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Chromatographic separation of fluorescent thiol adducts of 4-chloro-7-sulphobenzofurazan Use as substrates for enzymes of the mercapturic acid xenobiotic pathway

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Abstract

Fluorescent adducts of 4-chloro-7-sulphobenzofurazan with cysteine, cysteinylglycine, reduced glutathione and *N*-acetylcysteine were prepared. Adducts were separated by HPLC on a 3-mm Nova-Pak C_{18} reversed-phase column using isocratic elution with a solvent of acetonitrile–0.15 *M* phosphoric acid (5:95) buffered at pH 2.5. The adducts were detected using a fluorescence detector set at an excitation wavelength of 365 nm and an emission wavelength of 510 nm and an ultraviolet detector at 254 nm. The adduct of reduced glutathione was also formed by the action of the enzyme glutathione-*S*-transferase. This adduct acted as a substrate for the enzyme γ-glutamyltranspeptidase and the product of this reaction, the 4-chloro-7-sulphobenzofurazanyl derivative of cysteinylglycine, acted as a substrate for either dipeptidase or aminopeptidase M. The sequential enzymic effects could be detected by changes in the relative fluorescence intensity of the solutions to which the respective enzymes had been added but were more appropriately followed by changes in the HPLC elution profiles after enzymic treatment of solutions. © 1998 Elsevier Science B.V.

Keywords: 4-Chloro-7-sulfobenzofurazan; Mercapturic acid

1. Introduction

The mercapturic acid pathway is one detoxification mechanism for xenobiotic compounds in mammalian systems. The initial reaction is a nucleophilic attack of reduced glutathione on the electrophilic centre of the xenobiotic catalysed by the enzyme glutathione-*S*-transferase (GST). The second reaction in the sequence involves removal of the glutamate from the glutathione compound by γ-glutamyltranspeptidase (GGT) while glycine is then removed from the product of this reaction by a third enzyme which could be either dipeptidase (DP) or aminopeptidase M (APM). Prior to excretion in the urine, the cysteine compound is acetylated in the N position by cysteine-*S*-conjugate-*N*-acetyltransferase (CNAT).

1-Chloro-2,4-dinitrobenzene (CDNB) is a common substrate for estimating GST activity [1,2]. L-γ-Glutamate-*p*-nitroanilide may be used for GGT activity [3] while L-alanylglycine (Ala-Gly) and L-leucyl-*p*-nitroanilide may be used for estimation of DP and APM activity respectively [3]. CNAT activi-

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ty may be estimated by a radiolabelled acetyl transfer assay [4]. It would be very convenient if the sequential changes occurring to one xenobiotic compound could be detected and then used to follow the activity of each of the enzymes in the above reaction sequence. This aspect of analysis was examined in the present work using fluorescent adducts of the reduced forms of glutathione (GSH), cysteinylglycine (Cys-Gly), cysteine (CySH) and *N*-acetylcysteine (NAC) which were prepared with the nonfluorescent specific thiol probe 4-chloro-7-sulphobenzofurazan (SBf-Cl [5]). The adducts were then separated by high-performance liquid chromatography using fluorescence detection and were also examined as potential in vitro substrates for the various enzymes of the mercapturic acid pathway by following changes in the HPLC patterns.

2. Materials and methods

2.1. Chemicals

GSH, Cys-Gly, CySH, reduced dithiothreitol (DTT_{red}), SBf-Cl and GGT were purchased from Sigma (St Louis, Mo, USA). Other chemicals were of analytical grade.

2.2. Instruments

Absorbance readings were made with a Varian-Techtron 635 recording spectrophotometer. pH measurements were made with a Metrohm AG620 pH meter. Fluorescence estimations were made on a Shimadzu SP 500 spectrophotofluorimeter.

2.3. High-performance liquid chromatography

A 3-mm Nova-Pak C_{18} reversed-phase column (150×3.9 mm) was used with a flow-rate of 1 ml min^{-1}. Isocratic elution was used with a solvent of acetonitrile–0.15 *M* phosphoric acid (5:95) buffered at pH 2.5.

A Waters liquid chromatograph with M-45 pump, model 441 fixed wavelength detector (254 nm) and 10-mm flow cell was used for ultraviolet detection of SBf-Cl and all adducts. Fluorescence readings were made using a Varian HPLC system including a 9012 pump and 9070 detector set at 365 nm for excitation and 510 nm for emission throughout. Absorbance readings were also made at 380 nm using a using a Jasco 510 system and a 490 variable detector.

2.4. Enzymes

The supernatant fluid, obtained after centrifuging rat liver homogenate at 100 000*g* for 1 h, was used as a crude source of GST. Alternatively the enzyme was purified to homogeneity by affinity chromatography [6]. GGT was obtained from a commercial source (Sigma type II) and prepared as a 1 mg ml^{-1} solution in purified water.

A mixture of APM and DP was prepared from rat kidney microvillae by a standard procedure [3]. This preparation may also have contained some GGT but its presence was not significant in these experiments.

2.5. Enzyme reactions

GST reactions were effected by mixing 0.33 ml of 3 m*M* SBf-Cl solution with 0.1 ml of 10 m*M* GSH in a total volume of 1.23 ml of 0.1 *M* phosphate buffer pH 7 containing 2 m*M* EDTA. GST (0.1 ml) preparation was added and the solution incubated at 37°C. Changes in fluorescence were recorded. Additions of other reagents were made to this reaction mixture and other enzymic mixtures described below as required.

For GGT reactions the SBf-GS adduct was prepared as above but in 1.13 ml. When equilibrium was obtained, the enzyme was inactivated in a boiling water bath for 2 min, the mixture cooled to 37°C and 0.1 ml of 10 m*M* $ZnCl_2$ and 0.1 ml of GGT solution was added and the fluorescent changes followed. Amino acids were sometimes added at 1 m*M* to act as acceptor molecules for glutamate.

DP/APM reactions were followed spectrofluorometrically using SBf-Cys-Gly, prepared enzymatically as described above and heated to inactivate the GGT. Enzyme preparation (0.1 ml) was added and incubations conducted at 37°C in a total volume of 1.33 ml.

Samples (100 ml) were taken, mixed with 100 ml of 1 *M* HCl and duplicate 50-μl samples injected into the Waters HPLC port or duplicate 20-μl samples into the injection port of the Varian Instrument.

2.6. SBf-Cl adduct formation

Adducts of SBf-Cl with CySH, NAC, Cys-Gly and DTT_{red} were prepared at 37°C by mixing 0.33 ml of 3 m*M* SBf-Cl in 0.1 *M* borate buffer pH 9.2 with 0.1 ml of 1 *M* NaOH and 0.23 ml of distilled water containing excess (13.3 fold) thiol. The final pH was 11.8. Development of fluorescence was recorded. After the reaction reached completion the solution was neutralized with 0.1 ml of 1 *M* HCl and diluted to 1.33 ml with 0.2 *M* phosphate buffer pH 7.

In some experiments adducts were formed by heating SBf-Cl and thiols together for 20 min at pH 9.2 and 60°C, the temperature used for formation of ABD-F derivatives [7].

3. Results

3.1. Formation of thiol adducts

GSH, NAC, Cys-Gly and CySH formed yellow and fluorescent adducts on alkaline treatment (Fig. 1, curves B–E respectively). This pH (11.8) was chosen for adduct formation because all the SBf-Cl had disappeared (UV detection) suggesting that it was completely complexed as a thiol adduct. The high pH also favoured scission of some of the disulphide group [8] and maintained the resulting thiol as an anion.

Fluorescence also developed when GSH was treated with GST at pH 7 (Fig. 1, curve A). CySH, DTT_{red} NAC and mercaptoethanol (300 nmol) did not react with SBf-Cl at pH 7, but on treatment with GST, DTT_{red} increased the enzymic reaction with GSH by 4 percent and mercaptoethanol decreased the rate of reaction by 38 percent. DTT_{red} was sometimes added at a concentration of 1.2 m*M* to maintain reduced GSH. It did not affect GST activity at this concentration.

The thiol adducts showed minor variations in the maximum excitation and emission wavelengths but the excitation wavelength for all adducts was maintained at 365 nm and the emission wavelength at 510 nm for consistency.

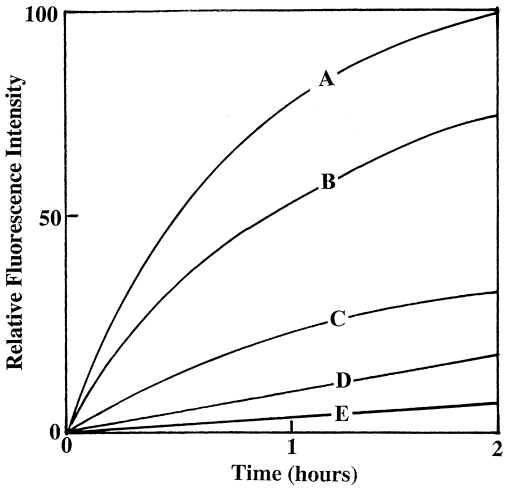

Fig. 1. Development of fluorescence of thiol compounds with SBf-Cl. Curve A represents a mixture of 300 nmol of SBf-Cl and 4 μmol of GSH at pH 7 in the presence of 150 μg of protein from the supernatant fluid of rat liver homogenate. Curves B, C, D and E represent the same amounts of GSH (B), NAC (C), Cys-Gly (D), CySH (E) with 300 nmol of SBf-Cl at pH 11.8.

3.2. Ultraviolet spectra and molar absorbancies

On reaction with thiols, the absorbance of solutions containing SBf-Cl increased significantly at 254 nm and slightly at 325 nm. A typical spectrum for the reaction with GSH is shown in Fig. 2 with the short dashes showing the spectrum of the underivitised reagent (SBf-Cl) and the long dashes the spectrum of the GSH adduct. A broad absorption band developed which extended from 350 nm beyond 400 nm. Similar changes were also observed after the enzyme catalysed reaction. The absorbance at 380 nm of an 0.75 m*M* solution of each adduct

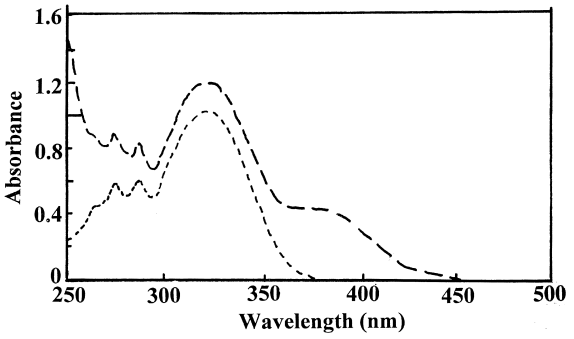

Fig. 2. Ultraviolet visible absorption spectrum of SBf-Cl (- - -) and SBf-GS (– – –) prepared by reaction with 600 nmol of GSH at pH 11.8.

was approximately 1 and quantitative estimates of adduct concentrations were made from the molar absorbancies which were estimated as 1.4×10^3 M^{-1} cm^{-1} for SBf-GS and SBf-DTT_{red} and 1.56×10^3 M^{-1} cm^{-1} for SBf-NAC, SBf-Cys and SBf-Cys-Gly after checking that the SBf-Cl had been consumed in formation of adduct.

Respective standard deviations of 2.2%, 2.3%, 1.6% 7.2% and 4.8% were obtained after chemical formation of GSH, NAC, DTT_{red}, Cys-Gly and CySH derivatives (8 readings each). The latter two readings are probably too dispersed for accurate quantitative estimation of these thiols. The standard deviation for the enzymic formation of SBf-GS was 8% which is considered acceptable for an enzymic reaction.

3.3. HPLC of thiol adducts

The thiols of the mercapturic acid pathway were separated by HPLC using 5% acetonitrile in 0.15 *M* phosphoric acid (pH 2.5) and were detected by fluorescence (Fig. 3). The respective peaks corresponded to each of the adducts chromatographed separately or fluorescent peaks detected when the adducts were formed by heating at 60°C and at pH 9.2. Apart from the NAC adduct, they also corresponded to peaks obtained in enzymic reactions (see below). These results extend the number of benzofurazan derivatives effectively separated by HPLC and permitted the use of the adducts for some biological purposes. The adduct of SBf-NAC consistently exhibited a shoulder of fluorescence in chromatograms. The origin of this shoulder could not be determined.

Adducts and SBf-Cl could also be detected at 254 nm but fluorescence was preferred because other material, absorbing at 254 nm, was sometimes detected. This occurred especially when SB-NAC was formed by heating at 60°C where small peaks with retention times 2.45 and 4.73 min were observed. These peaks were not detected with fluorescence detection nor with the detector set at 380 nm nor when the NAC adduct was formed at pH 11.8. They probably represent side reactions formed on heating or dimers of NAC. Ultraviolet absorbing material has also been observed when the benzofurazan derivative SBD-F, was heated with other thiols [9].

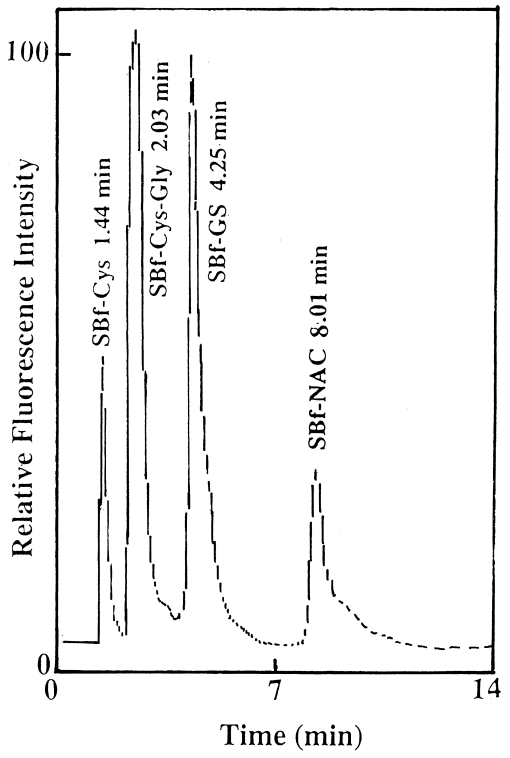

Fig. 3. HPLC chromatograms of SBf-Cl derivatives of mercapturic acid thiol compounds detected by fluorescence detector system

3.4. Fluorescence detection of enzyme activities

The relative fluorescence intensity of 0.75 m*M* SBf-GS at pH 7 was given an arbitrary value of 100. In comparison SBf-NAC was 35, SBf-Cys-Gly was 22 and SBf-Cys was 11. These results suggested that it might be possible to use these compounds to follow the mercapturic acid pathway reactions from changes in relative fluorescence intensity after enzyme treatment of compounds.

At pH 7, a mixture of SBf-Cl, GSH and GST developed fluorescence steadily as shown in Fig. 1 (curve A). The expected substantial fall in fluorescence occurred when SBf-GS was a substrate for GGT. The rate of decrease was accelerated by addition of glycine, methionine, glutamine and glycylglycine (by 21, 26, 48 and 48 percent respec-

tively) which acted as acceptor molecules for glutamate transfer in this reaction. Further treatment of the solution of SBf-Cys-Gly, with the DP/APM mixture resulted in a further loss of fluorescence consistent with the relative fluorescence intensity values indicated above but proportionally much lower than the fluorescence change which occurred on transition of SBf-GS to SBf-Cys-Gly. These changes were a poor indicator of DP/APM activity and since HPLC analysis identified the reaction products completely, it was considered more desirable for following in vitro enzyme reactions.

Only the product of GST activity (Fig. 4A) was identified by its fluorescence using HPLC. The spontaneous reaction was also detected but was much lower than that observed using CDNB [1,2]. On the other hand both the disappearance of SBf-GS and appearance of SBf-Cys-Gly, the product of GGT activity, could be identified and estimated (Fig. 4B). In this reaction there was also a small amount of SBf-Cys formed thus indicating an impurity in the GGT preparation. The disappearance of SBf-Cys-Gly or the appearance of SBf-Cys in the DP/APM reaction could also be identified and estimated in samples of reaction mixtures (Fig. 4, frame C). The thiol compounds DTT_{red}, GSH and CySH (at 1 m*M*) inhibited this reaction by 96.4, 96.8 and 94.6 percent respectively. These results suggest that the hydrolytic reaction was due to the DP component of the enzyme mixture [10]. HPLC is a useful method for following mercapturic acid pathway enzyme activities on these benzofurazan derivatives.

Activity of the acetylating enzyme cysteine-*S*-conjugate-*N*-acetyltransferase was not detected from either an expected increase in fluorescence or by HPLC analysis.

4. Discussion

The mercapturic acid pathway detoxifies xenobiotic compounds by four enzymic reactions. The xenobiotic compound SBf-Cl may be metabolised sequentially to SBf-GS, then SBf-Cys-Gly and finally to SBf-Cys in vitro by the first three enzyme systems of this pathway. Each of the reactions may be detected either by changes in relative fluorescence or preferably by HPLC patterns using a fluorescence detector. The fourth possible reaction of the mercapturate pathway, the formation of SBf-NAC from SBf-Cys, was not detected. SBf-Cys is quite soluble in aqueous solution while cysteine-*S*-conjugate-*N*-acetyltransferase usually acetylates more lipophilic adducts [4].

Several benzofurazan derivatives react with GSH [5,7,9,11–13]. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) is a general reagent for amino acids [5] but also reacts with the thiol group of GSH and has been used as a second substrate for placental

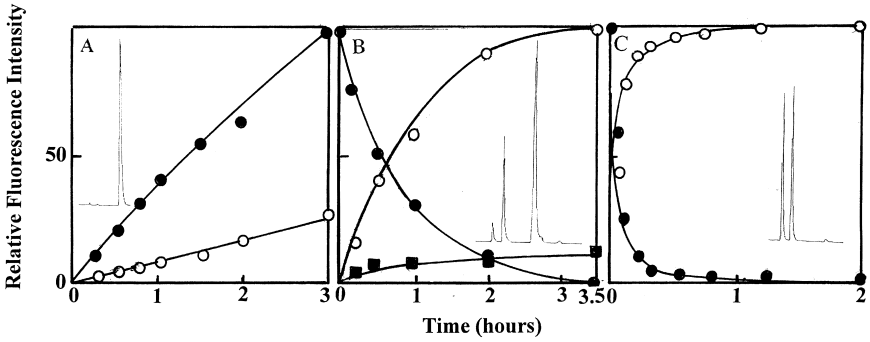

Fig. 4. Use of HPLC to follow the enzymic sequences of GST, GGT and DP/APM. Frame A is SBf-Cl plus GSH and (●) is GST catalysed while (○) is the spontaneous reaction. Frame B is SBf-GS treated with the GGT preparation and (●) is the disappearance of SBf-GS while (○) is the appearance of Sbf-Cys-Gly and (■) indicates the appearance of SBf-Cys. Frame C is SBf-Cys-Gly following DP/APM treatment and (○) represents the formation of SBf-Cys and (●) the disappearance of SBf-Cys-Gly. Insets in each frame indicate representative HPLC traces of each enzyme reaction.

GST with an optimum pH of 6.5 [11]. NBD-Cl is mutagenic [5]. SBf-Cl, on the other hand is non-mutagenic [5], is thiol specific [5] and clearly acts as a second substrate for rat liver GST. The pH optimum is about 8. Compared with CDNB [1,2], it shows a relatively low spontaneous reaction with GSH at pH 7. This suggests that it may be suitable for toxicological and detoxification studies using GST and the mercapturic acid pathway. By contrast the spontaneous reaction of GSH with ABD-F [7,13], which yields the same adduct with GSH, but which is more electrophilic, had reached the level shown by the enzyme catalysed reaction in 20 min at pH 7. SBf-Cl has also been shown to be a specific substrate for one isozyme only of four isoforms of GST of the larvae of *Galleria mellonella* [14]. A combination of NBD-Cl, SBf-Cl and other benzofurazan derivatives may prove to be of very great value in defining GST enzyme and isoform types and parameters.

The enzymic reactions described in this work may also be useful in examining potential activators and inhibitors of the enzymes of this pathway because the products show specific fluorescent properties. Most chemical additives are unlikely to have fluorescent properties at the same excitation and emission wavelengths and at the same retention times as the adducts of this work. It is possible that any of these three enzyme systems, which use SBf-substrates, will have a role in toxicological and excretion studies of drugs and other compounds. For instance, the analgesic acetaminophen is metabolised to *N*-acetylbenzoquinoneimine *via* a cytochrome P_{450} mechanism [15]. *N*-Acetylbenzoquinoneimine reacts with GSH and high doses of acetaminophen can deplete hepatic GSH so that the residual metabolite may then react with thiol groups of hepatic proteins to increase hepatic necrosis [15]. Some preliminary results indicate that high concentrations of acetaminophen ($0.25–5\ mg\ ml^{-1}$) can inhibit rat liver supernatant fluid GST activity on SBf-Cl between 10 and 82 percent. Since SBf-Cl does not react spontaneously with thiols (other than GSH) at pH 7, its thiol adducts may have a role in both in vitro studies and in vivo perfusion studies particularly if combined with radiolabelled GSH in the CySH residue. SBf-Cl may also have a role in histochemical studies involving GST.

5. Abbreviations

ABD-F = 4-(Aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole
APM = Aminopeptidase M
CNAT = Cysteine-*S*-conjugate-*N*-acetyltransferase
Cys-Gly = Cysteinylglycine
CySH = Cysteine
DP = Dipeptidase
DTT_{red} = Reduced dithiothreitol
GGT = γ-Glutamyltranspeptidase
GSH = Reduced glutathione
GST = Glutathione-*S*-transferase
NAC = *N*-Acetylcysteine
NBD-Cl = 7-Choro-4-nitrobenzo-2-oxa-1,3-diazole
SBD-F = Ammonium 7-fluoro 2,1,3-benzoxadiazole-4-sulphonate
SBf-Cl = 4 Chloro-7-sulphobenzofurazen

SBf-Cys, SBF-Cys-Gly, SBf-GSH and SBf-NAC= respective adducts of cysteine, cysteinylglycine, reduced glutathione and *N*-acetylcysteine with SBf-Cl.

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