Journal of Chromatography, 474 (1989) 435–440
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 537

Note

An improved method for the separation and quantification of glutathione S-transferase subunits in rat tissue using high-performance liquid chromatography

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(Received December 23rd, 1988)

The glutathione S-transferases are a family of isoenzymes that play an essential role in the biotransformation of xenobiotics. They catalyse the conjugation of glutathione with numerous electrophilic agents, but also perform a number of other functions in cellular metabolism, *e.g.*, reduction of organic hydroperoxides, steroid isomerization and binding of non-substrate hydrophobic ligands such as bile acids, bilirubin and a number of drugs[1,2]. These enzymes have been found in numerous species and in many organs of mammals[2]. They exist as dimers and each dimer consists of two identical (homo-dimer) or two different (hetero-dimer) subunits with molecular weights in the region of 25 000 daltons. Each subunit has a characteristic enzymatic activity, which is additive in the different dimers[3]. Glutathione S-transferases are usually isolated from different sources with affinity chromatography, using epoxy-activated Sepharose to which S-hexylglutathione is linked. This method has to be used carefully, however, since some isoenzymes might not be bound. For example, rat glutathione S-transferase 5-5 does not bind to the S-hexylglutathione-Sepharose matrix[4]. Separation of the individual isoenzymes is usually performed with chromatofocusing, *e.g.*, on a Pharmacia fast protein liquid chromatography (FPLC) system[4]. Recently, Farrants *et al.*[5] described a method for the separation of glutathione S-transferase subunits using high-performance liquid chromatography (HPLC). This method appears very suitable for development into a system for the quantification of subunits in small amounts of sample or samples with low transferase activity. However, in the method described, the subunit peaks are rather broad and baseline resolution is poor, especially for the rat subunits 3, 4, 7 and 2. Chromatograms also suffer from a significant increase of the baseline during an analysis.

In this paper an improved method is presented for the separation and quantification of glutathione S-transferase subunits. Baseline separation of rat subunits 1, 2, 3, 4 and 7 is performed within 30 min.

EXPERIMENTAL

Chemicals and reagents

S-Hexylglutathione was obtained from Sigma (St. Louis, MO, U.S.A.). Epoxy-activated Sepharose 6B was obtained from Pharmacia/LKB (Uppsala, Sweden). Cen-

0021-9673/89/$03.50

tricon-10 microconcentrator tubes were from Amicon (Danvers, MA, U.S.A.). HPLC-grade acetonitrile was obtained from Promochem (Wesel, F.R.G.) and HPLC-grade trifluoroacetic acid (TFA) from Baker (Deventer, The Netherlands).

Isolation and purification of glutathione S-transferases

Cytosol of liver, kidney, lung and testis was prepared by homogenizing tissue with three volumes of 0.01 *M* Tris–HCl/0.14 *M* KCl pH 7.4 with a Potter-Elvehjem tissue homogenizer and centrifuging at 105 000 *g* for 75 min. Cytosol of the small intestinal mucosa was prepared by a modification of the method described by Borm *et al.*[6].

S-Hexylglutathione was linked to epoxy-activated Sepharose 6B as described by Mannervik and Guthenberg[7]. Rat glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 were isolated and purified from rat liver and kidney using S-hexylglutathione affinity chromatography and chromatofocusing on a Pharmacia FPLC system equipped with a Mono-P HR 5/20 column, as described previously[8]. The protein content of the purified isoenzymes was determined with the Lowry assay, using bovine serum albumin (BSA) as a standard. The identification and estimation of purity of the isoenzymes was performed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing using the Pharmacia PhastSystem.

For the determination of glutathione S-transferase subunits in different tissues, 1 ml of cytosol was applied to a S-hexylglutathione affinity column (2.5 cm × 1 cm I.D.) equilibrated with 10–15 ml of 10 m*M* Tris–HCl/1 m*M* EDTA/0.2 m*M* dithiothreitol, pH 7.8. After rinsing the column with 15 ml of equilibration buffer containing 0.2 *M* NaCl, the glutathione S-transferases were eluted with 6 ml equilibration buffer containing 0.2 *M* NaCl and 2.5 m*M* S-hexylglutathione. The flow-rate was 0.5–0.7 ml/min. The recovery of transferase activity in the eluate ranged from 86 (small intestine) to 99% (liver). Enzyme activities were determined using 1-chloro-2,4-dinitrobenzene as a second substrate, according to Habig *et al.*[9].

High-performance liquid chromatography

A modular HPLC system was assembled with a 2150 HPLC pump (LKB), a 2152 LC controller (LKB), a 2156 solvent conditioner (LKB) and a 2140 Rapid Spectral Detector (LKB) operating at 214 nm. Peak areas were integrated with Nelson Analytical Model 2600 chromatography software. The separation of glutathione S-transferase subunits was performed with a Vydac 201 TP 5 (200 mm × 3 mm I.D.) chromatography column (Chromsep system, Chrompack, The Netherlands) and a gradient of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient consisted of a linear gradient from 35 to 45% (v/v) solvent B in 18 min, followed by a linear gradient from 45 to 55% (v/v) solvent B in 5 min and isocratic elution at 55% solvent B for 7 min. The flow-rate was 0.6 ml/min. From the S-hexylglutathione eluates of liver and testis, 50 µl were directly injected on the HPLC column. Eluates of kidney and lung were concentrated ten times and the eluate of the small intestine twenty times, using Centricon-10 microconcentrator tubes, before injection of 50 µl.

RESULTS

Elution profiles

Fig. 1A shows a typical chromatogram of a standard mixture of rat glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 each in a concentration of 50 μg/ml. Under the denaturating conditions employed, the dimers dissociate and the individual subunits are separated. Subunits 3, 4, 7, 2 and 1 were eluted with retention times of 15.0, 16.5, 17.2, 19.3 and 24.5 min respectively (capacity factors, $k' = 13.1, 14.6, 15.3, 17.3$ and 21.8), as established by injecting the purified homodimers. Subunit 1 separates into two major peaks because of its microheterogeneity, as observed before[5,10]. In Fig. 1B–F, chromatograms of glutathione S-transferase subunits derived from rat liver, kidneys, testis, lung and small intestinal mucosa are presented. In liver, subunits

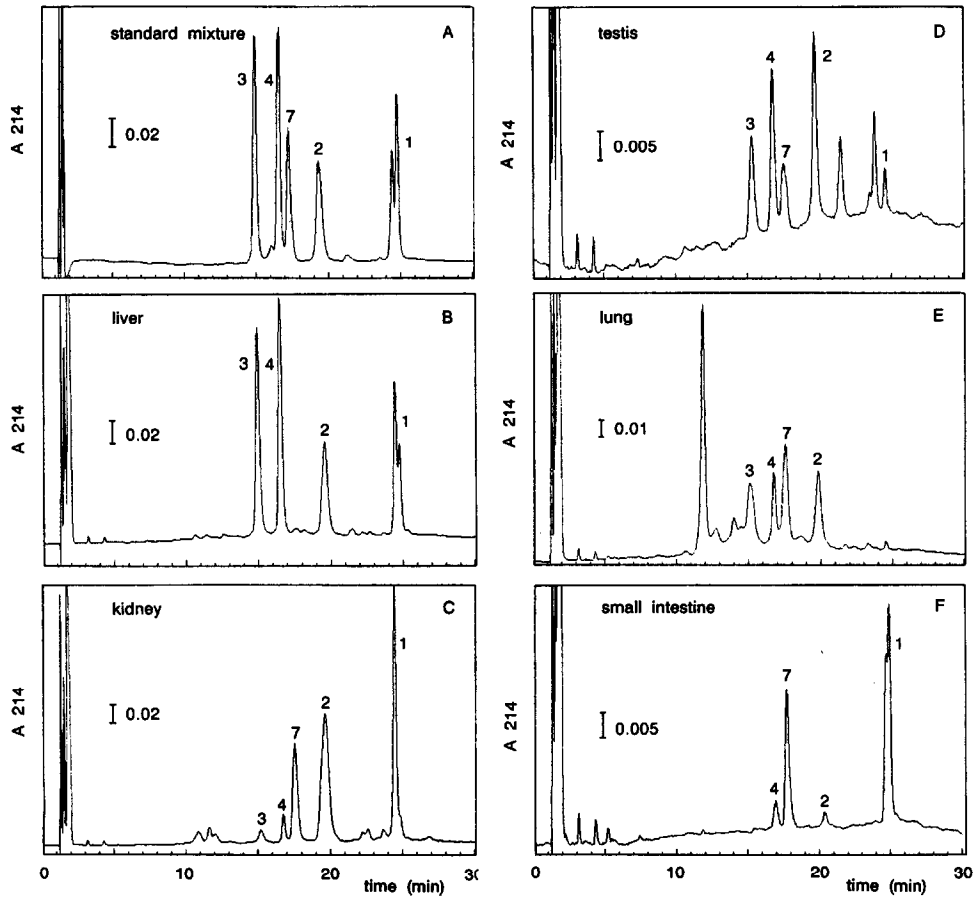

Fig. 1. Elution profile of a standard mixture of glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 in a concentration of 50 μg/ml (2.5 μg injected) (A), and elution profiles of S-hexylglutathione eluates of 2.1 mg liver (B), 2.1 mg testis (C), 16.7 mg kidney (D), 16.7 mg lung (E) and 0.53 mg small intestinal protein (F). The HPLC conditions are described in Experimental.

TABLE I

CONCENTRATION OF GLUTATHIONE S-TRANSFERASE SUBUNITS IN RAT LIVER, KIDNEY, TESTIS, LUNG AND SMALL INTESTINAL MUCOSA

Preparation of cytosol, isolation of glutathione S-transferases and HPLC separation and quantification of subunits were performed according to the procedures described in Experimental. The HPLC elution profiles of the different tissues are presented in Fig. 1B–F.

Organ	*Subunit concentration*				
	3	*4*	*7*	*2*	*1*
Liver[a]	1494	1661	–	1433	1245
Kidney[a]	26	33	217	414	239
Testis[a]	224	283	240	515	51
Lung[a]	85	65	148	126	–
Small intestine[b]	–	0.47	2.96	0.40	3.72

[a] Expressed in μg/g tissue.
[b] Expressed in μg/g protein.

3, 4, 2 and 1 are the only major components. In kidney, subunits 3 and 4 are present in minor amounts, while subunits 7, 2 and 1 are the major components. In liver, subunit 7 exists only in trace amounts[11].

In testis, a number of isoenzyme subunits exist. Subunits 3, 4, 7, 2 and 1 are clearly identified. Subunit 1 is present in minor amounts. Between subunits 2 and 1, at retention times of 21.5 and 24 min respectively, two peaks are present. The elution profile is in good agreement with that of seminiferous tubulus of rat testis as described by Farrants *et al.*[5], the peak at 21.5 min probably being due to subunit 6.

Fig. 1E shows the isoenzyme pattern of lung cytosol. Subunits 3, 4, 7 and 2 are major components. Subunit 1 seems to be almost absent. At 12 min, however, an unknown component is eluted, which is abundant in lung. It is different from the subunits 1, 2, 3, 4, 6 and 7. It cannot be identified with subunit 5, because the latter has no affinity for the S-hexylglutathione-Sepharose matrix[4]. Further investigation will be needed to identify this component. Fig. 1F presents the elution profile of the small intestinal mucosa. Subunits 7 and 1 are the major components, while subunit 4 and 2 exist only in minor amounts. Subunit 3 seems to be totally absent. The subunit concentration, calculated with the standard mixture of Fig. 1A, in the different organs is presented in Table I.

Linearity, precision and sensitivity of the assay

To estimate the relationship between the amount of isoenzyme injected and the integrated peak area, four different concentrations of a mixture containing isoenzymes 1-1, 2-2, 3-3 and 4-4 in the range from 15 to 90 μg/ml were injected on the HPLC column. The correlation coefficient varied between 0.997 and 0.999. The intercepts did not differ significantly from zero.

To determine the precision of the assay, the subunit concentration was determined in a pooled liver cytosol on different days, running the whole procedure. The interassay variation for the subunits 1, 2, 3 and 4 was 4, 4, 10 and 13%, respectively.

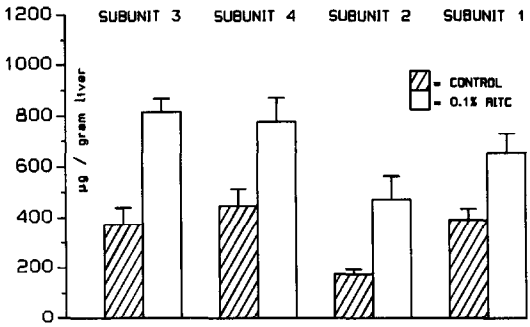

Fig. 2. Subunit composition in a rat control group (n = 5) and a group who received 0.1% allyl isothiocyanate (AITC) in the diet for 4 weeks (n = 5). Isolation of isoenzymes and separation of subunits were performed according to the procedures described in Experimental. Means and standard deviations are presented.

The method described is sensitive, detection of 50 ng (2 pmol) of the glutathione S-transferase subunits (absorption 0.002) being possible.

DISCUSSION

An improved HPLC method has been developed for the separation of glutathione S-transferase subunits. A Vydac TP reversed-phase column was used: the wide pores in TP silica make it ideal for the separation of large bio-molecules. Using the system outlined above, baseline separation between subunits 1, 2, 3, 4 and 7 can be performed within 30 min. The composition of the subunits was determined in rat liver, kidney, testis, lung and small intestinal mucosa, and was found to be in good agreement with the results obtained by Hayes and Mantle[11], who used immunoblotting for subunit identification. The method described is not only suitable for the quantification of subunits in different tissues, even in small samples or samples with low transferase activity, but can also be applied in experiments to estimate which subunits are induced by xenobiotics. For example, in Fig. 2 the subunit contents in liver are presented of a feeding study with rats, consisting of a control group and a group who received 0.1% of allyl isothiocyanate in the diet for 4 weeks, respectively. Subunits 2 and 3 were induced 169 and 119% by the allyl isothiocyanate, while subunits 1 and 4 were induced to much lower extents (67 and 74% respectively). Finally, in addition to other procedures, the method can be applied for the identification of isoenzymes and determining impurities during isolation and purification procedures.

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