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HPLC determination of glutathione and L-cysteine in pharmaceuticals after derivatization with ethacrynic acid

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Abstract: Ethacrynic acid and its methyl ester are proposed as useful pre-chromatographic derivatization reagents for the HPLC analysis (UV detection) of reduced glutathione (GSH) and L-cysteine. The optimum experimental conditions for the thiol derivatization, the removal of the excess reagent by liquid–liquid or solid-phase extraction and the reversed-phase chromatographic separations of the thiol adducts were investigated. The method was applied to the HPLC determination of GSH and L-cysteine in commercial formulations and proved to be suitable for the HPLC determination of oxidized glutathione (GSSG) after reduction to GSH using dithiothreitol (DTT).

Keywords: *Glutathione; L-cysteine; ethacrynic acid and its methyl ester; pre-chromatographic derivatization; liquid chromatography.*

Introduction

Ethacrynic acid, [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy]acetic acid, is a diuretic drug [1–3] structurally characterized by the presence of α, β-unsaturated aryl ketone moiety reactive towards sulphydryl groups [2, 3]. The ability of ethacrynic acid to react with aliphatic thiols, affording 'saturated' thiol adducts with modified UV spectral properties, was conveniently exploited to devise selective difference UV spectrophotometric analyses [4, 5]; moreover, it was the rational basis for its application as a pre-chromatographic derivatization reagent in liquid chromatographic (HPLC) analysis of thiols of pharmaceutical [6] and cosmetic [7] interest.

The need for selective, inexpensive thiol reagents suitable for the routine HPLC analysis of bioactive aliphatic thiols led us to further examine the application field of ethacrynic acid. In fact, when the analysis does not require extreme sensitivity, this compound may constitute a convenient alternative to the known fluorogenic thiol reagents (bromobimanes, halogenobenzofurazans, maleimides and aroylacrylic compounds) [8–10], which are generally expensive (when commercially available). In addition, derivatization with ethacrynic acid allows simple, basic HPLC instrumentation (UV detection) to be used.

Thus, with a view to extending and improving the use of ethacrynic acid as a thiol pre-chromatographic derivatization reagent, the present work was concerned with the following objectives: (a) HPLC determination of reduced glutathione (GSH) and L-cysteine in pharmaceutical formulations after derivatization with ethacrynic acid and its methyl ester; (b) removal of the reagent excess from the reaction mixture by selective liquid–liquid or solid-phase extraction procedures and (c) application of the method to the determination of oxidized glutathione (GSSG) after reduction with 1,4-dithiothreitol (DTT).

Experimental

Chemicals

Ethacrynic acid was purchased from Sigma (St Louis, MO, USA), glutathione (GSH), oxidized glutathione (GSSG), L-cysteine and dithiothreitol (DTT) were from Fluka (Buchs, Switzerland). All other chemicals were obtained from Farmitalia C. Erba (Milan, Italy). Organic solvents of HPLC grade and de-ionized, double-distilled water were used for the chromatographic separations.

A 0.3 M buffer solution (pH 7.4) was prepared by adding 2 M sodium hydroxide sol-

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ution to 0.3 M potassium dihydrogen phosphate solution up to the desired pH value. Similarly, a 0.05 M triethylammonium phosphate (TEA) buffer solution (pH 3.0) was obtained from 0.05 M triethylamine aqueous solution and phosphoric acid.

The reagent (ethacrynic acid) solution (1.2 mg ml^{-1}) was prepared daily in the pH 7.4 buffer solution; it proved to be stable for 3 days in the freezer.

Stock solutions of GSH and GSSG were made in double-distilled water, while L-cysteine solutions were made in aqueous EDTA disodium salt solution (1 mg ml^{-1}). These solutions were maintained in the freezer for 3 days.

Internal standard solutions were prepared as follows: methyl- and propyl-*p*-hydroxybenzoate (80 μg ml^{-1}) in methanol–0.05 M TEA phosphate (pH 3.0) 60:40 (v/v) and ethyl *p*-hydroxybenzoate (10 μg ml^{-1}) in acetonitrile. A dithiothreitol solution (1×10^{-3} M) was prepared in water.

Apparatus

The HPLC system consisted of a Varian 5020 chromatograph and a Hewlett–Packard 1040A photodiode array detector connected to a HP 79994A work station.

Manual injections were made using a Rheodyne 7125 injection valve (20 μl loop). Chromatographic separations were performed on 5 μm Hypersil C_{18} column (150 or 250 × 4.6 mm i.d.) at ambient temperature using the following binary mixtures: (a) 0.05 M TEA phosphate (pH 3.0)–methanol (42:58, v/v) for the routine analysis of GSH and L-cysteine derivatized with ethacrynic acid; (b) 0.05 M TEA phosphate (pH 3.0)–methanol (56:44, v/v) for the separation of GSH and L-cysteine derivatized with ethacrynic acid; and (c) 0.05 M TEA phosphate (pH 3.0)–acetonitrile (53:47, v/v) for the analysis of GSH derivatized with the methyl ester of ethacrynic acid. The flow rate was 1 ml min^{-1} and the detector wavelength was set at 270 nm. IR spectra were taken in Nujol mull with a Perkin–Elmer 298 IR spectrophotometer. ^{1}H-NMR spectra were recorded on a Gemini 300 spectrometer (Varian). Solid-phase extraction was performed on Bond-Elut cartridges (C_{18} sorbent; 300 mg) using the Baker 10SPE system connected to a water aspirator. The SPE columns were conditioned by rinsing with 6 ml of methanol and 3 ml of water in succession.

Ethacrynic acid methyl ester

The esterification of ethacrynic acid was carried out by reaction with methanol–hydrochloric acid according to a described method [11]. The crude product was purified by column chromatography on silica gel using chloroform as the mobile phase to give a colourless oil. IR spectrum (cm^{-1}): 1765 (CO ester), 1665 (CO ketone), 1585, 1210, 1080, 1000, 800, 755. ^{1}H-NMR ($CDCl_3$) (ppm, δ): 1.13 (t, 3H) and 2.45 (q, 2H), CH_3CH_2; 3.81 (s, 3H), —$COOCH_3$; 4.76 (s, 2H) —OCH_2CO—; 5.60 (s, 1H) and 5.94 (s, 1H), CH_2=C; 6.78 (d, 1H) and 7.13 (d, 1H), 2H Arom. UV spectrum (methanol) λ = 269 nm ($\epsilon = 3.114 \times 10^3$).

The solutions of ethacrynic acid methyl ester for the pre-chromatographic derivatization of thiols were prepared in acetonitrile (concentrations under derivatization) and maintained at ambient temperature.

Derivatization procedure

Ethacrynic acid reagent. Procedure A: into a centrifuge tube 1 ml of thiol solution was reacted with 1 ml of the reagent solution at ambient temperature for 25 min. Then 0.3 ml of 8.5% phosphoric acid and 0.5 ml of internal standard (propyl paraben) solution were added and the resulting clean solution was injected into the chromatograph.

Procedure B: the reaction was carried out as in Procedure A; then, 0.3 ml of 8.5% phosphoric acid and 1.5 ml of methylene chloride were added. The mixture was vortexed for 1 min and then centrifuged for 2 min. A 1.0 ml aliquot of the aqueous layer was mixed with 0.5 ml of the internal standard (propyl paraben) solution and then injected into the chromatograph.

Procedure C: the reaction was carried out as in Procedure A; then, 0.3 ml of 8.5% phosphoric acid were added and 1 ml of the reaction mixture was applied to the conditioned SPE column (C_{18} sorbent). The thiol adducts retained were then eluted with 3 × 1 ml of 0.05 M TEA phosphate (pH 3.0)–methanol (70:30, v/v).

Ethacrynic acid methyl ester reagent. Into a centrifuge tube, 0.2 ml of thiol solution were treated with 0.5 ml of pH 7.4 buffer solution (0.3 M) and 0.2 ml of the reagent solution (0.8 mg ml^{-1}) at ambient temperature for 30 min. Then, 1 ml of water was added and the

mixture was extracted with *n*-hexane (2 × 1 ml) using centrifugation for the phase separation. The aqueous phase was acidified with 0.15 ml of 8.5% phosphoric acid, 0.2 ml of the internal standard (ethyl paraben) solution were added and the resulting solution, filtered through a 0.45 μm filter, was injected into the chromatograph.

Calibration graphs

GSH. (A) Standard GSH solutions (15–90 μg ml^{-1}) in water were subjected to the derivatization with ethacrynic acid (Procedure A). The peak-height ratio of derivatized GSH to the internal standard (propyl paraben) was plotted against the corresponding GSH concentration to obtain the calibration graph. Alternatively, the Procedure B for the derivatization with ethacrynic acid was followed:

(B) Standard GSH solutions (75–350 μg ml^{-1}) were subjected to the derivatization with the methyl ester of ethacrynic acid. The peak-height ratio of derivatized GSH to the internal standard (ethyl paraben) was plotted against the corresponding GSH concentration to obtain the calibration graph.

L-Cysteine. Standard solutions of L-cysteine (10–60 μg ml^{-1}) were prepared in EDTA sodium salt (1 mg ml^{-1}) solutions and then subjected to the derivatization with ethacrynic acid (Procedure A). The usual procedure was then followed to obtain the calibration graph.

Analysis of pharmaceutical formulations

For the analysis of GSH coated tablets, a powdered sample containing approximately 12 mg of GSH, was extracted with ethanol–water (1:1, v/v) with stirring for 15 min. The extract was filtered and diluted with water to give a final GSH concentration of about 60 μg ml^{-1}.

When commercial solution or lyophilized products were analysed, appropriate dilution or dissolution were made with water to provide final solutions containing about 75 μg ml^{-1} of GSH.

For L-cysteine, a powder sample, equivalent to about 0.4 mg of thiol, was extracted with ethanol–water (1:1, v/v) for 20 min and the extract was filtered and diluted with EDTA sodium salt solution (1 mg ml^{-1}) to obtain a final cysteine concentration of about 40 μg ml^{-1}.

The resulting sample solutions were then subjected to the derivatization step with ethacrynic acid (Procedure A) or its methyl ester and analysed by HPLC. The GSH or L-cysteine content in each sample was determined by comparison with a standard solution of GSH (60 μg ml^{-1}) or L-cysteine (40 μg ml^{-1}).

Analysis of oxidized glutathione (GSSG)

To 0.1 ml of aqueous GSSG solution (1.3×10^{-4} M) into a centrifuge tube, 0.2 ml of pH 7.4 buffer solution and 0.1 ml of DTT solution (1×10^{-3} M) in water were added. The reaction mixture was then maintained at ambient temperature in the dark for 1 h. Subsequently, the mixture from the reduction was directly subjected to the derivatization with ethacrynic acid or its methyl ester. In particular, the reaction mixture was treated with 0.2 ml of ethacrynic acid solution (pH 7.4) at room temperature for 30 min, then 1 ml of water, 0.3 ml of 8.5% phosphoric acid and 1 ml of methylene chloride were added. The mixture was centrifuged for 2 min and the aqueous layer was injected into the chromatograph. Alternatively, the reaction mixture was treated with 0.2 ml of ethacrynic acid methyl ester solution (1.25 mg ml^{-1}) in acetonitrile and the procedure described under 'derivatization' was followed.

Results and Discussion

The work concerned the search for the optimum conditions for the derivatization of GSH and L-cysteine with ethacrynic acid and its methyl ester, the removal of the reagent excess from the reaction mixture and the reduction of GSSG to GSH prior to the derivatization and HPLC procedures.

Derivatization reaction

The reaction (Fig. 1), involving the sulphydryl function of the drugs and the reactive vinyl group of the reagents I and Ia, was carried out at pH 7.4 and ambient temperature to give thiol adducts of structure II. Under these mild conditions, the reaction between ethacrynic acid and L-cysteine in EDTA solution was complete after about 10 min, while the reaction with GSH was complete over about 20 min, with or without EDTA (Fig. 2). EDTA solution was found to be essential to obtain stable aqueous L-cysteine solutions and subsequent high derivatization yield. The effect of the reagent concentration on the reaction course

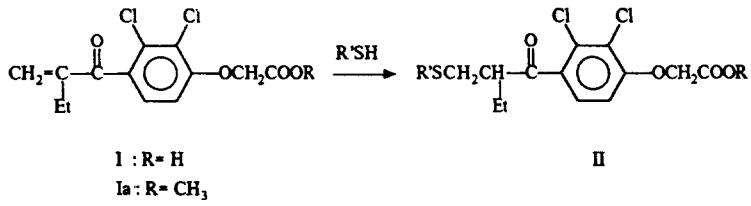

Figure 1
Reaction of ethacrynic acid (I) and its methyl ester (Ia) with aliphatic thiols.

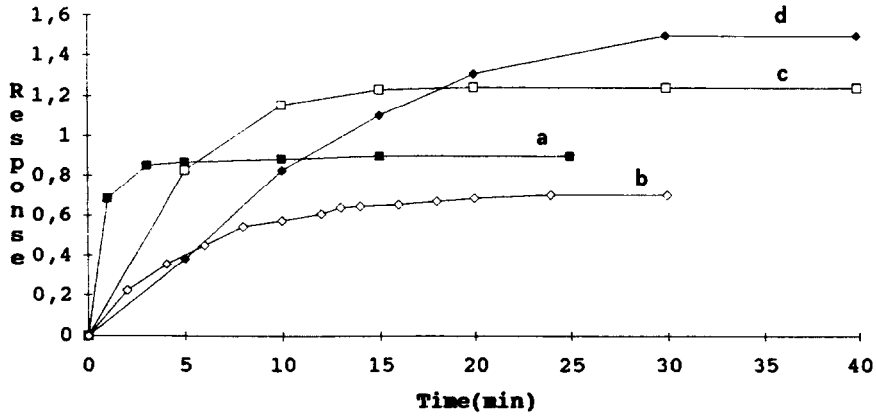

Figure 2
Reaction course in the derivatization of L-cysteine (a) and GSH (b) with ethacrynic acid (I) and in the derivatization of L-cysteine (c) and GSH (d) with the methyl ester of ethacrynic acid (Ia) at ambient temperature and pH 7.4. Response: peak-height ratios of thiol adducts to internal standard.

was evaluated on GSH. The maximum reaction yield was observed using a reagent to thiol molar ratio >3 and higher molar ratios did not affect the reaction.

When the methyl ester of ethacrynic acid was used as the reagent, the reaction course was comparable (Fig. 2), but a higher reagent to thiol molar ratio (>15) was required. With both reagents I and Ia, it was necessary to acidify the reaction mixture with diluted phosphoric acid to improve the stability and the chromatographic behaviour of the thiol adducts.

Chromatography

Chromatographic separations of the GSH and L-cysteine adducts with ethacrynic acid and its methyl ester were carried out in reversed-phase mode using a Hypersil C_{18} column. The mobile phase composition was adjusted according to the derivatization reagent used or according to whether the resolution of the thiol adducts was required or not.

Routine analysis of GSH and L-cysteine in commercial dosage forms, where these thiols are not associated, involved their derivatization with ethacrynic acid (Procedure A) and subsequent HPLC analysis using the binary mixture methanol–0.05 M TEA phosphate (pH 3) (58:42, v/v) as the mobile phase. These chromatographic conditions were suitable for a rapid HPLC assay of GSH or L-cysteine (Fig. 3), but were unable to provide separated peaks for the two thiol adducts. Their complete resolution (R_s = 2.1) was achieved using a mobile phase with a higher percentage of the aqueous pH 3.0 buffer (56%, v/v). Under these conditions, L-cysteine (t_R = 10.4) and GSH (t_R = 12.2) can be identified and simultaneously determined, but the analysis time became too long owing to the high retention of the hydrophobic excess reagent (t_R = 56.0). Similar results were obtained when the thiol adducts from the methyl ester of ethacrynic acid were chromatographed (Fig. 4).

Thus, in order to offer a useful and practical HPLC method of general application suitable for the simultaneous analysis of GSH and L-cysteine, the selective removal of the reagent excess from the reaction mixture was devised. The introduction of a simple liquid–liquid extraction step using methylene chloride (Pro-

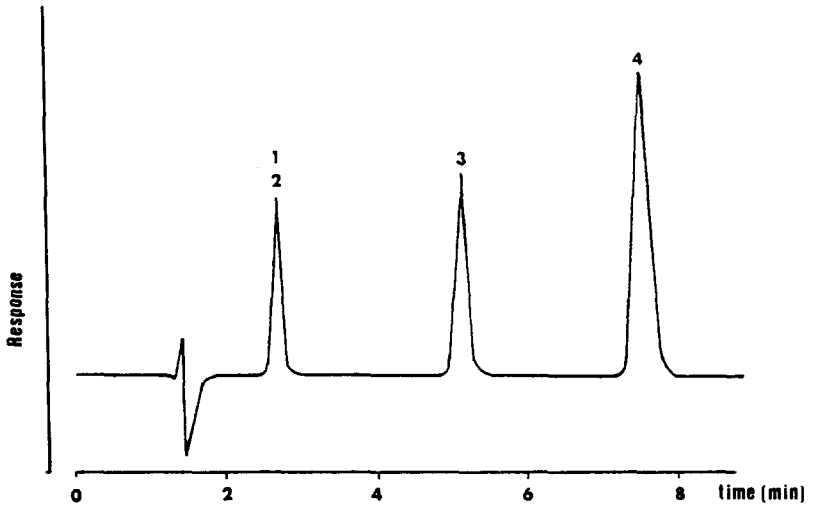

Figure 3
HPLC separation of derivatized L-cysteine (1) and GSH (2), propyl paraben (I.S.) (3) and ethacrynic acid (the reagent excess) (4). Column: Hypersil C_{18} (5 μm); mobile phase: methanol–TEA phosphate (pH 3.0; 0.05 M) (58:42, v/v) at a flow rate of 1 ml min^{-1}. Detection at 270 nm.

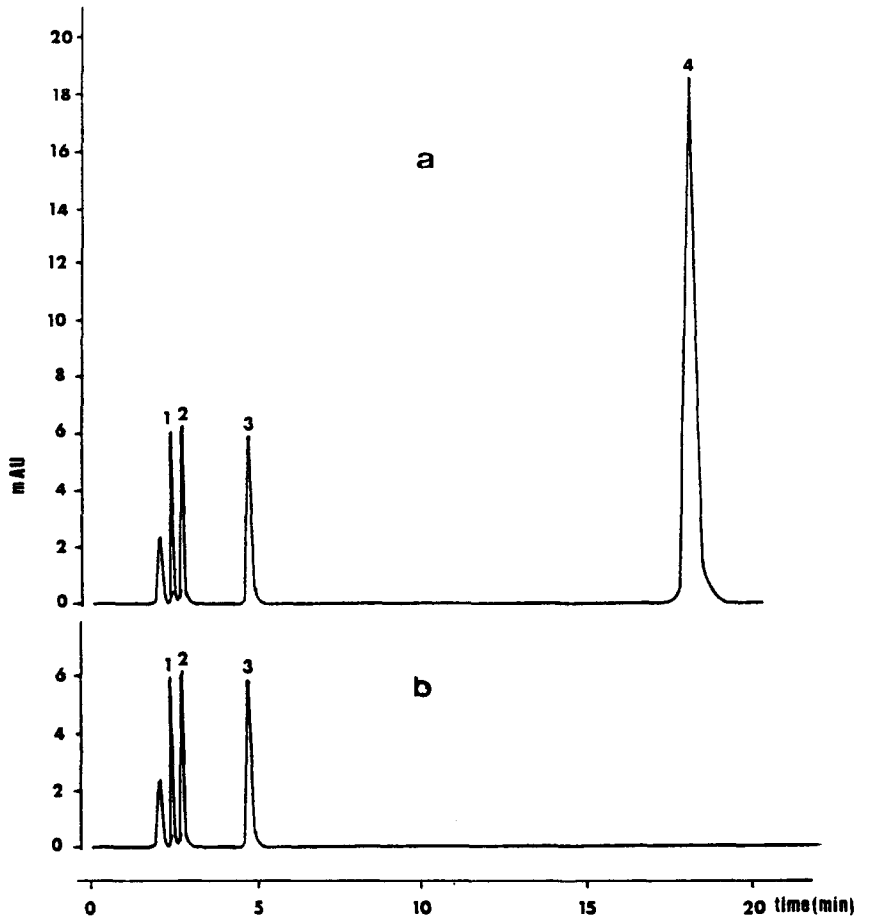

Figure 4
HPLC separation of GSH (1) and L-cysteine (2) derivatized with the methyl ester of ethacrynic acid (Ia), ethyl paraben (I.S.) (3) and the excess reagent (Ia) (4). (a) Reaction mixture directly injected; (b) reaction mixture subjected to liquid–liquid extraction with *n*-hexane. Column: as in Fig. 3; mobile phase: TEA phosphate (pH 3.0; 0.05 M)–acetonitrile (53:47, v/v) at a flow rate of 1 ml min^{-1}. Detection at 270 nm.

cedure B) proved to be convenient for obtaining an adequate (essentially quantitative) elimination of the excess reagent without significant losses of the more hydrophilic thiol adducts which are maintained in the upper aqueous phase.

Alternatively, the reaction mixture was subjected to a solid-phase (C_{18} sorbent) extraction (Procedure C). The SPE column retained the reagent excess while the thiol adducts were selectively eluted with a binary mixture of appropriate composition. The use of the methyl ester of ethacrynic acid as derivatization reagent was found to improve the selectivity of the liquid–liquid extraction. In fact, the methyl ester, due to its high lipophilicity, was quantitatively removed from the reaction mixture with *n*-hexane without any loss of the thiol adducts (Fig. 4). This procedure, however, appears more laborious than the procedure B with ethacrynic acid.

Analysis of pharmaceutical formulations

Glutathione (GSH), N-(N-L-γ-glutamyl-L-cysteinyl)glycine, is a cellular nucleophile and reductant tripeptide playing an essential protective role within the cell (protection of SH groups, reactivation of SH enzymes, conjugation with toxic chemicals or metabolites and detoxification of endogenous peroxides) [12, 13]. It is used in the treatment of poisoning and in hepatitis [1]. L-cysteine is an aliphatic thiol amino acid used in the treatment of leg ulcers [1].

A number of HPLC methods have been reported for the analysis of GSH [8, 9, 14–21], its derivatives [28] and cysteine [8, 14, 17, 19, 20] in biological samples, while limited reports concern their HPLC determination in pharmaceutical dosage forms [22, 23]. On account of the growing interest in the new formulations of GSH, this work was predominantly directed to the analysis of this thiol drug.

Using ethacrynic acid as derivatization reagent, according to Procedure A and under the chromatographic conditions of Fig. 3, a rapid HPLC determination of GSH and L-cysteine was accomplished. In this application, the reagent excess was not removed, propyl paraben was the internal standard and linear relationship between the peak-height ratio (analyte to internal standard) and the analyte concentration was obtained for both the thiol drugs (Table 1). For GSH, a linear calibration graph was also obtained following the derivatization Procedure B, involving a liquid–liquid extraction step (Table 1). When the methyl ester of ethacrynic acid was used for the determination of GSH, the chromatographic conditions were as in Fig. 4, and a linear calibration graph was obtained (Table 1) using ethyl paraben as the internal standard. In all applications, the measurements were made at 270 nm, the absorption maximum wavelength of the thiol adducts [5, 6], and the results obtained are summarized in Table 2. As can be seen, the drug content in each commercial formulation was in close agreement with the declared content. The accuracy of the method was evaluated by recovery studies by analysing samples spiked with 25% of the thiol content, and as shown, quantitative recoveries of the added were obtained with good precision. Amino compounds and hydroxy compounds contained in certain formulations did not interfere and simplified chromatograms were obtained. As for the sensitivity, the detection limit ($S/N = 3$) of the method was about 0.5 μg ml^{-1} for the solution to be subjected to derivatization, equivalent to about 3 pmoles of the GSH adduct injected.

As far as the sample preparation is concerned, ultrasonication for the drug dissolution should be avoided because it proved to be responsible for some degradation of the thiol drugs.

Analysis of oxidized glutathione (GSSG)

In pharmaceutical and biological systems glutathione (GSH) easily undergoes oxidation to the corresponding disulphide GSSG. A growing interest in the biological role of thiols and related disulphides has spurred the development of several chromatographic methods to evaluate the redox status of these compounds.

Simultaneous determinations of GSH and GSSG have been proposed based on HPLC with electrochemical [18, 20, 21, 24] and UV [22] detection. Some assays relied on the conversion of the disulphides to the parent thiols using reductants such as dithiothreitol [25, 28], tributylphosphine [26, 27] or $NaBH_4$[19] and subsequent derivatization to form a detectable fluorophore or chromophore. According to the latter analytical approach, it was considered of interest to verify the applicability of ethacrynic acid to the determination of GSSG after reduction to GSH.

To this end sodium borohydride, tributyl-

Table 1
Data for calibrations graphs (n = 6) for the HPLC determination of GSH and L-cysteine after derivatization with ethacrynic acid (I) and its methyl ester (Ia)

Thiol	Reagent	Slope*	Intercept	Correlation coefficient	Concentration range (mg ml^{-1})
GSH	I†	32.10	0.027	0.9998	0.015–0.090
	I‡	9.33	0.004	0.9999	0.030–0.120
	Ia	3.30	0.006	0.9990	0.075–0.350
L-cysteine	I	24.34	0.025	0.9996	0.010–0.060

*Peak-height ratio.
†Procedure A.
‡Procedure B.

Table 2
Assay results for the HPLC determination of glutathione (GSH) and L-cysteine in commercial formulations after derivatization with ethacrynic acid (I) and its methyl ester (Ia). The results are the average of five determinations and are expressed as a percentage of the claimed content (Found) or of the added amount (Recovery)

Thiol	Dosage* forms	Reagent	Found (%)	RSD (%)	Recovery (%)	RSD (%)
GSH	Tablets	I	99.4	1.70	99.0	0.85
		Ia	99.5	1.60	99.2	0.90
	Lyophilized A	I	98.7	0.85		
		Ia	99.2	1.00		
	Lyophilized B	I	99.1	1.15	99.3	1.20
L-Cysteine	Powder	I	99.4	2.1	100.7	1.15

*Other ingredients: (1) Tablets: formulation (Detoxicon) no longer commercially available, containing aminoacetic acid, glucuronolactone, ascorbic acid, acetyl methionine, inositol, starch, saccharose and other excipients; (2) lyophilized B: aminoacetic acid, uridine 5′-diphosphate and cyanocobalamin; (3) powder: neomycin sulphate, bacitracin Zn, glycine, DL-threonine, starch and magnesium oxide.

phosphine and dithiothreitol (Cleland's reagent) were assessed as reducing agents. In our experiments, dithiothreitol provided the best results, yielding an essentially quantitative conversion of GSSG to GSH calculated as a percentage of the thiol content theoretically available from the disulphide. The optimum molar ratios for the reduction and derivatization reactions were as follows: GSSG/DTT = 0.1 and DTT/reagent = 0.12.

After the reduction step, the reaction mixture was directly subjected to the derivatization with ethacrynic acid or its methyl ester, without significant interferences from the excess reagent.

Conclusions

Ethacrynic acid was confirmed to be a useful, commercially available reagent for the pre-chromatographic derivatization of aliphatic bioactive thiols and analysis by HPLC–UV. Under the mild reaction conditions used (pH 7.4 and ambient temperature) the method displays good selectivity towards the thiol function. When required, the excess reagent can be removed from the reaction mixture by simple liquid–liquid or solid-phase extraction. The methyl ester of ethacrynic acid does not offer significant advantages over the parent acid. The methyl ester permits a more selective removal of the reagent excess, but the acid provides a more rapid and practical analysis procedure. Both ethacrynic acid and its methyl ester are suitable for the HPLC determination of oxidized glutathione (GSSG) after conversion to reduced glutathione (GSH) using dithiothreitol (DTT).

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