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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CYTOSOLIC GLUTATHIONE S-TRANSFERASE ACTIVITY WITH STYRENE OXIDE

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay for measuring cytosolic glutathione S-transferase activity with styrene oxide is described. After incubating lung or liver cytosol with reduced glutathione and styrene oxide, unreacted styrene oxide is extracted into ethyl acetate. An aliquot of the aqueous phase is evaporated to dryness and reconstituted in the mobile phase for HPLC analysis. The two glutathione conjugates of styrene oxide [S-(1-phenyl-2-hydroxyethyl)glutathione and S-(2-phenyl-2-hydroxyethyl)glutathione] are separated in less than 10 min; quantitation of transferase activity is based on the comparison of the UV absorbance of the two conjugates at 254 nm with synthetic conjugate standards. As little as 1 nmole of either conjugate can be quantitated with good precision. This assay has advantages over previously published methods for measuring styrene oxide glutathione S-transferase activity as it does not depend on the use of relatively unstable and expensive radiolabelled substrates.

INTRODUCTION

Cytochrome P450-dependent metabolism of a number of chemicals results in the formation of highly reactive, electrophilic intermediates that can interact with cellular macromolecules. These interactions have been implicated in toxicities ranging from cellular necrosis to cancer [1, 2]. Alternatively, these highly reactive intermediates can form adducts with reduced glutathione (GSH), a process thought to represent a major detoxification pathway for many electrophiles [3]. In an effort to understand the mechanisms by which certain chemicals are toxic to specific target organs, several studies have examined the role of glutathione conjugation versus other competing detoxification pathways. These studies have often been hampered by the lack of reliable methods for quantitating the glutathione adducts.

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Glutathione adduct formation with styrene oxide has been used extensively as an in vitro measure of glutathione S-transferase activity [4—6]. Styrene oxide also has been used in isolated perfused organs where attention has focused on the relative importance of epoxide hydration and glutathione conjugation in the overall metabolic fate of this epoxide [7, 8].

Previous methods for measuring glutathione transferase activity with styrene oxide have relied on the use of either carbon-14- or tritium-labelled epoxide, which are currently available only by custom preparation. Quantitation of transferase activity was based on the amount of radioactivity remaining in the aqueous phase after extraction of the unreacted substrate. The sensitivity of this radiochemical assay is limited by high background levels of radioactivity in incubations with boiled enzyme. This problem can be corrected, however, by purifying the substrate just prior to use [9].

Cytosolic glutathione transferase activity also has been measured by utilizing [^{35}S]glutathione followed by thin-layer chromatographic separation of the styrene oxide—glutathione adducts from ^{35}S-labelled reduced and oxidized glutathione [10].

This report presents a rapid, reliable high-performance liquid chromatographic (HPLC) procedure for measuring glutathione S-transferase activity with styrene oxide, which has advantages over previous assays because it does not depend upon the use of relatively unstable and expensive radiolabelled substrate. Quantitation of transferase activity is based on comparison of the UV absorbance of the two styrene oxide—glutathione conjugates eluting from the column with synthetically prepared styrene oxide conjugate standards.

EXPERIMENTAL

Chemicals

Styrene oxide was purchased from Aldrich (Milwaukee, WI, U.S.A.). Reduced glutathione was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were of analytical-reagent grade or better.

Animals

Male Sprague-Dawley rats (150—225 g) were obtained from Hilltop Breeding Labs. (Chatsworth, CA, U.S.A.) and were kept for at least 5 days before use. Animals were allowed food and water ad libitum.

Preparation of lung and liver cytosol fraction

Animals were killed by decapitation and the lungs were perfused via the pulmonary artery with ice-cold heparinized saline. All further procedures were carried out at 0—4°C. Lungs and livers were removed, rinsed with ice-cold buffer (0.02 *M* Tris—1.15% potassium chloride, pH 7.4), minced and homogenized in 3 volumes of Tris—potassium chloride buffer. After centrifugation at 9000 *g* for 20 min, the supernatant was centrifuged at 105,000 *g* for 1 h. Endogenous glutathione was removed by chromatographing the supernatant on a Sephadex G-25 column previously equilibrated with 0.05 *M* sodium phosphate buffer, pH 7.4 [11]. Protein concentrations were determined by the method of Lowry et al. [12] using bovine serum albumin as standard.

Incubations

A 2-ml incubation contained lung or liver cytosolic protein (at specified protein concentrations), 1 m*M* reduced glutathione and 0.05 *M* sodium phosphate buffer, pH 7.8. Styrene oxide (1.0 m*M* in 20 μl of acetonitrile) was added prior to transferring the incubation vessels from ice to a shaking incubator at 37°C. In time-course studies, enzyme and glutathione were preincubated for 2 min prior to adding styrene oxide. Reactions were terminated by adding 3 ml of ice-cold ethyl acetate and the contents of each vessel were transferred to centrifuge tubes. Unreacted styrene oxide was extracted into ethyl acetate and discarded and an aliquot of the aqueous phase (100—200 μl) was evaporated to dryness under nitrogen for HPLC analysis. The rate of non-enzymatic glutathione conjugate formation was monitored in identical incubations containing boiled cytosolic enzyme.

HPLC analysis

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) M6000A pump, U6K injector and M440 UV detector (fixed wavelength at 254 nm). Samples were reconstituted in a small volume of mobile phase (20% methanol—1% glacial acetic acid—79% water) and an aliquot was injected on to a 30 × 0.39 cm μBondapak C_{18} column (10-μm packing material, Waters Assoc.) at a flow-rate of 1.5 ml/min.

Preparation of standards

Styrene oxide—glutathione conjugate standards were synthesized and partially purified as described by Ryan and Bend [7]. The methanol eluate from the XAD-2 column, which contained both glutathione conjugates, was rotary evaporated and the conjugates were separated by semi-preparative HPLC (Waters Assoc. μBondapak C_{18} column, 30 × 0.78 cm) and collected. After removing the solvent by rotary evaporation, the remaining oily product was dissolved in a small volume of water. The addition of methanol resulted in the precipitation of colorless crystals. Reference standards of each conjugate were prepared in distilled water and were stable for at least 6 months when frozen.

Identification of the conjugates

The two conjugates were separated and purified from incubations of styrene oxide, glutathione and liver cytosolic enzyme by HPLC on a semi-preparative C_{18} column as described above. Solvent was removed by rotary evaporation and the product was dissolved in ^2H_2O for NMR analysis. Proton NMR spectra of each conjugate were recorded on a Brucker WM 250-mHz spectrometer. Structural assignments were based on previously reported spectra [13].

RESULTS AND DISCUSSION

Aliquots of the aqueous phase from incubations containing native cytosolic protein, glutathione and styrene oxide analyzed by HPLC revealed two UV-absorbing peaks that eluted at 5.7 and 6.8 min (Fig. 1A). In identical incubations with boiled cytosolic protein, only a small amount of each conjugate

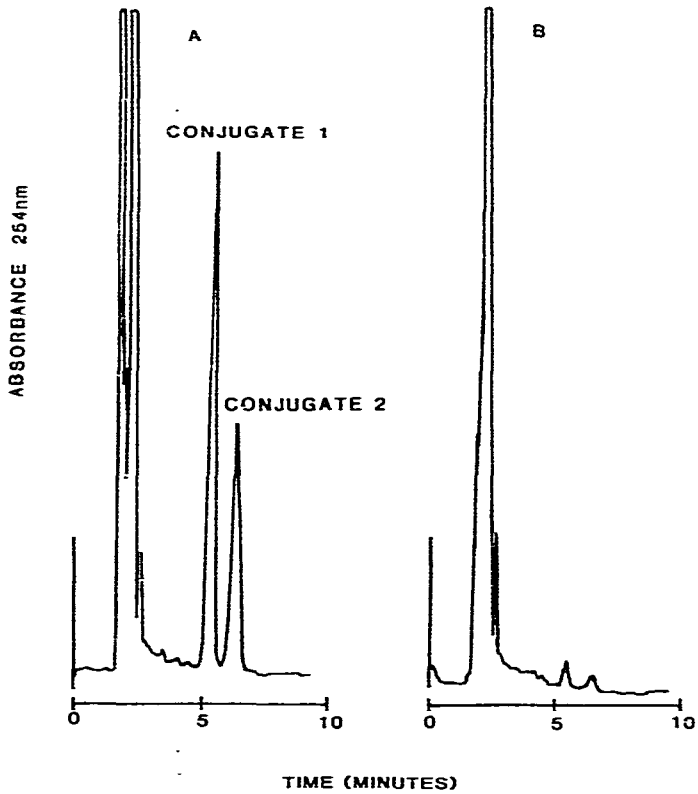

Fig. 1. HPLC profile of 50 μl of the aqueous phase prepared from an incubation containing styrene oxide (1.0 m*M*), reduced glutathione (1.0 m*M*) and (A) 1.0 mg of native liver cytosol or (B) 1.0 mg of boiled liver cytosol. The UV absorbance was monitored at 254 nm. Detector sensitivities were 0.01 and 0.005 a.u.f.s. for A and B, respectively.

was detectable (Fig. 1B). The two peaks were completely absent from incubations lacking styrene oxide or glutathione (data not shown). Extracts prepared from incubations of lung cytosolic enzyme yielded chromatograms which were similar to those obtained with liver cytosolic enzyme (Fig. 1). Synthetically prepared styrene oxide—glutathione conjugates also eluted at 5.7 and 6.8 min.

Separation and quantitation of the two styrene oxide—glutathione conjugates can also be achieved on C_{18} Radial-Pak columns (Waters Assoc., 10 × 0.8 cm) using 35% methanol—1% glacial acetic acid—64% water containing 5 m*M* heptanesulfonic acid at a flow-rate of 3 ml/min. Under these conditions, the elution times of the two conjugates formed from styrene oxide and glutathione were 5.8 and 6.6 min.

Preparative-scale isolation of the two styrene oxide—glutathione conjugates followed by NMR analysis showed conjugate 1 to be S-(1-phenyl-2-hydroxyethyl)glutathione and conjugate 2 to be S-(2-phenyl-2-hydroxyethyl)glutathione, thus confirming the earlier studies of Seutter-Berlage et al. [14], Pachecka et al. [15] and Watabe et al. [13], showing that conjugation of glutathione with styrene oxide occurs at both electrophilic carbon atoms.

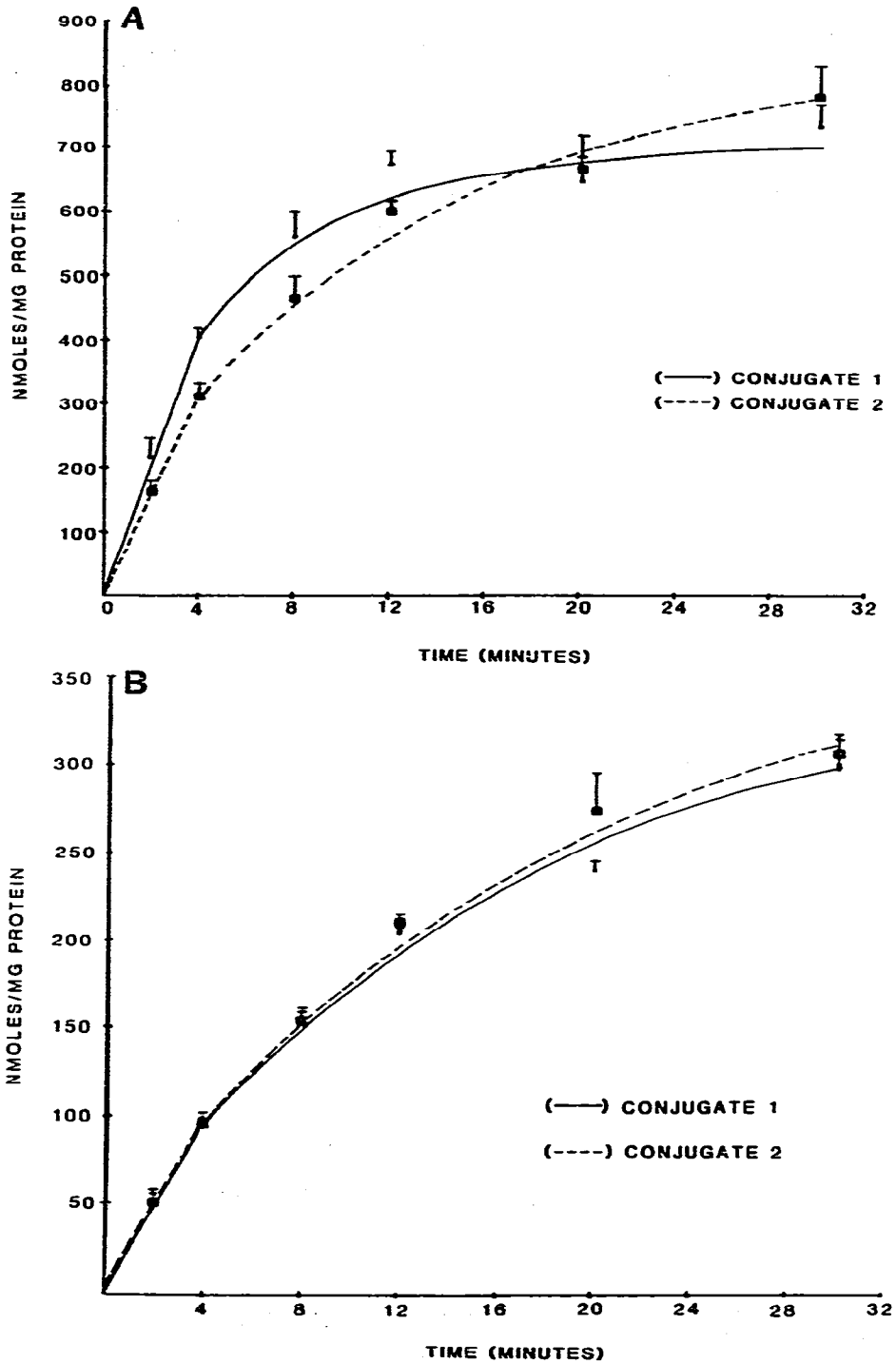

Fig. 2. Time course of formation of conjugates 1 and 2 in incubations containing (A) liver or (B) lung cytosolic enzymes (1 mg), styrene oxide (1.0 m*M*) and glutathione (1.0 m*M*). The rate of non-enzymatic conjugate formation has been subtracted from each value. Points are the means ± standard errors of the means for triplicate incubations.

Calibration graphs, prepared by plotting the product of peak height × absorbance units full-scale versus the amount of synthetically prepared styrene oxide—glutathione conjugate injected, were linear from 0.4 to 4 μg for each conjugate, with correlation coefficients > 0.9996.

The rates of formation of both S-(1-phenyl-2-hydroxyethyl)glutathione and S-(2-phenyl-2-hydroxyethyl)glutathione were linear for 4 min when either liver (Fig. 3A) or lung (Fig. 3B) was used as an enzyme source. Liver cytosolic

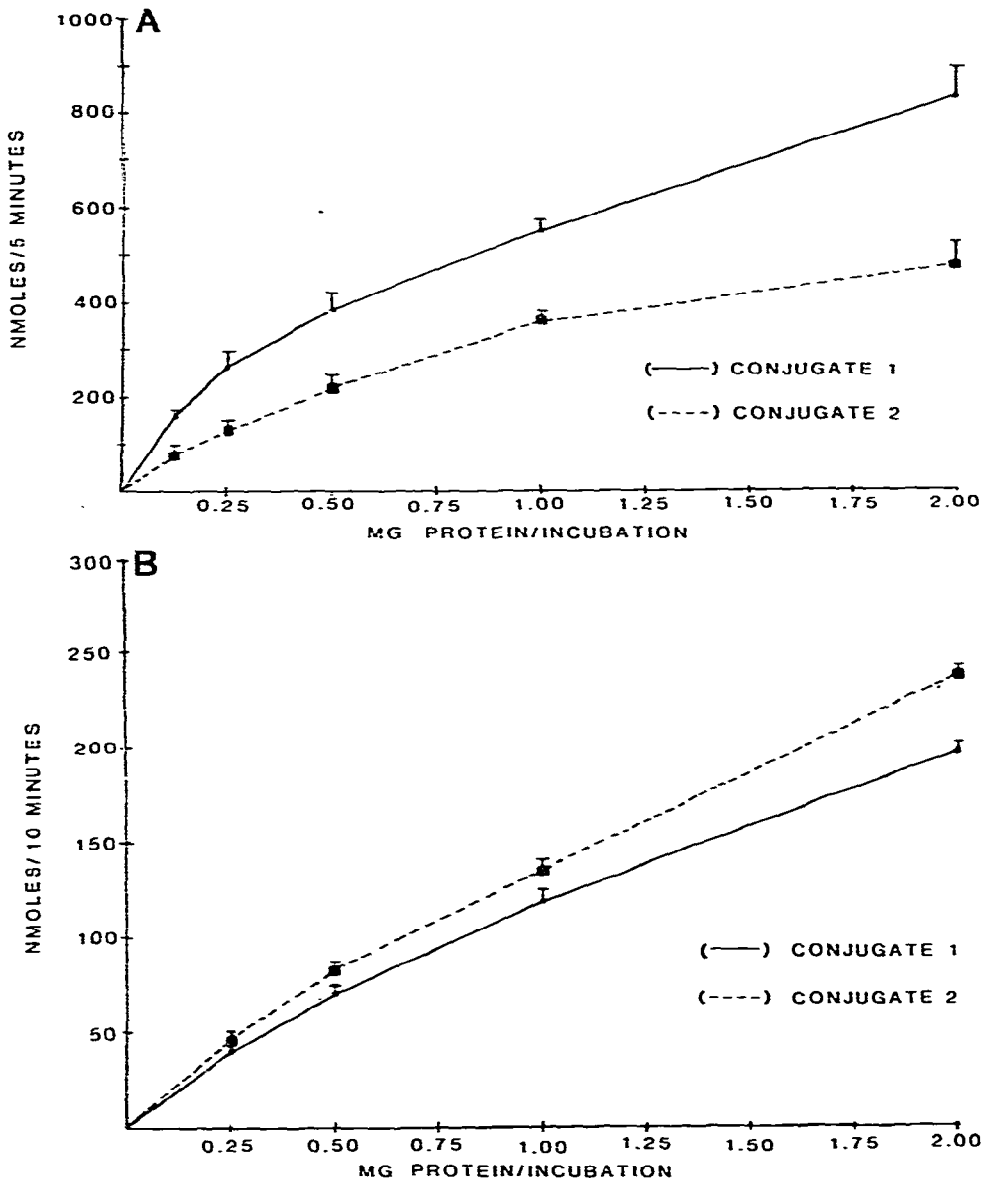

Fig. 3. Effect of protein concentration on the rate of conjugate formation in incubations containing (A) liver or (B) lung cytosolic protein, styrene oxide (1.0 m*M*) and glutathione (1.0 m*M*). The rate of non-enzymatic conjugate formation has been subtracted from each value. Points are the means ± standard errors of the means for triplicate incubations.

enzymes consistently produced conjugate 1 at faster rates than conjugate 2, while pulmonary cytosolic enzymes produced the isomers at approximately equal rates. The ratio of the rates of formation of the two conjugates was not affected by styrene oxide concentration or by the pH of the incubation (6.0—9.0). The rates also were not affected by the type of buffer used (phosphate or Tris). At pH 7.5, non-enzymatic conjugate formation occurred at less than 5% of the enzymatic rate.

Liver cytosolic transferase activities measured with this HPLC method are in good agreement with those of previous studies [4, 5, 16], while activities from lung cytosolic transferase were slightly higher than those reported previously [16]. This may be due to the increased specificity of this method, thereby resulting in lower background levels, or because the lungs were perfused in the current study to remove extraneous blood.

Fig. 3A shows that the formation of both conjugates was linear with protein to 0.25 mg in incubations of liver cytosolic enzymes and to 0.50 mg with lung cytosolic enzymes. These results are consistent with those of studies using radiolabelled styrene oxide [4, 5].

Hence the method presented here provides a rapid means of quantitating cytosolic glutathione S-transferase activity with styrene oxide. Unlike previous methods, the proposed assay does not require the use of radiolabelled substrates, and it is sufficiently sensitive to detect conjugate formation in tissues which generally have low activity, such as lung.

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