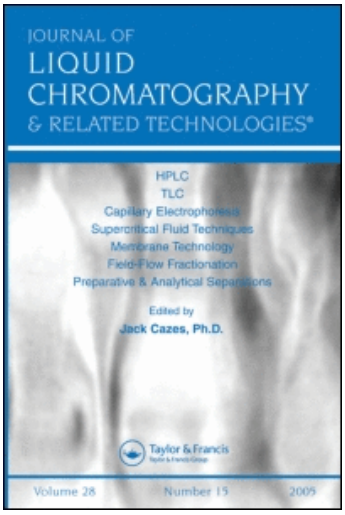

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A. Conchillo[a]; F. Camps[a]; A. Messeguer[a]
[a] Department of Biological Organic Chemistry, C.I.D.(C.S.I.C.) J. Girona, Barcelona, Spain

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VERSATILITY OF REVERSED PHASE CHROMATOGRAPHIC TECHNIQUES ON THE SEPARATION AND PURIFICATION OF COMPLEX GLUTATHIONE CONJUGATES MIXTURES FROM SYNTHETIC ORIGIN

A. CONCHILLO, F. CAMPS, AND A. MESSEGUER*
Department of Biological Organic Chemistry
C.I.D.(C.S.I.C.)
J. Girona, 18-26
08034 Barcelona, Spain

ABSTRACT

Reversed phase chromatographic techniques have shown to be versatile and convenient tools for separation and purification of the *cis* [**c-GSH 1** (3*S*,4*R*) and **c-GSH 2** (3*R*,4*S*)] and *trans* [**t-GSH 1** (3*R*,4*R*) and **t-GSH 2** (3*S*,4*S*), see Figure 1a] diastereomeric pairs of adducts derived from the reaction of glutathione with 3,4-epoxyprecocene II (**1**), a model of bioactive epoxide. Combination of analytical and semipreparative HPLC, Sep-Pack cartridges and open column flash chromatography, enabled the separation and eventual isolation of the above compounds at microgram, milligram, or gram scales. In addition, an open column procedure has also been developed for easy removal of salts and water from eluates in the above separations.

INTRODUCTION

The advent of techniques based on reversed phase chromatography has constituted a major improvement in chemical and biochemical analysis. These techniques, particularly high performance liquid chromatography (HPLC), have shown to be a valuable tool for the monitorization of both polar and high molecular weight compounds.

In addition, reversed phase chromatography has also become a routine technique in organic and bioorganic chemistry laboratories. However, in these cases its use is mainly restricted to analytical or preparative HPLC whereas all other chromatographic procedures, specially those working with open columns, are still currently carried out with normal phase. For example, extension of the flash chromatography technique developed by Clark-Still and coworkers (1) to the reversed phase has received so far little attention (2), in spite of its potential application to a wide range of common separations and purifications in the work-up of synthetic procedures.

As an example of the above potential and the versatility that the use of reversed phase can offer, we report herein our results on the separation and purification of adducts formed in the reaction of glutathione with 3,4-epoxyprecocene II (1), a compound postulated as responsible for the cytotoxic activity elicited by precocenes in both invertebrate (3) and vertebrate (4) species. These conjugates have been prepared as standards for their putative detection as metabolites from the detoxication of the above epoxide in biological matrices (5).

The synthesis and structural characterization of these glutathione adducts and their stereochemical correlation with the other conjugates related to the mercapturic acid pathway (i.e. those derived from cysteine and *N*-acetylcysteine) has been recently studied in our laboratory (6,7). As shown in Figure 1a, reaction of glutathione with racemic epoxide **1** at pH

8.4 gives a mixture of four adducts, i.e. the *cis* [**c-GSH 1** (3*S*,4*R*) and **c-GSH 2** (3*R*,4*S*)] and *trans* [**t-GSH 1** (3*R*,4*R*) and **t-GSH 2** (3*S*,4*S*)] diastereomeric pairs, in a *cis* : *trans* 29 : 71 isomeric ratio, while working under strong basic medium the *trans* adducts could be stereoselectively obtained (6). In fact, it was not possible to find suitable reaction conditions leading to the stereoselective formation of the corresponding *cis* adducts and the highest amount of these compounds was obtained by working under the pH conditions indicated above. Therefore, it was necessary to undertake this model separation of the four diastereomers at both analytical and preparative scales to obtain pure standards. Fortunately, as described below, a proper combination of reversed phase chromatography methods showed to be efficient for our purpose.

MATERIALS AND METHODS.

Reaction of glutathione with epoxide **1** was carried out by adding dropwise a solution of **1** (0.20 g, 0.85 mmol) in tetrahydrofuran (20 mL) over 50 mL of a $NaHCO_3$ solution of glutathione (0.52 g, 1.7 mmol) adjusted at pH 8.4 (7), and stirring the mixture at room temperature. When the reaction was completed (30 min, HPLC monitoring, 93% conversion yield), the volume was concentrated under vacuum to 20 mL and the residue was extracted with diethyl ether (3 x 20 mL) to remove the *cis* : *trans* mixture of 3,4-dihydro-3,4-diols concomitantly formed during the reaction, in a 2 and 5%, respectively. Finally, the aqueous extract was reduced to 10 mL to give a solution containing the *cis* : *trans* mixture of glutathione adducts at a concentration of 34 mg/mL.

HPLC analyses (part **A**, see Fig. 1a) were performed with a Waters modular system, provided with two Model 510 pumps, an Automated Gradient Controller, a U6K injector, a Data

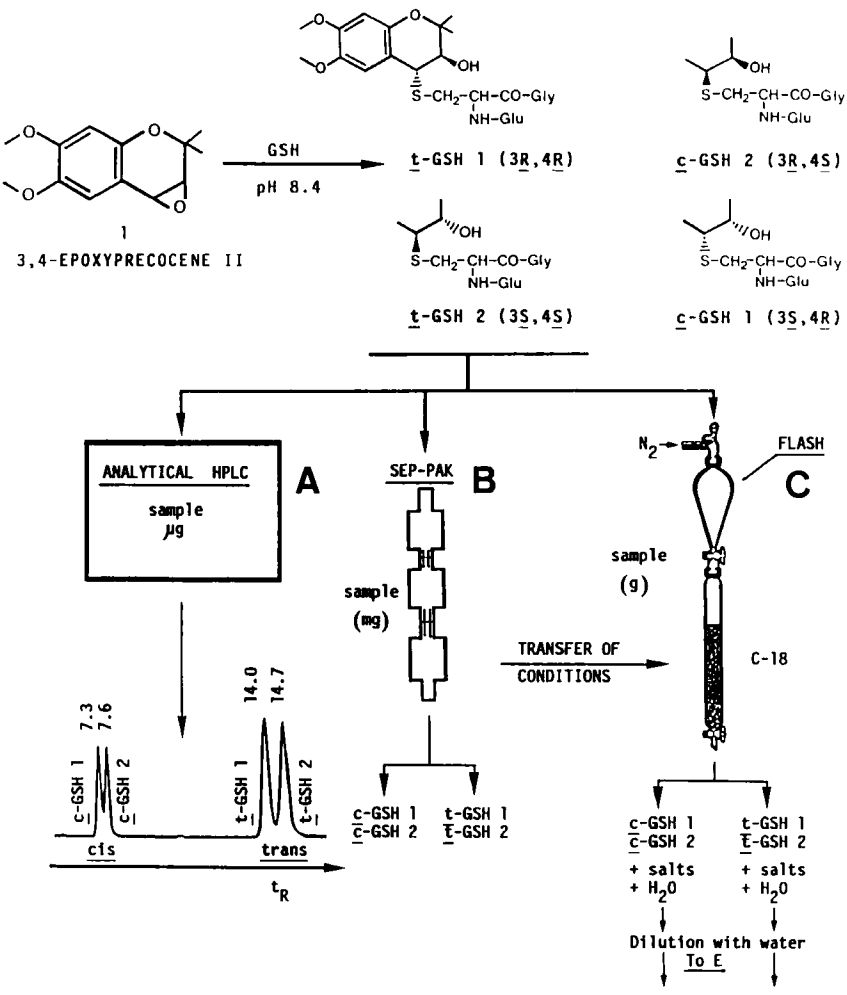

FIGURE 1a. Schematic representation of methodology used for analysis and separation of *cis* and *trans* isomeric glutathione adducts derived from 3,4-epoxyprecocene II.

Module 730 and a UV Model 481 detector. For analytical separations a Hypersil ODS (10 x 0.47 cm, 5 μ, Tracer Analítica, Barcelona, Spain) column was used. Elution conditions are given where appropriate and detection was set at 295 nm.

Sep-Pak separations (part **B**): a set of three C-18 Sep-Pak cartridges (Waters Associates) was assembled with special care in eliminating death volumes by cutting the inlet and outlet tubings. The minicolumns were washed with methanol (20 mL), water (40 mL) and buffer solution (0.1 M formic acid-triethylamine, pH 3.1, 40 mL). Then an aliquote part of the glutathione adduct mixture (45 μL, 1.5 mg, acidified to pH 3 with 25 μL of 2N HCl) was placed on the top of the Sep-Pak assembly and eluted with a gradient from 95 : 5 to 50 : 50 of the pH 3.1 buffer solution: (3 : 1 : 1 water : acetonitrile : tetrahydrofuran) .

Preparative flash chromatography (part **C**): 30 g of C-18 reversed phase (55-105 μ, Waters Associates) was packed with methanol and nitrogen pressure into a flash chromatography column. Washing and activation were carried out by successive elutions with methanol (200 mL), water (200 mL) and standard buffer solution at pH 3.1 (200 mL). Then an aliquote fraction of the crude reaction mixture of glutathione adducts (0.34 g) was acidified to pH 3 with 2N HCl and placed onto the column. Elution was performed under nitrogen pressure (1.5 Kg, to give a flow of 18 mL/min, approximately)) with a similar solvent gradient to that indicated for the Sep-Pak separations.

HPLC at semipreparative scale (Fig. 1b, Part **D**): a Spherisorb ODS-2 column (15 x 1 cm, 5 μ, Waters Associates) was used. The eluent was a 80:20 mixture of 0.075 M formic acid-triethylamine buffer at pH 3.1 : (3 : 1 : 1 water : acetonitrile : tetrahydrofuran) at 4.3 mL/min, for the case of the *trans* diastereomers and a 85:15 mixture of the 0.1 M buffer solution at pH 3.1 and the same organic modifier, operating at 4 mL/min, for the separation of the *cis* diastereomeric pair.

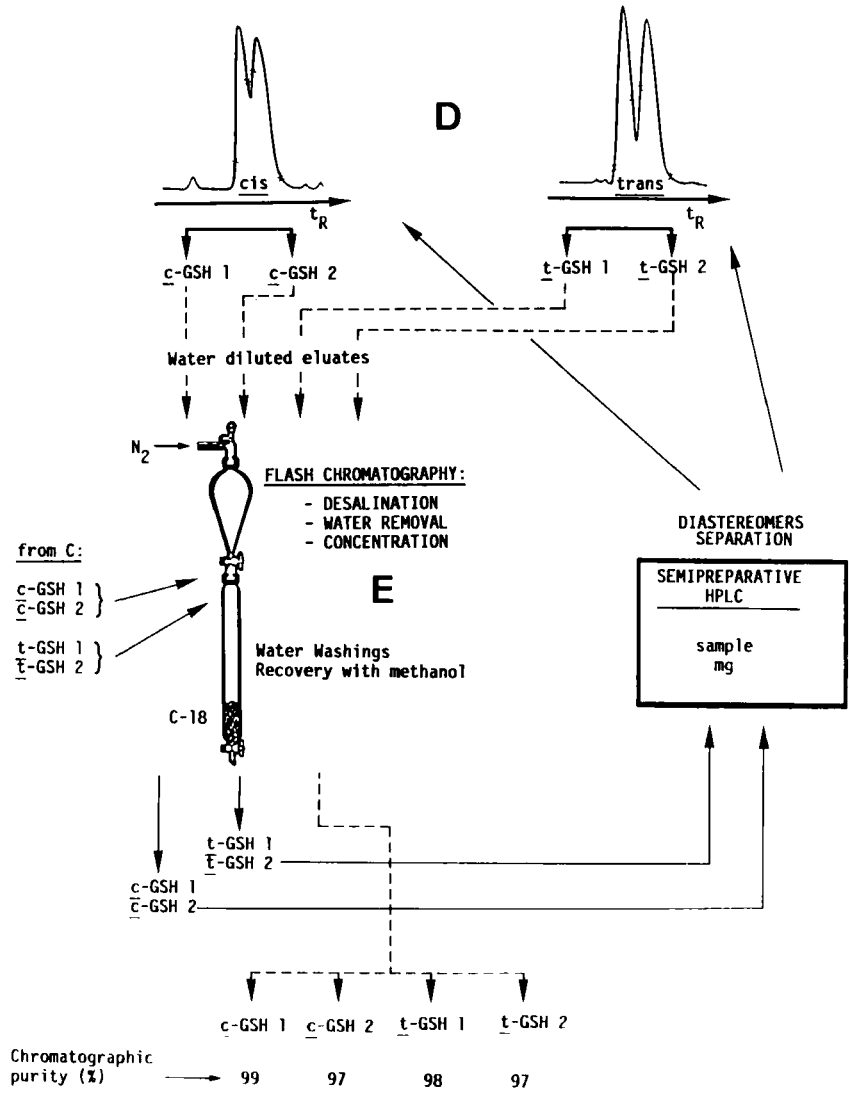

FIGURE 1b. Schematic representation of methodology used for diastereomers separation and purification, desalination and eluates concentration of isomeric glutathione adducts derived from 3,4-epoxyprecocene II.

Eluates desalting and concentration (Part E): 10 g of C-18 reversed phase (55-105 μ, Waters Associates) was packed with methanol and nitrogen pressure into a column similar to that used for flash chromatography. The column was conditioned by successive elutions with methanol (50 mL), water (100 mL) and standard buffer solution at pH 3.1 (50 mL). Then an eluate volume diluted with water up to a content of approximately 5-8% of organic modifier was passed through the column. After washing with water, elution with a small amount of methanol yielded the corresponding conjugates.

RESULTS AND DISCUSSION

As indicated above, the use of reversed phase techniques for the separation and purification of the mixture of glutathione conjugates was carried out at different levels, which are schematically illustrated in Figures 1a and 1b.

A. Obtention of a HPLC profile for the mixture of conjugates (microgram scale). In a parallel study we have undertaken the analytical resolution of all the adducts derived from epoxide **1** and the thiols related to the mercapturic acid pathway. The satisfactory separation of the *cis* and *trans* glutathionyl diastereomeric pairs was a particular case of this problem and it was accordingly solved. Basically, the polar and acid-base character of these adducts forced the use of eluent mixtures containing buffered solutions at pH within the range 3 to 5, combined with a water:acetonitrile modifier. Under these conditions, the separation of the *cis* pair from the corresponding *trans* was easily accomplished. However, a good resolution for each diastereomeric pair within the same chromatogram demanded additional optimization assays. After several attempts, addition of a stronger organic modifier (tetrahydrofuran) in small proportion and adjustment of pH at 3.1 led to α values of 1.04-1.05 for each diastereomeric pair in an analysis completed within 15 minutes. The optimal eluent

was a 80 : 20 0.075 M formic acid-triethylamine buffer at pH 3.1 : (3 : 1 : 1 water : acetonitrile : tetrahydrofuran) mixture, working at 1.5 mL/min. Under these conditions, the *cis* pair of diastereomers **c-GSH 1** and **2** eluted at 7.3 and 7.6 min, respectively, while the *trans* **t-GSH 1** and **2** appeared at 14.0 and 14.7 min, respectively (see Figure 1a).

B. Separation of the cis pair of diastereomers from the corresponding trans pair by using Sep-Pak cartridges (milligram scale, Fig. 1a). The satisfactory resolution achieved in the HPLC separation of the *cis* pair of GSH diastereomers from the corresponding *trans* pair (α = 1.9), opened the possibility to scale up the procedure to the semipreparative and preparative levels. Thus, assembly of three Sep-Pak cartridges and elution at 5 mL/min with a solvent gradient similar to that used for the analytical separations (see above), but starting with mixtures with lower eluent power, enabled the processing of 1.5 mg samples of the crude reaction mixture of GSH conjugates, to afford the *cis* diastereomers (95% purity by HPLC, 93% yield) and the corresponding *trans* pair (100% purity by HPLC, 95% yield). In addition, the same cartridge assembly was used for desalting and concentration of the former collected eluates in a similar manner as that described in Part **E** (see below).

C. Separation of the cis pair of diastereomers from the corresponding trans pair by flash column chromatography (gram scale). As stated above, the bibliography on the use of reversed phase flash chromatography is rather scarce. Thus, Kühler and Lindstein (7) studied a convenient system for preparative separations working with chemically bonded phases, especially those based on modifications of the *n*-octadecyldimethylsilyl moiety. Likewise, Hostettmann et al. reported only two examples on the use of this technique, i.e. for purification of urine samples containing Na^+/K^+ ATPase

inhibitors using C-18 reversed phase (400 μ) and in the isolation of an antibacterial α-bromoenone from marine sponges by using 25-40 μ C-18 reversed phase (8).

In our case, results from Sep-Pak assays led us to test the separation of the *cis* pair of GSH diastereomers from the *trans* pair at the gram scale by using an open column flash chromatography (for conditions, see Materials and Methods). Accordingly, when 0.34 g of the original crude reaction mixture containing the GSH conjugates in a 29:71 *cis:trans* isomeric ratio were processed through the column, excellent separations were obtained. Thus, for the most unfavorable case, i.e. the *trans* pair of diastereomers, the 96% of the recovered sample has the 100% purity. In the case of the corresponding *cis* pair, the 97% of the recovered sample had a 100% purity (Fig. 1a).

In addition, this flash chromatography procedure permitted an easy separation of the unreacted glutathione present in the crude reaction mixture. Actually, the thiol tripeptide could be eluted by using the initial solvent mixture, with the highest aqueous content. Therefore, this chromatographic procedure appears to be also valuable for the simple removal of salts and polar reagents involved in this class of reactions.

Finally, it is worth of note that simple washing of the column with methanol, followed by a reconditioning procedure similar to that routinely carried out with HPLC columns, permitted the continued reuse of the above column without significant loss of its efficacy.

D. Separation of each diastereomeric pair of GSH conjugates by HPLC at semipreparative scale (Fig. 1b). Working under the conditions described in Materials and Methods, the *cis* diastereomers, **c-GSH 1** (3*S*,4*R*) and **c-GSH 2** (3*R*,4*S*) were separated and isolated in 99 and 97% purity, respectively. Similarly, the corresponding *trans* isomers **t-GSH 1** (3*R*,4*R*) and **t-GSH 2** (3*S*,4*S*) were obtained in 98 and 97% purity, respectively.

Although the above separations were not simple to carry out, yields and diastereomeric purity achieved were satisfactory enough. In this context, the choice of formic acid as component of the buffer solution deserves some additional comments. Actually, trifluoroacetic acid has been recommended for this purpose, since its low pK_a leads to a rapid formation of ionic pairs (9). However, the presence of this acid in the buffer eluent mixture, in a separation at a semipreparative scale performed at pH 3, as in the present case, restricted the charge to 0.75 mg of substrate per injection, in comparison with the 3.0 mg used when formic acid was the component of the buffer solution. It is possible that at that pH, the buffering capacity of the halogenated acid (pK_a 0.6) is diminished. Therefore, the injection of relatively large amounts of samples with an acid-base character, such as those of GSH conjugates, could cause punctual ruptures of the buffer effect, which would eventually lead to peak broadening with concomitant loss of resolution. Evidently, these effects could hardly be observed in analytical separations, since the injected amount is much lower. In fact, although we had also good separation profiles for the above conjugates at pH 5, similar effects to those observed for trifluoroacetic acid at pH 3 were detected in this case for formic acid (pK_a 3.7), which led us to discard the use of these alternative eluent conditions at the semipreparative scale.

E. Eluates desalting and concentration (Fig. 1b). An important drawback of the purifications carried out by reversed phase chromatography techniques is the isolation of compounds from the collected eluates. These eluates, usually containing high proportions of water and eventually salts coming from buffers, require time consuming treatments, such as liophylization, combined with extractions and concentrations under vacuum, to remove polar solvents and inorganic compounds and then leave the expected analytes either

isolated or in an organic solvent solution. In many cases, all these treatments lead to significant recovery losses of analytes due to different reasons (thermolability, hydrolysis, etc).

To circumvent these problems, a modified use of conventional reversed phase chromatography can also be a valuable tool. Recently, Kirk and Rajagopalan (10) reported the application of C-18 Sep-Pak cartridges to clean up aldosterone sulphate solutions from inorganic salts, although the low capacity of the cartridges dissuaded their use at a preparative scale. In our case, the same flash chromatography system utilized for separations at the gram scale was succesfully used for a rapid and mild removal of salts and a further concentration of the different GSH conjugates in a low amount of an organic solvent.

As example of this application, a 500 mL volume of collected eluates from a preparative flash column processing (see part C), which contained the buffer components and an approximately 85% water content, was cleaned up of salts and concentrated to a volume of 30 mL of 100% methanol. For this purpose, a similar column to that used for the preparative separation but containing only one third of the reversed phase (i.e. 10 g), was conditioned as described above. Then the original solution was diluted to 1 Lt with water to reduce the organic solvent contents to a 5-8%, and it was eluted in 500 mL fractions through the column. In this manner, salts and eluents were collected while the corresponding pair of GSH conjugates remained in the column. After washing with additional water (125 mL), elution with a small volume of methanol (30 mL) afforded the respective *cis* or *trans* conjugates, salt free, and concentrated in an organic solvent. Recovery yields with this procedure were within 85-90%, and neither extractions nor concentrations under vacuum or liophylization were needed.

In conclusion, the results herein reported show how the versatility of reversed phase chromatography techniques can

be advantageously applied for performing analytical, semipreparative or preparative separations of reaction mixtures of polar and structurally related compounds, using a single eluent system. In addition, the same cartridges and columns employed for these separations can also be used for removal of salts and polar reagents present in these mixtures or for desalting and concentration of collected eluates containing the purified reaction products. Finally, despite being the cost of reversed phases certainly much higher than that of normal phases, it is also true that the former can be reutilized for many times without a significant loss of efficacy following a simple washing and reconditioning procedure.

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