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INHIBITION OF SOLUBLE GLUTATHIONE *S*-TRANSFERASE BY DIURETIC DRUGS

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Abstract—Glutathione transferases are believed to play an important protective role in the various tissues of animals and man by catalysing the glutathione conjugation of electrophilic drugs and electrophilic drug metabolites. Many of these compounds have the potential to react with vital cellular macromolecules in the absence of this enzyme system. We have investigated the interaction of a number of high ceiling diuretics with the glutathione transferases contained in the cytosolic fraction of the rat liver. Of bumetanide, ethacrynic acid, furosemide, indacrynic acid and tienilic acid, only ethacrynic acid was conjugated with glutathione. Further experiments revealed that ethacrynic, indacrynic and tienilic acids are all potent inhibitors of glutathione *S*-aryltransferase. Glutathione *S*-alkyltransferase and glutathione *S*-epoxide transferase were also inhibited by the diuretics, but to a lesser extent than glutathione *S*-aryltransferase. The diuretics giving the greatest inhibition of these reactions were chemically related to ethacrynic acid. The concept where inhibition of glutathione-*S*-transferase by a drug may enhance its own toxicity is considered. This mechanism has also the potential of enhancing the toxicity of other concurrently administered drugs which normally require glutathione *S*-transferase for detoxication.

The serious side-effects of commonly used diuretics have been relatively few, but tienilic acid, which is chemically related to ethacrynic acid, has resulted in serious complications, including fatal hepato-toxicity [1]. Tienilic acid was withdrawn from clinical use early in 1980, because of its suspected involvement in fatal cases of liver damage. Subsequently tienilic acid has been implicated in 24 fatal and over 300 non-fatal cases of liver toxicity.‖ The decision to withdraw the drug from use has remained controversial [1] and at least in France its marketing and use has continued. Meanwhile the mechanism of toxicity has remained obscure. It would be most important to understand the mechanisms involved, since tienilic acid was a very promising uricosuric diuretic. A better understanding of the toxic mechanism may permit future development of a similar drug without the same toxic effects.

The toxicity of a large number of drugs and other organic chemicals depends on the relative balance between the metabolic activation and detoxication processes. One of the most important detoxicating enzyme systems in the liver is the glutathione *S*-transferase system (EC 2,5,1,18) in its multiple forms [3, 4]. The glutathione conjugation is the first step leading to usually non-toxic and readily excretable water-soluble compounds [5]. Inhibition of the glutathione *S*-transferase system could have an effect comparable to the effect of depleting cellular glutathione as seen in paracetamol toxicity [6]. In the presence of reactive intermediates, vital cellular macromolecules would be poorly protected if the glutathione *S*-transferase system was greatly inhibited. Ethacrynic acid is known to interact with glutathione *S*-transferase in two ways, firstly as a substrate and secondly by binding covalently to glutathione *S*-transferase [7]. We have investigated the interaction of tienilic acid and several other diuretics (Fig. 1) with glutathione *S*-transferase.

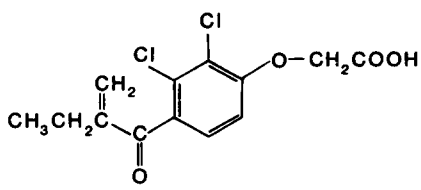
MATERIALS AND METHODS

Reagents. The chemicals used were the highest quality available commercially. Reduced glutathione, 1-chloro-2,4-dinitrobenzene, 3,4-dichloronitrobenzene, *p*-nitrobenzyl chloride, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St Louis, MO). Bumetanide was from Leo Pharmaceutical Products Ltd. (Balerup, Copenhagen, Denmark); ethacrynic and indacrynic acids were from Merck Sharp & Dohme (West Point, PA): furosemide was from Hoechst Roussel Pharmaceuticals Pty Ltd. (Melbourne, Victoria, Australia and tienilic acid was from Smith Kline & French Lab (Philadelphia, PA).

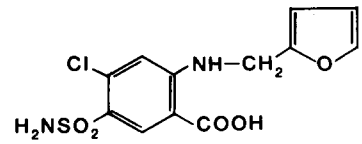
Cytosolic fraction. Livers of young, sexually mature, male Sprague–Dawley rats (220–250 g) were used as the source of the cytosolic fraction. The cytosolic fraction was prepared by homogenizing the livers in 4 vol. of ice-cold phosphate buffer (pH 7.4). This homogenate was centrifuged for 20 min at 10,000 *g*. The resulting supernatant was then centrifuged at 100,000 *g* for 1 hr. The supernatant resulting

§ To whom correspondence should be addressed.

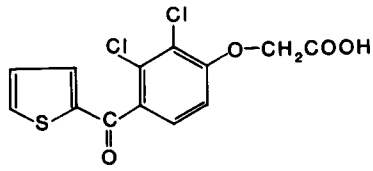
‖Twenty-four deaths now reported in U.S. Selacryn patients says Smith Kline [2].



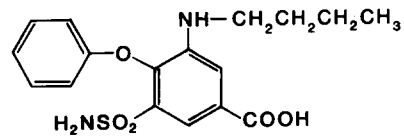
Ethacrynic acid



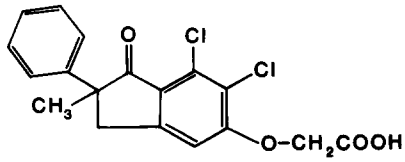
Furosemide



Tienilic acid



Bumetanide



Indacrynic acid

Fig. 1. Structures of high ceiling diuretics.

Table 1. Inhibition of soluble glutathione *S*-transferase activities by diuretics

	S-epoxide transferase* (nmole/mg protein/min) ± S.E.	*S*-aryltransferase† (nmole/mg protein/ min) ± S.E.	*S*-alkyltransferase‡ (nmole/mg protein/ min) ± S.E.	*S*-alkyltransferase§ (nmole/mg protein/ min) ± S.E.
Control	138.0 ± 5.0 (100)	1433.0 ± 38.0 (100)	52.0 ± 1.2 (100)	371 ± 6.0 (100)
Bumetanide	99.0 ± 3.0 (71)	955.0 ± 10.0 (67)	44.2 ± 1.3 (85)	311.0 ± 3.0 (84)
Furosemide	92.0 ± 4.0 (67)	968.0 ± 24.0 (68)	43.1 ± 1.3 (83)	282.0 ± 11.0 (76)
Tienilic acid	79.0 ± 4.0 (57)	603.0 ± 22.0 (42)	3.5 ± 0.2 (6)	188.0 ± 4.0 (51)
Ethacrynic acid	63.0 ± 3.0 (45)	37.0 ± 0.28 (3)	1.3 ± 0.002 (2)	192 ± 1.0 (52)
Indacrynic acid	76.0 ± 1.0 (55)	392 ± 16.0 (27)	3.1 ± 0.6 (6)	139.0 ± 8.0 (37)

The activity remaining after inhibition is presented as per cent of the uninhibited activity in parentheses.

* The *S*-epoxide transferase assays were carried out at 37° and pH 6.5. The substrate [1,2-epoxy-3-(*p*-nitrophenoxy)-propane] concentration was 0.5 mM. The absorbance change was monitored at 360 nm.

† *S*-Aryltransferase assays were carried out at 25° and pH 6.5. The substrate (1-chloro-2,4-dinitrobenzene) concentration was 1.0 mM. The absorbance change was monitored at 340 nM.

‡ *S*-Aryltransferase assays were carried out at 25° and pH 8.0. The substrate (3,4-dichloronitrobenzene) concentration was 1.0 mM. The absorbance change was monitored at 344 nm.

§ *S*-Alkyltransferase assays were carried out at 25° and pH 6.5. The substrate (*p*-nitrobenzyl chloride) concentration was 0.5 mM. The absorbance change was monitored at 310 nm.

In each case the inhibitor (diuretic) concentration was 0.2 mM. All values are means of four determinations.

from this centrifugation was used as the cytosolic fraction.

Enzyme assays. The inhibition of glutathione S-transferase activities by tienilic acid and the other diuretics shown in Fig. 1 was determined by using the cytosolic fraction as the glutathione S-transferase preparation and selected substrates representing alkyl- (*p*-nitrobenzyl chloride), aryl- (1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene) and epoxide–glutathione conjugation (1,2-epoxy-3[*p*-nitrophenoxy]-propane). The determinations were carried out kinetically in the presence of the diuretics under the conditions shown in Table 1. These assay methods were based on methods published by Habig *et al.* [5]. The incubation temperatures were those shown in Fig. 1 and the same as those used by Habig *et al.* [5]. Apart from one assay (epoxide–glutathione conjugation), it was not possible to use physiological incubation temperature because the reactions were not linear with respect to time sufficiently long. For this reason the S-aryltransferase and S-alkyltransferase activities were measured at 25°. Glutathione conjugation with ethacrynic acid was determined by monitoring absorbance changes at 270 nm. The ethacrynic acid concentration was 0.25 mM. The assay was carried out at 25° and pH 6.5 (0.1 M potassium phosphate) [5]. Glutathione conjugation of the diuretics was also determined by the disappearance of free sulfhydryl groups. This was done by incubating the diuretics in the presence of the rat liver cytosolic fraction and reduced glutathione, and after 15 min incubation depletion of the free sulfhydryl groups was measured by the method published by Ellman [8].

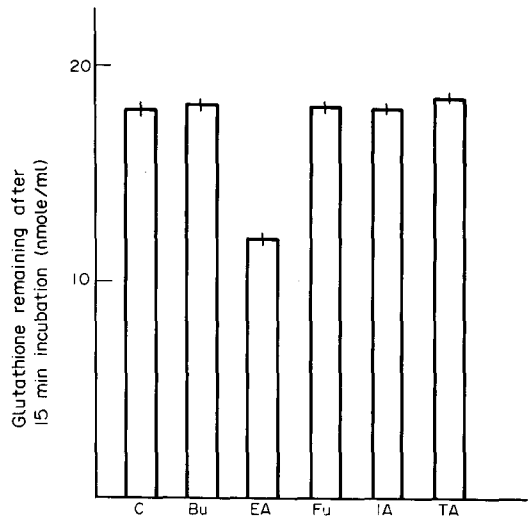


Fig. 2. Depletion of reduced glutathione in the presence of various high ceiling diuretic drugs. C = Control, Bu = bumetanide, EA = ethacrynic acid, Fu = furosemide, IA = indacrynic acid, TA = tienilic acid. The concentration of the diuretic drugs was 0.19 mM. The glutathione concentration was 2.5 mM and 50 μl of the rat liver 100,000 *g* supernatant was added to the incubation medium with a total volume of 2.15 ml. The incubations were carried out at pH 6.5, 7.5 and 8.5 for 15 min. The incubation temperature was 25°. The data are presented as the mean (± S.E.) of four determinations. Only data obtained at pH 7.5 are shown above.

RESULTS

The effect of diuretics on glutathione S-*transferases*

Ethacrynic acid inhibited the *in vitro* glutathione S-epoxide transferase by more than 50% (Table 1). Tienilic acid and indacrynic acid also caused very substantial inhibition of this reaction. Bumetanide and furosemide had less effect. The glutathione S-aryltransferase (1-chloro-2,4-dinitrobenzene) was strongly inhibited by tienilic acid and indacrynic acid and particularly so by ethacrynic acid (Table 1), but was less affected by bumetanide and furosemide.

Ethacrynic acid, tienilic acid and indacrynic acid caused almost complete inhibition of glutathione S-aryltransferase when 3,4-dichloronitrobenzene was used as the substrate, as shown in Table 1. Bumetanide and furosemide had relatively less effect on the glutathione S-aryltransferase.

As for the other glutathione S-transferase activities, glutathione alkyl S-transferase was also inhibited more strongly by the diuretic drugs structurally related to ethacrynic acid (Table 1), although the degree of inhibition was much less than observed with 3,4-dichloronitrobenzene as a substrate (Table 1).

It was possible to measure spectrally the glutathione conjugation of ethacrynic acid (data not presented). Habig *et al.* [5] have previously reported glutathione conjugation of ethacrynic acid. A similar procedure, measuring absorbance changes from 200 to 500 nm with other diuretics, failed to detect any evidence of glutathione conjugation. Also when glutathione depletion was used as a measure of glutathione conjugation, only ethacrynic acid caused glutathione depletion (Fig. 2). None of the other diuretics caused a depletion of glutathione in the presence of reduced glutathione and the rat liver cytosolic fraction (Fig. 2), suggesting that they are not substrates of this enzyme system *per se*. Altering the pH of the incubation medium over the range 6.5–8.5 did not affect these results.

DISCUSSION

Covalent binding and toxicity of a metabolically activated drug, such as paracetamol, follows the depletion of glutathione [6]. In this study we suggest that the protection of vital cellular macromolecules could be reduced if the glutathione conjugation of reactive intermediates was inhibited by other means than glutathione depletion. This may occur if the glutathione S-transferases were extensively inhibited. Our experiments show that the diuretics which are halogenated phenoxyacetic derivatives are strong inhibitors of glutathione S-transferases. Tienilic acid inhibits the *in vitro* conjugation of the epoxide used in this study by 50%. Indacrynic acid also appears to meet the structural requirements to act as a strong inhibitor of some glutathione S-transferases. Bumetanide and furosemide have less effect on these reactions.

The inhibition of glutathione S-aryltransferase is

strongly dependent on the substrate used (Table 1), but in each case the phenoxyacetic acid diuretics and indacrynic acid inhibited the reactions more strongly than bumetanide and furosemide. According to recent work, there are at least six forms of glutathione *S*-transferases. These are the A, B, C, E, AA and M forms [4]. The A and C forms catalyse the arylglutathione *S*-conjugation of 3,4-dichloronitrobenzene. The glutathione *S*-transferase activity of these two forms can be almost totally abolished by the halogenated phenoxyacetic acid diuretics (tienilic and ethacrynic acids) and indacrynic acid.

However, the strong inhibition of glutathione *S*-transferases alone cannot explain the toxicity of tienilic acid, as ethacrynic acid and indacrynic acid are also strong inhibitors of the glutathione *S*-transferases, but they have not exhibited toxicity similar to that of tienilic acid. One possible explanation may be found in the structural differences of the diuretic drugs (Fig. 1). The furan and the thiophene ring-containing diuretics have a potential of forming an epoxide which is highly reactive. The furan ring of furosemide has been reported to be metabolically activated and in high doses it is hepatotoxic [9]. The activation of thiophene ring is also likely. Should such an activated metabolite require glutathione *S*-transferase for its detoxication, the combined property of having a thiophene ring and being a strong inhibitor of glutathione *S*-transferase could make such a compound potentially very toxic.

The toxicological significance of these results remain to be clarified. It is known that paracetamol requires glutathione *S*-transferases A and C for its detoxication. Consideration ought to be given to the possibility that some diuretic drugs and commonly used analgesics such as paracetamol could have enhanced toxicity when administered together. Further work is being undertaken to investigate the toxicological significance of these findings.

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