	0.538	0.570
Asn	0.569	-	0.570
GSH	0.575	0.519	0.410
Ala	0.650	0.656	0.675
Gln	0.781	0.794	0.810
Val	1.000	1.000	1.000
Tyr	1.081	1.088	1.110
Met	1.156	1.163	1.150
Leu	1.156	1.163	1.150
Ile	1.156	1.163	1.150
Met	1.156	1.163	1.150
Pro	1.206	1.250	1.150
Trp	1.256	1.250	1.200
Phe	1.256	1.313	1.200

of the longer migration distance. The following amino acids showed well defined spots: Lys, Arg, His, Glu, Gly, Ala, Gln, Val, Tyr and Pro.

The order of several components have changed in phenol -0.5% SDS = 7:3 solvent system. The migrations of GSH and GSSG, Asp, Glu and Cys have decreased and those of Lys, Arg, His have increased. His, Glu and Arg run with the same velocity. The relative migration distances are presented in the Table I. The oxidized form of glutathione could be separated again, but the reduced form migrated near the threonine.

In order to separate and quantify the GSH, the concentration of SDS has been enhanced. Developing the plates in phenol-1% SDS = 7:3, the two forms of glutathione have been separated from amino acids, the position of GSSG has not changed. The GSH was localized as a well separated spot under the glycine. The relative migration distances of the components are shown in the Table I. Beside the two forms of glutathione the following amino acids were separated: Asp, Glu, His, Ser, Gly, Ala, Gln, Val and Tyr. The localization of glutathione forms and amino acids both from standard mixture and biological samples are shown in Fig. 1. Lys and Cys migrated with the same velocity, and Asn, Thr and Arg could not be separated from each other in this solvent system.

Fig. 2 shows the densitometric calibration curve for the quantitative evaluation of GSH and GSSG. The most preferable range for the determination is about 1-10 µg GSH and 1-5 µg GSSG in a spot.

In biological samples the reduced glutathione is easily converted into its oxidized form in solution or during the development. To avoid this effect, the samples were bubbled

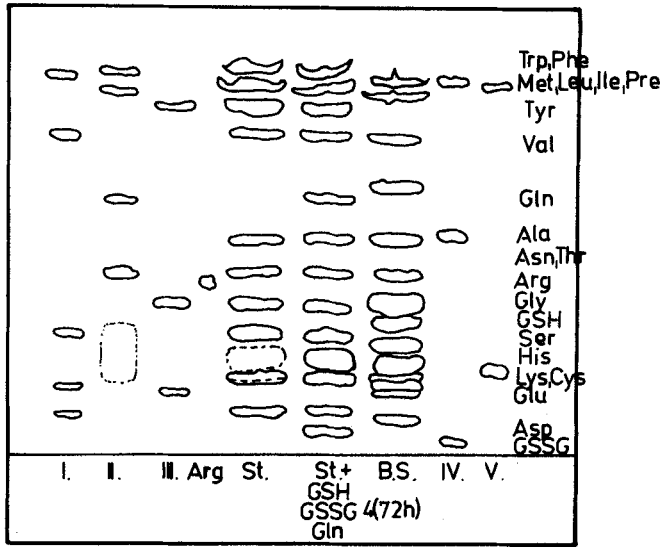

Fig. 1. Separation of reduced (GSH) and oxidized (GSSG) glutathione from amino acid mixture and biological sample on Merck 5548 HPTLC plate, in phenol-1% SDS= =7:3 solvent system. Flow rate: 2.92×10^{-3} cm^3/sec.

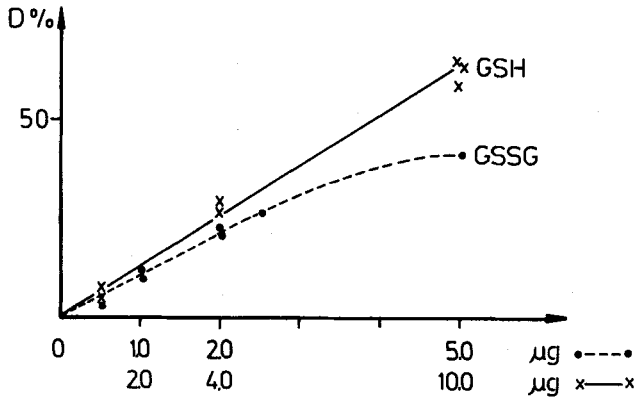

Fig. 2 Calibration curve for reduced and oxidized form of glutathione evaluated in videodensitometer.

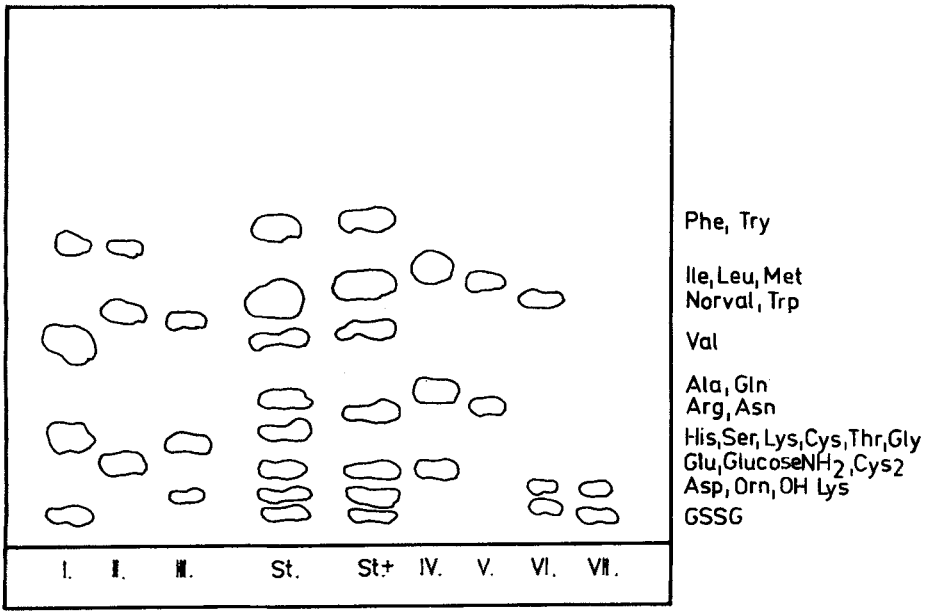

Fig. 3 Development of glutathione and amino acids in normal chamber on Merck 5554 Kieselgel 60 F_{254} plate, in phenol-1% SDS solvent system. Running time: 9 hours.

with oxigen. By this way the total glutathione content might be determined quantitatively by videodensitometer.

These findings suggest that the two forms of glutathione can be separated from amino acid mixtures in phenol-1% SDS = = 7:3 solvent system by OPTLC method. Comparing the development of samples in pressurized ultra-micro chamber with normal chamber (Fig. 3) it can be stated, that the previous method is suitable especially for solvent systems having a high viscosity. The slow migration can be reduced from 8-9 hours to 50 minutes in the case of our solvent system, decreasing the possibility of diffusion of components.

Development in phenol-water = 7:3 solvent system the reduced form of glutathione can not be separated from asparagine. The SDS changes the selectivity of the original solvent and the relative position of several components has changed. It is true especially for basic amino acids and GSH. Ahrland et al. (9) found that the titration curve of silica gel resembles that of a weakly acidic ion exchanger. A suppose is, that the detergent depletes the ion exchange capacity of layer and its partitional feature has been achieved. By this way the separation depends first of all on the polarity and size of different components.

According to our results a modified phenol-water solvent system containing SDS proved to be suitable for the separation of two forms of glutathione, especially for the oxidized form. The later showed a low migration velocity and could be localised as a well separated spot. On the basis of this phenomenon the application of OPTLC method might afford a possibility for rapid estimation of GSSG. By this way several amino acids, namely Asp, Glu, His, Ser, Gly, Ala, Gln, Val and Tyr could be separated from the glutathione forms and quantified, too.

The time of development was decreased from 9 hours to 50 minutes by application of OPTLC method, comparing it with the development in normal chamber. Reducing the running time, the possibility of diffusion was strongly limited. The oxidation of GSH in the biological samples prior to application might take possible the quantitative determination to escape the oxidation during the development. This method can be applied for rapid analysis of glutathione and amino acids in clinical and industrial practice.

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