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EFFICACY OF PARACETAMOL-ESTERIFIED METHIONINE VERSUS CYSTEINE OR METHIONINE ON PARACETAMOL-INDUCED HEPATIC GSH DEPLETION AND PLASMA ALAT LEVEL IN MICE

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Abstract—The effect of paracetamol-*N*-acetyl-DL-methionate (PAM) in preventing paracetamol-induced hepatic glutathione (GSH) depletion and hepatic cell damage assessed by plasma ALAT level, was compared to those of concomitantly administered paracetamol and *N*-acetyl-L-cysteine (NAC) or *N*-acetyl-DL-methionine (NAM) and paracetamol 400 mg/kg (P) alone. PAM, NAM and NAC reduced hepatic GSH depletion compared to P. The concomitant administration of GSH precursors in either form apparently maintained hepatic cell integrity as evaluated by plasma ALAT compared to predose and 16 hr control measurements. No statistically significant difference between PAM, NAM and NAC was observed. In group P a statistically significant, but transitory, rise in plasma ALAT level following dosage was seen. NAC was more effective than PAM and NAM in the prevention of GSH depletion 1 hr after dosing but was less effective in promoting *de novo* GSH synthesis towards 16 hr. There was no statistically significant difference between PAM and NAM with respect to effect on GSH depletion or hepatic cell integrity. PAM and NAM increased the GSH level significantly above control level 16 hr after dosing. PAM is rapidly cleaved to paracetamol and methionine following dosage as shown by the observed plasma paracetamol level. PAM compares favourably in hepatoprophylactic effect, to concomitant administration of equimolar doses of free *N*-acetyl-DL-methionine added to the paracetamol formulation.

In contrast to the safety of the commonly used analgesic paracetamol (acetaminophen, USA) used in therapeutic doses stands the serious hepatotoxicity associated with overdosage [1]. The hepatotoxicity of paracetamol is believed to be mediated by a very reactive arylating metabolite formed by the cytochrome P-450-dependent mixed function oxidase system [2, 3]. It is generally assumed that *N*-acetyl-*p*-benzoquinoneimine (NAPQI) is the reactive metabolite of paracetamol as it covalently binds to protein and reacts with hepatic glutathione (GSH) to form a GSH conjugate [4]. GSH precursors such as methionine, cysteine and *N*-acetyl-cysteine have been demonstrated to be effective in preventing experimental paracetamol-induced hepatotoxicity in animals and in the clinical management of severe paracetamol poisoning [5].

Prophylactic addition of a GSH precursor to the paracetamol formulation has been proposed as a means of enhancing the detoxifying capacity of the liver in cases of paracetamol overdosage [6]. SUR 2647 is the *N*-acetyl-DL-methionine ester of paracetamol. The aim of the present study was to compare the efficacies of *N*-acetyl-L-cysteine or *N*-acetyl-DL-methionine added to the paracetamol formulation versus SUR 2647 with respect to hepatic GSH depletion and cell damage assessed by plasma ALAT (GPT) level in mice.

MATERIALS AND METHODS

Animals. Male Bom:NMRI mice of 35–50 g, age > 20 days to allow a stable GSH level [7], generally kept five in each cage and familiarized with laboratory surroundings for at least 2 weeks were used. Lighting in animal quarters was 12:12 dark:light cycles, humidity 50–55% and temperature 20–21°. Commercial pelleted rat diet (EWOS, Södertälje, Sweden) and tap water was offered *ad libitum* until commencement of the experiment, when only water was allowed.

Drugs. Paracetamol (*N*-acetyl-*p*-aminophenol), SUR 2647 (paracetamol-methionate, *p*-acetamidophenyl 2-carbamoyl, 4-methylthiobutanoate) were gifts from Sterling-Winthrop, Surrey, U.K. *N*-acetyl-L-cysteine and *N*-acetyl-DL-methionine were bought from Sigma Chem. Co., St. Louis, MO, U.S.A. Drugs were suspended in 1% w/v aqueous methylcellulose, grade 15, solution (NMD, Oslo, Norway) freshly made within 1 hr prior to each experiment and continuously stirred until administration by gastric tube to the animals. Treatment groups receiving paracetamol (P), paracetamol plus *N*-acetyl-L-cysteine (NAC) or *N*-acetyl-DL-methionine (NAM) and SUR 2647 (PAM) were established. A total of 120 mice were used. Included were two groups of five methylcellulose treated mice

sacrificed at 0 hr and 16 hr as controls (C) and four mice sacrificed at 1 hr, three mice at 4 hr and three mice at 8 hr for the assessment of plasma paracetamol level after PAM dosage. The dose of paracetamol administered was 400 mg/kg in all groups. The doses of *N*-acetyl-L-cysteine and *N*-acetyl-DL-methionine, free or esterified to paracetamol in the SUR 2647 formulation, were equimolar to the administered paracetamol dose.

Sampling procedure. Blood samples were obtained by heart puncture during methoxyflurane (Penthrane®, Abbott Lab. Ltd., Kent, U.K.) inhalation anaesthesia and transferred to glass tubes containing 65 U.S.P. units of sodium heparine (Venoject®, Terumo Co., Tokyo, Japan). After centrifugation with 3000 rpm at 4° for 8 min in a Beckman TJ-6R cooled centrifuge (Beckman Instruments Inc., Fullerton, CA, U.S.A.), plasma was frozen at −20° for one week awaiting ALAT (GPT) or paracetamol analysis. Storage under these conditions has been shown not to reduce ALAT activity [8]. After sacrifice by cervical dislocation the animals were autopsied and the liver frozen at −20° pending reduced glutathione (GSH) analysis.

GSH assay. GSH was assayed as reduced non-protein sulfhydryl using a slightly modified method of Ellman [9] with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), Sigma). After thawing, 1 g of liver was homogenized in 9 ml cold 0.1 M sodium phosphate buffer (pH 7.2). Two milliliters of the homogenate was thoroughly mixed with 2 ml of 4% 5-sulfosalicylic acid (Sigma) and centrifuged (Wifug Type X1, Stockholm, Sweden) at 4000 rpm for 10 min at 18°. Half-milliliter aliquots of these supernatants were added to 4.5 ml 0.1 mM Ellman's reagent (dissolved in 0.1 M sodium phosphate buffer, pH 8.0) and used for GSH assay. Unless otherwise stated all solutions were kept ice-cold. The absorbance of each sample was determined by a Beckman Model 35 spectrophotometer at 412 nM.

ALAT assay. ALAT was determined by a Gemsaec Fast Analyzer with Autoloader (Electro-Nucleonics Inc., Fairfield, NJ, U.S.A.). Using the principle of Karmen *et al.* [10, 11] with standardized optimal reagents; UV-GPT Working Solution, α-ketoglutaric acid (J. T. Baker Chem. B.V., Deventer, Holland) and Seronorm™ (Nyegaard & Co. A/S, Oslo, Norway).

Paracetamol assay. Plasma paracetamol concentrations were assessed using a high pressure liquid chromatograph (Waters Ass. Inc., Milford, MA, U.S.A.) operating at 254 nm with a U6K loop injection system [12]. A flow rate of 1.5 ml/min was employed. The retention times for the internal standard metacetamol (*N*-acetyl-*m*-aminophenol, Sterling-Winthrop) and paracetamol were 11.1 and 6.4 min, respectively.

Statistical analysis. A two-way analysis of variance (BMDP2V-statistical package, UCLA, CA, U.S.A.) test with time of sacrifice as one factor and treatment as the other. The treatments are compared with respect to time average (i.e. the average value over all the sacrifice times), and with respect to time course (i.e. the way the values change with time). Statistically, the latter comparison corresponds to a test of interaction between the treatment factor and the time factor. A logarithmic transformation of ALAT data was made to achieve a more symmetric distribution before statistical analysis [13]. A one-way analysis of variance (BMDP2V) was used for analysis of ALAT and GSH values from the test groups and the control group at 16 hr.

RESULTS

Plasma paracetamol concentrations following PAM dosage

To demonstrate the bioavailability of free paracetamol after the administration of PAM the plasma paracetamol concentration was measured 1, 4 and 8 hr after dosage. Figure 1 demonstrates that PAM is apparently rapidly cleaved to paracetamol and methionine and that the effect on the liver is not mediated by the intact paracetamol-methionate complex.

Comparison of the effects of treatment groups P, NAC, NAM and PAM on hepatic GSH content

Figure 2 shows the time development of the hepatic GSH depletion after peroral administration of the test substances. Hepatic GSH content compared to pre-dose level following the administration of paracetamol 400 mg/kg (P) was by 1 hr 26%, 4 hr 20% and by 8 hr 20%. GSH content in the NAC group was by 1 hr 75%, 4 hr 81% and by 8 hr 79%. By 1 hr 42%, by 4 hr 78% and by 8 hr 66% of pre-dose hepatic GSH content was seen in the NAM group. GSH content was by 1 hr 54%, by 4 hr 56% and by 8 hr 62% in the PAM group. By 16 hr GSH content was 77% in the C group compared to the pre-dose level, indicating a normal decline in hepatic GSH over the day in mice withheld from food.

The analysis of variance showed that the time average of group P was significantly lower than that of the other groups (P values <0.001). There was no statistically significant difference between the time averages of groups NAC, NAM and PAM (P = 0.55). There was, however, a significant difference between the time courses of groups NAC, NAM and PAM (P = 0.05). From Fig. 2 it can be seen that this is caused by an increasing level of hepatic GSH

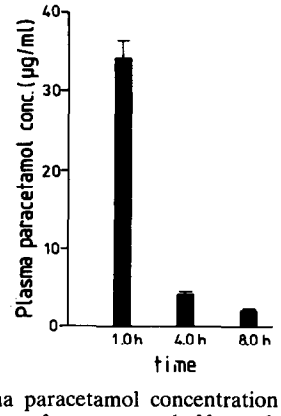

Fig. 1. Plasma paracetamol concentration is shown following dosage of paracetamol *N*-acetyl-DL-methionate (PAM) equivalent to 400 mg paracetamol/kg to male Bom:NMRI mice. The results are shown as means of 3–4 mice ± 1 S.D.

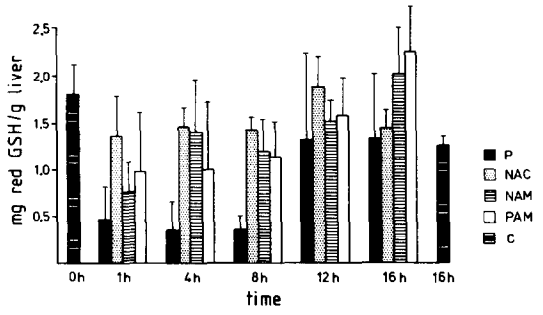

Fig. 2. Time development of hepatic GSH following administration of paracetamol (P), equimolar doses of paracetamol and *N*-acetyl-L-cysteine (NAC) or *N*-acetyl-DL-methionine (NAM) and paracetamol *N*-acetyl-DL-methionate (PAM). 400 mg/kg paracetamol in all groups. Methylcellulose treated animals were controls (C) at 0 and 16 hr. Results are shown as means of 3–5 mice ± 1 S.D.

towards the end of the experimental period for groups NAM and PAM compared to the rather constant hepatic GSH level of group NAC. To establish the effect of the GSH precursors on the GSH synthesis the treatment groups were compared with a control group (C) 16 hr after dosing. There was a statistically significant difference between the five groups ($P < 0.025$). From Fig. 2 one sees that this is due to the higher GSH level in the NAM and PAM groups.

Statistical analysis showed no significant difference between group NAM and group PAM with respect to time average ($P = 0.94$) and time course ($P = 0.68$). A 90% confidence interval was made for the average difference (over time) between the two groups. The confidence interval was -0.01 ± 0.25 (-0.26–0.24). This means that a "true" difference of up to about 0.25 mg red. GSH/g liver could be consistent with the observed results.

ALAT assessment of the effects of treatment groups P, NAM, NAC and PAM on the liver

The time average of group P was significantly higher than those of treatment groups NAC, NAM and PAM (all $P < 0.001$). However, it is of interest to note the apparent latency time evolving from 0 to

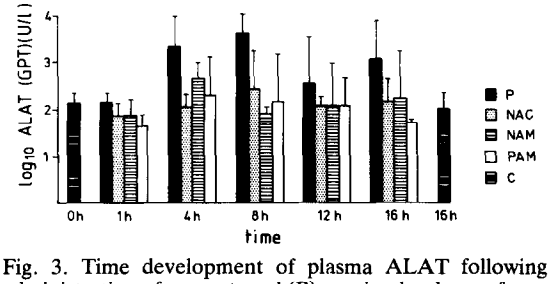

Fig. 3. Time development of plasma ALAT following administration of paracetamol (P), equimolar doses of paracetamol and *N*-acetyl-L-cysteine (NAC) or *N*-acetyl-DL-methionine (NAM) and paracetamol *N*-acetyl-DL-methionate (PAM). 400 mg/kg paracetamol in all groups. Methylcellulose treated animals were controls (C) at 0 and 16 hr. Results are shown as $\log_{10}$ means of 3–5 mice ± 1 S.D.

Table 1. The survival rates of mice following administration of 400 mg/kg paracetamol (P), equimolar doses of paracetamol and *N*-acetyl-DL-cysteine (NAC) or *N*-acetyl-DL-methionine (NAM) and paracetamol-*N*-acetyl-DL-methionate (PAM)

0	1 hr	4 hr	8 hr	12 hr	16 hr
P	5/5	5/5	5/5	3*/5	5/5
NAC	5/5	5/5	5/5	5/5	5/5
NAM	5/5	5/5	5/5	5/5	4*/5
PAM	5/5	5/5	5/5	5/5	5/5

The predetermined number of animals to be sacrificed per time point was five.

* Spontaneous deaths occurred irrespective of medication 7–8 hr after dosing.

1 hr before the elevation of the ALAT level in the P group seen at 4 hr, while a rapid GSH depletion was seen as soon as 1 hr after dosing. No statistically significant difference was found between the time averages of groups NAC, NAM and PAM ($P = 0.60$). No significant difference was found between the groups with respect to time course ($P = 0.67$). The time development of the ALAT values of the treatment groups is shown in Fig. 3. At 16 hr after dosing there was no statistically significant difference between group P and groups NAC, NAM, PAM and C with respect to ALAT values.

Table 1 shows the survival rate of mice within each treatment group per time point of sacrifice. All but three animals survived the trial period irrespective of treatment group. The loss of three animals may have induced a slight bias in the comparisons.

DISCUSSION

Data reviewed by Jollow [14] indicate that the reactive hepatotoxic metabolite of paracetamol is formed in the liver after both non-toxic and toxic doses of the drug. The reactive metabolite is preferentially detoxified by conjugation with hepatic GSH. To maintain cellular integrity it is imperative that the hepatic cells do not exhaust their GSH content [15].

The concept of two GSH pools in the liver has been proposed [16]. With freshly isolated rat hepatocytes a mitochondrial GSH pool with a long half-life (30 hr) and a cytoplasmic pool with a short half-life (2 hr) have more recently been observed [17]. The mitochondrial pool may represent the stable pool of GSH observed *in vivo* [18]. The labile cytoplasmic GSH pool is assumed to be principal in xenobiotic conjugation and is readily mobilized. The steady-state hepatocellular GSH concentration is maintained through GSH synthesis, inter-organ GSH export, the rate of utilization through interconversion of GSH and GSH disulfide and GSH conjugation with exogenous and endogenous compounds [19]. Hepatic GSH synthesis is largely limited by the intracellular availability of its main precursor L-cysteine. In addition, the liver has the unique ability to convert methionine to cysteine [20].

These facts offer the possibility of effective therapeutic measures in case of paracetamol overdosage.

Several reports have demonstrated the ability of GSH precursors such as cysteine and methionine to prevent paracetamol-induced hepatic damage in experimental animals if administered within a reasonable time after the toxic insult [21–24]. In 1974 McLean [6] proposed to include a GSH precursor into the paracetamol formulation to prevent the hepatotoxic effects of paracetamol overdosage including the therapeutic difficulties associated with the necessity of an early treatment. *N*-acetyl-DL-methionine is a cheap and palatable methionine analog [25] which, esterified to paracetamol, gives a tasteless powder contrasting with the bitter taste of free paracetamol.

In our study the addition of *N*-acetyl-L-cysteine was found to have a faster onset of action than the inclusion of methionine in either form, with respect to counteracting hepatic GSH depletion. The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine and L-glycine is a two-step enzymatic reaction [20]. The sequential conversion of methionine to GSH through the cystathione pathway involves the synthesis of cysteine which thereafter is converted to GSH via the two-step enzymatic reaction [25]. The better result with *N*-acetyl-L-cysteine within the early phase of the trial may reflect cysteine as being a more readily (i.e. faster) convertible GSH-precursor. It is also of interest to note the difference in hepatic GSH content between the groups by 16 hr. This difference suggests that there is a time-dependent difference between cysteine and methionine in the way they prevent paracetamol-induced hepatic GSH depletion. This observation also lends support to the view that the mechanism of action of GSH precursors indeed is the facilitation of *de novo* GSH synthesis as suggested in mice [24] and rats [26]. On the other hand, *N*-acetyl-cysteine has been reported to inhibit gastric emptying in mice and thus afford protection from concomitant perorally administered paracetamol [27]. Neither this claim nor the hypothesis that *N*-acetyl-L-cysteine protects due to drug interaction resulting in slowed gastric emptying, potentiation of the sulphate pathway or marked stimulation of conjugative metabolism is substantiated by a recent extensive study by Corcoran *et al.* [28].

N-acetyl-L-cysteine, deacetylating rapidly to L-cysteine [29, 30], was chosen as a positive control because of its known preference as a GSH substrate. This use of a single isomer will offer advantages compared to the racemic mixture of *N*-acetyl-DL-methionine as most biochemical processes are stereoselective [31]. Despite earlier reports to the contrary more recent findings does not confirm that D- and L-isomers of methionine are equally utilized in man and experimental animals [25]. Furthermore, it has been shown *in vitro* that the presence of L-methionine exerts an inhibitory effect of the hydrolysis of *N*-acetyl-D-methionine in racemic mixtures [32]. Also the unavailability of *N*-acetyl-D-methionine, in contrast to *N*-acetyl-L-methionine, as a source of dietary methionine has been demonstrated in rats [33]. These factors may influence the effectivity of *N*-acetyl-DL-methionine as a GSH precursor and may explain the initial difference in effect compared to *N*-acetyl-L-cysteine.

However, the GSH repleting characteristics of *N*-acetyl-DL-methionine plus paracetamol (NAM) and paracetamol-*N*-acetyl-DL-methionate (PAM) with respect to time course and time average was not statistically different. Hence, it can be inferred that the esterification of *N*-acetyl-methionine to paracetamol apparently does not limit the absorption of methionine compared to free methionine. The observation of no apparent loss of *de novo* GSH synthesis following PAM compared to NAM dosage, and the plasma paracetamol profile after PAM dosage, indicate that PAM is rapidly cleaved to its paracetamol and methionine constituents in the gastrointestinal tract or by serum enzymes.

Plasma ALAT levels may rise considerably due to leakage from the cytoplasm of hepatocytes in case of acute chemically induced toxicity. However, even the administration of 1000 mg/kg of paracetamol perorally to rats gives a transitory elevation of ALAT culminating 48–72 hr after dosing [34]. A correlation between serum ALAT levels and hepatic necrosis after p.o. paracetamol administration has been demonstrated [35]. The administration of 400 mg/kg paracetamol causes a significant transitory elevation of plasma ALAT starting 4 hr after dosing, denoting a debut of hepatic cell damage which apparently subsides at 16 hr.

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