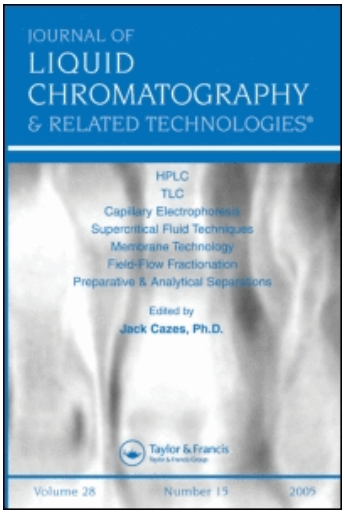

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HPLC Analysis of the Isomeric Thioether Metabolites of Styrene Oxide

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HPLC ANALYSIS OF THE ISOMERIC THIOETHER METABOLITES OF STYRENE OXIDE

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ABSTRACT

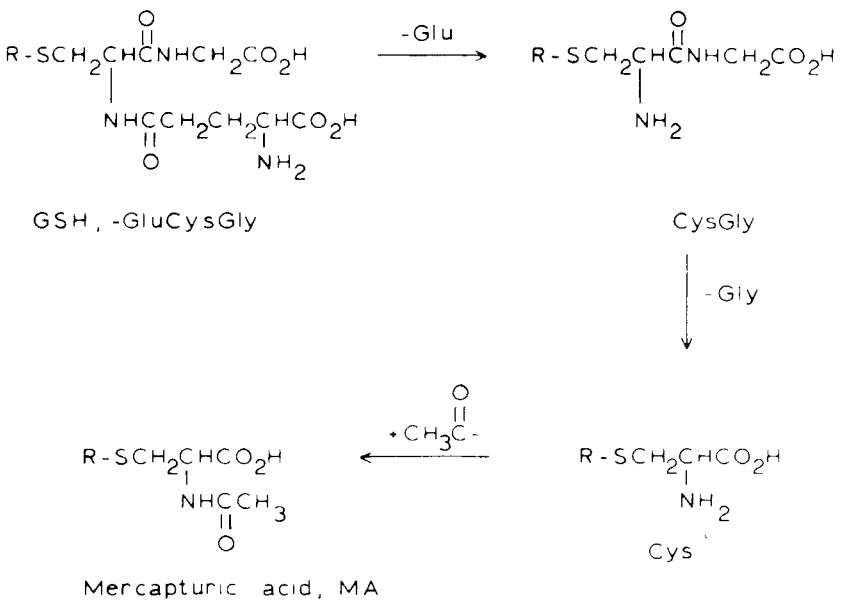
An efficient separation of the isomeric thioether metabolites of styrene oxide was achieved under reversed-phase conditions. The column was eluted isocratically with 15% methanol in buffered solutions of phosphoric acid-tris-hydroxymethylaminomethane. The thioether conjugates were separated by class, and the order of elution was cysteine, cysteinylglycine, glutathione, and N-acetylcysteine. The effect of pH and buffer salt concentration on the HPLC separation was examined. Optimal conditions for a separation were either found at low pH (pH 3 or 4) or neutral pH, both at a high buffer salt concentration (75mM). The positional isomers and stereoisomers comprising each amino acid conjugate sample were separated into two peaks. The variations in k' and α observed with changes in pH were interpreted as reflecting the degree of interaction of the ionizable groups in the amino acid residue and the hydrophobic portion of the molecule. This interaction was found to be strongly influenced by the relative stereochemistry of the benzylic carbon center, thus allowing the separation of diastereoisomeric thioethers.

INTRODUCTION

The reaction of glutathione (GSH) with electrophilic chemicals constitutes an important part of the detoxication mechanisms available to many species. Depending upon the reactivity of the electrophile involved, the conjugation reaction with GSH may

occur with or without enzyme catalysis (1). The catalytic function is performed by a group of enzymes known collectively as the GSH transferases (2).

The measurement of the enzymatic activity of the GSH transferases is an important parameter as it constitutes an expression of the metabolic potential of a given biological system. This interest in the GSH transferases is reflected in the several procedures which have been developed in order to monitor the activities of these enzymes toward different electrophiles. These assay procedures are based on spectral differences between reactants and products (3), or in the use of radiolabelled substrates, either thiol (4) or electrophile (5). The latter procedure requires separation of excess labelled substrate from the incubation mixture by a solvent partition or by chromatographic procedures such as paper and thin-layer chromatography (tlc). One of the most widely used radioactive assays utilizes styrene oxide as the substrate (5,6). Total levels of enzyme activity are determined by extracting excess oxide from the incubation medium, and the assumption is made that all remaining radioactivity in the aqueous layer represents GSH conjugates. An improvement over the extraction procedure is the use of silica gel tlc (7) which allows distinction among the major metabolites resulting from the in vivo transformation of the GSH conjugates, namely the cysteinylglycine (CysGly), cysteine (Cys) and N-acetylcysteine (mercapturic acid, MA) thioethers (Scheme 1, and Figure 1). This assay is satisfactory for most cases, a major drawback being that it is operationally lengthy.



Scheme 1

The use of high-performance liquid chromatography (HPLC) for the analysis of mixtures of GSH conjugates has been reported recently. The system utilizes a reversed-phase column and the separation mechanisms are based on ionic suppression, using acetic acid as modifier (8), or on an ion-pair mechanism with alkylammonium or sulphonate counter ions (9). The HPLC analysis of the conjugates derived from an epoxide substrate has not been previously described in detail. Our interest in the chemical and biological aspects of epoxide metabolism by the GSH transferase system prompted us to explore the separation of the different thioether metabolites derived from the enzymatic conjugation of styrene oxide with GSH in detail.

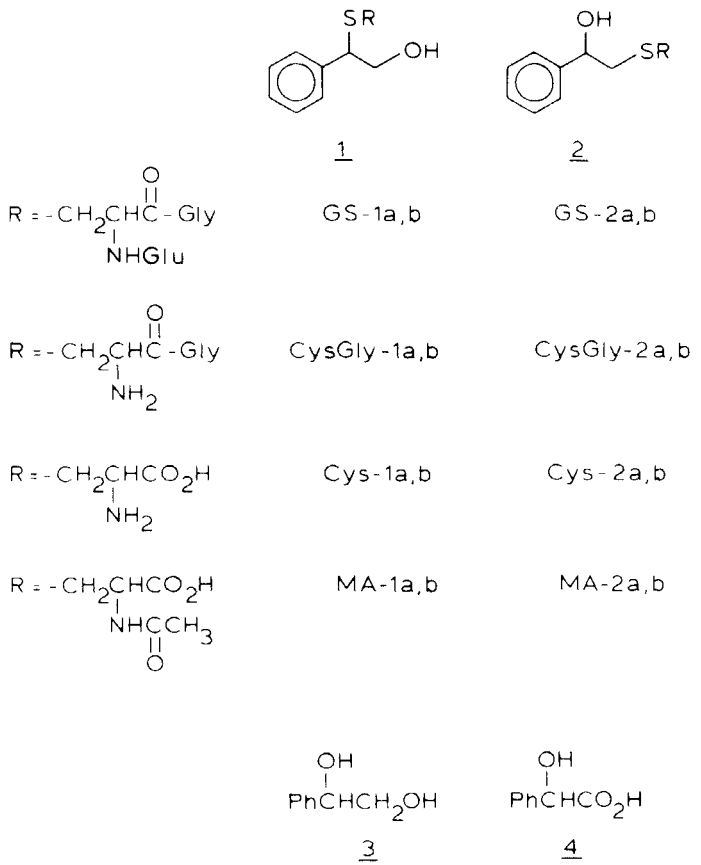


FIGURE 1. Thioether metabolites derived from styrene oxide. Benzylic thioether (1) and benzylic alcohol (2) isomers; a and b denote diastereomeric forms. Also included are styrene glycol (3) and mandelic acid (4).

Our preliminary findings indicated immediate advantages of the HPLC system over the tlc procedure, namely the shorter time required for analysis and the higher resolution achieved under the reversed-phase conditions utilized (10). The superior resolution achieved by HPLC allowed the separation and identifi-

cation of positional isomers resulting from the epoxide ring opening reaction (Fig. 1, 1 and 2) as well as the presence of diastereoisomers for each positional isomer (Fig. 1, 1a and 1b). The initial set of conditions developed has been applied to the analysis of *in vitro* and *in vivo* samples of styrene oxidesulfur conjugates (10,11). In the present study we explore the effect of pH and ionic strength on the HPLC separation of the different thioether metabolites of styrene oxide.

MATERIALS AND METHODS

The thioether compounds were available from previous studies. The stereochemical assignments, chemical synthesis and structural properties are the subject of other publications (10,12). The peaks listed in Table 1 were individually collected and characterized by ^{13}C NMR (10). The presence of diastereomers was implied from the doubling of signals on the ^{13}C NMR spectra and further verified by reaction of optically pure styrene oxide with GSH. The notation used in Figure 1 implies the existence of two diastereoisomers for each positional isomer present (i.e. 1 and 2). No attempts are made in this text to correlate diastereoisomers to the corresponding optical forms of styrene oxide.

The equipment used consisted of Waters Associates M6000A pumps, model 440 UV absorbance detector, model U6K injector and a model 660 solvent programmer. The columns used were μ-Bondapak C_{18} (0.39 x 30 cm) equipped with a pre-column packed with Corasil Bondapak C_{18}. The solvent mixtures consisted of 75 mM H_3PO_4 (prepared from 85% H_3PO_4, ca. 5 ml per liter) neutralized to the

indicated pH with tris-(hydroxymethyl)aminomethane (Tris-base). The pH values were measured with a Corning 125 pH meter equipped with a Markson combination glass electrode, before addition of organic solvent.

RESULTS

The use of trialkylammonium-based buffer systems in HPLC was originally introduced by Rivier (13). This buffering reagent has proven a most useful tool for the analysis of amino acids and polypeptides. Our initial work utilizing Rivier's procedure, which calls for triethylamine as the basic component in the buffer eluent, produced good results. A recurring problem however, was the instability of this organic base in air and light. This decomposition process produces UV absorbing impurities which must be removed by distillation, preferably immediately before use. In searching for another, more stable organic base than triethylamine, we discovered that tris-(hydroxymethyl)-aminomethane (Tris-base) produced results very comparable to those obtained with triethylammonium phosphate. The Tris-phosphate solutions are sufficiently transparent to allow detection at 254 nm, and the chemical stability of Tris-base makes it a desirable choice for routine analysis. The work described in this study was conducted with Tris-phosphate buffers although as indicated above, similar results may be obtained with the triethylamine-phosphoric acid system.

The separation of the thioether derivatives of styrene oxide (Fig. 1) was originally accomplished (10) by using a reversed-

phase (μ-Bondapak C_{18}) column and a 75 mM solution of phosphoric acid adjusted to pH 3.1-3.5 with organic base. A methanol gradient provided a clean separation of the desired compounds.

It was clearly established that separation of diastereoisomeric forms was possible and that the order of elution of the different thioether derivatives was, at pH 3.5, directly proportional to the number of amino acid residues in the molecule, i.e. the order of elution under those conditions was Cys, CysGly and GS. This latter finding suggested that the separation mechanism was strongly influenced by the amino acid residue. Consequently, factors affecting the ionizable portion of the molecule should be reflected in the separation of a given mixture of thioether conjugates. Hence, the most obvious variables to explore were the effect of pH and ionic strength on the HPLC profile. The separation conditions were determined by initially selecting an isocratic solvent combination which provided baseline separation of GSH conjugates 1 and 2 (Fig. 1, GS-1 and GS-2), and then holding the amount of organic solvent constant, but varying the pH from 3 to 7 in one pH unit increments. The lower limit used (pH 3) was based on the observation that at lower pH, decomposition of the benzylic alcohols 2 takes place readily. This was particularly true with 0.1% phosphoric acid solution (pH 2.2), a popular buffering system for polypeptide purification (14).

The chemical (non-enzymatic) reaction of the thiol amino acid-GSH, CysGly, Cys, NAcCys-with styrene oxide produces a mixture of positional isomers consisting of 1 (70%) and 2 (30%) (10,12).

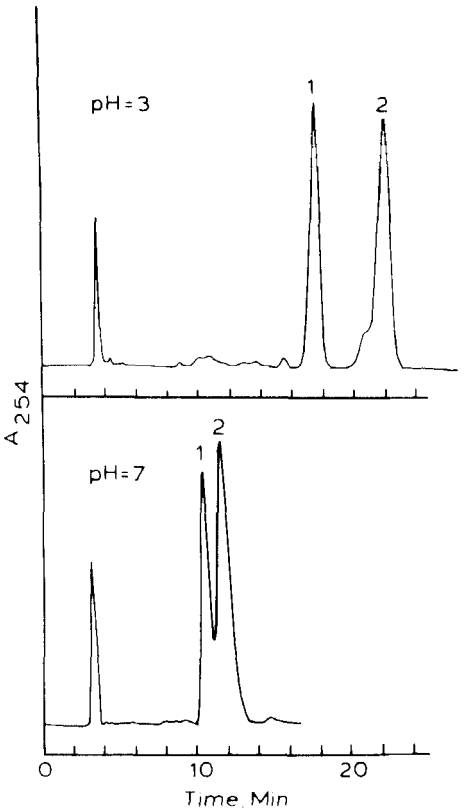


FIGURE 2. Isocratic separation of the glutathione conjugates of styrene oxide. Conditions were: one μ-Bondapak C_{18} column (0.39 x 30 cm) eluted at 1 ml/min with 15% MeOH/75 mM H_3PO_4 buffered with Tris-base, upper trace pH 3; lower trace, pH 7; 0.1 AUFS. Peak 1 is GS-P1 and peak 2 is GS-P2. GS-P1 contains GS-1a; GS-P2 contains GS-1b, GS-2a, and GS-2b.

The isocratic separation of the GSH conjugates of styrene oxide, GS-1 and GS-2, at pH 3 and pH 7 is illustrated in Fig. 2. This sample consists of two positional isomers (GS-1 and GS-2) for each of which two diastereoisomeric forms (GS-1a,b and GS-2a,b) are possible. This mixture of stereoisomers is resolved

into two peaks (GS-P1 and GS-P2). The benzylic thioether diastereoisomers (GS-1a and GS-1b) are clearly resolved emerging in peaks GS-P1 and GS-P2 respectively, while the benzylic alcohol diastereoisomers (GS-2a and GS-2b) emerge in the late eluting peak (GS-P2) together with GS-1b. Optimal conditions for the separation of these particular glutathione conjugates are at pH 3 or pH 4, both of which provide baseline separation. At pH 5 the resolution drops drastically and does not deteriorate further with increasing pH (Table 1).

The isocratic separation at pH 3 and pH 7 of a mixture containing all of the thioether conjugates of styrene oxide (Fig. 1) is illustrated in Fig. 3. The cysteine (Csy) and cysteinylglycine (CysGly) samples, as is the case for GSH, are resolved into two peaks. In these samples, however, the isomeric composition of the peaks relative to GSH is reversed. In the cysteine case, the first eluting peak (Csy-P1) contains a benzylic thioether isomer (i.e. Cys-1a) and the benzylic alcohol diastereoisomers (Cys-2a and Cys-2b); the remaining benzylic thioether diastereoisomer (Cys-1b) elutes in the second peak (Cys-P2). The cysteinylglycine compounds behave similarly with the two peaks (CysGly-P1 and CysGly-P2) containing an identical isomer distribution as for Cys. It is noteworthy that for each amino acid conjugate the benzylic thioethers 1a and 1b are consistently separated. This finding is relevant since this is the predominant positional isomer (80-90%) produced in the enzymatic conjugation of styrene oxide with GSH (10).

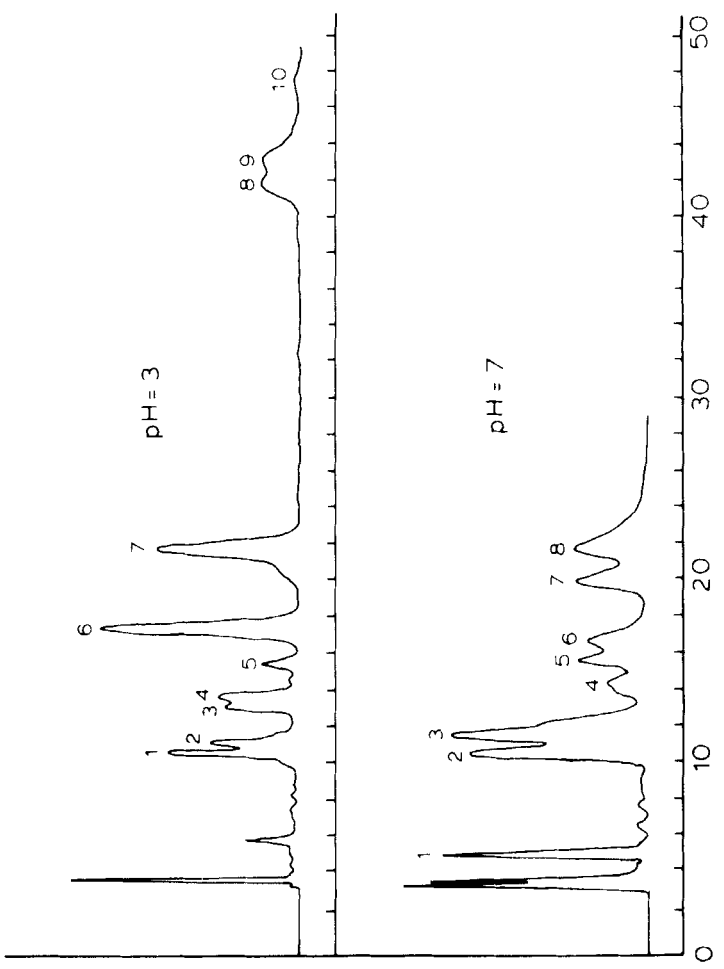


FIGURE 3. Isocratic separation of a mixture of the thioether conjugates of styrene oxide, styrene glycol, and mandelic acid. Conditions as described in Fig. 2. Peak composition: pH 3, 1, mandelic acid; 2, Cys-P1; 3, Cys-P2; 4, CysGly-P1, 5, styrene glycol; 6, GS-P1 and CysGly-P2; 7, GS-P2; 8, MA-P1; 9, MA=P2; 10, MA-P3; pH 7, 1, mandelic acid, 2, Cys-P1 and GS-P2; 3, Cys-P2 and GS-P2; 4, styrene glycol; 5, Cys-Gly-P1; 6, CysGly-P2, MA-P1; 8, MA-P2.

TABLE 1

The Capacity Factor (k^1) as a Function of pH

Sample/pH[a]	3	4	5	6	7	% Δk' at pH 7[b]
GS-P1	4.3	3.1	2.4	2.4	2.4	-44
GS-P2	5.7	3.8	2.8	2.6	2.7	-52
Styrene glycol	3.7	3.7	3.5	3.6	3.7	0
CysGly-P1	3.1	2.7	2.6	3.0	4.0	+29
CysGly-P2	4.3	3.6	3.4	3.7	4.3	0
Cys-P1	2.4	2.3	2.3	2.4	2.5	+4
Cys-P2	3.0	2.9	2.8	2.9	3.0	0
Mandelic acid 4	2.2	1.2	0.6	0.7	0.5	-77

a - 75mM H_3PO_4; b - Relative to pH 3.

TABLE 2

The Effect of pH on α for Diastereomeric Pairs (e.g. GS-1a and GS-1b)

Sample/pH	3	4	5	6	7	3[a]
GSH	1.32	1.22	1.16	1.08	1.12	1.31
CysGly	1.35	1.33	1.3	1.23	1.07	1.37
Cys	1.25	1.26	1.2	1.2	1.2	1.28

a - 10 mM phosphate solution, all others were 75 mM.

The variation of k' (capacity factor) with pH for the different conjugates is summarized in Table 1.

The changes introduced were not entirely predictable. The GSH conjugates showed a drastic decrease in retention times (44% and 52%) at pH 7 relative to pH 3. The cysteine compounds were for the most part unaffected, and interestingly, only one of the cysteinylglycine peaks (CysGly-P1) showed a significant increase (29%) in retention. Styrene glycol, as anticipated was not affected by changes in pH, and mandelic acid exhibited the most dramatic decrease (77%) in retentivity of all compounds examined. The effect of pH on the separation factor (α value) for diastereoisomeric pairs is illustrated in Table 2.

TABLE 3

The Effect of Salt Concentration on k'. Buffer Solutions at the Indicated Acid Concentrations were Adjusted to pH 3 with Tris-base

Sample	10 mM H_3PO_4	75 mM H_3PO_4	%Δk'
GS-P1	3.8	4.3	+13
GS-P2	5.0	5.7	+14
Styrene glycol 3	3.2	3.7	+15
CysGly-P1	2.7	3.1	+15
CysGly-P2	3.7	4.3	+16
Cys-P1	2.1	2.4	+14
Cys-P2	2.7	3.0	+11
Mandelic acid	0.7	2.2	+214

a - Increase at 75 mM H_3PO_4 relative to 10 mM H_3PO_4.

The trend parallels that for the k' values, that is a decreasing value for α with increasing pH. The notable exception again is the cysteine compounds for which α remains relatively constant throughout the tested pH range. For the cysteinylglycine isomers, a significant decrease in α at pH 7 is consistent with the increased k' observed for only one of the peaks (Table 1).

The effect of the buffer salt concentration was examined at pH 3 and it is described in Table 3.

Operating at 10 mM phosphate concentration caused a general decrease in retention times. This decrease was essentially constant for all the sulfur compounds and styrene glycol (3); mandelic acid showed a disproportionate decrease in retentivity. The changes in retentivity listed in Table 3 are expressed as percentage increase at the high molarity buffer relative to the low molarity buffer. It is worth reemphasizing that the salt concentration had no effect on α, over the ranges tested.

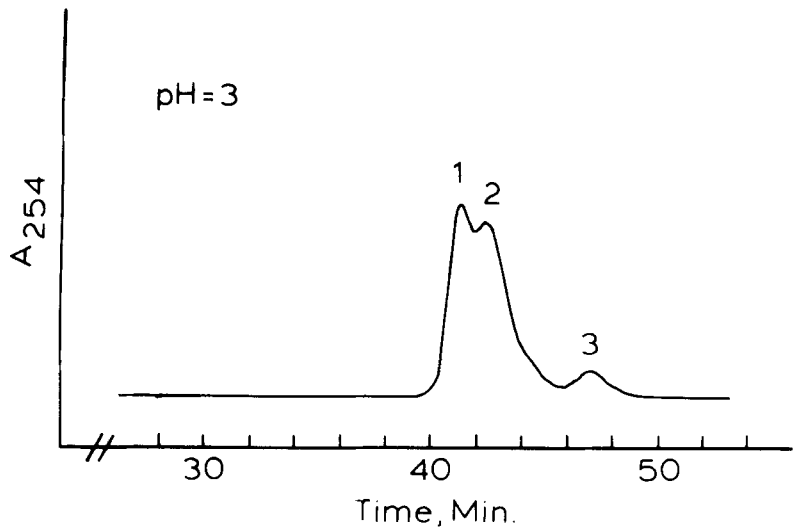


FIGURE 4. Isocratic separation of the mercapturic acid conjugates (MA) of styrene oxide. Conditions as described in Fig. 2 (pH 3). Peak identification: 1, MA-P1; 2, MA-P2; 3, MA-P3. MA-P1 contains MA-1a; MA-P2 contains MA-1b and MA-2b; MA-P3 contains MA-2a.

TABLE 4
The Effect of pH and Salt Concentration on k' for the Mercapturic Acid Isomers

Sample/pH	3	3[a]	4	5	6	7
MA-P1	11.8	10	7.0	5.6	5.5	5.4
MA-P2	12.1		7.5	6.1	6.0	6.0
MA-P3	13.5					

a - 10 mM H_3PO_4, all others 75 mM H_3PO_4.

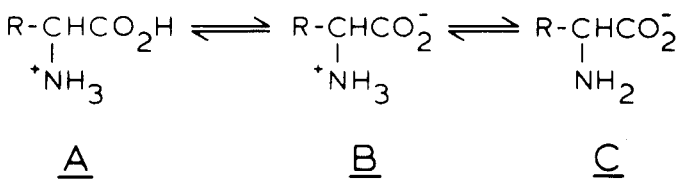
The chromatographic behavior of the N-acetylcysteine (mercapturic acid) conjugate isomers of styrene oxide is summarized in Table 4.

The effect of pH on k' is similar to that found for the GSH compounds. The best separation is achieved at pH 3 where three

of the four possible peaks are distinguishable on the trace (Fig. 4). At pH 4 only two peaks are resolved, a further drop in k' is found at pH 5 and these latter values remain unchanged up to pH 7. Interestingly the α obtained for MA-P1 and MA-P2 at pH 4 is not affected by subsequent increases in pH. The isomeric composition of the resolved peaks is as follows: at pH 3, MA-P1 and MA-P2 contain benzylic thioether isomers (MA-1a and MA-1b); a benzylic alcohol (MA-2a) elutes in the third peak (MA-P3) while the other diastereomeric benzylic alcohol (MA-2b) coelutes with MA-1b in the second peak. The two peaks separated at pH 4-7 each contain a benzylic thioether diastereoisomer. The location of the benzylic alcohols (MA-2a,b) was not established. The effect of buffer concentration on the separation is more pronounced for this isomer mixture. The resolution obtained at pH 3 is lost entirely when the phosphate concentration is decreased to 10 mM (Table 4).

DISCUSSION

The chromatographic behavior of the thioether metabolites of styrene oxide may be interpreted by a structural model in which hydrophobic interactions are modulated by equilibria of the ionizable groups in the molecules. The hydrophobic interactions are considered to be due largely to the affinity of the least polar end of the conjugate (phenyl substituent) for the hydrocarbon bonded-phase of the column. The ionic forms of an amino acid equilibrium in aqueous solution may be represented as in Scheme 2.



A B C

Scheme 2

The relative concentrations of the three ionic forms are pH dependent, and for the range used in this study (pH 3-7) all three forms are expected to participate in the separation mechanism. The pKa values for the carboxyl groups lie between 3. and 3.5 and those of the amino group are between 8. and 8.5. These values are anticipated to change slightly due to the presence of organic solvent.

It has been demonstrated by Kroeff and Pietrzyk that for a series of dipeptides, differences in k' values for acidic and basic elements are related to the differences in position of the charged species relative to the non-polar substituents, and not to the degree of ionization (pKa) of the amine and carboxyl group of the amino acids (15). This type of analysis is also applicable to the interpretation of the separation mechanism(s) of the compounds involved in the present study. The conjugates are separated by class, and the order of elution is Cys > CysGly > GSH which correlates with the relative distance of the charged groups to the non-polar end of the molecule.

For Cys-1 and Cys-2 the close proximity of the ionic groups and a slightly lower pKa for the carboxyl group combine to render

these compounds insensitive to pH changes; ionic dissociation is established at pH 3 and is not drastically affected by increases in pH. For GS-1 and GS-2 the distance between the participating groups is greater; the effect of the α-amino acid should not be as strong, and it apparently allows participation of the carboxyl group of the Gly residue. This effect is reflected in the k' where at pH 5 this group is presumably fully ionized and the α-amino acid is in the zwitterion form (B); consequently the capacity factor remains essentially constant (Table 1). Kroeff and Pretrzyk reported minimum values for k' at the isoelectric point (form B) of a given dipeptide (15).

A similar argument may be used to explain the HPLC behavior of the N-acetylcysteine compounds (MA) and mandelic acid, where decreasing k' values parallel the increased ionization of the carboxyl group with increasing pH (Tables 1 and 4). The effect of distance is also exemplified in these two cases. The decrease in k' for mandelic acid is much more dramatic than for MA-1 and MA-2 which argues for a stronger inhibition by the ionized carboxyl group in the mandelic acid case. Styrene glycol (3), as anticipated is not affected by pH (Table 1).

The CysGly samples do not fall in line with the HPLC behavior observed for Cys and GS derivatives. Capacity factors for these compounds are maximal at the two ends of the pH range studied (Table 1). Additionally, a net increase in k' is observed for one of the peaks, CysGly-P1. A unique feature of the CysGly is that it has an isolated (Cys) amino group, which in terms of proximity to the hydrophobic portion of the molecule should be

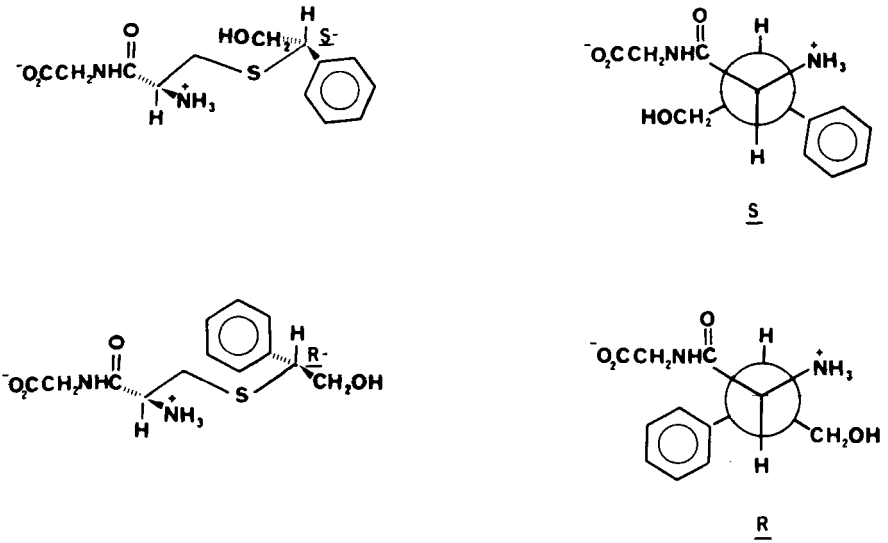


FIGURE 5. Structural projections of the diastereomeric benzylic conjugates of CysGly. The R and S denote the configuration at the benzylic carbon center.

more effective in influencing the separation than the $-CO_2H$. As mentioned earlier, a separation mechanism based only on differences in ionization constants is not plausible. It follows that the anomalous behavior of the CysGly compounds must be primarily explained on the basis of the interaction of the proximate ammonium group with the phenyl ring substituent (Fig. 5). The extent of this interaction will be strongly influenced by the relative configuration at the benzylic center in CysGly-1. From the favorable conformations available to CysGly-1a and CysGly-1b, it is conceivable that for one diastereoisomer the ammonium group is placed on the same side of the molecule as the hydrophobic portion. This diastereoisomer would have a small k' at low pH,

since when the amine is fully protonated the hydrophobic surface available for binding is decreased. In turn this allows for participation of the carboxyl group in Gly since the decline in k' is significant from pH 3 to pH 5 (Table 1). A gradual increase in k' with increasing pH would be consistent with a decrease in the concentration of protonated amine and the subsequent increase in hydrophobic area available for binding to the column. The other diastereoisomer with the charged group farther away from the phenyl ring portion, would have a larger k' at low pH as a result of decreased inhibition by the amine function (relative to the first diastereoisomer), and since the hydrophobic sites are more available for binding the effect of the carboxyl group in the k' is not expected to be as pronounced. An increase in k' would follow at higher pH for the reason stated earlier. The chromatographic pattern observed for CysGly-P1 accomodates the first case described, while CysGly-P2 fits the second model (Fig. 5). Small changes in k' for Cys-P1 and GS-P1 are also suggestive of this type of conformational control in hydrophobic binding.

The failure to separate the benzylic alcohol diastereoisomers (2) in Cys, CysGly-, and GS-2 conjugates is more likely related to the increased distance between the ionizable groups and the chiral center in 2. The fact that partial separation for MA-2 occurs at pH 3 (Fig. 4) with 75 mM but not 10 mM buffer indicates a partition mechanism operating on a neutral molecule (see below).

The effect of salt concentration is summarized in Table 3. A net decrease in k' is evident for all compounds at lower ionic

strength buffer. This decrease at 10 mM buffer concentration is nearly constant (13-16%) except for mandelic acid. The α values for pairs of diastereoisomers are not affected (Table 3) by decreased salt concentration. The conclusion is that higher salt concentration favors increased interaction with the column bonded phase by a "salting-out" effect. Of the thioether compounds the mercapturic acids are most strongly affected demonstrating a net loss of separation (Table 4) at lower ionic strength buffer.

From these data one might speculate, that under the conditions used (pH 3-7 and alkylammonium buffer system), the influence of $-^{+}NH_3$ in hydrophobic interactions is stronger than that of $-CO_2H$. A clear example is Cys versus MA; the latter, with a blocked amine group elutes much later and is more susceptible to ionic strength changes. It is possible that by operating at higher pH (pH 9) where the only ionized species would be $-CO_2^{-}$, the effect of this group in conformational control would be felt; predictably the mercapturic acids would be more strongly affected. It is also possible that the organic base forms ion-pairs with the carboxyl function, and in this way nullifies some of its ionic effect.

The information derived form this study is not limited to thioether metabolites of styrene oxide. For example, in developing an HPLC assay for other sulfur conjugates it is clear that optimal conditions for separation occur at pH 3 or 4. If substrate lability is incompatible with acidic conditions, then working at pH 7 is more desirable than at the intermediate pH 5 or pH 6. At

pH 7 the concentration of zwitterion β should be less and participation by unionized amine groups becomes more favorable.

For styrene oxide metabolites, an isocratic separation at pH 3 or 4 allows separation of the major metabolites commonly found (11), although the CysGly overlaps with GS under these conditions. However, the presence of CysGly may be inferred by isocratic elution at pH 7; if determined to be present then gradient conditions allow separation of all the compounds at either acidic (pH 3 or 4) or neutral pH.

The analysis described allows the rapid estimation of GSH transferase activities with styrene oxide as substrate and the separation and quantitation of intermediary mercapturic acid derivatives of this alkene oxide, while providing valuable structural and stereochemical information concerning each class of thioether metabolites.

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