ABSTRACT:
The distribution, metabolism, and clearance of trimethylolpropane phosphate (TMPP), a potent, bicyclophosphate, \( \gamma \)-aminobutyric acid-ergic convulsant, were studied in male Fischer-344 rats. Intraperitoneal administration of TMPP was compared with oral gavage with respect to rates of absorption, distribution, and clearance. Distribution of TMPP to major body tissues was evaluated for the first 24 hr after administration or, in the case of regional brain distribution, immediately after the first TMPP-induced clinical seizure. Samples purified from the urine, feces, and bile of rats exposed to TMPP, as well as from rat liver microsomes incubated with TMPP in vitro, were analyzed for possible phase I and phase II metabolism, using HPLC. The disposition and clearance of TMPP in the blood and major body tissues were measured. TMPP was found to be well distributed to highly vascularized tissue compartments, with little retention >24 hr after administration. TMPP was eliminated through the urine and feces as the parent compound, with no evidence of phase I or phase II metabolism. TMPP was rapidly cleared from the blood during the first 30 min after exposure, with slower clearance of >87% of the drug during the following 8-hr period and >99.5% clearance by 100 hr after injection. Repeated daily exposure to TMPP for up to 5 successive days resulted in no measurable accumulation in the brain or other major tissue compartments. Possible mechanisms for TMPP-induced, short- and long-term, neurobehavioral modulation are discussed.

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1 Abbreviations used are: MIL-L-23699, Military Specification L-23699 hydraulic fluid; GABA, \( \gamma \)-aminobutyric acid; GTCS, generalized tonic-clonic seizure(s); CNS, central nervous system; TMPP, trimethylolpropane phosphate; PIC, picrotoxin; PTZ, pentylenetetrazol; PE, polyethylene.
solvents used for HPLC analysis were obtained from Fisher Scientific Co. (Pittsburgh, PA). All other chemicals used in this study were of analytical grade and were obtained from Sigma.

Animals. Eighty-four male Fischer-344 rats, weighing 250–300 g at the time of testing, were acquired from Charles River Breeding Laboratories (Raleigh, NC). Animals were individually housed in polycarbonate, shoebox, double-sized, hanging cages. Fresh pelleted food (type 5008; Purina Mills, St. Louis, MO) and conditioned (by reverse osmosis) water were available ad libitum. Room air temperature and humidity were maintained at 23°C (±10%) and 55% (±10%), respectively. Rodent cage racks were located within air mass displacement units that provided a constant supply of High-Efficiency Particulate Absolute (HEPA)-filtered air. Electronically controlled, multispectrum, fluorescent light was provided on a 12/12-hr light/dark cycle. For the oral treatment, rats were transferred to metabolism cages, wherein urine and feces were collected for determination of elimination. Immediately after sacrifice, rats were deprived of food for the 16 hr before experimental procedures. For metabolism studies, rats were transferred to metabolism cages (Sigma-Aldrich Research Co., St. Louis, MO) for the collection of urine and feces.

Tissue Distribution Experiments. Forty-two rats were injected ip with 1 μCi of [14C]TMPP (0.075 mg, approximately 0.25 mg/kg), a dose insufficient to induce GTCS. During the first 6 hr after injection, groups of six rats each were sacrificed at 60-min intervals. The remaining six rats were sacrificed 24 hr after TMPP administration. To determine the regional distribution in the brain, eight additional rats were administered a convulsive dose of 2 μCi of [14C]TMPP ip (0.15 mg, approximately 0.5 mg/kg) and were sacrificed by decapitation as soon as GTCS were observed (4–20 min after administration). To assess possible tissue accumulation of TMPP after repeated administrations, 15 additional rats were given 2 μCi of [14C]TMPP daily for 1–5 consecutive days. Groups of three rats each were sacrificed 24 hr after either one, two, three, four, or five daily TMPP administrations. After drug administration, the rats were housed in metabolism cages, wherein urine and feces were collected for determination of elimination. Immediately after sacrifice, liver, spleen, kidney, heart, a peritoneal fat sample, and five brain regions (cerebellum, brainstem, hippocampus, striatum, and cerebral cortex) were dissected and homogenized in cold normal saline solution (10%, w/v) for 3 min, using a tissue homogenizer (model PT-10; Brinkman, Luzern, Switzerland). An aliquot of 1 mL, combined with 15 mL of scintillation cocktail (ScintiVerse; Fisher Scientific), was counted in a liquid scintillation counter (model 1600-TR; Packard Co., Downers Grove, IL). After quench correction, results were computed as nanograms of TMPP per gram of wet weight.

Metabolic Disposition Experiments. Biliary Excretion. Isolated, perfused, liver tissue specimens, prepared according to the method previously described (Miller et al., 1951; Wyman et al., 1995), were used to determine the biliary excretion of TMPP and possible metabolites. The experiment was repeated five times, with liver preparations from five rats. Each liver was surgically exposed during deep diethyl ether anesthesia, and the common bile duct was cannulated with PE-240 tubing filled with the perfusate, and an outflow cannula was inserted into the right atrium. The liver was then removed with the diaphragm and placed in a container filled with warm saline solution. The preparation was placed in an humidified atmosphere (95% O2/5% CO2 mixture) and was perfused with Krebs-Henseleit-Ringers solution (118 mM NaCl, 4.7 mM KCl, 10 mM CaCl2·10H2O, 1.2 mM MgSO4·7H2O, 1.2 mM KH2PO4, 1.2 mM NaHCO3, 11.5 mM dextrose, pH 7.4), at the rate of 25 mL/min, at 37°C. The perfusate was supplemented with 5 mM sodium taurocholate to maintain bile flow throughout the perfusion period. After an equilibration period of 30 min, 1 μCi of [14C]TMPP was added to the perfusate. The effluent from the outflow cannula was continuously returned to the reservoir. Perfusion of the liver in the closed perfused system was continued for a 1-hr period. The viability of the preparation during this period was determined by measuring the oxygen consumption of the liver. Pooled samples were kept frozen for subsequent analysis of bile content.

In Vivo Incubation of Liver Microsomes. Liver microsomes from six rats were prepared using the standard isolating procedure (Lowry et al., 1951; Cinti et al., 1972). Each liver was removed immediately after sacrifice, minced, and homogenized in a 20% (w/v) mixture of 10 mM Tris-HCl containing 250 mM sucrose (pH 7.4), using six strokes of a motor-driven homogenizer (PowerGen; Fisher Scientific). Nuclei and cell debris were removed by centrifugation at 100,000g for 20 min. In an ice bath, CaCl2 was added to the postmitochondrial supernatant to achieve a final concentration of 8 mM. Microsomes were pelleted by centrifugation at 25,000g for 30 min and were washed twice with 150 mM KCl in 10 mM Tris-HCl (pH 7.4). Protein concentrations were estimated by the method of Lowry et al. (1951), using bovine serum albumin as the standard. The preparation was carried out at 4°C. [14C]TMPP (1 μCi, 50 mg), in an assay volume of 800 mL containing 250 mL of sodium phosphate (0.4 M, pH 7.4), 50 mL of NAD (10 mM), 50 mL of NADP (10 mM), 50 mL of ATP (50 mM), 50 mL of glucose-6-phosphate (50 mM), 30 mL of MgCl2 (0.1 M), glucose-6-phosphate dehydrogenase (20 units), and 100 mL of microsomes (50 mg of protein/mL) was incubated at 37°C for 60 min in a metabolic shaker. At the end of the incubation period, the reaction was stopped by transfer to a boiling water bath for 5 min. The precipitated protein was centrifuged in a microcentrifuge (Beckman, Fullerton, CA), and the supematant was stored frozen for further analysis.

HPLC Analysis. Urine and feces samples from rats used in the clearance and excretion studies described above were collected, pooled, and stored at −20°C until processing for HPLC analysis. Urine was centrifuged at 12,000g for 30 min. The supernatant was lyophilized, extracted with chilled methanol, maintained at −70°C for 30 min before centrifugation at 12,000g for 30 min, and dried in a vacuum. The dried material was extracted with a minimal amount of water, treated with activated charcoal, and centrifuged at 12,000g for 30 min. The supernatant was filtered through a 0.45-μm filter, dried in an evaporator (model SC 110; Speed Vac, Farmingdale, NY), reconstituted in a minimal amount of water, and analyzed using HPLC. Feces were weighed and homogenized in distilled water. The fecal homogenates were processed identically to the urine samples with the exception that, before charcoal treatment, feces samples were passed through three, serially arranged, C18 columns (catalog number 51910; Sep-Pak, Waters Associates, Milford, MA) and washed with water until no radioactivity was detected in the elutriate. The samples were separated on an anion exchange column (150 × 4.5 mm; particle size, 10 μm) using an HPLC system (Beckman). Samples were isocratically eluted with a mixture of sodium bicarbonate (2.8 mM) and sodium carbonate (2.2 mM), at a flow rate of 1 mL/min (3000 pounds/ft²), at ambient temperature. Fractions were collected at the rate of 1 mL/min, a 100-μl aliquot was mixed with 15 mL of scintillation cocktail and counted.

Clearance and Excretion Experiments. Clearance from the blood. In the excretion in the urine and feces were measured after ip or oral gavage administration of TMPP. Three rats were used for each route of administration. [14C]TMPP at a dose level of 1 μCi (approximately 0.25 mg/kg) was given ip or by oral gavage using PE-100 tubing, followed by 5 mL of water. After drug administration, rats were housed in metabolism cages for collection of urine and feces. A small incision was made at the base of the tail, allowing periodic withdrawal of 20 μl of blood using an Eppendorf pipette with a heparinized tip. Samples were drawn at 10-min intervals for the first 1 hr after drug administration and then were drawn every 30 min for up to 6 hr. After 6 hr, rats were transferred to metabolism cages, where they remained housed for 3 days, with ad libitum access to food and water. Urine and feces were collected when available, and the time of collection, sample weight, and body weight were recorded. Urine and feces samples were processed as previously described.

Statistics. Statistical comparison of the differences between means of treatment groups was accomplished using one-way analysis of variance, followed by the Levene test of homogeneity and the post hoc Bonferroni t test, as appropriate. Differences between means were considered to be statistically significant at p < 0.05. Graphical representations of group means include SE bars.

Results. The clearance of [14C]TMPP from the blood after a single systemic (ip) administration is depicted in fig. 1. TMPP was absorbed from the peritoneal cavity, reaching a peak blood concentration of 1.6 μg/mL of blood 20 min after injection, and was cleared from the blood to a concentration of 0.1 μg/mL of blood within 35 min after administration. During the next 5 hr, TMPP concentrations in the blood remained relatively constant, at approximately 0.1 μg/mL.
Fig. 1. $^{14}$C]TMPP concentrations in the blood during the first 6 hr after a single ip administration of 1 μCi (0.075 mg).

Fig. 2. Clearance of $^{14}$C]TMPP (nanograms per gram of wet weight) in liver, spleen, kidney, heart, brain, and peritoneal fat during the first 6 hr after ip administration of 1 μCi (0.075 mg).

Fig. 3. $^{14}$C]TMPP concentrations (nanograms per gram of wet weight) in the whole brain and cerebellar, striatal, hippocampal, cortical, and brainstem regions during the first GTCS induced by 2 μCi of $^{14}$C]TMPP (0.15 mg).

Fig. 4. Clearance of $^{14}$C]TMPP (nanograms per gram of wet weight) in liver, spleen, kidney, heart, brain, and peritoneal fat during the first 6 hr after ip administration of 1 μCi (0.075 mg).

Fig. 5. Cumulative concentrations of $^{14}$C]TMPP detected in the analyzed tissue compartments of five different rat groups (N = 3) sacrificed 24 hr after the series of five daily injections.

Discussion

Although systemically administered GABAergic convulsants (PTZ or PIC) are routinely used in animal studies for the investigation of neural mechanisms of epileptogenesis or preclinical trial evaluation of antiepileptic drugs, limited information exists concerning their pharmacokinetic and pharmacodynamic properties. In an evaluation of the short- and long-term neurotoxicity risks from single or repeated exposures to the GABAergic bicyclophosphate convulsant TMPP, pharmacokinetic and pharmacodynamic profiles, after two routes of administration, were developed for Fischer-344 rats.

Because the compound is water soluble, with limited lipophilic properties (Gearhart et al., 1990a, 1990b; Narayanan and Carpenter, 1993), TMPP can be administered ip, dermally, or by oral gavage. When administered ip, GTCS were observed within 4–20 min after administration of TMPP doses of ≥0.40 mg/kg (Higgins and Gardier, 1990; Rossi et al., 1990, 1993) in every male or female Fischer-344, Sprague-Dawley, or Long-Evans rat challenged.

Within 20 min after ip injection of 1 μCi, $^{14}$C]TMPP reached maximal concentrations in the blood (approximately 150 ng/ml); within 30 min, 13% was found to be cleared through the urine. Thirty

The clearance of TMPP through the excreted urine and feces after a single ip dose of 1 μCi of $^{14}$C]TMPP is shown in fig. 4. Within 30 min after administration, approximately 20% of the total administered $^{14}$C]TMPP dose was recovered from the excreted urine. Within 30 hr after injection, approximately 83% of the total administered TMPP was accounted for in the excreted urine and feces. Within 100 hr after administration, >99.5% of the administered TMPP was recovered.

Examination of HPLC elution profiles indicated that the $^{14}$C]TMPP standard eluted as a major peak (corresponding to fractions 19–23) and a minor peak (corresponding to fractions 5–7). Samples purified from urine, feces, and bile from TMPP-treated rats and from in vitro incubations of TMPP with microsomes exhibited peaks identical to those of the standard, suggesting that TMPP undergoes no in vivo metabolic transformation.

Determination of blood concentrations of $^{14}$C]TMPP after a single oral gavage administration of TMPP (1 μCi) revealed that $^{14}$C]TMPP reached a concentration of 100 μg/ml of blood within 15 min after oral gavage, with a mean concentration that remained relatively constant throughout the next 6 hr. Within 72 hr after gavage, >99.5% of the TMPP dose was recovered in the excreted urine and feces.
minutes after administration, the highest concentration in the analyzed tissue compartments was found in the kidneys, with lesser but approximately equal concentrations detected in the brain, liver, spleen, and heart. Peritoneal fat exhibited the lowest deposited concentration of TMPP, a result consistent with the lipophilicity of the compound. After reaching maximal compartmental concentrations, TMPP tissue levels remained relatively constant for the first 6 hr after administration.

The TMPP serum half-life (i.e. the time required for the blood concentration to decline to 50% of the maximal measured concentration) was calculated to be approximately 20 min. These results approximate those previously reported for PIC (Ko and Husein, 1971; Hun, 1976; Razman and Levy, 1985) but differ from those reported for PTZ (Soto-Otero et al., 1989), which exhibits a substantially longer serum half-life. This longer blood retention may partially account for the observed 10-fold difference in the concentration of PTZ necessary to induce GTCS, in comparison with TMPP.

Intraperitoneally administered [14C]TMPP was found to be eliminated through the urine (>90%), feces (9–10%), and liver bile (<1%), indicating the urine as the major route of clearance. Clearance routes and times for TMPP approximate those reported for PTZ (Ko and Husein, 1971; Hun, 1976; Razman and Levy, 1985; Loscher et al., 1991) and PIC (Soto-Otero et al., 1989), suggesting that, although the three compounds share similar degrees of lipophilicity and tissue retention, pharmacological potency is not directly related to the overall terminal half-lives of the compounds.

Clearance of [14C]TMPP was also investigated after oral gavage. Although significant amounts of TMPP were observed in the serum during the first 15–20 min after gavage, these concentrations were approximately 5% of those observed during a similar time period after ip administration. Additionally, serum TMPP concentrations after oral gavage did not exhibit the rapid decline to near-baseline levels observed after ip administration but remained relatively stable for the first 6 hr after administration. Similar to the observations for ip injection of TMPP, the drug exhibited nearly complete clearance within 72 hr after gavage administration, with the primary route of excretion remaining the urine.

The repeated administration (generally every 48–72 hr) of subconvulsive doses of a number of GABAergic convulsants (e.g. PTZ, PIC, and endosulfan) has been shown to induce GTCS within 10 weeks (Mason and Cooper, 1972; Ono et al., 1990; Gilbert, 1992), although initial administrations produced negligible convulsive effects (chemical kindling paradigm). In this laboratory, however, we found that TMPP (0.25–0.275 mg/kg) administered ip three times each week for 10 weeks did not induce kindling of GTCS in adult male Fischer-344 or Sprague-DawleyCD rats (Rossi et al., 1994). The pharmacological basis of chemical kindling has yet to be definitively established, although it appears possible that tissue accumulation of the convulsant compound may be a contributing factor. To investigate the possibility of tissue accumulation of TMPP, rats in the present study were administered a convulsive dose of [14C]TMPP for 1–5 consecutive days and then were evaluated for TMPP accumulation in major tissue compartments, including the brain, 24 hr after each administration. Although there was some variance in tissue compartment levels of TMPP after two to four administrations, there was no significant difference in TMPP concentrations 24 hr after the final (fifth) TMPP injection, compared with levels detected 24 hr after the first administration. Although these results do not conclusively demonstrate that chemical kindling is dependent on tissue accumulation of convulsants, they may partially explain our previous failure to chemically induce kindling using repeated TMPP injections. Interestingly, pharmacokinetic studies involving multiple administrations have not been reported for convulsants that have been successfully used to induce chemical kindling of GTCS.

Finally, Rossi et al. (1995) demonstrated long-term behavioral sensitization after one to four doses of TMPP, as measured by test paradigms including schedule-induced polydipsia, appetitive approach behavior, and juvenile play behavior. Because TMPP has been hypothesized to exert its short-term pharmacological effects (i.e. electroencephalographic paroxysms and behavioral convulsions) through modulation of the GABA inhibitory system (Bowery et al., 1976; Higgins and Gardier, 1990), but the parent compound was eliminated from the brain within 100 hr, it seems apparent that any long-term consequences of acute TMPP exposure are unrelated to the persisting presence of the parent compound in the CNS. The inability to identify any metabolic conversion of TMPP in the present study complicates elucidation of the neural mechanisms producing the previously reported long-term effects on neurobehavioral capacity. Lindsey et al. (1998), perfusing TMPP directly into the nucleus accumbens of rats, reported significant decreases in extracellular levels of dopamine and

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**Fig. 4.** Clearance of [14C]TMPP in the excreted urine and feces of rats during the 6 days (144 hr) after ip injection of 1 μCi (0.075 mg).

**Fig. 5.** Relative presence/accumulation (nanograms per gram of wet weight) of [14C]TMPP in liver, kidney, brain, spleen, heart, and peritoneal fat 24 hr after the last of one to five daily ip doses of 2 μCi (0.15 mg).
norepinephrine in the nucleus accumbens that were temporally consistent with the onset of significant changes in neurobehavioral activity that persisted beyond the clearance of TMPP from the brain. This finding implicates TMPP in the long-term modulation of monoamine systems. Similarly, it was shown by Lin et al. (1998) that repeated systemic administration of subconvulsive doses of TMPP before daily, nonkinding, electrical stimulation of the amygdala resulted in long-term sensitization of several limbic pathways, as measured by enhanced evoked responses to electrical stimulation within those systems. It appears, that although TMPP exerts its acute convulsive effects by direct modulation of the GABA system, its long-term sensitization effects may reflect indirect but persistent modulation of non-GABA neurotransmitter systems. The pharmacokinetic profile of TMPP presented in this report may prove useful both in identifying the mechanisms of the acute neurotoxicity of convulsants and in further elucidating the mechanisms of such poorly understood phenomena as long-term sensitization of the CNS, chemical kindling, and epileptogenesis.

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References


