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Acute Methanol Toxicity in Minipigs[1]

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The pig has been proposed as a potential animal model for methanol-induced neuro-ocular toxicosis in humans because of its low liver tetrahydrofolate levels and slower rate of formate metabolism compared to those of humans. To examine the validity of this animal model, 12 4-month-old female minipigs (minipig YU) were given a single oral dose of water or methanol at 1.0, 2.5, or 5.0 g/kg body wt by gavage (n = 3 pigs/dose). Dose-dependent signs of acute methanol intoxication, which included mild CNS depression, tremors, ataxia, and recumbency, developed within 0.5 to 2.0 hr, and resolved by 52 hr. Average maximum methanol concentrations in plasma, of 3100 ± 700 (SD), 6200 ± 2300, and 15,200 ± 900 μg/ml were reached within 0.5 to 4 hr following methanol administration in animals given 1.0, 2.5, or 5.0 g methanol/kg, respectively. The mean initial elimination half-lives of methanol were 9.0 ± 1.6, 22.4 ± 6.1, and 18.9 ± 4.3 hr, for 1, 2.5, and 5.0 g/kg doses, respectively. In 3 minipigs, a transient increase in plasma formate concentration (1.74–3.40 mEq/liter vs control = 0.5 ± 0.3 mEq/liter) occurred 4 to 30 hr following methanol administration. Methanol- and formate-dosed pigs did not develop optic nerve lesions, toxicologically significant formate accumulation, or metabolic acidosis. Based on results following a single dose, female minipigs do not appear to be overtly sensitive to methanol and thus may not be a suitable animal model for acute methanol-induced neuro-ocular toxicosis.

Methanol exposure may result in neurobehavioral (Infurna and Weiss, 1986), teratological (Infurna and Weiss, 1986; Nelson *et al.*, 1985), neurodevelopmental (Nelson *et al.*, 1985), reproductive (Cooper *et al.*, 1992), and neuro-ocular effects. The toxicity of methanol in humans is characterized by central nervous system depression, weakness, headache, vomiting, severe metabolic acidosis (McMartin *et al.*, 1980; Clay *et al.*, 1975), optic disc edema (Hayreh *et al.* 1977), and bilateral necrosis of the putamen (Koopmans *et al.*, 1988; Ley and Gali, 1983; Sharpe *et al.*, 1982). Clinical consequences of these lesions, which typically occur following a single accidental or intentional ingestion of methanol, are blindness or Parkinsonian-like motor disease (Ley and Gali, 1983).

In nonhuman primates, methanol is metabolized to formaldehyde predominantly by hepatic alcohol dehydrogenase (McMartin *et al.*, 1975). In nonhuman primates, formaldehyde has also been shown to be reactive with a half-life in blood on the order of minutes, and is not thought to be directly involved in the production of methanol toxicosis (McMartin *et al.*, 1979). Formaldehyde is metabolized by a glutathione-mediated pathway, involving formaldehyde dehydrogenase, to formic acid, which at physiological conditions dissociates to formate and hydrogen ions (Tephly and McMartin, 1984). Although methanol may have direct toxic effects, formate is considered to be the toxic metabolite of methanol, and the accumulation of formic acid with the subsequent development of metabolic acidosis and blindness is characteristic of methanol poisoning in sensitive species (e.g., humans, nonhuman primates) (McMartin *et al.*, 1977, 1980). Furthermore, administration of formic acid to monkeys reproduces optic disc edema with the loss of the papillary light reflex, features that are also characteristic of methanol toxicity in humans (Martin-Amat *et al.*, 1978). Conversely, in resistant species (e.g., rats, mice), methanol administration does not result in formate accumulation, metabolic acidosis, or blindness (Tephly and McMartin, 1984).

The rate at which methanol-derived formate accumulates to toxic levels following methanol exposure is primarily influenced by the rate of formate metabolism. Formate metabolism is dependent upon the activities of formyltetrahydrofolate synthetase as well as methenyltetrahydrofolate dehydrogenase and their cosubstrate, tetrahydrofolate (Black *et al.*, 1985; Johlin *et al.*, 1987). Susceptible species appear to have lower liver tetrahydrofolate concentrations, slower formate metabolism, and thus increased sensitivity to methanol when compared to resistant species such as

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rats. Like humans, young swine and micropigs have low hepatic tetrahydrofolate concentrations and appear to metabolize formate at a rate slower than that observed in rats (Makar *et al.*, 1990; Tephly *et al.*, 1992).

It is predicted that the proposed heavier reliance on methanol-based automotive fuels will result in an increased incidence of blindness and deaths in people resulting from accidental methanol ingestion (Litovitz, 1988). The animal models available to study methanol-induced neuro-ocular toxicity have been limited to nonhuman primates (Martin-Amat *et al.*, 1977) and to monkeys and rodents made folate-deficient by nutritional or pharmacologic manipulation (McMartin *et al.*, 1977; Eells, 1991). Pigs represent an attractive animal model for the study of methanol-induced neurotoxicity. It would be predicted that pigs would accumulate formate following methanol exposure based upon their reported low levels of hepatic tetrahydrofolate concentrations and slow rate of formate metabolism (Makar *et al.*, 1990; Tephly *et al.*, 1992). The actual sensitivity of swine to methanol and methanol-induced neuro-ocular toxicity is unknown. This study was, therefore, performed to evaluate the Yucatan minipig as a potential nonprimate model for methanol neuro-ocular toxicosis by exploring the toxicity and pharmacokinetics of methanol and formate in these animals.

MATERIALS AND METHODS

Chemicals. Methanol (high-performance liquid chromatography grade) was obtained from Sigma Chemical Co. (St. Louis, MO). Sodium pentobarbital was purchased from Abbott Laboratories (North Chicago, IL), ketamine hydrochloride from Aveco (Fort Dodge, IA), halothane from Fort Dodge Laboratories (Fort Dodge, IA), and tropicamide from Alcon Laboratories (Ft. Worth, TX). Formate dehydrogenase was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Hog kidney acetone powder and all other chemicals were purchased from Sigma and were of the highest available purity.

Animals. Fourteen, 4-month-old, 8.5 to 13.0 kg, female minipigs (Minipig YU, Charles Rivers Breeding Laboratories, Wilmington, MA) were used. Minipigs were housed individually in raised stainless-steel runs with tenderfoot flooring. They were fed a commercially available, pelleted, pig and sow diet (Wayne Feed Division, Chicago, IL) meeting National Research Council nutritional specifications for swine. Food and water were available *ad libitum* except for the 12 hr immediately prior to surgery or methanol administration. A 12-hr light/dark cycle was provided throughout the study.

Catheter implantation. Following premedication with atropine (0.04 mg/kg, sc), ketamine (10 mg/kg, im), and xylazine (5 mg/kg, im), anesthetic induction was achieved with 1.5 to 2.0% halothane. Animals were maintained on 1 to 1.5% halothane in a closed circuit system. External jugular vein and carotid artery catheters were surgically implanted approximately 48 hr prior to methanol or formate administration using the methods described by Smith and coworkers (1989). A 75-cm long, single lumen, medical-grade silicone elastomere tube (Silastic, Dow Corning, Midland, MI) with an outside diameter of 2.2 mm and an inside diameter of 1.0 mm was used for all catheterizations. Catheters were exteriorized through a dorsal skin incision and placed into a vinyl pouch sutured to the skin (Wittry *et al.*, 1990).

Methanol administration. A single oral dose of methanol (20% v/v in sterile water) was given by gavage at 0, 1.0, 2.5, or 5.0 g/kg (n = 3 pigs per dose). Control animals were given a volume of water equivalent to the highest volume given to the methanol-dosed group.

Formate administration. To examine whether formate accumulation alone would result in neuro-ocular toxicosis, a formate buffer (sodium formate:formic acid, 10:1, 0.5 M, pH 7.4) was given (425 mg formate/kg) intravenously every 4 hr for 32 hr to two additional minipigs. The dose of formate given was anticipated to produce blood formate concentrations similar to those seen in formate-poisoned monkeys that subsequently developed neuro-ocular toxicity (Martin-Amat *et al.*, 1978).

Animal monitoring and assessment of toxicity. Before and each 24 hr after methanol dosing, funduscopic examinations were performed by indirect ophthalmoscopy after inducing pharmacologic mydriasis with topical tropicamide. In some pigs, retinal vascular permeability was also assessed 24 to 96 hr after methanol administration by conventional fluorescein angiography (Bellhorn, 1973). Angiographic findings were recovered on high-speed color film using a hand-held fundus camera (Kowa RC 2, Kowa, Japan). Animals were monitored continuously for the development of clinical signs during the first 8 hr after methanol or formate administration. They were subsequently observed for clinical signs at least every 4 hr for the first 48 hr after dosing and then every 8 hr until completion of the study. Serial neurologic and physical examinations were performed more frequently in animals that developed clinical signs.

Blood gas analysis. Routine arterial blood gas analyses were performed every 6 hr after methanol administration using a commercially available blood gas analyzer (IL 1306, Instrumentation Laboratories, Lexington, MS).

Methanol and formate pharmacokinetics. Heparinized venous blood (1 ml) was collected at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, 16, 32, and 64 hr after administration of methanol or formate. All samples were kept frozen until analyzed. Blood methanol concentration was determined by gas chromatography–flame ionization detection using the methods described by Pollack and Kawagoe (1991). A Hewlett-Packard 5880A gas chromatograph (Kennett Square, PA) equipped with a 30 m × 0.25 mm column (J&W DB-Wax, J&W Scientific, Folsom, CA) and a 1 m × 0.53 mm deactivated silica precolumn were used. Temperatures were as follows: injector, 70°C; detector, 325°C; column oven, 105°C. These conditions produced retention times of 1.9 and 2.1 min for acetonitrile (external standard) and methanol, respectively. Blood formate concentration was determined spectrophotometrically using the enzymatic method of Cook *et al.* (1991).

Liver (total) folate determination. Liver samples from control pigs were prepared as described by McMartin and coworkers (1981). Folates were assayed in their monoglutamate form after hydrolysis with hog kidney polyglutamate hydrolase prepared from hog kidney acetone powder (Lin and Lester, 1985). Total hepatic folate was determined using a commercially available homogenous enzyme immunoassay (Microgenics Corporation, Concord, CA) described by Khanna and coworkers (1989).

Pathology. Animals were euthanatized 10 days after methanol administration. Minipigs were tranquilized with chlorpromazine (0.3 mg/kg, iv) and anesthetized by the intravenous administration of pentobarbital (20.0 mg/kg). Following induction of deep anesthesia, a liver biopsy specimen was obtained from control minipigs through abdominal laparotomy. The liver wedge was frozen in liquid nitrogen and stored at −80°C until analyzed for folate. The aorta was then cannulated through the left ventricle with a 13-mm o.d. plastic catheter, and 2 liters of 0.9% saline containing 2000 IU heparin/liter at a temperature of 37°C were infused through the catheter by gravity at a pressure equivalent to 120–150 mm water. Blood and saline escaped from the vascular system through a 2- to 3-cm incision in the right auricle. When the escaping saline became clear, the infusion was changed to 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), also at 37°C. Each pig was perfused with 4.0 liters of this fixative. The total fixative perfusion time ranged from 20 to 35 min.

A necropsy was performed immediately on each fixed animal. The following tissues were collected for histologic examination: brain (cerebrum, cerebellum, and brain stem), spinal cord (cervical and lumbar intumescences), eye (optic nerve and retina), kidney, liver, lung, heart, adrenal, pancreas, and spleen. Tissues were stored overnight in 2.5% glutaraldehyde in 0.035 M phosphate buffer (pH 7.4) at 4°C, processed by standard procedures, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Data analysis. Because of the small sample size (n = 3 per dose group), no formal statistical analysis was performed. Data are reported as means ± SD. Peak plasma concentration (C_{max}) and time to peak plasma concentration (t_{max}) were determined by inspection of the methanol plasma concentration vs time results. The half-life ($t_{1/2}$) of methanol was estimated from the slope of the terminal phase of the log plasma concentration–time plot fitted by the method of least squares. Following the initial intravenous dose of formate, the $t_{1/2}$ of formate was estimated from the slope of the log plasma concentration–time plot and was also fitted by the method of least squares.

RESULTS

Clinical signs of acute toxicosis developed within 0.5 to 2 hr of methanol administration and resolved by 52 hr. The clinical signs of methanol toxicosis included mild to severe CNS depression (8 of 9 pigs), ataxia (8/9), recumbency (2/9), and tremors (1/9). Once they had recovered from the initial effects, the minipigs remained asymptomatic until they were euthanized. With each increasing methanol dose, the time until the onset of clinical signs decreased, and the duration and severity of clinical effects increased. No clinical signs were apparent in the control minipigs given water.

Clinical signs consistent with ocular toxicity were not observed in any of the minipigs given methanol. Their pupillary light reflexes and menace responses remained normal, and the pigs appeared to maintain the ability to negotiate around objects. No significant changes in the optic nerves and retinal vessels were observed using funduscopic examination or fluorescein retinal angiography. The 10-day histologic evaluation of one of the minipigs given the highest methanol dose (5.0 g/kg) revealed multifocal degeneration of the outer retinal layers (Fig. 1). The accumulation of amorphous cellular debris in the outer nuclear layer resulted in the elevation of the adjacent sensory retina. However, no histopathologic lesions consistent with methanol-related toxicosis were seen in the eyes of other methanol-treated or control minipigs. Putamen lesions were not observed in brain cross-sections from either methanol-treated or control minipigs.

A dose-dependent increase in blood methanol concentration was observed. Mean peak plasma methanol concentrations (±SD) of 3100 ± 700, 6200 ± 2300, and 15,200 ± 900 μg/ml occurred 0.5 to 4 hr after administration of 1.0, 2.5, or 5.0 g methanol/kg, respectively (Fig. 2). The mean initial elimination $t_{1/2}$ of methanol was 9.0 ± 1.6, 22.4 ± 6.1, and 18.9 ± 4.3 hr for animals given 1.0, 2.5, or 5.0 g methanol/kg, respectively. The terminal phase elimination $t_{1/2}$ of methanol could not be determined.

Although a slight decrease in blood pH was noted in the minipigs given 5.0 g/kg methanol (Fig. 3), neither depletion of blood bicarbonate nor formic acidemia occurred. In three of the minipigs (from the 1 and 2.5 g/kg dose groups), a transient, dose-independent increase in plasma formate concentration (1.74–3.40 mEq/liter) developed between 4 and 30 hr after methanol gavage (Fig. 4); endogenous blood formate concentrations in control minipigs were 0.53 ± 0.3 mEq/liter.

Minipigs given intravenous formate developed depression, polyuria, and polydipsia between 36 and 40 hr after the initial formate dose. Neither animal given formate developed metabolic acidosis or significant formate accumulation (data not shown). Minipigs given formate had normal pupillary light reflexes and menace responses and did not develop clinical signs consistent with optic nerve or retinal involvement. No significant changes in the optic nerves and retinal vessels were observed using funduscopic examination or fluorescein retinal angiography. No histopathologic lesions consistent with formate-related toxicosis were seen in the eyes of minipigs given formate. The initial formate elimination $t_{1/2}$ was 50 and 112 min for the two minipigs given formate intravenously (Fig. 5).

The average total hepatic folate concentration in the control minipigs was 17.5 ± 2.2 nmol/g of liver (n = 3).

DISCUSSION

The use of the pig as an animal model of methanol poisoning in man has been suggested by Makar and coworkers (1990). They based their hypothesis on the known association between formate accumulation and the subsequent development of metabolic acidosis and blindness in species sensitive to methanol poisoning. Formate is converted by 10-formyltetrahydrofolate synthetase to 10-formyltetrahydrofolate. 10-Formyltetrahydrofolate is subsequently metabolized by 10-formyltetrahydrofolate dehydrogenase to carbon dioxide (Johlin *et al.*, 1989). The rate of formate metabolism, therefore, is dependent on adequate levels of hepatic folic acid, especially its tetrahydrofolate form (Johlin *et al.*, 1987). As demonstrated by Makar and coworkers (1990), some pigs have low liver tetrahydrofolate concentrations and a decreased rate of formate metabolism, suggesting an increased sensitivity to methanol toxicosis. In our study, however, a single oral dose of 1.0 to 5.0 g/kg of methanol failed to result in formate accumulation sufficient to induce toxicity. The dose at which methanol-induced neuro-ocular toxicity occurs in pigs remains unknown. The doses of methanol used in this study are comparable to or greater than the minimal lethal oral dose (1.0 gram/kg) of methanol in humans (Roe, 1982). There are a number of cases in which adult humans have survived the ingestion of 500 to 600 ml of methanol (Naraqi *et al.*,

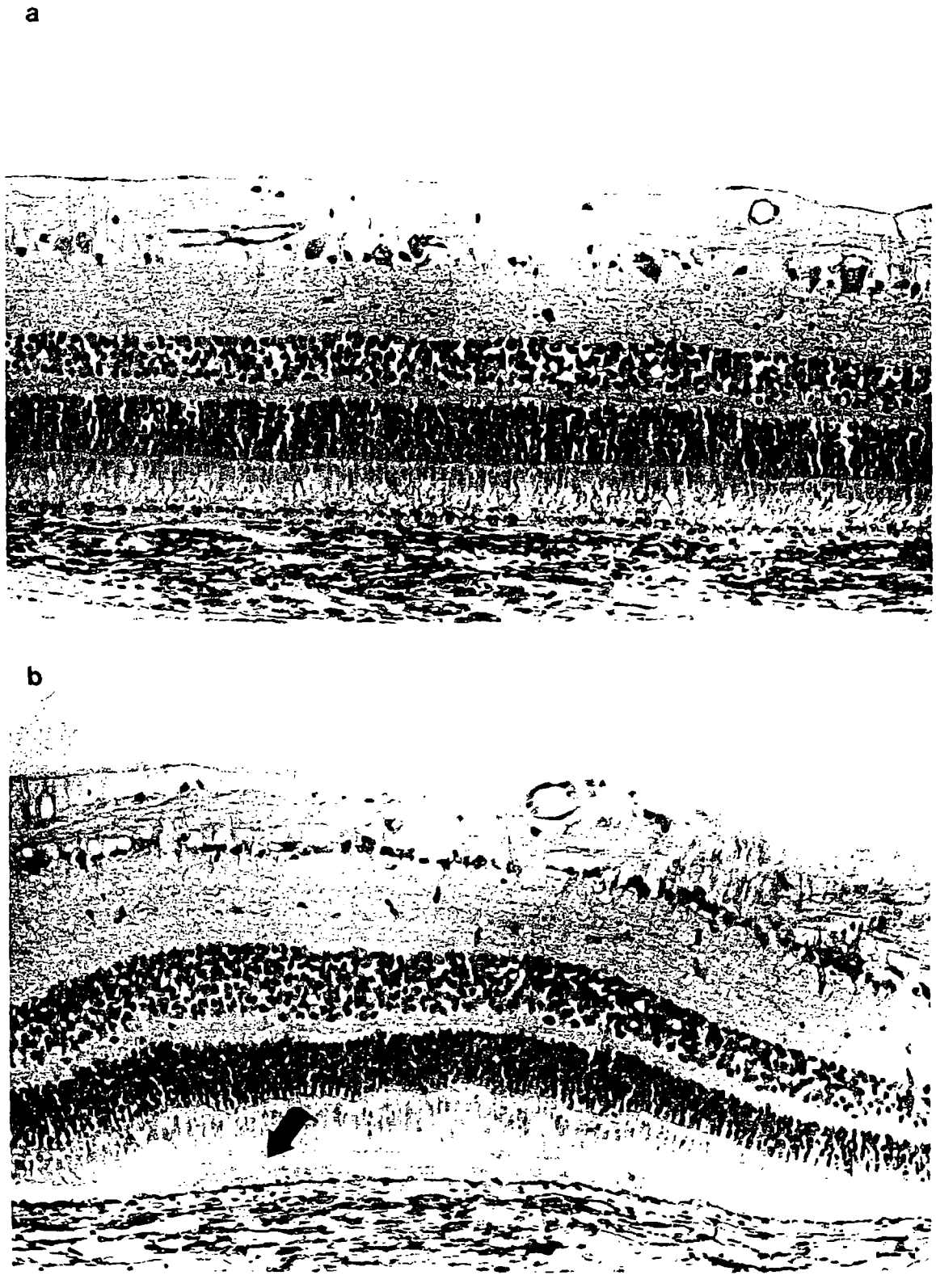

FIG. 1. Multifocal retinal degeneration of the outer retinal layers (b) in a minipig given methanol (5.0 g/kg) 10 days earlier, compared to control (a). Accumulation of amorphous cellular debris (arrow) in the outer nuclear layer resulting in the elevation of the adjacent sensory retina is also present. (HE, 40×).

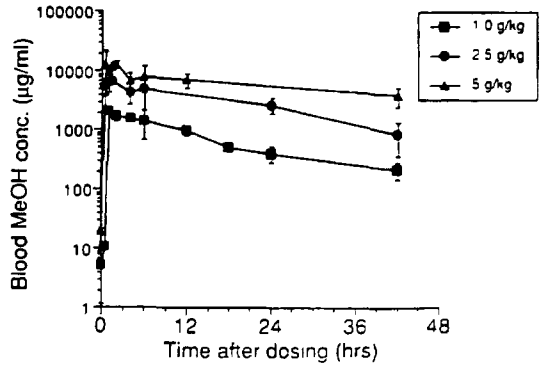

FIG. 2. Blood methanol concentration following a single oral dose of methanol (n = 3 minipigs per dose). Error bars ($\bar{x}$ ± SD) not visible are within the symbol used.

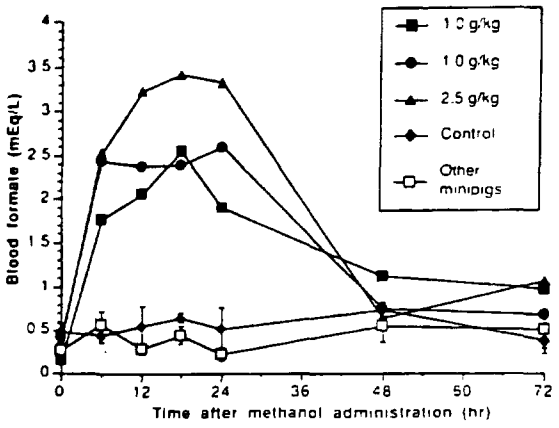

FIG. 4. Individual animal blood formate concentrations following a single oral dose of methanol. Only three minipigs, two from the lowest (1.0 g/kg) and one from the intermediate (2.5 g/kg) methanol dose groups, had increased blood formate levels following methanol administration. All other minipigs did not have increased blood formate concentrations, including three minipigs given 5.0 g methanol/kg and the remaining animal from each of the lower (1 or 2.5 g/kg) methanol dose groups.

1979). Even among sensitive humans, there is a tremendous amount of variability in the methanol dose–response.

The oral absorption and total body clearance of methanol in these minipigs appeared similar to those observed in humans. Whether unusual methanol distribution or metabolism occurs in minipigs is unknown. Minipigs rapidly absorbed the methanol and developed maximal blood methanol concentrations (range, 1600 to 30,300 µg/ml) that were comparable to those reported in humans following methanol ingestion. These plasma methanol concentrations appear higher than what would be expected based on methanol kinetics in other species. Although maximal blood methanol concentrations following acute lethal methanol exposure in humans are often undetermined, blood methanol levels in excess of 1000 µg/ml are commonly reported within 24 to 48 hr of methanol ingestion (Jacobsen *et al.*, 1982; Naraqi *et al.*, 1979; Kane *et al.*, 1968; Swartz *et al.*, 1981). The initial methanol elimination half-lives (9 to 22 hr) observed in the minipig were also comparable to those reported following methanol ingestion in humans (17 to 27 hr) without ethanol or dialysis treatment (Kane *et al.*, 1968). Finally, as in humans, methanol administration was associated with transient CNS depression and ataxia that paralleled their blood methanol concentrations.

Ultimately, however, no significant accumulation of formate occurred in any of the methanol-treated minipigs in this study. Blood formate levels in excess of 10 mEq/liter are reported in humans with neuro-ocular toxicosis following methanol ingestion (Sejersted *et al.*, 1983; McMartin *et al.*, 1980). Although there was a mild decrease in the blood pH of the minipigs given the highest methanol dose (5 g/kg), none of the methanol-treated minipigs developed a degree of metabolic acidosis or bicarbonate depletion consistent with methanol poisoning in humans. Minipigs in this study given the highest methanol dose (5 g/kg) had blood bicarbonate levels 72 hr after methanol ingestion (25.5

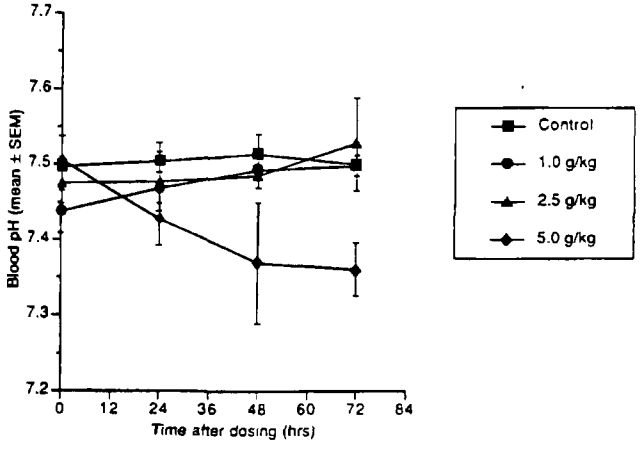

FIG. 3. Blood pH following a single oral dose of methanol (n = 3 minipigs per dose).

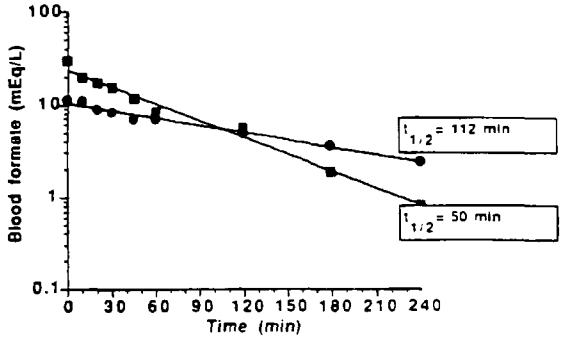

FIG. 5. Initial formate elimination following an intravenous administration of buffered formate (425 mg/kg) in two minipigs.

± 1.5 mEq/liter) that were similar to those of control minipigs (27.8 ± 1.4 mEq/liter). For comparison, a blood pH less than 7.2 with a blood bicarbonate concentration less than 15 mmol/liter commonly occurs in methanol-poisoned humans with blurred vision (Jacobsen *et al.*, 1982; Sejersted *et al.*, 1983). The significance of this decrease in blood pH in the highest dose group of pigs in the present study is unknown.

Susceptible species have lower total liver folate concentrations and slower formate metabolism, and thus increased sensitivity to methanol when compared to resistant species such as rats. For example, total liver folate concentration in humans (15.8 ± 0.8 nmol folate/g of liver) and monkeys (25.5 ± 1.2 nmol folate/g of liver) are lower than those observed in rats (25.3 ± 0.9 nmol folate/g of liver) and mice (60.9 ± 2.1 nmol folate/g of liver) (Johlin *et al.*, 1987). Previously reported values (5.1 ± 1.2 nmol/g of liver) for total liver folate concentration in young swine (Makar *et al.*, 1990) and micropigs (8.2 ± 0.6 nmol/g of liver; Tephly *et al.*, 1992) were lower (17.5 ± 2.2 nmol/g of liver) than those determined for control minipigs used in this study. These findings are consistent with the lack of formate accumulation observed in the minipigs. However, even within this strain of minipig, formate elimination was variable. In this present study, one minipig given formate directly had an initial rate of formate elimination ($t_{1/2}$ = 50 min) that was similar to that reported for rats (Johlin *et al.*, 1987), while a second minipig had a much slower initial rate of formate elimination ($t_{1/2}$ = 112 min) similar to that reported for young female swine ($t_{1/2}$ = 87 ± 18 min; Makar *et al.*, 1990) and micropigs ($t_{1/2}$ = 74.1 ± 6.0 min; Tephly *et al.*, 1992). Humans have low (15.8 ± 0.8 nmol/g of liver) total liver folate concentrations (Johlin *et al.*, 1987) that result in slow formate elimination, but this elimination rate of formate in humans is also variable ($t_{1/2}$ = 60 to 120 min; McMartin *et al.*, 1980). These results suggest that strain differences as well as differences between individual animals in formate metabolism may exist.

Most importantly, none of the minipigs given methanol developed clinical signs of ocular toxicity, although one minipig given the highest methanol dose (5 g/kg) did have histopathologic evidence of multifocal retinal degeneration. Histopathologic evidence of retinal damage following methanol administration has been reported in nitrous oxide-treated methanol-exposed rats (Murray *et al.*, 1991). Electroretinographic alterations suggestive of retinal involvement have also been reported following methanol administration to mice (Carricaburu *et al.*, 1979), monkeys (Potts *et al.*, 1955), and folate-deficient rats (Eells, 1991). Interestingly, in this study, retinal degeneration in this minipig developed in the absence of significant formate accumulation (maximal blood formate = 1.8 mEq/liter). These results, albeit limited, suggest that methanol may be acting as a direct retinal toxicant or, alternatively, that regional (ocular) methanol metabolism to formate occurs at a rate sufficient to induce retinal toxicity. Whether formate in vitreous humor accumulated to toxic levels in this animal is unknown. However, vitreal accumulation (in excess of blood concentrations) of formate has been reported in rats (Eells, 1991). It is possible that similar lesions may have been present at earlier times in the other methanol-treated minipigs. However, no microscopic evidence of retinal damage or repair was observed.

Since acute methanol-induced neuro-ocular toxicosis often develops in humans following a single oral ingestion of methanol (>0.4 to 1 g/kg), it does not appear that the Yucatan minipig on a normal folate diet will serve as a sufficiently sensitive animal model to study this syndrome or to evaluate effective means of therapeutic intervention. Similarly, rhesus monkeys given a single oral dose (0.5 to 6.0 g/kg) of methanol comparable to those given to the minipigs used in this study survived (Cooper and Felig, 1961). As with other animal models including primates (Martin-Amat *et al.*, 1977), it is still possible that the administration of repeated doses of methanol to minipigs could result in sufficient formate accumulation to result in neuro-ocular toxicity.

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16. ABSTRACT

The pig has been proposed as a potential animal model for methanol-induced neuro-ocular toxicosis in humans because of its reported low liver tetrahydro folate levels and therefore, slower formate metabolism as compared to humans. To determine the validity of the animal model, minipigs were given a single oral dose of methanol or water. Dose-dependent signs developed including mild CNS depression, tremers, and ataxia, however, these subjects failed to develop optic nerve lesions, toxicologically-significant formate accumulation or metabolic acidosis. Methanol and formate pharmacokinetics are also discussed.

17. KEY WORDS AND DOCUMENT ANALYSIS

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