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REACTION OF *o*-PHTHALALDEHYDE WITH AMINO ACIDS AND GLUTATHIONE

APPLICATION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION

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SUMMARY

Experimental conditions were found for the preparation of stable fluorescent adducts of *o*-phthalaldehyde with glutathione and its metabolites: glutamine, glutamic and aspartic acids, γ-glutamylglutamine and γ-glutamylglutamylglutamine. The structure of the glutathione isoindole derivative obtained was confirmed by NMR studies. The procedure was applied to reversed-phase high-performance liquid chromatographic separation of the previous compounds. The method was extended to glutathione and "total glutathione" determinations.

INTRODUCTION

Recent studies showed that glutamine and glutathione (GSH) metabolism may play an important rôle in the biochemistry of antimalaria[1,2] and anticancer[3] drugs. GSH regulation in normal cells after cytotoxic insult will be very useful in determining therapeutic research. One of the factors which regulate the GSH level in the cell is the availability of the precursor amino acids glutamate and glutamine[4]. In this context, studies carried out in our laboratory called for a specific and sensitive analytical method for characterizing and evaluating GSH in the presence of glutamate, glutamine and their metabolites aspartate and peptides γ-glutamylglutamine and γ-glutamylglutamylglutamine.

High-performance liquid chromatography (HPLC) with fluorescence detection is probably one of the more suitable means for the analysis of biological material since it provides both efficient separation and selective detection. Therefore, we searched for a reaction suitable for precolumn derivatization of thiol and amino acids and isocratic elution which is recommended for precise quantitation and routine analysis.

The reaction of *o*-phthalaldehyde (OPT) and 2-mercaptoethanol (2ME), introduced by Roth[5] for fluorometric detection of α-amino acids, was applied to reversed-phase HPLC with postcolumn[6] and precolumn[7] derivatization. On other hand, OPT was used as a reagent for a fluorometric assay[8,9], and the determination of

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thiols by HPLC with postcolumn derivatization (with OPT and taurine) was reported[10]. In this paper, we demonstrate that GSH and amino acids can be separated using the same precolumn derivatizing procedure and the technique is extended to GSH and "total GSH" determinations.

The mechanism of the reaction of OPT and thiol with primary amines was studied by Simons and Johnson[11]. In this work the structure of the isoindole derivative of GSH obtained is confirmed by NMR spectroscopy.

EXPERIMENTAL

Apparatus

Chromatographic experiments were carried out using Kontron LC14 pumps in combination with a single-valve injector (Model 7125, Rheodyne) and a Kratos Fluoromat FS 950 fluorimeter. (The excitation filter had maximum transmittance at 360 nm and the emission filter a cut-off value at 410 nm.) All separations were monitored on a LiChrosorb RP-18 column (7 μm, 250 mm × 4 mm) (Merck). A low-capacity guard column (Chrompack RP, 10 mm × 3 mm) was connected between the injector and the column. The flow-rate was 1 ml/min.

^{1}H NMR spectra at 270 MHz were obtained on a Brucker HX 270 in ^2H_2O. Chemical shifts are expressed in ppm with the following notations indicating the multiplicity of the signal: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. The coupling constants are expressed as J values in Hertz. Two-dimensional correlation spectroscopy yields the connectivities between spins through the scalar couplings. The basic two-pulse Jeener sequence was used[12].

Mass spectra (DCI/NH_3, DCI is desorption chemical conisation) were recorded on a Nermag X1010.

Chemicals and reagents

Chemicals, solvents and reagents were of the highest purity grade available (Prolabo). Water used for the eluent preparation was distilled twice. L-Glutamyl-glutamine and OPT were from Sigma, L-glutamic, L-aspartic acids and glutamine from Janssen, L-glutamyl peptides from Bachem.

Derivatization reagent

The derivatization reagent was obtained by mixing 1 ml of 80 mM OPT solution (54 mg dissolved in 0.5 ml methanol, volume adjusted to 5 ml with 0.1 M NaOH) and 1 ml of 98 mM 2ME solution (35 μl in 5 ml of 0.1 M NaOH).

Standard solutions

Amino acids and peptides were dissolved to 10 mM in water, except for GSH which was dissolved in 0.035 M perchloric acid (pH 1.5) to prevent formation of glutathione disulphide[13].

Derivatization procedure

Aliquots of 50 μl of standard solutions were added to 50 μl derivatization reagent and the final volume was adjusted to 150 μl. After vortexing for 10 s, the mixture was incubated for 2 min and 25 μl were injected for HPLC.

For GSH estimation, 30 μl of standard solution were treated by 50 μl of 40 mM OPT and the final volume adjusted to 150 μl with 0.1 M NaOH.

For total glutathione, an aliquot of the standard solution was preincubated for 2 min with 25 μl of 98 m*M* 2ME and then treated with 25 μl of 80 m*M* OPT.

For UV and fluorescence determinations, amino acids and peptides were treated with a 2000-fold excess of the derivatization reagent prepared in 0.05 *M* borate buffer (pH 12).

For NMR and mass spectra, the samples were prepared as follows: to 4 ml of 10 m*M* GSH or amino acid solution in water were added 0.5 ml of 80 m*M* OPT and 0.4 ml of 98 m*M* of 2ME. After stirring for 30 min at 0°C, the mixture was lyophilized. An aliquot of the product obtained was dissolved in ^2H_2O just before measurement.

Glutamate adduct, **2a.** UV (ethanol): λ_{max} 232, 260 (sh), 338 nm ($\varepsilon = 5.0 \cdot 10^3$ l mol^{-1} cm^{-1}). Fluorescence (borate buffer pH 8 or 12): λ_{ex} 345 nm, λ_{em} 450 nm. ^{1}H NMR: δ 1.92, m, 2H ($2H_{10}$); 2.32, m, 1H (H_9); 2.52, m, 1H (H'_9); 2.65, t, J=6, 2H ($2H_{14}$); 3.45, t, J = 6, 2H ($2H_{13}$); 5.45, dd, J = 10, J' = 4, 1H (H_8); 7.0, m, 2H (H_5, H_6); 7.5, s, 1H (H_3); 7.62, m, 1H (H_4, H_7).

Aspartate adduct, **2b.** UV (ethanol): λ_{max} 231, 260 (sh), 338 nm ($\varepsilon = 3.3 \cdot 10^3$ l mol^{-1} cm^{-1}). Fluorescence (0.05 *M* borate, pH 8 or 12): λ_{ex} 345 nm, λ_{em} 450 nm. ^{1}H NMR: δ 2.7, m, 2H ($2H_{14}$); 3.02, m, 2H ($2H_9$); 3.45, t, J = 6, 2H ($2H_{13}$); 5.8, dd, J = 10, J' = 4, 1H (H_8); 6.95, m, 2H (H_5, H_6); 7.45. s, 1H (H_3); 7.6, m, 2H (H_4, H_7).

Glutathione adduct, **3.** UV (ethanol): λ_{max} 231, 265 (sh), 340 nm ($\varepsilon = 2.7 \cdot 10^3$ l mol^{-1} cm^{-1}). Fluorescence (borate buffer pH 8 or 12): λ_{ex} 355 nm, λ_{em} 430 nm. ^{1}H NMR: δ 1.9, m, 1H (H_{10}); 2.36–2.6, m, 3H (H_{10}, $2H_9$); 2.87, dd, J = 15, J' = 12, 1H (H_{14}); 3.2, dd, J = 15, J' = 3.5, 1H (H_{13}); 3.52, q, J = 17, 2H ($2H_{17}$); 4.77, dd, J = 12, J' = 3, 1H (H_{13}); 5.4, dd, J = 13, J' = 5, 1H (H_8); 7.0, m, 2H (H_5, H_6); 7.5, s, 1H (H_3); 7.6, m, 2H (H_4, H_7). Mass spectrum (DCI/NH_3, m/z and relative intensity): 406 (M+1, 9); 392(6), 362(5), 180(14), 152(19), 134(37), 118(27), 101(20), 78(27), 70(100).

Glutathione. ^{1}H NMR (^2H_2O): δ 2.0, m, 2H ($2H_{10}$); 2.37, m, 2H ($2H_9$); 2.77, dd, 1H (H_{14}); 3.11, dd, 1H (H'_{14}); 3.63, t, 1H (H_8); 3.77, s, 2H ($2H_{17}$); 4.55, m, 1H (H_{13}).

RESULTS

OPT in the presence of 2ME has been reported to react with primary amines and amino acids to give fluorescent 1-alkylthio-2-alkyl substituted isoindoles[11]. The parameters which affect the fluorescence properties of isoindole have been examined[14]. Using borate buffer (pH 9.5), the procedure was applied to precolumn derivatization of amino acids and to the separation of isoindole derivatives by reversed-phase HPLC[7].

On other hand, OPT was reported to react with thiols, and glutathione was assayed by the reaction with excess of OPT at room temperature in pH 8 buffer[8]. Fluorometric determination of thiols by anion-exchange chromatography with postcolumn derivatization with OPT and taurine at pH 10 was described[10].

Structure of fluorescent adducts

The structure of the fluorescent product obtained in the reaction of OPT and a thiol with primary amine has been determined[11]. Compared to the results cited above, the UV, fluorescence and ^{1}H NMR spectral data (*cf.*, Experimental section) obtained from the isolated OPT and 2ME adducts of glutamate and aspartate established the isoindole ring structures **2a** and **2b** (Fig. 1).

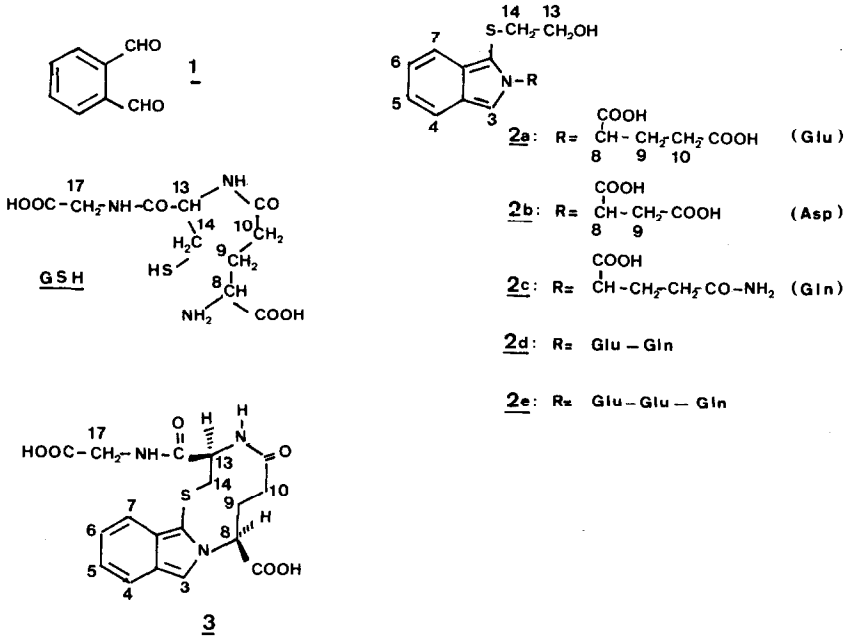

Fig. 1. Structure of *o*-phthalaldehyde (OPT) (**1**), amino acids (**2**) and glutathione (**3**) derivatives.

The similarity of the reaction conditions and the spectral properties of the product formed to those of the isoindoles suggests that the fluorescent OPT-GSH derivative is an isoindole where GSH supplies the thiol and amine functional groups[14]. We succeeded in isolating this compound and confirmed on the basis of spectroscopic data the structure **3**. The fluorescence (λ_{ex} 345 nm, λ_{em} 450 nm) and UV spectra (λ_{max} 231, 340 nm) were characteristic of the isoindole ring system[11]. Support for this structure was provided by ^{1}H NMR studies: the aromatic region displayed a 2:1:2 proton pattern as previously reported[11]: peaks at δ 7.62 (m, 2H); 7.5 (s, 1H), 7.0 (m, 2H). The signals corresponding to the protons of alkyl substituents were assigned by comparison with the spectrum of GSH (see Experimental section). The deshielding of the H_8 of glutamate observed (δ 5.4, dd) can be attributed to the aromatic ring current in the isoindole[11].

To obtain complete information about chemical shifts and proton–proton coupling, a two-dimensional correlation spectroscopy (COSY) experiment was done (Fig. 2). This confirmed the assignment of the various signals, the coupling between protons H_8 and H_9 of glutamate (δ 2.36–2.60, m, 2H) and the inequivalence of the two protons H_{17},H_{17}', of glycine (δ 3.52, q).

Furthermore, the structure was confirmed by the CI mass spectrum of **3** which showed peak at m/z = 406 corresponding to the molecular weight (M + 1). Complementary studies will be necessary to determine the structure of the fragment ions observed.

Determination of conditions for fluorescence derivatization

As previously described[14], the initial fluorescence intensity of the amino

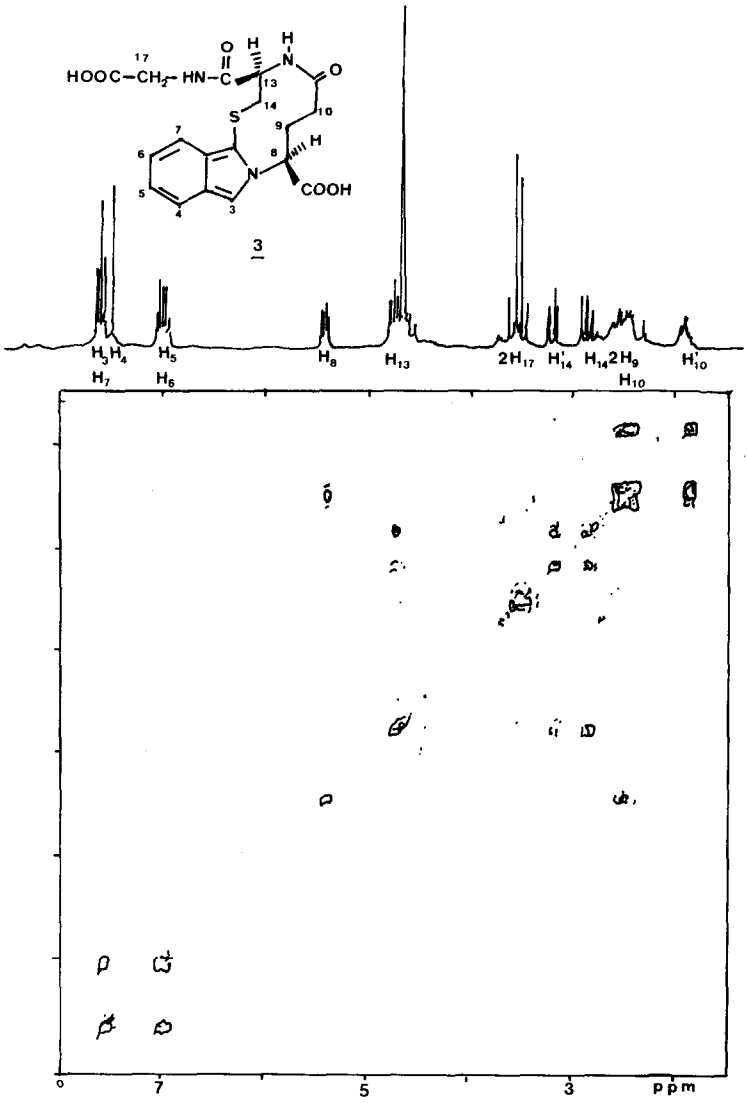

Fig. 2. Two-dimensional correlation spectrum for the OPT glutathione derivative in ^2H_2O. See Experimental section for detailed ^1H NMR data.

acid-OPT adduct **2** is relatively pH insensitive. However, the stability of these products is pH dependent: at pH 12, **2** was stable up to 30 min, but at pH 8 a 20–30% decrease of the fluorescence intensity was observed. In contrast, the stability of the GSH adduct, **3**, was unaffected by pH: the fluorescence intensity remained quite constant, for more than 30 min, either at pH 8 or 12. So, we chose to carry out the derivatization reaction at pH 12.

Addition of 1 equiv. of amino acid or peptide to an equimolar amount of OPT and 2ME yielded quantitatively, after 10 min, the adduct. In the presence of a 200-fold excess of the reagent the reaction was complete instantaneously (< 1 min). Using these

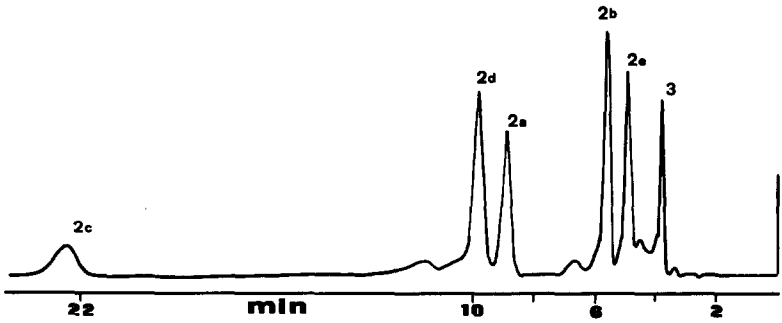

Fig. 3. Chromatogram showing the separation of glutathione (3), γ-glu-glh-gln (2e), aspartate (2b), glutamate (2a), γ-glu-gln (2d) and glutamine (2c). See Experimental section for column and mobile phase conditions.

conditions, we checked that the fluorescence response of the glutamic acid derivative was linear up to 20 μM, and unaffected by GSH. The fluorescence intensity due to the isoindole structure of the GSH derivative is not affected by 2ME.

So, the following experimental conditions for derivatization were chosen: in a final volume of 150 μl, mixed solutions of amino acids, peptides and GSH were added to 50 μl of 0.1 *M* NaOH containing 40 mM OPT and 50 mM 2ME. The mixture was vortexed (10 s) and, after 2 min, used for HPLC analysis.

Optimization of separation

Derivatized amino acids and peptides **2**, **3** were separated on a reversed-phase column using an isocratic system of acetonitrile and sodium acetate solution.

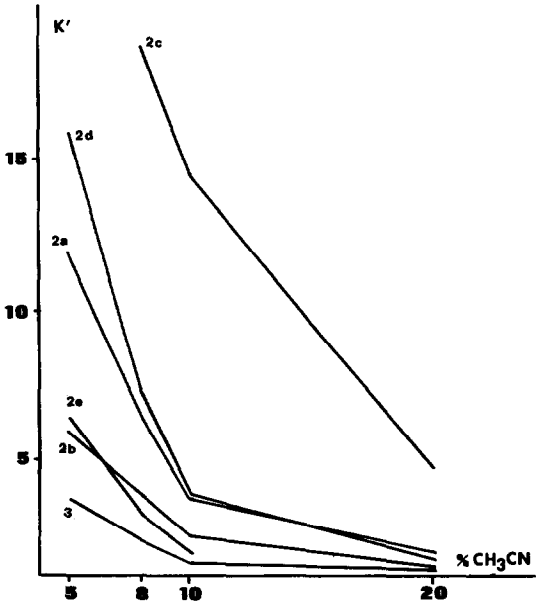

Fig. 4. The effect of the acetonitrile concentration on the capacity factors. The mobile phase contains various levels of acetonitrile in 0.3 *M* sodium acetate, pH 7.4. Compound numbers as in Fig. 1.

Optimization of the separation was obtained after studying the effect of the nature and the concentration of the ionic medium, the pH and the acetonitrile concentration. The mobile phase conditions leading to the best separation (Fig. 3) were: 0.3 *M* sodium acetate (pH 7.4) containing 8% acetonitrile. The criteria were the resolution, stability of the fluorescence intensity and the analysis speed. As shown in Fig. 4, the capacity factors of all derivatives decreased with acetonitrile concentration, and a change in the acetonitrile percentage in the mobile phase can be advantageous for studying a mixture without glutamine.

Glutathione estimation

The technique described has been applied to the estimation of GSH. The isoindole derivative of GSH was obtained after OPT treatment of standard solutions prepared in perchloric acid to prevent oxidation of GSH to disulphide (GSSG). The calibration graph, defined by a least squares linear regression of standard concentrations *versus* the peak height, was linear over the range 5–250 p*M* (correlation coefficient 0.999, slope 0.2522, *y* intercept −0.0253). The detection limit was estimated to be 0.1 p*M* using a signal-to-noise ratio of 5. The repeatability of fluorescence intensity was evaluated by injecting consecutively seven times a standard solution containing 165 p*M* of GSH: the coefficient of variation obtained was 0.040.

The method has been extended to the evaluation of "total glutathione" comprising GSH and GSSG. 2ME was added to a test solution of GSSG, which was rapidly and totally converted into GSH. After 2 min, OPT was added and the derivative obtained subjected to HPLC analysis: a fluorescence intensity corresponding to 2 equivalents of GSH was observed.

GSSG reduction by 2ME and derivatization by OPT gave a linear response in the HPLC analysis over a range of 2.5 to 37.5 p*M* (correlation coefficient 0.999, slope 0.2474, *y* intercept 0.013). The two lines corresponding to GSH and GSSG [expressed as 2(GSH)] estimations are identical.

DISCUSSION

Our data demonstrate that GSH, amino acids (glutamic, aspartic acids, glutamine) and peptides (γ-glutamylglutamine and γ-glutamylglutamylglutamine) can be separated by reversed-phase HPLC using the same precolumn derivatizing procedure. This technique may be extended to the separation of GSH and other amino acids, for example the precursors cysteine, glycine and the intermediate biosynthetic dipeptide, γ-glutamylcysteine.

In cells, oxidation of GSH leads to the formation of GSSG and intracellular GSH is maintained in the reduced state by GSSG reductase linked to the NADPH–NADP system. The method described is applicable to the estimation of GSH and "total glutathione" comprising GSH and GSSG. The sensitivity (0.1 p*M* detected) allows the detection of picomole quantities of GSH.

Furthermore using two successive determinations, the first one after preincubation of the sample in the presence of 2ME and the second one after treatment with a mixture of 2ME and OPT, it is possible to evaluate the glutathione present as GSSG according to:

$$(\text{GSSG}) = \frac{(\text{total glutathione}) - (\text{GSH})}{2}$$

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