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Study of chromatographic parameters for glutathione S-transferases on an high-performance liquid chromatography affinity stationary phase

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Abstract

The chromatographic parameters were examined for recombinant glutathione S-transferases (GSTs) on a new HPLC affinity packing containing the immobilized ligand S-octylglutathione. The k' values of both rA1-1 and rP1-1 were determined under isocratic conditions with increasing concentrations of the mobile phase ligand S-butylglutathione. Plots of $1/k'$ vs. S-butylglutathione concentration were non-linear which is consistent with a bivalent model for the association of these dimeric enzymes and the stationary phase. Low flow-rates were found to be decisive in obtaining good resolution of the isoenzymes, and at 0.10 ml/min it was possible to obtain baseline resolution of rP1-1, rA1-1 and rM1a-1a using shallow, linear gradients of GST competitive inhibitors. Association constants were determined from solution phase kinetics assuming a rapid equilibrium random Bi Bi mechanism. Solution phase association constants provide an approximate guide for the selection of ligands useful in this affinity phase HPLC separation of GST isoenzymes. A good fit ($r^2 = 0.998$) for the rA1-1 binding data was obtained using the solution phase binding constant but this was not the case for rP1-1. A comparison of the selectivities for the separation of rP1-1, rA1-1 and rM1a-1a was made using the GST competitive inhibitors S-hexylglutathione, S-butylglutathione and γ-glutamyl-(S-hexyl)cysteinyl-phenylglycine as mobile phase modifiers. The association constants determined in solution did not always predict the elution order of the recombinant GSTs (rGSTs) using the mobile phase inhibitors. Yields of active rGSTs from the column were 90%, 88% and 61% for rP1-1, rA1-1 and rM1a-1a, respectively. This technique was used in the fractionation of GSTs in placental and liver cytosols.

1. Introduction

Glutathione S-transferases (GSTs, E.C. 2.5.1.18) are a group of cytosolic enzymes which catalyze the conjugation of electrophilic xenobiotics with glutathione via the free sulfhydryl group present in this abundant intracellular tripeptide [1]. This process generally leads to the detoxification of the xenobiotics since the resulting adducts are normally metabolized to mercapturates and excreted. These human cytosolic enzymes belong to a supergene family comprised of at least four multigene classes Alpha, Mu, Pi and Theta. The enzymes exist as dimers with monomeric molecular masses between 23 and 27 kD and have a range of isoelectric points from 4.8 to 8.9 [2].

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These dimeric enzymes are named by class followed by an Arabic numeral designation of subunit composition. For example A1-1 designates a homodimer of the Alpha class consisting of two monomeric subunits of type A1 [3].

Current chemotherapy often fails due to the appearance of tumor cells resistant to chemotherapeutic agents [1,4]. In some cases elevated levels of the GSTs have been associated with the resistant state [5,6]. Because the expression of GSTs in cancerous and in normal human tissues varies widely in both level and type, techniques to examine isoenzyme profiles are needed for more accurate determination of the role of GSTs in drug resistance. Traditionally, the batch purification of GSTs is performed by affinity chromatography [7–9]. Affinity chromatography is also used to separate a range of GSTs by using isocratic and/or gradient elution with a counter ligand in the mobile phase. Partial resolution of GST homodimers and heterodimers has previously been accomplished using this technique with either S-hexylglutathione or glutathione as the affinity ligand bound to agarose [10–12]. In principle, the elution order of GSTs from an affinity column can be controlled by varying the type of counter ligand in the mobile phase [13–16]. Changes in selectivity resulting from a simple change in a mobile phase additive potentially make this a very powerful separation technique.

We recently developed an affinity HPLC stationary phase for the separation of GSTs using gradients of GST inhibitors [17]. In this report we study the interactions of recombinant GSTs (rGSTs) with this stationary phase in order to gain a better understanding of the parameters required to optimize chromatographic separation of GSTs. The parameters studied are the valence of interaction between the rGSTs and the stationary phase [14,15], and the effect of different mobile phase inhibitors [13–15,18], flow-rate and gradient steepness [14] on elution. These parameters are applied to optimize the separation of GSTs from two different human tissues.

2. Experimental

2.1. Reagents

Iodobutane, iodooctane, sodium borohydride, 1,4-butanediol diglycidyl ether and EDTA were purchased from the Aldrich (Milwaukee, WI, USA). Glutathione, dithiothreitol, 1-chloro-2,4-dinitrobenzene (CDNB), Tween-20 and phenylmethylsulfonyl fluoride were obtained from the Sigma (St. Louis, MO, USA). Recombinant GST enzymes rA1-1, rP1-1, and rM1a-1a were obtained from B. Mannervik (University of Uppsala, Sweden). HEMA BIO 1000 (10 μm) was purchased from Melcor Technologies (Sunnyvale, CA, USA)

2.2. Synthesis of peptides

S-butylglutathione and S-octylglutathione were synthesized by the method of Vince *et al.* [19]. The tripeptide γ-glutamyl-(S-hexyl)cysteinyl-phenylglycine (TER102) was synthesized as previously reported [20]. All peptides had greater than 90% purity when analyzed by reversed-phase HPLC and had acceptable elemental analyses.

2.3. Chromatography

The HPLC system consisted of one or two HPXL pumps equipped with 10 ml/min titanium pump heads, a Rheodyne 7125-081 titanium injector and a Dynamax UV-C detector (Rainin Instrument, Woburn, MA, USA). At the detection wavelength of 280 nm, an intensity of 1000 mV is equivalent to 1 OD unit. All pathways in contact with the mobile phase were of either titanium or biocompatible polymer construction.

For affinity chromatography a gradient was formed with a static mixing tee (Upchurch Scientific, Oak Harbor, WA, USA). When chromatography was performed in the isocratic mode, one port in the static mixing tee was plugged. A biocompatible 250 p.s.i. (1724 kPa) back-pressure regulator (Upchurch Scientific) was placed between the injector and the mixing tee. The

back-pressure regulator was placed in-line to insure proper function of the HPLC pump check valves at the low pressure generated at low flow-rates.

Fractions (0.8 min/fraction) were collected with a Gilson FC203 fraction collector (Rainin Instrument) into a polystyrene, 96-well plate (Costar, Cambridge, MA, USA, Catalog No. 9017). To reduce protein adsorption, the 96-well plate was pretreated with a solution of 0.1% Tween-20 for 16 h, washed with deionized water and air dried.

To determine the fraction of activity retained on the column, the unretained and retained protein peaks, as determined by absorbance at 280 nm, were collected in preweighed 1.5 ml polypropylene tubes. The volume of the fraction collected was determined by weight difference. Activities were then determined for the collected fractions.

2.4. Determination of valence of interaction

To determine the valence of binding of rA1-1 and rP1-1 to the affinity stationary phase, a series of zonal elution, isocratic runs were made at 0.1 ml/min with mobile phases containing 200 m*M* sodium chloride, 10 m*M* sodium phosphate (pH 6.0) and varying concentrations of the eluting ligand S-butylglutathione (Fig. 1). The column was equilibrated with at least 40 ml of a mobile phase containing S-butylglutathione before determining the elution times of rGSTs. The elution time of the recombinant protein (20 μl injection volume) was first determined in the mobile phase with the lowest concentration of S-butylglutathione. Subsequent elution times were determined with sequentially increasing concentrations of the eluting ligand. Capacity factors (k′) were calculated by using the elution time of ovalbumin as t_o.

2.5. CDNB conjugating activity

GST activity, as determined by the color change following conjugation of CDNB with glutathione, was measured at 340 nm with a Tmax Plate Reader (Molecular Devices, Menlo Park, CA, USA). Aliquots (5 or 10 μl) of fractions were placed in the wells of a 96-well plate and mixed with 190 μl of a standard reaction solution. Standard reaction conditions were 200 m*M* sodium phosphate (pH 6.8) with 1 m*M* glutathione and 1 m*M* CDNB at 32°C [21].

2.6. Determination of enzyme K_i values

The kinetic constants (K_i) for rP1-1, rA1-1 and rM1a-1a in the presence and absence of the inhibitors S-butylglutathione, S-hexylglutathione, TER102, or S-octylglutathione were determined from CDNB conjugating activity measurements at 10 m*M* sodium phosphate, 200 m*M* sodium chloride (pH 6) as described above and fitting the data using Hanes–Woolf plots [21].

The simplest mechanism for the conjugation of CDNB (A) and glutathione (B) by GST in the presence of an inhibitor (I) is shown below.

$$E + A \rightleftharpoons EA$$

$$E + B \rightleftharpoons EB$$

$$EA + B \rightleftharpoons EAB$$

$$EB + A \rightleftharpoons EAB$$

$$E + I \rightleftharpoons EI$$

$$EAB \rightarrow E + P$$

It is assumed that the binding of A and B are independent and that the inhibitor, I, is purely competitive with both A and B. The relevant dissociation constants based on these assumptions are $K_a = [E][A]/[EA] = [EB][A]/[EAB]$, $K_b = [E][B]/[EB] = [EA][B]/[EAB]$, and $K_i = [E][I]/[EI]$. Assuming steady-state kinetics, Eq. 1 can be derived for this rapid equilibrium random Bi Bi mechanism [22].

$$v = \frac{V_{max}[B]/(1 + K_a/[A])}{K_b\{1 + [I]/K_i(app)\} + [B]} \quad (1)$$

where

$$K_i(app) = K_i\frac{[A]}{K_a}(1 + K_a/[A]) \quad (2)$$

The apparent dissociation constant, $K_i(app)$,

for an inhibitor was determined in the presence of an inhibitor by varying the glutathione concentration (B) and holding the CDNB (A) concentration constant at 1 m*M*. In a separate series of experiments the value of K_a was determined in the absence of inhibitor by varying the CDNB concentration while maintaining the glutathione concentration at 1 m*M*. The dissociation constants for CDNB were found to be 1.8 m*M*, 0.65 m*M* and 1.6 m*M* for rA1-1, rM1a-1a and rP1-1 respectively. The K_i values for the inhibitors were then calculated from the experimental values K_i(app), K_a and [A] = 1 m*M*. In Table 1 are listed the association constants K_{asso} (reciprocal of K_i) for the inhibitors. The inhibitors listed in Table 1 were found to be purely competitive with glutathione for all the rGSTs investigated and competitive with CDNB for rA1-1. All four inhibitors showed mixed inhibition with CDNB for rM1a-1a. All of the inhibitors were competitive with CDNB for rP1-1, except for TER102 which showed mixed inhibition.

2.7. Determination of activity retained on the affinity column

The percentage of activity retained was determined as a function of ionic strength of the loading buffer for the rGSTs. For these experiments the GST was diluted (1:5) in loading buffer (10 m*M* sodium phosphate, pH 6.0 with or without 200 m*M* sodium chloride) and injected onto the column (5 × 0.46 cm I.D.). Then the column was eluted for 5 min at 0.5 ml/min with loading buffer and the unretained protein collected. The flow-rate was increased to 1.0 ml/min with a mobile phase of 200 m*M* sodium chloride (10 m*M* sodium phosphate, pH 6). From 5 to 15 min a linear gradient of 5 m*M* S-hexylglutathione in 200 m*M* sodium chloride (10 m*M* sodium phosphate, pH 6) was used to elute retained GST from the column. The fraction containing eluted protein was collected and the activity determined. The percent of activity retained on the column was determined using the relationship: % activity retained = [activity retained/(activity retained + activity unretained)] × 100.

To determine the total recovery of protein from the column, injections were made in a mobile phase of 5 m*M* S-hexylglutathione, 200 m*M* sodium chloride and 10 m*M* sodium phosphate (pH 6.0). Under these conditions all the rGSTs were unretained. The area (280 nm) of the peak eluted in the 5 m*M* S-xylglutathione mobile phase was compared with the sum of the areas for the unretained and retained peaks described in the previous paragraph.

The amount of enzyme activity recovered after injection in 200 m*M* sodium chloride, 10 m*M* sodium phosphate (pH 6.0) from the affinity column was determined using the gradient previously described. A stock solution of the enzyme was prepared and one-half injected onto the column. Factions corresponding to the elution of protein were collected and the activity determined. The column was then disconnected, the injection loop filled using the other half of the enzyme stock solution and the enzyme collected in approximately the same volume of mobile phase as the other collected fractions. Again the activity was determined. The percent of activity recovered was calculated using the relationship: %activity recovered = activity eluted from the column/activity injected on the column.

Table 1
Solution phase association constants of ligands with GSTs

Ligand	K_{asso} (μM^{-1})		
	rA1-1	rM1a-1a	rP1-1
S-Hexylglutathione	1.3	1.2	0.17
S-Butylglutathione	0.02	0.11	0.07
TER102	0.34	0.08	6.3
S-Octylglutathione	4.0	4.0	0.65

2.8. Influence of eluting ligand

The three rGSTs were injected onto the column in a mobile phase of 10 m*M* sodium phosphate (pH 6.0). Gradients of S-hexylglutathione (0–5 m*M*), S-butylglutathione (0–20 m*M*) or TER102 (0–20 m*M*) as indicated in Fig. 2 were used to elute the rGSTs.

2.9. Influence of flow-rate and gradient steepness

Flow-rate and the gradient steepness were investigated to determine their influence on resolution of rP1-1 and rA1-1 (Fig. 3) using S-hexylglutathione (0–5 m*M*) gradients in 10 m*M* sodium phosphate (pH 6) and 200 m*M* sodium chloride. The gradient duration was varied inversely with the flow-rate so that their product (gradient steepness) was constant. For these flow-rate studies, the gradient duration was 25 min for measurements at 0.2 ml/min, 50 min at 0.1 ml/min and 167 min at 0.03 ml/min (Fig. 3a). Next the gradient steepness was varied while the flow-rate was maintained at 0.1 ml/min (Fig. 3b). For both studies the samples were loaded at 0.1 ml/min in 10 m*M* sodium phosphate (pH 6) for 9 min and the washing step was from 10–14 min with 10 m*M* sodium phosphate (pH 6) containing 200 m*M* sodium chloride. Gradients commenced at 15 min.

2.10. Tissue treatment

Human liver samples were obtained from the Cooperative Human Tissue Network (Columbus, OH and Birmingham, AL, USA) and stored at −80°C. Samples were slightly thawed, minced with scissors and homogenized at 25% (w/v) in buffer containing 10 m*M* Tris hydrochloride (pH 7.8), 1 m*M* EDTA, 1 m*M* dithiothreitol and 0.10 m*M* phenylmethylsulfonyl fluoride using an OMNI stator generator homogenizer (Marietta, GA, USA). Cytosol was prepared by ultracentrifugation at 105 000 *g* for 35 min at 4°C in a Beckman Optima TL-100 tabletop ultracentrifuge (Fullerton, CA, USA). Protein concentrations were determined using a 96-well plate Coomassie dye binding assay (Bio Rad, Richmond, CA, USA) read on a Tmax Plate Reader with bovine serum albumin as a standard [9].

2.11. Affinity matrix

The synthesis of the affinity matrix follows the general procedure of Sundberg and Porath [23]. HEMA BIO 1000, 1,4-butanediol diglycidyl ether and 0.6 *M* sodium hydroxide containing 2 mg/ml of sodium borohydride (0.03:1:1, w/v/v) were mixed overnight. The particles were filtered and washed with water, ethyl alcohol and acetone.

To 700 mg of the dried particles were added S-octylglutathione (75 mg) dissolved in 3.5 ml of 0.5 *M* sodium carbonate. The suspension was mixed for approximately 90 h. After filtration the particles were washed with (i) 1 *M* sodium chloride, 0.1 *M* sodium phosphate (pH 9), (ii) 1 *M* sodium chloride, 0.1 *M* sodium acetate (pH 4.5), (iii) water, (iv) ethyl alcohol and (v) acetone.

2.12. Packing of columns

The affinity material (650 mg) was slurried in 20 ml of water and was packed at high pressure into stainless-steel columns 5 × 0.46 cm I.D. or 3 cm (Supelco, Bellefonte, PA, USA). The column frits were 2 μm (average pore diameter) titanium encased in a CTFE ring (Upchurch Scientific, Oak Harbor, WA, USA). A Haskell (Burbank, CA, USA) DSTV122 liquid pump was used to provide the drive solvent (water) during the packing process. The columns were packed at 2000 p.s.i. (13 790 kPa) with 50 ml of water and then 4000 p.s.i. (27 579 kPa) with 50 ml of water. In some cases 5 × 0.3 cm I.D. Omni glass columns with stainless-steel frits (Rainin Instrument) were used. These columns were packed by connecting a 5-ml syringe to the outlet and pulling an aqueous slurry of the stationary phase into the column.

3. Results and discussion

Two models for the interaction of a GST with the stationary phase were considered. The first model assumes only monovalent interactions between a protein molecule and a ligand.

$$E + I \overset{K_1}{\rightleftharpoons} EI$$

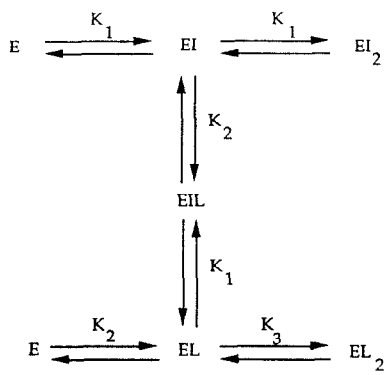
$$E + L \overset{K_2}{\rightleftharpoons} EL$$

In this scheme E is the protein (GST), I is the mobile phase eluting ligand (S-butylglutathione) and L is the immobilized affinity ligand (S-octylglutathione). From the treatment of Walters [24,25] for this model, the inverse of the capacity factor as a function of mobile phase ligand concentration is given by:

$$\frac{1}{k'} = \frac{V_M}{K_2 m_L}(1 + K_1[I]) \quad (3)$$

In Eq. 3, $K_1 = [EI]/[E][I]$, $K_2 = \{EL\}/[E]\{L\}$, m_L = number of moles of affinity ligand in column and is the product of $A \cdot \{L\}$ and V_M is the total volume of mobile phase in the column. The symbol $\{\}$ indicates surface concentration and A is the column surface area [24,25]. Eq. 3 predicts that a plot of $1/k'$ *vs.* [I] should be a linear relationship. Clearly from Fig. 1, such a linear relationship is not exhibited for either rA1-1 or rP1-1.

The second model assumes that a protein molecule can interact with two molecules of ligand. This divalent model is represented as:



For the divalent model, the inverse of the capacity factor as a function of the mobile phase ligand concentration is given by [25].

$$\frac{1}{k'} = \frac{V_M}{K_2 m_L} \frac{(1 + K_1[I])^2}{2(1 + K_1[I]) + K_3\{L\}} \quad (4)$$

In this model an assumption is made that the two binding sites on the protein are identical and independent even when E is adsorbed on the stationary phase. The association constant K_1 is assumed, therefore, to be the same for the binding of I to E, EI and EL. The equilibrium constant K_3, which represents the equilibrium constant for the binding of EL to a second immobilized ligand, may be highly sensitive to steric effects. This condition is not necessary, however, and a divalent model has been proposed in which the equilibrium constant for the binding of the second immobilized affinity ligand is the same as for the first ligand [16]. The solid lines shown in Fig. 1 are computed based on Eq. 4. An association constant of $K_1 = 0.02\ \mu M^{-1}$ is used to generate the curve ($r^2 = 0.998$) for rA1-1. For the rP1-1 the curve ($r^2 = 0.976$) is generated using an association constant of $K_1 = 0.6\ \mu M^{-1}$.

The interaction of recombinant GSTs with this affinity stationary phase is best described by the divalent model. Several of the assumptions used in the derivation of Eq. 4 are reasonable for these enzymes. GSTs are known to be dimeric enzymes with each subunit containing one active site. Extensive kinetic data strongly suggest that the two active sites per dimer are kinetically independent [26]. Thus we propose that two affinity ligands (S-octylglutathione) bound to the stationary phase as well as two mobile phase ligands (S-butylglutathione) are able to interact with a GST dimer during the affinity chromatographic process.

The small deviations seen between the positions of the experimental points in Fig. 1 and the curves calculated from Eq. 4 may arise because of invalid assumptions used to derive this equation. In particular the assumption that K_1 best describes the binding of inhibitor to the EL complex may be incorrect. Enzymatic activities often decrease when the protein is attached to a surface [27,28] indicating that association constants may be different for the protein in solution than for the same protein attached to a surface.

In principle, the relevant association constants for mobile phase and stationary phase ligands can be calculated from kinetic inhibition constants. On the basis of the simple mechanistic model proposed here, the relevant association constants were determined (Table 1). The use of these experimentally determined association constants to calculate binding curves (Fig. 1) and to predict the elution order of proteins (Fig. 2) was

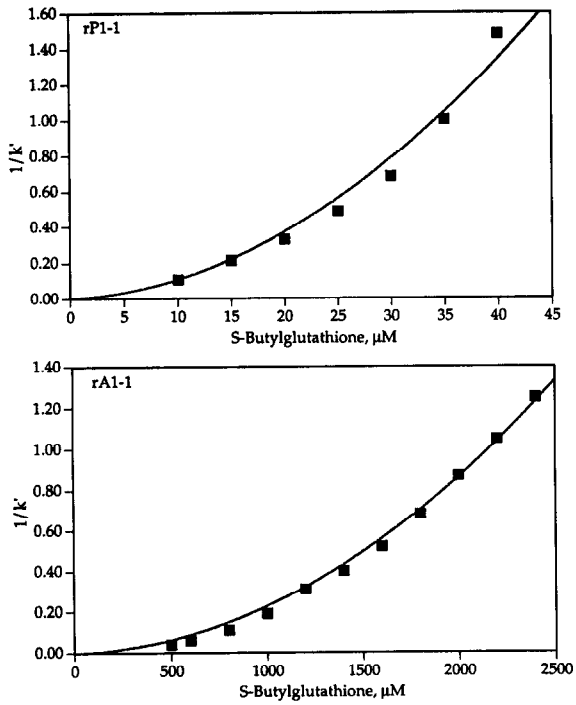


Fig. 1. $1/k'$ *vs.* S-butylglutathione concentration for rP1-1 and rA1-1. The k' values for the proteins were determined at increasing concentrations of S-butylglutathione in 10 m*M* sodium phosphate and 200 m*M* sodium chloride (pH 6). Flow-rate: 0.1 ml/min. Detection: 280 nm. Columns: 3 × 0.46 cm I.D. stainless steel for rP1-1, 5 × 0.46 cm I.D. stainless steel for rA1-1.

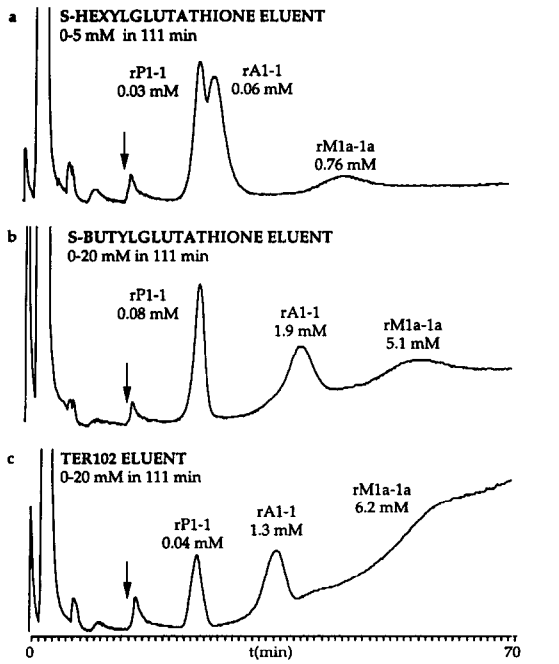


Fig. 2. Effect of eluting ligand on rGST elution. Gradients of S-hexylglutathione (a), S-butylglutathione (b) or TER102 (c) were used to elute the rGSTs. Mobile phases: A, 10 m*M* sodium phosphate (pH 6); B, 200 m*M* sodium chloride in A; C, S-hexylglutathione (5 m*M*), S-butylglutathione (20 m*M*) or TER102 (20 m*M*) in B. Gradient : 0–6 min, A, 0.1 ml/min; 7–14 min, B, 0.4 ml/min; 15–70 min, 0–57.3% C, 0.1 ml/min. Detection: 280 nm. Arrow marks the beginning of the eluent gradient. Column: 5 × 0.3 cm I.D. glass.

only partially successful. The curve for rA1-1 in Fig. 1 was calculated from the kinetically derived association constant and gave a good fit to the experimental points ($r^2 = 0.998$). The best fit curve for rP1-1 was calculated using an association constant of 0.6 μM^{-1} ($r^2 = 0.976$). Curves for rP1-1 data calculated using the kinetically derived association constant, 0.07 μM^{-1}, gave a very poor fit ($r^2 = 0.90$).

As expected, the ligand (S-hexylglutathione) which binds most strongly with the GSTs (Table 1) was the most effective ligand for the elution of the GSTs (Fig. 2). S-hexylglutathione eluted all three GSTs at concentrations less than 0.8 μM while the other two ligands required 5.1 μM (S-butylglutathione) or 6.2 μM (TER102). The order of rGST elution with S-hexylglutathione did not correspond, however, to the kinetically determined association constants. Examination of Table 1 indicates that rA1-1 and rM1a-1a exhibit equal solution phase association constants with S-hexylglutathione (the mobile phase ligand) and with S-octylglutathione (the stationary phase ligand). One might expect that these two rGSTs should nearly co-elute when S-hexylglutathione was used as the eluting ligand. The two proteins are clearly well separated using this mobile phase ligand (Fig. 2a). By a similar argument, rM1a-1a would be expected to be eluted before rA1-1 in a mobile phase containing S-butylglutathione but the order is in fact reversed (Fig. 2b). Interestingly, the order of elution using TER102 does correspond to that predicted from the kinetic association constants.

At least two reasons may be advanced to

explain the failure of kinetically derived solution phase association constants to predict chromatographically determined association constants or elution order. First the association constants of rGSTs with the immobilized S-octylglutathione may not be the same as those measured in solution. Because the immobilized S-octylglutathione imparts a net negative charge to the phase, the rGSTs see a different ionic environment when binding to an immobilized ligand than when binding to the ligand free in solution. The isoelectric points [2] of rP1-1 (p*I* 4.8), rM1a-1a (p*I* 6.6) and rA1-1 (p*I* 7.9) vary widely and at pH 6, the negatively charged rP1-1 will be repelled from the stationary phase while rA1-1 will be attracted. In addition, compared to binding in free solution, restricted orientation of the immobilized ligand could affect its ability to attain the same docking orientation in the catalytic site. The degree of restriction may vary between the various recombinant enzymes. Several investigators have found similar association constants for proteins to a ligand whether the ligand is free in solution or immobilized [15,18] while others have found differences of a factor of ten [29]. Second, the kinetically derived association constants may not be a valid measure of the true association constants. We have assumed the simplest mechanistic model for this complex enzymatic reaction. More complicated mechanisms have been proposed for the GSTs, and it has been postulated that mechanisms may vary among the isoenzymes [30,31]. Our observation that the four ligands studied showed mixed inhibition with CDNB for the GST rM1a-1a and competitive inhibition with rP1-1 and rA1-1 does support these mechanistic complexities. At best one may only use kinetically derived association constants for the qualitative prediction of elution order of GSTs.

Flow-rate and gradient steepness had a significant effect on this separation. The best separations were done at flow-rates considerably lower than are normally used in HPLC (Fig. 3). Low flow-rates are required due to a characteristic of affinity chromatography which relates to the slow dissociation rates of many protein–ligand complexes. Much faster kinetics are exhibited in other types of chromatography such as reversed-phase [14,32,33]. In addition, other

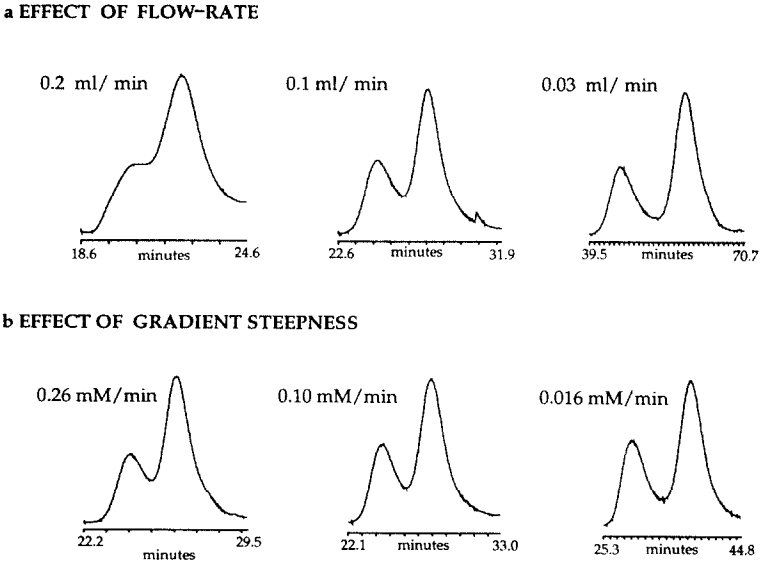


Fig. 3. Effect of flow-rate (a) and gradient steepness (b) on the separation of rP1-1 from rA1-1. Mobile phases: A, 10 m*M* sodium phosphate (pH 6); B, 200 m*M* sodium chloride in A; C, S-hexylglutathione (5 m*M*) in B. Samples were loaded in A, 0–9 min, 0.1 ml/min; washed in B, 10–14 min, 0.8 ml/min; and the gradient of 0–100% C commenced at 15 min. For gradient conditions see Experimental. Detection: 280 nm. Column: 5 × 0.3 cm I.D. glass.

diffusion-based processes responsible for band dispersion are sensitive to the molecular size of the analyte. Large molecules, such as proteins, produce large reduced velocities at normal HPLC flow-rates resulting in large plate heights [34]. Slow dissociation rates and high reduced velocities lead to large plate heights and relatively wide bands. At smaller reduced velocities these band widths can be minimized.

The slow dissociation rates of enzyme–stationary phase complexes can also be invoked to explain the increasing broadness of the peaks eluting later in the gradient (Fig. 2). Unlike gradients in reversed-phase chromatography where the peak width is generally constant regardless of the position in the gradient [35], here peak width increases with increasing retention. We postulate that M1a-1a elutes later in the gradient than rP1-1 because rM1a-1a binds more tightly to the affinity phase, and this difference in the association constants results from a smaller dissociation rate constant for the rM1a-1a/stationary phase complex than for the rP1-1/stationary phase complex. On the basis of this argument, at equal flow-rates the rM1a-1a peak will have a greater peak volume than the rP1-1 peak volume.

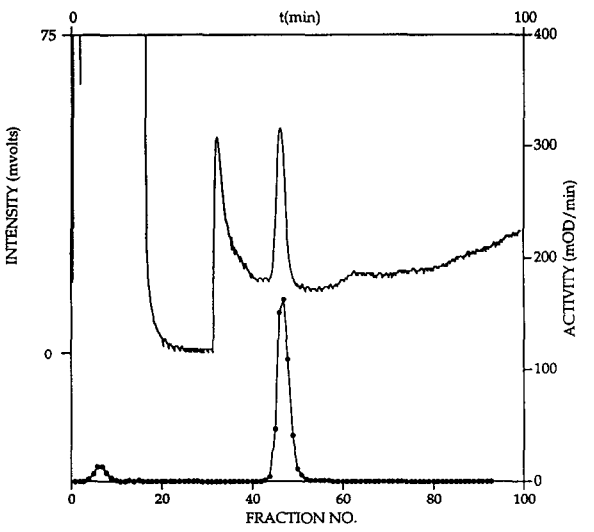


Fig. 4. Separation of GSTs from placental cytosol. Mobile phases: A, 10 m*M* sodium phosphate (pH 6); B, 200 m*M* sodium chloride in A; C, S-butylglutathione (20 m*M*) in B. Gradient : 0–12 min, A, 0.1 ml/min; 12–24 min, B, 0.4 ml/min; 24–116 min, 0–100% C, 0.1 ml/min. Detection: 280 nm (——). Activity (-●-). Column: 5 × 0.3 cm I.D. glass.

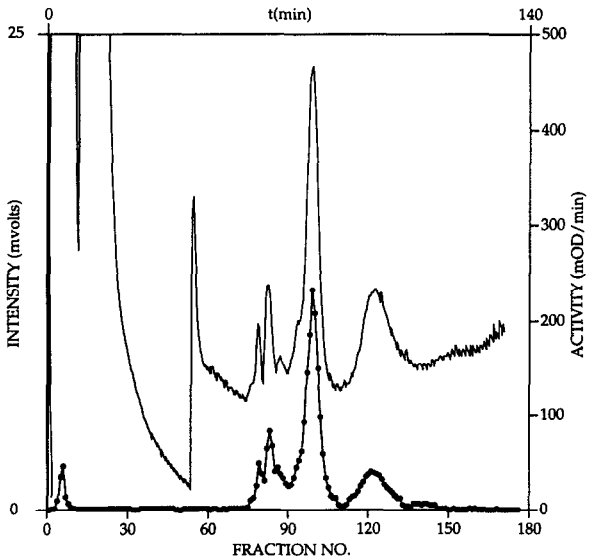


Fig. 5. Separation of GSTs from liver cytosol. Mobile phases: A, 10 m*M* sodium phosphate (pH 6); B, 200 m*M* sodium chloride in A; C, S-butylglutathione (20 m*M*) in B. Gradient : 0–5 min, A, 0.1 ml/min; 5–50 min, B, 0.4 ml/min; 50–146 min, 0–100% C, 0.1 ml/min. Detection: 280 nm (——). Activity (-●-). Column: 5 × 0.3 cm I.D. glass.

The ionic strength of the loading buffer had little effect upon the amount of rGST activity retained on the column. The activities retained for rP1-1, rM1a-1a and rA1-1 were 100%, 93% and 97%, respectively at low ionic strength (10 m*M* phosphate) and 100%, 99% and 95%, respectively at high ionic strength (200 m*M* sodium chloride, 10 m*M* phosphate). Yields as determined from peak areas at 280 nm of total protein eluted from the column were greater than 95% for the rGSTs. The percent activity eluted from the column under the higher ionic strength conditions were 90%, 88% and 61% for rP1-1, rA1-1 and rM1a-1a respectively.

Human cytosol obtained from human placenta and human liver was injected onto the affinity column and the GSTs in these samples fractionated. The separations were performed at the slow flow-rates and with gradients of S-butylglutathione found to be optimum for the model studies with rGSTs. For human placenta (Fig. 4) greater than 96% of the activity was eluted in

one peak which probably corresponds to the human P1-1. Previous investigators have shown that the GST in human placenta is predominately P1-1 [2]. For human liver (Fig. 5) greater than 97% of the activity was separated into at least four GST isoenzymes. Human liver is reported to contain multiple forms of GSTs [36].

In conclusion, solution phase inhibitory potency provides an approximate guide for selection of ligands useful in affinity HPLC separations of GST isoenzymes. Following empirical verification and adjustment, an optimized pair of immobilized and eluting ligands has been defined for resolving the major species of GSTs in human tissues.

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