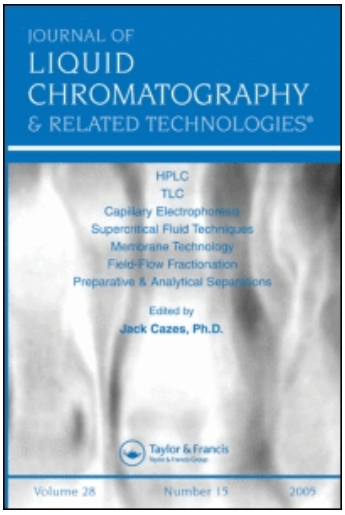

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HPLC SEPARATION OF DIASTEREOMERIC ADDUCTS OF GLUTATHIONE WITH SOME K-REGION ARENE OXIDES.

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ABSTRACT

The diastereomeric glutathione (GSH) adducts of the K-region arene oxides phenanthrene 9,10-oxide, pyrene 4,5-oxide, (±)-benz[a]anthracene 5,6-oxide, and (±)-benzo[a]pyrene 4,5-oxide were separated by reversed phase liquid chromatography. Chromatographic conditions involved an organic base, Tris-base or diethylenetriamine (DETA), neutralized to pH 3 with phosphoric acid, and an alcohol, methanol or 1-propanol, as modifier. For (±)-benzo[a]pyrene 4,5-oxide, the use of DETA and 1-propanol provided a complete stereochemical profile of the thioether conjugates derived from this arene oxide. For the GSH adducts of (±)-benz[a]anthracene 5,6-oxide complete separation was achieved under two sets of chromatographic conditions; methanol and 1-propanol enhanced the selectivity of the system for different sets of diastereomers. For both arene oxides, the GSH adducts with S-configuration eluted earlier than the R-diastereomers.

INTRODUCTION

A growing body of evidence indicates that a close relationship exists between the stereochemistry of oxides derived from polynuclear aromatic hydrocarbons and the expression of mutagenic and carcinogenic acitivity (1). This stereo-dependence is also manifested in the detoxication processes involving these metabolites. Stereopreference in the hydrolysis of

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arene oxides to trans-dihydrodiols by epoxide hydrolase has been reported (2). Equally important, the glutathione transferase enzymes have also been shown to be highly stereospecific in catalyzing the reaction of epoxides with glutathione (3). The ability to analyze for the different stereoisomers formed in the reaction of arene oxides with glutathione becomes a limiting factor in the interpretation of experimental data.

Analytical conditions for the glutathione (GSH) adducts of (±)-benzo[a]pyrene 4,5-oxide (BPO) have been reported (3). This HPLC analysis has been successfully employed in the stereochemical analysis of the enzymatic reaction of GSH with this arene oxide. More recently these analytical conditions were applied in the determination of the stereochemistry of oxidation of benzo[a]pyrene by a purified cytochrome P-450 preparation (4). In the present report we describe conditions for the analysis of diastereometric GSH adducts of phenanthrene 9,10-oxide, pyrene 4,5-oxide, (±)-benz-[a]anthracene 5,6-oxide (BAO) and improved conditions for the separation of thioethers derived from (±)-BPO.

MATERIALS AND METHODS

Phenanthrene 9,10-oxide and pyrene 4,5-oxide were prepared by literature procedures (5). Racemic BPO and BAO were obtained from the Midwest Research Institute, Kansas City, MO. Samples of optically pure BPO were prepared by a published procedure (1). The GSH adducts were prepared by reaction of GSH with the corresponding epoxide in methanol solution (6). The structures were verified by ^{13}C-nmr and mass spectral analysis with the exception of the benz[a]anthracene products. Supporting evidence for the latter comes from mechanistic considerations and similarities with (±)-BPO. A sample of purified GSH adducts of (+)- and (-)-BAO were generously provided by Dr. Richard N. Armstrong, Department of Chemistry, University of Maryland, College Park, MD 20742. Assignment of absolute stereochemistry to the GSH adducts of pyrene 4,5-oxide and phenanthrene 9,10-oxide was not complete at this time.

The chromatographic conditions employed involved two systems. System 1, Waters Associates M6000A pump, model U6K injector, model 440 UV absorbance detector (254 nm), RCM-100 radial compression system equipped with a 5 micron C_{18} Radial-PAK (8 mm ID x 10 cm) and a Brownlee 5 micron RP-18 pre-column. The eluent was 75 mM phosphoric acid adjusted to pH 3 with either Tris-base or diethylenetriamine (DETA). Methanol was used as modifier in the concentration indicated in the figure legends. System 2, DuPont Gradient LC system 8822 with absorbance detector (254 nm), model 7125 Rheodyne injector, 5 micron Zorbax C_8 column (4.6 mm ID x 25 cm) equipped with a Brownlee RP-8 5 micron pre-column. The eluent consisted of 5 mM DETA neutralized to pH 3 with concentrated phosphoric acid. 1-Propanol was used as modifier in the concentrations indicated in the figure legends.

RESULTS AND DISCUSSION

The structures of the compounds of interest are shown in Fig. 1. The relative stereochemistry of the hydroxyl and thioether groups is depicted as trans in accordance with the known mechanism of nucleophilic ring opening of epoxides (7). The symmetric nature of phenanthrene 9,10-oxide and pyrene 5,6-oxide excludes the possibility of positional isomers while for the unsymmetrical epoxides (±)-BPO and (±)-BAO two positional isomers are possible. The nomenclature used in Fig. 1 is as follows: The numbers identify regioisomers, e.g., for (±)-BPO 3 refers to a 5-glutathionyl-adduct and 4 to a 4-glutathionyl isomer; for (±)-BAO 5 refers to a 5-glutathionyl and 6 to a 6-glutathionyl regioisomer. Because of the chiral nature of GSH each regioisomer will consist of a pair of diastereomers, identified by the letters a and b following the compound number.

For the symmetrical K-region arene oxides of phenanthrene and pyrene, the diastereomeric adducts formed on reaction with GSH are 1a,b and 2a,b (Fig. 1). In both cases, separation of the two possible diastereomers was readily achieved as shown in Fig. 2. Assignment of relative stereochemistry to the individual diastereomers was not available.

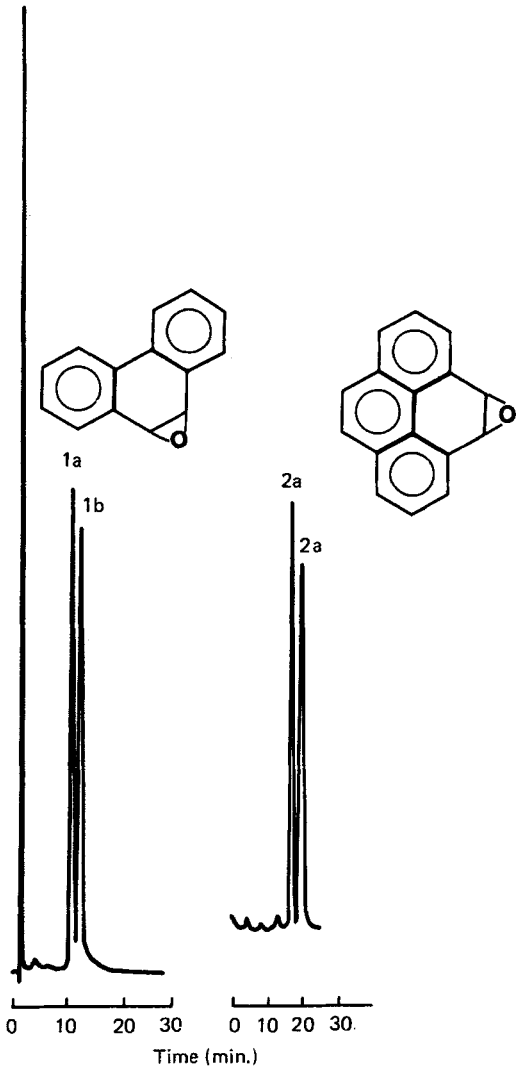

Figure 1. Structures of the GSH adducts of phenanthrene 9,10-oxide (1), pyrene 4,5-oxide (2), (±)-benzo[a]pyrene 4,5-oxide (3 and 4), and (±)-benzo[a]anthracene 5,6-oxide (5 and 6). Absolute stereochemistry is not implied. The letters designated diastereomers for each regioisomer.

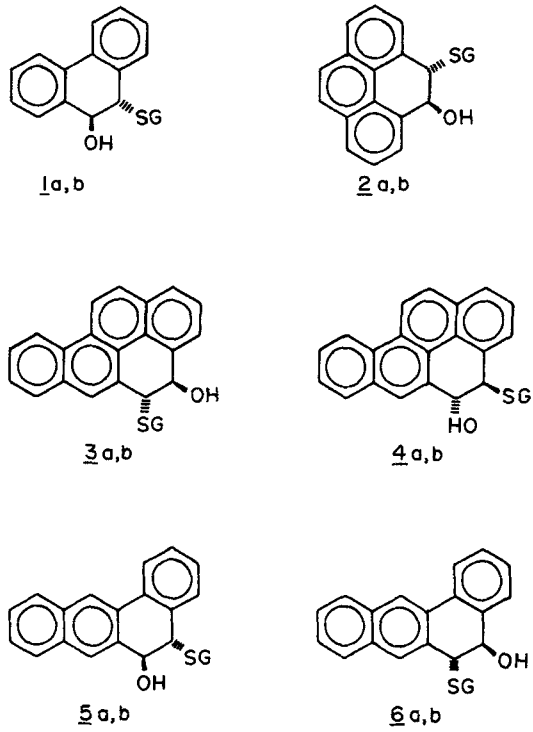

Figure 2. HPLC profile for the diastereomeric GSH adducts of phenanthrene 9,10-oxide (1a and 1b) and pyrene 4,5-oxide (2a and 2b). System 1, Tris-phosphate pH 3/30% MeOH, flow rate 3 ml/min.

For (±)-BPO there are two possible positional isomers (e.g. 3 and 4, Fig. 1). Because this arene oxide is capable of existing in enantiomeric forms, a total of four diastereomers are formed on reaction of racemic BPO with GSH. The separation originally developed for the GSH adducts of (±)-BPO provided baseline separation for two of the four diastereomers (Fig. 3a). These analytical conditions found immediate application in establishing the stereochemical course of the enzymatic reaction of GSH with this arene oxide (3). The first two eluting peaks, 3a and 4a in Fig. 3a, represent a single diastereomer each originating from a different enantiomer of (±)-BPO (8). Thus, compound 3a, a 5-glutathionyl regioisomer,

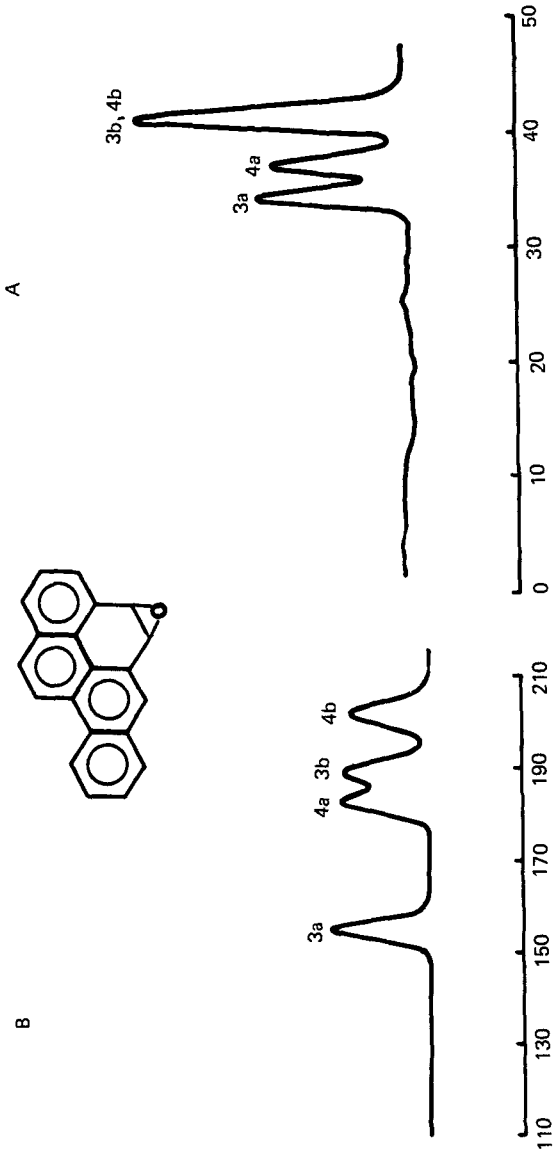

Figure 3. HPLC profile for the diastereomeric GSH adducts of (±)-benzo[a]-pyrene 4,5-oxide. Trace A, Tris-phosphate pH 3/40% MeOH, flow rate 3 ml/min. Trace B, system 2, DETA-phosphate pH 3/9% 1-propanol, flow rate 2 ml/min. Peak identification: 3a, (4S,4S)-5-SG; 4a, (4S,5S)-4-SG; 3b, (4R,5R)-5-SG; 4b, (4R,5R)-4-SG.

originates from (4S,5R)-BPO and it follows that the absolute configuration of 3a is (4S,5S); compound 4a, a 4-glutathionyl regioisomer, originates from (4R,5S)-BPO and it has absolute configuration (4S,5S). The third eluting peak in Fig. 3a contains the remaining two diastereomers, a 4-glutathionyl isomer (from (4S,5R)oxide) and a 5-glutathionyl isomer (from (4R,5S)oxide), both with absolute configuration (4R,5R). Separation of the latter set of diastereomers was required for further studies on the stereochemical aspects of the enzyme catalyzed reaction. The approach followed was to affect the selectivity of the system by changes in the eluent. Two modifications were introduced: first, the use of diethylenetriamine (DETA) in the preparation of the buffer was found beneficial (9); second, the use of 1-propanol as organic modifier had a profound effect on the selectivity of the column. As shown on Fig. 3b separation of all the diastereomeric GSH adducts of (±)-BPO was achieved with the modifications described above. The diastereomers shown coeluting in Fig. 3a are now identified as 3b, a 5-glutathionyl isomer with absolute configuration (4R,5R), and 4b, a 4-glutathionyl isomer with absolute configuration (4R,5R). The HPLC on Fig. 3b represents a complete stereochemical profile of the GSH adducts of (±)-BPO.

The situation with (±)-BAO was as complicated as with BPO with two positional isomers and a total of four diastereomers. As shown in Fig. 4, conditions were developed which allow the analysis of all four diastereomers. Trace 4a shows separation of three peaks in the ratio of 2:1:1, the modifier used in this case was methanol. Trace 4b shows a different profile, three peaks in the relative ratio 1:1:2, when 1-propanol was used as modifier. Structural and stereochemical assignments for the GSH adducts of BAO, based on HPLC analysis of authentic GSH conjugates of optically pure BAO (see Materials and Methods), were as follows: (5R,6S)-BAO on reaction with GSH gave rise to a 5-glutathionyl isomer, absolute configuration (5S,6S), identified as 5a, and a 6-glutathionyl isomer, absolute configuration (5R,6R), identified as 6b; (5S,6R)-BAO

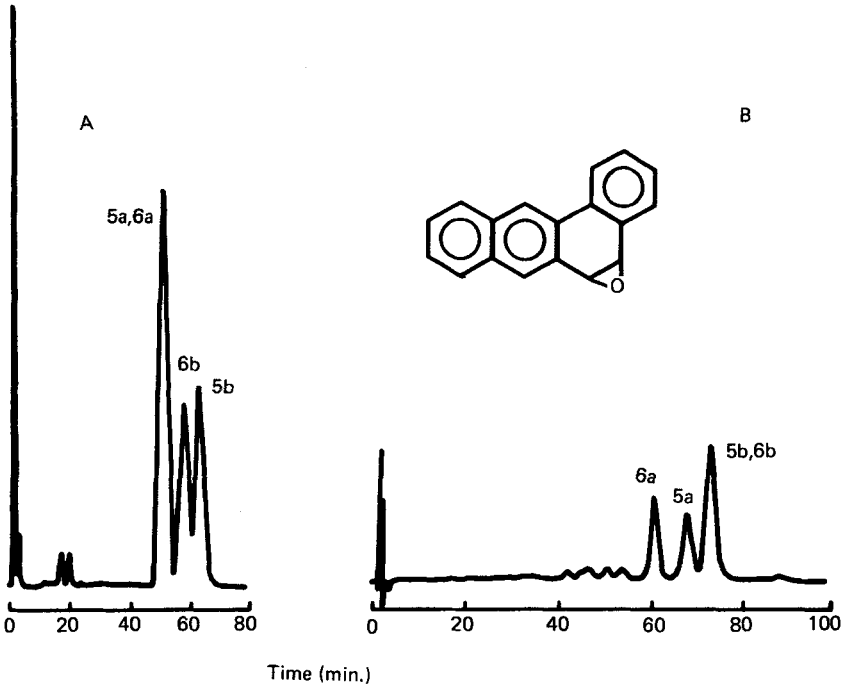

Figure 4. HPLC profile for the diastereomeric GSH adducts of (±)-benz[a]-anthracene 5,6-oxide. Trace A, system 1, Tris-phosphate pH 3/ 30% MeOH, flow rate 3 ml/min. Trace B, system 2, DETA-phosphate pH 3/9% 1-propanol, flow rate 2 ml/min. Peak identification: 6a, (5S,6S)-6-SG; 5a, (5S,6S)-5-SG; 6b, (5R,6R)-6-SG; 5b, (5R,6R)-5-SG.

formed a 5-glutathionyl adduct, absolute configuration (5R,6R), identified as 5b, and a 6-glutathionyl adduct, absolute configuration (5S,6S), identified as 6a. Elution profiles for all these diastereomers is illustrated in Fig. 4. A recent report in the literature described an HPLC separation of the GSH conjugates of BAO (10). The profile reported in that paper resembled that obtained in the present work with methanol as modifier. A direct extrapolation to the present work is not possible since we have shown that the separation is quite sensitive to solvent effects. The conditions used in that report (10) (3 micron ODS-2 column, 8% CH_3CN/20%

MeOH/72% 100 mM Tris-phosphate pH 2.5) are substantially different from the ones used in the present study. The conditions described here allow separation of all diastereomers by choosing the appropriate solvent conditions.

The two bonded phases used is this study, C-8 Zorbax and Waters C-18, were comparably effective in resolving these compounds. The differences observed in selectivity are more directly correlated to the effect of the organic modifiers. The use of an organic base remains a prime requirement (9), and DETA would seem to be the base of choice for these compounds.

The observed change in column selectivity as a function of organic modifier is the combined result of two effects: 1) the nature of the interaction between the solvent and the bonded phase; 2) solute-solvent interactions. The first effect involves a comparison between methanol and 1-propanol. Higher molecular weight alcohols are reported to change the selectivity of reversed-phase bonded phases by a loading effect, the hydrophobic alcohols effectively saturate the HPLC column (11). How this effect may help discriminate among stereoisomers is not predictable. The second effect is perhaps more easily correlated with the problem at hand. Diastereomers differ in the spatial arrangement of their substituents and consequently are expected to have different stable conformations. This stable conformer population may be significantly affected by solvent interactions, and it has been reported (7) that for dihydro derivatives of polynuclear aromatic hydrocarbons a shift from diequatorial to diaxial conformers occurs in high polarity solvents. The use of 1-propanol, a stronger eluent than methanol, decreases the amount of organic modifier (9% vs 30% methanol) required for elution, with the resulting mobile phase being more aqueous and hence more polar. This change in polarity could have an effect on the relative conformer populations of the GSH adducts of (±)-BPO and (±)-BAO (12).

Although it is not possible at this time to clarify the observed selectivity effects, two important observations emerge from these studies.

First, the order of elution of sets of regioisomers, 3 and 4 for BPO and 5 and 6 for BAO is consistent in that diastereomers with S-configuration emerge earlier than those with R-configuration; 3a and 4a for BPO (Fig. 3b), and 5a and 6a for BAO (Fig. 4b). Second, the order of elution of diastereomers with the same absolute configuration at the carbon-bearing sulfur, i.e. 3a and 4a for BPO, 5a and 6a for BAO, is determined by the proximity of the peptide residue to the most hydrophobic portion of the molecule. Thus, on 3a the peptide residue is adjacent, and presumably interacts by inhibiting binding of the "naphthalene residue" to the bonded phase (13), while in 4a this group is more available for binding; similarly, 3b elutes earlier than 4b. A similar analysis in BAO shows that 5a elutes earlier than 6a, and 5b is less retained than 6b. These observations on the influence of stereochemistry in the separation of thioether derivatives of K-region arene oxides are in accordance with those reported for the GSH adducts of styrene oxide (14). In the latter, thioethers with S-configuration eluted earlier than the R-diastereomers. It is possible that the order of elution under the conditions described here, represent a general rule for this type of compounds. If such were the case, it should be possible to assign relative configuration, based on the HPLC elution profile of GSH adducts of epoxides of unknown stereochemistry. A prerequisite would be the ability to separate the anticipated number of diastereomers. For phenanthrene 9,10-oxide and pyrene 4,5-oxide this conditions is met and it is tempting to assign the S-configuration to the first eluting diastereomer in each case (Fig. ?).

The conditions described here provide a tool for the stereochemical analysis of the glutathione transferase catalyzed reactions with arene oxides and, in the case of metabolically formed epoxides, for the stereochemistry of oxidation of polynuclear aromatic hydrocarbons by the cytochrome P-450 dependent monoxygenase system (4).

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