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Journal of Chromatography A, 663 (1994) 53–63

JOURNAL OF CHROMATOGRAPHY A

Examination of glutathione S-transferase isoenzyme profiles in human liver using high-performance affinity chromatography

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(First received September 22nd, 1993; revised manuscript received December 1st, 1993)

Abstract

A method for the examination of the glutathione S-transferase isoenzyme profiles in human liver using a new HPLC affinity support is described. Liver cytosol was injected directly onto an HPLC column (5 × 0.46 cm) containing a support with a covalently bound affinity ligand (S-octylglutathione) specific for the isoenzymes. Contaminating cytosolic proteins were removed in a washing step. The isoenzymes were eluted with a linear gradient of a different affinity ligand in the mobile phase. Coinciding with the affinity ligand gradient, a salt gradient (0–200 m*M* sodium chloride) was applied. In this manner the isoenzymes were fractionated into the enzymatically active homodimers and heterodimers. The classes of the affinity fractionated isoenzymes were determined by SDS-PAGE and ELISA while the subunit content was determined by reversed-phase chromatography. For one liver three Alpha class isoenzyme subunits, forming three heterodimers and two homodimers, were detected. Five livers were examined, and the homodimer A1–1 was found to be the predominant glutathione S-transferase isoenzyme. Minor amounts of Pi and Mu class isoenzymes were also detected. This non-denaturing high-performance affinity chromatography method reduced analysis time by a factor of ten when compared to other affinity analysis methods for the glutathione S-transferases.

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 subunit molecular masses between 23 000 and 27 000 and have a range of isoelectric points from 4.8 to 8.9. Both heterodimers and homodimers exist, but heterodimers only form from subunits within the same class [2]. A dimeric enzyme is named by class followed by an Arabic numeral designation of the subunit composition. For example A1–2 designates a heterodimer of the Alpha class consisting of the monomeric subunits A1 and A2 [3].

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0021-9673/94/$07.00
SSDI 0021-9673(93)E1286-9

Individual isoenzymes show significant preference for particular compounds, including some in use as cancer chemotherapeutics [4–9]. Current chemotherapy often fails due to the appearance of tumor cells resistant to the chemotherapeutic agents [1,10]. Elevated levels of the GSTs have been associated with this resistant state [11–13] and as a predictor of early cancer recurrence [14]. This accumulated body of evidence suggests that GSTs are important in several aspects of cancer therapy. To substantiate this idea, improved methods are needed for determining the types and levels of the native isoenzymes.

Previously described sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separations of human GSTs allow the determination of class but not of subunit composition. Similarly, prior work has shown that the sensitive, high-resolution reversed-phase HPLC analysis of GSTs identifies many of the known subunits but gives no information as to the dimeric composition [15–17]. Both separation techniques are conducted under denaturing conditions which is of concern with regard to the discovery of any new GST isoenzymes in the genetically diverse human population. A number of ion-exchange [18–20] and chromatofocusing [21,22] methods have been described for the analysis of GSTs, and in some cases intact, enzymatically active dimers have been isolated. Before the application of any of these analytical techniques, a batch purification of the GSTs from cytosol is required. This partial purification is traditionally performed by affinity chromatography [23–25].

A method for the separation of a range of GSTs in dimeric, active form is affinity chromatography using isocratic and/or gradient elution with a counter ligand. This method combines the purification of the GSTs from the cytosol by affinity chromatography and the separation of the individual GSTs. 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 [26–28]. Due to the large particle diameter and slow flow-rates inherent with agarose gels, the fractionation time for these studies was approximately 25 h [29] which explains why this technique is not used for the routine analysis of the GSTs. Appreciable savings in time and increases in sensitivity would be anticipated if this affinity technique were performed on a small particle stationary phase [30]. An HPLC column containing immobilized glutathione as the affinity ligand has been described for the preparative purification of GSTs [31], but no attempt was made to separate the individual enzymes.

This report describes the affinity separation of GSTs from human liver as active dimers using a novel HPLC method. A stationary phase with a GST affinity ligand (S-octylglutathione) covalently linked to an HPLC particle was developed and used to determine liver profiles of this important family of detoxifying isoenzymes.

2. Materials and methods

2.1. Reagents

Iodobutane, iodooctane, sodium borohydride, 1,4-butanediol diglycidyl ether and EDTA were purchased from Aldrich (Milwaukee, WI, USA). Glutathione, dithiothreitol, 1-chloro-2,4-dinitrobenzene (CDNB), Tris, Tween-20, *p*-nitrophenyl phosphate and phenylmethylsulfonyl fluoride were obtained from Sigma (St. Louis, MO, USA). Alkaline phosphatase labeled goat anti-rabbit IgG was purchased from Kirkegaard and Perry (Gaithersburg, MD, USA). Recombinant GST enzymes rA1–1, rP1–1, rM1a–1a and rM1b–1b were obtained from B. Mannervik (University of Uppsala, Uppsala, Sweden), and rA2–2 was obtained from A. Townsend (Bowman Gray School of Medicine, Winston-Salem, NC, USA). Rabbit antisera against rA1–1, rP1–1 and rM1a–1a were obtained from rabbits, immunized with the appropriate recombinant GST, from BAbCo (Richmond, CA, USA). 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.* [32]. The tripeptide γ-glutamyl-(*S*-benzyl) cysteinyl-β-alanine (TER106) was synthesized as previously reported [33]. 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 two HPLX 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). For both reversed-phase (214 nm) and affinity chromatography (280 nm), 1000 mV were equivalent to 1 absorbance unit.

For reversed-phase analyses a 1.2-ml titanium, dynamic mixer (Rainin Instrument) was used to form the gradient. A 25 × 0.46 cm Vydac (Hesperia, CA, USA) reversed-phase column 218ATP54 with a mobile phase of water and acetonitrile containing 0.1% trifluoroacetic acid (Pierce Chemical, Rockford, IL, USA) was used to separate the GST subunits. The elution times of P1, M1a, M1b, A1 and A2 were determined by injection of the appropriate recombinant GST.

For affinity chromatography the gradient was formed with a static mixing tee (Upchurch Scientific, Oak Harbor, WA, USA). A biocompatible 100 p.s.i. (1 p.s.i. = $6.9 \cdot 10^3$ Pa) back-pressure regulator (Upchurch Scientific) was placed between the injector and the mixing tee. The back-pressure regulator was placed in-line to insure the proper function of the HPLC pump check valves at the low pressure generated during affinity chromatography. All pathways in contact with the mobile phase were of either titanium or biocompatible polymer construction. 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.

2.4. Tissue treatment

Human liver samples were obtained from the Cooperative Human Tissue Network (Columbus, OH and Birmingham, AL, USA) and stored at − 80°C. Liver tissues were designated with the prefix L affixed to a three digit number followed by the suffix CA (cancer) or N (normal). Samples were slightly thawed, minced with scissors and homogenized at 25% (w/v) in buffer containing 10 m*M* Tris–HCl (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 135 000 *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 (Molecular Devices, Menlo Park, CA, USA) with bovine serum albumin as a standard [25].

2.5. Pooled GST fractions

To obtain the total pooled population of GSTs from cytosol, a batch purification was performed using a Sepharose-6B affinity matrix with S-hexylglutathione ligand in a 96-well microplate equipped with a membrane on the bottom surface [25]. GSTs were eluted with S-hexylglutathione.

2.6. Gel electrophoresis

SDS-PAGE was performed according to standard methods [34] at a 12.5% (w/v) acrylamide concentration. Proteins were visualized after electrophoresis by silver staining [35].

2.7. CDNB conjugating activity

GST enzymatic activity was determined by measuring the conjugation of CDNB with glutathione at 340 nm with a Tmax Plate Reader. Aliquots (10 μl) of fractions from affinity chromatography or from fractions collected to determine activity recoveries 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 30°C [36].

2.8. Recovery of CDNB conjugating activity

Cytosol was diluted (1 to 50) into 200 m*M* sodium phosphate (pH 6.8) and 10 μl of this solution was used to determine the true activity. Unretained activity was determined from the column effluent collected during the loading step. The eluate containing the GSTs was collected as one fraction and the activity determined. A correction factor [25] to compensate for the inhibition of GST activity by the S-butylglutathione eluent was determined by comparing the true cytosolic activity with the activity of cytosol diluted (1 to 50) in a solution containing a concentration of S-butylglutathione equivalent to that in the GST eluate. This equivalent concentration of S-butylglutathione was obtained by collecting a fraction containing S-butylglutathione from a blank gradient. The time interval of collection of the fraction from the blank gradient was identical to the time interval for collection of the GST fraction from affinity chromatography. The ratio of true cytosolic activity to inhibited cytosolic activity was used to correct the activity of the eluted GST fraction. For the recovery studies the GSTs were collected over an interval of 10 min.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Aliquots of 50 μl of PBS (140 m*M* sodium chloride, 37 m*M* potassium dihydrogen phosphate, 11.2 m*M* disodium hydrogen phosphate, pH 7.2) were pipetted into each well of a Nunc MaxiSorp 96-well plate (VWR Scientific, Brisbane, CA, USA). To each well was added an aliquot (usually 10 μl) from the corresponding well of the collection plate from the affinity separation. The ELISA plate was then covered and left overnight at room temperature. The next day, contents of the plate were shaken out and the plate was blocked with 5% non-fat dry milk in PBS, 200 μl/well, for 1 h at 37°C or 2 h at room temperature. The plate contents were shaken out and the plate was washed once with a PBS solution with 0.05% Tween 20. To each well, 50 μl of the appropriate rabbit antisera diluted in the PBS–Tween solution was added and incubated for 1 h at 37°C. Plates were then washed three times with PBS–Tween, and alkaline phosphatase labeled goat anti-rabbit IgG, diluted 1:2500 in PBS–Tween, was added, 50 μl to each well. This was also incubated for 1 h at 37°C, and the plate was then washed three times with deionized water. Finally, color was developed by the addition of 100 μl/well of *p*-nitrophenyl phosphate, 1 mg/ml in 10 m*M* diethanolamine and 0.5 m*M* magnesium chloride. After color development (typically 30 min), the absorbance was read at 405 nm.

2.10. Affinity matrix

The synthesis of the affinity matrix follows the general procedure of Sundberg and Porath [37]. 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 was 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.

2.11. Packing of HPLC 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 (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) DSTV-122 liquid pump was used to provide the drive solvent (water) during the packing process. The columns were packed at 2000 p.s.i. with 50 ml of water and then 4000 p.s.i. with 50 ml of water.

3. Results

The chromatogram and activity profile for a typical affinity separation of GSTs from a human liver cytosol (L006N) is shown in Fig. 1. During loading (0–10 min) and washing (10–35 min) steps, GSTs were bound to the column and contaminating proteins were removed. Sodium chloride was required during the washing step to facilitate the elution of the contaminating proteins presumably adsorbed to the stationary phase by ionic interactions. GSTs were then eluted (50–120 min) with a gradient of the glutathione analog TER106. In subsequent affinity chromatograms only the portion of the separation corresponding to the activity profile for the retained GSTs is shown.

The average recovery of CDNB conjugating

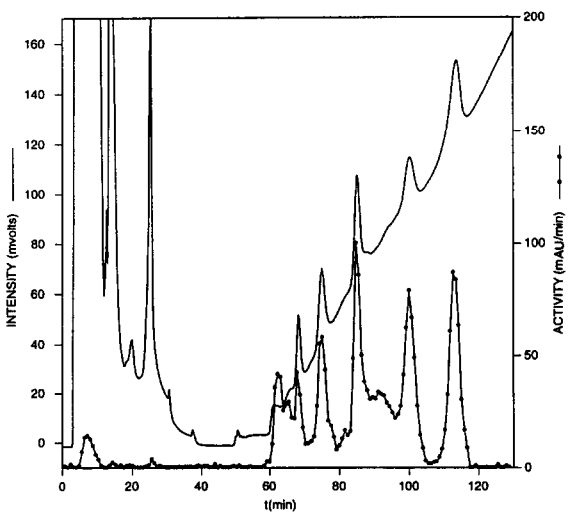

Fig. 1. Profiles of CDNB conjugating activity (●) and intensity at 280 nm (—) for the affinity separation of liver L006N using TER106 as the eluting ligand. Mobile phase: (A) 10 m*M* sodium phosphate (pH 6), (B) 200 m*M* sodium chloride in A, (C) 40 m*M* TER106 in B. Gradient: 0–10 min, 100% A, 0.1 ml/min; 10–30 min, 100% B, 0.4 ml/min; 30–35 min, 100% B, 1 ml/min; 35–50 min, 100% A, 1 ml/min; 50–108 min, 100% A to 48% C, 0.1 ml/min; 108–150 min, 48% to 100% C, 0.1 ml/min.

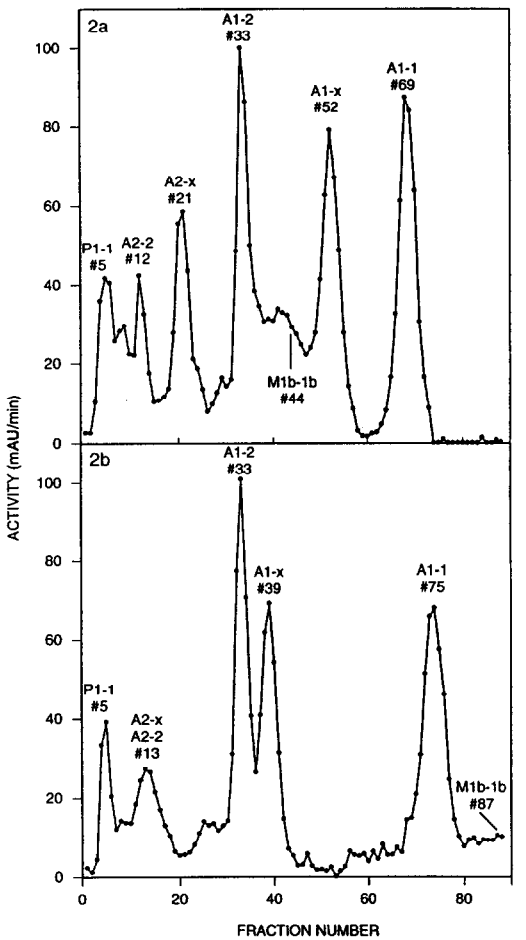

Fig. 2. Profiles of the CDNB conjugating activity for the affinity separations of liver L006N using different eluting ligands. (a) Eluting ligand TER106 (40 m*M* in buffer C); (b) eluting ligand S-butylglutathione (20 m*M* in buffer C). See Fig. 1 for the gradient profile. Fraction No. 1 in Fig. 2 corresponds to 59.2 min in Fig. 1.

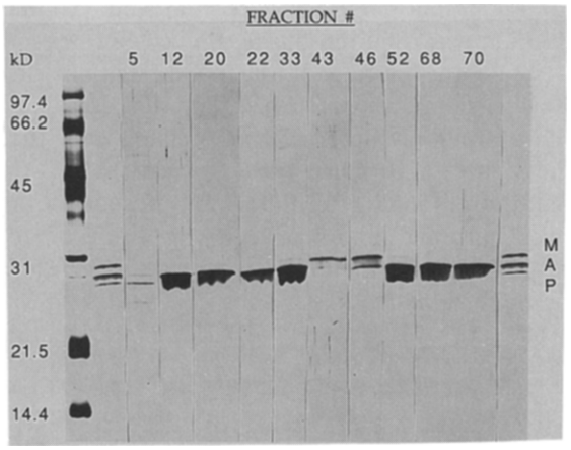

Fig. 3. SDS-PAGE of selected fractions from the affinity separation of L006N using TER106 as the eluting ligand. Lanes 4–13 contain aliquots from the corresponding fractions of Fig. 2a. Lanes 3 and 14 contain a mixture of the recombinant M1a–1a (M), A1–1 (A) and P1–1 (P).

activity during an affinity separation was 76%. The activity recoveries for three liver samples were 66% (L002N), 60% (L002CA) and 103% (L004CA). The proportion of unretained activity for the three livers varied from 3 to 13%. The reproducibility of the GST affinity extraction was determined by comparing the amounts of unretained activity from a series of repetitive injections of the same cytosol (L005N). On the first day, two affinity separations were performed and the amount of unretained activity was 2.5 and 2.3%. In a separate series of experiments made two days later the amount of unretained activity was 2.6, 2.6 and 3.5%.

Fig. 2 shows the GST isoenzyme content of the major peaks eluted with TER106 (Fig. 2a) or with S-butylglutathione (Fig. 2b). Note that fraction No. 1 in Fig. 2a corresponds to 59.2 min of Fig. 1. The GST isoenzyme content of the peaks in Fig. 2a was identified from the analysis of the collected fractions by SDS-PAGE, ELISA and reversed-phase chromatography while in the case of Fig. 2b the fractions were analyzed by SDS-PAGE and reversed-phase chromatography. The class of the GST in the fractions was determined by SDS-PAGE and ELISA with representative data shown in Figs. 3 and 4, respectively. There appeared to be some cross-

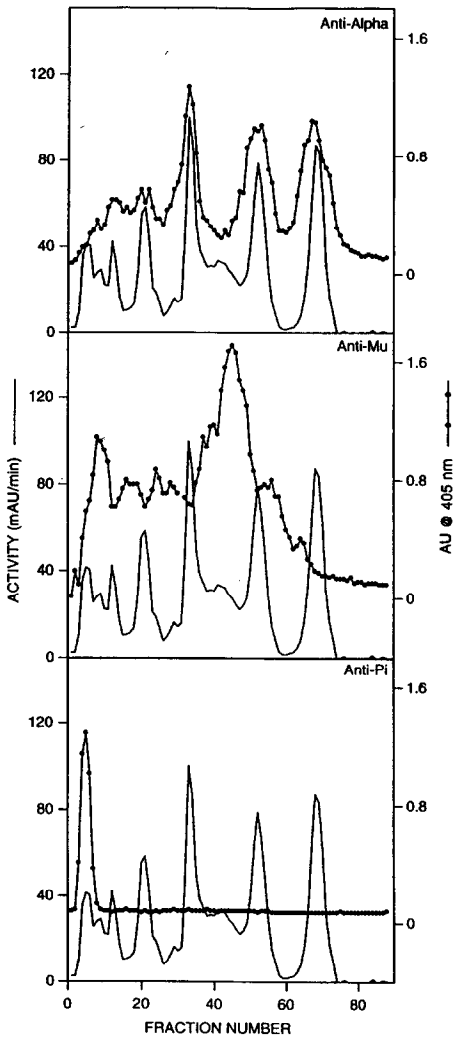

Fig. 4. ELISA analysis (●) and CDNB conjugating activity (—) of fractions from the affinity separation of L006N using TER106 as the eluting ligand. On separate plates the aliquots from the corresponding fractions of Fig. 2a were analyzed with anti-Alpha, anti-Mu or anti-Pi IgG. See Materials and Methods for experimental details.

reactivity of the anti-Mu IgG with Alpha isoenzymes in fraction Nos. 9 and 52. This cross-reactivity was not investigated further. The major Mu peak as detected by ELISA was centered at fraction No. 44 in agreement with the SDS-PAGE and reversed-phase chromatography results.

To assess the subunit composition of the isoenzymes, the fractions corresponding to the

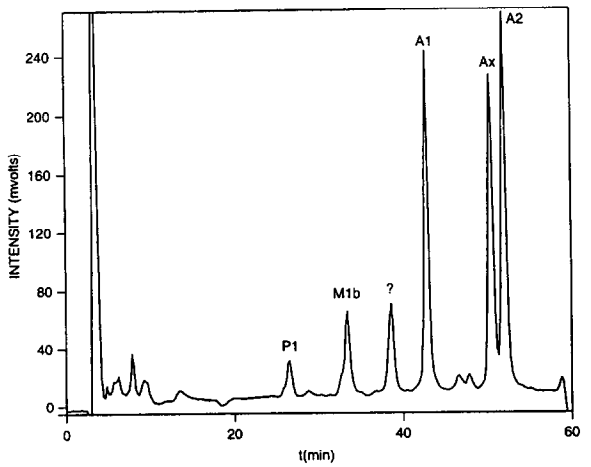

Fig. 5. Reversed-phase chromatogram of the pooled GSTs of liver L006N. Mobile phase: (A) acetonitrile with 0.1% trifluoroacetic acid, (B) water with 0.1% trifluoroacetic acid. Gradient: 41% A to 55% A in 55 min at 1 ml/min.

major peaks found in Fig. 2 were individually analyzed by reversed-phase chromatography. Subunits were identified by comparison of their retention times with the retention times of recombinant GSTs. Liver L006N contained a protein component which eluted between A1 and A2 during reversed-phase chromatography (Fig. 5) and which had an elution time not corresponding to any of the recombinant enzyme standards. This protein component was tentatively assigned as Ax (see Discussion). Representative chromatograms are shown in Fig. 6 for fractions Nos. 21 and 33 of the TER106 elution. Affinity peaks assigned as containing heterodimers showed two peaks of equal area corresponding to the two subunits in the isoenzyme (Fig. 6). For affinity peaks containing homodimers, only one major peak was detected by reversed-phase chromatographic analysis.

The isoenzyme content of five liver cytosols, shown in Table 1, was determined from affinity chromatographic analysis using S-butylglutathione as the eluting ligand. Peak assignments for the affinity separations were based on retention of the isoenzymes identified for L006N, as described above (Fig. 2b). Isoenzyme identities were further substantiated (see Discussion) from the subunit content determined by the

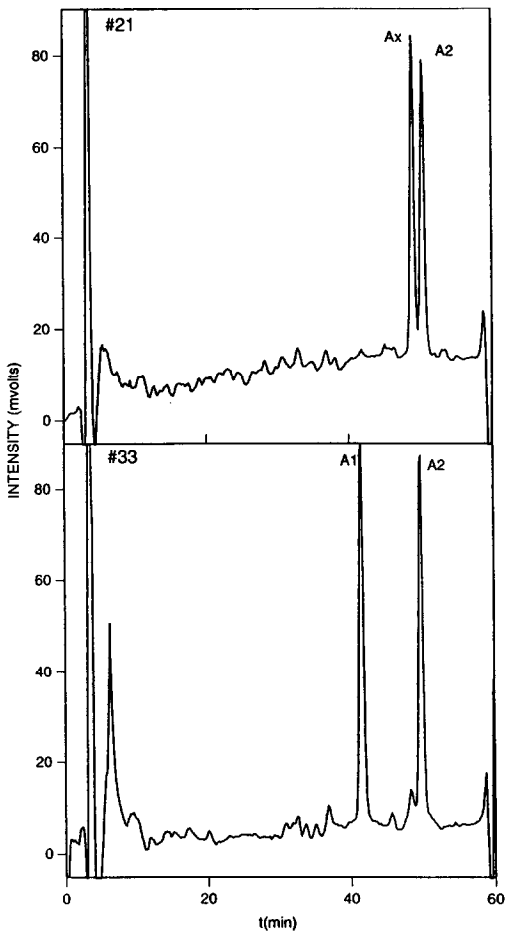

Fig. 6. Reversed-phase chromatograms of fractions Nos. 21 and 33 from the affinity separation of liver L006N using TER106 as the eluting ligand. Fraction numbers correspond to the fractions of Fig. 2a. See Fig. 5 for chromatographic conditions.

reversed-phase analysis of pooled GSTs derived from S-hexylglutathione–Sepharose 6B extractions of the cytosols [25]. The reversed-phase chromatogram from the pooled GST extract of L006N, corresponding to the affinity separation shown in Fig. 2b, can be seen in Fig. 5. Peak assignments were based on the retention of rGSTs. The peak between M1b and A1 and centered at 39 min, which was observed for all five livers, has not been identified and was not investigated further. A peak at a similar retention time, also unidentified, has been reported by Van Ommen *et al.* [16]. Minor

Table 1
GST profiles of human liver

Liver	Isoenzyme content[a]						Subunit content[b]				
	P1–1	A2–x A2–2	A1–2	A1–x	A1–1	M1b–1b	P1	M1b	A1	Ax	A2
L006	+[c]	++	++	++	++	+	+	+	++	++	++
L007	+	++	++	–	++	–	+	–	++	–	++
L004	+	++	–	++	++	+	+	+	++	++	+
L005	+	–	–	+	++	++	+	+	++	+	+
L002	–	+	+	–	++	+	+	+	++	–	+

[a] The GST isoenzyme content of a liver as determined by affinity analysis. Buffers: (A) 10 m*M* sodium phosphate (pH 6.0); (B) 200 m*M* sodium chloride in A; (C) 20 m*M* S-butylglutathione in B. Conditions: 0–5 min, A, 0.1 ml/min; 5–21 min, B, 1.5 ml/min; 21–34 min, A, 1 ml/min; 34–35 min, A, 0.1 ml/min; 35–155, 0–42% C, 0.1 ml/min.
[b] The GST subunit content of the liver as determined by reversed-phase analysis.
[c] + + = Greater than 10% of total peak area; + = less than 10% of total peak area; – = not detected.

amounts of M1a were also observed at 28 min for two of the livers, L005N and L002N (data not shown). The GST subunit composition, along with the isoenzyme content, is shown in Table 1 for the five liver cytosols.

4. Discussion

A significant saving in GST analysis time was achieved by performing the separation on the novel HPLC packing described here. Liver samples were analyzed in 2 h, more than ten times faster than similar separations using Sepharose-6B based affinity supports [29]. Washing and eluting steps were significantly shorter in duration with the HPLC method. Although the washing step in the separation shown in Fig. 1 was conducted at 0.4 and 1.0 ml/min, the HPLC packing is capable of withstanding higher pressures. In the analysis of the five human livers (Table 1) advantage was taken of this pressure stability by conducting the washing step at 1.5 ml/min thereby reducing the washing time by 9 min. In preliminary experiments we have found that flow-rates of 2 ml/min during the washing step can be tolerated in this system with no loss of affinity bound GST isoenzymes.

The affinity separation of the GST isoenzymes described here depended upon the simultaneous application of two concentration gradients. One gradient consisted of a linearly increasing concentration of either TER106 (Fig. 2a) or S-butylglutathione (Fig. 2b), both known competitive inhibitors of the GSTs [36]. The general mechanism for this type of chromatographic separation has been reviewed [38]. In the loading step, a GST complexes with an affinity ligand covalently linked to the stationary phase. As the concentration of the competitive inhibitor (TER106 or S-butylglutathione) increases during the elution step, the GST increasingly partitions into the mobile phase and finally elutes. The order of elution of a mixture of GSTs is a complex function of their affinities for both the immobilized affinity ligand and the competitive inhibitor in the mobile phase. For instance a change in elution order was observed when comparing the difference in GST elution profiles using two different eluting ligands. Elution with S-butylglutathione as compared with elution with TER106 shifted the Mu class isoenzyme to a longer retained peak relative to the Alpha class peaks. In addition, the S-butylglutathione elution resulted in the co-elution of A2–x and A2–2 which were resolved with TER106 elution. Note that twice the concentration of TER106 to S-butylglutathione was needed to produce similar chromatograms, reflecting their differing affinities for the isoenzymes [36].

Second, a salt gradient was also applied, coinciding with the ligand gradient. Without this

gradient the first three components (P1–1, A2–x and A2–2 in Fig. 2b) co-eluted. The salt gradient may be acting to overcome ionic interactions between the proteins and the stationary phase bound affinity ligand, S-octylglutathione, which was expected to have a net negative charge at pH 6.0. Another interpretation of the salt effects is possible. The chloride ion may act as a competitive inhibitor of the GSTs and thus affect their retention on the affinity stationary phase. Small anions have been shown to act as competitive inhibitors in another enzyme system [39].

The recovery of CDNB conjugating activity eluted with an affinity ligand from the affinity column averaged 76% for three livers while the average yield of total activity (affinity ligand eluted activity plus unretained activity) was 83%. The yield of CDNB conjugating activity from the affinity column described here was comparable to that described in previous reports. Reported yields of the recovery of CDNB conjugating activity from affinity chromatography of tissues from various sources have varied from 30–95% [31]. For human liver cytosol eluted from agarose columns using S-hexylglutathione as the affinity ligand, yields varied from 84 [40], 80–100 [41], to 51% [42] while in a study of two livers using the affinity ligand S-octylglutathione on agarose yields were 47 and 67% [22].

There are several possible reasons for the variation in the fraction of unretained activity. Competitive inhibitors of GSTs may exist in liver and these competitive inhibitors may cause a decrease in the binding of isoenzymes to the column. The concentrations and types of these competitive inhibitors may vary from liver to liver, giving rise to the observed inconsistency of unretained activities for different livers. In addition Hayes [29] has noted that different affinity matrices have different selectivities. The affinity matrix described in this report may not bind certain GST isoenzymes. The levels and amounts of these non-binding GST isoenzymes can be expected to vary depending on the liver source, and this variation could give rise to the observed inconsistencies in yield. Variation in the unretained activity from different rat livers processed by affinity chromatography has been noted [28].

The class of GST isoenzymes in the major peaks were identified by both SDS-PAGE (Fig. 3) and ELISA (Fig. 4). The subunit composition of the isoenzymes was determined by reversed-phase HPLC (Fig. 6). Liver L006N appears to contain three forms of Alpha subunits. The reversed-phase chromatogram of the GSTs isolated from liver L006N shows a peak which elutes between A1 and A2 (Fig. 5). We have tentatively designated this later eluting protein as subunit Ax. This designation was based on the following evidence. The SDS-PAGE analysis (Fig. 3) of the TER106 affinity fraction No. 21 containing A2–x and fraction No. 52 containing A1–x (Fig. 2a) showed subunits with molecular masses characteristic of Alpha class subunits. The reversed-phase HPLC analysis indicated equal amounts of subunits A1 and A2 in fraction No. 33 which indicates that this analysis method is valid for identifying heterodimers (Fig. 6). Equal amounts of subunits A2 and Ax in fraction No. 21 (Fig. 6), is consistent with heterodimer formation. In addition a dimer of A1 and Ax is detected by reversed-phase chromatography in fraction No. 52 (data not shown). We surmise that Ax is capable of forming a heterodimer with Alpha class subunits. To date heterodimers have only been found to form among members of the same class [2]. The association of Ax with A1 and A2 suggests that this subunit belongs to the Alpha class of GSTs. Moreover, the ELISA analysis of the affinity HPLC fractions (Fig. 4) shows that the fractions containing Ax are only reactive with the anti-Alpha IgG.

Human liver cytosol has previously been analyzed for both GST subunit [16,41,43] and dimeric content [21,22,40,42,44]. Using an isoelectric focusing technique [41], six different Alpha subunits were identified. From reversed-phase HPLC analysis of GST subunits, two Alpha subunits were identified in human liver [16,43]. In one report of dimer isolation by chromatofocusing [40], 13 forms of GST were found, but no attempt was made to differentiate them into classes. Other investigators [22,42,44] of GST dimers in human liver have found two types of Alpha subunits which form two homodimers and one heterodimer. For L006N we have identified

three subunits which form two homodimers (A1–1 and A2–2) and three heterodimers (A2–x, A1–x, and A1–2). Interestingly the potential homodimer Ax–x was not identified in this liver.

For the examination of five livers, S-butylglutathione was chosen as the eluting ligand because it separated Mu class GSTs from the Alpha class. The five human livers were examined for dimeric content by affinity chromatography and for subunit content by reversed-phase chromatography (Table 1). The amount of cytosol injected onto the affinity column was equivalent to approximately 20 mg of liver. In previous reports [16,21,22,41–44] the predominant forms of GST in human liver were A1 subunits and A1–1 dimers. This was the case for the five livers examined in this report. When appreciable amounts of Ax or A2 subunits were detected, heterodimers with A1 were also found. For L007N, which had appreciable amounts of subunit A2, both the homodimer A2–2 and the heterodimer A1–2 as well as A1–1 were detected. Human liver L005N which had A1 and Ax but only a minor amount of A2 exhibited A1–x and A1–1, but no Ax–x was detected. Minor amounts of P1 were detected by reversed-phase analysis in all five samples and were also detected by affinity analysis in four of the five livers. Previous investigations also showed Pi to be a minor component in human liver [41,42]. Small amounts of the Mu class GSTs were detected by the reversed-phase method. Due to poor peak shape in affinity chromatography, the detection of the Mu class is difficult when it constitutes only a minor portion of the total GST content.

This new affinity chromatography system using HPLC technology has significant advantages over conventional soft gel affinity systems for the analysis of the GST dimeric content of tissues. The time of analysis is reduced by a factor of ten. In addition the sensitivity for this affinity system is greater because the peak volumes for HPLC affinity are only about 0.5 ml compared to 50–100 ml when using conventional soft gels [29]. This technique also has the advantage of combining both sample cleanup and analysis into a single step. Other techniques such as reversed-phase HPLC, chromatofocusing or electrophoresis require a separate affinity step to batch purify the GSTs before the final analysis.

Affinity chromatography using gradients of a counter ligand in the eluent is a powerful separation technique. Variation of the biospecific ligand in the eluent results in chromatographic separations exhibiting different selectivities. The different selectivities observed are due to the unique set of binding constants that exist among ligands in their associations with various isoenzymes. This was the case for the GSTs when comparing elution profiles using TER106 or S-butylglutathione as the counter ligand. Many more ligands interact with GSTs [36], and any of these could give a unique separation allowing a custom separation to be developed. In particular, the analytical methods reported here can be readily scaled-up for preparative isolation of particular GSTs of interest.

5. Acknowledgements

The authors thank Larry M. Kauvar for comments on this manuscript. This work was supported in part by a Small Business Innovative Research grant to L.M.K. from the US National Science Foundation (No. ISI-9022271).

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