CHAPTER 5

GSH DEPLETION IN RAT HEPATOCYTES:
A MIXTURE STUDY WITH \(\alpha,\beta\)-UNSATURATED ESTERS

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ABSTRACT

GSH depletion is often reported as an early cytotoxic effect, caused by many reactive organic chemicals. In the present study, GSH depletion in primary rat hepatocytes was used as an in vitro effect-equivalent to measure the toxic potency of α,β-unsaturated esters (acrylates and methacrylates). When these compounds were administered as a mixture, GSH depletion was found to be dose additive. The results of the mixture study show that GSH depletion may be a useful effect-equivalent for the risk assessment of mixtures of α,β-unsaturated esters. To get more insight in the underlying mechanisms of GSH depletion, the metabolism of two esters was investigated in greater detail. One of them, allyl methacrylate was found to be metabolized to acrolein. This metabolic pathway can explain the high potency of allyl methacrylate to deplete GSH despite its low intrinsic chemical reactivity.
INTRODUCTION

Risk assessment of mixtures is recognized as an important issue in environmental and human toxicology (1, 2) because most exposures are complex in nature. From air or water samples, typically hundreds of xenobiotic chemicals are isolated (1, 3). For many compounds in such complex mixtures, the chemical structure remains unknown. On the other hand, many commercially available substances are in fact complex mixtures (e.g. petroleum products or polymer products like paints or adhesives). They consist of mixtures of structurally related chemicals with different physico-chemical and toxicological properties. Often, only a tentative characterization is available for such mixtures, like the range of boiling point or the average chain length. To provide a scientific framework for effect assessment of mixtures, sumparameters for toxic effects of similar acting compounds have been proposed. A well known example of this strategy is the use of toxic equivalent factors (TEF) for the assessment of the effect of dioxin-like compounds (4, 5). Other effect equivalents are the inhibition percentage of acetylcholinesterase, which is used for pesticide exposure assessment (6) or the critical body burden which is used in aquatic toxicology as a measure for narcosis potency (7, 8). Effect equivalents are generally based on internal target concentrations. To link a sumparameter with an external exposure concentration, a physiologically based pharmacokinetic (PBPK) model can be used, which can account for differences in kinetics of each compound in a mixture. This combination was used by Verhaar et al. (2, 9) for the risk assessment of jet fuel in a workplace exposure scenario.

Recently, Frederick et al. (10) established a PBPK model that uses time integrated glutathione (GSH) depletion as a effect equivalent for toxic effects of ethyl acrylate, an α,β-unsaturated ester. Because many other reactive chemicals are known to cause GSH depletion, and because GSH depletion is well established as a cytotoxic effect it may be worthwhile to test whether GSH depletion can be used as an effect equivalent for mixtures of reactive chemicals. α,β-unsaturated esters are used in various combinations in polymer chemistry (11), and a mixture toxicity model for these compounds may improve their risk assessment. Many of these chemicals are Michael acceptors and react easily with biological thiols to form a covalent bond (12-16). Metabolism of α,β-unsaturated esters was shown to occur by hydrolysis and by conjugation with GSH (10, 11, 17-20). Toxicity tests in rodents and fish showed that toxic effects can be organ specific (10, 15, 19-24) and that differences in chemical reactivity alone seems not enough to explain differences in potency (25, 26).

In the present study, we used primary rat hepatocytes to measure GSH depletion caused by 11 individual α,β-unsaturated esters and two mixtures. The objective of this study is to
test, if effect data from individual chemicals could be used to predict the effects of mixtures and if GSH depletion might serve as an effect equivalent for these compounds in further studies. Hepatocytes were chosen as a model system, because the liver is the primary producer of GSH and because hepatocytes contain many enzymes that are related to GSH metabolism (27). It should be noted however, that because of their high metabolic activity and their intrinsic high GSH concentration, hepatocytes may be more resistant to GSH depletion than cells from target organs, like e.g. gills gastro-intestinal tract or respiratory tract. The effect of two mixtures was tested for dose additivity (28) and for response additivity (29).

The concept of an effect equivalent on the basis of GSH depletion might be applicable across different mechanisms. Based on the experimental EC_{50} data in hepatocytes, we wanted to get some more insight in the underlying mechanistic aspects of GSH depletion. In particular, the low EC_{50} of allyl methacrylate, which is in contrast to the low chemical reactivity of this compound suggested that different modes of action were present within our test set. To get an idea about alternative mechanisms of GSH depletion, we compared allyl methacrylate with the strongest Michael acceptor in the set, diethyl fumarate using a number of biochemical parameters. Dose response curves for cellular GSH levels were compared with the concentration of a lipid peroxidation marker (malondialdehyde) and with the production of acrolein and acetaldehyde, both being probable metabolites of allyl methacrylate and diethyl fumarate, respectively.

**MATERIALS AND METHODS**

*Chemicals*

The following chemicals were used: o-phthalaldehyde (OPA), purchased from Arcos (‘s Hertogenbosch, The Netherlands), reduced glutathione (GSH), pentafluorobenzyl-hydroxylamine (PFB), acetaldehyde, acrolein, 1,1,3,3-tetraethoxypropane, NaWO_{4}, 3,4-dichlorotoluene, ethyl acrylate, 2-hydroxyethyl acrylate, isobutyl acrylate, diethyl fumarate, allyl methacrylate, benzyl methacrylate, 2-ethoxyethyl methacrylate, tetrahydrofurfuryl methacrylate, isobutyl methacrylate and methyl methacrylate from Fluka Sigma-Aldrich (Zwijndrecht, The Netherlands) and isopropyl methacrylate from Pfalz&Bauer (Waterbury, CT).
Animals

Male Wistar(U::Wu) rats were fed *ad libitum* with a grain-based diet and had free access to drinking water.

Cell culture and exposure

Hepatocytes were isolated by whole liver perfusion using the two step collagenase technique as described by Seglen (30). The cells were incubated at 37°C in air-tight 50 ml tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) at a density of 8*10^5 cells/ml. Initial culture medium consisted of Williams’ E medium, supplemented with 0.1 M HEPES, 26 mM NaHCO₃, 2 mM L-glutamine, 1 µM insulin, 10 µM hydrocortisone, 70 µM gentamycine and 3 % newborn calf serum (NCS). After 3 hours a cell monolayer was formed and initial culture medium was removed and replaced by culture medium without NCS. Non-attached and dead cells were thereby washed off. Exposure of hepatocytes started 24 hours after isolation. The old medium was removed and replaced by culture medium without NCS in which the tested chemical had been dissolved. Geometric dilution series with a factor of two and five concentration steps were used. The hepatocytes were harvested after 4 hours of exposure. Culture medium was removed and an aliquot was used to determine LDH activity. For some chemicals, samples of culture medium were frozen and stored at -20°C for subsequent analysis of aldehyde production. The cell monolayer was suspended with a TritonX-100 solution (0.5%) and homogenized by vortexing the cell-suspension for 10 min. Aliquots of the homogenate were used to determine LDH activity, reduced GSH concentration and protein content.

Protein content

Protein content of the homogenate was measured according to Bradford (31).

Cell viability

The viability of the cells was assessed by lactate dehydrogenase (LDH) leakage. The percentage of LDH leakage was determined by comparing LDH activity in the culture medium with total LDH activity in the culture flask (medium + cell homogenate). LDH activity was measured according to Bergmeyer et al.(32).

GSH measurement

Cell homogenates were precipitated with 5% trichloroacetic acid, centrifuged at 10000 g and the supernatant was kept frozen at -20°C until analysis (max. 48 hours). The supernatant was diluted 1:10 with destilled water and reduced glutathione was measured by RP-HPLC
with post-column OPA derivatisation as described by Fujita et al. (33) and modified by Freidig et al. (13, 25). Cellular GSH concentrations were calculated as nmol/mg protein.

**Analysis of aldehydes in culture medium**

Three aldehyde products from metabolism of unsaturated esters as well as from endogenous metabolism of the hepatocytes were analyzed in the culture medium of exposed cells. Extraction of the medium, derivatisation with PFB and analysis of the aldehydes on a gas chromatograph with electron capture detector was performed according to DeZwart et al. (34), with the following minor adjustments: PFB-derivates were extracted from aqueous solutions with cyclohexane using 3,4-trichlorotoluene as internal standard. Standard solutions of acrolein and acetaldehyde were prepared in water. Standard aqueous solutions of malondialdehyde were prepared from 1,1,3,3-tetraethoxypropane according to DeZwart (34). Aqueous standards were derivatized along with the samples and tentative identification and quantification of the three aldehydes in the culture medium were achieved using retention times and responses of the pure compounds.

**Calculations**

Two dose response curves for GSH depletion and LDH leakage (each concentration in duplicate) were measured for each tested ester using hepatocytes from different isolations. For GSH depletion, EC50 values and slopes were fitted from each dose-response curve using the sigmoidal dose-response algorithm of Prism software (GraphPad Software, San Diego, CA) given in equation 1 with (C) being the nominal exposure concentration. Because many compounds caused only a partial LDH leakage at the highest tested concentration, LOECs (p<0.05, one tailed) were used to assess changes in cell viability.

\[
GSH(\% \text{ of control}) = \frac{100}{1 + 10^{-\text{slope}(\log(\text{EC}_{50})-\log(C))}}
\]

(EQ 1)

**Mixture toxicity**

Two different models, dose addition and independent response addition were used to test for a possible additivity of GSH depletion. Two equitoxic mixtures of six esters were prepared based on their individual EC50 for GSH depletion. Concentrations of the compounds were transformed to toxic units (TU) according to equation 2 and each of the six compounds was added at equal TU(i) (equitoxic mixtures) to the mixture. The potency of a mixture is thereby given by the \(\Sigma\)TU, defined in equation 3 (28, 35).
\[ TU(i) = \frac{\text{conc}(i)}{EC_{50}(i)} \]  

(EQ 2)

\[ \sum_{i=1}^{n} TU(i) = \sum_{i=1}^{n} TU(i) \]  

(EQ 3)

Each mixture was tested in a geometric dilution series starting with a \( \Sigma TU \) of 6. Dose response curves were recorded for GSH depletion and LDH leakage as described for the individual compounds. If a mixture is dose additive, a \( \Sigma TU \) of 1 is expected to cause 50% GSH depletion. Furthermore, a model for response additivity was applied to the experimental data, to compare the dose addition model with. The probabilistic addition model describing independent joint action (29, 36) was chosen to describe a mixture situation where each compound would deplete GSH by an independent pathway. Percentages of GSH depletion, caused by individual compounds below their EC_{50} were estimated from fitted sigmoidal dose response curves and used as effect probabilities, \( P_i \). To calculate the effect probability of the mixture, \( P_{mix} \), the \( P_i \)'s of each compound in the mixture were added up according to equation 4.

\[ P_{mix} = 1 - [(1 - P_1)(1 - P_2)\ldots(1 - P_n)] \]  

(EQ 4)

RESULTS

**Single chemical tests**

A series of \( \alpha,\beta \)-unsaturated esters, which were known to react chemically with GSH (13) were tested for their potency of inducing GSH depletion in-vitro. EC_{50} values for GSH depletion of single chemicals after 4 hours are presented in table 1. They span a range from 0.14 mM for allyl methacrylate to 7.42 mM for methyl methacrylate. Slope factors for the dose response curves (table 1) were found to vary between 0.8 and 4.0. Cell viability, as determined by LDH leakage, was not affected by a concentration causing 50% depletion of GSH, except for two compounds (allyl- and isobutyl methacrylate). However, at higher exposure concentrations more esters were found to decrease the cell viability during the 4 hour assay.

**Mixture tests**

Two mixtures, each containing six esters, were tested under identical conditions as the single substances. Their compositions is given in table 1. Dose - effect relations for both mixtures are presented in figure 1 a and b. The \( \Sigma TU \) was used as dose - equivalent on the x-
Table 1: \(\alpha,\beta\)-unsaturated esters tested for induction of GSH depletion in hepatocytes. \(EC_{50}\) and slope factors were fitted using a sigmoidal dose-response curve (equation 1).

<table>
<thead>
<tr>
<th>Substance</th>
<th>(k_{\text{GSH}})</th>
<th>GSH depl</th>
<th>LDH-leakage</th>
<th>Included in mixture:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{[M}^{-1}\text{min}^{-1}])</td>
<td>(\text{EC}_{50})</td>
<td>slope</td>
<td>LOEC</td>
</tr>
<tr>
<td>Allyl methacrylate</td>
<td>0.51</td>
<td>0.14</td>
<td>0.02</td>
<td>1.5</td>
</tr>
<tr>
<td>2-Hydroxyethyl acrylate</td>
<td>50.00</td>
<td>0.28</td>
<td>0.18</td>
<td>2.2</td>
</tr>
<tr>
<td>Diethyl fumarate</td>
<td>112.00</td>
<td>1.44</td>
<td>0.65</td>
<td>2.0</td>
</tr>
<tr>
<td>Isobutyl acrylate</td>
<td>42.00</td>
<td>1.70</td>
<td>0.38</td>
<td>3.4</td>
</tr>
<tr>
<td>Ethyl acrylate</td>
<td>40.00</td>
<td>1.85</td>
<td>1.21</td>
<td>2.9</td>
</tr>
<tr>
<td>Benzyl methacrylate</td>
<td>0.33</td>
<td>2.36</td>
<td>0.49</td>
<td>0.8</td>
</tr>
<tr>
<td>2-Ethoxethyl methacrylate</td>
<td>0.25</td>
<td>2.73</td>
<td>1.11</td>
<td>2.0</td>
</tr>
<tr>
<td>Tetrahydrofurfuryl methacrylate</td>
<td>0.30</td>
<td>3.05</td>
<td>0.24</td>
<td>1.7</td>
</tr>
<tr>
<td>Isobutyl methacrylate</td>
<td>0.19</td>
<td>5.00</td>
<td>1.93</td>
<td>1.3</td>
</tr>
<tr>
<td>Isopropyl methacrylate</td>
<td>0.00</td>
<td>5.35</td>
<td>1.08</td>
<td>3.4</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>0.20</td>
<td>7.42</td>
<td>1.21</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Italic SE-values were determined from one dose-resp. curve
axis. Mixture 1 was found to be dose additive for GSH depletion ($EC_{50} = 1.00 \, \Sigma TU$, 95% confidence interval = 0.49-2.01 $\Sigma TU$), whereas mixture 2 was more potent than predicted by dose additivity ($EC_{50} = 0.58 \, \Sigma TU$, 95% confidence interval = 0.39-0.86 $\Sigma TU$). The model for response addition underestimated the potency for both mixtures, as can be seen in figure 1. Cell viability was only affected at the highest tested concentration for both mixtures. LOECs for LDH-leakage were 5.3 $\Sigma TU$ for mix 1 and 6.6 $\Sigma TU$ for mix 2, respectively.

Comparison of two esters

Allyl methacrylate (AMA) and diethyl fumarate (DF) differ in chemical reactivity toward GSH, DF being 200 times more reactive than AMA (table 1). However, AMA was found to be 10 times more potent than DF depleting GSH in vitro. We chose to investigate the effect of these two compounds on primary rat hepatocytes in greater detail, to see whether different modes of action could be identified that cause GSH depletion. Figure 2 a and b show the dose dependent GSH depletion and cell viability of hepatocytes exposed to both substances. For AMA, loss of cell integrity measured by LDH leakage was parallel with a decrease in cellular GSH. Loss of GSH might therefore be caused by an increasing damage of cell membranes. The lowest exposure concentration of DF caused a GSH induction with a factor 2.5 above levels of unexposed hepatocytes (32 ± 7 nmol/mg protein). Increasing DF concentrations depleted GSH but there was no effect on cell viability.

Three aldehydes were determined in the culture media of exposed cells. Malondialdehyde (MAD) was used as a marker of lipid peroxidation in AMA and DF exposed cells, whereas acetaldehyde and acrolein were used as an indicator for the metabolism pathways of DF and AMA, respectively. Figure 2 c shows that in AMA exposed cultures, MDA concentration increases together with LDH leakage. For DF exposed cells no increase of MDA-production was detected for the tested concentrations. Acrolein, which was suspected to be formed by alcohol dehydrogenase after hydrolysis of AMA, was detected above background levels at the two highest exposure concentrations (Figure 2 d). Because cell integrity was partially lost at these two concentrations, acrolein could have been formed by cytosolic enzymes that leaked into the medium. In the medium of DF exposed cells, acetaldehyde concentrations up to 50 $\mu$M were detected (figure 2 e). This oxidative metabolic product of DF was most probably formed in the cells because hepatocytes exposed to DF showed no sign of membrane damage or leakage.
Figure 2 a-e: Dose-effect curves for allyl methacrylate and diethyl fumarate. Both compounds induce GSH depletion (2 a), but vary in LDH leakage (2 b), lipid peroxidation as measured by malondialdehyde production (2 c) and formation of the oxidized hydrolysis product (2 d and e).
DISCUSSION

GSH depletion by α,β-unsaturated esters

Chemicals with α,β unsaturated carbonyl groups are known to cause GSH depletion in rat livers (12). Accordingly, all esters tested in the present investigation depleted GSH in primary rat hepatocytes. Their potency to deplete GSH was only weakly correlated with their chemical reactivity with GSH (table 1). Two compounds (allyl methacrylate and 2-hydroxyethyl acrylate) were substantially more potent than the other compounds. EC$_{50}$ for the other nine esters differ only by a factor of 5 although their chemical reactivity, given in table 1, differs by more than 2 orders of magnitude. It can be concluded that chemical reactivity with GSH is not the predominant mechanism that leads to GSH depletion in isolated hepatocytes. Moreover, because most esters share a relatively high effect concentration (1-10 mM) and because they are easily hydrolyzed by hepatic carboxylases (10), the metabolites can be expected to play an important role. There are a number of processes apart from pure chemical reactivity, that can govern the extent of depletion of GSH in the hepatocytes.

First, fast enzyme-catalyzed conjugation of the less reactive methacrylates by glutathione transferases (GST) might be responsible for the small difference in observed EC$_{50}$. Enzymatic conjugation by GST was described for ethyl acrylate in rat liver (16). However, no information is available about the selectivity of GST towards acrylates and methacrylates. Second, fast enzymatic hydrolysis of the esters can decrease the extent of GSH-depletion. In vivo inhibition of hydrolysis with tri-orthotolyl phosphate caused an increase of thioether production in rats exposed to methyl acrylate and methyl methacrylate (37) and increased the extent of depletion of non-protein thiols in various tissues of rats exposed to ethyl- and methyl acrylate (38). Third, metabolites resulting from hydrolysis that form or accumulate in cells can have an impact on the GSH level. This seems to be the case for allyl methacrylate. Allyl alcohol, a possible product of AMA metabolism was found to produce GSH depletion in vitro (39-41) and in vivo in rats (42, 43). This was explained by oxidation of allyl alcohol to acrolein by alcohol dehydrogenase. Acrolein, the reactive metabolite, was shown to be approximately 100 times more reactive with GSH than any of the esters tested in this work (25). In the present investigation, cells exposed to allyl methacrylate produced MDA, an indicator of lipid peroxidation. MDA was also found in allyl alcohol exposed cells (44-47). Furthermore, acrolein was detected in the culture medium. Neither acrolein nor MDA were found in DF exposed cells. We therefore suggest, that the low EC$_{50}$ for GSH depletion of allyl methacrylate is caused by its metabolite acrolein which is formed through hydrolysis and subsequent oxidation. This is in agreement with findings about the toxicity
of other esters of allyl alcohol (48, 49). The induction of GSH, observed at the lowest exposure concentration of DF (figure 2 a) may be caused by the “electrophilic counterattack” as described by Talalay and co-workers (50-53). They reported that many chemicals with unsaturated carboxyl and carbonyl groups were able to co-induce a battery of phase 2 enzymes in hepatoma cells at very low concentrations.

Formation of acid equivalents by ester-hydrolysis in the cell could be another mechanism by which GSH is depleted. Lowering of intracellular pH from 7.35 to approx. 7.05 was found to cause GSH depletion in hepatocarcinoma cells (54). Acrylic acid itself was furthermore found to induce membrane permeability transitions in isolated mitochondria (55). This, in turn could lead to an energy deprivation and subsequently to a loss of reduced glutathione.

**GSH depletion of mixtures**

In the mixture experiment it was clearly shown, that GSH depletion of α,β-unsaturated esters is an additive effect in hepatocytes. Mixtures of compounds, diluted to 10-16% of their individual effect concentration induced a comparable GSH depletion as one compound at 100% of its EC₅₀. Two additivity models, dose addition and response addition were used to predict the effect of mixtures of α,β-unsaturated esters. Both mixtures were well predicted with dose addition. Response addition, which should be able to predict the joint effect of compounds with independent modes of action (29) underestimated the potency of both mixtures. It may be, however that the effects at low concentrations were underestimated with the current test protocol using a dilution series of a factor two.

**Use of GSH depletion as effect equivalent in risk assessment**

The advantage of an effect equivalent above a target concentration is, that it allows to aggregate the effect of several compounds. This study clearly shows, that GSH depletion in hepatocytes can be used as additive effect-equivalent for the toxic effects of acrylic and methacrylic acid esters in primary rat hepatocytes. The use of GSH depletion data from individual esters made it possible to predict the potency of a complex mixture. For a translation to in-vivo effects, information about the toxicokinetics of each compound in the mixture is needed to define their target tissue concentrations. Verhaar et al. (9) showed, how an existing PBPK model can be adapted to predict target tissue concentrations of compounds of jet fuel mixture with varying physico-chemical properties. Using such a distribution model together with a toxicodynamic model for GSH depletion (10, 56), it may be possible to predict the GSH depletion due to a complex mixture of e.g. acrylic monomeres.
REFERENCES


