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Glutathione depletion in a liver microsomal assay as an *in vitro* biomarker for reactive metabolite formation

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Glutathione (GSH) plays a major role in cytoprotection, acting as a nucleophile trap for reactive species derived from xenobiotics. This has led to the development of an assay for the detection of reactive species generated by liver microsomal metabolism of xenobiotics. This assay has been used extensively to study reactive metabolites which initiate toxicity through a direct (non-immunological) mechanism, but there are few data on its ability to detect reactive metabolites that initiate toxicity through neo-antigen formation, or to detect xenobiotics that cause GSH loss by oxidation mediated by a redox cycling process. Accordingly, the ability of rat and human liver microsomes to metabolize xenobiotics to GSH-depleting metabolites has been investigated further. Of the five neo-antigen-forming xenobiotics tested, four (amodiaquine, phenobarbitone, procainamide, and sulphanilamide) displayed GSH reactivity that was either dependent or independent (amodiaquine) on metabolism. The other neo-antigen-forming xenobiotic (carbamazepine) was inactive in all microsomal samples tested. Four quinones believed to exert toxcity through arylation (1,4-benzoquinone) and/or redox cycling (duroquinone, menadione, mitomycin c) displayed GSH reactivity, as did nitrofurantoin and diquat, two other redox cycling xenobiotics. Induction of the mixed function oxidase system with Aroclor afforded little advantage when using rat liver microsomes, whilst there was considerable inter-individual variation in the ability of human liver microsomes to mediate metabolism-dependent GSH depletion. It is concluded that the liver microsome GSH depletion assay may be of general utility as a screen for a number of xenobiotic-derived reactive species.

Keywords: reactive metabolite, glutathione, liver, neo-antigens, redox cycling.

Introduction

The toxicity of many xenobiotics is mediated by an initial cytochrome P450-dependent generation of a reactive electrophilic metabolite that binds covalently to nucleophilic sites of proteins (Pirmomahed *et al*. 1994, Cohen *et al*. 1997). In many instances, this protein binding leads directly to toxicity through processes involving disturbance of Ca^{2+}-homeostasis and/or mitochondrial function (Gregus and Klaassen 1996). The thiol group of cysteine is a common nucleophilic target on proteins for attack by these electrophilic metabolites, whilst conjugation of these metabolites with the abundant cellular cysteine-containing tripeptide glutathione (GSH) serves as a detoxication pathway (Reed 1985). As a consequence of this, the cellular GSH level falls on generation of xenobiotic reactive metabolites.

This GSH reactivity has been used (Mulder and Le 1988, Garle and Fry 1989) as the basis of an assay for detection of electrophilic metabolites of xenobiotics, in which liver microsomes (source of the P450 enzymes), cofactors, substrate, and added GSH are incubated together, and generation of reactive metabolite detected

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by either production of a GSH conjugate or loss of GSH (the 'GSH depletion assay'). A large number of xenobiotics have now been tested in this GSH depletion assay (Garle and Fry 1989, Fry *et al*. 1993, Wilkinson and Fry 1995, Garle *et al*. 1999, Hammond *et al*. 1999), the accumulated results of which suggest that this assay may be useful as a screening method for detection of xenobiotics that elicit toxicity through the mechanism described above.

It is recognized (Pirmohamed *et al*. 1994, Leeder 1998) that covalent binding of reactive electrophilic xenobiotic metabolites to proteins may initiate toxicity by an alternative mechanism, by which the modified protein acts as a neo-antigen that elicits cytotoxic antibody production. In addition, GSH may act in an alternative cytoprotective capacity by acting as a scavenger of free radicals generated during the redox cycling of xenobiotics (Reed 1985, Yu 1994), which is typically catalysed by another component of the P450 system, NADPH-cytochrome P450 reductase.

In light of the encouraging results obtained to date with the GSH depletion assay, we have now sought to determine if its use could be extended to detection of neo-antigen-forming reactive metabolites and redox-cycling chemicals.

Materials and methods

Chemicals

Diquat was obtained from Greyhound Chromatogrpahy and Allied Chemicals, Birkenhead, UK, and mitomycin c was a generous gift from Kyowa Hakko (UK) Limited. All other test chemicals and reagents required for the GSH and other assays were obtained from the Sigma Chemical Company, Dorset, UK, with the exceptions of 7-ethoxycoumarin and 7-ethoxyresorufin, which were synthesized as described previously (Mayer *et al*. 1977, Aitio 1978).

Animals and treatment

Male Wistar rats, approximately 180–200 g in weight, were obtained from the University of Nottingham Medical School Animal Unit. Control (untreated) rats were given free access to a commercial diet and drinking water. Treated rats were given a single i.p. injection of 500 mg kg^{-1} Aroclor 1254 in arachis oil 5 days prior to killing, and fasted for 18 h prior to killing, according to a standard protocol (Maron and Ames 1983).

Preparation of rat liver microsomes

Liver homogenates were prepared from the pooled livers of six rats per group using a Braun-type homogenizer. These homogenates were centrifuged at 10 000 *g* at 4 °C for 20 min. The supernatant was removed and to this was added 100 mM calcium chloride at a volume equivalent to 20 % of the measured fraction. This was mixed and left to stand on ice for 10 min prior to centrifugation at 28 000 *g* for 15 min. The supernatant was discarded and the pellet resuspended in 1.15 % (w/v) KCl and recentrifuged at 28 000 *g* for 15 min. The final pellet was resuspended in sucrose–EDTA–Tris buffer (0.25 M–5.4 mM–20 mM, pH 7.4), such that one ml of suspension contained the equivalent of 500 mg of liver. Microsomes were stored at –80 °C until use.

Preparation of human liver microsomes

Human liver material was obtained as surgical waste from patients undergoing liver resections for the removal of secondary carcinomas. Tissue samples that were macroscopically free of tumour were placed in ice-cold 0.25 % (w/v) sucrose in a closed, insulated container for transportation to the laboratory. Rough dissection of the tissue was performed, and the resultant liver pieces were frozen rapidly in liquid nitrogen, and stored at –80 °C overnight until preparation of microsomes as described for rat liver. Further details of the patients are given in table 1.

GSH depletion assay

The GSH depletion assay was performed as previously described (Garle and Fry 1989), with the modification that a greater volume of microsomal suspension was used (75 µl rather than 25 µl described in the original report).

Table 1. Source of human liver microsomes, and some relevant parameters.

	Subject				
	1	2	3	4	5
Sex (M/F)	F	F	F	M	M
Age (years)	76	60	50	73	73
Protein content (mg g^{-1} liver)	18.0	13.5	13.5	9.5	11.5
EROD activity (pmol min^{-1} mg^{-1} protein)	189	37.5	28.5	11.0	86.0
ECOD activity (pmol min^{-1} mg^{-1} protein)	25	20	15	26	17
NADPH-cytochrome c reductase activity (μmol min^{-1} mg^{-1} protein)	0.59	1.26	0.58	0.83	0.93

Briefly, the incubation mixture (0.25 ml) contained liver microsomes (75 μl), 0.2 M phosphate buffer (pH 7.4), 30 mM $MgSO_4$, 0.5 mM NADP, 5 mM isocitric acid, isocitric dehydrogenase (1 unit ml^{-1}), GSH (200 μM), and substrate added in solvent (water or methanol). Tubes were incubated at 37 °C for 30 min in a shaking water bath. The reaction was stopped by the addition of 10% TCA (0.25 ml). The amount of GSH remaining in the protein-free supernatant was measured by the methods of Sedlack and Lindsay (1968) or Hissin and Hilf (1976).

The results are expressed as loss of GSH in nmol GSH per mg protein per 30 min, and corrected for loss of GSH arising in the absence of substrate.

Other assays

Protein was measured by the method of Lowry *et al*. (1951). Cytochrome P450 content was measured by the method of Omura and Sato (1964). NADPH-cytochrome c reductase activity was measured by the method of Gibson and Skett (1994), whilst ethoxyresorufin O-deethylase (EROD) and 7-ethoxycoumarin O-deethylase (ECOD) activities were measured as described by Fentem and Fry (1991), using substrate concentrations of 5 and 10 μM respectively.

Presentation and analysis of results

Unless otherwise stated, the results are presented as the mean ± SEM of four experiments performed on the same batch of microsomes. Statistical analyses were performed using the INSTAT program, with probabilities of less than 0.05 deemed to be significant.

Results

The levels of GSH depletion recorded for 13 xenobiotics are presented in table 2. The full range of compounds could be tested with only two of the five human liver samples. Two compounds previously reported to give a large and moderate response in this assay, particularly after enzyme induction—3-hydroxyacetanilide (3-HA) and paracetamol respectively—were used as reference compounds. Both compounds produced measurable GSH depletion in all four microsomal samples, those in rat liver microsomes being significantly elevated in samples isolated from Aroclor-treated animals. Preliminary studies confirmed that the depletions obtained by both compounds were concentration- and metabolism-dependent, the latter determined by a lack of depletion when cofactors were omitted from the incubation mixture (data not shown as this has been demonstrated in a previous paper by Garle and Fry (1989)).

Incubation of liver microsomes with benzoquinone, a quinone that is reduced chemically by GSH with the formation of oxidized glutathione, also produced appreciable GSH depletion in all microsomal samples tested, the magnitude of which was either not affected or was increased when cofactors were omitted from the incubation.

Table 2. GSH reactivity of xenobiotics in liver microsomal incubations.

Xenobiotic[a]	Source of liver microsomes			
	Untreated rat	Aroclor-treated rat	Human 1	Human 2
	GSH depletion (nmol mg^{-1} protein per 30 min)			
3-Hydroxyacetanilide	14.7 ± 0.9[b]	35.6 ± 0.3*	9.3 ± 0.6	8.2 ± 1.6
Paracetamol	11.1 ± 2.0	19.4 ± 0.5*	29.3 ± 1.6	7.8 ± 0.4
Benzoquinone	20.4 ± 0.1	10.5 ± 0.5	43.3 ± 0.9	20.3 ± 0.6
	(36.1 ± 0.4)[c]	(13.7 ± 0.7)	(24.4 ± 1.3)	(48.2 ± 4.3)
Sulphanilamide	18.5 ± 1.4	12.0 ± 0.8	5.9 ± 1.0	11.3 ± 1.3
	(0)			
Procainamide	8.8 ± 1.4	7.5 ± 1.6	16.6 ± 3.5	7.1 ± 1.3
	(0.9 ± 0.5)			
Amodiaquine	5.5 ± 0.4	6.4 ± 0.9	14.8 ± 0.6	19.3 ± 2.7
	(5.9 ± 2.4)	(10.6 ± 0.8)	(37.6 ± 0.6)	(25.6 ± 0.6)
Carbamazepine	0	0	0	0
Phenobarbitone	5.9 ± 0.5	2.8 ± 0.6*	0	4.0 ± 1.6
	(0)			
Nitrofurantoin	34.2 ± 0.1	25.5 ± 0.6	49.6 ± 0.0	67.9 ± 0.7
	(25.2 ± 1.5)	(13.4 ± 0.8)	(30.0 ± 1.7)	(18.5 ± 1.1)
Diquat	21.3 ± 1.7	14.9 ± 0.1*	15.9 ± 0.6	32.4 ± 1.0
	(0)			
Menadione	21.4 ± 0.5	34.9 ± 0.0	49.3 ± 1.2	98.8 ± 0.0
	(41.0 ± 0.0)	(31.3 ± 0.0)	(71.6 ± 0.6)	(53.5 ± 0.6)
Mitomycin c	15.5 ± 0.6	13.9 ± 0.4	20.4 ± 1.8	29.1 ± 0.9
	(1.6 ± 0.5)			
Duroquinone	12.5 ± 0.1	7.7 ± 0.6*	5.0 ± 0.8	23.1 ± 1.0
	(0)			

[a] Xenobiotics were tested at a final concentration of 1 mM, with the exceptions of benzoquinone (0.1 mM), mitomycin c (0.15 mM), and duroquinone (0.3 mM).

[b] GSH reactivity is reported as nmol GSH depleted mg^{-1} protein per 30 min, and the results represent the mean ± SEM of four experiments performed on each batch of microsomes.

[c] When performed, the results of incubations run in the absence of cofactor mix are indicated within parentheses.

* Where indicated, results obtained with liver microsomes isolated from Aroclor-treated rats are significantly different with those from untreated rats ($P < 0.05$ or less; unpaired t-test with or without Welch's correction for unequal variances as appropriate).

Neo-antigen-forming xenobiotics

Sulphanilamide, procainamide, amodiaquine, and phenobarbitone displayed GSH reactivity in both sets of rat liver microsomes, although the level of reactivity was not enhanced by Aroclor treatment; indeed, it was reduced in the case of phenobarbitone. Carbamazepine produced no detectable GSH depletion with any of the microsomal samples tested. Sulphanilamide, procainamide, amodiaquine, and phenobarbitone also elicited a GSH depletion response in incubations containing human liver microsomes, the magnitudes of which were broadly similar

to those determined with rat liver microsomes. The GSH reactivity associated with procainamide, sulphanilamide and phenobarbitone appeared to be metabolism-dependent, as omission of cofactors led to a 90–100 % reduction in the GSH depletion response (see table 2). Amodiaquine appeared to be a direct GSH depletor, as omission of cofactors either had no effect on the response or, with some microsomal preparations (Aroclor-treated rat, human 1 and 2), enhanced the response. Concentration-dependent responses were obtained with these compounds; an example with procainamide is presented in figure 1.

Redox-cycling xenobiotics

The five redox-cycling xenobiotics studied—menadione, diquat, mitomycin c, duroquinone, and nitrofurantoin—produced a concentration-dependent depletion of GSH, the magnitudes of which were again similar when using rat or human liver microsomes (see figure 2 for data on mitomycin c as an example of the concentration dependence). Use of liver microsomes from Aroclor-treated rats led to an elevation in GSH depletion mediated by menadione, but small reductions with the four other redox-cycling xenobiotics. The GSH depletion mediated by diquat, mitomycin c, and duroquinone appeared to be metabolism-dependent, as judged by the loss of response when cofactors were omitted from the incubation. The reactivity of menadione appeared to be metabolism-independent, whereas the reactivity of nitrofurantoin appeared to be only partially dependent on metabolism, as omission of cofactors only partially reduced the reactivity.

Variation between human liver microsome samples

Although the only two human liver microsome samples for which we were able to study all 10 test compounds showed similar activity, we wished to have a clearer idea of the extent of variability between different subjects. To study this, a limited range of xenobiotics, encompassing metabolically-activated compounds (paracetamol, procainamide, phenobarbitone) and redox-cycling compounds

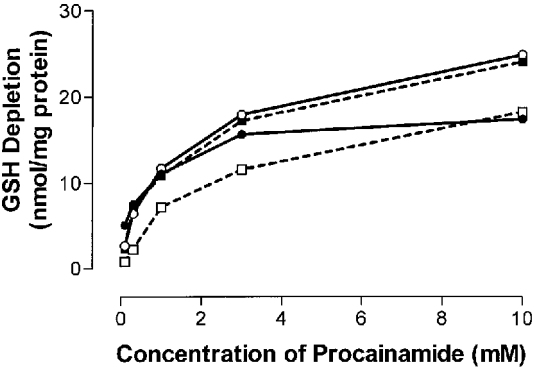

Figure 1. GSH depletion mediated by procainamide in the presence of liver microsomes isolated from untreated rats (●), rats treated with Aroclor (○), and human subjects 1 (■) and 2 (□). The error bars have been omitted for clarity, but were typically 10 % or less of the mean value.

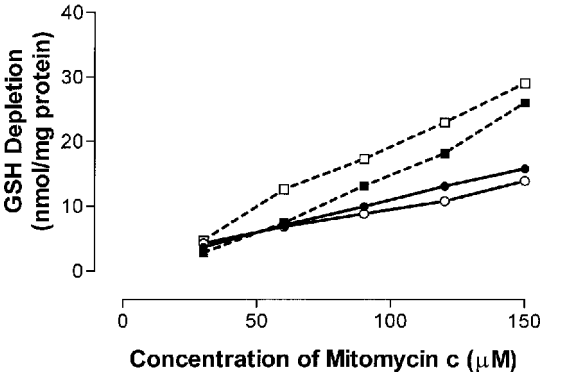

Figure 2. GSH depletion mediated by mitomycin c in the presence of liver microsomes isolated from untreated rats (●), rats treated with Aroclor (○), and human subjects 1 (■) and 2 (❐). The error bars have been omitted for clarity, but were typically 10% or less of the mean value.

(diquat, nitrofurantoin) was studied in five different human liver microsome samples (figure 3). Subjects numbered 1 and 2 in the figure were those given the same number in table 2. In addition, the EROD, ECOD and NADPH cytochrome c reductase activities of the microsome samples were also measured (table 1). EROD activity displayed a wide inter-subject variabilty, whereas the other two enzyme activities displayed much more modest variability. For each of the substrates tested, a variation in GSH reactivity of at least two-fold was identified, which for diquat was as large as four-fold. There appeared to be no correlation between any of the GSH depletion responses across the subject samples.

Discussion

The results presented in this report provide further support for the use of the GSH depletion assay as a screen for chemically-reactive species. In previous reports we have indicated the utility of this assay for detection of reactive metabolites which bind to protein thiol groups. In this report we indicate that the assay can also detect reactive metabolites that have been reported to exert toxicity through neo-antigen formation, and xenobiotics that initiate redox cycling.

In agreement with our previous report (Garle and Fry 1989), GSH depletion mediated by 3-HA and paracetamol was enhanced following induction of the P450 system (with Aroclor in this study). However, the absolute levels of depletion (per mg protein) are lower in these studies than those reported in the previous paper. We ascribe this to the slightly modified incubation system used in this study (greater volume of microsome suspension), the modification being carried out to increase the actual loss of GSH in the reaction tube. It is apparent from this that the extent of GSH depletion is not linearly dependent on protein content.

Sulphanilamide, procainamide, amodiaquine, phenobarbitone, and carbamazepine have been reported (Kitteringham *et al*. 1988, Maggs *et al*. 1988, Uetrecht 1988, Pirmohamed *et al*. 1994, Leeder 1998) to be metabolized to reactive metabolites that may bind to cellular protein and so initiate hypersensitivity-type toxicity through neo-antigen formation. Of these xenobiotics, only carbamazepine

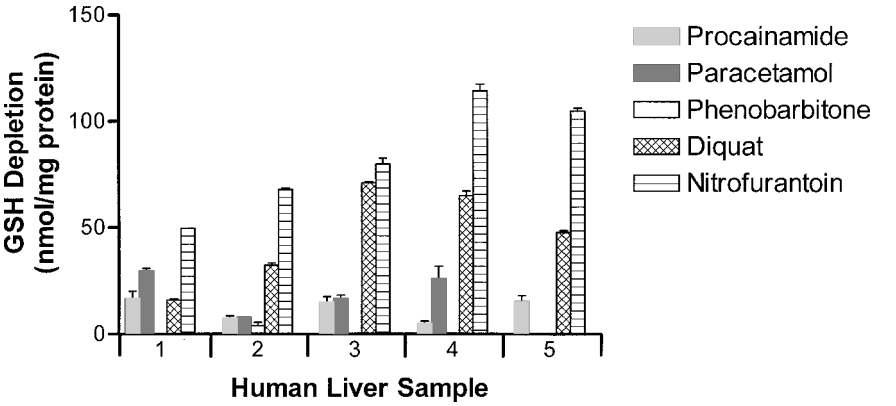

Figure 3. Levels of GSH depletion mediated by 1mM concentrations of procainamide, paracetamol, phenobabitone, diquat, and nitrofurantoin in five human liver microsome samples. The results are expressed as the mean ± SEM, $n = 4$. Phenobarbitone elicited no response in samples 1, 3, and 4, whilst he limited supply of sample 5 precluded analysis of the effects of paracetamol and phenobarbitone.

failed to elicit GSH depletion in rat liver microsomes (and also human liver microsomes). Of the remaining four compounds, the GSH depletion mediated by sulphanilamide, procainamide and phenobarbitone appeared to be dependent on an active P450/reductase system, as omission of cofactor mix produced a marked (90–100%) reduction in the level of GSH depletion. The depletion mediated by amodiaquine was not metabolism-dependent as judged by a lack of effect of omission of cofactors. This is consistent with the report of Maggs *et al*. (1988) that amodiaquine can be converted to chemically reactive species by autooxidation in neutral solution under air, although activation through enzymic means can also occur.

With sulphanilamide, procainamide and phenobarbitone, Aroclor treatment did not enhance the extent of GSH depletion; rather, in the case of phenobarbitone, it led to a significant decrease in activity. This suggests that activation of these xenobiotics is mediated by constitutive forms of P450 rather than those induced by Aroclor (forms 1A1, 1A2, and 2B1). In the case of procainamide, this suggestion is consistent with the observation (Lessard *et al*. 1996) that its activation to the reactive form (a hydroxylamine) is mediated principally by the constitutive CYP2D family. There appears to be no published information on the CYP isoforms responsible for activation of sulphanilamide or phenobarbitone. The present findings of GSH reactivity of reactive species derived from amodiaquine, sulphanilamide, and phenobarbitone are in accord with the observations of Kitteringham *et al*. (1988) that covalent binding of reactive products derived from these drugs was inhibited in the presence of GSH.

Carbamazepine did not display GSH reactivity in any of the microsomal preparations at the concentrations used. It is believed that carbamazepine is activated by CYP forms to an unstable arene oxide which may be inactivated through the action of epoxide hydrolase, and it has been suggested (Friedman *et al*. 1994) that this inactivation pathway may be deficient in patients predisposed to carbamazepine hypersensitivity. It is thus possible that a failure to detect GSH

reactivity is due to an efficient inactivation of the reactive metabolite in the microsomal preparations used.

The GSH reactivity of four quinones—1,4-benzoquinone, duroquinone, menadione, and mitomycin c—have also been evaluated in this assay system. 1,4-Benzoquinone is an arylating quinone in that it reacts with GSH by a Michael addition with the formation of a glutathione conjugate (O'Brien 1991, Butler and Hoey 1992). Duroquinone is a redox cycling quinone that exerts toxicity, in part, by causing oxidation of reduced glutathione to the dimer, GSSG (O'Brien 1991, Butler and Hoey 1992). Menadione is a mixed arylating and redox cycling quinone (O'Brien 1991, Miura *et al*. 1992), with the latter predominating in the cytotoxicity associated with this compound (Thor *et al*. 1988). The principal route by which mitomycin c exerts toxicity is that of reductive activation to yield a semiquinone which then causes GSH depletion by both arylation and redox cycling (O'Brien 1991, Sharma and Tomasz 1994).

All four quinones produced appreciable GSH depletion in the assay system. Depletion mediated by duroquinone and mitomycin c was dependent on activity of the P450/reductase systems, as reactivity was markedly reduced in the absence of cofactors. GSH depletion mediated by benzoquinone and menadione did not appear to be dependent on either of these systems. Indeed, in the majority of microsomal preparations studied, omission of cofactors enhanced the level of GSH depletion. The results obtained with 1,4-benzoquinone, duroquinone, and mitomycin c are consistent with the known arylating/redox cycling properties discussed above. The results obtained with menadione appeared rather surprising, given the predominant role ascribed to redox cycling and oxidative stress in the toxicity of this compound, and point to a major role of a direct arylating mechanism (i.e. no requirement for metabolism) for GSH reactivity in the present assay system. Once again, the level of GSH reactivity of each of these four compounds appeared to be similar in rat and human liver preparations.

The GSH reactivity of two other compounds—diquat and nitrofurantoin—believed to exert their toxicity through redox cycling and subsequent oxidative stress (Ross 1989) were also determined in the assay system. GSH reactivity of diquat was dependent on activity of the P450/reductase systems, as indicated by a lack of reactivity in the absence of cofactors, whereas that for nitrofurantoin was only partially dependent on the P450/reductase systems. This suggestion of a dual mechanism in the GSH reactivity of nitrofurantoin is consistent with evidence (Sliva *et al*. 1993) of both redox cycling/oxidative stress and covalent binding to protein (and associated protein thiol depletion), the latter being independent of activation by cytochrome P450 (Minchin *et al*. 1986). Induction of the P450 system by Aroclor treatment was without effect on these responses, which seemed to be greater with the human liver preparations.

The variability between human liver preparations was assessed further with a panel of five human liver samples and a limited range of substrates – procainamide, paracetamol, phenobarbitone, diquat, and nitrofurantoin (figure 3). In addition, ECOD and EROD activities were assayed to determine the functional state of the P450 system in the samples, whilst NADPH-cytochrome c reductase activity was assayed to determine the reducing capacity of the samples required for the one-electron reduction of the redox-cycling compounds. Inter-individual variation in enzyme activity was particularly marked with EROD, in agreement with the findings of others (Forrester *et al*. 1992, George *et al*. 1995). Inter-subject variations

were also apparent in the levels of GSH depletion, although there appeared to be no correlation between the level of GSH depletion and the cytochrome P450 or cytochrome P450 reductase activity. Furthermore, the sample in which the greatest response occurred for a particular substrate varied between substrates. Thus, sample 1 produced the greatest response with procainamide and paracetamol, sample 2 with phenobarbitone, sample 3 with diquat, and sample 4 with nitrofurantoin. No response to phenobarbitone could be detected in three of the four samples analysed (numbers 1, 3 and 4), in spite of appreciable levels of P450 metabolism being determined with procainamide and paracetamol, and by EROD and ECOD activities.

In conclusion, the results of the present study indicate that the GSH depletion assay based on the use of a liver microsomal incubation system is capable of detecting reactive species derived from drugs which elicit toxicity though a number of different mechanisms involving perturbation of GSH homeostasis. However, it is recognized that this assay will not detect reactive species from xenobiotics which do not react with GSH, but which may interact with other nucleophilic sites such as lysine residues on proteins (as discussed previously, Garle and Fry 1989). These results also indicate that human liver microsomes can be successfully used as the metabolic component in this assay. Taken together with our previous data obtained with the GSH depletion assay (see Introduction), these results indicate that the assay may be of broad utility in the screening of reactive species generated from new chemical entities.

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