Toxicology, 52 (1988) 287–295
Elsevier Scientific Publishers Ireland Ltd.

COMPARISON OF THE EFFECTS OF ASCORBYL PALMITATE AND L-ASCORBIC ACID ON PARACETAMOL-INDUCED HEPATOTOXICITY IN THE MOUSE

DIANA JONKER[a], VICTORIA S. LEE, RICHARD J. HARGREAVES and BRIAN G. LAKE**

The British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS (United Kingdom) and [a]Department of Toxicology, Agricultural University of Wageningen, De Dreijen 12, 6703 BC, Wageningen (The Netherlands)

(Received November 20th, 1987)
(Accepted March 17th, 1988)

SUMMARY

The effects of ascorbyl palmitate (ASCP) and free L-ascorbic acid (LAA) on the hepatotoxicity of paracetamol (acetaminophen) and the in vivo covalent binding of reactive paracetamol metabolites to hepatic proteins has been studied in male MF1 mice. The oral administration of [^{3}H(G)]paracetamol (600 mg/kg) resulted in covalent binding to hepatic proteins, a depletion of hepatic non-protein sulphydryl (NPS) groups after 2 h, and a marked elevation of plasma alanine aminotransferase (ALAT) activity after 24 h. The co-administration of paracetamol and ASCP (1412 mg/kg, equivalent to 600 mg/kg free LAA), but not paracetamol and LAA (600 mg/kg), significantly reduced covalent binding of paracetamol metabolites at 2 and 4 h after treatment. In addition ASCP, but not LAA, significantly reduced the depletion of NPS groups and the elevation of plasma ALAT activity. ASCP also completely prevented the 35% mortality observed at 24 h in paracetamol treated mice. These results demonstrate that ASCP, but not LAA, when co-administered orally with the analgesic is an effective inhibitor of paracetamol-induced hepatotoxicity in the mouse. The mechanism by which ASCP prevents liver injury appears to involve destruction of reactive paracetamol metabolites which is associated with a sparing action on hepatic reduced glutathione levels.

***Address all correspondence and reprint requests to:* Dr. Brian G. Lake BIBRA, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, U.K.

Abbreviations: ALAT, alanine aminotransferase; ASCP, ascorbyl palmitate; GSH, reduced glutathione; LAA, L-ascorbic acid; NPS, non-protein sulphydryl.

0300-483X/88/$03.50

Key words: Ascorbyl palmitate; Covalent binding; Hepatotoxicity; L-ascorbic acid; Mouse; Paracetamol

INTRODUCTION

Paracetamol (acetaminophen, 4-acetamidophenol) is a widely used non-narcotic analgesic and antipyretic which is safe at therapeutic doses. However, at high doses paracetamol is known to cause hepatic necrosis in experimental animals such as the mouse and hamster [1], and in man [2]. The classic studies of Mitchell and coworkers [3–6] demonstrated that paracetamol-induced liver injury is mediated through reactive paracetamol metabolite(s) which are generated by cytochrome *P*-450 dependent mixed function oxidase enzymes. These reactive metabolite(s) bind covalently to tissue macromolecules resulting in cell death and tissue necrosis. Several workers [7–10] have suggested that the reactive metabolite derived from paracetamol is *N*-acetyl-*p*-benzoquinoneimine (acetamidoquinone). At low doses of paracetamol this metabolite is detoxified by conjugation with reduced glutathione (GSH), but at high doses GSH levels become markedly depleted resulting in the covalent binding of paracetamol metabolites to hepatic proteins.

Many studies have been conducted into the inhibition of paracetamol-induced liver injury. Paracetamol hepatotoxicity is reduced in mice given inhibitors of cytochrome *P*-450 dependent enzyme activities such as piperonyl butoxide [3] and metyrapone [11]. Similarly, protective effects have been reported in animals treated with various sulphur-containing compounds including cysteine, cysteamine, methionine and *N*-acetylcysteine. These agents may act in various ways including serving as glutathione precursors to replete hepatic GSH levels, destroying reactive paracetamol metabolites – either by forming stable adducts or by reduction back to the parent drug – and by inhibiting cytochrome *P*-450 dependent enzyme activities [10–14].

A number of studies have also examined the potential usefulness of LAA (Vitamin C) as a protective agent against paracetamol-induced liver injury. LAA is known to inhibit the covalent binding of paracetamol metabolites to mouse and hamster hepatic microsomes in vitro [9,10,15,16]. Hargreaves et al. [17] studied the effects of several ascorbate formulations in vivo and demonstrated that ASCP, but not free LAA, reduced the hepatotoxicity of paracetamol in the mouse. However, in other studies with the mouse Raghuram et al. [18] reported that LAA treatment resulted in a reduction of paracetamol-induced mortality, whereas Romero-Ferret et al. [19] observed no protective effect. Sodium ascorbate has been reported not to protect against paracetamol-induced hepatotoxicity in the Syrian hamster [20].

In this study we have investigated some possible mechanisms by which ASCP inhibits paracetamol-induced liver injury. Further, in view of the conflicting results reported for the effects of free LAA and its derivatives, we have performed some additional comparative studies between LAA and ASCP.

MATERIALS AND METHODS

Chemicals

Paracetamol (B.P. grade) was kindly supplied by Beecham Products Research Department, Weybridge, Surrey, U.K. LAA (>99% pure) was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K.; ASCP (99% pure) from Ventron Gmbh, Karlsruhe, Germany and Gum Tragacanth from Sigma Chemical Co., Poole, Dorset, U.K. [^{3}H(G)]Paracetamol (spec. act. 18.9 Ci/mmol, >98.5% pure) was obtained from NEN Research Products, Stevenage, Herts, U.K.

Animals and treatment

Male MF1 mice (25–30 g) were obtained from HARLAN OLAC Ltd., (Bicester, Oxon, U.K.) and housed in plastic cages with wire mesh floors. The accommodation was maintained at 22 ± 2°C with a relative humidity of 45–70%. Mice were allowed free access to water and R and M No. 1 diet (Special Diet Services Ltd., Witham, Essex, U.K.). After acclimatizing the animals to the experimental conditions for 7 days, mice were given single oral doses of [^{3}H(G)]paracetamol (600 mg/kg; 12 μCi/animal) alone or in combination with LAA (600 mg/kg) or ASCP (1412 mg/kg, equivalent to 600 mg/kg free LAA). Controls received corresponding quantities (20 ml/kg) of the 0.56% (w/v) Gum Tragacanth vehicle. Food was withdrawn after treatment and groups of mice were killed at intervals of 2–24 h by exsanguination under ether anaesthesia.

Biochemical investigations

Livers were excised into ice-cold 0.154 M KCl containing 50 mM Tris–HCl buffer (pH 7.4) and homogenised (0.1 g fresh tissue/ml) using a Potter-type, Teflon-glass motor driven homogeniser (A.H. Thomas Co., Philadelphia, PA, U.S.A.). Whole homogenate NPS groups were determined by the method of Ellman [21] as modified by Sedlak and Lindsay [22]. A portion of the whole homogenate was treated with an equal volume of 10% (w/v) trichloroacetic acid and macromolecular bound [^{3}H(G)]paracetamol metabolites determined by exhaustive solvent extraction of the pellet as described previously [16]. Radioactivity was determined by scintillation counting and protein by the method of Lowry et al. [23] employing bovine serum albumin as standard. Plasma samples were assayed [24] for activity of ALAT.

Statistical analysis

Statistical evaluation of data was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

RESULTS

Effect on covalent binding of paracetamol metabolites

The oral administration of 600 mg/kg [^{3}H(G)]paracetamol to mice resulted

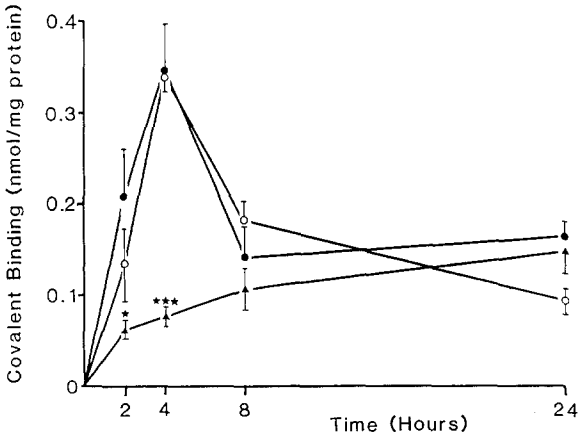

Fig. 1. Effect of LAA and ASCP co-administration on the in vivo covalent binding of reactive paracetamol metabolites to mouse hepatic proteins. Mice were given single oral doses of [^{3}H(G)]paracetamol (600 mg/kg) alone (●) or in combination with LAA (600 mg/kg; ○) or ASCP (1412 mg/kg; ▲) and the extent of covalent binding determined after various time intervals. Each point represents the mean ± S.E.M. of 5 mice. Results significantly different from mice given paracetamol alone are: *$P < 0.05$; ***$P < .0.001$.

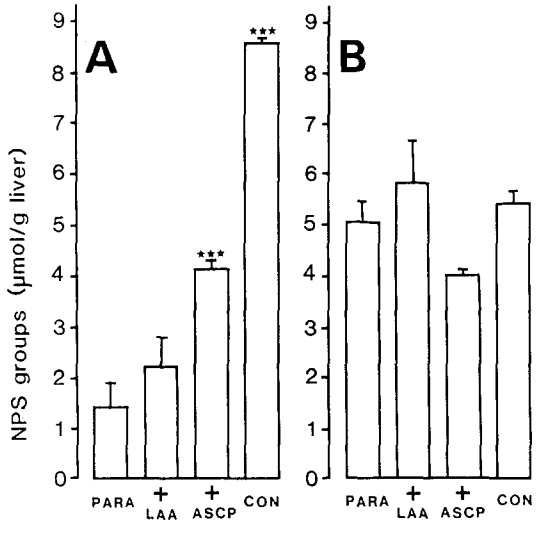

Fig. 2. Effect of paracetamol alone or co-administered with LAA or ASCP on mouse hepatic NPS groups. Mice were given single oral doses of paracetamol (600 mg/kg) alone (PARA) or in combination with 600 mg/kg LAA (+LAA) or 1412 mg/kg ASCP (+ASCP). Control animals (CON) received Gum Tragacanth and NPS groups were determined 2 h (A) and 24 h (B) after treatment. Each point represents the mean ± S.E.M. of 5 mice. Results significantly different from mice given paracetamol alone are: ***$P < 0.001$.

in covalent binding of paracetamol metabolites to mouse liver proteins, with peak levels occurring after 4 h (Fig. 1). The co-administration of 600 mg/kg LAA, which represents a toxin to protective agent molar ratio of 1:0.86, had no effect on paracetamol metabolite covalent binding at any time interval examined. In contrast, co-administration of ASCP (1412 mg/kg, equivalent to 600 mg/kg free LAA) significantly reduced the covalent binding of paracetamol metabolites at 2 and 4 h to 31% and 22%, respectively, of the levels observed in mice given paracetamol alone (Fig. 1).

Effect on paracetamol-induced hepatotoxicity

Compared to control mice paracetamol administration markedly reduced hepatic NPS groups, known to consist largely of reduced GSH [25], from 8.6 to 1.4 μmol/g liver 2 h after treatment (Fig. 2A). Whereas NPS groups in mice co-administered paracetamol and LAA were not significantly different from those in animals given paracetamol alone, co-administration with ASCP significantly reduced the fall in tissue NPS groups. After 24 h there were no significant differences in hepatic NPS groups between mice given paracetamol alone and the other treatment groups (Fig. 2B).

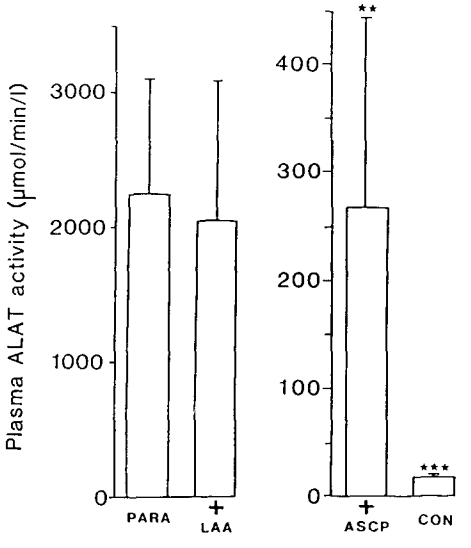

Fig. 3. Effect of paracetamol alone or co-administered with LAA or ASCP on mouse plasma ALAT. Mice were given single oral doses of paracetamol (600 mg/kg) alone (PARA) or in combination with 600 mg/kg LAA (+LAA) or 1412 mg/kg ASCP (+ASCP). Control animals (CON) received Gum Tragacanth and plasma ALAT activity was determined 24 h after treatment. Each point represents the mean ± S.E.M. of 12 mice. Note the use of 2 sets of y axes for expression of enzyme activity. Results significantly different from mice given paracetamol alone are: $^{**}P < 0.01$; $^{***}P < 0.001$.

TABLE I

EFFECT OF LAA AND ASCP ON PARACETAMOL-INDUCED MORTALITY IN THE MOUSE

Treatment[a]	Deaths after 24 h[b]
Paracetamol (600 mg/kg)	6/17
Paracetamol (600 mg/kg) plus LAA (600 mg/kg)	3/17
Paracetamol (600 mg/kg) plus ASCP (1412 mg/kg)	0/13
Control (Gum Tragacanth)	0/13

[a]Compounds were administered as single oral doses.
[b]The first figure refers to the number of animals dead after 24 h and the second figure to the number of animals dosed.

Paracetamol treatment resulted in a marked elevation of plasma ALAT activity from 15 to 2240 μmol/min per litre at 24 h (Fig. 3). Treatment with LAA had no protective effect, whereas ASCP administration substantially reduced the elevation of plasma ALAT activity.

At the dose level employed in these studies paracetamol administration resulted in a 35% mortality after 24 h (Table I). Whilst some deaths were also observed in mice co-administered LAA, ASCP treatment completely protected against paracetamol-induced mortality. Previous studies have demonstrated that this protective effect of ASCP is not due simply to a delayed onset of mortality [17].

DISCUSSION

The results described in this paper demonstrate that ASCP, but not LAA, had a significant protective effect both against the in vivo covalent binding of reactive paracetamol metabolites and against paracetamol-induced hepatotoxicity in the mouse.

The data for protection against liver injury and subsequent mortality by ASCP confirms our previous studies [17] and more recent investigations by Mitra et al. [26]. Our results also help to clarify the apparently conflicting reports on the effects of ascorbate formulations on paracetamol-induced liver injury. Several groups have not observed any protective effect of LAA or sodium ascorbate against paracetamol-induced liver injury in the mouse and hamster [17,19,20]. These findings and the present results are in contrast with those of Raghuram et al. [18] who reported that LAA protected against paracetamol-induced mortality in the mouse. The apparent divergence between the effects of LAA and ASCP may simply be due to differences in the absorption and subsequent bio-availability to the liver — as free LAA — of the 2 ascorbate forms relative to the time course of the hepatotoxic process. Clearly, this explanation for the protective effect of ASCP could be readily tested by comparing the hepatic pharmacokinetic profiles of orally

administered paracetamol, ASCP and LAA in the mouse. In previous studies on the effect of ascorbate formulations on paracetamol-induced liver injury there have been many differences in important experimental conditions including the use of both oral and i.p. administration, different times of administration of ascorbate and paracetamol and different ratios of paracetamol to ascorbate [17–20,26]. These differing experimental designs may also have contributed to the controversy surrounding the protective effect of ascorbate formulations.

The observation that ASCP, presumably active as free LAA, significantly reduced the in vivo covalent binding of reactive paracetamol metabolites to liver proteins is in agreement with previous in vitro studies with mouse and hamster hepatic microsomal preparations [9,10,15,16]. LAA does not form a conjugate with *N*-acetyl-*p*-benzoquinoneimine, the putative reactive intermediate derived from paracetamol, but rather is thought to reduce this intermediate back to paracetamol thereby preventing covalent binding [9,10]. Thus as suggested by in vitro studies [6], LAA (derived from ASCP) probably acts in vivo by supplementing GSH in scavenging reactive paracetamol metabolites after they have been generated by cytochrome *P*-450 dependent enzymes, and not, for example, by inhibiting reactive metabolite formation. Indeed, a sparing effect of ASCP treatment on hepatic GSH levels was observed in this study and in vitro studies [16] have demonstrated additive inhibitory effects of LAA and GSH on the covalent binding of reactive paracetamol metabolites.

One possible application suggested by the present results would be to incorporate ASCP into paracetamol formulations to reduce the risk of liver injury in humans following the ingestion of large doses of the analgesic. A similar role has been proposed for methionine [27], which is used for the clinical treatment of paracetamol poisoning [28,29]. However, further studies are required to assess the relative merit [30] of ASCP against methionine as a suitable "built-in antidote" for pharmaceutical preparations of paracetamol.

ACKNOWLEDGEMENT

We are grateful to Dr S.D. Gangolli, Director of Research at BIBRA, for support and encouragement.

REFERENCES

1 D.C. Davis, W.Z. Potter, D.J. Jollow and J.R. Mitchell, Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci., 14 (1974) 2099.

2 L.F. Prescott, N. Wright, P. Roscoe and S.S. Brown, Plasma paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. Lancet, 1 (1971) 519.

3 J.R. Mitchell, D.J. Jollow, W.Z. Potter, D.C. Davis, J.R. Gillette and B.B. Brodie, Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J. Pharmacol. Exp. Ther., 187 (1973) 185.

4 D.J. Jollow, J.R. Mitchell, W.Z. Potter, D.C. Davis, J.R. Gillette and B.B. Brodie, Acetamino-

phen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. J. Pharmacol. Exp. Ther., 187 (1973) 195.
5 W.Z. Potter, D.C. Davis, J.R. Mitchell, D.J. Jollow, J.R. Gillette and B.B. Brodie, Acetaminophen-induced hepatic necrosis. III. Cytochrome *P*-450-mediated covalent binding *in vitro*. J. Pharmacol. Exp. Ther., 187 (1973) 203.
6 J.R. Mitchell, D.J. Jollow, W.Z. Potter, J.R. Gillette and B.B. Brodie, Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exp. Ther., 187 (1973) 211.
7 D.J. Miner and P.T. Kissinger, Evidence for the involvement of *N*-acetyl-*p*-quinoneimine in acetaminophen metabolism. Biochem. Pharmacol., 28 (1979) 3285.
8 J.A. Hinson, L.R. Pohl, T.J. Monks and J.R. Gillette, Acetaminophen-induced hepatotoxicity. Life Sci., 29 (1981) 107.
9 D.C. Dahlin, G.T. Miwa, A.Y.H. Lu and S.D. Nelson, *N*-acetyl-*p*-benzoquinone imine: a cytochrome *P*-450-mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 1327.
10 G.B. Corcoran, J.R. Mitchell, Y.N. Vaishnav and E.C. Horning, Evidence that acetaminophen and *N*-hydroxyacetaminophen form a common arylating intermediate, *N*-acetyl-*p*-benzoquinoneimine. Mol. Pharmacol., 18 (1980) 536.
11 E.B. Nelson, M. Montes and M. Goldstein, Effectiveness of metyrapone in the treatment of acetaminophen toxicity in mice. Toxicology, 17 (1980) 73.
12 F.D. Harvey and T.E. Levitt, Experimental evaluation of paracetamol antidotes. J. Int. Med. Res., 4 (Suppl.) 4 (1976) 130.
13 J.M. Tredger, H.M. Smith, M. Davis and R. Williams, Effect of sulphur-containing compounds on paracetamol activation and covalent binding in a mouse hepatic microsomal system. Toxicol. Lett., 5 (1980) 339.
14 M.C. Savides and F.W. Oehme, Acetaminophen and its toxicity. J. Appl. Toxicol., 3 (1983) 96.
15 J.A. Hinson, L.R. Pohl, T.J. Monks, J.R. Gillette and F.P. Guengerich, 3-Hydroxyacetaminophen: a microsomal metabolite of acetaminophen. Evidence against an epoxide as the reactive metabolite of acetaminophen. Drug Metab. Dispos., 8 (1980) 289.
16 B.G. Lake, R.A. Harris, J.C. Phillips and S.D. Gangolli, Studies on the effects of L-ascorbic acid on acetaminophen-induced hepatotoxicity. I. Inhibition of the covalent binding of acetaminophen metabolites to hepatic microsomes *in vitro*. Toxicol. Appl. Pharmacol., 60 (1981) 229.
17 R.J. Hargreaves, J.G. Evans, D. Pelling and K.R. Butterworth, Studies on the effects of L-ascorbic acid on acetaminophen-induced hepatotoxicity. II. An *in vivo* assessment in mice of the protection afforded by various dosage forms of ascorbate. Toxicol. Appl. Pharmacol., 64 (1982) 380.
18 T.C. Raghuram, D. Krishnamurthi and R. Kalamegham, Effect of Vitamin C on paracetamol hepatotoxicity. Toxicol. Lett., 2 (1978) 175.
19 C. Romero-Ferret, G. Mottot, J. Legros and G. Margetts, Effect of Vitamin C on acute paracetamol poisoning. Toxicol. Lett., 18 (1983) 153.
20 M.G. Miller and D.J. Jollow, Effect of L-ascorbic acid on acetaminophen-induced hepatotoxicity and covalent binding in hamsters. Evidence that *in vitro* covalent binding differs from that *in vivo*. Drug Metab. Dispos., 12 (1984) 271.
21 G.L. Ellman, Tissue sulfhydryl groups. Arch. Biochem. Biophys., 82 (1959) 70.
22 J. Sedlak and R.H. Lindsay, Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellmans reagent. Anal. Biochem., 25 (1968) 192.
23 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193 (1951) 265.
24 G. Bechtler, UV-Assay, automated method (for glutamate-pyruvate transaminase), in H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. 2, Academic Press, New York, 1974, p. 758.
25 M.S. Moron, J.W. DePierre and B. Mannervik, Levels of glutathione, glutathione reductase and glutathione S-transferase in rat lung and liver. Biochim. Biophys, Acta, 582 (1979) 67.

26 A. Mitra, S.L. Morris and D.R. Bourcier, Protection of acetaminophen induced hepatotoxicity in mice by ascorbic acid esters. Toxicologist, 6 (1986) 2.
27 P.J. Neuvonen, O. Tokola, M.-L. Toivonen and O. Simell, Methionine in paracetamol tablets, a tool to reduce paracetamol toxicity. Int. J. Clin. Pharmacol. Ther., 23 (1985) 497.
28 L.F. Prescott, J. Park, G.R. Sutherland, I.J. Smith and A.T. Proudfoot, Cysteamine, methionine and penicillamine in the treatment of paracetamol poisoning. Lancet, 11 (1976) 109.
29 P. Crome, J.A. Vale, G.N. Volans, B. Widdop and R. Goulding, Oral methionine in the treatment of severe paracetamol (acetaminophen) overdose. Lancet, 11 (1976) 829.
30 R. Williams, Paracetamol hepatotoxicity: a built-in antidote? Hepatology, 6 (1986) 751.