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Coupled affinity–reversed-phase high-performance liquid chromatography systems for the measurement of glutathione S-transferases in human tissues

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Abstract

HPLC affinity and reversed-phase modes were coupled for the direct measurement of glutathione S-transferases (GSTs) in cytosol extracts. Two coupling designs were examined. In the sequential configuration the affinity column served to extract the isoenzymes which were then eluted directly onto the reversed-phase column as a single fraction. Subsequent separation in the reversed-phase mode provided a GST profile based on the subunit composition of the isoenzymes as a whole. In the second configuration (rapid sampling configuration), gradient elution was performed in the affinity mode resulting in resolution of the intact isoenzymes. The eluate from the affinity separation was sampled in continuous, repetitive intervals and automatically subjected to ongoing reversed-phase analysis. This multidimensional approach provided information on the GST subunit content and also gave information about the distribution of the subunits among individual isoenzymes, thereby forming a basis for the determination of the actual isoenzymatic composition of the GSTs. In both configurations, events were automated and co-ordinated through the use of computer and multiport switching valves. Examples of GST separations from these procedures are shown for human lung and liver tissues. A comparison of the GST subunit analyses from normal and cancer lung tissue excised from the same patient showed substantial elevations of GSTs in the cancer sample. Two-dimensional affinity–reversed-phase analysis of a human liver sample illustrates the utility of the technique for determining the isoenzymatic organization of GST subunits. The criteria for extending two-dimensional analysis to more complex GST mixtures are discussed.

1. Introduction

Glutathione S-transferases (GSTs) are a widely occurring family of isoenzymes which show large compositional variations among different tissues in both type and amount [1–4]. Variations in the levels of individual isoenzymes and the overall GST enzymatic activity have also been found in cancer tissues in comparison with surrounding, normal tissue [5]. In addition, correlations have been reported between elevated GST levels and drug resistance in cells exposed to various cancer drugs *in vitro* [6]. Since alkylating agents used in cancer treatment are electrophilic compounds which are susceptible to GST-catalyzed glutathione conjugation, the presence of elevated GSTs in cancer tissue has been postulated as contributing to drug resistance which often develops in the course of chemotherapy [5,7,8]. Inhibition of GSTs has been proposed as a means of lowering drug resistance by reducing the GST metabolism of cancer drugs, thereby

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rendering such drugs more effective [5,7,9,10]. This approach would have the added benefit of potentiating the action of the drug such that lower concentrations of these toxic compounds might be effective in cancer treatment.

Cytosolic GSTs are categorized according to four classes: Pi (P), Alpha (A), Mu (M) and Theta (T) [11,12]. The isoenzymes are comprised of two subunits with each subunit possessing one catalytic site which is involved in the conjugation of glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) to widely diverse classes of electrophilic compounds. Multiple subunit forms have been identified for some classes. These include A1 and A2 from Alpha; M1a, M1b, M2, M3, M4, and M5 from Mu; and T1 and T2 from Theta. Subunits within a class form both homodimers (A1-1, A2-2, M1a-1a, M1b-1b, M2-2, M3-3, etc.) and heterodimers (A1-2 and M1-2), but assembly of isoenzymes from different classes of subunits has not been observed to date. Pi is generally recognized as existing as a single isoenzyme, P1-1, although reports of Pi variants have also been published [3].

Analysis of GSTs has been performed most effectively by using reversed-phase HPLC following an affinity chromatography step to extract the enzymes from tissue homogenate [13,14]. The affinity step has usually been carried out with glutathione or S-hexylglutathione immobilized on agarose beads. GST extraction has also been performed using a commercially available HPLC column possessing immobilized glutathione [15]. We have recently developed an HPLC GST affinity packing in order to facilitate the extraction and separation of GSTs as part of a larger study concerned with selective inhibition of GST isoenzymes. In this context we also explored the unique selectivities obtained for isoenzyme separations generated from gradient elutions using structural variants of the GST substrate glutathione as the eluting ligand [16].

Reports have appeared in which coupled systems are described for affinity extractions followed by reversed-phase analysis [17–19] and for two-dimensional analysis using coupled modes exhibiting complementary selectivities [20–22]. The potential of and criteria for optimizing multidimensional separations have also been reviewed [23,24]. This report describes two system designs for coupling a GST affinity column, possessing an S-octylglutathione affinity ligand, to a reversed-phase column. The goal of one design, referred to as the sequential design, was to streamline the extraction and reversed-phase steps through coupling and automation. The reversed-phase separation, using this approach, provides information on the GST subunit content of the sample based on dissociation of GST isoenzymes into their subunits under reversed-phase conditions. The second design, referred to as rapid sampling, is multidimensional in that the eluate provided from a separation of GST isoenzymes in the affinity mode is subjected to repetitive analysis in the reversed-phase mode. The multidimensional approach provides an analysis of the subunit content of the GSTs and also gives information on how the subunits are organized into individual isoenzymes.

2. Experimental

2.1. Reagents

Iodobutane, iodooctane, sodium borohydride, ethanolamine and 1,4-butanediol diglycidyl ether were purchased from Aldrich (Milwaukee, WI, USA). Tris, EDTA, dithiothreitol, sodium chloride, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB) and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate, HPLC-grade water and acetonitrile were from VWR Scientific (Brisbane, CA, USA). Trifluoroacetic acid was obtained from Pierce (Rockford, IL, USA). Recombinant GST (rGST) enzymes rA1-1, rP1-1, rM1a-1a, rM1b-1b and M2-2 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).

2.2. Synthesis of peptides

S-Butylglutathione and S-octylglutathione

were synthesized by the method of Vince *et al.* [25]. The tripeptide γ-glutamyl-(*S*-benzyl) cysteinyl-β-alanine (TER106) was synthesized as previously reported [26]. All peptides had greater than 90% purity when analyzed by reversed-phase HPLC and had acceptable elemental analyses.

2.3. HPLC Apparatus

Model HPXL pumps, pump heads, a Rheodyne 7125-081 titanium injector, a 1.2-ml titanium dynamic mixer, Dynamax UV-C detectors, and Dynamax HPLC Method Manager for HPLC control and data acquisition were purchased from Rainin (Woburn, MA, USA). Eight-port high-pressure valves mounted on two-position electric actuators were obtained from Valco (Houston, TX, USA). Six-port low-pressure selection valves of polyether ether ketone (PEEK) construction, mounted on electric actuators, were obtained from Upchurch Scientific (Oak Harbor, WA, USA). A static mixing tee of PEEK construction and biocompatible 250 p.s.i. (1 p.s.i. = 6894.76 Pa) back-pressure regulators were obtained from Upchurch Scientific. For affinity chromatography all pathways in contact with the mobile phase were of either titanium or biocompatible polymer construction.

2.4. Tissue extraction

Human liver and lung samples were obtained from the Cooperative Human Tissue Network (Columbus, OH, USA and Birmingham, AL, USA) and stored at −80°C. Samples were slightly thawed, minced with scissors and homogenized in buffer (1 g per 4 ml total volume) 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 (Molecular Devices, Menlo Park, CA, USA) with bovine serum albumin as a standard [27].

2.5. Measurement of enzymatic 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 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 [10].

2.6. HPLC columns

Reversed-phase analyses with the sequential design were carried out with a C_8, 5 μm, 250 × 4.6 mm column (7105-00) from Baker (VWR Scientific). Reversed-phase analyses with the rapid sampling design were performed with a C_{18}, 5 μm, 50 × 4.6 mm column (218ATP5405) from The Separations Group (Hesperia, CA, USA). HEMA BIO 1000 (Tessek 0001620005), a 10-μm polymeric HPLC support based on copolymerization of 2-hydroxymethacrylate and ethylene dimethacrylate, was obtained from Melcor Technologies (Sunnyvale, CA, USA).

The synthesis of the affinity matrix followed the general procedure of Sundberg and Porath [28]. The mixture HEMA BIO 1000–1,4-butanediol diglycidyl ether–0.6 *M* sodium hydroxide containing 2 mg/ml of sodium borohydride (0.03:1:1, w/v/v) was mixed overnight. The product was filtered and washed with water, ethanol and acetone. A 700-mg amount of the derivatized support was combined with a solution of S-octylglutathione (75 mg) dissolved in 3.5 ml of 0.5 *M* sodium carbonate and 60 μl of ethanolamine, pH 10.5. The suspension was mixed for approximately 90 h. After filtration the final product was washed with the following, in the order shown: 1 *M* sodium chloride in 0.1 *M* sodium phosphate (pH 9); 1 *M* sodium chloride in 0.1 *M* sodium acetate (pH 4.5); water; ethanol; and acetone.

A 90-mg amount of the affinity packing was slurried in 20 ml of water and packed at high pressure into stainless-steel columns, 30 × 2.1 mm. (Supelco, Bellefonte, PA, USA). The column frits were 2 μm (average pore diameter) titanium encased in a CTFE ring (Upchurch Scientific). 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. (140 bar) with 50 ml of water and then 4000 p.s.i. (275 bar) with 50 ml of water.

2.7. HPLC system designs

The HPLC system design for the sequential configuration is shown in Fig. 1. Two pumps are associated with each column enabling gradient elution in both modes. Valve V1, a six-port selection valve mounted on an electric actuator, controls delivery of the loading, wash and regeneration solvents to the affinity column through pump P1. The affinity eluent is delivered through pump P2, and the affinity gradients are formed in the low-volume (3 μl) static mixing tee M1. The sample is introduced through the manual injection valve IN1. Reversed-phase solvents are delivered through pumps P3 and P4 which are mixed in the dynamic mixer M2 (1.2 ml). The affinity method is controlled from the computer. Since the software is not capable of exerting simultaneous control of affinity and reversed-phase pumps, the reversed-phase method is directed through (programmable) pump P3 with P4 subordinate to P3. A second manual injection valve, IN2, permits the option of introducing samples directly onto the reversed-phase column which can be used independently of the affinity system. Back-pressure regulators (250 p.s.i.) were installed in-line in order to insure proper function of the pumps' check valves at the low flow-rates existing at various times during the procedure.

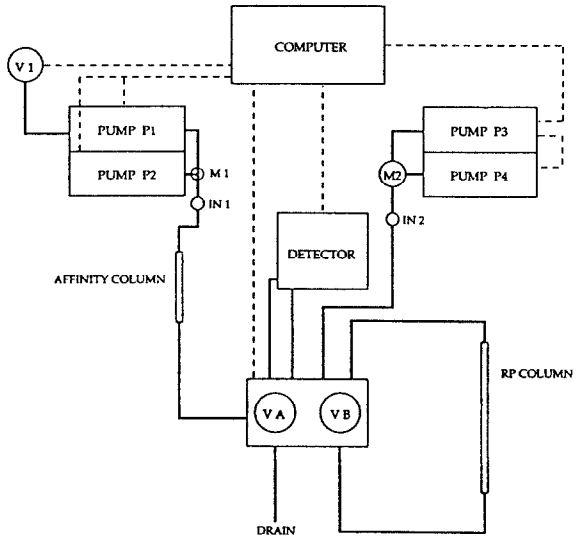


Fig. 1. HPLC design for sequential coupling of GST affinity and reversed-phase modes. M1 = Static low volume gradient mixer; M2 = dynamic gradient mixer; V1 = low pressure 6-port selection valve; IN1, IN2 = manual injection valves; VA, VB = 8-port, 2-position switching valves; solid lines = plumbing; broken lines = electrical connections. See text for complete description.

Valves VA and VB are two-position, eight-port, electrically actuated switching valves and co-ordinate changes in flow paths which occur during the procedure. The system plumbing in relation to VA and VB is shown in Fig. 2. Five of the eight ports from each valve were required for this design. The position of these valves and V1 is controlled from the computer which also signals initiation of the reversed-phase method stored in P3 and acquires data from the detector.

The HPLC system design for the rapid-sampling configuration, shown in Fig. 3, resembles the sequential configuration with a few fundamental differences. One switching valve and two detectors are required. Data from the detectors are acquired on two computer channels. The switching valve, shown in Fig. 4, is slightly modified from a design by Bushey and Jorgenson [20] in that one of the loops is incorporated into a manual injection valve thereby permitting independent sample introduction onto the reversed-phase column.

2.8. Chromatography

The determination of GST isoenzymes using the sequential design involves three stages: (1) extraction of the isoenzymes from the sample;

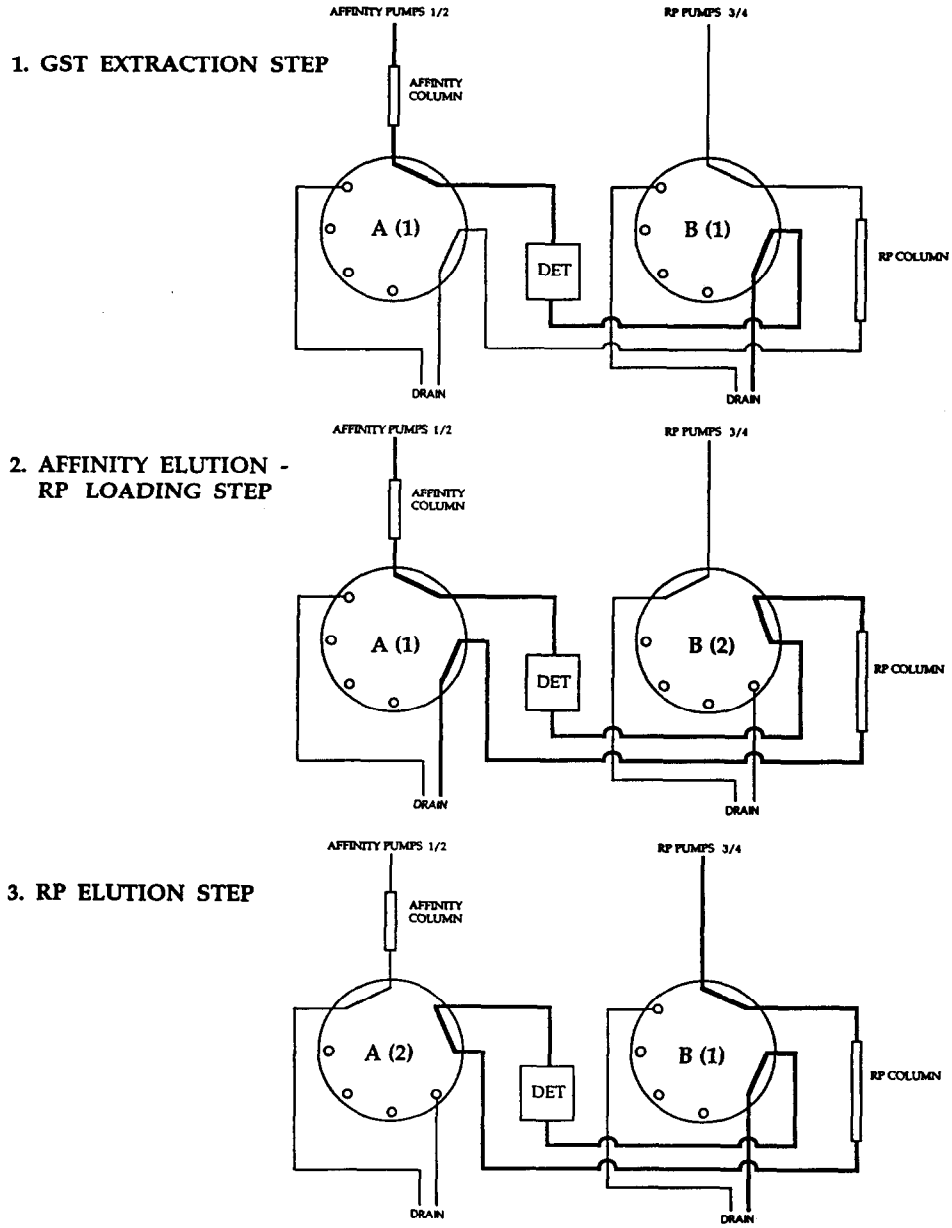


Fig. 2. Valve design for sequential coupling of GST affinity and reversed-phase modes. Positions of valves A and B are given for the three stages of the procedure. Use of a single detector permits acquisition of both affinity and reversed-phase data as a single chromatogram. DET = Detector. See text for detailed description.

(2) elution of the isoenzymes as a single fraction from the affinity column onto the reversed-phase column; (3) separation of the mixture of isoenzymes into their constituent subunits in the reversed-phase mode. These processes, which are co-ordinated by the positions of valves VA and VB, are summarized in Fig. 2 and described in detail by the method shown in Table 1.

During the first step, when valves VA and VB are both in position 1, the sample is introduced

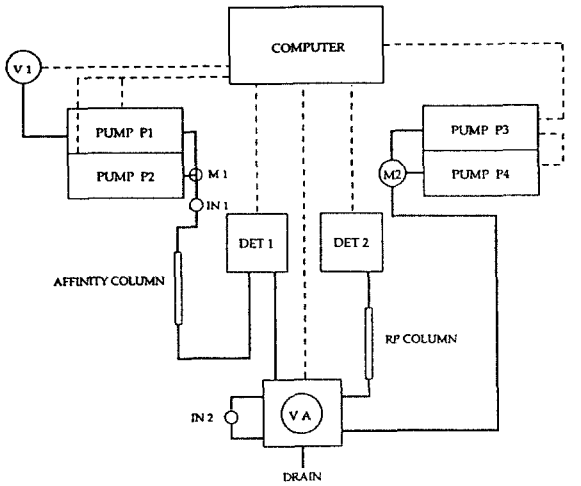


Fig. 3. HPLC design for rapid sampling coupling of GST affinity and reversed-phase modes. See Fig. 1 and text for description of components.

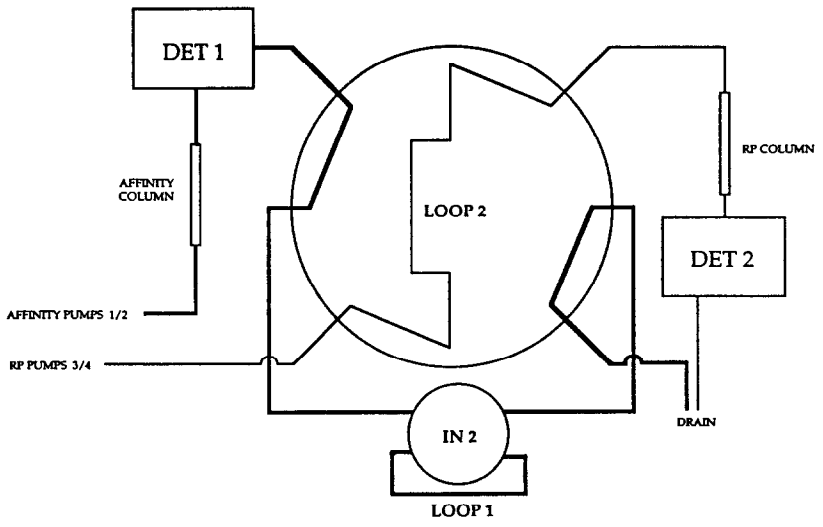
onto the affinity column in loading buffer A (solvent compositions are shown in Table 1) where the isoenzymes are extracted from the sample. Following a salt wash in buffer B, the isoenzymes are eluted from the affinity column with buffer C which, for this study, contained the affinity eluent S-butylglutathione. At about the same time valve VB is changed to position 2 which has the effect of directing the affinity eluate, containing the GST isoenzymes, onto the reversed-phase column. During the GST loading step the reversed-phase solvents from pumps P3 and P4 are diverted to drain. It is important to note that during the loading step the flow cell in the detector experiences back-pressure from the reversed-phase column. Consequently, the loading step was carried out at flow-rates consistent with pressure specifications of the flow cell.

Following the elution of the GSTs from the affinity column, which is monitored by the detector at 280 nm, VA and VB are simultaneously switched to positions 2 and 1, respectively. As a result the affinity and reversed-phase columns are de-coupled, and the effluent from the affinity column is diverted to drain. Also, the solvents from pumps P3 and P4 are redirected onto the reversed-phase column, and the effluent from the reversed-phase column replaces the affinity effluent in the detector flow cell. When the valves are switched a signal is also sent from the computer instructing pump P3 to initiate the reversed-phase method. About 2 min later the detector wavelength is changed to 214 nm and, subsequently, a signal is sent from the reversed-phase method to re-zero the detector.

During the reversed-phase separation (third stage of the analysis), the affinity column is subjected to a cleaning and regeneration procedure which is directed from the affinity method. At the conclusion of the reversed-phase gradient valve VA is returned to position 1, the detector wavelength is returned to 280 nm, and the regenerated affinity column is prepared to accept the next sample. The injection can be made prior to reversed-phase regeneration, which can be completed during the first part of the affinity procedure when the columns are de-coupled.

The affinity method for the rapid-sampling analysis resembles the sequential approach except that the GST elution is carried out using a long, shallow gradient. As the affinity eluate leaves the affinity column it passes into either loop 1 or loop 2 (1 ml volume), depending on the position of the switching valve, shown in Fig. 4. At regular intervals the valve position is changed as directed by the affinity program. When this occurs the reversed-phase flow path is directed through the most recently filled loop, sweeping the sample in that loop onto the reversed-phase column. At the same time the reversed-phase method, stored in pump P3, is initiated. While the reversed-phase analysis is performed, affinity eluate enters the other loop in preparation for the next reversed-phase analysis. The result is that all of the affinity eluate is collected as discrete fractions which are subjected to reversed-phase analysis. In order to obtain a detailed reversed-phase analysis of the affinity eluate, the reversed-phase analysis time must be fast. The cycle time in this study, including separation time and column regeneration, was 6 min. Since elution of GSTs from the affinity column was performed at 20 μl/min, the affinity eluate was sampled in continuous 120-μl fractions. The volume of the sampling loops (1 ml) was sufficient to insure complete capture of the affinity fractions.

VALVE POSITION 1 (FILL LOOP 1)



VALVE POSITION 2 (FILL LOOP 2)

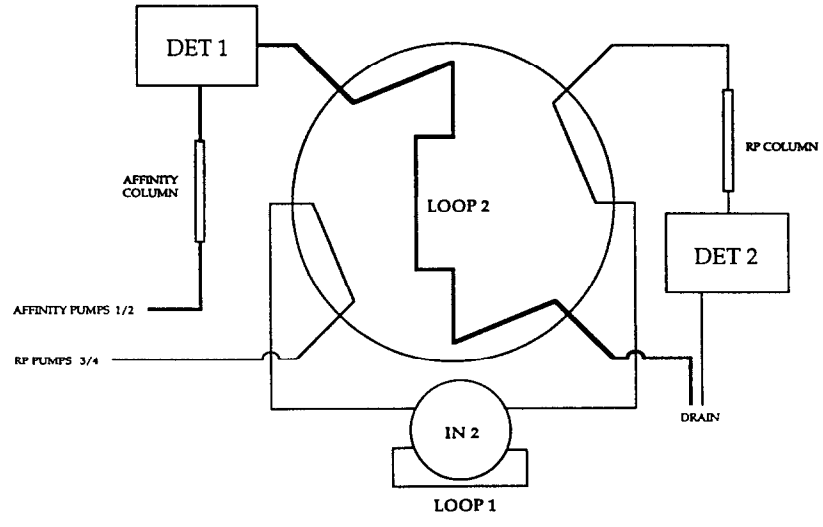


Fig. 4. Valve design for rapid sampling coupling of GST affinity and reversed-phase modes. Two valve positions permit continuous sampling of affinity eluate in loops 1 and 2 (volume 1 ml) and comprehensive reversed-phase analysis of fractionated isoenzymes. See text for details.

3. Results

The sequential design employs a single detector arranged such that the eluate from both affinity and reversed-phase columns passes through the flow cell. A similar feature, whereby

Table 1
HPLC method for coupled affinity–reversed-phase analysis of GSTs (sequential design)

Time (min)	VA[a]	VB[a]	Flow-rate (ml/min)[b]		Mobile phase	
			Affinity	RP	Affinity[c]	RP[d]
0	1	1	0.04	0.25	A	20.0% B′
5.00			0.04		B	
6.00			1.00			
17.00			1.00			
17.50			0.02			
20.00					B	
22.00		2				
26.00					C	
32.00					C	
32.02					B	
39.00[e]	2	1				
32.02			0.02	0.25	D	
39.50			1.00			
41.00				1.00		
42.00[f]						20.0% B′
47.00						42.0% B′
54.50					A	
59.50			1.00			
59.75			0.04			
93.00						50.7% B′
95.00	1		0.04			
95.50			1.00			
97.50			1.00			
98.00			0.04			

Mobile phase buffers, affinity column: 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; D, 1.0 *M* sodium chloride in A. Mobile phase solvents, reversed-phase column: A′, 0.1% trifluoroacetic acid in water; B′, 0.1% trifluoroacetic acid in acetonitrile. Separate programs direct the flow of solvents through the affinity and reversed-phase columns and are controlled from the computer and pump P3, respectively. The GST extraction and wash occurs at 0–20 min. GST elution and reversed-phase loading occurs at 20–39 min, and the reversed-phase elution takes place at 39.02–95 min. See text for details.

[a] VA and VB represent 2-position, 8-port switching valves described in Fig. 2 and the text. The values 1 and 2 correspond to valve positions as shown in Fig. 2.

[b] Changes in flow-rate occur over a linear gradient between the times indicated.

[c] Mobile phases A, B and D were selected in a step at the times indicated. The change between B and C occurred in a linear gradient over 6 min.

[d] Changes in reversed-phase mobile phase occur over a linear gradient between the times indicated.

[e] At 39.00 min an output signal from the computer initiates the reversed-phase program stored in pump C. Reversed-phase mobile phase composition and flow-rate are controlled from this program.

[f] At 42.00 min an output signal is sent from the reversed-phase program stored in pump C to detector auto-zero.

data acquired from separations on two coupled columns are presented in a single chromatogram, was recently reported [29]. The first part of the chromatogram, obtained from the sequential design, depicts a separation of GST isoenzymes in the affinity mode, and the reversed-phase separation of GST subunits appears in the second part. Such a chromatogram, obtained from the injection of a standard mixture of five recombinant GST isoenzymes, is shown in Fig. 5. Peak assignments were made from individual injections of the isoenzymes in the mixture.

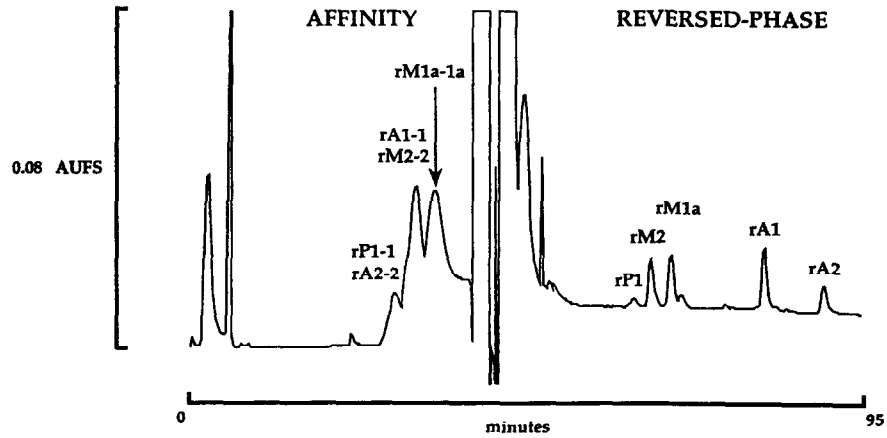


Fig. 5. Separation of a mixture of recombinant GST isoenzymes using the sequential design. Partial separation of the intact isoenzymes was achieved in the affinity mode, appearing in the first part of the chromatogram. Complete resolution of isoenzymatic subunits was achieved in the reversed-phase mode, shown in the second part of the chromatogram. Sample, 25 μl containing rP1-1 (2.3 μg), rM1a-1a (2.3 μg), rM2-2 (1.1 μg), rA1-1 (1.7 μg) and rA2-2 (1.7 μg); affinity column, S-octylglutathione immobilized onto a 10-μm polymeric support, 30 × 2.1 mm; reversed-phase column, Baker C_8, 5 μm, 250 × 4.6 mm; detection, 280 nm and 214 nm for affinity and reversed-phase, respectively. Mobile phases and the complete method program are given in Table 1.

Under the conditions of the affinity elution (0–20 m*M* S-butylglutathione over 6 min) only limited resolution of the intact isoenzymes was observed in the affinity mode. However, three distinct isoenzyme populations can be seen: an early eluting mixture of rA2-2 and rP1-1, a middle population containing rA1-1 and rM2-2, and a strongly retained band containing rM1a-1a. The small peak preceding the GST bands corresponds to the switch in the position of valve VB at 22 min. The strong absorption in the middle of the chromatogram results from an abrupt shift in mobile phase following the valve changes at 39.00 min (affinity to reversed-phase solvents) and from elution of S-butylglutathione from the reversed-phase column early in the method.

The peaks eluted from the reversed-phase column and appearing in the second part of the chromatogram represent rGST subunits generated from the isoenzymes observed in the affinity separation. Since the sample was comprised entirely of homodimeric isoenzymes, each of which dissociates into a single subunit under reversed-phase conditions, each subunit peak in the reversed-phase separation corresponds to one of the isoenzymes in the sample mixture. For example, rP1-1 generates a single rP1 peak in the reversed-phase chromatogram. The five subunit peaks are clearly resolved and easily distinguished.

Fig. 6 shows a comparison of chromatograms obtained from sequential GST analysis of lung tissue cytosols derived from cancer and normal tissue taken from the same patient. The peak areas are expressed as mV s mg^{-1} of injected cytosolic protein. In this case the sample protein concentrations were comparable (9.3 *versus* 9.4 mg protein per ml cytosol) so that the two chromatograms can be compared directly. It is clear from the reversed-phase profiles that the GST subunit content is substantially elevated for all components in the cancer sample compared to the normal sample. Both P1 and A2 subunits exist at greater than 2.5 × the normal concentrations. The concentrations of M1a and A1 are even more amplified in the cancer sample with M1a occurring at 30 × the normal concentration. The peak labeled X, the identity of which is under investigation, has been observed in this laboratory in other lung samples as well as in cytosol derived from breast tissue and in numerous cell lines grown in culture.

Fig. 7 shows the results of a GST analysis of

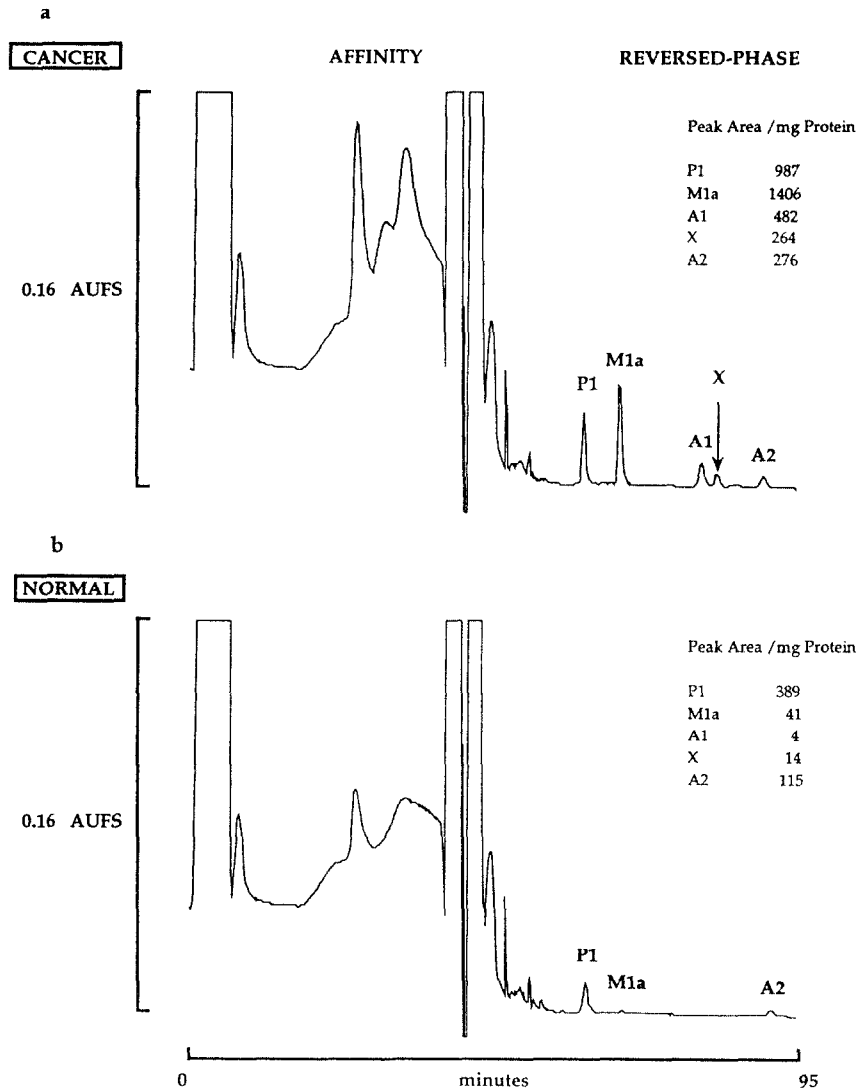


Fig. 6. Comparison of GST subunit analyses for cytosol derived from cancer (a) and normal (b) lung tissues excised from the same patient. The analyses were obtained from using the sequential design and are expressed as peak areas per mg of injected protein. The same amount of protein was injected from each sample permitting direct graphic comparison of peaks. A 200-μl volume of cytosol, diluted (1:2) in loading buffer, was injected. Other conditions as in Fig. 5. The peaks corresponding to A1 and X in the normal sample, which occur in very small amounts, are not apparent at this attenuation.

human liver cytosol, using the rapid sampling approach. The affinity separation is depicted vertically on the left, and the reversed-phase analysis of each 6-min fraction is shown horizontally alongside the corresponding affinity fraction. A reversed-phase chromatogram obtained for a standard mixture of rGSTs appears at the bottom. Three major bands appear in the affinity chromatogram, at about 25, 37 and 79 min. Reversed-phase analysis of the band at 25 min showed a single peak corresponding in retention to the A2 subunit, indicating that this affinity band represents the A2-2 homodimer. Reversed-phase analysis of the affinity eluate between 73

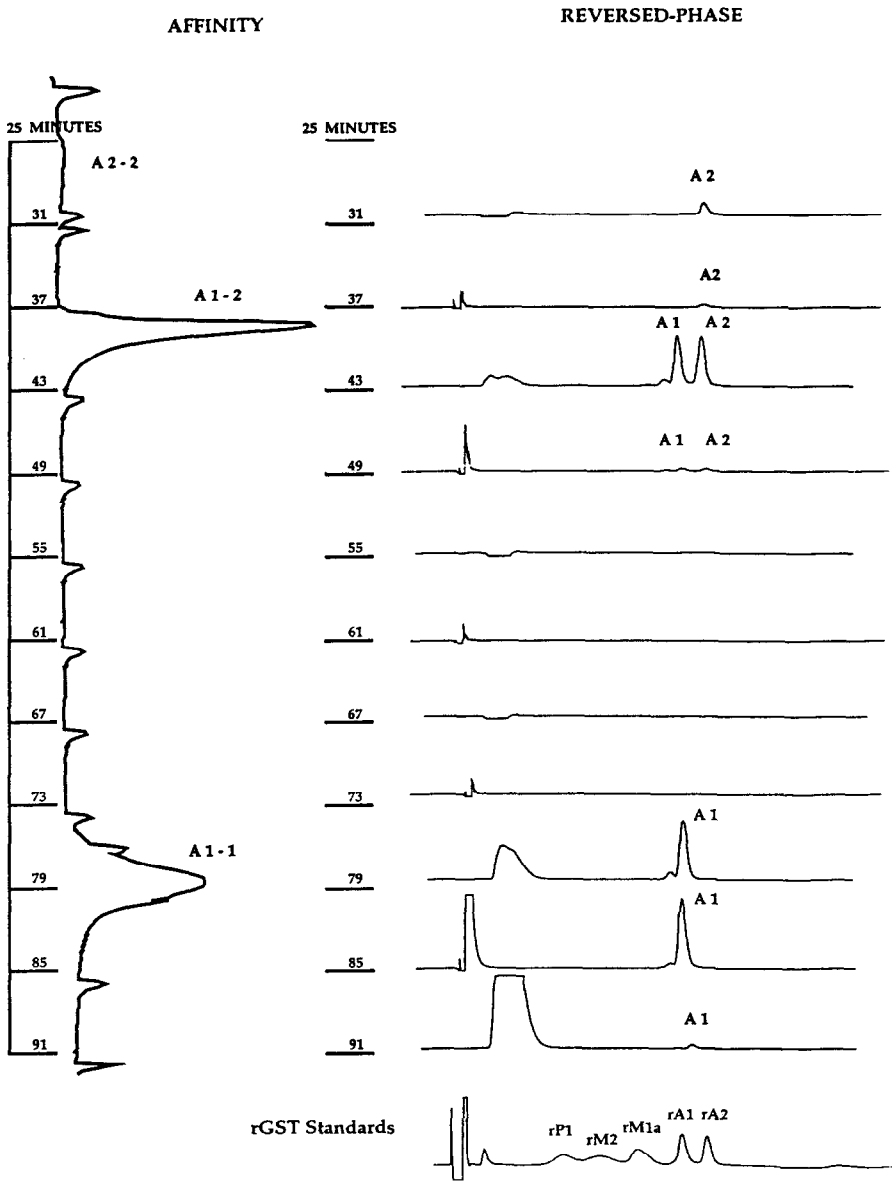


Fig. 7. Two-dimensional affinity, reversed-phase analysis of GSTs in human liver cytosol using the rapid sampling design. See text for description. Mobile phases, detection wavelengths and affinity column as in Fig. 5 and Table I. Reversed-phase column, Vydac C_{18}, 5 μm, 50 × 4.6 mm; sample volume 180 μl cytosol (17.4 mg protein/ml); affinity method: 0–2 min, A buffer (10 m*M* sodium phosphate, pH 6), 0.1 ml/min; 2–17 min, B buffer (200 m*M* sodium chloride in A); 17–20 min, B buffer, 0.02 ml/min; 20–127 min, 0–28% C buffer (20 m*M* S-butylglutathione in B), 0.02 ml/min. Reversed-phase method, 0–1 min, 44–45% B′ (0.1% trifluoroacetic acid in acetonitrile; A′ 0.1% trifluoroacetic acid in water); 1–4 min, 45–62% B′; 4–6 min, 44% B′; flow 2 ml/min. Reversed-phase analysis of affinity eluate was performed every 6 min beginning with affinity fraction 25–31 min.

and 91 min showed a single major GST peak, corresponding to subunit A1, suggesting that the affinity band represents the A1-1 isoenzyme.

Reversed-phase analysis of the affinity eluate between 37 and 49 min revealed two major peaks of approximately equal area, apparently

corresponding to the A1 and A2 subunits. Since these subunits originate from neither the A1-1 nor A2-2 isoenzymes, which were detected in separate affinity fractions, this affinity band must correspond to the A1-2 heterodimer. Equal areas would be anticipated for the pure A1-2 isoenzyme since their molar extinction coefficients are nearly the same. A minor, unidentified reversed-phase peak, eluting just prior to A1, also accompanies the A1 subunit wherever it appears and suggests some associated species distinct from the A2 subunit.

The bands eluting early in the reversed-phase chromatograms probably represent compounds in the affinity eluent, primarily S-butylglutathione. The regular alternations in the background, appearing near the beginning of the reversed-phase gradient, apparently depend on the position of the switching valve and could result from some difference in the two flow paths. Spikes appearing at regular intervals in the affinity chromatogram correspond to valve changes.

4. Discussion

The sequential approach for GST analysis essentially provides a reversed-phase analysis for the subunit content of a mixture of isoenzymes loaded onto the reversed-phase column as a single fraction. The coupled design represents a substantial improvement in comparison with methods employing separate agarose-based affinity extractions. The automated system, through computer control, streamlines all of the steps associated with the affinity extraction and efficiently transfers the extract to the reversed-phase column. As a result, handling errors are eliminated as is the labor associated with the individual manipulations inherent in a manual approach. The affinity and reversed-phase systems can also be used independently by de-coupling the two columns (valves VA and VB positioned as described for step 1 or step 3 in Fig. 2). In the de-coupled configuration the affinity portion of the system may be used to purify GST isoenzymes for studies other than reversed-phase analysis. Conditions for achieving isoenzyme separations on this affinity column have been described elsewhere [16]. Also, the two independent gradient systems can also be used for other purposes so that the equipment need not be dedicated to GST analysis.

Relative standard deviations, determined for the retentions of P1, M1a, M1b, M2, A1 and A2 in the reversed-phase separation of the sequential design, indicated less than 5% overlap of the statistical envelopes surrounding the most closely eluting pairs of subunits in this group. Independent characterization of GST affinity eluate compositions, through immunochemical procedures and sodium dodecyl sulfate–polyacrylamide gel electrophoresis [16], was used to further corroborate the subunit identities. The efficacy of the affinity column for extracting GSTs was also examined. Injections of cytosol derived from 20 cell lines (containing mostly Pi GST) and 40 lung specimens showed, respectively, that an average of 1.8% and 5.9% of the enzymatic activity in the samples was unretained by the affinity column (data not shown). These measurements and those above indicate that efficient GST extractions are achieved on the affinity column and that the resolution obtained under the conditions described in Table 1 permit peak assignments with a high degree of confidence. From the samples examined thus far, we estimate that the GST content found in 1–2 mg of tissue should be easily detected, with variations expected according to tissue type and individual GST content. The amount of tissue obtained from a needle biopsy would thus prove sufficient for determination of GST analytical profiles using this procedure.

In reversed-phase separations of GSTs, the appearance of only one subunit from within a given GST class necessarily suggests its organization as the homodimeric isoenzyme. However, when two or more subunits from the same class are present, as is often the case, their distribution among isoenzymes cannot be deduced from the information obtained through the sequential approach. In order to determine the actual isoenzyme composition, the isoenzymes must first be at least partially separated from each

other. If this can be achieved the separated isoenzymes can then be identified through reversed-phase analysis. The rapid sampling design addresses this problem by co-ordinating a two-dimensional separation comprised of an affinity mode, by which isoenzymatic resolution is effected, and a reversed-phase mode for determining the subunit composition of the separated isoenzymes. This is illustrated in Fig. 7 for the separation of three A isoenzymes in liver cytosol. In this case the isoenzymes A1-1, A1-2 and A2-2 were fully resolved in the affinity mode and their identities confirmed by subunit analysis in the reversed-phase mode. Examination of this sample with the sequential design would have shown two peaks in the reversed-phase analysis corresponding to the total A1 and A2 subunit content but would have provided no information on the distribution of the subunits among the isoenzymes.

The GST isoenzyme content of the liver sample profiled in Fig. 7 is relatively simple, and the three A isoenzymes were easily separated. Often, however, more complex GST mixtures are encountered in which several Mu class subunits may be present in addition to the A1 and A2 subunits. We have observed mixtures containing various combinations of P1, A1, A2, M1a, M1b and M2, and several other Mu class subunits (M3, M4 and M5) have been reported elsewhere [6]. In addition to A1 and A2, we have also encountered samples containing a third A subunit, designated as Ax. In one sample these A subunits were distributed among five isoenzymes [16]. Adding to this complexity is the anticipation that new GST forms would be expected in any broad screening of the genetically diverse human population.

Although gradient separations of complex mixtures of GST isoenzymes in the affinity mode tend to produce overlapping bands, the identity of the overlapping isoenzymes can often be deduced from the subunit information obtained from the reversed-phase separation. For example, the subunit composition of an affinity fraction containing two isoenzymes of different classes necessarily suggests the identity of the isoenzymes. This is a consequence of isoenzymes assembling from among subunits within only the same class. In some cases isoenzyme content can be determined even when overlap of same class isoenzymes occurs. For the A mixture of Fig. 7, if affinity conditions were such that A1-2 and A2-2 overlapped (or even co-eluted) and the A1-1 band were still clearly distinguishable, reversed-phase analysis of the (distinct) A1-1 band would show a single peak for the A1 subunit. The A1 peak area for the A1-2, A2-2 mixture would then be attributable to heterodimeric association with an equal amount of A2, and the remaining A2 peak area would have to arise from the A2-2 homodimer.

In some cases, even with a two-dimensional analysis, failure to adequately resolve same-class isoenzymes in the affinity mode would prevent their identification. When this occurs, changing the eluting ligand for the affinity separation can improve resolution sufficiently for isoenzyme determination. As part of a larger study concerned with molecular recognition, this laboratory has measured dissociation constants for binding between GST isoenzymes and a variety of glutathione analogues [10]. Sets of dissociation constants for a collection of GST isoenzymes are distinct for each structural variant of glutathione. For chromatographic affinity systems which employ competitive inhibitors as immobilized and eluting ligands, retention of an enzyme represents a balance between the affinities displayed by the immobilized and free ligands for the enzyme [30]. The relative retentions (selectivity) for a mixture of isoenzymes in a defined system depends on the unique set of enzyme–ligand dissociation constants for that system, *e.g.*, a mixture of GST isoenzymes in the S-octylglutathione, S-butylglutathione system described here. By changing the eluting ligand, significant shifts in selectivity can be achieved commensurate with the relative changes in the binding constants between the isoenzymes in the mixture and the new ligand.

A potent shift in selectivity of this kind has been observed for liver samples which were found to contain five A isoenzymes: A1-1, A1-2, A2-2, and the previously unreported forms designated as A1-x and A2-x [16]. Affinity sepa-

rations, using S-butylglutathione as the eluting ligand under conditions similar to those described above (see affinity conditions in Fig. 7), failed to resolve A2-2 from A2-x and showed overlap of A1-2 with A1-x. Two-dimensional analysis based on this affinity separation would fail to provide the isoenzymatic distribution of subunits Ax and A2. By eluting with the glutathione analogue γ-glutamyl-(S-benzyl) cysteinyl-β-alanine instead of S-butylglutathione, all five isoenzymes were baseline resolved. Such an affinity separation would simplify subunit identification in a manner analogous to that described for the sample of Fig. 7.

Since all five Alpha isoenzymes in the above sample can be baseline resolved, they could, in principle, be distinguished on the basis of their separation in the affinity mode. It should be noted, however, that differentiation of isoenzymes in the affinity mode is complicated by several factors. Although data are limited at present, variations for retentions in the affinity mode appear to be wider than those in the reversed-phase mode. For example, single injections of cytosol from five different liver samples on the affinity column, using conditions similar to those of Fig. 7, gave a relative standard deviation for A1-1 of 4.6% (unpublished results) compared to 1.4% for retention of A1 on the reversed-phase column of Fig. 5 (10 injections from a single sample of liver cytosol). In addition, resolution in the affinity mode also appears to be limited by low peak capacities. Mu isoenzymes in particular tend to produce relatively wide bands in this affinity system [16]. These factors suggest that confident statistical differentiation of some isoenzymes may not be possible when based solely upon their retention times in the affinity mode. In addition, since peptide eluents interfere with detection at lower, more sensitive wavelengths, identification of minor isoenzymes, which may be present in low concentration, could be limited to subunit delineation from the reversed-phase separation.

The affinity and reversed-phases described in this report exhibit complementary selectivities such that the resolution provided by the 4-min reversed-phase separation (6 min with regeneration time) is sufficient for unequivocal differentiation of many of the subunit mixtures likely to be encountered in the affinity eluate. For example, P1-1 and A2-2, which exhibit similar selectivities in the affinity mode (see affinity separation of Fig. 5), would show widely separated P1 and A2 subunits under the reversed-phase conditions of Fig. 7. A1 and M2, whose homodimers also exhibit similar affinity selectivities, are also widely separated on the reversed phase. Some isoenzyme mixtures, however, could exhibit similar selectivities in the affinity mode and release subunits which also show similar retention on the reversed-phase. For example, subunit M1b, which elutes between M1a and A1 [16], would overlap with those subunits when the steep reversed-phase gradient described for Fig. 7 is used. Overlap of any of the isoenzymes in the affinity separation possessing the M1b subunit (M1b-1b or M1b-2, for example) with those possessing the M1a or A1 subunits could pose difficulty in obtaining confident identification of these subunits based on the reversed-phase separation. The GST subunits P1, M1a, M1b, M2, A1, A2 and Ax can be unequivocally resolved using shallower gradients like the one described for the sequential analysis in Fig. 5. However, if it is assumed that there are practical limits on the resolution obtainable in the affinity mode, as the reversed-phase analysis time increases, the number of affinity fractions that may be examined declines with a concomitant loss in definition. Also, the degree of resolution (and overall separation time) required in the affinity mode for reversed-phase identification of isoenzymes depends on the speed of the reversed-phase analysis. The importance of fast analysis time in the second mode for maximizing overall resolution in a two-dimensional separation has been discussed elsewhere [20]. In order to address these factors we are currently examining high-resolution, non-porous supports for speeding the reversed-phase analysis and other affinity eluents for producing diverse GST selectivities in the affinity mode.

In multidimensional chromatography, overall resolution improves as differences increase in the mechanisms by which separations occur in each

of the modes; the value of combining orthogonal modes in maximizing resolution has been discussed in detail [20,23,24,31,32]. By this measure, the combination of affinity and reversed-phase modes examined in this study provides an excellent basis for a two-dimensional separation since their respective chromatographic mechanisms are fundamentally different. We believe that these principles can be extended beyond the analysis of GST compositions for the elucidation of other complex mixtures of closely related isoenzymes. We are currently engaged in widespread screening of normal and cancer tissues for GST subunit compositions, using the sequential approach described in this study. The information obtained from the kinds of subunit mixtures encountered will provide the criteria for further development of two-dimensional separations based on the principles discussed above.

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