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Inhibition of Dapsone-induced Methaemoglobinaemia in the Rat Isolated Perfused Liver

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Abstract—We have investigated the disposition of dapsone (DDS, 1 mg) in the rat isolated perfused liver in the absence and the presence of cimetidine (3 mg). After the addition of DDS alone to the liver there was a monoexponential decline of parent drug concentrations and rapid formation of DDS-NOH (within 10 min) which coincided with methaemoglobin formation ($11{\cdot}7 \pm 3{\cdot}0\%$, mean ± s.d.) which reached a maximum ($22{\cdot}6 \pm 9{\cdot}2\%$) at 1 h. The appearance of monoacetyl DDS (MADDS) was not apparent until 30-45 min. Addition of cimetidine resulted in major changes in the pharmacokinetics of DDS and its metabolites. The AUC of DDS in the presence of cimetidine ($1018{\cdot}8 \pm 267{\cdot}8$ μg min mL^{-1}) was almost three-fold higher than control ($345{\cdot}0 \pm 68{\cdot}1$ μg min mL^{-1}, $P < 0{\cdot}01$). The half-life of DDS was also prolonged by cimetidine compared with control ($117{\cdot}0 \pm 48{\cdot}2$ min vs $51{\cdot}2 \pm 22{\cdot}9$, $P < 0{\cdot}05$). The clearance of DDS ($3{\cdot}0 \pm 0{\cdot}55$ mL min^{-1}) was greatly reduced in the presence of cimetidine ($1{\cdot}03 \pm 0{\cdot}26$ mL min^{-1} $P < 0{\cdot}01$). The $AUC_{0\text{-}3h}$ for DDS-NOH ($28{\cdot}3 \pm 21{\cdot}2$ μg min mL^{-1}) was significantly reduced by cimetidine ($8{\cdot}1 \pm 3{\cdot}40$ μg min mL^{-1}, $P < 0{\cdot}01$). In contrast, there was a marked increase in the $AUC_{0\text{-}3h}$ for MADDS ($32{\cdot}7 \pm 25{\cdot}8$ μg min mL^{-1}) in the presence of cimetidine ($166{\cdot}0 \pm 26{\cdot}5$ μg min mL^{-1} $P < 0{\cdot}01$). The methaemoglobinaemia associated with DDS was reduced to below 5% by cimetidine. Hence, a shift in hepatic metabolism from bioactivation (*N*-hydroxylation) to detoxication (*N*-acetylation) caused by cimetidine, was associated with a fall in methaemoglobinaemia. These data suggest that the combination of DDS with a cytochrome P450 inhibitor might reduce the risk to benefit ratio of DDS.

Dapsone (DDS) has been used in leprosy therapy for over 40 years and remains part of modern multidrug regimens (Shepard 1982). It is also a malarial prophylactic in combination with pyrimethamine (maloprim; Bruce-Chwatt 1982). The compound is effective in controlling dermatitis herpetiformis (Swain et al 1983) and disorders characterised by polymorphonuclear leucocyte infiltration (Lang 1979). More recently, DDS has been incorporated in the treatment regimes for *Pneumocystis carinii* in AIDS patients (Green et al 1988). DDS is extensively metabolised in man through *N*-hydroxylation to DDS hydroxylamine (DDS-NOH, Israili et al 1973, Coleman et al 1989) and by acetylation to monoacetyl DDS (MADDS, Gelber et al 1971). MADDS may also undergo hydroxylation to form MADDS-NOH (Uetrecht et al 1988; Grossman & Jollow 1988). Adverse reactions to DDS include fatal agranulocytosis (Levine & Weintraube 1968; Ognibene 1970) and, far more commonly, methaemoglobinaemia (Cohen et al 1968) and haemolysis (DeGowin et al 1966). The latter reactions are dose dependent (DeGowin et al 1966) and occur to some extent in all patients receiving the drug (Zuidema et al 1986). All the adverse effects of DDS have been attributed to the hydroxylamine metabolites (Kramer et al 1972; Glader & Conrad 1973; Weetman et al 1980). DDS induced haemotoxicity is especially severe in patients suffering from deficiencies in NADH-dependent methaemoglobin reductase (Ganer et al 1981), glucose-6-phosphate dehydrogenase or glutathione reductase activities (Zuidema et al 1986).

Studies in the intact rat have illustrated that selective inhibition of the oxidative metabolism of DDS with cimetidine leads to the abolition of methaemoglobin formation (Coleman et al 1990a). The rat isolated perfused liver preparation is a useful and versatile experimental system with which to investigate the hepatic component of drug metabolism interactions (Mihaly et al 1982, 1985; Coleman et al 1985). We wish to evaluate the effect of inhibition of cytochrome P450 on the disposition of DDS, its acetylated metabolite, and most importantly, DDS-NOH. However, the instability of DDS-NOH in the presence of oxygen (Coleman et al 1989) causes it to be difficult to assay by conventional solvent or solid phase extraction. Previous methods of analysis of this metabolite have involved complete oxidation to its nitro derivative, hence measuring the sum of the hydroxylamine and nitroso intermediate species (Uetrecht et al 1988). In order to directly measure the hydroxylamine, a simple methanol protein precipitation method has been developed. The rat perfused liver system does not contain the normal contaminants of whole plasma, hence facilitating the analysis of DDS-NOH by this method. In addition, this experimental model employs human erythrocytes as oxygen carriers, therefore illustrating the toxic effect of DDS-NOH on the human red cell.

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Materials and Methods

Chemicals

4,4′-Diaminodiphenyl sulphone (DDS) was supplied by Sigma Chemical Co. (Poole, Dorset). Cimetidine was a gift from Smith, Kline and Beecham (Welwyn Garden City, Herts). Monoacetyl DDS and mono-propionyl DDS (the internal standard) were kindly supplied by Dr S. A. Ward, Liverpool School of Tropical Medicine. All other chemicals, including HPLC solvents, were of reagent grade and were obtained from BDH Chemicals Ltd (Poole, Dorset).

Animals

Male Wistar rats obtained from the Department of Pharmacology and Therapeutics breeding colony (230–275 g) were housed in well ventilated cages and kept at a room temperature of approximately 24°C. They were allowed to feed on a pelleted Labsure CRM diet (Biosure Ltd, Cambridgeshire, UK) plus free access to water.

Rat isolated livers

Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and their livers were isolated as follows: the bile duct was cannulated with polypropylene tubing (o.d. = 0·61 mm i.d. = 0·28 mm) and the liver was cleared of connective tissue before cannulation of the portal vein (Argyle medicut, 14G, Sherwood Industries) which was then flushed with heparinized saline (50 units mL^{-1}). The inferior vena-cava was tied off at the renal vein and cava cannulated with polypropylene tubing (o.d. = 1·9 mm i.d. 1·4 mm) beyond the hepatic vein. The liver was removed and placed on a glass platform inside a humidified, thermostatically (37°C) controlled environment. Livers were then perfused in a constant flow (15 mL min^{-1}) recirculating system as described previously (Mihaly et al 1982). The perfusate (100 mL) comprised 10% washed human erythrocytes, 1% (w/v) bovine serum albumin (Sigma Chemicals) and 0·1% glucose in a standard electrolyte solution (Krone et al 1974). The principal indices of liver viability (Mihaly et al 1982) were: steady oxygen consumption (1·7–2·2 μmol (g liver)$^{-1}$ min^{-1}) sustained bile flow (0·2–0·6 mL h^{-1}) and constant perfusion pressure (6–8 cm H_2O).

Protocol

The disposition of DDS (1 mg) dissolved in dimethyl sulphoxide (5 μL) was studied alone (n = 5, group A) and in the presence of cimetidine (3 mg, dissolved in 400 μL 0·05 M HCl, n = 5, group B). Both compounds were added as bolus doses directly into the liver reservoir. Cimetidine was administered to livers in group B 30 min before the administration of DDS. Samples (0·8 mL) were removed from the perfusate pre-dose, then at 5, 10, 15, 20, 25, 30, and 45 min, 1, 1·5, 2, 2·5 and 3 h. Each sample vial contained ascorbic acid (40 μL 100 mM) dissolved in 0·9% NaCl (saline) to preserve the DDS-NOH. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. Before the centrifugation (10 000 *g*, 2·5 min) of the samples and separation of perfusate plasma and red cells, 0·4 mL of whole perfusate was removed for methaemoglobin determination. The remainder of the perfusate plasma was stored at −20°C until assayed. Bile was collected at hourly intervals into preweighed vials also containing ascorbate (5 mM). Sodium taurocholate was infused at 30 μM h^{-1} to maintain bile flow. Perfusate gases (CO_2 and O_2) were measured pre (sampling via the portal inflow) and post (sampling via the hepatic vein outflow) liver at t = 0, t = 1 h and t = 3 h. At 3 h, the livers were flushed with saline, blotted dry, weighed and frozen at −20°. Before assay for DDS, MADDS and DDS-NOH, the livers were thawed and then homogenized in three times their weight of ice-cold 0·007 M phosphate buffer (pH 7·5 containing 1·15% KCl and 5 mM ascorbate) using a teflon-in-glass homogenizer.

Analytical procedures

DDS, MADDS and DDS-NOH were analysed as follows: to samples of perfusate plasma (0·2–0·4 mL) contained in 1·5 mL capacity microcap tubes (L.I.P. Equipment and Services, West Yorkshire, UK) was added an aqueous solution of the internal standard (0·1 mg mL^{-1} 8–16 μL) and methanol (0·330 mL). Methanol was thus added in a 5:3 ratio to the samples. The samples were briefly vortexed and then centrifuged for 10 min at 10000 *g*. The clear supernatant was removed from the sample and an aliquot (0·1 mL) injected on to the HPLC.

The method was based on that of Uetrecht et al (1988), but extensively modified. It was developed on a Spectra-Physics Liquid Chromatograph. The system consisted of an SP 8700 solvent delivery system, an SP 8750 organiser module equipped with a Rheodyne injection system and a Pye Unicam LC3 UV absorbance detector. Chromatographic separation was achieved using a Waters μ Bondapak C_{18} stainless steel column (30 cm × 0·39 cm i.d. 10 μm, Waters Associates, Hartford, Cheshire). The mobile phase comprised water–acetonitrile–acetic acid–triethylamine (79:21:1:0·05 v/v) and was continually degassed with helium. For the analysis of DDS, MADDS and DDS-NOH, a linear gradient was employed from 0 to 14·2 min, ending at 64·5: 35·5: 1: 0·05 v/v. The flow rate was maintained throughout at 1·2 mL min^{-1}. The eluent was monitored at 254 nm. The respective retention times of DDS-NOH, DDS, MADDS and internal standard were 7·1, 8·2, 9·5 and 13 min. DDS-NOH was stabilised in solution in 5 mM ascorbic acid. This ensured 95% preservation of the compound through the steps of the assay at DDS-NOH concentrations of up to 0·8 mg mL^{-1} in perfusate plasma. Addition of ascorbate to the mobile phase was unnecessary as the phase had been thoroughly helium degassed before assay. Standard curves in the range 0·2 μg mL^{-1} − 0·8 mg mL^{-1} were prepared by adding known quantities of DDS-NOH, DDS and MADDS to drug-free perfusate plasma or liver homogenate containing 5 mM ascorbate. Samples were analysed as described above and the peak height ratio of compound to internal standard was plotted against the corresponding weight ratios. Peak height ratios of experimental samples were also determined and the concentrations calculated from the standard curves.

Recovery of the compounds were estimated by comparing the peak height obtained from an assayed sample (either perfusate or homogenate) with that from a standard solution containing the same amount of each compound. Analytical recoveries of DDS, DDS-NOH and MADDS in perfusate plasma were 84·2 ± 6·6%, 77·2 ± 5·3% and 54·0 ± 5·7%, respectively. The recovery from liver homogenate for DDS was 68·0 ± 4·2%. A number of interfering peaks in liver homogenate which coeluted with DDS-NOH and MADDS made attempts to reproducibly assay these compounds unsuccessful.

The intra- and inter-assay precision data were determined for all three compounds by replicate assays of the same perfusate plasma sample. At 400 ng mL^{-1} intra-assay variations for DDS, DDS NOH and MADDS were 9·8, 9·8 and 8·8%. Inter-assay variation was determined to be 3·8, 7·2 and 4·3%, respectively. Intra-assay precision at 800 ng mL^{-1} for DDS, DDS NOH and MADDS was 7·3, 4·6 and 8·6%;

inter-assay variation at 800 ng mL^{-1} was 7·2, 6·3 and 5%, respectively. The limits of detection were 190, 210, and 280 ng mL^{-1} for DDS, DDS-NOH and MADDS from a 200 μL perfusate plasma sample. All calibration curves showed linearity (r = 0·99) for each compound. The methaemoglobin level relative to total haemoglobin in the perfusate samples was measured using the spectrophotometric technique of Harrison & Jollow (1986).

Pharmacokinetic calculations and statistical analysis

The area under the curves ($AUC_{0\text{-}3\ h}$) for the perfusate concentration- time data of DDS, DDS-NOH and MADDS were each calculated from t = 0 to t = 3 h by the use of the trapezoidal rule (Gibaldi & Perrier 1982). AUC from 3 h to infinity was calculated for DDS by the ratio C_3/β where C_3 was the perfusate concentration of DDS at time 3 h. The area under the curve from zero-infinity (AUC) was obtained from the sum of the two areas. When DDS was added to the liver system alone, the elimination rate constant (β) was obtained by least squares regression analysis of the perfusate DDS concentration-time data for all 12 time points, as DDS under-went a monoexponential decline. However, when DDS was given to the rat isolated perfused liver in the presence of cimetidine, a biexponential decline was observed, hence regression analysis was carried out on the post distributive perfusate DDS concentration-time data from 30 min to 180 min. In both cases the terminal phase half-life ($t_{\frac{1}{2}}$) was calculated from the ratio $0{\cdot}693/\beta$. Clearance was calculated from the equation:

$$CL = \frac{Dose}{AUC}$$

Volume of distribution (V_d) was obtained from the relationship:

$$V_d = \frac{CL \times t_{\frac{1}{2}}}{0{\cdot}693}$$

Statistical analysis between groups was made by the Wilcoxon Rank-Sum Test. Data are supplied in the text as mean ± s.d. and presented graphically as mean ± s.e.m. Statistical significance was set at the $P < 0{\cdot}05$ level.

Results

Liver oxygen consumption

The consumption of oxygen measured at 0, 1 and 3 h post drug addition in the livers to which DDS alone was added (t = 0, 1·8 ± 0·41; t = 1 h, 1·86 ± 0·35; t = 3 h, 1·8 ± 0·5 μmol O_2 (g liver)$^{-1}$ min^{-1}) did not significantly differ from those preparations dosed with cimetidine and DDS (t = 0 1·95 ± 0·16; t = 1, 2·0 ± 0·1; t = 3 h, 1·9 ± 0·31 μg mol O_2 (g liver)$^{-1}$ min^{-1}). Overall, oxygen consumption of all 10 liver preparations were within acceptable limits of viability (1·5–2 μM O_2 (g liver)$^{-1}$ min^{-1}, Mihaly et al 1982) throughout the study period.

Perfusate distribution of DDS, DDS-NOH and MADDS

The addition of DDS (1 mg) to the livers (Fig. 1) resulted in a decline of the parent drug and rapid production of DDS-NOH (within 10 min of drug addition; Fig. 2). However,

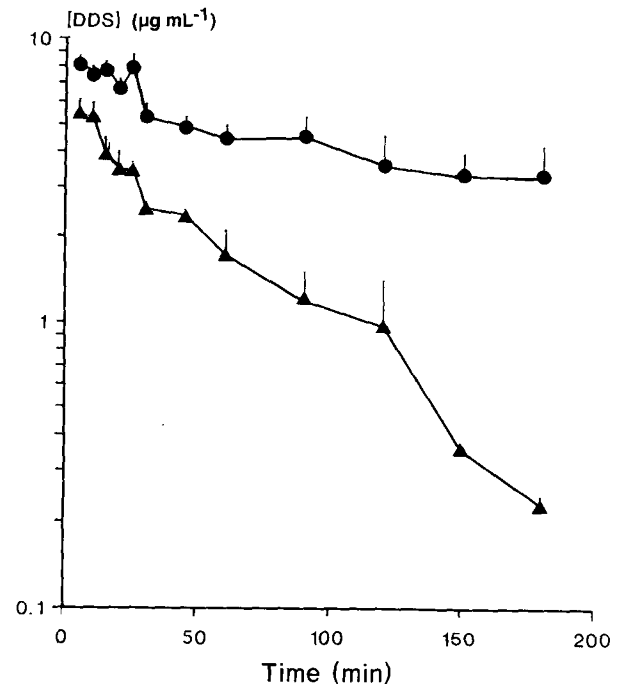

FIG. 1. The disposition of DDS in the liver perfusate plasma after the addition of DDS (1 mg, n = 5) alone (▲) or concurrently with cimetidine (3 mg, n = 5, ●).

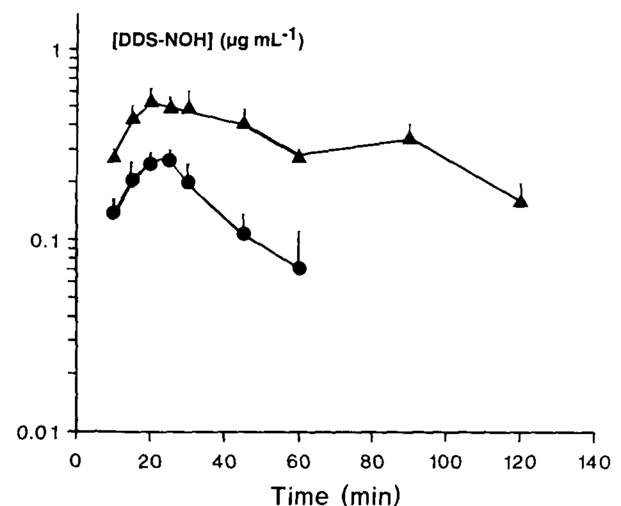

FIG. 2. The disposition of DDS-NOH in the liver perfusate plasma after the addition of DDS (1 mg) alone (▲) or concurrently with cimetidine (3 mg, n = 5, ●).

MADDS did not become detectable until between 30 and 45 min post dose (Fig. 3). Prior addition of cimetidine resulted in marked changes to the pharmacokinetics of DDS and its metabolites in the livers. In the presence of cimetidine (Fig. 1), the AUC of DDS (1018·8 ± 267·8 μg min mL^{-1}) was almost three-fold higher than that of DDS alone 345·0 ± 68·1 μg min mL^{-1}, $P < 0{\cdot}01$). In addition, the half-life of DDS alone (51·2 ± 22·9 min) was significantly ($P < 0{\cdot}05$) prolonged in the presence of cimetidine (117·0 ± 48·2 min). The clearance of DDS alone (3·00 ± 0·55 mL min^{-1}) was almost

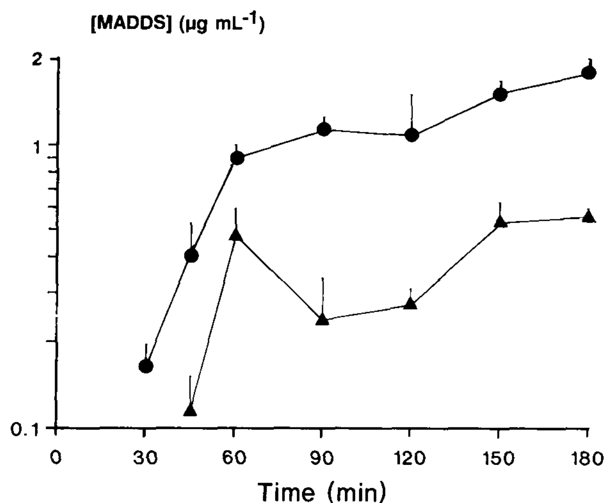

FIG. 3. The disposition of MADDS in the liver perfusate after the addition of DDS (1 mg, n = 5) alone (▲) or concurrently with cimetidine (3 mg, n = 5, ●).

three fold higher than in the presence of cimetidine (1·03 ± 0·26 mL min^{-1}). There was no significant change in the volume of distribution (V_d) between the two groups (209·0 ± 56·2 mL vs 166·6 ± 50·0 mL).

The $AUC_{0-3\,h}$ for DDS-NOH (28·3 ± 21·2 μg min mL^{-1}) was significantly ($P<0·01$) reduced in the presence of cimetidine (8·13 ± 3·40 μg min mL^{-1}, Fig. 2). In contrast, (Fig. 3) there was a marked increase in the $AUC_{0\,3}$ h for MADDS after the co-administration of cimetidine

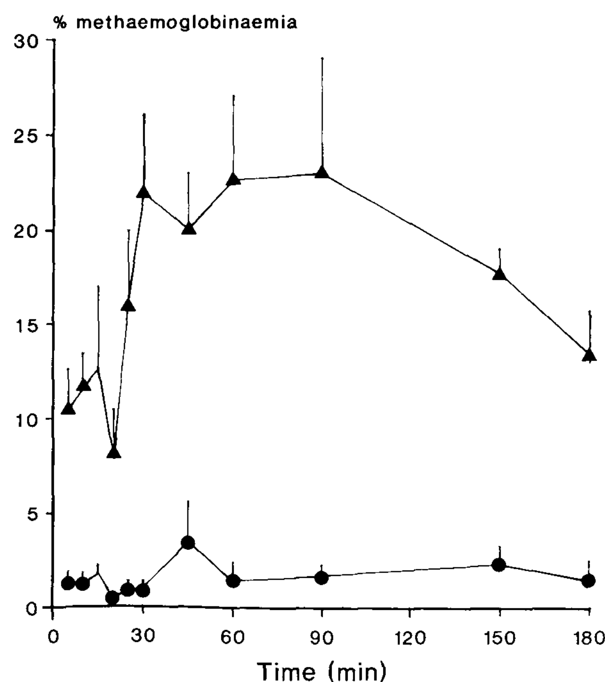

FIG. 4. Methaemoglobinaemia plotted against time in the perfused liver after addition of DDS (1 mg, n = 5) alone (▲) or concurrently with cimetidine (3 mg, n = 5 ●).

(166·0 ± 26·5 μg min mL^{-1}) compared with that of MADDS (32·7 ± 25·8 μg min mL^{-1}) after the addition of DDS alone.

The addition of DDS to the livers caused a marked and sustained methaemoglobinaemia (Fig. 4) which appeared within 10 min of drug addition (11·7 ± 3·0%) and coincided with the formation of DDS-NOH. Methaemoglobin concentrations reached a peak at 1 h (22·6 ± 9·2%). However, after the addition of cimetidine with DDS to the livers, methaemoglobin formation was virtually abolished, levels not exceeding 5% throughout the study period.

Biliary disposition of DDS and its metabolites

The biliary elimination of DDS, DDS-NOH and MADDS was relatively low. Although there was no significant difference between the groups in biliary DDS-NOH elimination (0·42 ± 0·32% dose mL^{-1} bile), significantly more ($P<0·05$) DDS (4·0 ± 1·54% dose mL^{-1} bile) was eliminated in bile in the absence of cimetidine compared with the 0·83 ± 0·8% dose mL^{-1} bile eliminated in the presence of cimetidine. Chromatograms of bile collected from the liver preparations dosed with DDS revealed a number of peaks which eluted close to the void volume. As these peaks were absent in blank bile they may have been highly polar unidentified derivatives of DDS. MADDS was eliminated in bile in very low concentrations (0·14 ± 0·14% dose mL^{-1} bile) after DDS alone. However, the compound could not be detected in bile after the administration of cimetidine. Less than 8% (7·34 ± 2·16%) of the dose of DDS was measured in whole liver homogenate at 3 h. However, the presence of cimetidine did not significantly affect this value (5·98 ± 1·88%).

Discussion

In the present study DDS was cleared from the liver at approximately 20% of the hepatic perfusate flow (900 mL h^{-1}). In comparison with quinine (87%, Coleman et al 1990b), primaquine (75%, Ward et al 1985), antipyrine (20%, Mihaly et al 1985) and pyrimethamine (8·6% Coleman et al 1985) DDS may be considered to be of low to intermediate clearance. DDS was excreted unchanged in bile only to a small extent and only a minor fraction of the dose remained in the liver as unchanged drug (~8%) compared with over 25% and >60% for pyrimethamine and mefloquine respectively (Coleman et al 1985, 1988).

Metabolism of DDS to DDS-NOH was rapid and could be observed within 10 min of drug addition. The appearance of this metabolite coincided with the rapid formation of considerable methaemoglobinaemia which has also been observed in the intact rat (Grossman & Jollow 1988). In the intact rat only a relatively small proportion of the dose of DDS appeared to have been converted to DDS-NOH. However, this metabolite is unstable (Uetrecht et al 1988; Coleman et al 1989) and is also thought to undergo extensive phase II conjugation (Israili et al 1973). Therefore it may be that, in-vivo, measured levels may underestimate the true quantities formed. Although DDS-NOH has not been quantified in human plasma so far (Zuidema et al 1986), a number of studies have investigated the capacity of DDS-NOH to cause methaemoglobinaemia (Cucinell et al 1972; Kramer et al 1972, Israili et al 1973). Aryl hydroxylamines

are known to undergo rapid redox cycling to aryl nitroso species within red cells (Kiese 1966), which in the case of DDS results in methaemoglobin formation (Modderman et al 1983). Studies with procainamide and benzene nitroso species indicate that these derivatives may either react with glutathione (Uetrecht 1984), or be recycled to the hydroxylamine by reduction with either NADPH or ascorbate (Becker & Sternson 1980). This process is thought to cause cumulative red cell toxicity (Grossman & Jollow 1988). In addition, DDS-NOH has been shown to cause red cell shrinkage through potassium chloride transport stimulation (Haas & Harrison 1989). These processes may contribute to the characteristic haemotoxicity of DDS-NOH.

The presence of cimetidine caused a marked reduction in DDS clearance from 20 to 6·6% of liver perfusate flow. This was also associated with a four-fold reduction in the AUC of DDS-NOH, and more importantly, the abolition of methaemoglobin formation. Cimetidine is a well characterized and potent inhibitor of the oxidative metabolism of a number of compounds in both man and the rat (Pelkonen & Puurunen 1980; Rendic et al 1984). It is likely, that in the present study, cimetidine has inhibited the *N*-hydroxylation of DDS and hence reduced the hydroxylamine-derived toxicity of DDS. However, the formation of DDS-NOH was not totally abolished by cimetidine. It may be that the levels of methaemoglobin formed by the low concentrations of DDS-NOH measured in the presence of cimetidine were within the detoxifying capacity of the erythrocytes. In normal physiological conditions, small quantities of methaemoglobin are formed continually within red cells but are reduced by NADH-methaemoglobin reductase to haemoglobin. However, oxidation of haemoglobin by hydroxylamines of DDS is thought to rapidly overtake this process in a dose dependent manner (Modderman et al 1983; Zuidema et al 1986).

The appearance of detectable MADDS concentrations in perfusate was delayed considerably in both the present study and the whole rat (Grossman & Jollow 1988). It may be that the acetylation of DDS is a much slower process than the *N*-hydroxylation of the drug in the rat. The presence of cimetidine was associated with a large increase in the acetylation of DDS in the perfused liver. It is likely that the lack of oxidative metabolism of DDS caused its greater availability for cytosolic acetylation, which resulted in a marked increase in MADDS formation. Cimetidine does not inhibit the acetylation of DDS in man (Wright et al 1984) which is in broad agreement with the present study. There is no evidence that MADDS itself is toxic. However, MADDS may be *N*-hydroxylated in man to MADDS hydroxylamine, which is as haemotoxic as DDS-NOH (Israili et al 1973). The co-administration of an inhibitor of cytochrome P450 with DDS would also prevent the hydroxylation of MADDS and DDS would thus be cleared to the non-toxic acetylated metabolite.

Dapsone is administered in combination with rifampicin and clofazimine for leprosy therapy (Zuidema et al 1986). Clofazimine has a long half-life and undergoes little metabolism (Levy 1976) and is not thought to appreciably influence DDS disposition (Venkatesan et al 1980). Rifampicin which is largely deacetylated in man, also induces hepatic metabolizing enzymes thus reducing skin and nerve levels of DDS (Peters et al 1977, 1978). This may promote resistance to DDS (Zuidema et al 1986). The co-administration of cimetidine or any other suitable inhibitor of oxidative metabolism would probably not greatly affect clofazimine disposition or interfere with the deacetylation of rifampicin but would inhibit the formation of toxic metabolites of DDS. In addition, cimetidine has been shown to rapidly reverse the effect of rifampicin enzyme induction on antipyrine metabolism in man (Feely et al 1984). Hence the reduction in DDS tissue levels might be avoided with concurrent cimetidine treatment.

In summary, addition of cimetidine to the rat isolated perfused liver significantly reduced the haemotoxicity of DDS by reducing its clearance to toxic hydroxylamine derivatives. This was associated with a concomitant increase in the acetylation of DDS. Co-administration of a reversible inhibitor of oxidative drug metabolism with compounds which undergo *N*-hydroxylation may result in fewer adverse reactions without change in pharmacodynamic activity.

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