Quantification of human hepatic glutathione *S*-transferases

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Human hepatic glutathione *S*-transferase (GST) subunits were characterized and quantified with the aid of a recently developed h.p.l.c. method. In 20 hepatic tissue specimens the absolute amounts of the basic Class Alpha subunits B1 and B2, the near-neutral Class Mu subunits μ and ψ and the acidic subunit π were determined. The average total amount of GST was 37 μ g/mg of cytosolic protein, with the Class Alpha GST being the predominant class (84 % of total GSTs), and π as the sole representative of the Class Pi GSTs present in the lowest concentration (4 % of total GSTs). Large inter-individual differences were observed for all subunits, with variations up to 27-fold, depending on the subunit. For the Class Alpha GST-subunits B1 and B2, a biphasic ratio was observed. The genetic polymorphism of the subunits μ and ψ was confirmed by h.p.l.c. analysis, and correlated with the enzymic glutathione conjugation of *trans*-stilbene oxide and with Western blotting of cytosols, using a monoclonal anti-(Class Mu GST) antibody. Of the 20 livers examined, ten contained only μ , whereas the occurrence of ψ alone, and the combination of μ and ψ , were found in only one liver each.

INTRODUCTION

The glutathione *S*-transferases (GSTs) are an interesting family of isoenzymes, best known for their role in the detoxification of electrophilic (alkylating) xenobiotics and intermediates [1]. However, a number of endogenous functions (prostaglandin synthesis, bile acid binding etc.) have been reported [1]. Although most abundantly present in the liver, GST can be detected in most organs. Apart from one monomeric microsomal form, all GSTs are dimeric cytosolic proteins, with both hetero- and homodimeric subunit compositions occurring.

The isoenzymes of GST have been divided into three classes, Alpha, Mu and Pi. Similarities exist within the same class, on the basis of both physiological functions (e.g. substrate specificity) and structural aspects (*N*-terminal amino acid sequence similarity, immunological cross-reactivity and isoelectric points [1,2]). Two closely related human Class Mu GSTs have been described, the GSTs μ and ψ [3,4]. A genetic deficiency has been observed for the human Class Mu GST: in approx. 50 % of all livers examined, no near-neutral GSTs were observed [5]. This deficiency has been shown to be the result of a specific gene deletion [6].

GST isoenzymes are known to be induced by a large variety of compounds. This phenomenon has been studied extensively in laboratory animals (see, e.g. [7,8]). Little is known, however, about the exact concentrations and inducibility of the GSTs in man. The main aim of the present study was to analyse quantitatively the human hepatic GST subunits by means of a recently developed h.p.l.c. method [9,10]. This method of analysis reveals the inter-individual differences (both genetically and environmentally determined), on the basis of subunit composition, thus bypassing heterodimer/homodimer complications.

MATERIALS AND METHODS

Chemicals

Both [³H]- and [³⁵S]-glutathione were obtained from NEN—du Pont, Dreiech, Germany; *trans*-stilbene oxide was from Aldrich, Milwaukee, WI, U.S.A.; Polybuffers, epoxy-activated

Sephacrose 6B and Phastgel materials were from Pharmacia, Uppsala, Sweden. *S*-Hexylglutathione was purchased from Sigma, St. Louis, MO, U.S.A.; the conjugated second antibodies were from Dakopatts, Copenhagen, Denmark. H.p.l.c.-grade acetonitrile was from Promochem, Wesel, Germany. H.p.l.c.-grade trifluoroacetic acid was from Baker, Deventer, The Netherlands. *S*-Hexylglutathione-coupled Sepharose 6B was synthesized by the method of Mannervik & Guthenberg [11].

Origin of human tissues

The liver tissue which was used for the purification of the GST isoenzymes was obtained at autopsy from an adult kidney donor. Specimens used for quantification and characterization of the Class Mu polymorphism were obtained either at autopsy from kidney donors or from surgical biopsies. All tissues were frozen at –80 °C within 12 h of clinical death; blood circulation was maintained until the moment of removal of the organ.

Purification of human GST isoenzymes

Class Alpha and Mu GSTs were purified from human liver by *S*-hexylglutathione affinity chromatography, followed by chromatofocusing, as previously described [12]. An LKB 2150 h.p.l.c. pump equipped with a Pharmacia Mono P HR 5/20 f.p.l.c. chromatofocusing column was used for the separation of the isoenzymes. By using a chromatofocusing column in combination with the f.p.l.c. system, i.e. small particle size, combined with a moderately high pressure (approx. 20 MPa), a high resolution was obtained. A pH gradient was obtained by eluting with 90 ml of Pharmacia Polybuffer 96 (diluted 1:20, v/v), followed by elution with Polybuffer 74, also diluted 1:20. For the purification of the Class Alpha GSTs, cytosol was used which contained no Class Mu transferases. Isoenzyme π was purified from a thoroughly perfused placenta by *S*-hexylglutathione affinity chromatography as mentioned above, and f.p.l.c. chromatofocusing using a gradient of pH 7–4, as created by Polybuffer 74, diluted 1:20. Purity was judged on the basis of isoelectric focusing and PAGE, as well as by the h.p.l.c. method described below.

H.p.l.c. analysis of GST subunits

The human GST subunits were separated and quantified using an LKB 2150 h.p.l.c. system. Separation was achieved on a Vydac 201 TP 5 [200 mm $\times$ 3 mm (int. diam)] column (Chrompack, Middelburg, The Netherlands), using acetonitrile and water, both containing 0.1 % trifluoroacetic acid, as eluting solvents. A gradient from 35 to 45 % acetonitrile in 18 min, followed by a gradient to 55 % acetonitrile in 5 min, and finally a 5 min isocratic elution at 55 % acetonitrile, were used. The flow rate was 0.6 ml/min. Detection was performed at 214 nm with an LKB 2140 rapid spectral detector, and peak-area integration was achieved by using Nelson analytical model-2600 chromatography software. The method proved to be very reproducible in terms of elution time and yield.

For the determination of the GST subunit composition of small liver samples with the above-described h.p.l.c. method, 1 ml of cytosol (5–15 mg of protein) was applied to a *S*-hexylglutathione–Sephacrose 6B affinity column and eluted as described previously [12]. The eluate was concentrated to a volume of approx. 100 μ l by using Centricon 10 micro-concentration tubes (Amicon, Danvers, MA, U.S.A.), and 50 μ l of this concentrate was injected for h.p.l.c. analysis. The absolute amounts of the various GST subunits were calculated from the integrated peak of the h.p.l.c. chromatograms, using purified homodimeric isoenzymes for calibration and correction for the loss (usually less than 10 %) of activity during the affinity-chromatography procedure. The minimal amount of wet liver tissue necessary to identify and quantify the individual subunits was approx. 25 mg.

Other analytical procedures

Western blotting, using horseradish peroxidase as staining antibody, was performed as described previously [13]. A monoclonal antibody raised against the human Class Mu GST isoenzymes was used, which showed no cross-reactivity with

either the Class Alpha or the Class Pi GSTs, but showed equal activity against μ and ψ [14].

Assays

Measurement of the enzymic activity of GSTs towards the glutathione conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) was adapted from Habig *et al.* [15], using 1 mM-glutathione and 1 mM-CDNB, and detection of product formation at 340 nm. A new procedure for the measurement of the conjugation of *trans*-stilbene oxide was developed, using radiolabelled glutathione instead of the (not commercially available) labelled epoxide. The enzyme (approx. 10 pmol) was incubated for 4 min at 37 °C with 0.5 μ mol of either ^3H (sp. radioactivity 4.0 Ci/mol) or ^{35}S (sp. radioactivity 3.26 Ci/mol)-labelled glutathione and *trans*-stilbene oxide (12.5 nmol) in 100 μ l of 0.25 M-Tris/HCl, pH 7.2. In preliminary experiments it was established that, under these conditions, the rate of conjugation was linear with time and protein concentration. The reaction was terminated by cooling to 0 °C. A 50 μ l portion of the incubation mixture was subjected to h.p.l.c. analysis, using a Hypersil ODS 100 mm $\times$ 3 mm Chrompack column, eluted with a linear gradient of 20–95 % (v/v) methanol in 0.02 M-Tris/phosphate, pH 2.5, in 15 min, followed by an isocratic elution with 95 % methanol for 15 min, at a flow rate of 0.4 ml/min. Quantification of the conjugation was achieved by collection of the conjugate fractions and subsequent determination of the amount of eluted radioactivity by liquid-scintillation counting. K' (elution volume/void volume) values for glutathione, the glutathione conjugate and *trans*-stilbene oxide were 1.1, 18 and 25 respectively.

RESULTS

H.p.l.c. separation of the human GST subunits

By using wide-pore reversed-phase h.p.l.c., all major human GST subunits were completely separated within 30 min (Fig. 1a).

Fig. 1. H.p.l.c. separation of human GST subunits present in four human liver specimens after purification by *S*-hexylglutathione affinity chromatography

The chromatograms show the genetic polymorphism of both Class Mu subunits: (a) μ and ψ are both present; (b) μ and ψ are both absent; (c) only π is present; and (d) only ψ is present. For h.p.l.c. conditions, see the Materials and methods section.

Fig. 2. H.p.l.c. separation of the near-neutral Class Mu GST subunits, as purified by chromatofocusing

(a) Subunit μ , as obtained from the pH 6.3 f.p.l.c. fraction; (b) subunits μ and ψ , as obtained from the pH 6.0 f.p.l.c. fraction; and (c) subunit ψ , as obtained from the pH 5.7 f.p.l.c. fraction.

With purified GST homodimers, the retention times of the GST subunits were determined as 20.7–21.2 min for π , 22.2–22.5 min for μ , 22.9–23.1 min for ψ , 23.9–24.2 min for B1 and 24.7–25.0 min for B2. The 214 nm detection allowed analysis and quantification of GST subunits with a lower detection limit of 2 pmol per subunit, i.e. a sensitivity which equals that of Western blotting or silver staining after electrophoresis. In all samples, an unknown protein eluted just before the B1 subunit.

H.p.l.c. analysis of the three Class Mu GSTs, as purified from a liver which contained both the μ and ψ forms, demonstrated the dissociation into subunits (Fig. 2). Both the μ homodimer and the ψ homodimer were eluted as single peaks (Figs. 2a and 2c), whereas the μ - ψ heterodimer showed both the μ peak and the ψ peak (Fig. 2b). The same phenomenon was observed for the B1-B1, B1-B2 and B2-B2 isoenzymes (results not shown).

Quantification of the individual GST subunits in human liver

GST of 20 liver specimens were purified from cytosol on 2 ml S-hexylglutathione affinity columns, and the eluates were concentrated and subjected to h.p.l.c. analysis and quantification, as described in the Materials and methods section. The absolute amounts of the individual GST subunits are presented in Table 1.

GST comprises an average of 3.7% of the total amount of cytosolic protein, with the B1 subunit being the most abundantly present: 18.6 μ g of B1/mg of cytosolic protein, 51% of the amount of hepatic GST. The amount of B1 subunit was about twice the amount of B2. Subunit π is present in minor amounts in almost all human livers; it accounts for 3.5% of the total amount of GST. In the livers in which subunit μ was present, its average concentration was 6.5 μ g/mg of cytosol, whereas subunit ψ showed a similar concentration in the two livers where it was present. The presence or absence of these two subunits did not affect the concentration of any of the other GST subunits (Table 1).

All subunits showed a rather large inter-individual variation. For example, subunit μ showed, apart from the genetic polymorphism, a 14-fold variation in concentration. For π , B1 and B2, an approx. 27-, 7- and 21-fold variation was observed. In terms of total amount of GST, the variation was less pronounced and was comparable with the variation in the specific activity of the cytosolic GST mixture towards CDNB. A correlation coefficient of 0.74 was calculated between this specific activity and the relative amount of total GST protein in cytosol. It is noteworthy that the variation in total amount of cytosolic GST is much smaller for μ -negative livers than for μ -positive livers. No relation could be established between this variation and either sex or age, partly because of the absence of information on a number of specimens.

The ratio between the two basic subunits showed a remarkable feature: of the 20 livers assayed, 12 showed an average ratio between B1 and B2 of 1.6 ± 0.3 , whereas seven other samples could be grouped with an average ratio of 3.8 ± 0.6 , and one sample had an extreme ratio of 10.5 (Fig. 3).

Detection of genetic polymorphism of Class Mu GST by h.p.l.c.

Fig. 1 presents examples of chromatograms of livers containing both μ - and ψ -subunits, only one of the two subunits, and neither of these subunits. From Table 1 an absence of both subunits is evident in 40% of the individuals, with the μ -subunit occurring in 55% of the samples and the ψ -subunit in 10%. Only one of the 20 livers investigated contained both the μ - and the ψ -subunit. These results were confirmed by immunoblot analysis of the SDS/polyacrylamide gels of the corresponding liver cytosols, using a monoclonal antibody selectively reacting with human GST μ - and ψ -subunits (Fig. 4). Furthermore, the enzymic activity of the cytosols and affinity-purified GST mixtures towards the glutathione conjugation of *trans*-stilbene oxide corresponded with the presence or absence of Class Mu GSTs. Livers containing μ and/or ψ displayed an activity of 14.8 ± 8.8 munits/mg of cytosolic protein ($n = 6$), whereas in the absence of these subunits, a rate of conjugation of *trans*-stilbene oxide of 0.31 ± 0.31 munit/mg of cytosolic protein was measured ($n = 5$). The purified μ and ψ homodimers and the μ - ψ heterodimer displayed approximately the same specific activity towards *trans*-stilbene oxide (1.54, 1.39 and 1.73 units/mg of protein respectively).

DISCUSSION

Although it is known that hepatic GSTs are inducible and some isoenzymes are subject to genetic polymorphism, the

Table 1. Quantification of the GST subunits present in 20 human liver specimens

The GST subunits of 20 human liver samples were quantified after small-scale affinity purification and subsequent separation of the subunits h.p.l.c., with peak area integration at 214 nm. Below, averages are presented with s.d. values and with, for the μ and ψ subunits, only the positive livers taken into account (*, not detectable). The specific activity (S.A.) is expressed as nmol of CDNB conjugated/min per mg of cytosolic protein.

Specimen no.	Amount of subunit ($\mu\text{g}/\text{mg}$ of cytosolic protein)					Specific activity (units/mg)
	π	μ	ψ	B1	B2	
1	1.36	6.05	5.88	14.61	12.29	40.19
2	5.07	1.30	*	37.70	40.53	89.94
3	0.93	9.92	*	17.21	10.43	38.49
4	3.28	18.45	*	19.33	14.10	55.16
5	1.75	4.16	*	12.62	8.98	27.51
6	*	9.69	*	47.02	15.11	71.82
7	0.15	*	*	25.30	6.59	32.04
8	0.52	*	5.39	20.31	1.94	28.16
9	1.87	*	*	21.53	11.79	35.19
10	0.48	*	*	14.72	14.01	29.21
11	0.44	*	*	22.42	5.09	27.95
12	0.24	2.33	*	6.97	4.39	13.93
13	*	5.79	*	23.59	14.65	44.03
14	0.51	2.76	*	11.71	3.08	18.06
15	3.71	5.81	*	14.17	2.92	26.61
16	1.75	5.72	*	23.48	12.37	43.32
17	4.09	*	*	20.76	5.55	30.40
18	1.30	*	*	13.18	8.14	22.62
19	0.91	*	*	20.99	12.16	34.06
20	2.14	*	*	19.26	9.47	30.87
Average ...	1.5	6.5	5.6	20.3	10.7	41.2
s.d. ...	1.5	4.8	0.4	9.0	8.2	19.1
Average for μ -negative livers only ...	1.4	0	0	19.8	9.1	30.3
s.d. ...	1.3			4.0	3.3	3.9

Fig. 3. Frequency distribution of the GST subunits B1 and B2 in human livers

The subunit concentration was determined by the described h.p.l.c. method in 20 livers (see Table 1). The distribution is presented as the ratio between subunits B1 and B2.

quantification of the individual human hepatic GST subunits reveals unexpectedly high inter-individual differences for all major subunits. This variation will be of major importance for substrates which are relatively selective for certain isoenzymes. The CDNB conjugation, as measured for the 20 assayed hepatic cytosols, does not express this variation, owing to the fact that CDNB is a rather aspecific substrate, with only a 3-fold higher specific activity found for the most effective isoenzymes (Class Mu GSTs) as compared with the least effective isoenzymes (Class Alpha GSTs) [1]. For other substrates a more than 1000-fold difference in selectivity is observed between certain isoenzymes, which thus more easily reveals inter-individual variations [1]. The ratio between the two basic subunits B1 and B2 shows an interesting frequency distribution. The number of samples used in the present study is too small to identify positively a (genetic) polymorphism in the distribution, but the observed trend deserves further attention.

The human Class Mu family is encoded by the polymorphic *GST1* locus, displaying three alleles, named *GST1-0*, *GST1-1* and *GST1-2* [5]. The phenotypes correspond to the expression of GST μ (alleles *GST1-1/GST1-1* and *GST1-1/GST1-0*), GST ψ (alleles *GST1-2/GST1-2* and *GST1-2/GST1-0*), both μ and ψ (alleles *GST1-1/GST1-2*), and the absence of both Class Mu subunits (alleles *GST1-0/GST1-0*) [5,16]. The frequency distribution for the occurrence of these phenotypes has been described after screening a number of individuals (liver or blood samples) on the basis of electrophoretic, immunological or catalytic properties [5,14,17,18–21]. The most recent reports show an occurrence of the null phenotype (no μ or ψ) of approx.

Fig. 4. Immunodetection of class Mu GST in hepatic cytosols

After SDS/PAGE (10% polyacrylamide), hepatic cytosols and purified GST were subjected to Western blotting. The left part of the blot was stained with Amido Black. Marker proteins (68, 44, 29 and 20 kDa respectively from top to bottom) and *S*-hexylglutathione-Sepharose-purified hepatic GST (1.4 µg) are shown in slots 1 and 2 respectively. The right part of the blot was incubated with a monoclonal antibody against Class Mu GST. Purified hepatic GST (0.7 µg) is shown in slot 3. In slot 4 rat liver cytosol is present, and in slots 5–14 human hepatic cytosols (25 µg of protein) from ten different individuals are shown. The numbers 5–14 correspond to the numbers of Table 1. The purified GST of slot 3 corresponds with the cytosol of slot 5.

40%, i.e. the same percentage as presented in this paper. Data on the frequency distribution of μ and ψ are less abundant, since most investigators were not able to discriminate between μ and ψ (either catalytically or immunologically). By using starch-gel electrophoresis, three bands were revealed, corresponding with the three class Mu isoenzymes [5,17,21]. The percentage of individuals possessing both μ and ψ is similar to reported values (6% [16]). The frequency distributions of μ and ψ vary with the various reports.

The method used in this paper to determine the subunit composition of human GST mixtures is of potential value for a number of applications: the quantification of induction of GST isoenzymes at the protein level, determination of the GST isoenzyme patterns of different tissues, routine characterizations during purification, homodimer/heterodimer characterizations, etc. No heterodimer/homodimer equilibria need to be taken into account, since dissociation into monomeric subunits is achieved. One of the advantages of the h.p.l.c. method for quantification is that inactivation of the isoenzymes does not interfere with the method. For example, the enzymic activity towards conjugation

of *trans*-stilbene oxide of the Class Mu subunits showed a rather poor correlation with the amount of μ subunits quantified by h.p.l.c. (ranging from 1.5 to 4.2 units of *trans*-stilbene oxide/mg of GST μ and/or ψ , as present in the cytosol samples), most likely due to partial loss of activity during storage or working-up procedures.

Correlations between, on the one hand, environmental factors (e.g. occupational exposure, nutrition, smoking, medication, etc.), age and gender, and, on the other hand, the concentrations of the individual subunits, could not be established, owing to the relatively small number of subjects and the lack of information on individual histories. Since the variations observed are very large, these relationships need to be explored. Furthermore, it is clear that the large inter-individual variation of GST subunits will be of major consequence in the individual risk assessment towards alkylating xenobiotics, including, e.g., alkylating cytostatics. Therefore this subject certainly deserves further attention.

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