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On the Use of Diazepam and Pro-diazepam (2-Benzoyl-4-chloro-*N*-methyl-*N*-lysylglycin anilide), as Adjunct Antidotes in the Treatment of Organophosphorus Intoxication in the Guinea-pig

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Abstract—Diazepam and pro-diazepam (2-benzoyl-4-chloro-*N*-methyl-*N*-lysylglycin anilide) have been used as adjunct antidotes to pyridostigmine and atropine against the organophosphate, soman, in the guinea-pig. Both added significant protection to the pyridostigmine/atropine treatment. Animals pretreated with diazepam, 60 min before soman, were "better" protected than animals given an equimolar dose of pro-diazepam therapeutically 1 min after soman. A pretreatment with diazepam for three days further increased the protection. A therapeutic dose of pro-diazepam, 1 min after soman, gave no further protection, to the three day diazepam pretreatment. The serum concentrations of diazepam (given i.p.) and desmethyldiazepam (given i.m.) were determined by GLC after diazepam (i.p.) and pro-diazepam (i.m.) were given. The protection, relative to the control, provided by the diazepam pretreatment (60 min before and for three days before soman) correlated linearly, r = 0·9898, with the serum values of diazepam achieved at these times. Our data suggest that diazepam as adjunct to pyridostigmine and atropine administered as pretreatment gives a "safer" protection, than an equimolar dose of pro-diazepam given therapeutically.

Organophosphorus compounds like soman (pinacolyl methyl phosphonofluoridate) act as irreversible acetylcholinesterase (AChE) inhibitors. Pretreatment with the reversible AChE inhibitor pyridostigmine and treatment with atropine and a reactivating oxime like obidoxime, are the current standard protocol used to protect against organophosphorus intoxication in man (Gall 1981). The anticonvulsant diazepam has been shown to provide additional protection, when given as adjunct to the pyridostigmine, atropine and oxime treatment in several animal models (Lipp 1972; Johnson & Wilcox 1975; Boskovic 1981; Inns & Leadbeater 1983). Diazepam has also been shown to reduce or suppress the irreversible brain damage that normally results from soman intoxication (Martin et al 1985).

Diazepam is virtually insoluble in water, and its rapid therapeutic use therefore calls for intravenous injection. The dipeptide derivatives of 2-aminobenzophenones have been used as pro-drugs for benzo-1,4-diazepines (Hassall et al 1977; Hirai et al 1978; Fujimoto et al 1980 a, b; Wong & Bymaster 1983). These pro-drugs are readily soluble in water and may thus be injected intramuscularly (i.m.). The problem of rapid administration of diazepam, may also be overcome by its prophylactic use (Puu & Sellström 1987).

We have compared the antidotal effect against soman intoxication of pro-diazepam given therapeutically with that of diazepam given prophylactically, to the guinea-pig. As chronic diazepam will eventually lead to tolerance (Haig & Feely 1988), it was of interest to compare its efficacy when given before and after a three day diazepam "treatment" of guinea-pigs. Accordingly, serum values of diazepam and desmethyldiazepam were determined following the i.m. injection of diazepam or the i.p. injection of its pro-drug, 2-benzoyl-4-chloro-methyl-*N*-lysylglycin anilide, in the guinea-pig.

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Materials and Methods

Animals

Male Dunkin-Hartley guinea-pigs (Möllegaards, Landskrona, Sweden) were housed for at least two weeks before use. They were allowed free access to food and water and maintained on a 12 h alternating light/dark cycle, with artificial light between 18.00 and 06.00 h. At the time of experimentation they weighed 250–500 g.

Chemicals

Pyridostigmine, (F. Hoffman-La Roche and Co Ltd, Lot 68115) was used as a 1·1 mg mL^{-1} aqueous solution for injection. Diazepam (Diazemuls, Kabivitrum) was injected in solution of 5 mg mL^{-1}. Atropine sulphate was injected as a 200 mg mL^{-1} aqueous solution. The pro-drug, pro-diazepam, was a lysylglycin derivative of a 2-benzoyl-4-chloro-*N*-methyl anilide synthesized in this department according to Sugasawa et al (1978) and Hirai et al (1980). An aqueous solution of pro-diazepam, 8·4 mg mL^{-1}, and atropine, 200 mg mL^{-1}, was injected. Diazepam and pro-diazepam were, accordingly, given in equimolar doses. Soman was injected as an aqueous solution, freshly prepared, at a concentration corresponding to 1 mL kg^{-1}, for each dosage group. At higher soman doses, above 0·12 mg kg^{-1}, appropriate volumes of a 10 mM aqueous solution of soman were used.

Ro 7-9957 (5-(2-fluorophenyl)-1,3-dihydro-7-iodo-1-methyl-2H-1,4-benzodiazepin-2-one) and Ro 7-9749 (5-(2-flurophenyl)-1,3-dihydro-7-iodo-2H-1,4-benzodiazepin-2-one) were generous gifts of Roche Products.

Acute toxicity

The LD50 was estimated according to Weil (1952). Six

different treatments, according to the following have been used:

Group 1 (control): four dosages of soman (s.c.).

Group 2 (P+A): 0·1 mg kg^{-1} pyridostigmine (i.p., −60 min), 20 mg kg^{-1} atropine (i.m., +1 min), four dosages of soman (s.c., 0 min).

Group 3 (P+A+D): 5 mg kg^{-1} diazepam (i.p., −60 min), 0·1 mg kg^{-1} pyridostigmine (i.p., −60 min), 20 mg kg^{-1} atropine (i.m., +1 min), Four dosages of soman (s.c., 0 min).

Group 4 (P+A+pD): 0·1 mg kg^{-1} pyridostigmine (i.p. −60 min), 20 mg kg^{-1} atropine (i.m., +1 min), 8·4 mg kg^{-1} pro-diazepam (i.m., +1 min), four dosages of soman (s.c., 0 min).

Two groups of animals were treated for three days with diazepam and thereafter challenged with soman and additional antidotes. Diazepam was given (i.p.) 2·5 mg kg^{-1} at 09.00 and at 15.00 h, daily.

Group 5 (P+A+3×D): 3×2×2·5 mg kg^{-1} diazepam (i.p., −60 min, last injection), 0·1 mg kg^{-1} pyridostigmine (i.p., −60 min), 20 mg kg^{-1} atropine (i.m., +1 min), Four dosages of soman (s.c., 0 min).

Group 6 (P+A+3×D+pD): 3×2×2·5 mg kg^{-1} diazepam (i.p., −60 min, last injection), 0·1 mg kg^{-1} pyridostigmine (i.p., −60 min), 20 mg kg^{-1} atropine (i.m., +1 min), 8·4 mg kg^{-1} pro-diazepam (i.m., +1 min), four dosages of soman (s.c., 0 min).

Four guinea-pigs and at least four doses of soman with a constant ratio between the dose levels were used. The mortality was recorded 24 h after soman administration.

Blood sampling

Four different protocols were used. Protocol 1: animals were given diazepam (5 mg kg^{-1} i.p.). Protocol 2: pro-diazepam (8·4 mg kg^{-1} i.m.) 1 h after diazepam (5 mg kg^{-1} i.p.). Protocol 3: diazepam (2×2·5 mg kg^{-1}, i.p. for three days) or Protocol 4: pro-diazepam (8·4 mg kg^{-1} i.m.) 1 h after a three days diazepam (2×2·5 mg kg^{-1}) treatment.

Animals were made unconscious by a blow to the head and a blood sample withdrawn from the heart. Serum and blood was separated by centrifugation. The serum was transferred to separate tubes and further processed for chemical analysis.

Gas chromatographic analysis of diazepam and des-methyldiazepam

Standard and calibration solutions. Stock solutions, 1 mg mL^{-1}, of diazepam, des-methyldiazepam and the two internal standards, Ro 7-9957 and Ro 7-9749, were prepared in methanol and were stored in a freezer. Stock solutions were diluted with xylene for direct gas chromatographic injection or with methanol before adding to serum.

Fresh calibration solutions were prepared daily, by adding 5–500 ng (a maximum volume of 10 μL) each of diazepam and des-methyldiazepam in methanol to 1·0 mL serum. These standards were then extracted according to the same procedure as the samples.

Extraction procedure

To 1·0 mL of serum, or of serum with added standard, was added 6·0 mL of benzene, containing 0·2 μg mL^{-1} each of the internal standards Ro 7-9957 and Ro 7-9749. The mixture was vigorously shaken in a Vortex apparatus for 1 min and then centrifuged (Wifug-X) for 10 min at 4000 rev min^{-1} to separate the two phases. A sample of 5·0 mL of the organic phase was evaporated under nitrogen at 50–60°C and the residue dissolved in 100 μL of xylene. The recovery was approximately 90% for both diazepam and des-methyldiazepam.

At least one blank was prepared daily, by working up blank serum according to the above extraction procedure. The blanks did not contain any impurities which could interfere with the diazepam/des-methyldiazepam quantification.

Analytical method

The samples were analysed on an HP 5880 gas chromatograph, equipped with a nitrogen-phosphorous sensitive detector. A fused silica column, OV-101, Hewlett Packard, Avondale, PA, 17·5 m × 0·2 mm with a 0·25 μm methylsilicon phase (not crosslinked), with a 1·0 m retention gap at the injector end, was used. A volume of 1·0 μL was injected splitless (splitless period 1·0 min) at an injector temperature of 250°C. The three level oven temperature was programmed as follows: Level 1: 110°C for 2 min, 25° min^{-1} to 262°C, 262°C for 0·5 min, Level 2: 2° min^{-1} to 264°C, Level 3: 10° min^{-1} to 275°C, 275°C for 2 min. The detector temperature was 280°C. The results were corrected for extraction recovery and possible matrix effects, using the standard samples.

Results

The acute toxicity, 24 h, of soman (s.c.) was assessed in guinea-pigs given various antidotal treatments (Fig. 1). The experiments showed pyridostigmine and atropine to give significant protection against soman intoxication. They also showed diazepam, given in addition to pyridostigmine and atropine, to provide additional protection. The i.p. injection of diazepam 1 h before soman, resulted in the most reproducible protection. The protection provided by an i.m. injection of pro-diazepam, 1 min post soman exposure, was less reproducible and not significantly different from that given by pyridostigmine and atropine alone. Pro-diazepam given after the soman also provided less protection than diazepam given 1 h before. The most effective protection was provided when animals treated with diazepam for three days were given pyridostigmine and atropine in combination with the soman challenge (See Materials and Methods for the

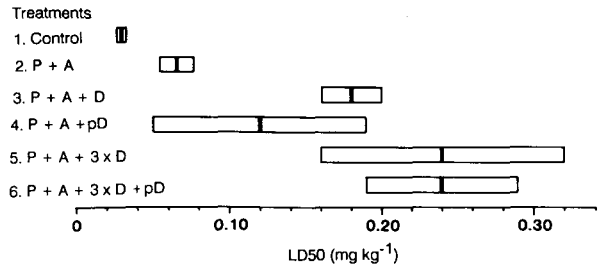

FIG. 1. The acute toxicity of soman is illustrated as a function of various treatments (1–6). For details on the treatment see Materials and Methods. Soman was given s.c. and survival after 24 h was recorded. LD50 values according to Weil (1952) ± their interval of 95% confidence are given.

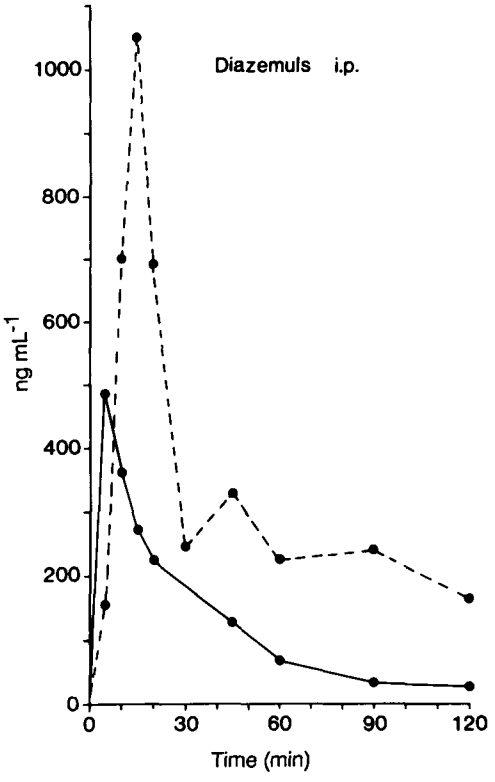

FIG. 2. Diazepam (●—●) and des-methyldiazepam (●---●) in guinea-pig serum following an i.p. injection of 5 mg kg^{-1} Diazemuls. Points represent the mean from 2–4 animals.

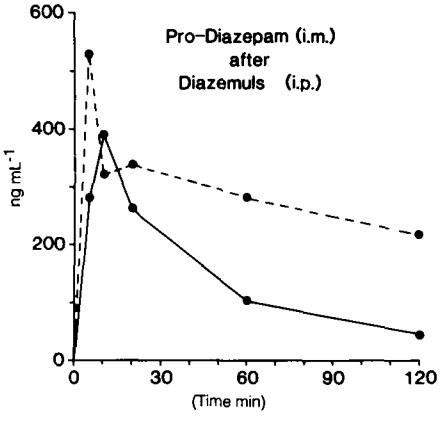

FIG. 3. Diazepam (●—●) and des-methyldiazepam (●---●) in guinea-pig serum after i.m. injection of 8·4 mg kg^{-1} pro-diazepam given 1 h after an i.p. injection of Diazemuls 5 mg kg^{-1}. Points represent the mean from two animals each.

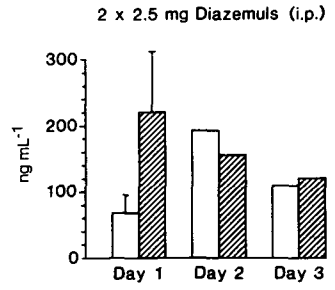

FIG. 4. Diazepam (open columns) and des-methyldiazepam (hatched columns) in guinea-pig serum 1 h after daily injections (i.p.) of 2 × 2·5 mg kg^{-1} of Diazemuls. The values represent the mean ± s.d. from two and four animals, respectively.

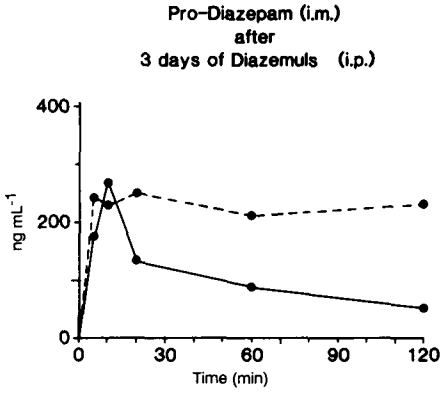

FIG. 5. Diazepam (●—●) and des-methyldiazepam (●---●) in guinea-pig serum following an i.m. injection of pro-diazepam (8·4 mg kg^{-1}). The animals were given 2 × 2·5 mg Diazemuls (i.p.) for three days, pro-diazepam was given 60 min after the last injection of Diazemuls. Points represent the mean from two animals each.

schedule). No further protection was achieved when a therapeutic pro-diazepam treatment was added to the "diazepam pre-treatment protocol".

Serum concentrations of diazepam and des-methyldiazepam in animals given an i.p. injection of diazepam or an i.m. injection of pro-diazepam or both are presented in Figs 2–4.

Diazepam was rapidly absorbed into the circulation and metabolized to des-methyldiazepam following its i.p. injection (Fig. 2). Significant values of the active substances were present still after 60 min.

Pro-diazepam was also rapidly absorbed into the circulation and metabolized to diazepam and des-methyldiazepam, following its i.m. injection (Fig. 3). Pro-diazepam was given 60 min after the i.p. injection of diazepam as we wanted to compare the metabolism of pro-diazepam before (Fig. 3) and after (Fig. 5) a three day treatment with diazepam, without using more animals. This means that some diazepam and des-methyldiazepam was already present in the serum as pro-diazepam was injected. In spite of that, the des-methyldiazepam levels initially achieved were not as high after i.m. pro-diazepam as after i.p. diazepam injection. At a later stage, however (60 and 120 min), the des-methyldiazepam and diazepam levels remained higher in animals that received i.m. pro-diazepam and i.p. diazepam compared with those that received only i.p. diazepam.

Some guinea-pigs were treated with diazepam (2 × 2·5 mg) daily for three days and thereafter given an i.m. injection of pro-diazepam. During the three day treatment, serum levels of diazepam and des-methyldiazepam were measured daily (Fig. 4). Samples were taken 60 min after the i.p. injection of diazepam. On day two and three the difference between diazepam and des-methyldiazepam observed on day one was no longer present, i.e. the levels of diazepam were increased and the levels of des-methyldiazepam decreased.

Pro-diazepam was injected 60 min following the last diazepam treatment. The serum levels of diazepam and des-methyldiazepam were followed for 120 min. The initial rise in

serum levels of diazepam and especially of des-methyldiazepam was less pronounced (Fig. 5), compared with that obtained after the i.p. injection of diazepam (Fig. 2). This is similar to the case when pro-diazepam was given 60 min after the single i.p. injection of diazepam (Fig. 3). The metabolism of diazepam and des-methyldiazepam seems to occur at the same rate in animals treated with diazepam for three days (Fig. 5) as in animals treated only once with diazepam (Fig. 3).

Discussion

Diazepam, like other benzodiazepines, has a wide spectrum of effects. When used as an adjunct antidote against organophosphorus intoxication, it is normally referred to as an anticonvulsant and many clinicians consider it the first choice for the initial control of seizures (see Haig & Feely 1988). It was also successfully tried as an adjunct antidote in the organophosphorus intoxication (Lipp 1972; Johnson & Lowndes 1974; Johnson & Wilcox 1975).

There has been some controversy about the relation between the serum concentration of diazepam, its active metabolites and their pharmacological effects (Kanto et al 1979). In spite of this, Fink et al (1976) demonstrated a linear relation between the amount of EEG activity and serum diazepam concentration, while Paul et al (1979) demonstrated a relation between benzodiazepine receptor occupancy and the anticonvulsant effect of diazepam.

In the present study we have tried to correlate the efficacy of diazepam as an adjunct antidote to pyridostigmine and atropine with its serum level in the guinea-pig. This species was chosen, since it has been advocated that organophosphorus intoxication, as well as antidotal treatment, in the guinea-pig closely resembles that seen in primates (rhesus monkey) compared with mice and rats (Inns & Leadbeater 1983).

The pharmacokinetics of diazepam in the guinea-pig have to some extent been investigated (see Garattini et al 1973). The initial metabolism of diazepam in the guinea-pig is dominated by N_1-demethylation whereas in man C_3-hydroxylation is also significant. Further, the binding of desmethyldiazepam to plasma proteins is lower in the guinea-pig than in man (Klotz et al 1979).

The time-course profiles reported (Fig. 2) following the i.p. injection of diazepam, are as might be expected considering that uptake into blood probably involves a first pass of the liver. The serum values of diazepam and des-methyldiazepam following the i.m. injection of pro-diazepam (Fig. 3) resembles those obtained after the i.p. injection of diazepam, i.e. relatively high levels of des-methyldiazepam were present. The high extent of metabolism encountered should be viewed from the background that high peptidase activity is necessary for the conversion of pro-diazepam into diazepam in the liver, i.e. diazepam is formed in the same compartment as where it is mainly metabolized (Fujimoto et al 1980a). Serum levels of diazepam and des-methyldiazepam recovered from animals given diazepam for three days showed a tendency towards an increased concentration of diazepam and a decreased concentration of des-methyldiazepam on days 2 and 3. (Fig. 4). If the three days exposure to diazepam in any way induced changes in the metabolism of diazepam, these were not significant enough to result in an altered time-course for serum levels of diazepam and des-methyldiazepam following the i.m. injection of pro-diazepam (Fig. 5).

The additional protection provided against acute soman toxicity by diazepam in this study (Fig. 1), corroborate with previous observations (e.g. Inns & Leadbeater 1983). Only in the experiment where pro-diazepam was given 1 min after the soman injection, was the protection achieved not significantly different from that obtained when pyridostigmine and atropine were given alone. The large variation encountered in the experiments with pro-diazepam, probably reflects variations in the time-course of intoxication. The first sign of symptoms appear between 30 and 60 s after soman administration so there may be considerable variations in the circulatory status at the time of pro-diazepam injection.

We also attempted to correlate the additional protection provided by diazepam, when added to the pyridostigmine/atropine pre-treatment, with the serum levels of diazepam achieved in these treatments. The protection ratio (LD50 treated/LD50 control), for treatment 2, 3 and 5 (Fig. 1) correlated linearly with the serum content of diazepam, 60 min after an i.p. injection (Fig. 2, 5), $r = 0{\cdot}9898$. Since this analysis involves both animals given diazepam acutely and for three days, this correlation may indicate that diazepam provides as much protection after a three day treatment as when given by a single i.p. injection, i.e. there is no "desensitization" or development of tolerance during this short treatment.

In conclusion, using the guinea-pig we have confirmed the usefulness of diazepam as an adjunct antidote to pyridostigmine and atropine in organophosphorus intoxication. We suggest that pretreatment with diazepam, for up to three days, gives a "safer" protection, than an equimolar dose of pro-diazepam given therapeutically. Although our investigation indicates that even higher doses of diazepam may provide better protection, its side effects, such as drowsiness or an impaired motor function, will become a dose-limiting factor in man.

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